UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA
DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR IV

TESIS DOCTORAL

Epidemiological and Molecular Analysis of Virulence and Antibiotic Resistance in *Acinetobacter baumannii*

Análisis Epidemiológico y Molecular de la Virulencia y la Antibiorresistencia en *Acinetobacter baumannii*

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Elias Dahdouh

DIRECTORA

Mónica Suárez Rodríguez

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Bajo la dirección de la doctora

Mónica Suárez Rodríguez

Madrid, Diciembre de 2016

First and foremost, I would like to thank God for the continued strength and determination that He has given me. I would also like to thank my father Abdo, my brother Charbel, my fiancée, Marisa, and all my friends for their endless support and for standing by me at all times. Moreover, I would like to thank Dra. Monica Suarez Rodriguez and Dr. Ziad Daoud for giving me the opportunity to complete this doctoral study and for their guidance, encouragement, and friendship. Additionally, I would like to acknowledge the help and support given by Dr. Jesus Mingorance, Dr. Belen Ortaz, Dr. Carmen San Jose, Dr. Bruno Gonzalez Zorn, Dr. Alicia Aranaz, Dña Sonsoles Pacho, Dña. Rosa Gimez-Gil, Dña. Micheline Hajjar, and "los Brunos". This work would not have been realized if not for the wonderful support given by all these professors, colleagues, family, and friends. Finally, I would like to acknowledge the scientific journals and scientific conferences that have accepted the various parts of this work for publication.

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Dª Mónica Suárez Rodríguez, Profesora Titular de Universidad del Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid,

CERTIFICA:

Que la Tesis Doctoral titulada "Análisis Epidemiológico y Molecular de la Virulencia y la Antibiorresistencia en *Acinetobacter baumannii*", que presenta el Titulado en Máster en Ciencias Biomédicas, D. Elias Dahdouh, ha sido realizada bajo mi dirección en el Departamento de Sanidad Animal de la Facultad de Veterinaria dentro del programa de tercer ciclo "Bioquímica, Biología Molecular, y Biomedicina", y estimamos que cumple todos los requisitos necesarios para optar al grado de Doctor por la Universidad Complutense de Madrid.

Madrid, Noviembre de 2016

Fdo.: Dra. Mónica Suárez Rodríguez

“Ask, and it shall be given you; seek, and ye shall find; knock, and it shall be opened unto you: For every one that asketh receiveth; and he that seeketh findeth; and to him that knocketh it shall be opened.”

The Bible - Matthew 7:7-8

To my beloved Missa, amazing father, and wonderful brother.

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Ia. RESUMEN

Resumen Corto

Acinetobacter baumannii es un patógeno nosocomial versátil implicado en importantes infecciones como la neumonía asociada a ventilación mecánica, infecciones del torrente sanguíneo, del tracto urinario, de heridas y de quemaduras en pacientes críticamente enfermos. Se han encontrado elevadas tasas de resistencia a muchos grupos de antibióticos en esta especie, incluyendo carbapenemas. La adquisición de resistencias se debe a su genoma elástico. Por ejemplo, la adquisición de OXAs es uno de los mecanismos más comunes en *A. baumannii* en su resistencia a carbapenemas, y las cepas resistentes a este antibiótico están asociadas con algunos clones internacionales. *A. baumannii* expresa factores de virulencia de una manera diferente en las diferentes cepas y algunos estudios muestran una relación entre virulencia y antibiorresistencia, que aún no está muy desarrollada. En esta Tesis Doctoral, se investiga la relación entre clonalidad, virulencia, y antibiorresistencia en cepas aisladas en España y el Líbano, dos países de la cuenca Mediterránea. Nuestro objetivo con este estudio es apoyar a los expertos en el control de infecciones, y proporcionar las herramientas necesarias para combatir la propagación de cepas resistentes a diferentes antibióticos. Además, con todo ello intentamos comprender mejor la compleja relación entre virulencia y resistencia antibiótica.

Cincuenta y nueve cepas de *A. baumannii* fueron aisladas del HU-LP (España) y 90 del SGH-UMC (Líbano). Se identificaron las cepas utilizando tiras API, amplificando por PCR genes de OXA-51, y mediante análisis por MALDI-TOF MS. Se analizó la resistencia antibiótica de las cepas según la guía de CLSI, se determinó la clonalidad por PFGE y se realizó la amplificación diferencial de genes "housekeeping". Los genes de carbapenemasas se detectaron por PCR y la formación de biofilms, hemólisis, movilidad, actividad proteolítica, y tiempos de generación se detectaron fenotípicamente. A continuación, se llevó a cabo la secuenciación del operón *pmr*CAB y el genoma de cepas resistentes a colistina. Además, se investigaron los patrones de formación de biofilms, después de cultivar las cepas en soportes de acero inoxidables, mediante recuento de las células adheridas y microscopía confocal.

Los resultados obtenidos muestran tasas muy altas de resistencia a antibióticos, especialmente a carbapenemas, en HU-LP, surgiendo la necesidad inmediata de intervención con programas de control de infección y de administración de antibióticos. El clon internacional II fue el más común, y la familia OXA-24 fue la más frecuente en este hospital. Se encontraron 7 pulsotipos distintos por PFGE, que fueron responsables de la mayoría de las

infecciones en HU-LP, demostrando la capacidad que tienen sólo algunos clones de producir infecciones recurrentes. Los perfiles de virulencia fueron muy variables entre las cepas, pero se encontraron asociaciones entre el clon internacional II y OXA-23, y un mayor nivel de virulencia en las cepas del HU-LP. Los otros clones y OXAs en este set se asociaron con una menor virulencia.

Se encontraron también altas tasas de resistencia a carbapenemas en SGH-UMC. El clon internacional II y la familia OXA-23 fueron los más frecuentes en este hospital. No se encontraron asociaciones entre los OXAs, clonalidad, y virulencia en el set de cepas libanesas. Esto indica que estas asociaciones son locales y es necesario realizar estudios preliminares en cada hospital. Las asociaciones entre virulencia, clonalidad y OXAs pueden emplearse, sin duda, para modificar los tratamientos y los protocolos de control de estas infecciones. Sin embargo, encontramos asociaciones entre los factores de virulencia en el set de cepas libanesas, y una investigación más profunda a este respecto puede conducir a una mejor comprensión de los mecanismos patogénicos de *A. baumannii*.

Se detectaron dos mutaciones distintas en PmrB (P233S y deleción de Ile), que fueron responsables de causar resistencia a colistina en dos cepas distintas. La primera mutación se ha descrito en otros estudios y en nuestro trabajo se muestra su relación con la producción de sideróforos y la actividad proteolítica. La segunda mutación ha sido detectada por primera vez en nuestros estudios de Tesis Doctoral y se ha comprobado que no afecta a la virulencia. Además, en este estudio se ha detectado el gen *bla*$_{\text{GES-5}}$ por primera vez en *A. baumannii*, indicando el intercambio de carbapenemasas entre diferentes especies.

Durante la investigación de la relación entre los patrones de formación de biofilms y perfiles de antibiorresistencia, se observó una relación entre cepas formadoras rápidas de biofilms y susceptibilidad a aminoglicósidos. Se detecta pigmentación para algunas cepas que no fue asociada con la producción de sideróforos. Además, se estudió la relación entre resistencia a carbapenemas y la formación de biofilms. Se encuentra una relación entre formaciones densas de biofilms y cepas resistentes a carbapenemas. Analizar un mayor número de cepas puede confirmar nuestros resultados preliminares y puede ayudar una mejor comprensión de la relación entre la resistencia antibiótica y formación de biofilms.

En resumen, se demuestra que existen tasas de resistencia a carbapenemas muy elevadas, así como la predominancia del clon internacional II en HU-LP y SGH-UMC. Se muestra también la predominancia de OXA-24 en HU-LP en comparación con la predominancia de OXA-23 en SGH-UMC. A continuación, se describe una asociación entre clonalidad y virulencia en las cepas de HU-LP, que será necesario verificar a nivel local, ya que se encuentra una diferencia en las asociaciones de los diferentes hospitales. Se analiza, además, una mutación previamente descrita y se describe, por primera vez, una nueva mutación en PmrB, que origina resistencia a colistina. Mientras que la primera afecta la virulencia, la segunda no muestra un efecto sobre ella. Finalmente, se observa una relación preliminar entre formaciones densas de biofilms y resistencia a carbapenemas.

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Resumen Largo

Acinetobacter baumannii es un cocobacilo Gram negativo, aerobio, no fermentador, catalasa positivo, y oxidasa negativo, que está incluido en la familia *Moraxellaceae*. Esta bacteria esta capaz de utilizar varias fuentes de carbono y crece fácilmente en medios de cultivo comunes. Se considera *A. baumannii* como un organismo ubicuo en la naturaleza, ya que se encuentra en muestras de origen ambiental y animal. *A. baumannii* forma parte del complejo *Acinetobacter calcoaceticus – Acinetobacter baumannii* (ACB) y puede formar parte de la microbiota normal de los seres humanos. Se han desarrollado varias técnicas para la identificación de *A. baumannii* que incluyen pruebas bioquímicas, secuenciación de la región espaciadora 16S-23S en el ARN del ribosoma, y desorción-ionización de matriz asistida por láser de tiempo de vuelo espectrometría de masas (con abreviación común MALDI-TOF MS). Es importante tener en cuenta que, puesto que la identificación mediante pruebas bioquímicas sólo puede caracterizar el complejo ACB, es necesario complementar estas pruebas con la amplificación del gen *bla*$_{\text{OXA-51-like}}$, que es intrínseco en *A. baumannii*.

El patógeno *Acinetobacter baumannii* está implicado en varias infecciones nosocomiales que incluyen neumonía asociada a ventilación mecánica, infecciones del torrente sanguíneo, infecciones del tracto urinario, e infecciones de heridas y de quemaduras. *A. baumannii* está especialmente implicada en la infección de pacientes en las Unidades de Cuidados Intensivos (UCI) y puede resultar en tasas de mortalidad de hasta el 43% de estos

pacientes. Se encuentran muchos mecanismos intrínsecos de resistencia en *Acinetobacter baumannii* frente a un gran número de antibióticos y su genoma es muy elástico, permitiéndole adquirir resistencia frente a un número aún mayor de antibióticos. La resistencia natural de *A. baumannii* limita severamente las opciones de tratamiento y deja poco margen de tratamiento mediante el uso de antibióticos con suficiente eficiencia en el curso de infecciones causadas por este patógeno. Además, la gran capacidad de adaptación de este organismo resulta muy a menudo en una mayor resistencia a otros antibióticos, hasta que el número de opciones de tratamiento se limita de forma preocupante. Mientras en las últimas décadas se hablaba de cepas resistentes a dos o tres grupos de antibióticos, hoy en día se pueden encontrar bases de datos repletas de estudios que muestran resistencia a todos los grupos de antibióticos exceptuando un par, e incluso cepas resistentes a todos los antibióticos. La elevación en la tasa de resistencia a carbapenemas es especialmente alarmante, ya que estos antibióticos se reservan para su uso en el tratamiento de pacientes gravemente enfermos e infectados con cepas resistentes a múltiples antibióticos. Estudios a nivel mundial muestran que la tasa de resistencia frente a carbapenemas en cepas de *A. baumannii* ha aumentado del 41% al 63% en sólo 4 años. Estudios recientes también muestran que la tasa de resistencia a carbapenemas puede alcanzar el 93% en algunos países europeos y en regiones de Oriente Medio. En España, la tasa de resistencia a carbapenemas es notoriamente más alta que en otros países europeos. Los estudios muestran que la susceptibilidad a carbapenemas ha disminuido desde el 33,8% hasta el 19,3% durante diez años. En el Líbano, un estudio nacional muestra que la tasa de resistencia a carbapenemas es el 88% en las cepas clínicas de *A. baumannii*. Estas elevadas tasas ponen en peligro la continuidad en el uso de esta crucial familia de antibióticos en el futuro. El dato más alarmante es que las cepas resistentes a carbapenemas son las responsables de tasas de mortalidad muy elevadas en pacientes infectados.

Acinetobacter baumannii tiene la capacidad de adquirir resistencia frente a varios antibióticos a través de la trasferencia horizontal de genes que codifican beta-lactamasas y a través de varias mutaciones en su genoma y la regulación de la expresión de genes intrínsecos. La familia más común de carbapenemasas encontrada en *A. baumannii* es la familia de oxacilinasas (OXAs). En particular, las familias OXA-23, OXA-24, y OXA-58 son las más frecuentes entre cepas de *A. baumannii* resistentes a carbapenemas. Estas OXAs se encuentran distribuidas por todo el mundo y están asociadas con algunos clones internacionales que han demostrado una importante capacidad para evadir el tratamiento antibiótico. El clon internacional II, anteriormente conocido como clon europeo II, es el clon más frecuente a nivel

mundial. Las cepas de este clon normalmente son resistentes a varios grupos de antibióticos y entre ellas se pueden encontrar las tres familias de OXA, siendo común encontrar más de una familia de OXA en la misma cepa. Además de este clon, los clones internacionales I y III y varios otros clones internacionales con resistencia a más de tres grupos de antibióticos se encuentran distribuidas muy frecuentemente a nivel mundial.

La prevalencia global de cepas de *A. baumannii* resistentes a carbapenemas ha empujado a los profesionales clínicos a reutilizar la colistina y experimentar su uso en terapias, combinándola con diferentes antibióticos. La colistina fue un antibiótico poco utilizado debido a sus efectos adversos a nivel renal, pero hoy en día se utiliza cada vez más por su eficacia en el tratamiento de infecciones con cepas resistentes a carbapenemas. Sin embargo, ciertas cepas de *A. baumannii* han podido desarrollar resistencia a colistina durante el tratamiento con este antibiótico. Aunque esta resistencia aparece de forma esporádica y dispersa, se han detectado varios brotes causados por cepas resistentes a colistina y todos los demás antibióticos en varios países del mundo. En España, un estudio muestra que el 40,67% de las cepas de *A. baumannii* aisladas durante seis años resultaron resistentes a colistina. La causa de la resistencia a colistina en *A. baumannii* es, en la mayoría de los casos, una mutación genética que da lugar a modificaciones en el lipopolisacárido (LPS), la molécula responsable del efecto de la colistina. Mutaciones en los genes *lpx* que provocan la pérdida del Lipido A, el ancla del LPS, causan resistencia a colistina, alteraciones en la virulencia, y mayor susceptibilidad a otros antibióticos en *A. baumannii*. Estas mutaciones son frecuentes entre cepas que desarrollan resistencia a colistina *in-vitro.* Las mutaciones en el operón *pmr*CAB, que codifica para ciertas moléculas que actúan como un sistema regulador del sensor quinasa que modifica el Lipido A en respuesta a estímulos ambientales, también producen resistencia a colistina en *A. baumannii*. Estas mutaciones son frecuentes entre cepas que desarrollan resistencia a colistina durante el tratamiento con este antibiótico.

La patogenicidad de *A. baumannii* todavía no está completamente descrita, pero varios factores de virulencia se han asociado con su capacidad de causar enfermedades y persistir en el hospital. La formación de biofilms es uno de estos factores identificados en *A. baumannii* y resulta en el secuestro de este organismo y su protección frente al efecto de los antibióticos y desinfectantes. Por eso, este factor se ha considerado como un factor de virulencia y un factor de persistencia al mismo tiempo en *A. baumannii*. Además, se han detectado diferentes patrones de formación de biofilms en las diferentes cepas y clones de *A. baumannii* estudiadas

hasta el momento. Esta bacteria también es capaz de producir sideróforos, lo que le permite captar hierro de su ambiente. La superproducción de sideróforos puede disminuir notablemente las reservas de hierro del paciente, por lo que este factor está considerado como un factor de virulencia. Además, se ha observado que algunas cepas clínicas de *A. baumannii* son capaces de producir hemólisis en agar sangre mediante la producción de hemolisinas. Estas enzimas pueden causar la lisis de las células rojas en la sangre, con la subsiguiente liberación de los grupos hemo, portadores de hierro. La producción de enzimas proteolíticas está también descrita en *A. baumannii* y está considerada como otro factor de virulencia en este organismo. Más adelante, aunque *A. baumannii* se consideraba un organismo no móvil, algunos estudios demostraron que ciertas cepas clínicas son capaces de tener movilidad de superficie. Todos estos factores pueden estar expresados en combinaciones que en ocasiones originan cepas con altos niveles de virulencia, capaces de producir altas tasas de mortalidad. Sin embargo, varios estudios muestran que la expresión fenotípica de estos factores puede variar mucho entre las diferentes cepas por razones que aún no son completamente conocidas.

Parece existir una relación entre virulencia y resistencia antibiótica en *A. baumannii*. Algunos estudios muestran que la expresión fenotípica diferencial de la virulencia entre las diferentes cepas puede relacionarse con perfiles de resistencia frente a varios antibióticos. Además, genes que codifican ambos determinantes de virulencia y antibiorresistencia pueden coexistir en el mismo elemento genético móvil. Las interacciones complejas entre estos dos fenómenos no están bien estudiadas en *A. baumannii*. Sin embargo, varias moléculas se están investigando actualmente, debido a sus efectos anti-virulencia, con el posible objetivo de aplicarlas junto con los antibióticos en el tratamiento de infecciones.

En esta Tesis Doctoral, cepas clínicas de *A. baumannii* aisladas de España y el Líbano, dos países mediterráneos con altas tasas de resistencia a carbapenemas, fueron caracterizadas fenotípica y genotípicamente en términos de antibiorresistencia, clonalidad, virulencia, y el efecto de estos factores sobre el crecimiento de las células. Además, se investigaron las causas de resistencia a colistina en dos sets distintos de *A. baumannii* que habían desarrollado resistencia a este antibiótico durante el tratamiento. A continuación, se investigaron los patrones de formación de biofilms de algunas cepas seleccionadas con diferentes perfiles de antibiorresistencia para determinar si existe una relación entre estos patrones y la resistencia a ciertos antibióticos. Nuestro objetivo es proporcionar a médicos y especialistas en control de infecciones, herramientas epidemiológicas e información sobre posibles asociaciones entre los

complejos mecanismos de antibiorresistencia y virulencia en *A. baumannii*. Estos datos pueden ayudar a obtener un mayor éxito terapéutico y protocolos más enfocados al control de las infecciones. Si se implementa correctamente, esta información puede ser clave para reservar la eficacia de los antibióticos más importantes para su uso en el futuro, limitar la propagación de cepas resistentes a varios grupos de antibióticos, y comprender de una manera más clara la relación existente entre los diferentes factores que provocan el gran éxito patogénico de *A. baumannii*.

Para realizar este estudio, 59 cepas clínicas de *A. baumannii* fueron recogidas en el Hospital Universitario – La Paz (HU-LP) en Madrid, España y 90 cepas del Hospital Saint Georges – University Medical Center (SGH-UMC) en Beirut, Líbano. Se realizó la identificación de las cepas mediante pruebas bioquímicas, la amplificación del gen *bla*$_{\text{OXA-51}}$, y, cuando fue necesario, por MALDI-TOF MS. A continuación, se determinó el perfil de antibiorresistencia según la guía de "Clinical and Laboratory Standards Institute". Más adelante, se determinó la clonalidad de las cepas llevando a cabo PCRs para distintos genes "housekeeping" y comparando el perfil de amplificación con datos publicados y, cuando fue necesario, por Pulsed-Field Gel Electrophoresis (PFGE). Además, se amplificaron por PCR los genes *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{OXA-58}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM}}$, y *bla*$_{\text{KPC}}$. La formación de biofilms se determinó en tubos de poliestireno, tras teñirlos con 1% de cristal violeta. La hemólisis se determinó mediante cultivo de las cepas en agar sangre durante seis días consecutivos. La producción de sideróforos se evaluó mediante el ensayo de CAS en medio líquido. La actividad proteolítica se analizó realizando el ensayo de azoalbúmina. La movilidad en superficie se evaluó inoculando la superficie de un gel de agar Luria Bertani (LB) al 0,3% tras 14 horas de incubación. Los tiempos de generación se determinaron para ciertas cepas con distintos perfiles de antibiorresistencia durante su cultivo de 8 horas en caldo de LB. El análisis estadístico se realizó utilizando varios programas para determinar las posibles relaciones entre los diferentes factores estudiados.

Los datos obtenidos muestran tasas muy altas de resistencia a carbapenemas en HU-LP, alcanzando el 84,75%. Se detecta también altas tasas de resistencia a otros antibióticos en el 80% de estas cepas. El clon internacional II fue el más frecuente y se detectó en el 71,19% de las cepas. También se detectaron los clones internacionales I y III en el 8,47% y el 6,78% de las cepas, respectivamente, mientras el 13,56% de las cepas no pertenecieron a ningún clon internacional y una cepa pertenecí al grupo internacional 14. El análisis de PFGE muestra que

estas cepas se encuentran distribuidas en 7 grupos distintos y que 5 aislados no pertenecieron a ningún grupo. La familia OXA-24 fue la más frecuente entre las cepas de HU-LP y se encuentra en el 62,71% de estas cepas mientras las familias OXA-23 y OXA-58 se encontraron en el 1.86% y el 13.56% de las cepas, respectivamente. Estos resultados muestran que la familia OXA-24 continúa siendo la más frecuente en España, donde fue descrita por primera vez.

En la mayoría de los casos, los perfiles de virulencia obtenidos muestran una gran variación entre las cepas. En las cepas del HU-LP, el 69,5% fue positivo para la producción de sideróforos, el 84,4% para formaciones fuertes de biofilms, y el 54,2% para hemólisis. La actividad proteolítica entre estas cepas fue 26,6 ± 8,4 U/L y los tiempos de generación fueron desde 0,324 ± 0,027 hasta 0,666 ± 0,037 horas. Muy pocas cepas fueron positivas para movilidad en superficie. Sin embargo, se encuentran algunas relaciones estadísticas entre los factores estudiados. En las cepas del HU-LP, las cepas que pertenecieron al clon internacional II fueron asociadas positivamente con hemólisis, producción de sideróforos, y formaciones fuertes de biofilms ($p<0,05$). En comparación, las cepas que pertenecieron a los clones internacionales I y III han mostrado menores niveles de virulencia. De forma similar, las cepas positivas para la familia OXA-24 fueron positivamente asociadas con estos mismos factores de virulencia ($p<0,05$). Estas asociaciones pueden ser muy útiles para médicos y especialistas en control de infección en HU-LP, ya que les permiten desarrollar tratamientos más agresivos dirigidos a estos factores cuando aparezca una cepa del clon internacional II y positiva a OXA-24. Además, se puede utilizar la asociación negativa entre virulencia, el resto de clones internacionales y las otras familias de OXA para limitar el uso de antibióticos de amplio espectro, si la condición del paciente lo permite, y de esta manera reservar estos antibióticos para su uso en el futuro. Una ventaja adicional de este estudio es que la determinación de la clonalidad y la presencia de las familias de OXAs se pueden realizar de forma fácil y rápida utilizando dos PCR multiplex, de manera que se aprovechen las asociaciones entre estos factores y la virulencia, para adaptar los tratamientos a los resultados obtenidos.

El 90% de las cepas del SGH-UMC fueron resistentes a carbapenemas y el 85,6% fue también resistente a otros antibióticos. El clon internacional II fue detectado en el 88,9% de las cepas mientras el 6,7% de las cepas pertenecieron al grupo internacional 4, el 2.2% al grupo 14, el 1,1% al grupo 10, y una cepa no perteneció a ningún clon internacional. En las cepas aisladas del SGH-UMC, la familia OXA-23 se encuentra en el 93,8% de las cepas resistentes a carbapenemas mientras OXA-24 se encuentra en sólo dos cepas y OXA-58 no se encuentra

en ninguna. Los datos de los dos hospitales muestran que hay una tasa de resistencia a carbapenemas muy elevada en ambos lugares. Este hecho necesitaría una intervención inmediata y rápida mediante la implantación de protocolos adecuados de control de infección y programas de administración de antibióticos que asegurasen que estos antibióticos importantes siguen siendo una opción válida en el futuro. Además, estos resultados muestran la gran propagación del clon internacional II, que se encuentra con una elevada y alarmente frecuencia en dos países distanciados geográficamente.

En las cepas del SGH-UMC, el 57,8% fue positivo para producción de sideróforos, el 85,6% para formaciones fuertes de biofilms, y el 47,6% para hemólisis. La actividad proteolítica fue 17,7 ± 9,5 U/L y los tiempos de generación fueron desde 0,262 ± 0,021 hasta 0,653 ± 0,049 horas. La mayoría de las cepas estudiadas han sido positivas para movilidad en la superficie. A pesar de haber encontrado asociaciones entre clonalidad, OXAs, y virulencia en las cepas españolas, estas asociaciones no fueron detectables entre las cepas libanesas. Esto sugiere que las asociaciones presentes en las cepas aisladas del HU-LP no son aplicables a las cepas en otros hospitales sino que sólo se pueden utilizar de manera local en el mismo hospital. Además, esto sugiere la necesidad de realizar investigaciones preliminares en cada hospital sobre la relación entre virulencia, clonalidad y resistencia antibiótica antes de poder aprovechar esta asociación. Otro factor que puede haber evitado la detección de asociaciones entre las cepas libanesas es que dichas cepas han sido mucho más homogéneas en comparación con las cepas españolas. Sin embargo, se encuentran asociaciones estadísticas entre movilidad, producción de sideróforos, y formación de biofilms en las cepas estudiadas. Investigar estas relaciones en el futuro puede conducir a un mejor nivel de comprensión de las complejas interacciones entre estos factores, así como entre virulencia y resistencia antibiótica.

A continuación, se secuenciaron *pmr*CAB y el genoma completo en dos sets de cepas que habían desarrollado resistencia a colistina durante el tratamiento, para tratar de determinar la causa de esta resistencia. El análisis genómico muestra que dos mutaciones distintas en *pmr*CAB pueden causar esta resistencia. La primera mutación (P233S en PmrB) es una conocida causa de resistencia a colistina en *A. baumannii*. Cuando se compara con su cepa sensible, esta mutación parece tener un efecto sobre la virulencia, ya que la cepa resistente fue positiva para la producción de sideróforos y mostraba casi la mitad de actividad proteolítica. Esta observación puede relacionarse con el papel de PmrB en respuesta a cambios en los niveles de hierro en el ambiente, pero se necesitan más investigaciones sobre el efecto exacto de esta

mutación a PmrB antes de obtener conclusiones sobre su efecto sobre el metabolismo de la célula. Le segunda mutación encontrada (Δ19Ile en PmrB) en el segundo set fue descrita por primera vez en nuestro estudio y no parece tener efecto sobre la virulencia de la cepa. Mas investigaciones en el futuro sobre los cambios exactos que produce esta mutación en PmrB pueden ayudar a comprender mejor el mecanismo de resistencia a colistina en *A. baumannii*. El segundo set de cepas fue positivo para el gen *bla*$_{\text{GES-5,}}$ también descrito por primera vez en *A. baumannii* en este trabajo. La presencia de este gen en *A. baumannii* es un indicador importante del intercambio de genes de resistencia entre las diferentes especies bacterianas.

Los patrones y tasas de formación de biofilms se investigaron también para ciertas cepas con distintos perfiles de resistencia, con el objetivo de determinar si existe una asociación entre resistencia a ciertos antibióticos y patrones específicos de formación de biofilms. Esto se realizó mediante cultivo de las cepas en soportes de acero inoxidables y recuento de las células viables tras 5, 12, y 24 horas de incubación. Además, se realizó un análisis de la composición de ciertas cepas utilizando microscopia confocal. La investigación dio lugar a la identificación de tres patrones de formación de biofilms tras 5 horas de incubación: formadores rápidos, formadores intermedios, y formadores lentos. El primer grupo mostraba una alta tasa de células adherentes en soportes de acero inoxidables y se trataba de cepas susceptibles a aminoglicósidos. Sin embargo, no todas las cepas susceptibles a esta familia de antibióticos formaban parte del grupo de formadores rápidos de biofilms. Todas las cepas resistentes a más de dos grupos de antibióticos fueron formadores rápidos o intermedios de biofilms, pero algunas cepas sensibles a carbapenemas se encuentran en estos grupos. El grupo de formadores lentos incluía sólo una cepa que fue susceptible a carbapenemas. Durante el experimento, algunas cepas mostraron una pigmentación que no estaba relacionada con la producción de sideróforos. A continuación, dos cepas pigmentadas y resistentes a carbapenemas y dos cepas sin pigmentación y sensibles a carbapenemas fueron analizadas por microscopia confocal. No se detectó ninguna relación directa que explicase la formación de pigmentación, pero las imágenes obtenidas mostraron una relación entre las cepas resistentes a carbapenemas y la formación de biofilms densos y que cubren todo el substrato tras 24 horas de incubación. Las cepas sensibles a carbapenemas mostraron formaciones de biofilms débiles y dispersas que no cubrían todo el substrato tras 24 horas de incubación. La realización de este experimento utilizando más cepas puede confirmar nuestra observación preliminar que establecería una relación entre formaciones densas de biofilms y resistencia a carbapenemas. Esta relación puede ser muy útil para su uso clínico, ya que los especialistas podrían predecir el patrón de

formación de biofilms utilizando los datos de resistencia antibiótica y, en consecuencia, adaptar sus tratamientos.

En resumen, se encontraron tasas muy altas de resistencia a carbapenemas en ambos HU-LP y SGH-UMC, donde el clon más frecuente fue el clon internacional II. Se detectaron siete distintos pulsotipos en HU-LP, además de cepas aisladas con resistencia a varios antibióticos capaces a producir infecciones. El gen *bla*$_{\text{OXA-24}}$ fue el más frecuente entre las cepas españolas, mientras que *bla*$_{\text{OXA-23}}$ fue el más frecuente entre las cepas libanesas. Estos datos sugieren una necesidad inmediata de implementación de programas de control de infección y administración de antibióticos para disminuir la prevalencia de estas cepas de *A. baumannii* en dichos hospitales. Los perfiles de virulencia en las dos poblaciones fueron muy variados, detectándose una asociación entre cepas españolas del clon internacional II y *bla*$_{\text{OXA-24}}$ por un lado, y una mayor virulencia, por otro lado. Esta asociación no se detectó entre las cepas libanesas, lo que sugiere que estas asociaciones son locales, y se necesita una mayor investigación sobre la presencia de estas asociaciones en cada hospital antes de poder aprovechar esta información para modificar tratamientos basándose en la clonalidad y detección de OXAs. Además, la mutación P233S en PmrB, previamente descrita en cepas de *A. baumannii* resistentes a colistina, se ha encontrado en este trabajo y parece tener un efecto sobre la producción de sideróforos y la actividad proteolítica. Investigar el efecto exacto de esta mutación en el futuro puede ayudar a comprender la relación entre virulencia y resistencia a colistina. Se detectó además una segunda mutación en PmrB (ΔIle19) en otra cepa de *A. baumannii,* descrita por primera vez en nuestro estudio. Esta mutación parece que no afecta a la virulencia de la cepa e investigar su efecto sobre la función de PmrB puede ayudar a comprender más en profundidad los mecanismos de resistencia a colistina en *A. baumannii*. Finalmente, se detectó una asociación preliminar entre formaciones densas de biofilms y resistencia a carbapenemas. Esta hipótesis necesita un estudio más profundo, utilizando un mayor número de cepas, antes de consolidarla y poder utilizarla clínicamente. Precisamente, su interés clínico radica en la posibilidad de modificar los tratamientos aplicando el conocimiento de estas asociaciones de factores, que se podrían conocer de forma rápida mediante los datos de resistencia antibiótica.

Ib. SUMMARY

Short Summary

Acinetobacter baumannii is a highly versatile nosocomial pathogen that is implicated in several nosocomial infections among critically ill patients and results in high mortality rates. These infections include ventilator associated pneumonia, bloodstream infections, urinary tract infections, and burn wound infections. Multi-Drug Resistant (MDR), and especially Carbapenem Resistant *Acinetobacter baumannii* (CRAB) isolates are also sharply increasing in frequency, forcing clinicians to revert to the use of colistin. This organism has numerous intrinsic resistance mechanisms, virulence determinants, and an elastic genome that allows it to acquire resistance to almost all antimicrobial agents rather easily. The acquisition of oxacillinases (OXAs) is one of the most common mechanisms of acquiring carbapenem resistance. Moreover, some clinically important International Clones (ICs) with MDR profiles are disseminated all over the world. *A. baumannii* is known to differentially express virulence determinants and few studies hint at their relationship with antimicrobial resistance. However, this relationship is not extensively investigated. In this Doctoral Thesis, the relationship between clonality, virulence, and antimicrobial resistance is investigated in two sets of clinical isolates obtained from Spain and Lebanon, two countries in which the rate of CRAB isolates is notoriously high. Our aim is providing clinicians and infection control specialists with tools that could be used to assess the infecting strain based on initial clinical data that could improve the chances of successful therapy and limit the spread of MDR and CRAB isolates. Additionally, it is our aim to better understand the interaction between virulence and antibiotic resistance.

Fifty nine clinical *A. baumannii* isolates were obtained from Hospital Universitaro - La Paz (HU-LP), Spain and 90 from Saint Georges Hospital - University Medical Center (SGH-UMC), Lebanon. Identification was performed using 20NE API strips, PCR amplification of *bla*$_{\text{OXA-51-like}}$, and Matrix Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). Susceptibility testing was performed according to CLSI guidelines and clonality analysis was performed by tri-locus sequence typing and Pulsed-Field Gel Electrophoreis (PFGE). PCR for the detection of common carbapenemases was then performed and biofilm formation, hemolysis, siderophore production, surface motility, proteolytic activity, and doubling times were phenotypically determined. Additionally, sequencing of the *pmr*CAB operon and the whole genome was performed for two sets of isolates that acquired colistin resistance during therapy. Also, the patterns of biofilm formation

for isolates with different susceptibility profiles were determined through growth on steel coupons and Confocal Laser Scanning Microscopy (CLSM) analysis.

Our data show very high rates of antimicrobial resistance, especially to carbapenems, in both hospitals, indicating an immediate need for intervention though antimicrobial stewardship programs and infection control protocols. IC II was predominant in both countries while OXA-24-like was predominant in HU-LP as opposed to the predominance of OXA-23-like in SGH-UMC. PFGE analysis showed that strains pertaining to 7 pulsotypes were responsible for most infections. This shows the ability of a handful of clones to cause repeated infections and the international spread of ICs and OXAs. The virulence profiles were highly variable among the different isolates. However, among the Spanish set, IC II and OXA-23-like were associated with increased virulence as opposed to ICs I and III and the other OXAs. This association was absent among the Lebanese set, suggesting the locality of the association and the need for preliminary local analyses before being able to exploit the relationship between these factors in the modification of treatment approaches and infection control protocols. Nevertheless, associations between the different virulence factors was evident and further investigating this relationship could help better understand the poorly understood pathogenicity of *A. baumannii*.

Genomic analysis of colistin resistant strains showed that P233S and deletion of Ile in PmrB cause colistin resistance. The former mutation is known and was shown in our study to affect virulence while the latter mutation is novel and had no effect on virulence. Moreover, *bla*$_{\text{GES-5}}$ was identified for the first time in *A. baumannii* among these isolates indicating inter-species exchange of carbapenemases. Investigating the patterns of biofilm formation showed a possible relationship between aminoglycoside sensibility and fast rates of biofilm formation and between carbapenem resistance and dense biofilm formations. These relationships, however, are preliminary and require further investigations using larger pools of isolates.

In conclusion, this study shows high rates of CRAB isolates and predominance of IC II in HU-LP and SGH-UMC. It also shows a predominance of OXA-24-like in HU-LP as opposed to OXA-23-like in SGH-UMC. An association between virulence and clonality seems to exist but needs to be determined on a local since different associations were detected in the different hospitals. Additionally, a previously identified and a novel mutation in *pmr*B conveyed resistance to colistin but only the P233S mutation had an effect on virulence. Finally, a

preliminary relationship between dense biofilms and carbapenem resistance has been identified.

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Extended Summary

Acinetobacter baumannii is a Gram-negative aerobic, non-fastidious, non-fermenting, catalase-positive, oxidase-negative coccobacillus that is classified under the family *Moraxellaceae*. This bacterium can utilize a wide range of carbon sources and is easily grown on common laboratory media. It is thought to be ubiquitous in nature where it has been isolated from environmental, animal, and insect specimens. *A. baumannii* is part of the *Acinetobacter calcoaceticus*– *Acinetobacter baumannii* (ACB) complex and could be part of the human normal flora. Numerous techniques have been developed over the years for the identification of *A. baumannii* that range from biochemical tests to the sequencing of the 16S-23S ribosomal RNA spacer. Matrix Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) has also been employed in the identification of this organism. Additionally, though traditional biochemical identification of *A. baumannii* can only reach the level of identifying the ACB complex, the subsequent amplification of the intrinsic *bla*$_{\text{OXA-51-like}}$ is indicative of *A. baumannii*.

It was shown that *Acinetobacter baumannii* is implicated with nosocomial infections that include ventilator associated pneumonia, bloodstream infections, urinary tract infections, and burn wound infections. *A. baumannii* is especially implicated in infecting patients in the Intensive Care Unit (ICU) and produces mortality rates among these patients that could be as high as 43%. *Acinetobacter baumannii* has a wide range of intrinsic resistance mechanisms to antimicrobial agents and a highly elastic genome that allows it to acquire resistance to even more antibiotics. The natural resistance of *A. baumannii* severely limits treatment options to just a handful of antimicrobial agents that retain a good activity against this pathogen. Nevertheless, the high capacity for adaption of this organism often results in the acquisition of resistance and limits even more the available treatment option. While in previous decades, reports of Multi-Drug Resistant (MDR) *A. baumannii* isolates were considered alarming, we find nowadays databases full of reports of Extensively-Drug Resistant (XDR) and even Pan-Drug Resistant (PDR) isolates. The increase in the rate of resistance towards carbapenems is especially alarming since these agents are usually the treatment of choice for critically ill

patients infected with MDR organisms. Resistance to carbapenems among *A. baumannii* isolates have been found to increase from 41% to 63% over just a four-year period in large-scale studies. Moreover, recent global studies have reported carbapenem resistance rates among *A. baumannii* to be as high as 93% in certain European and Middle Eastern regions. A nation-wide study in Lebanon that included nine hospitals over a one-year period showed a rate of carbapenem resistance of 88%. In Spain, the rate of carbapenem resistance is notoriously higher than in other European countries where susceptibility to imipenem was shown to drop from 33.8% to 17.3% over ten years. These high rates of carbapenem resistance seriously endanger the continued use of this valuable family of antimicrobial agents for the treatment of future infections. To make matter worse, resistance to carbapenems has been associated with even higher mortality rates among infected individuals.

This versatile organism is able to acquire resistance to various antimicrobial agents through the acquisition of beta-lactamases by horizontal gene transfer and through a wide range of mutations in its genome, in addition to regulation of expression of several intrinsic genes. The most common group of beta-lactamases that are found to convey resistance to carbapenems in *A. baumannii* are the oxacillinases (OXAs). OXA-23-like, OXA-24-like, and OXA-58-like are the most commonly detected OXAs among Carbapenem Resistant *Acinetobacter baumannii* (CRAB) isolates. These OXAs are globally disseminated and are associated with certain International Clones (ICs) that have shown much success as pathogens all across the globe. IC II, formerly known as European Clone II, is the most widely worldwide disseminated clone. This MDR clone is reported to harbor all three types of OXAs and sometimes more than one OXA at a time. IC I and III, in addition to several other MDR clones have also been reported to be present across large geographical regions around the globe.

The global prevalence of CRAB isolates has forced clinicians to revert to the use of colistin and experimenting with different combination therapies that often contain this antibiotic. Colistin is an antimicrobial agent that was not used for a long time in treatment due to its nephrotoxic effects. However, it has returned to clinical use since it has shown very promising results in terms of treating MDR and XDR infections. Nevertheless, *A. baumannii* infections resistant to this antibiotic have been reported to emerge during therapy. Though reports are normally sporadic and dispersed, outbreaks caused by PDR isolates that have acquired colistin resistance have been reported from several countries. In Spain, one study even showed that 40.67% of *A. baumannii* isolates collected over six years were colistin resistant.

Most reported cases of colistin resistance trace back the cause of resistance to one of two main sets of genes, both resulting in the modification of the Lipopolysaccharide (LPS) molecule, the target of colistin. Mutations in the *lpx* genes that result in the loss of Lipid A, the LPS anchor, have been reported to cause colistin resistance, in addition to alteration of virulence and increased susceptibility to other antimicrobial agents. The second set of genes are within the *pmr*CAB operon which codes for sensor kinase regulatory molecules that result in modifications of lipid A in response to environmental stimuli. While the former mutations are commonly reported from *in-vitro* studies, the latter mutations are commonly reported among clinical isolates that have acquired colistin resistance during therapy.

The pathogenicity of *A. baumannii* is still not yet fully understood but several virulence factors have been identified as part of its disease-causing ability and to contribute to its persistence in the hospital environment. Biofilm formation is one virulence factor identified in *A. baumannii* that is known to sequester this organism and protect it from the effect of antimicrobial agents and disinfectants. This factor therefore contributes to both the virulence and persistence of *A. baumannii*. Moreover, varying rates and patterns of biofilm formation have been reported among different *A. baumannii* isolates and clones. Iron uptake through the production of siderophores is another factor of virulence in this organism. Siderophores capture iron from the environment and their overproduction could result in iron-deprivation in the host. Hemolysis is another related virulence factor observed in some *A. baumannii* isolates that produce hemolysins. These enzymes in turn cause lysis of red blood cells and the subsequent release of the iron-containing hemes to the surroundings. *A. baumannii* is also known to produce proteolytic enzymes that exert exoprotease activity and add to the virulence of this organism. Additionally, though *A. baumannii* has been long thought of as a non-motile, several clinical isolates have shown surface motility. All these factors could act together in the production of virulent isolates that are capable to attain relatively high mortality rates. Nevertheless, several studies showed that the phenotypic expression of these factors could vary greatly between different isolates due to reasons that are not yet fully understood.

A relationship seems to exist between virulence and antimicrobial resistance in *A. baumannii*. A few studies reported the differential expression of the various virulence factors produced by *A. baumannii* and have linked it to MDR profiles. Moreover, genes encoding virulence determinants and those encoding resistance determinants have been found to co-exist on mobile genetic elements. The complex interaction between virulence and resistance has not

been extensively investigated in *A. baumannii.* Nevertheless, several molecules are being investigated for their anti-virulence effect in an attempt to use them in the clinical setting in conjunction with antimicrobial therapy.

In this Doctoral Thesis, clinical *A. baumannii* isolates obtained from Spain and Lebanon, two Mediterranean countries with notoriously high rates of carbapenem resistance, are phenotypically and genotypically characterized. The characterization is in terms of antimicrobial susceptibility, clonality, virulence determinants, and effect on cell growth. Moreover, molecular investigation was performed for two sets of isolates that acquired colistin resistance during therapy in order to determine the cause of this resistance. Finally, the rates of biofilm formation for a subset of isolates was performed in order to determine whether or not a link between antimicrobial susceptibility and biofilm formation patterns exist. Our aim is to provide clinicians and infection control specialist with epidemiological tools and insight on the complex that mechanisms that govern resistance and virulence in *A. baumannii* that could result in improved clinical success and more targeted infection control protocols. If properly implemented, such information could be key for safeguarding antimicrobial agents for future use, limiting the spread of MDR clones, and better understanding the interplay between the different factors that make *A. baumannii* such a successful nosocomial pathogen.

To that end, 59 clinical *A. baumannii* isolates were collected from Hospital Universitario – La Paz (HU-LP) in Madrid, Spain and 90 isolates from Saint Georges Hospital – University Medical Center (SGH-UMC) in Beirut, Lebanon. Identification of the isolates was performed by biochemical testing using 20NE API strips, amplification of the *bla*$_{\text{OXA-51-like}}$ gene by PCR, and where applicable, by Matrix Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). Antibiotic Susceptibility Testing (AST) was then performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Clonality analysis was performed by tri-locus sequence typing and, when possible, by Pulsed-Field Gel Electrophoresis (PFGE). PCRs for the detection of *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24-like}}$, *bla*$_{\text{OXA-58-like}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM}}$, and *bla*$_{\text{KPC}}$ were also performed. Biofilm formation in polystyrene tubes after staining with 1% crystal violet was then determined. Hemolysis by growth on blood agar plates for 6 consecutive days and siderophore production using the CAS liquid assay were also determined. Also, proteolytic activity using the azoalbumin assay and surface motility through the inoculation of the surface of 0.3% Luria Bertani (LB)-Agar were performed. Doubling times through growth in LB broth were then determined for selected isolates with

varying profiles. Statistical analysis was performed in an attempt to determine associations between the different factors that were determined. Additionally, sequencing of the *pmr*CAB operon and whole-genome sequencing for two sets of isolates that developed resistance to colistin during therapy were performed in order to determine the cause of this resistance. Finally, the rates and patterns of biofilm formation were investigated for a subset of isolates with different AST profiles in order to determine whether or not a relationship between these factors exist. This was performed by counting viable cells grown on steel coupons and Confocal Laser Scanning Microscopy (CLSM).

In this study, a very high rate of carbapenem resistance was encountered in both HU-LP and SGH-UMC. These rates were, respectively, 84.75% and 90%. Both sets of isolates also showed high rates of cross-resistance to other antimicrobial agents that reached 80% for the Spanish isolates and 85.6% for the Lebanese set. Two isolates from the Spanish set of isolates were resistant to colistin whereas only one isolate was colistin resistant among the Lebanese set. This low resistance rate provides a valuable alternative for the treatment of infections caused by CRAB isolates, when no other options are available. IC II was the most prevalent clone in both hospitals where it represented 71.19% of the isolates from HU-LP and 88.9% of those from SGH-UMC. ICs I and III where also detected in HU-LP where they represented 8.47% and 6.78% of the isolates, respectively. 13.56% of the Spanish set of isolates did not pertain to any IC and one isolate pertained to tri-locus PCR group 14. Among the Lebanese set, 6.7% of the isolates pertained to tri-locus PCR group 4, 2.2% to group 14, 1.1% to group 10, and one isolate did not pertain to any defined clone. PFGE analysis showed the presence of 7 distinct pulsotypes among the Spanish set of isolates and 5 sporadic isolates that did not pertain to any cluster.

OXA-24-like was predominant among the HU-LP isolates at 62.71% whereas OXA-23-like and OXA-58-like were detected in 1.86% and 13.56% of the isolates, respectively. On the other hand, 93.8% of the CRAB isolates were positive for OXA-23-like while OXA-24-like was only detected in two isolates and OXA-58-like was not detected. These results demonstrate the extremely high rates of carbapenem resistance among *A. baumannii* isolates in both these hospitals. This warrants immediate intervention through the application of adequate infection control protocols and antibiotic stewardship programs in order to safeguard these valuable antimicrobial agents for future use. Moreover, these results show the predominance of IC II in two geographically distinct regions, highlighting the ability of this

clone to disseminate across vast distances. Additionally, these results show the continued high prevalence of OXA-24-like in Spain, where it was first discovered, as opposed to the high prevalence of OXA-23-like in the Lebanese hospital.

For the most part, the virulence profiles obtained showed great variation between the isolates. Nevertheless, a few statistical associations were made. Among the Spanish set of isolates, isolates pertaining to IC II were positively associated with hemolysis, siderophore production, and strong biofilm formation ($p<0.05$). In comparison, isolates pertaining to ICs I and III in this set were associated with reduced virulence. Similarly, harboring *bla*$_{\text{OXA-24-like}}$ was positively associated with hemolysis, siderophore production, and strong biofilm formation ($p<0.05$). These associations could prove to be very useful in the clinical setting were clinicians and infection control specialists in HU-LP could design more aggressive approaches towards isolates of IC II, and especially those harboring *bla*$_{\text{OXA-24-like}}$. Moreover, if the patient condition permits it, clinicians might opt for restraining from using broad spectrum antibiotics for isolates of ICs I and III which contributes to their safeguarding for future use. An additional advantage for the implementation of such approach is that clonality and determination of which carbapenemase is present could be easily performed in three quick multiplex PCR reactions.

Despite the associations encountered among the Spanish set of isolates, no statistical association between clonality, the presence of a particular OXA, and any specific virulence factor was detected among the Lebanese set of isolates. This suggests that the data obtained from the HU-LP isolates could not be projected for other hospitals but rather should be used locally in that hospital. Additionally, this suggests that a preliminary investigation in each hospital is needed before being able to successfully predict what virulence factors an isolate with a specific genotype could express. Moreover, another factor that could have contributed to the lack of detection of these associations is the higher level of homogeneity among the isolates as compared to the Spanish set of isolates. Nevertheless, statistical associations between motility, siderophore production, and biofilm formation have been detected among the Lebanese set of isolates. Further investigating these associations could help better understand the complex interactions between antimicrobial resistance and virulence.

Among the Spanish set of isolates, 69.5% produced siderophores, 84.4% produced strong biofilms, and 54.2% showed hemolysis. These percentages are somewhat similar to those detected among the Lebanese isolates, where 57.8% produced siderophores, 85.6% produced strong biofilms, and 47.8% showed hemolysis. Proteolytic activity for the Spanish

set of isolates (26.6 ± 8.4 U/L) was only slightly higher than that of the Lebanese isolates (17.7 ± 9.5 U/L). Moreover, the doubling times for the selected isolates in both populations, though not associated with any other factor, showed similar ranges (0.324 ± 0.027 to 0.666 ± 0.037 hours for the Spanish isolates and 0.262 ± 0.021 to 0.653 ± 0.049 hours for the Lebanese isolates). However, very few Spanish isolates showed surface motility as opposed to the majority of the Lebanese isolates that showed diffusion patterns on the surface of LB-agar plates, indicating surface motility. This finding further contributes to the "locality" of virulence since a large difference was observed between the two populations in regard to motility, although the majority of both populations pertained to the same IC.

Genomic analysis of the two sets of isolates that developed colistin resistance throughout the course of treatment revealed two mutations in *pmr*CAB that led to colistin resistance. The first mutation was previously reported from various studies and is the P233S mutation in PmrB. This mutation seems to affecting siderophore production and proteolytic activity in the colistin resistant isolate where it was positive for siderophore production and had almost half the proteolytic activity of its sensitive counterpart. The fact that PmrB plays a role in responding to iron changes in the surrounding environment could explain this observation. However, the exact interactions of PmrB with the various factors involved in the expression of these virulence factors require further investigation before determining the exact effect PmrB is having on the cellular metabolism. The second mutation that was detected was a deletion of isoleucine in PmrB in the colistin resistant strain. This mutation does not seem to affect the virulence determinants of the resistant strain, as compared to its sensitive counterparts, and is not previously reported in the literature. Investigating the exact effect this mutation has on PmrB could help better understand the mechanism of colistin resistance in *A. baumannii*. Moreover, one of these two sets of isolates harbored $bla_{\text{GES-5}}$. This gene has not been previously identified in *A. baumannii* and could be an indicator of inter-species exchange of resistance genes.

Investigating the biofilm formation rates and patterns for isolates with different AST profiles showed that three distinct rates of biofilm formation were evident after 5 hours of incubation. The first group had a high rate of attached cells on steel coupons and isolates of this group were susceptible to aminoglycosides. However, not all isolates that were susceptible to aminoglycosides pertained to this group. The second group was consisted of intermediate biofilm formers and harbored most of the tested isolates. All of the isolates that showed MDR

and XDR profiles were part of these two groups, although these groups also contained some carbapenem sensitive isolates. The third group harbored a single carbapenem sensitive isolate and had a slow rate of biofilm formation. Some isolates showed pigmentation while performing the experiments that was not associated with siderophore production. Therefore, analysis by CLSM was performed for two pigment-forming carbapenem resistant and two pigment-non-forming carbapenem sensitive isolates in order to further investigate the composition of their biofilms. Though no direct explanation was encountered for the pigmentation, the carbapenem resistant isolates showed more voluminous biofilms on steel coupons after 24 hours. These results, though preliminary show an association between dense biofilm formations and carbapenem resistance. Performing similar analyses on larger pools of isolates could reveal clinically important associations that could be useful for infection control specialists and clinicians in the development of biofilm eradication protocols based on AST data.

In conclusion, a very high rate of CRAB isolates was detected in both HU-LP and SGH-UMC with a predominance of IC II in both hospitals. Isolates pertaining to seven distinct pulsotypes, in addition to sporadic isolates were able to produce MDR and XDR infections in HU-LP. *bla*$_{\text{OXA-24-like}}$ was predominant among the Spanish set of isolates whereas *bla*$_{\text{OXA-23-like}}$ was predominant among the Lebanese set. These findings suggests an urgent need for intervention in an attempt to decrease the prevalence of CRAB isolates in these hospitals. Though virulence profiles showed high variability in both sets of isolates, IC II and *bla*$_{\text{OXA-24-like}}$ were associated with increased virulence in the Spanish set of isolates but not in the Lebanese set. This suggests that local studies need to be performed before being able to clinically employ associations between virulence, clonality, and antibiotic resistance. Additionally, the previously reported P233S mutation in PmrB was found to convey colistin resistance in one set of isolates. This mutation seems to affect mechanisms of siderophore production and proteolytic activity. A novel ΔIle19 mutation was detected in another colistin resistant isolate that developed resistance during therapy which did not seem to affect the virulence factors tested for. Finally, a preliminary association between dense biofilm formations and carbapenem resistance has been detected in a subset of isolates. Further consolidation of this association using larger pools of isolates could prove useful in the prediction of biofilm formation patterns in *A. baumannii* from AST data. This information, in turn, could be valuable for clinicians and infection control specialists in the development of targeted eradication protocols.

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II. LIST OF ABBREVIATIONS

A. baumannii: *Acinetobacter baumannii*

A/S: Ampicillin/Sulbactam

AAC: Aminoglycoside Acetyltransferases

ABC: ATP-Binding Cassette

ACB: *Acinetobacter calcoaceticus– Acinetobacter baumannii*

Acinetobacter spp.: *Acinetobacter* species

ADC: *Acinetobacter* Derived Cephalosporinase

AFLP: Amplified Fragment Length Polymorphism

AK: Amikacin

AME: Aminoglycoside Modifying Enzymes

ANT: Adenyltransferases

APH: Phosphoryltransferases

ARDRA: Amplified Ribosomal DNA Restriction Analysis

AST: Antibiotic Susceptibility Testing

BES: N-bis-(2-hydroxymetyl)-2-aminoethanesulfonic acid

BHI: Brain Heart Infusion Broth

C1-7: Clusters 1-7

CARB: Carbenicillin-hydrolyzing Beta-lactamase

CAS: Chrome Azurol S

CC: Clonal Complex

CCCP: cyanide 3-chlorophenylhydrazone

CCNU: Critical Care Nursing Unit

CFP: Cefepime

CFU: Colony Forming Unit

CHEF: Contour-Clamped Homogeneous Electric Field

CHEF: Contour-Clamped Homogeneous Electric Field

CIP: Ciprofloxacin

CLSI: Clinical and Laboratory Standards Institute

CLSM: Confocal Laser Scanning Microscopy

COL: Colistin

CRAB: Carbapenem-Resistant Acinetobacter baumannii

CSF: Cerebrospinal Fluid

CT: Cardio/Thoracic

CTX-M: Cefotaximase

CTZ: Ceftazidime

CVC: Central Venous Catheterization

DNA: Deoxyribonucleic Acid

EDTA: Ethylenediaminetetraacetic

EM: Emergency

EPS: Extracellular Polymeric Substances

ESBL: Extended Spectrum Beta-Lactamases

G: Gentamicin

GES: Guiana Extended Spectrum

GS: General Surgery

GTP: Guanosine Triphosphate

HDTMA: HexaDecyl TriMethyl Ammonium bromide

HO: Hematology/Oncology

HU-LP: Hospital Universitario-La Paz

I: Intermediately resistant

IC: International Clone

ICU: Intensive Care Unit

IM: Internal Medicine

IMI: Imipenem

IMP: Imipenem metallo-beta-lactamase

KPC: *Klebsiella pneumoniae* Carbapenemase

LAMP: Loop-Mediated Isothermal Amplification

LB: Luria-Bertani

LEV: Levofloxacin

LPS: Lipopolysaccharides

MALDI-TOF MS: Matrix Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry

MATE: Multi-drug and Toxic compound Extrusion

MBL: Metallo-Beta-Lactamase

MDR: Multi-Drug Resistant

MER: Meropenem

MFS: Major Facilitator Superfamily

MIC: Minimum Inhibitory Concentration

MIC: Minimum Inhibitory Concentrations

MIN: Minocycline

MLST: Multi-Locus Sequence Typing

mRNA: Messenger Ribonucleic Acid

MV: Mechanical Ventilation

NCBI: National Center for Biotechnology Information

NDM: New Delhi Metallo-beta-lactamase

NE: Nephrology

NEO: Neonatal

NGS: Next-Generation Sequencing

OD: Optical Density

OmpA: Outer Membrane Protein A

ORF: Open Reading Frame

OXA: Oxacillinase

P/T: Piperacillin/Tazobactam

PBA: Phenylboronic Acid

PBP: Penicillin Binding Protein

PCR/ESI-MS: Polymerase Chain Reaction / Electrospray Ionization - Mass Spectrometry

PCR: Polymerase Chain Reaction

PCR-RFLP: Polymerase Chain Reaction – Restricted Fragment Length Polymorphism

PDR: Pan-Drug Resistant

PER: Pseudomonas Extended-Resistant

PFGE: Pulsed Field Gel Electrophoresis

PIP: Piperacillin

qPCR: Quantitative Polymerase Chain Reaction

QS: Quorum Sensing

R: Resistant

RAPD: Random Amplified Polymorphic DNA

RE: Restriction Enzyme

RE: Resuscitation

RNA: Ribonucleic Acid

RNA: Ribonucleic Acid

RND: Resistance-Nodulation-cell Division

RPM: Round Per Minute

RPP: Ribosomal Protection Protein

S: Susceptible

SBA: Sheep Blood Agar

SEM: Scanning Electron Microscopy

SGH-MC: Saint Georges Hospital-Medical Center

SHV: Sulfhydryl Variable

SIM: Seoul Imipenemase

SMR: Small Multi-drug Resistance

SNP: Single Nucleotide Polymorphism

SNP: Single Nucleotide Polymorphism

SS: Stainless Steel

ST: Sequence Type

T/S: Trimethoprim/Sulfamethoxazole

TEM: Temoneira

TIC: Ticarcillin

TO: Tobramycin

tRNA: Transfer Ribonucleic Acid

TSA: Tryptone Soy Agar

UC: Urethral Catheterization

UPGMA: Unweighted Pair Group Method with Arithmetic Mean

USA: United Stated of America

UTI: Urinary Tract Infection

VAP: Ventilator Associated Pneumonia

VEB: Vietnamese Extended-spectrum Beta-lactamase

VIM: Verona Integron-encoded Metallo-beta-lactamase

WGS: Whole-Genome Sequencing

XDR: Extensively-Drug Resistant

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Table 1. Clinical data of the patients harboring the isolates; Risk factors that may contribute to biofilm formation, previous antibiotic treatment, primary infection prior to bacteremia, underlying disease, and clinical outcome of the patient.

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V. INTRODUCTION

1. CHARACTERISTICS OF *Acinetobacter baumannii*

1.1. History of the Genus *Acinetobacter*

Acinetobacter is a genus that is currently classified under the family *Moraxellaceae*, which also includes the genera *Moraxella, Psychrobacter*, and other related organisms. This family is within the order *Pseudomonadales* of the class *Gammaproteobacteria* (Rossau *et al.,* 1991). Nevertheless, the genus *Acinetobacter* has undergone extensive taxonomic changes ever since it was first discovered in 1911. This is mainly because members of this genus can adapt to most substrates by using different catabolic pathways and thus resulting in confusion while interpretating biochemical tests (La Scola *et al.,* 2006). The first bacterium belonging to this genus was identified in 1911 by a Dutch microbiologist named Martinus Willem Beijerinck. The isolate was obtained from an environmental sample and was called *Micrococcus calcoaceticus* back then (Beijerinck, 1911). Since then, and up until the 1970s, several species and genera were described that later turned out of to be part of the *Acinetobacter* genus. These species were summarized by Henriksen in 1973 and they include *Achromobacter anitratus, Achromobacter mucosus, Alcaligenes haemolysans, Bacterium anitratum, Diplococcus mucosus, Herellea vaginicola, Micrococcus calcoaceticus, Mima polymorpha, Moraxella lwoffi, Moraxella lwoffi var. glucidolytica,* and *Neisseria winogradskyi* (Henriksen, 1973). Many common characteristics were found among those bacteria until, in 1968, it became widely accepted to group them under the current genus name, *Acinetobacter*, after an extensive review of their phenotypic characteristics (Baumann *et al.*, 1968). This, in turn, led to the official acknowledgement of this genus and its separation from the genus *Achromobacter* by the Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria in 1971 (Lessel, 1971). Interestingly, around that time, the first clinical isolate pertaining to *Acinetobacter* was isolated from the Intensive Care Unit (ICU) in 1969 (Stirland *et al.,* 1969). Subsequently, this genus was listed in Bergey's Manual of Systematic Bacteriology with only one species described (Lautrop, 1974) and the "Approved List of Bacterial Names" with the description of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii* (Skerman, 1980).

In 1986, DNA-DNA hybridization studies resulted in the description of 12 species within the genus *Acinetobacter* that had more than 70% DNA-DNA relatedness. These species included *Acinetobacter baumannii, Acinetobacter calcoaceticus, Acinetobacter haemolyticus, Acinetobacter johnsonii, Acinetobacter junii,* and *Acinetobacter lwoffii* (Bouvet *et al.*, 1986). Nevertheless, not all of the discovered species were given names at the time. In subsequent

years, a total of 31 genomic species have been identified, 17 of which were given species names. The rest were referred to as "Genomic Species" followed by a number that could vary, depending on the nomenclature scheme followed (Bouvet and Jeanjean, 1989; Tjernberg and Ursing, 1989; Nishimura *et al.,* 1988, Nemec *et al.,* 2001; Nemec *et al.,* 2003; Carr *et al.,* 2003). In 2012, due to the increase in the amount and accuracy of identification techniques, the number of genomic species identified rose to 36 and a total of 27 species were given a valid name (Peleg *et al.,* 2012). Nowadays, the identification of new species pertaining to the genus *Acinetobacter* is still occurring in addition to giving species names to the previously described genomic species after exhaustive phenotypic and genotypic characterization (Nemec *et al.,* 2015; Nemec *et al.,* 2016). Currently, 51 species within this genus have been identified and given valid or provisional names (Al Atrouni *et al.,* 2016a).

The early studies using DNA-DNA hybridization also showed that within the genus *Acinetobacter*, the species *Acinetobacter calcoaceticus, Acinetobacter baumannii*, Genomic Species 3 and Genomic Species 13TU shared a high degree of similarity among each other as compared to the others (Tjernberg and Ursing, 1989; Bouvet and Grimont, 1986). These four species later became known as the *Acinetobacter calcoaceticus– Acinetobacter baumannii* (ACB) complex, a term first coined by Gerner-Smidt *et al.,* (1991). More recent studies have further confirmed the relatedness of these species using advanced molecular techniques, such as comparison of the sequences of housekeeping genes (Périchon *et al.,* 2014) and whole-genome analysis (Touchon *et al.,* 2014). Moreover, using similar techniques, Genomic Species 3 has been identified as *Acinetobacter pittii* whereas Genomic Species 13TU was identified as *Acinetobacter nosocomialis* (Nemec *et al.,* 2011). In addition to the aforementioned species that make up the ACB complex, two additional Genomic Species were identified as being part of this complex. These Genomic Species were called "Close to 13TU" and "Between 1 and 3" due to their similarities to the respective genomic species. A recent study has further characterized Genomic Species "Close to 13TU" and called it *Acinetobacter seifertii* (Nemec *et al.,* 2015). Members of the ACB complex could not be easily distinguished one from another using routine biochemical tests. As a result, throughout different times in history, infections caused by members of this complex were referred to as being caused by *Acinetobacter calcoaceticus, Acinetobacter baumannii*, or the ACB complex. Nevertheless, with the advancement of identification techniques, *Acinetobacter baumannii* was identified as the member of this complex most frequently isolated from human infections (Peleg *et al.,* 2008).

Figure 1 shows a visual representation of the timeline of the complicated history of the *Acinetobacter* genus.

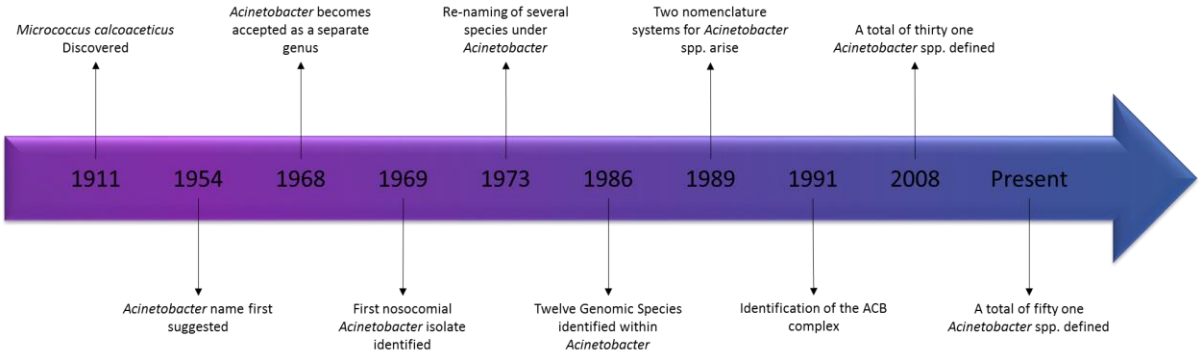


Figure 1. Timeline of the history of the genus *Acinetobacter*.

1.2. General Characteristics of *Acinetobacter* spp.

The word *Acinetobacter* was first proposed by Brisou and Prevot (1954) from the Greek word "Akinetos" which literally translates to "non-motile". This name was suggested in order to separate the non-motile species from the motile ones within the genus *Achromobacter* and grouping the non-motile species under the new genus, *Acinetobacter.* This was because members of this genus failed to show motility by traditional motility testing (Brisou and Prevot 1954) and were considered as non-motile organisms for a long time. Nevertheless, more recent studies have shown that some strains within the *Acinetobacter* genus are able to display surface, or twitching, motility (Antunes *et al.*, 2011). The motility of *Acinetobacter* spp. will be discussed in later sections.

Acinetobacter spp. are easily grown on common solid and liquid growth media. On solid agars, they have a similar appearance to *Enterobacteriaceae* where they show smooth, grayish-white colonies that could sometimes be mucoid and have a diameter of 1.5 to 3 mm after an overnight incubation (Peleg *et al.,* 2008). Figure 2 shows the shape of *Acinetobacter baumannii* colonies after growth on Blood Agars. Most species in this genus grow well in temperatures ranging from 25°C to 45°C, with optimal growth at 37°C for species implicated with human infections. Environmental species, however, seem to prefer temperatures that are below 30°C (Antunes *et al.*, 2011). Biochemically, most members of the *Acinetobacter* genus can produce acid from glucose, galactose, mannose, rhamnose, lactose, and xylose. They are also positive for the Simon's citrate test but are not able to produce acid from mannitol and

sucrose. Moreover, these species are negative for the indole, nitrate, esculin hydrolysis, production of H_2S, and Voges-Proskauer tests (Constantiniu *et al.*, 2004). *Acinetobacter* spp. have also been found to be able to degrade xenobiotic compounds such as alkanes, herbicides, and even some pharmaceutical compounds (Tenover, 2006; Antunes *et al.*, 2014).

Members of the genus *Acinetobacter* share several characteristics among one another. They appear by Gram staining as Gram-negative coccobacilli that could be in diploid formation or chains of variable length. However, they are difficult to de-stain and therefore could be mistakenly observed as Gram-negative or Gram-positive cocci (Peleg *et al.*, 2008). These organisms are also strictly aerobic, non-fastidious, non-fermenting, catalase-positive, oxidase-negative, and have a G+C content that varies from 39% to 47% (Rossau *et al.*, 1991).

Figure 2. *Acinetobacter baumannii* colonies grown on Blood Agar (adapted from image obtained from http://www.slideshare.net/alfredobambang/acinetobacter-28595602).

1.3. Identification of *Acinetobacter* spp.

Studies have shown that it is very difficult to differentiate members of the *Acinetobacter* genus one from another based solely on simple routine biochemical tests (La Scola *et al.*, 2006; Fournier and Richet, 2006). Nevertheless, a wide array of more advanced tests have been developed for the identification of *Acinetobacter baumannii*, the main pathogen of this genus (Peleg *et al.*, 2008). The DNA-DNA hybridization scheme first proposed by Bouvet and Grimont (1986) remains a valid identification method for the detection of several *Acinetobacter* species. In subsequent years, several additional tests were developed and successfully applied

for the identification of *Acinetobacter* at the species level. One such test is Amplified Ribosomal DNA Restriction Analysis (ARDRA). This test relies on the amplification by Polymerase Chain Reaction (PCR) of the bacterium's 16S ribosomal DNA and its subsequent digestion by four different Restriction Enzymes (REs). The digested DNA would then be electrophoresed on agarose gels and the pattern generated by REs would be visualized and compared to previously identified patterns, allowing for the identification of the species (Vaneechoutte *et al.,* 1995). This test has proven to be reliable in the identification of several *Acinetobacter* species, including *Acinetobacter baumannii* (Kong *et al.,* 2011).

A technique similar to ARDRA that is used for identification of *Acinetobacter* species is PCR-Restricted Fragment Length Polymorphism (PCR-RFLP). In this technique, the 16S ribosomal RNA gene is amplified by PCR and then digested by the *Hae*III RE. The fragments are then run on agarose gels, visualized, and compared to known patterns of *Acinetobacter* spp. (Lu *et al.,* 2000). This technique successfully detects *Acinetobacter baumannii* isolates, even those missed by automated detection through growth in blood cultures (Rohit *et al.,* 2016). Amplified Fragment Length Polymorphism (AFLP) is another useful technique for the identification of *Acinetobacter* spp. that produces high resolution DNA fingerprints. In this technique, the entire bacterial genome is digested with two REs (*Eco*RI and *Mse*I), resulting in a large number of fragments that would vary, depending on the amount and locations of Single Nucleotide Polymorphisms (SNPs). Adapters would then be ligated to the fragments and selective amplification would occur (based on the adapters for one of the REs). The amplified fragments would then be labeled and electrophoresed on agarose gel in order to produce DNA fingerprints and identify the species (Janssen *et al.*, 1997).

Ribotyping, a technique somewhat similar to AFLP has also been successfully used in the identification of *Acinetobacter* spp. (Gerner-Smidt, 1992). This technique relies on the endonuclease digestion of whole genomes by REs. The fragments would then be separated by electrophoresis, transferred by Southern Blot, and hybridized with radiolabeled ribosomal operon probes. The visualized bands would correspond to the multiplicity of ribosomal RNA in each sample and would generate a unique pattern corresponding to a specific species (Bouchet *et al.*, 2008). Another technique that is used in the identification of *Acinetobacter* spp. is restriction analysis of the 16S-23S ribosomal RNA spacer sequence. This technique is very similar to ribotyping with the difference being that the digestion by the RE *Eco*RI would

be carried out on PCR products of the 16S-23S ribosomal RNA spacer region, using the respective probes (Dolzani *et al.,* 1995).

All the previously described techniques are accurate in identifying members of the genus *Acinetobacter* at the species level. However, they are highly laborious and time consuming. This makes them, despite being still used in reference laboratories, not suitable for routine diagnostic labs (Peleg *et al.,* 2008). With the advancement of technology and interest in members of this genus, especially in the pathogenic *Acinetobacter baumannii*, numerous less laborious techniques have been developed. One such technique is the genetic sequencing of the 16S-23S ribosomal RNA gene spacer region. This technique relies on the amplification of the spacer region between the aforementioned genes and its subsequent sequencing by automated sequencers. The obtained sequences would then be compared to known sequences in the National Center for Biotechnology Information (NCBI) database and the species would be identified (Chang *et al.,* 2005). An offshoot from this technique that is widely used nowadays is the sequencing of the 16S ribosomal DNA gene and identification based on the comparison with published sequences in the NCBI database. Identification by this method is becoming increasingly used due to the increase in the available sequences in the NCBI database. This technique is fast and simple and has shown very good accuracy in identifying bacterial species (Loon *et al.,* 2016). Moreover, recent advances in sequencing techniques and next-generation sequencing led to labeling whole-genome sequencing as the definitive identification, epidemiological, and genomic investigative technique (Karah *et al.,* 2012). This technique is, however, expensive and requires access to next-generation sequencing systems.

Another fast technique that could identify the species in around 4 hours is PCR Electrospray Ionization Mass Spectrometry (PCR/ESI-MS). In this technique, seven housekeeping genes are amplified by PCR and then ESI-MS is performed in order to determine the number of adenines, thymines, guanines, and cysteines in each sample. This would, in turn, reflect the identity of each species (Ecker *et al.,* 2006). Mass spectrophotometry is also used for the identification of bacterial isolates through Matrix Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). In this technique, bacterial isolates are grown in liquid cultures and then concentrated by centrifugation. The samples are then properly processed and placed in the mass spectrophotometer. The spectra obtained would be compared to reference spectra in databases that are normally provided by manufacturers, and the identity of the isolate would be determined (Romero-Gómez *et al.,* 2012). These techniques, though

fast, require the presence of a mass spectrophotometer that is limited in availability in most laboratories.

In order to facilitate the identification of bacterial species in clinical laboratories, several manual and semi-automated commercial tools have been developed over time. These tools, that include the API 20NE™ (BioMérieux) manual test, and the semi-automated Vitek 2™ (BioMérieux), Phoenix™ (Becton Dickinson), and MicroScan WalkAway™ (Beckman Coulter) systems, have been implicated in the identification of *Acinetobacter* spp. in clinical laboratories (Bernards *et al.*, 1995; Bernards *et al.,* 1996; Horrevorts *et al.*, 1995). They rely on a wide array of phenotypic tests and provide fast and reproducible methods for the identification of a wide range of bacterial species. However, they largely depend on the databases provided by the manufacturers for identification. Although these databases are updated on a regular basis, the identification does not cover all possible species and none of these tools is specifically tailored for the identification of the complex species within the genus *Acinetobacter* (Karlowsky and Richter, 2015). Nevertheless, the ACB complex could be differentiated from the rest of the *Acinetobacter* spp. using these commercial tools (Peleg *et al.*, 2008).

A major breakthrough in the identification of *Acinetobacter baumannii*, and its separation from other species within this genus, was the discovery of the intrinsic *bla*$_{\text{OXA-51-like}}$ gene (Héritier *et al.*, 2005a; Turton *et al.*, 2006a). This gene was shown to only convey carbapenem resistance when the insertion sequence IS*Aba1* was adjacent to it (Turton *et al.*, 2006b). However, the presence of this gene in the vast majority of *Acinetobacter baumannii* isolates led to the wide acceptance of its amplification as indicitave of *A. baumannii* and its use as a rapid and accurate method for identifying this species (Tomaschek *et al.*, 2016; Turton *et al.*, 2006a). Finally, a combination of any number of the aforementioned techniques, when possible, could capitalize on the strengths of each one of them and reach a fast, cost-effective, and reliable method for the identification of *Acinetobacter baumannii*. One such combination could be the use of API 20 NE for the identification of the ACB complex, followed by PCR for the detection of *bla*$_{\text{OXA-51-like}}$ for the identification of *Acinetobacter baumannii*. The choice of which technique(s) to use largely depends on the availability of funds, speed required, and the purpose of the identification.

1.4. Natural Habitat of *Acinetobacter* spp.

Acinetobacter spp. could be isolated after enrichment from a wide range of soil and surface water specimens. Additionally, members of this genus that are generally less implicated with human disease have been isolated from vegetables with varying rates that could reach up to 51% (Berlau *et al.,* 1999a; Houang *et al.,* 2001). Moreover, *Acinetobacter* spp. in general, and occasionally *Acinetobacter baumannii* in particular, have been isolated from animal specimens (Francey *et al.,* 2000; Vaneechoutte *et al.,* 2000) and body lice specimens obtained from homeless persons (La Scola and Raoult, 2004). The presence of these organisms in these different habitats led to the consideration of this genus as ubiquitous in nature (Baumann, 1968; Fournier and Richet, 2006). Nevertheless, a systematic study that evaluates the prevalence of the main human pathogen of this genus, *Acinetobacter baumannii*, was not performed in order to confirm the ubiquitous distribution of this species in nature (Peleg *et al.,* 2008).

Acinetobacter spp. have been identified as part of the normal micrbiota among healthy individuals in studies dating back to 1997. The most prevalent species known to colonize the skin and mucous membranes of healthy humans are *Acinetobacter lwoffii*, *Acinetobacter johnsonii, Acinetobacter junii, Acinetobacter pittii, Acinetobacter radioresistens, Acinetobacter nosocomialis, Acinetobacter variabilis*, and, to a much lesser extent, *Acinetobacter baumannii* (Seifert *et al.,* 1997; Berlau *et al.,* 1999b; Chu *et al.,* 1999). Moreover, seasonal variation in skin colonization among medical staff by *Acinetobacter* spp. has been demonstrated. The prevalence of these species was shown to be higher in summer as compared to winter, mirroring the seasonal variation observed for *Acinetobacter baumannii* infections (Chu *et al.,* 1999; McDonald *et al.,* 1999). *Acinetobacter johnsonii, Acinetobacter guillouiae* (formerly genomic species 11), and rarely *Acinetobacter baumannii*, were also reported from fecal samples of healthy humans indicating their presence in the normal human gut flora (Dijkshoorn *et al.,* 2005; Nemec *et al.,* 2010).

2. PATHOGENESIS AND VIRULENCE OF *Acinetobacter baumannii*

Pathogenicity is defined as the ability of a bacterium to cause disease, whereas virulence is the degree to which a bacterium is pathogenic (Beceirt *et al.,* 2013). In humans, although several members of the genus *Acinetobacter* have been implicated with a certain degree of pathogenicity (Seifert *et al.,* 1993; Seifert *et al.,* 1994), *Acinetobacter baumannii* remains the key pathogenic organism of this genus (Gonzalez-Villoria and Valverde-Garduno

2016). Although community acquired pneumonia caused by *Acinetobacter baumannii* has been documented, especially among patients with a history of alcohol abuse (Kroeger *et al.,* 2007), the vast majority of infections caused by this organism are hospital acquired infections (Peleg *et al.,* 2008). Moreover, *Acinetobacter baumannii* is considered as one of the ESKAPE pathogens, which are regarded as the leading cause of nosocomial infections all over the world. ESKAPE is an acronym that is short for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp. (Santajit and Indrawattana, 2016).

2.1. Virulence of *Acinetobacter baumannii*

Virulence could be assessed by testing for several factors that affect the host with different consequences (Antunes *et al.,* 2011). Part of what makes *Acinetobacter baumannii* successful as a nosocomial pathogen is its ability to express several virulence factors. Although the pathogenicity of this organism is not yet fully understood, numerous virulence factors have been attributed to it (Peleg *et al.,* 2012). In the following sections, the virulence factors that have been identified in *A. baumannii* will be presented.

2.1.1. Formation of Biofilms

Biofilms are a mesh constituted of pili and exopolysaccharides wherein bacterial populations can grow and exchange genetic material (Tomaras *et al.*, 2003; Doi *et al.*, 2015). They protect the bacterial cells they harbor against various hazards, such as antimicrobial agents and macrophage attacks (Ciofu and Tolker-Nielsen, 2011), in addition to stress conditions such as desiccation and disinfection (Lebeaux *et al.*, 2014). They make bacteria difficult to eradicate and contribute to the persistence of *A. baumannii* for prolonged periods of time in hospitals (Loehfelm *et al.*, 2008). This protective barrier creates a microenvironment where bacterial cells can exist in a wide range of physiological states and be protected from eradication (Stewart and Franklin, 2008; Longo *et al.,* 2014). Additionally, biofilms create an environment with a high density of microorganisms and thus facilitate horizontal transmission of genetic material that could contain genes of virulence and/or resistance (Aminov, 2011). Moreover, *A. baumannii* living in biofilms have been found to be able to communicate one with another through quorum sensing (Niu *et al.,* 2008). Through this communication, *A. baumannii* is able to induce the formation of the slow dividing persister cells in deep layers that are tolerant to antimicrobial agents (Bhargava *et al.,* 2014). These persister cells could

serve as a reservoir for the renewal of the bacterial population and are shielded from the action of the antibiotics by the biofilm itself. The freshly dividing cells could also pick up genetic material from their surroundings, possibly leading to the acquisition of resistance to antimicrobial agents. To add to all of this, bacterial cells on the topmost layers of a biofilm were shown to be able to detach from it (Derlon *et al.*, 2009). This would allow bacterial cells to spread and form new colonies, in addition to the reservoir that is formed within the biofilm that could serve as a source of repeated hospital outbreaks.

Expression of biofilms by *A. baumannii* clinical isolates, accompanied with changes in genetic expression, has been reported by several studies (Rodriguez-Baño *et al.*, 2008, Lee *et al.*, 2008, Longo *et al.*, 2014). The characterization of the complex mechanisms governing biofilm formation and its regulation is still ongoing (Liou *et al.*, 2014). Nevertheless, several genes have been associated with biofilm formation in *A. baumannii*. *CsuE, bap, OmpA*, and *pga*ABCD are some of these genes (Gaddy *et al.*, 2009; Tourton *et al.*, 2007; Loehfelm *et al.*, 2008; Choi *et al.*, 2009). Nevertheless, the absence of some of those genes has been reported among biofilm forming *A. baumannii* isolates indicating the presence of alternative compensatory mechanisms for biofilm formation (Gaddy *et al.*, 2009; De Breij *et al.*, 2009). Finally, a positive relationship between biofilm formation and antibiotic resistance has been described in several studies (Olsen, 2015; Gurung *et al.*, 2013; Ciofu and Tolker-Nielsen, 2011). Previous antibiotic use was also determined as a driving factor for the development of biofilms (Rodriguez-Baño *et al.*, 2008; He *et al*., 2015). Moreover, a study by Badmasti *et al.* (2015) showed that both antibiotic resistance and biofilm formation depend on genetic diversity and expression. Furthermore, they postulated that understanding the connections between these two phenomena has a threading potential for understanding the mechanisms of persistence of *A. baumannii* in the hospitals and its colonization of various hospital equipment, such as catheters and mechanical ventilators.

2.1.2. Proteolytic Activity and Siderophore Production

A. baumannii has the ability to produce proteases and iron-uptake molecules that negatively affect the patients' cells and metabolism. Production of phospholipase C and phospholipase D has been reported in this organism (Cerqueira and Peleg, 2011). While phospholipase C was found to have a modest cytotoxic effect on epithelial cells (Camarena *et al.*, 2010), Phospholipase D was found to be important for the bacterium's survival and growth

in human serum, in addition to invasion of epithelial (Jacobs *et al.,* 2010). Moreover, analysis of full genomes of *A. baumannii* isolates led to the prediction of the presence of exoprotease-related genes in certain Open Reading Frames (ORFs). This, in turn, led to the phenotypic detection of proteolytic activity among clinical *A. baumannii* isolates (Antunes *et al.,* 2011). The proteolytic and phospholipase enzymes add to the virulence of *A. baumannii* and demonstrate this species' ability to cause harm to the human host.

A. baumannii isolates are also able to produce siderophores, especially in iron-deficient media (Mihara *et al.,* 2004). Several genes for the production of siderophores were identified in this organism and they have been found to be upregulated upon iron depletion (Eijkelkamp *et al.,* 2011a). This ability is especially important in the host environment where iron is a very limited micronutrient that is usually bound up within hemes. Production of siderophores puts the bacterium in competition with the host for iron and could result in the deprivation of the patient from this crucial micronutrient (Wandersman and Delepelaire, 2004). Moreover, a link between pigmentation and virulence and/or antibiotic resistance was reported after an outbreak caused by a pigmented *A. baumannii* strain in Argentina (Vilacoba *et al.,* 2013). Since pigmentation was found to be related to the production of siderophores (Meyer *et al.,* 2000), one can assume that strong siderophore production was exhibited by these strains. This also further justifies the consideration of siderophore production as a virulence factor. In addition, iron has been described as being essential for the formation of biofilms, another important virulence factor in *A. baumannii* (Gentile *et al.,* 2014), indicating a possible interplay between production of siderophores and biofilm formation.

2.1.3. Hemolysis and Surface Motility

Hemolysis and motility are two factors that were previously thought to be non-existent in *A. baumannii*. Hemolysis among *Acinetobacter* spp. has been thought to be limited to *Acinetobacter haemolyticus* (Peleg *et al.,* 2008). Nevertheless, it has been demonstrated that some *A. baumannii* clinical isolates could actually show some *in-vitro* hemolytic activity on Sheep Blood Agar (SBA) plates after prolonged incubation times (Antunes *et al.,* 2011). Taken together, production of siderophores and hemolytic activity could result in the fast depletion of iron available to the host and the eventual overwhelming of the patient. Motility is another virulence factor that has long been thought to be absent from *Acinetobacter* spp. (Brisou and Prevot, 1954). Nevertheless, studies have shown that *A. baumannii* has the genes coding for

the Type IV pili secretion system (Peleg *et al.*, 2008). These pili are known to form a surface-associated movement through their extension and retraction. This type of bacterial motility is designated as twitching motility (O' Toole and Kolter, 1998). Recent studies have shown that, although not applicable to all isolates, several *A. baumannii* strains were able to phenotypically express this type of motility on the surface of agar plates (Mussi *et al.*, 2010; Antunes *et al.*, 2011). Moreover, this type of motility was associated with the ability to form biofilms (Mussi *et al.*, 2010).

2.1.4. Other Factors Contributing to Virulence in *Acinetobacter baumannii*

It has been shown that *A. baumannii* is able to bind to epithelial cells, colonize them and induce their apoptosis (Lee *et al.*, 2008). This induction is thought to be mediated by certain outer membrane proteins that are able to bind to the cells' death receptors, which in turn activate the caspase pathway. This pathway leads to the disintegration of the mitochondria and the subsequent release of cytochrome c and apoptosis-inducing factor, leading to cellular apoptosis (Choi *et al.*, 2005). One such outer membrane protein present among *A. baumannii* isolates is Outer Membrane Protein A (OmpA). Although OmpA is a porin that is involved in variety of cellular processes that allow the bacterium to survive and adapt to its environment, its implication in inducing apoptosis makes it an important virulence factor (Turton *et al.*, 2007).

Another virulence factor associated with *A. baumannii* is the production of lipopolysaccharides (LPSs) that aid in resistance to normal human serum (Luke *et al.*, 2010). LPSs are typically is formed of lipid A, an inner core, an outer core, and the O antigen. Lipid A is what anchors the LPS to the bacterium's outer membrane and is the most immunogenic part of LPSs (Moran, 2007). The inner and outer cores are non-repeated oligosaccharides that are proximal to lipid A, and the O antigen is a segment of repeated oligosaccharides of variable lengths (Raetz and Whitfield, 2002). In addition to the protective role LPSs play for the bacterial cell, these molecules, and especially the Lipid A segment, are able to induce overwhelming immune responses in the patient that are often fatal (Moran, 2007). On a similar note, *A. baumannii* was found to be able to produce the K1 capsular polysaccharide. This capsule was found to protect the bacterium against the harsh environment the human body provides, and thus acts as a major virulence factor (Russo *et al.*, 2010).

2.2. Nosocomial Infections Caused by *Acinetobacter baumannii*

A nosocomial infection is defined as an infection that occurs within forty eight hours after hospital admission, three days after discharge, or thirty days after surgery. It is estimated that one in ten patients admitted to a hospital are affected by a nosocomial infection (Inweregbu *et al.,* 2005). Patients in the ICU have been found to be particularly susceptible to nosocomial infections, mostly due to mechanical ventilation, invasive procedures, and their immunocompromised status (Chatterjee *et al.,* 2016).

A. baumannii has been implicated in nosocomial infections with alarming mortality rates from studies dating back to the 1970s (Glew *et al.,* 1977). This still holds true today where this pathogen is causing a wide range of infections, especially among critically ill patients. *A. baumannii* is most commonly implicated with bacteremia, Ventilator Associated Pneumonia (VAP), Urinary Tract Infections (UTIs), meningitis, and wound infections (Peleg *et al.,* 2008). VAP is inflammation in the lungs presenting 48 to 72 hours after endotracheal intubation of the patient. It is characterized by malaise, sputum-producing coughs, shortness of breath, fever, chills, and chest pain. Up to 20% of intubated patients suffer from VAP and this kind of infection is associated with a 2-fold increase in mortality, longer hospital stay, and higher hospital costs. Moreover, VAP is more common among critically ill patients and patients with several co-morbidities (van Diepen *et al.,* 2016). UTIs are infections of the urinary tract and are among the most commonly acquired types of infections in the community. UTIs can affect any of the organs of the urinary tract, including the bladder, kidneys, and urethra. Symptoms of UTIs include frequent urination, burning sensation during urination, and pain around the bladder region. Though these types of infections are generally self-limiting, the infecting agent could invade the urinary tract and cause complications that are usually limited by the administration of antimicrobial agents (Foxman, 2010).

Meningitis is the inflammation of the meninges, the tissue that engulfs the brain and spinal cord, and could also include infecting the area in-between the meningeal layers. Common symptoms indicative of meningitis are variations in body temperature (too high or too low), vomiting, stiff neck, fever, headache, confusion, light sensitivity, and low concentration ability. Meningitis could be life threatening and is associated with several co-morbidities, mainly since it involves organs in the central nervous system (Qian *et al.,* 2016). Wound infections are infection of the skin and soft tissue layers around an open wound. It can

present with a wide variation of features, depending on the infecting agent. *Acinetobacter* spp. are most commonly implicated with colonizing burn wound sites. These infections could also pose a challenge to infection control since they are capable of imbedding into the mattresses of patients and persist in the hospital for prolonged periods of time (Sherertz and Sullivan, 1985).

Bacteremia is defined as the presence of bacteria in the blood, regardless of the source. Clinically significant bacteremia manifests as fever, chills, and/or hypotension. Bacteremia could be transitory where symptoms disappear in under 8 hours, but could also progress to septic shock and disseminated intravascular coagulation which could be deadly. Almost all bacteremia cases caused by *A. baumannii* are nosocomial infections that occur around 20 days after hospitalization and could originate from any of the aforementioned primary infections (Cisneros *et al.,* 1996). Less frequent reports also show that *A. baumannii* is capable of causing endocarditis (Rizos *et al.,* 2007). This infection of the heart's endocardium could lead to the formation of blood clots, their dislocation, and subsequent clotting of arteries. Also few reports indicate that *A. baumannii* could cause and endophthalmitis (Levy *et al.,* 2005), an infection inside the eye that causes severe pain, redness of the eye, and may lead to loss of vision. Both endocarditis and endophthalmitis were mostly linked to previous surgical procedures and/or use of prosthetic heart valves and contact lenses, respectively.

Risk factors associated with infection by *A. baumannii* include long hospitalization times, use of central venous catheters, prior hospitalization, residence in nursing homes, and reduced immunity (Fishbain and Peleg, 2010). Although the discrimination between infection and colonization after the isolation of *A. baumannii* is difficult, the recovery of this organism is believed to be an indicator of severe illness and is associated with relatively high mortality rates (Perez *et al.,* 2007). Mortality rates for patients infected by *A. baumannii* in non-ICU wards range from 8% to 23% whereas mortality rates among infected patients in the ICU range from 10% to 43% (Fishbain and Peleg, 2010; McConnell *et al.,* 2013; Doi *et al.,* 2015). Some studies even demonstrate a mortality rate that is as high as 67.8% among patient with *A. baumannii* bacteremia (Cuang *et al.,* 2011). A retrospective study performed in USA showed that 6.78% of bloodstream isolates from patients undergoing severe sepsis pertained to *A. baumannii* (Zilberberg *et al.,* 2014). A similar study showed that 49.6% out of 131 patients with bacteremia caused by *A. baumannii* died (Shorr *et al.,* 2014). Interestingly, mortality rates caused by *A. baumannii* bacteremia was shown to be higher than those caused by other Gram-negative organisms, including *Klebsiella pneumoniae* (Robenshtok *et al.,* 2006). This shows

that, although the frequency of *A. baumannii* is relatively low as compared to other nosocomial pathogens, the high mortality rates associated with it makes it an opportunistic pathogen that is not be to taken lightly.

3. TREATMENT OPTIONS FOR *Acinetobacter baumannii*

Most available antimicrobial agents were successfully used in the treatment of *Acinetobacter* spp. infections in the 1970s (Bergogne-Bérézin *et al.,* 1996). However, increasing rates of resistance to various antimicrobial agents started being reported in the 1980s up until the early 1990s, when resistance to imipenem was first reported (Doi *et al.,* 2015). Due to the plethora of resistance mechanisms among *A. baumannii*, resistance to several antimicrobial agents could be found in the same isolate. This, in addition to resistance to many antimicrobial agents among other species, led to the classification of isolates as Multi-Drug Resistant (MDR) if they are non-susceptible to at least one agent in three or more antibiotic categories; Extensively-Drug Resistant (XDR) if they are non-susceptible to at least one agent in all but two or fewer antibiotic categories; and Pan-Drug Resistant (PDR) if they were non-susceptible to all antibiotic categories (Magiorakos *et al.,* 2011). The wide dissemination of MDR *A. baumannii* isolates has severely limited the treatment options for this organism nowadays (Zarilli *et al.,* 2013). Treatment of MDR *A. baumannii* infections, as well as other Gram-negative non-fermenters, is considered one of the greatest challenges of contemporary medicine (Mehrad *et al.,* 2015). The mechanisms of resistance to antimicrobial agents in *A. baumannii* will be discussed in later sections. In the following sections, the antimicrobial agents that are used nowadays for the treatment of *A. baumannii* infections are presented. These antimicrobial agents are summarized in Figure 3.

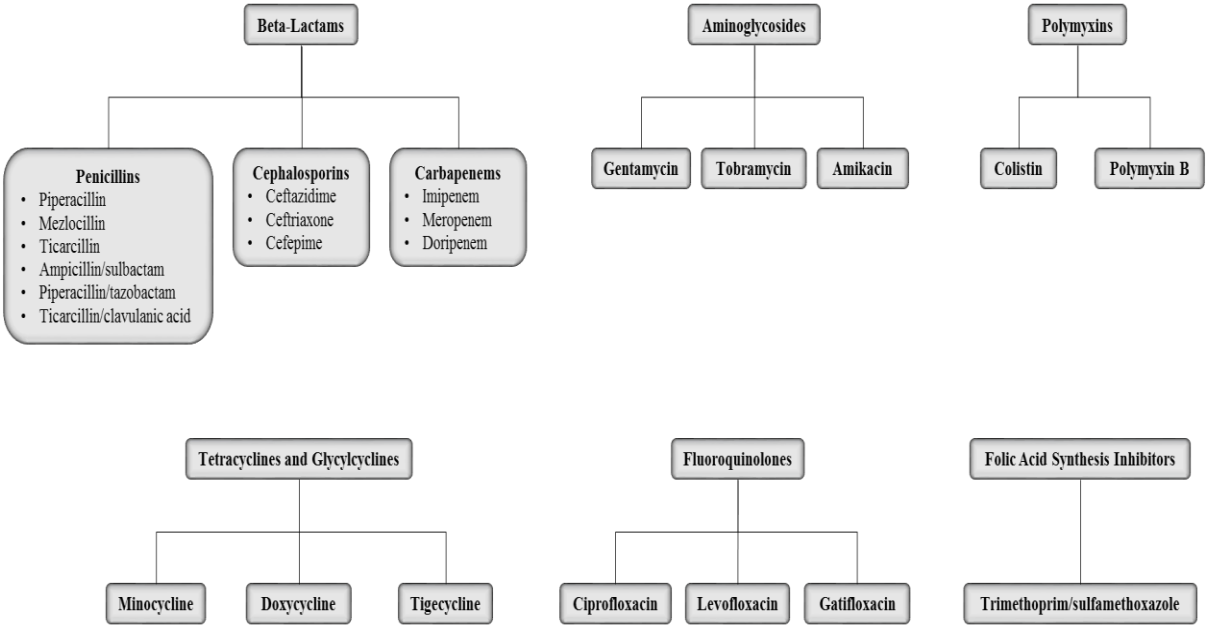


Figure 3. Antimicrobial agents recommended for the treatment of *A. baumannii* infections.

3.1. Beta-Lactams

Beta-lactams are a class of antimicrobial agents that encompass a wide range of bactericidal molecules routinely used in the treatment of bacterial infections (Elander, 2003). These agents are grouped into penicillins, cephalosporins, monobactams, and carbapenems (Dalhoff *et al.*, 2006). All these antimicrobial agents have in common a beta-lactam ring which acts as an analog of d-alanyl-d-alanine. They inhibit the synthesis of the bacterial peptidoglycan layer by binding with varying affinities to DD-transpeptidases, otherwise known as Penicillin Binding Proteins (PBPs). This, in turn, inhibits the cross-linking of the nascent peptidoglycan layer, which disrupts the synthesis of the cell wall (Fisher *et al.*, 2005). Moreover, due to the production of enzymes that hydrolyze beta-lactams (discussed later), several beta-lactamase inhibitors are being combined with beta-lactams. The most common combinations include amoxicillin with clavulanic acid, ampicillin with sulbactam, and piperacillin with tazobactam (Drawz and Bonomo, 2010).

Owing to the numerous natural resistance mechanisms present in *A. baumannii,* a limited number of beta-lactams are recommended for use against this organism. Of the many penicillins available for use in the clinical practice, only piperacillin, mezlocillin, ticarcillin, ampicillin/sulbactam, piperacillin/tazobactam, and ticarcillin/clavulanic acid are recommended for treatment. Moreover, monobactams are not recommended for the treatment of *A. baumannii* infections (CLSI, 2014). Ampicillin/sulbactam has shown promising clinical success against

A. baumannii infections, mainly due to the intrinsic antimicrobial activity of the beta-lactamase inhibitor sulbactam against this organism (Rafailidis *et al.*, 2007). Clinical response rates after use of this agent have been reported to range from 60% to 93% (Oliveira *et al.*, 2008; Wood *et al.*, 2002). However, decreasing rates of susceptibility to this antimicrobial agent are being increasingly reported, leading to its decreasing clinical success (Doi *et al.*, 2015).

Acinetobacter baumannii naturally produces a potent cephalosporinase which renders most cephalosporins obsolete against this bacterium (Hujer *et al.*, 2005). Nevertheless, the third generation ceftazidime and ceftriaxone, in addition to the fourth generation cefepime cephalosporins, retain good activity against *A. baumannii*. A global surveillance study showed that susceptibility to these antimicrobial agents in 2010 was 54.8%, 49.4%, and 65.5%, respectively. However, these rates decreased to 43.3%, 40.4%, and 41.3%, respectively in 2014 (Tärnberg *et al.*, 2016). A similar drop in susceptibility rates to cephalosporins among *A. baumannii* isolates has also been reported in another study (Chen *et al.*, 2015), suggesting that the continued use of these antimicrobial agents is threatened.

Carbapenems are the most potent broad spectrum antimicrobial agents within the beta-lactams. They are reserved for the hospitals and are usually used as last-line therapy and for the treatment of critically ill patients (Breilh *et al.*, 2013). The carbapenems include ertapenem, doripenem, imipenem, and meropenem. Ertapenem in not recommended for use against *A. baumannii* infections due to its limited efficacy against this organism and relatively narrower spectrum of activity (Oliver *et al.*, 2004). Doripenem has shown some *in-vitro* activity against *A. baumannii* however its *in-vivo* efficacy is still in question, making it not highly recommended for use (Drzewiecki *et al.*, 2012). Meropenem has a slightly reduced potency against *A. baumannii* as compared to imipenem (Oliver *et al.*, 2004). Nevertheless, both these antimicrobial agents are the most recommended carbapenems for the treatment of *A. baumannii* infections among critically ill patients (Goncalves-Pereira and Povoa, 2011). Moreover, these antimicrobial agents were shown to have favorable pharmacokinetic/pharmacodynamic properties (American Thoracic Society and Infectious Diseases Society of America, 2005). This led to the consideration of carbapenems as “last line agents” for the treatment of critically ill patients (Nowak and Paluchowska, 2016). However, the extensive use of these carbapenems lead to the inevitable steep rise of resistance against them among *A. baumannii* isolates throughout the past two decades (Chen *et al.*, 2015). A surveillance study across USA showed that 33% of nosocomial *A. baumannii* isolates were resistant to carbapenems (Hidron *et al.*,

2008). Another study from USA showed an increase in CRAB isolates from 1% in 2003 to 58% in 2008, as well as a two-fold increase in *A. baumannii* infections in that time period (Reddy *et al.*, 2015). A study conducted from 2004 to 2006 that spanned 266 centers across the globe showed that resistance to carbapenems was less than 26% in North America and Europe, 30.8% in Asia, 40.4% in the Pacific Rim, and ranged from 39.4% to 76.6% in Latin America (Reinert *et al.*, 2007). Nowadays, global resistance rates against carbapenems is 62.5% (Tärnberg *et al.*, 2016) with some studies reporting rates as high as 85% among MDR *A. baumannii* isolates (Perez *et al.*, 2010). In addition, by the time *A. baumannii* isolates acquire resistance to carbapenems, they often acquire resistance to several other antimicrobial agents as well (Doi *et al.*, 2015). Moreover, carbapenem resistance among *A. baumannii* isolates is significantly correlated with increased mortality rates (Nowak and Paluchowska, 2016). All of this has forced clinicians and researchers alike to search for alternative treatment approaches.

3.2. Aminoglycosides

Aminoglycosides are bactericidal concentration-dependent antimicrobial agents that act through the inhibition of protein synthesis (Gilbert, 1995). They are highly polar molecules that cross the bacterial outer membrane through a self-promoted uptake process after disruption of the LPS bridges. They then bind to the 30S subunit of the cytosolic, membrane-bound ribosomes. This, in turn, leads to the truncation of proteins being synthesized and/or mistranslation of mRNA, causing disruption of the cells' metabolic processes (Mingeot-Leclercq *et al.*, 1999). Although aminoglycosides have a wide spectrum of activity, their nephrotoxicity and ototoxicity resulted in the reluctance of clinicians in using them. Nevertheless, the increase in resistance against other antimicrobial agents and a revision of dosing regimens that attenuate toxicity have re-kindled their clinical use (Durante-Mangoni *et al.*, 2009).

Aminoglycosides that are recommended for use in the treatment of *A. baumannii* infections include gentamycin, tobramycin, and amikacin (CLSI, 2014). In general, *A. baumannii* seems to be more susceptible to tobramycin and amikacin than to gentamycin (Fishbain and Peleg, 2010). In addition to intravenous administration, the use of aminoglycosides in aerosol form has been approved for cystic fibrosis patients and has shown promising results among VAP patients (Hallal *et al.*, 2007). However, a great discrepancy in the detection of susceptibility to these antimicrobial agents has been reported, which depends

on the method used for the determination of the Minimum Inhibitory Concentration (MIC). This could result in falsely reporting isolates as being susceptible with often deadly consequences (Akers *et al.,* 2010). Despite the many concerns regarding the use of aminoglycosides, they retain their clinical usefulness due to the rise in resistance rates to other antimicrobial agents and their good activity against *A. baumannii* (Gounden *et al.,* 2009).

3.3. Polymyxins

Colistin is an amphipathic lipopeptide that belongs to the polymyxins class of antimicrobial agents. This molecule is cationic and acts upon Gram-negative bacteria through the interaction with lipid A and the subsequent disruption of the negatively charged outer membrane (Vaara *et al.,* 1985). Disruption of the stability of the membrane through the displacement of the divalent cations bridging the LPS molecules by colistin would in turn promote its self-uptake into the cell (Arroyo *et al.,* 2011). This would activate the second reported mechanism of antibacterial activity which acts through the induction of rapid killing of bacterial cells through the activation of a hydroxyl radical death pathway (Sampson *et al.,* 2012). Colistin is administered intravenously and has a rapid bactericidal effect that is concentration-dependent (Bergen *et al.,* 2012). Moreover, this antimicrobial agent is administered in an inactive form and it takes two to three days for its conversion in the plasma to its active form. In order to circumvent this, colistin is usually administered with a high loading dose and then treatment continues with maintenance doses (Mohamed *et al.,* 2012). Varying degrees of nephrotoxicity have been associated with colistin, which limits its administration at high doses (Bergen *et al.,* 2012). However, despite its nephrotoxic effects, clinicians are opting for its use against imipenem resistant MDR *A. baumannii* infections, mainly because it attains cure rates similar to carbapenems (Cai *et al.,* 2012). Nevertheless, *A. baumannii* isolates have been reported to acquire resistance to colistin during treatment and subsequently cause devastating outbreaks (Valencia *et al.,* 2009; Li *et al.,* 2006).

Recently, attempts to avoid nephrotoxicity caused by colistin have been made in VAP infections where this agent is delivered in aerosol form directly to the infection site. This method of delivery is usually combined with intravenous administration of colistin and has been associated with good bacterial clearance rates (Yapa *et al.,* 2014). However, its definitive effect on positive clinical outcomes is not yet fully established (Doi *et al.,* 2015). Additionally, Colistin has poor penetration into the Cerebrospinal Fluid (CSF) but ventriculitis and

meningitis could be cured by intrathecal or intraventricular administration (Karaiskos *et al.,* 2013). Finally, the clinical success of colistin led to an increased interest in using polymyxin B, a closely related antimicrobial agent. Polymyxin B is administered in its active form and has lower nephrotoxic effects as compared to colistin (Phe *et al.,* 2014). Nevertheless, its clinical efficacy in comparison with colistin is not yet fully investigated (Doi *et al.,* 2015).

3.4. Tetracyclines and Glycylcyclines

Minocycline and doxycycline are two tetracyclines that have been used for the treatment of *A. baumannii* infections (Wood *et al.,* 2003). Their value lies in their good clearance rates against Carbapenem-Resistant *Acinetobacter baumannii* (CRAB) infections and relative low toxicity (Chan *et al.,* 2010). Tetracyclines bind to the 30S ribosomal subunit and prevent the incorporation of the aminoacyl-tRNA into the A site, therefore inhibiting protein synthesis (Connell *et al.,* 2003). However, the wide dissemination of efflux pumps and Ribosomal Protection Proteins (RPPs) in *A. baumannii* protect this bacterium against tetracyclines and limits their therapeutic use (Doi *et al.,* 2015).

Tigecycline is the first antibiotic to be developed in a new class of antibiotics called glycylcyclines. It is a semi-synthetic derivative of minocycline and is not affected by most efflux pumps and RPPs that affect tetracyclines (Livermore, 2005). *In-vitro* resistance against tigecycline in *A. baumannii* is rare and usually results from over-expression of efflux pumps during treatment (Rumbo *et al.,* 2013). Tigecycline shows very good *in-vitro* activity against *A. baumannii* but clinical outcomes for patients treated with tigecycline have been sub-optimal, despite the *in-vitro* susceptibility. Moreover, its use at the currently approved dose in bacteremia was shown to not positively affect bacterial clearance from the blood (Kim *et al.,* 2013). All of this led to the lack of its recommended routine use against *A. baumannii* infections (CLSI, 2014). Nevertheless, its relatively low toxicity and positive clinical outcomes in certain cases make it useful, especially when other therapeutic options are severely limited (Chan *et al.,* 2010).

3.5. Fluoroquinolones

Fluoroquinolones are derived from quinolones, a fully synthetic class of antimicrobial agents (Suto *et al.,* 1992). They are able to enter the bacterial cell through porins and bind to DNA gyrase and topoisomerase IV. This would subsequently lead to cleavage of the bacterial

DNA resulting in double-strand DNA breaks and ultimately the fragmentation of the chromosome, and cell death (Hooper, 1999). They have also been shown to be able to exert their action on eukaryotic cells which results in toxicity, manifesting as neurotoxicity, cardiotoxicity, tendinopathies, and musculoskeletal pain (Strauchman and Morningstar, 2012). The second generation ciprofloxacin, third generation levofloxacin, and fourth generation gatifloxacin fluoroquinolones are recommended for use in the treatment of *A. baumannii* infections (CLSI, 2014). However, the use of fluoroquinolones in the treatment of the widely disseminated CRAB isolates is not recommended since resistance rates against these antimicrobial agents among CRAB isolates is as high as 97.9% (Huang *et al.*, 2015). Moreover, *A. baumannii* have been found to have a natural tendency to readily develop resistance to fluoroquinolones (Nwadike *et al.*, 2014), making their usefulness in the treatment of critically ill patients very limited.

3.6. Folic Acid Synthesis Inhibitors

Trimethoprim/sulfamethoxazole, also known as co-trimoxazole, is a folic acid synthesis inhibitor that acts through the inhibition of the dihydrofolate pathway, resulting in a bactericidal effect (Goldberg and Bishara, 2012). It consists of one part trimethoprim and five parts sulfamethoxazole since the synergistic effect between these two components is optimal at this ratio (Böhni, 1969). Sulfamethoxazole exerts its effect through competition with *p*-aminobenzoic acid and the subsequent disruption of *de novo* synthesis of dihydrofolate. Trimethoprim, on the other hand, competitively inhibits dihydrofolate reductase resulting in an inhibition of the production of tetrahydrofolate, the biologically active form of folic acid (Wormser *et al.*, 1982). This antimicrobial agent has a broad spectrum of activity and is used in the treatment of a wide range of infections. Nevertheless, its conventional use in *A. baumannii* is not recommended (Karageorgopoulos and Falagas, 2008). However, with the great increase in resistance among *A. baumannii* isolates, many clinicians are opting for its use when no other options are available. Reported sensitivity rates of *A. baumannii* isolates to trimethoprim/sulfamethoxazole range from 50% to 30% but the sensitivity among CRAB isolates is less than 20% (Falagas *et al.*, 2015). This makes it only useful in non-MDR *A. baumannii* isolates, if they show susceptibility to this antimicrobial agent.

3.7. Combination Therapies

The increasing rates of non-susceptibility of *A. baumannii* to antimicrobial agents and lack of development of new antibiotics resulted in attempts at optimizing the use of available antibiotics (Doi *et al.,* 2015). One such attempt is the use of combination therapies with synergistic antimicrobial agents, even if one of these agents is not recommended for use as monotherapy (Fishbain and Peleg, 2010). Rifampin is an antibiotic that works through the inhibition of RNA synthesis by binding to the RNA polymerase. Its use in monotherapy is not recommended for *A. baumannii* since rapid development of resistance is observed during treatment (Ng *et al.,* 2006). However, its combination with sulbactam, colistin, or imipenem showed promising results in terms of clearance rates and synergistic effects (Pachón-Ibáñez *et al.,* 2010). Similar synergistic effects have also been reported between the peptidoglycan synthesis inhibitor fosfomycin and colistin or sulbactam (Sirijatuphat and Thamlikitkul, 2014). A recent study also showed promising results when combining fosfomycin with amikacin (Leite *et al.,* 2016). Moreover, a surprising synergy between vancomycin, an antibiotic usually used for Gram-positive bacteria, and colistin showed promising results in its clearance rates of PDR *A. baumannii* isolates (Liu *et al.,* 2016).

Combining colistin with imipenem, sulbactam, or ampicillin/sulbactam showed better patient survival rates and bacterial clearance as compared to using each antibiotic separately for treating CRAB isolates (Batirel *et al.,* 2014). Moreover, triple therapy with colistin, sulbactam, and meropenem showed a synergistic effect against 96.7% of MDR *A. baumannii* isolates (Pongpech *et al.,* 2010). The combination between colistin and tigecycline failed to show a synergistic effect (López-Cortés *et al.,* 2014), but *in-vitro* synergy between imipenem and tigecycline has been demonstrated (Principe, *et al.,* 2009). Moreover, the combination of carbapenems with sulbactam also showed promising synergistic effects for the treatment of patients with CRAB infections (Lee *et al.,* 2007). Combination therapy with meropenem and ciprofloxacin (Ermertcan *et al.,* 2001) or minocycline (Tängdén, 2014) also showed some synergistic effect. Most of the aforementioned combinations are tested for *in-vitro* while *in-vivo* studies are still scarce and limited to a small number of patients. This makes combinations therapies, though promising, last resort options instead of standard treatment regimens (Doi *et al.,* 2015).

4. RESISTANCE TO ANTIMICROBIAL AGENTS

Resistance to antimicrobial agents is a phenomenon that was identified as soon as these molecules were discovered several decades ago. However, studies show that this phenomenon is an ancient one that was present in nature long before the discovery of antibiotics (D' Costa *et al.,* 2011). The mechanisms by which bacteria show resistance to antimicrobial agents are numerous and diverse. Most commonly, resistance to antimicrobial agents is mediated by enzymatic degradation of antibiotics, modifications of the target of antimicrobial agents, decreased permeability, and/or efflux of antibiotics (Coyne *et al.,* 2011). The resistance mechanisms relevant to *A. baumannii* will be discussed next.

4.1. Innate Mechanisms of Resistance in *Acinetobacter baumannii*

Acinetobacter baumannii innately possesses mechanisms of resistance to several antimicrobial agents. One such mechanism is harboring *AmpC*, the Ambler Class C cephalosporinase. This enzyme gives *A. baumannii* the ability to be resistant to most broad spectrum cephalosporins (Bou and Martínez-Beltrán, 2000a). Moreover, the presence of the insertion sequence IS*Aba1* upstream of this gene provides it with a strong promoter and causes a subsequent resistance to ceftazidime and cefepime (Héritier *et al.,* 2006). The group of cephalosporinases identified in this species has been labeled *Acinetobacter* Derived Cephalosporinase (ADC). Moreover, some allelic variants of ADCs have been found to have a broader spectrum of activity against cephalosporins than others, without the need of over-expression (Tian *et al.,* 2011b). *A. baumannii* also naturally harbors the Ambler Class D oxacillinase (OXA), *bla*$_{\text{OXA-51-like}}$ (Héritier *et al.,* 2005a). This OXA does not normally result in resistance to broad spectrum antibiotics unless it was over-expressed after association with IS*Aba1* (Turton *et al.,* 2006b). Additionally, the cellular membrane of *A. baumannii* was shown to be much less permeable to antimicrobial agents as compared to other Gram-negative bacteria (Obara and Nakae, 1991). This is mainly due to the small amount and size of porins this bacterium has on its outer membrane (Vila *et al.,* 2007). Finally, *A. baumannii* is known to constitutively express various efflux pumps at a basal level that play a role in its intrinsic resistance to several antimicrobial agents (Vila, 1999). The main intrinsic efflux pump identified in *A. baumannii* that contributes to its innate resistance is the adeIJK pump (Yoon *et al.,* 2015). The presence and regulation of efflux pumps in this organism will be discussed in the following section. All these factors contribute to the intrinsic resistance of *A. baumannii* to a wide range of antimicrobial agents and are represented in Figure 4.

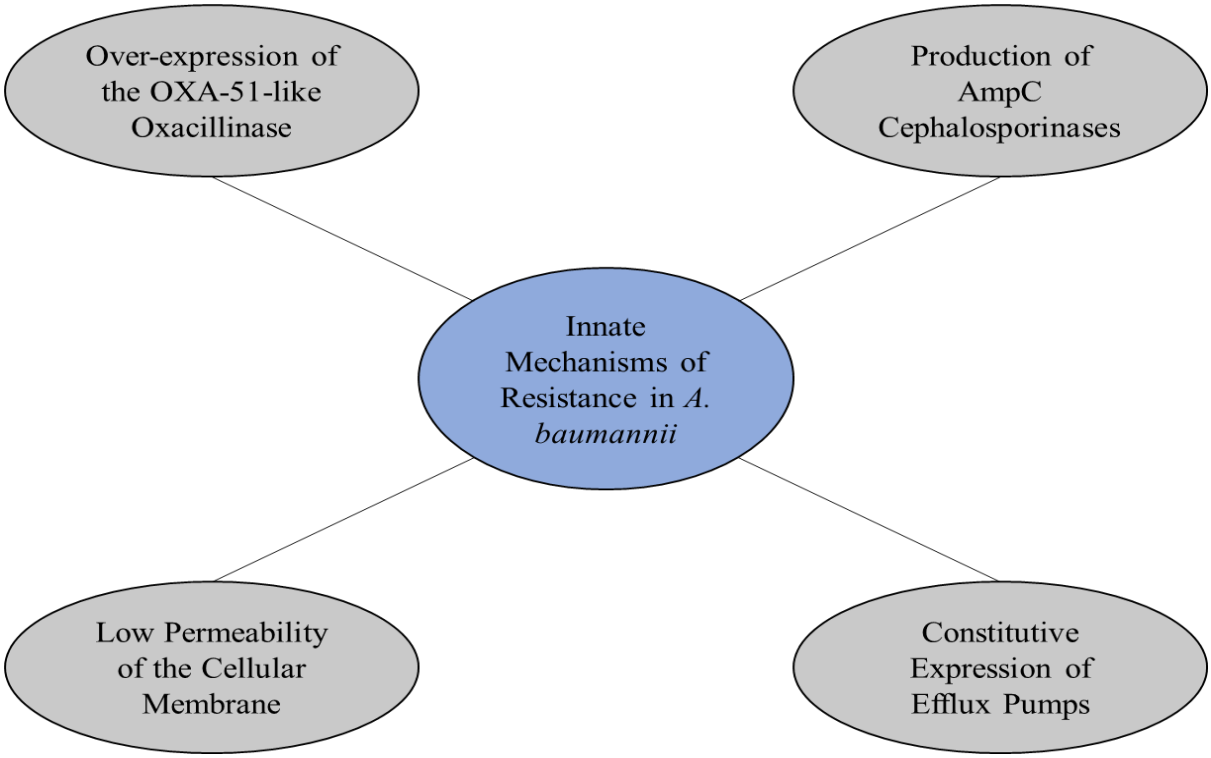


Figure 4. Innate Mechanisms of Antibiotic Resistance in *A. baumannii*

4.2. Acquired Mechanisms of Resistance in *Acinetobacter baumannii*

Resistance to antimicrobial agents could be acquired by several means. Mutations in endogenous genes could lead to the over-expression of certain cellular functions, such as efflux systems, and under-expression of others, such as outer membrane porins (Coyne *et al.*, 2011). Moreover, single mutations could cause an alteration of the antimicrobial agent's target, rendering it ineffective (Liou *et al.*, 2006). Finally, and most importantly, the horizontal transfer of mobile genetic elements, such as plasmids and transposons, carrying resistance genes lead to the acquisition and dissemination of antibiotic resistance (Goldstein *et al.*, 1983). Moreover, these mobile genetic elements could be incorporated into *A. baumannii*'s genome and form resistance islands that could contain up to 45 genes of resistance (Fournier *et al.*, 2006). Due to the plasticity of *A. baumannii*'s genome (Antunes *et al.*, 2014), this organism is able to acquire resistance to virtually all antimicrobial agents by different means which will be discussed next.

4.2.1. Non-Enzymatic Mechanisms of Resistance

Although the most common mechanism of resistance in *A. baumannii* is enzymatic degradation of antimicrobial agents, several non-enzymatic mechanisms exist. These include

over-expression of efflux pumps in combination with down-regulation of porins and modifications of the target of antibiotics (Doi *et al.,* 2015).

4.2.1.1. Efflux Pumps

Efflux pumps are part of bacteria's normal metabolism where they pump toxic material out of the cell and protect it from various environmental hazards (Vila *et al.,* 2007). There are five super-families of efflux pumps that have been associated with drug resistance. These are the ATP-Binding Cassette (ABC) transporters, Multi-drug and Toxic compound Extrusion (MATE), Resistance-Nodulation-cell Division (RND), Major Facilitator Superfamily (MFS), and Small Multi-drug Resistance (SMR) families (Coyne *et al.,* 2011). These families of efflux pumps are the most clinically relevant since they cross both inner and outer membranes and are able to extrude a wide range of substances (Nikaido, 2009). The ABC transporters utilize ATP for the expulsion of unwanted molecules whereas the other types of transporters are drug-proton antiporters that use the proton motive force as a source of energy for expulsion (Poole, 2002).

Over-expression of efflux pumps is implicated in the efflux of several antimicrobial agents, in addition to detergents, antiseptics, biocides, and dyes (Coyne *et al.,* 2011). Acquired resistance to macrolides, tetracyclines, and quinolones in association with over-expression of efflux pumps was reported since the 1990s (Barker, 1999). Although efflux pumps belonging to all super-families mediate certain degrees of resistance, those belonging to the RND super-family are the most commonly encountered among MDR *A. baumannii* isolates (Yoon *et al.,* 2016). These pumps are tightly regulated and several mutations in their transcriptional regulators lead to their overexpression (Yoon *et al.,* 2013). RND efflux pumps are formed of multiple components that are: an inner membrane protein that acts as the pump, a major fusion protein linked to it, and an outer membrane factor that allows the extrusion of substrates (Nikaido, 2009). The *ade*ABC operon was the first to be characterized in *A. baumannii* to code for an RND efflux pump (Magnet *et al.,* 2001). Prevalence of AdeABC efflux pumps is reported to range from 53% to 97% among clinical MDR *A. baumannii* isolates from regions all over the world (Coyne *et al.,* 2011). Up-regulation of this operon is involved in resistance to cefepime, cefpirome, and cefotaxime, in addition to other beta-lactams (Bratu *et al.,* 2008). This efflux system is also involved in resistance to aminoglycosides, tetracyclins, chloramphenicol, and trimethoprim (Damier-Piolle *et al.,* 2008). Moreover, up-regulation of

this pump is involved to a certain degree in resistance to carbapenems (Lee *et al.,* 2010). Finally, over-expression of AdeABC pumps were found to be able to extrude tigecycline, an antibiotic that resistant to expulsion by most other efflux systems (Yuhan *et al.*, 2016).

AdeIJK is another RND efflux pump that is intrinsic to *A. baumannii*. It works in synergy with the overexpression of AdeABC and is involved in resistance to penicillins, cephalosporins, monobactams, tetracyclines, tigecycline, fluoroquinolones, rifampin, lincosamides, novobiocin, chloramphenicol, trimethoprim/sulfamethoxazole, and fusidic acid (Damier-Piolle *et al.,* 2008). In addition to the intrinsic AdeIJK efflux pump, overexpression of AdeFGH is also involved in resistance to antimicrobial agents (Rosenfeld *et al.,* 2012). These agents include chloramphenicol, trimethoprim/sulfamethoxazole, fluoroquinolones, clindamycin, tigecycline, and tetracyclines (Coyne *et al.,* 2011). However, over-expression of AdeFGH is not very commonly reported as compared to the other RND efflux pumps (Yoon *et al.,* 2016). Another efflux pump involved in resistance to fluoroquinolones, trimethoprim, and chloramphenicol is AbeM, which belongs to the MATE superfamily (Su *et al.,* 2005). A recent study also showed that efflux systems that could be inhibited by cyanide 3-chlorophenylhydrazone (CCCP), a proton motive force inhibitor, are involved in colistin resistance (Ni *et al.,* 2016). In addition, the AbeS, an SMR efflux pump, is also able to expunge ammonium antiseptics, as well as erythromycin, chloramphenicol, fluoroquinolones, and novobiocin. This gives this bacterium a degree of resistance against disinfection as well as against several antimicrobial agents (Fournier *et al.,* 2006).

An association between antimicrobial resistance and resistance to several biocides has been described. It was shown that resistance to aminoglycosides, tetracyclines, fluoroquinolones, and carbapenems through efflux pumps makes *A. baumannii* more resistant to biocides (Fernández-Cuenca *et al.,* 2015). Nevertheless, the former study, and another recent study, suggest that some isolates have reduced virulence and fitness after over-expression of efflux pumps (Yoon *et al.,* 2016). Finally, *A. baumannii* has been shown to be able to acquire genes coding for efflux pumps from its surroundings (Coyne *et al.,* 2011). These pumps include TetA and TetB, members of the MFS superfamily. These pumps convey high level resistance to tigecycline and are detected in several MDR *A. baumannii* isolates (Vila *et al.,* 2007).

4.2.1.2. Down-Regulation of Porins

Another mechanism that works in synergy with the up-regulation of efflux pumps is the down-regulation of porins. This mechanisms acts by decreasing the permeability of the bacterium's membrane which prevents antimicrobial agents from re-entering the cell after expulsion and protects it against future exposure (Bonomo and Szabo, 2006). Porins are outer membrane proteins that form channels through which several molecules, including carbapenems, enter the bacterial cell (Peleg *et al.,* 2008). CarO is one such porin identified in *A. baumannii* whose down-regulation is associated with carbapenem resistance (Siroy *et al.,* 2005). One study showed that the disruption of the *carO* gene by mobile elements resulted in carbapenem resistance (Mussi *et al.,* 2005). Other outer membrane porins whose down-regulation has been involved in carbapenem resistance have also been identified in *A. baumannii*. These include porins homologous to OmpF and OprD found in *Pseudomonas aeruginosa* and to OmpW found in *Escherichia coli* and *Pseudomonas aeruginosa* (Peleg *et al.,* 2008).

Outbreaks of CRAB isolates associated with down-regulation of outer membrane proteins have been reported from USA (Quale *et al.,* 2003) and Spain (Bou *et al.,* 2000b) in the early 2000s. Additionally, the involvement of several porins, including CarO, has been found to work synergistically with carbapenem hydrolyzing enzymes in order to convey carbapenem resistance (Poirel and Nordmann, 2006). Another study showed that mutations in *carO* and *oprD*, accompanied by up-regulation of RND efflux pumps in *A. baumannii* could also lead to carbapenem resistance (Yang *et al.,* 2015). Analysis of outbreaks caused by CRAB isolates showed that all of those isolates had reduced *carO* expression contributing to carbapenem resistance in parallel to other mechanisms of resistance (Sen and Joshi, 2016). This suggests that decreased permeability of the membrane could act synergistically with other mechanisms of resistance and contribute to carbapenem resistance in *A. baumannii*.

4.2.1.3. Changes in the Target of Antimicrobial Agents

Another non-enzymatic mechanism of resistance found in *A. baumannii* is modification of the target of antimicrobial agents. Point mutations in the DNA gyrase and topoisomerase IV genes (*gyrA* and *parC*, respectively) result in the modification of the target of fluoroquinolones and the subsequent resistance to these antibiotics (Bonomo and Szabo, 2006). A recent study showed that the Ser83Leu in *gyrA* and Ser80Leu in *parC* mutations are the most common

among the topoisomerase genes mutations in *A. baumannii* (Güler and Eraç, 2016). Another study conducted in USA showed that all the 38 *A. baumannii* isolates responsible for two separate outbreaks were resistant to fluoroquinolones through mutations in *gyrA* and *parC*. The mutations caused the same amino acid changes as reported by the previous study (Warner *et al.*, 2016). A study investigating 34 fluoroquinolone-resistance *A. baumannii* isolates in China showed that 48.7% of them had single mutations in *gyrA*, 21.6% had single mutations in *parC*, and 5.4% had mutations in both genes (Sun *et al.*, 2015). Finally, a study from Sweden showed that all the *A. baumannii* isolates that were resistant to quinolones acquired this resistance through the aforementioned mutations (Karah *et al.*, 2016).

Resistance to aminoglycosides could be acquired by reduced permeability, efflux pumps over-expression, and amino acid substitutions in ribosomal proteins. However, the most potent form of acquiring resistance to these antimicrobial agents is through the alteration of their target by 16S rRNA methylases (Doi and Arakawa, 2007a). Of the ten 16S rRNA methylases identified in Gram-negative organisms, ArmA and RmtB seem to be the most prevalent among *A. baumannii* (Potron *et al.*, 2015). Analysis of the G+C content of the genes coding for these enzymes suggested that they are not intrinsic to this species but were rather acquired through horizontal gene transfer and incorporated into the genome (Doi *et al.*, 2007b). The RmtB methylase does not seem to be widely disseminated where it was detected in a few studies, one of which was in Vietnam (Tada *et al.*, 2013). ArmA, on the other hand, has been identified in *A. baumannii* isolates from regions all over the world.

Around 60% out of 101 clinical *A. baumannii* isolates were found to harbor *armA* in Vietnam (Tada *et al.*, 2013). In South Korea, a study across two hospitals showed that *A. baumannii* isolates that were highly resistant to aminoglycosides carried *armA* on a plasmid (Cho *et al.*, 2009). Another study from the same country showed that 90.32% of 31 *A. baumannii* isolates were positive for ArmA (Hong *et al.*, 2013). In Japan, ArmA was detected in highly aminoglycoside-resistant *A. baumannii* isolates obtained from 8 different patients (Yamada and Suwabe, 2013) whereas in China ArmA was detected in 221 out of 342 CRAB isolates (Zhou *et al.*, 2009). In Iran, 26% of amikacin-resistant *A. baumannii* isolates produced ArmA (Aghazadeh *et al.*, 2013) and 8.5% of amikacin-resistant CRAB isolates had ArmA in Algeria (Bakour *et al.*, 2014). This methylase was also found to be widely disseminated in Egypt were it was detected in 141 out of 150 aminoglycoside highly-resistant *A. baumannii* isolates (El-Sayed-Ahmed *et al.*, 2015). Moreover, all MDR *A. baumannii* isolates that had a

high level of resistance to aminoglycosides were found to produce ArmA in a study in Bulgaria (Strateva *et al.,* 2012). A single clone producing both OXA-23 and ArmA was detected in 19 out of 21 *A. baumannii* isolates in Italy (Brigante *et al.,* 2012) and four out of eleven *A. baumannii* MDR isolates produced ArmA in a study from Norway (Karah *et al.,* 2011a). A study from Sweden showed that all 16 isolates from the pool being studied that were highly resistant to aminoglycosides were positive for ArmA (Karah *et al.,* 2016). Investigating an outbreak in Latvia caused by 30 *A. baumannii* isolates showed that 26 of these isolates had this methylase (Saule *et al.,* 2013). In Pennsylvania, USA, five aminoglycoside highly-resistant *A. baumannii* isolates produced ArmA (Doi *et al.,* 2007a). All these reports are in agreement that ArmA conveys high-level resistance to aminoglycosides and is highly disseminated all around the globe.

Another form of modification of the target of antimicrobial agents is the ribosomal protection mechanism coded by several *tet* genes. In this mechanism, ribosomal protection proteins bind to the ribosome and sequester it from the action of tetracyclines, leading to resistance to these antimicrobial agents (Li *et al.,* 2013). This mechanism is very widely spread among *A. baumannii* isolates and works in synergy with efflux pumps in order to convey a very high level of resistance to tetracyclines (Potron *et al.,* 2015). However, tigecycline is able to evade this mechanism and show, at least *in-vitro*, good activity against *tet* harboring *A. baumannii* isolates (Hawkey and Finch, 2007). Nevertheless, mutations in the *trm* gene, which codes for a methyltransferase, were found to lead to resistance to tetracycline in *A. baumannii* through a mechanism that is still not well defined (Chen *et al.,* 2014). Finally, modifications of the many types of PBPs found in *A. baumannii*, the target of beta-lactams, have been found to convey low-level resistance to carbapenems (Gehrlein *et al.,* 1991). A study showed that, in addition to modification of PBPs, their reduced expression works in synergy with other mechanisms in order to convey low-level carbapenem resistance (Fernández-Cuenca *et al.,* 2003). However, data concerning the prevalence and exact mechanisms regarding modifications of PBPs in *A. baumannii* is very scarce (Nowak and Paluchowska, 2016).

4.2.2. Enzymatic Mechanisms of Resistance

Enzymatic degradation of antimicrobial agents is another mechanism by which bacteria become resistant to antimicrobial agents. More than 900 enzymes that degrade antimicrobial agents have been described in pathogenic bacteria (Mehrad *et al.,* 2015). Due to their genomic

plasticity, a large number of antibiotic-hydrolyzing enzymes have been detected among clinical *A. baumannii* isolates (Antunes *et al.*, 2014). Some *A. baumannii* isolates have even acquired enough enzymatic and non-enzymatic mechanisms so as to become resistant to all known antimicrobial agents (Leite *et al.*, 2016).

4.2.2.1. Beta-Lactamases

Among the numerous enzymes that hydrolyze antibiotics, beta-lactamases, which hydrolyze beta-lactams by breaking the amide bond of the beta-lactam ring, are the most common among Gram-negative bacteria (Xia *et al.*, 2016). Beta-lactamase enzymes are classified into Classes A, B, C, and D according to the ambler classification which is based on molecular structure (Ambler, 1980). Within this group of enzymes, the Extended Spectrum Beta-Lactamases (ESBLs) hydrolyze a broad range of beta-lactams, and carbapenemases are capable of hydrolyzing carbapenems (Weldhagen *et al.*, 2003). In the following sections, the prevalence of the different beta-lactamases, with an emphasis on carbapenemases, among *A. baumannii* isolates will be discussed. These enzymes are summarized in Figure 5.

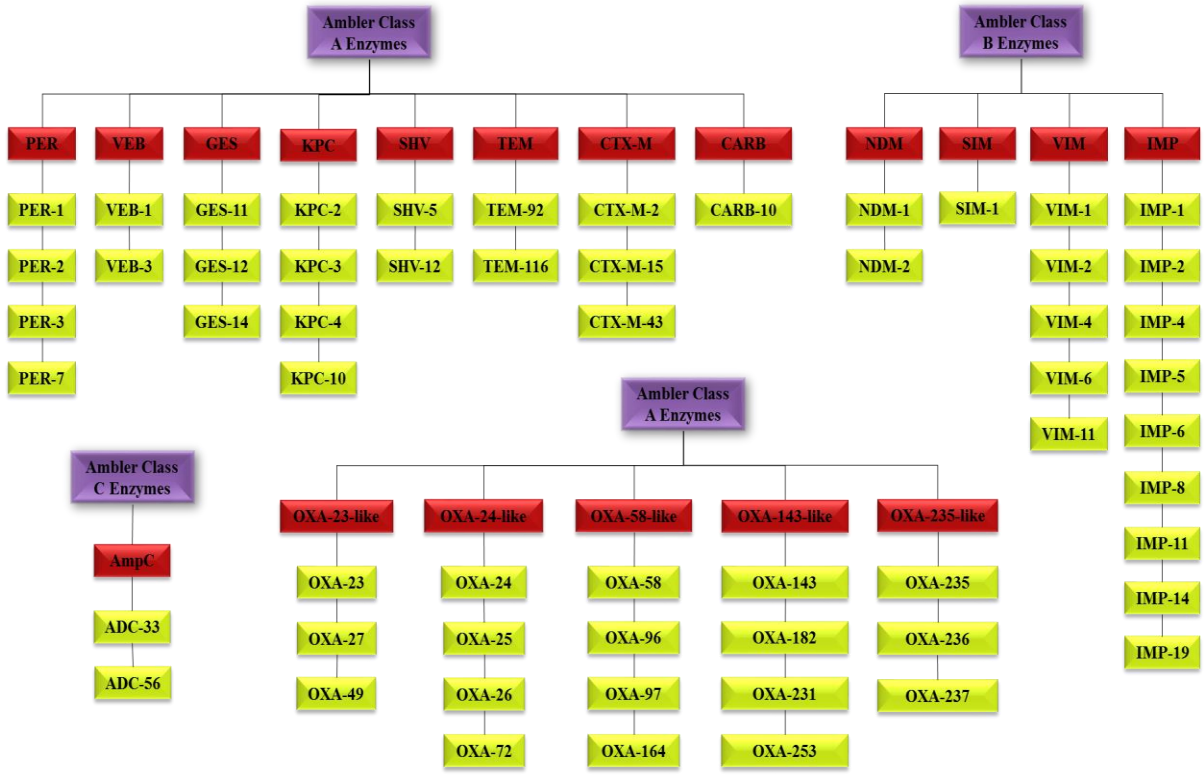


Figure 5. Acquired beta-lactamases detected in *A. baumannii* according to the Ambler classification system.

4.2.2.1.1. Ambler Class A Enzymes

Class A beta-lactamases are a set of enzymes that hydrolyze beta-lactams through the formation of an acyl enzyme and have an active site that contains serine (Bush and Jacoby, 2010). Numerous ESBLs of this class of beta-lactamases are extremely common among *Enterobacteriaceae* but only a few types are commonly found in *A. baumannii* (Potron *et al.*, 2015). The ESBL that was first identified in *A. baumannii* was the *Pseudomonas* Extended-Resistant (PER) ESBL, PER-1. PER-1 conveys resistance to ceftazidime and has a limited carbapenemase activity. It is detected in *A. baumannii* isolates spread across several countries around the world (Potron *et al.*, 2015). This enzyme was detected in 53 of 97 *A. baumannii* isolates in South Korea (Yong *et al.*, 2003) and 35 of 37 isolates in China (Zhang *et al.*, 2010). Another study from China that spanned across 11 teaching hospitals over six years showed that 77.8% of 221 *A. baumannii* isolates had PER-1 (Wang *et al.*, 2007). In Turkey, PER-1 was detected in 31% of *A. baumannii* isolates obtained from seven university hospitals (Kolayli *et al.*, 2005) whereas 51% of isolates in Iran produced this enzyme (Farajnia *et al.*, 2013). Additionally, two out of eight *A. baumannii* isolates obtained from four medical centers in Belgium were positive for PER-1 (Naas *et al.*, 2006b). One case of an isolate obtained from a Hungarian tourist returning from Egypt was also positive for this enzyme (Szabó *et al.*, 2008). In USA, PER-1 is present but was only detected in two out of seventy five isolates indicating a low prevalence in this country (Hujer *et al.*, 2006). PER-1 was also detected in isolated cases in India (Litake *et al.*, 2009), Romania (Naas *et al.*, 2007b), Russia, (Naas *et al.*, 2007a), and Bulgaria (Strateva *et al.*, 2008). A novel structure of PER-1 was also reported in a study performed on an *A. baumannii* isolate obtained from Kuwait (Opazo *et al.*, 2014). PER-2 is another enzyme detected in *A. baumannii* that has 86% amino acid identity with PER-1. However, its occurrence seems to be limited to South America (Pasterán *et al.*, 2006). PER-3 is yet another ESBL identified in *A. baumannii* isolates from Egypt (Al-Hassan *et al.*, 2013). One last variant, PER-7, was also identified in isolates obtained from the United Arab Emirates (Opazo *et al.*, 2012), France (Bonnin *et al.*, 2011a), and Sweden (Karah *et al.*, 2016).

Vietnamese Extended-spectrum Beta-lactamase (VEB) is another ESBL that was first detected among *A. baumannii* isolates in France (Poirel *et al.*, 2003). A subsequent study showed that VEB-1 is widely disseminated among *A. baumannii* isolates across the entire nation (Naas *et al.*, 2006a). In Belgium, six VEB-1 positive *A. baumannii* isolates were collected from 2003 to 2005 (Naas *et al.*, 2006b) whereas 10 out of 100 *A. baumannii* isolates

were VEB-1 positive in Iran from 2008 to 2009 (Farajnia *et al.*, 2013). VEB-1 was also detected in six *A. baumannii* isolates from Argentina (Poirel *et al.*, 2009) and its variant VEB-3 was detected in a single isolate from Taiwan (Huang *et al.*, 2008). The Guiana Extended Spectrum (GES) beta-lactamase is another type of beta-lactamase with varying degrees of hydrolytic activity towards carbapenems. GES-11, was first identified in an *A. baumannii* isolate in France showing reduced susceptibility to carbapenems (Moubareck *et al.*, 2009). A subsequent study in Belgium showed that GES-11, as well as two other variants, were present in 9 out of 125 MDR *A. baumannii* isolates obtained from 18 hospitals. The two other variants detected in this study were GES-12, which hydrolyzes ceftazidime, and GES-14, which hydrolyzes imipenem (Bogaerts *et al.*, 2010). A study in Kuwait targeted at 63 *A. baumannii* isolates surprisingly showed that all those isolates had GES genes on plasmids suggesting that the Middle East region could act as a reservoir for this ESBL. Of the 63 GES positive isolates, only one showed GES-14 whereas all the rest had GES-11 (Bonnin *et al.*, 2013). The GES-14 carbapenemase was also detected in France among *A. baumannii* isolates in 2011 (Bonnin *et al.*, 2011b). In a study performed in Turkey on 101 *A. baumannii* isolates collected over one year showed that 16 of them had GES-11 while 8 had another carbapenem hydrolyzing variant, GES-22 (Cicek *et al.*, 2014). Furthermore, GES-11 was detected among *A. baumannii* isolates in a study from Sweden (Karah *et al.*, 2011b) and another one from Tunisia (Charfi-Kessis *et al.*, 2014).

Another class A beta-lactamase that is the *Klebsiella pneumoniae* Carbapenemase (KPC). This enzyme could be spread through mobile genetic elements and has a high intrinsic activity against carbapenems (Naas *et al.*, 2008). Although this enzyme is commonly reported among *Enterobacteriaceae*, its dissemination among *A. baumannii* isolates is rare. Ten *A. baumannii* isolates from Puerto Rico were positive for KPC enzymes, seven of which had KPC-3 and the rest had KPC-2, KPC-4, and KPC-10, respectively (Robledo *et al.*, 2010). However, the presence of KPC in *A. baumannii* seems to still be scarce and limited to one geographical area (Martinez *et al.*, 2016). The Temoneira (TEM), Sulfhydryl Variable (SHV), and Cefotaximase (CTX-M) class A ESBLs that are widely disseminated among *Enterobacteriaceae* are also rarely detected in *A. baumannii* (Potron *et al.*, 2015). The plasmid-carried SHV-12 and TEM-116 ESBLs were found in three *A. baumannii* isolates in a study from the Netherlands (Naiemi *et al.*, 2005). Another seven-year study from Italy showed that 31 *A. baumannii* isolates carried the plasmidic TEM-92 ESBL (Endimiani *et al.*, 2007) while the chromosomal $bla_{\text{SHV-5}}$ was detected in one PDR *A. baumannii* isolate in New York City, USA (Naas *et al.*, 2007). The cefotaxime-hydrolyzing CTX-M-2 was detected in three *A.*

baumannii isolates from Japan (Nagano *et al.*, 2004) and five out of 49 isolates from USA (Adams-Haduch *et al.*, 2008). Another novel cefotaximase variant, CTX-M-43, was found to be disseminated among *A. baumannii* isolates in a multi-center study performed in several Bolivian hospitals (Celenza *et al.*, 2006). Two out of six CRAB isolates from India (Shakil and Khan, 2010) and two *A. baumannii* isolates obtained from Haiti (Potron *et al.*, 2011) were shown to be positive for CTX-M-15. Finally, a novel Carbenicillin-hydrolyzing Beta-lactamase (CARB), the chromosomally-coded, cefepime-hydrolyzing CARB-10, was identified in *A. baumannii* in France (Potron *et al.*, 2009). The distribution of Ambler class A beta-lactamases is summarized in Table 1.

Table 1. Worldwide Distribution of Ambler Class A Beta-Lactamases.

Africa	
Egypt	PER-3
Tunisia	GES-11
Asia	
China	PER-1
India	PER-1 and CTX-M-15
Iran	PER-1 and VEB-1
Japan	CTX-M-2
Kuwait	PER-1, GES-11, and GES-14
Russia	PER-1
South Korea	PER-1
Taiwan	VEB-3
Turkey	PER-1, GES-11, and GES-22
United Arab Emirates	PER-7
Europe	
Belgium	PER-1, VEB-1, GES-11, GES-12, and GES-14
Bulgaria	PER-1
France	PER-7, VEB-1, GES-11, GES-14, and CARB-10
Hungary	PER-1
Italy	TEM-92
Netherlands	SHV-12 and TEM-116
Romania	PER-1
Sweden	GES-11, PER-7
North America	
Haiti	CTX-M-15
Puerto Rico	KPC-2, KPC-3, KPC-4, and KPC-10
United States of America	PER-1, SHV-5, and CTX-M-2
South America	
Argentina	PER-2 and VEB-1
Bolivia	CTX-M-43

4.2.2.1.2. Ambler Class B Enzymes

Ambler class B enzymes are metalloenzymes that utilize a zinc ion at their active site in order to hydrolyze beta-lactams (Bush and Jacoby, 2010). These enzymes are referred to as

Metallo-Beta-Lactamases (MBLs), are very efficient at hydrolyzing carbapenems, and are not inhibited by clavulanic acid nor tazobactam (Nordmann *et al.,* 2012). Although MBLs are not the most common cause for carbapenem resistance in *A. baumannii*, the Verona Integron-encoded Metallo-beta-lactamase (VIM), Imipenem metallo-beta-lactamase (IMP), New Delhi Metallo-beta-lactamase (NDM), and Seoul Imipenemase (SIM) MBLs have been detected in several regions around the world (Potron *et al.,* 2015). Five VIM-1 positive isolates were detected among *A. baumannii* isolates obtained from two Greek hospitals between 2004 and 2005 (Tsakris *et al.,* 2006). In South Korea, a study across 28 hospitals showed that VIM-2 was present in 27 out of 267 *A. baumannii* isolates over a one-year period (Lee *et al.,* 2003). Eighteen out of 94 consecutive *A. baumannii* isolates obtained within a year were positive for VIM-2 in Kuwait (Al-Sweih *et al.,* 2012). In China, 8 out of 143 *A. baumannii* isolates were positive for VIM-2 (Wang *et al.,* 2015). Additionally, *A. baumannii* isolates producing VIM-4, VIM-6, and VIM-11 were detected in Italy (Mammina *et al.,* 2011), India (Potron *et al.,* 2015), and Taiwan (Huang *et al.,* 2008), respectively.

IMP-type MBLs seem to be disseminated among *A. baumannii* isolates throughout Eastern Asia and the Pacific Rim, but are not limited to that region (Potron *et al.,* 2015). In a one-year study in Japan, 30 *A. baumannii* isolates were found to be positive for IMP-1 and five were positive for IMP-2 (Shibata *et al.,* 2003). IMP-11 (Potron *et al.,* 2015) and IMP-19 (Yamamoto *et al.,* 2011) were also found among *A. baumannii* isolates in Japan. In southern India, 23 of 55 *A. baumannii* isolates were positive for IMP-1 (Uma Karthika *et al.,* 2009) whereas 8 of 35 isolates in Taiwan were positive for this enzyme (Chiu *et al.,* 2010). IMP-1 was detected in 27 of 38 *A. baumannii* isolates in South Korea (Lee *et al.,* 2003) and IMP-4 in 23 of 35 isolates in Hong Kong (Chu *et al.,* 2001). IMP-4 was also detected in one *A. baumannii* clinical isolate in Australia (Peleg *et al.,* 2007) and four isolates in Singapore (Koh *et al.,* 2007). In China, a single *A. baumannii* isolate producing IMP-8 was detected in a study that spanned six years (Wang *et al.,* 2007). Another study from Taiwan showed the presence of IMP-14 among *A. baumannii* isolates in that country (Kansakar *et al.,* 2011). In Kuwait, 8 of 94 *A. baumannii* isolates were positive for IMP-1 (Al-Sweih *et al.,* 2012) and IMP-6 was present in a single isolate in Brazil (Gales *et al.,* 2003). Another *A. baumannii* isolate was positive for IMP-5 in Portugal (Da Silva *et al.,* 2002) and one was positive for IMP-1 in Italy (Cornaglia *et al.,* 1999).

The SIM-1 carbapenemase seems to be widespread in South Korea, where it was first identified (Lee *et al.,* 2005). However, its dissemination seems to be limited to that country (Nowak and Paluchowska, 2016). NDM-1 was relatively recently discovered and was reported for the first time to be present among three *A. baumannii* isolates in India in 2010 (Karthikeyan *et al.,* 2010). NDM-1 was also detected in an *A. baumannii* isolate in Japan obtained from a patient that was transferred from India (Nakazawa *et al.,* 2013). In China, four *A. baumannii* isolates obtained from four different provinces were positive for NDM-1 and the *bla*$_{\text{NDM-1}}$ gene was present on transferrable plasmids (Chen *et al.,* 2011). One out of sixteen CRAB isolates collected over one year in Kenya was positive for NDM-1 (Revathi *et al.,* 2013) whereas 11 out of 47 CRAB isolates in Algeria were positive for this carbapenemase (Bakour *et al.,* 2014). Moreover, an outbreak of seven NDM-1 positive *A. baumannii* isolates was detected in France from January to May, 2013 and was traced back to two patients transferred from Algeria (Decousser *et al.,* 2013). Another case of an *A. baumannii* isolate carrying *bla*$_{\text{NDM-1}}$ from a patient transferred from Algeria was recorded in Belgium (Bogaerts *et al.,* 2012). Two *bla*$_{\text{NDM-1}}$ carrying *A. baumannii* isolates from a Balkan origin were detected in two different European countries; one in Switzerland (Poirel *et al.,* 2012) and the other in Germany (Pfeifer *et al.,* 2011). Single NDM-1 positive *A. baumannii* isolates were also detected in Slovenia (Bonnin *et al.,* 2012), Czech Republic (Krizova *et al.,* 2012), and Brazil (Pillonetto *et al.,* 2014). In Lebanon, four *A. baumannii* isolates producing NDM-1 were detected among patients wounded in the Syrian civil war (Rafei *et al.,* 2014b). NDM-2 was detected in one *A. baumannii* isolate from Egypt (Kaase *et al.,* 2011), two from United Arab Emirates (Ghazawi *et al.,* 2012), and five from Israel (Espinal *et al.,* 2011). The NDM-2 carrying isolates were shown to be clonally related in a subsequent study (Espinal *et al.,* 2013) suggesting that this region could act as a reservoir for NDM-2 producing *A. baumannii*. Finally, an extensive study showed that *bla*$_{\text{NDM-1}}$ most probably got transferred from *A. baumannii* to *Pseudomonas aeruginosa* and *Enterobacteriaceae* and not the other way around (Bonnin *et al.,* 2014). This suggests that *A. baumannii* could also act as a reservoir of resistance genes for other bacteria. Table 2 summarizes the above distribution of Ambler class B beta-lactamases.

Table 2. Worldwide Distribution of Ambler Class B Beta-Lactamases.

Africa	
Algeria	NDM-1
Egypt	NDM-2
Kenya	NDM-1
Asia	
China	VIM-2, IMP-4, IMP-8, and NDM-1
India	VIM-6, IMP-1, and NDM-1
Israel	NDM-2
Japan	IMP-1, IMP-2, IMP-11, and IMP-19
Kuwait	VIM-2 and IMP-1
Lebanon	NDM-1
Singapore	IMP-4
South Korea	VIM-2, IMP-1, and SIM-1
Taiwan	VIM-11, IMP-1, and IMP-14
United Arab Emirates	NDM-2
Europe	
Belgium	NDM-1
Czech Republic	NDM-1
France	NDM-1
Germany	NDM-1
Greece	VIM-1
Italy	VIM-4 and IMP-1
Portugal	IMP-5
Slovenia	NDM-1
Switzerland	NDM-1
Oceana	
Australia	IMP-4
South America	
Brazil	IMP-6 and NDM-1

4.2.2.1.3. Ambler Class C Enzymes

Ambler class C enzymes are beta-lactamases with a broad spectrum of activity against cephalosporins. They hydrolyze beta-lactams in a way similar to class A enzymes (Bush and Jacoby, 2010). No acquired genes producing this type of enzymes have been detected in *A. baumannii* (Potron *et al.*, 2015). This bacterium does, however, intrinsically produce AmpC, a class C cephalosporinase. Production of AmpC normally is at a low basal level and therefore does not contribute much to the resistance towards broad spectrum cephalosporins (Bou *et al.*, 2000a). However, insertion of IS*Aba1* upstream of the AmpC genes was found to increase their expression to the point of acquiring resistance to broad spectrum cephalosporins, but not to carbapenems (Héritier *et al.*, 2006). Additionally, some AmpC variants, such as ADC-33 (Rodríguez-Martínez *et al.*, 2010) and ADC-56 (Tian *et al.*, 2011b) have a broader spectrum of activity against cephalosporins. They have been found to hydrolyze ceftazidime and cefepime more efficiently. However, their clinical significance is yet to be defined (Potron *et al.*, 2015).

4.2.2.1.4. Ambler Class D Enzymes

Ambler class D enzymes are the most common cause of carbapenem resistance among *A. baumannii* isolates (Nowak and Paluchowska, 2016). They are a heterogeneous group of enzymes that are collectively referred to as Oxacillinases (OXAs). They work in a way similar to class A and class C enzymes, are able to hydrolyze cefalotin and amoxicillin, and are not inhibited by clavulanic acid (Poirel *et al.*, 2007). Several OXAs have been identified in *A. baumannii* and those that convey resistance to carbapenems are grouped into six families. These are the OXA-51-like, OXA-23-like, OXA-40/24-like, OXA-58-like, OXA-143-like, and OXA-235-like families (Evans and Amyes, 2014). The OXA-24-like and OXA-40-like enzymes were found to be the same group of enzymes but referred to differently in different studies (Tian et al., 2011a). These two identical groups will be referred to as OXA-24-like from here on. OXAs are known to often work synergistically with other mechanisms of resistance, such as reduced membrane permeability or increased efflux pump expression, in order to convey a high level of resistance to carbapenems (Poirel and Nordmann, 2006). *A. baumannii* intrinsically expresses OXA-51-like enzymes, but their expression alone does not convey resistance to carbapenems, unless over-expressed with the help of IS*Aba1* upstream of its gene (Turton *et al.*, 2006b). More than 95 chromosomally encoded enzymes have been described as belonging to the OXA-51-like family, to date (Nowak and Paluchowska, 2016). Two commonly encountered OXA-51-like alleles among CRAB nosocomial isolates are the OXA-66 and OXA-69 variants (Turton *et al.*, 2007). An outbreak of 31 CRAB isolates over-expressing OXA-66 was reported over a one-year period in Spain (Culebras *et al.*, 2010). OXA-51-like over-expressing CRAB isolates were also reported in 93.7% of isolates causing an outbreak in Korea that affected 77 patients (Chaulagain *et al.*, 2012). Nevertheless, carbapenem resistance resulting from over-expression of OXA-51-like is relatively infrequent (Nowak and Paluchowska, 2016).

The OXA-23-like family contains the enzymes OXA-23, OXA-27, and OXA-49 (Potron *et al.*, 2015). *bla*$_{\text{OXA-23-like}}$ genes are usually a part of a transposon and could be found on bacterial chromosomes or plasmids (Corvec *et al.*, 2007). This family of OXAs is the most prevalent among CRAB isolates causing nosocomial infections (Merino *et al.*, 2014). Although this family has intrinsic activity against carbapenems, enhanced carbapenemase activity is associated with the presence of IS*Aba1* upstream of *bla*$_{\text{OXA-23-like}}$ genes (Turton *et al.*, 2006b). OXA-23 in *A. baumannii* was first identified in an isolate obtained from Edinburg (Scaife *et*

al., 1995). Nowadays, OXA-23-like enzymes are reported with varying rates from countries all around the world among *Acinetobacter* spp., mainly owing to its wide dissemination through plasmid-mediated transfer (Evans and Amyes, 2014). OXA-23 was identified in a single isolate from Japan with IS*Aba1* upstream of its gene in a study that spanned from 2003 until 2011 (Kishii *et al.,* 2014). However, another multi-center study from that country showed that 23 out of 49 MDR *A. baumannii* isolates were positive for OXA-23-like (Tada *et al.,* 2014). Alarmingly, 97.7% of 221 CRAB isolates obtained from 11 Chinese medical centers were positive for OXA-23-like (Wang *et al.,* 2007). A more recent nation-wide study in China showed that 71.3% of 143 *A. baumannii* nosocomial isolates were positive for this enzyme (Wang *et al.,* 2015). A national survey in South Korea showed that 32.64% of CRAB isolates were positive for OXA-23-like (Lee *et al.,* 2009). Another study from that country showed that 80% of 190 CRAB isolates were positive for OXA-23-like (Kim *et al.,* 2010). In Australia, two *bla*$_{\text{OXA-23-like}}$ harboring *A. baumannii* isolates were detected in a 10-year study (Nigro *et al.,* 2011). On the other hand, in Brazil, 46 OXA-23-like positive *A. baumannii* isolates were collected from eight different hospitals in a two-year period (Cieslinski *et al.,* 2013). Another study from Brazil showed that 87.3% of 139 *A. baumannii* isolates with reduced carbapenem susceptibility were positive for OXA-23-like (Cortivo *et al.,* 2015). Thirty nine CRAB isolates harboring *bla*$_{\text{OXA-23-like}}$ were collected from three hospitals in Egypt over one year (Fouad *et al.,* 2013). Another study from Egypt showed that 50% of 40 CRAB isolates harbored this gene (Al-Agamy *et al.,* 2014). In Algeria, 23 out of 24 CRAB isolates obtained within a year from two university hospitals were positive for OXA-23-like (Touati *et al.,* 2012). Moreover, three OXA-23-like positive *A. baumannii* isolates were detected in Nigeria, one in South Africa, and fifty three in Madagascar (Olaitan *et al.,* 2013). Screening for OXA-23-like positive *A. baumannii* isolates among human lice and fecal samples in Senegal resulted in a total of nine positive isolates in one year. This suggests that human lice and stool samples in Senegal could act as a reservoir for *bla*$_{\text{OXA-23-like}}$ harboring CRAB isolates (Kempf *et al.,* 2012b). In a multi-center study across the Gulf Cooperation Council States (Saudi Arabia, United Arab Emirates, Oman, Qatar, Bahrain, and Kuwait), 91.45% of CRAB isolates were positive for OXA-23-like (Zowawi *et al.,* 2015). In northwestern Iran, the prevalence of OXA-23-like among CRAB isolates is reported to be 88.7% (Sohrabi *et al.,* 2012). Another study from that country showed that 68% of CRAB isolates infecting patients with burns had this carbapenemase (Pajand *et al.,* 2013). A more recent study that investigated 30 CRAB isolates from Iranian burn units showed that they all harbored *bla*$_{\text{OXA-23-like}}$ (Salimizand *et al.,* 2015).

A study across 10 ICUs in Italy from 2005 to 2009 showed an incidence of 71.1% of OXA-23-like among CRAB isolates (D'Arezzo *et al.*, 2011). In a similar study across 22 Italian hospitals from 2004 until 2009, 48 out of 202 CRAB isolates were positive for OXA-23-like (Mezzatesta *et al.*, 2012). A study performed in Poland from 2005 to 2010 showed that 47.12% of 104 *A. baumannii* isolates harbored *bla*$_{\text{OXA-23-like}}$ (Nowak *et al.*, 2012). Another study from Poland conducted from 2009 to 2011 showed that 27.9% of CRAB isolates were positive for OXA-23-like (Nowak *et al.*, 2014). In France, 11 CRAB isolates expressing OXA-23-like were collected over one year from several hospitals in Marseille (Kempf *et al.*, 2013). Moreover, a five-year study across 15 medical centers in Germany showed that 14 out of 25 CRAB isolates were positive for OXA-23-like (Schleicher *et al.*, 2013). Another study investigating 28 CRAB isolates in Sweden showed that 20 of these isolates were positive for OXA-23-like (Karah *et al.*, 2016). In Latvia, an outbreak caused by 35 OXA-23-like positive CRAB isolates across several medical centers was reported between 2008 and 2009 (Saule *et al.*, 2013). Sixteen of 28 CRAB isolates obtained from Serbia were reported to be positive for OXA-23-like (Novovic *et al.*, 2015). Additionally, 77.8% of 116 MDR *A. baumannii* isolates from Portugal were found to harbor *bla*$_{\text{OXA-23-like}}$ in one study (Manageiro *et al.*, 2012) and 63.4% of 213 CRAB isolates in another study harbored this gene (Grosso *et al.*, 2011). In Greece, 72.4% of CRAB isolates from were positive for this gene (Liakopoulos *et al.*, 2012) whereas a study from Finland reported that 33 out of 51 CRAB isolates harbored *bla*$_{\text{OXA-23-like}}$ (Pasanen *et al.*, 2014). Moreover, 9% of 64 CRAB isolates in a multi-center study in Croatia were positive for OXA-23-like (Vranić-Ladavac *et al.*, 2014). In Turkey, a surveillance study from 2000 to 2006 showed that 26 *A. baumannii* isolates were positive for OXA-23-like (Gur *et al.*, 2008). Another study from Turkey showed that 46.6% of CRAB isolates had this enzyme (Keyik *et al.*, 2014). A recent study across 24 hospitals distributed across Germany, Poland, Sweden, and Turkey showed that OXA-23-like was accountable for carbapenem resistance among 83.33% of CRAB isolates (Tomaschek *et al.*, 2016). Another study that characterized 35 *A. baumannii* strains isolated during 28 outbreaks in four Mediterranean countries (Italy, Turkey, Greece, and Lebanon) from 1999 to 2009 showed that OXA-23-like was present in one isolate from Italy and another from Turkey (Di Popolo *et al.*, 2011).

In USA, a study performed in the Miami area from 1994 to 2011 showed that 96 of 9,334 *A. baumannii* isolates were positive for OXA-23-like (Munoz-Price *et al.*, 2013). Another multi-center study in Ohio, USA showed that 18 of 39 *A. baumannii* isolates collected over one year were positive for OXA-23-like (Perez *et al.*, 2010). Yet another study from USA

investigating CRAB isolates obtained from wounded soldiers returning from Iraq showed that 40 out of 42 CRAB isolates had this enzyme (Huang *et al.*, 2012). In Canada, OXA-23-like producing CRAB strains caused an outbreak in 2012 (Ahmed-Bentley *et al.*, 2013). Moreover, a study by Mugnier *et al.* (2010) investigated 20 OXA-23-like positive *A. baumannii* isolates obtained from Vietnam, Thailand, New Caledonia, Australia, Réunion island, South Africa, Tahiti, Libya, Algeria, Egypt, Bahrain, United Arab Emirates, Brazil, France, and Belgium, indicating presence of this carbapenemase in these countries (Mugnier *et al.*, 2010). In Spain, an outbreak of OXA-23-like positive *A. baumannii* strains was reported between October 2010 and May 2011 that affected 17 patients (Mosqueda *et al.*, 2013). Another case of $bla_{\text{OXA-23-like}}$ harboring *A. baumannii* infecting a patient, with a history of hospitalization in Lisbon, was reported in Spain (Espinal *et al.*, 2013). Subsequently, another outbreak between 2011 and 2012 of OXA-23-like producing *A. baumannii* isolates obtained from 50 patients was reported in that country (Merino *et al.*, 2014). In Lebanon, a study from nine hospitals during 2012 showed that 97.89% of 142 CRAB isolates expressed OXA-23-like, indicating a very high prevalence of this enzyme in this country (Hamoudi *et al.*, 2015a). Another study from a Lebanese hospital showed that four *A. baumannii* isolates were positive for OXA-23-like in a three month period in 2011. In the same time frame one year later, seven CRAB isolates that were positive for OXA-23-like were detected (Hamoudi *et al.*, 2015b). Investigation of 42 *A. baumannii* strains collected from Lebanese hospitals between 2009 and 2012 showed that 28 out of 31 carbapenem resistant isolates had OXA-23-like (Rafei *et al.*, 2014a). In northern Lebanon, 65 out of 70 CRAB isolates were found to be positive for OXA-23-like in a two-year period (Rafei *et al.*, 2015b). Interestingly, another study from north Lebanon showed that five CRAB isolates that were obtained from fecal swabs from different livestock animals were all positive for OXA-23-like (Al Bayssari *et al.*, 2015). This indicates that livestock animals could act as a reservoir for CRAB isolates positive for this enzyme in Lebanon.

Another widely disseminated family is the OXA-24-like which consists of OXA-24, OXA-25, OXA-26, and OXA-72. This family could also be located on plasmids or the bacterial chromosome (Potron *et al.*, 2015). Much like OXA-23-like, this family of OXAs is disseminated among *A. baumannii* isolates all around the globe. The first OXA-24 enzyme was identified from *A. baumannii* isolated from Spain (Bou *et al.*, 2000c). Subsequently, in the early 2000s, OXA-24, OXA-25, and OXA-26 were characterized from *A. baumannii* isolates obtained from Spain, Belgium, Kuwait, and Singapore (Afzal-Shah *et al.*, 2001). OXA-72 is predominantly detected among *A. baumannii* isolates in Asian countries such as Japan, China,

South Korea, and Taiwan but is not limited to that region (Poirel *et al.,* 2010). In a study across 12 medical centers in Japan, 5 out of 49 MDR isolates were positive for OXA-72 (Tada *et al.,* 2014). In China, only one out of 221 CRAB isolates was positive for OXA-72 (Wang *et al.,* 2007). Another study across 23 Chinese provinces showed that 11 isolates were positive for OXA-24-like (Ji *et al.,* 2014). One *Acinetobacter baylyi* of 144 MDR *Acinetobacter* spp. was also positive for this carbapenemase in South Korea (Lee *et al.,* 2009). Moreover, 64.13% of 92 CRAB isolates obtained from a hospital in Taiwan over two years were positive for OXA-72 (Lu *et al.,* 2009). In Europe, OXA-72 positive *A. baumannii* strains were responsible for an outbreak in France that lasted for 14 months and affected 16 patients (Barnaud *et al.,* 2010). Another outbreak of *A. baumannii* strains positive for this enzyme was reported to affect 23 patients in Croatia (Goic-Barisic *et al.,* 2011). A study across several Lithuanian hospitals that investigated 444 *A. baumannii* isolates showed that 95% of CRAB isolates were positive for OXA-72 (Povilonis *et al.,* 2012). Isolated cases of CRAB isolates harboring *bla*$_{\text{OXA-72}}$ were also reported from Brazil (Werneck *et al.,* 2011; de Sá Cavalcanti *et al.,* 2013), Colombia (Montealegre *et al.,* 2012), and Spain (Candel *et al.,* 2010).

bla$_{\text{OXA-24-like}}$ has also been detected among 127 CRAB isolates obtained from Algeria (Olaitan *et al.,* 2012). In Egypt, 7.5% of 40 CRAB isolates were positive for OXA-24-like (Al-Agamy *et al.,* 2014). *bla*$_{\text{OXA-24-like}}$ was also detected in outbreak strains in Turkey that affected 22 patients (Sarı *et al.,* 2013). Moreover, five of 117 CRAB isolates obtained from the Cooperation Council for the Arab States of the Gulf were positive for OXA-24-like (Zowawi *et al.,* 2015). In Iran, one study showed that the prevalence of OXA-24-like among CRAB isolates was 1.6% (Sohrabi *et al.,* 2012) while another study showed a prevalence of 49% among isolates obtained from burn patients (Pajand *et al.,* 2014). Furthermore, investigation of 30 CRAB isolates obtained from that same country showed that 73% of them were positive for OXA-24-like (Salimizand *et al.,* 2015). In Lebanon, one *A. baumannii* isolate harboring *bla*$_{\text{OXA-24-like}}$ was reported from one hospital in 2012 (Hammoudi *et al.,* 2015b) and from two isolates in a multi-center study during that same year (Hammoudi *et al.,* 2015a). Another study investigating 31 CRAB isolates from different Lebanese hospitals detected *bla*$_{\text{OXA-24-like}}$ in one CRAB isolate (Rafei *et al.,* 2014a). Additionally, one OXA-24-like producing CRAB isolate was detected in a study across Italy, Greece, Turkey, and Lebanon from 1999 to 2009 (Di Popolo *et al.,* 2011).

The first OXA-24-like detected in USA among CRAB isolates was in 2005 (Lolans *et al.,* 2006). Subsequently, four *A. baumannii* isolates positive for OXA-24-like were identified in Ohio (Perez *et al.,* 2010), three in Pennsylvania (Tian *et al.,* 2011a), and thirteen in Miami (Munoz-Price *et al.,* 2013). CRAB isolates harboring *bla*$_{\text{OXA-24-like}}$ have been reported with varying incidences in countries all across Europe. In a study of 99 CRAB isolates from medical centers in Germany, Poland, Turkey, and Sweden, only two OXA-24-like positive *A. baumannii* were detected over six years (Tomaschek *et al.,* 2016). In Finland, only one out of 51 CRAB isolates was positive for OXA-24-like (Pasanen *et al.,* 2014). Six out of 24 isolates obtained in Sweden over one year were also positive for OXA-24-like (Karah *et al.,* 2016). Additionally, four *A. baumannii* isolates were detected in a study from Bulgaria (Todorova *et al.,* 2014) whereas in Croatia, 27% of 64 CRAB isolates were positive for OXA-24-like (Vranić-Ladavac *et al.,* 2014). Another study from Serbia showed that 82.14% of 28 CRAB isolates had this carbapenemase (Novovic *et al.,* 2015). In Poland, 46.15% of 104 CRAB isolates in one study (Nowak *et al.,* 2012) and 65.6% of 61 CRAB isolates in another were positive for this enzyme (Nowak *et al.,* 2014).

The family OXA-24-like seems to be highly prevalent among *A. baumannii* isolates in the Iberian Peninsula. In Portugal, a study across three medical centers showed that 65 of 162 CRAB isolates had OXA-24-like (Da Silva *et al.,* 2004). Another Portuguese study showed that 23.6% of 116 MDR *A. baumannii* isolates harbored *bla*$_{\text{OXA-24-like}}$ (Manageiro *et al.,* 2012). Yet another study from this country showed a prevalence of 22% of OXA-24-like positive isolates among CRAB isolates (Grosso *et al.,* 2011). In Spain, where OXA-24 was first identified in an outbreak of CRAB strains (Bou *et al.,* 2000c), the prevalence of this enzyme remains high nowadays. 42% of 83 CRAB isolates were positive for this enzyme in a study across 25 Spanish hospitals (Ruiz *et al.,* 2007). In 2006, a large outbreak caused by OXA-24-like producing CRAB isolates was reported in Madrid (Acosta *et al.,* 2011). Moreover, although *A. baumannii* mainly infects patients in the ICU, five CRAB isolates obtained from the internal medicine ward of a Spanish hospital over a six-week period were all positive for OXA-24-like (Tena *et al.,* 2013). Another study from Spain investigating 59 MDR *A. baumannii* isolates showed that 57.6% of these isolates had OXA-24-like (Villalón *et al.,* 2013). A comparative study performed in Spain showed that the prevalence of OXA-24-like rose among CRAB isolates from 48.7% in 2000 to 51.6% in 2010 (Mosqueda *et al.,* 2014). Another long-term study from this country showed that the prevalence of OXA-24-like among CRAB isolates in a single hospital was 40% (Villalón *et al.,* 2015).

The OXA-58-like family is composed of OXA-58, OXA-96, OXA-97, and OXA-164. This family of OXAs can be plasmid-borne or chromosomal and is associated with insertion sequence elements at the origin of its expression (Nowak and Paluchowska, 2016). These enzymes convey a low-level resistance to carbapenems by themselves but their over-expression, due to the presence of insertion sequences, leads to high-level resistance to these antimicrobial agents (Lopes *et al.,* 2012). Though not limited to Europe and Mediterranean countries, most OXA-58-like positive CRAB isolates seem to be reported from that region. The first detection of OXA-58 in *Acinetobacter baumannii* was in France in 2003 (Poirel *et al.,* 2005). Subsequently, six OXA-58-like positive CRAB isolates caused an outbreak in the same region in France among burn patients (Héritier *et al.,* 2005b). OXA-96 was identified in one CRAB isolate from Singapore (Koh *et al.,* 2007) and OXA-97 was identified in 19 isolates from Tunisia (Poirel *et al.,* 2008). Furthermore, OXA-164 was identified in the first six isolates out of thirteen CRAB isolates that caused an outbreak in Germany (Higgins *et al.,* 2010).

In China, OXA-58-like was identified in 7 out of 221 non-repetitive CRAB isolates (Wang *et al.,* 2007). More recently, the prevalence of this enzyme among 143 nosocomial *A. baumannii* isolates was shown to be 51% (Wang *et al.,* 2015). Additionally, OXA-58-like was detected in 32 isolates in a study that spanned across 23 Chinese provinces (Ji *et al.,* 2014). In Taiwan, only two out of 92 CRAB isolates collected over two years were positive for this enzyme (Lu *et al.,* 2009). Four non-repetitive *A. baumannii* isolates positive for OXA-58-like were also detected in USA in 2004 (Lopes *et al.,* 2012). Moreover, one OXA-58-like positive CRAB isolate was detected in the USA among soldiers returning from Iraq (Huang *et al.,* 2012). A study investigating 28 outbreaks affecting 484 patients in 20 hospitals across Greece, Turkey, Italy, and Lebanon showed that 27 of the 35 studied *A. baumannii* isolates were positive for OXA-58-like (Di Popolo *et al.,* 2011). Another study from Greece showed that 27.6% of 174 CRAB isolates had OXA-58-like and one additional isolate co-expressed this enzyme with OXA-23-like (Liakopoulos *et al.,* 2012). In Turkey, 17 OXA-58-like positive CRAB isolates were detected between 2000 and 2006 (Gue *et al.,* 2008). In another study from Turkey, 53.3% of 105 CRAB isolates were positive for OXA-58-like (Keyik *et al.,* 2014). Additionally, 11 out of 28 CRAB isolates from Serbia were positive for OXA-58-like (Novovic *et al.,* 2015). A study from two Egyptian hospitals over a period of four months showed that 5% of 40 CRAB isolates harbored *bla*$_{\text{OXA-58-like}}$ (Al-Agamy *et al.,* 2014). In Algeria, only one of 24 CRAB isolates was positive for OXA-58-like and another had both OXA-58-like and OXA-23-like (Touati *et al.,* 2012). In Lebanon, an outbreak caused by 17 MDR *A. baumannii*

isolates with plasmid-borne OXA-58-like was reported between 2004 and 2005 (Zarrilli *et al.,* 2008). Additionally, two CRAB isolates positive for OXA-58-like were detected in a study from 2009 to 2012 across several Lebanese hospitals (Rafei *et al.,* 2014a). One *A. baumannii* isolate harboring both *bla*$_{\text{OXA-58-like}}$ and *bla*$_{\text{OXA-23-like}}$ was also isolated from livestock animals in north Lebanon (Al Bayssari *et al.,* 2015).

Several studies in Italy have shown the dissemination of OXA-58-like in this country. One study showed that out of 111 MDR *A. baumannii* isolates obtained from 10 Italian ICUs, 22.8% were positive for OXA-58-like (D'Arezzo *et al.,* 2011). Another study from 22 hospitals in that country showed that 154 of 202 CRAB isolates were positive for OXA-58-like alone and an additional 21 isolates co-expressed OXA-58-like with OXA-23-like (Mezzatesta *et al.,* 2012). A study from Rome across eight ICUs showed that half of the 30 MDR *A. baumannii* isolates investigated produced OXA-58-like (Minandri *et al.,* 2012). In Germany, ten OXA-58-like producing *A. baumannii* isolates were detected in a study that spanned from 2005 to 2009 across 15 medical centers (Schleicher *et al.,* 2013). *bla*$_{\text{OXA-58-like}}$ harboring CRAB strains were also responsible for an outbreak that lasted two years and affected 28 patients in a French hospital (Barnaud *et al.,* 2010). In Finland, 8 out of 17 CRAB isolates were positive for OXA-58-like (Pasanen *et al.,* 2014) and 33% of 64 CRAB isolates were positive for this enzyme in a study from Croatia (Vranić-Ladavac *et al.,* 2014). A recent study that span across 24 hospitals in Poland, Sweden, Germany, and Turkey over 6 years showed that 11 of 84 CRAB isolates harbored *bla*$_{\text{OXA-58-like}}$ (Tomaschek *et al.,* 2016). In Spain, one study showed that 20% of 83 CRAB isolates were positive for OXA-58-like (Ruiz *et al.,* 2007). A more recent study showed that 57% of 46 *A. baumannii* isolates selected from 25 Spanish hospitals were positive for OXA-58-like (Fernández Cuenca *et al.,* 2012). In another study from that country, 20.3% of 59 MDR *A. baumannii* isolates were positive for this enzyme (Villalón *et al.,* 2013). A very similar rate was detected in another Spanish study where 20.5% of 101 CRAB isolates were positive for OXA-58-like (Mosqueda *et al.,* 2014). *bla*$_{\text{OXA-58-like}}$ was also identified in six CRAB strains that were part of three sequential outbreaks that took place in a Spanish hospital between 2009 and 2011 (Alvargonzalez *et al.,* 2014). Finally, analysis of *A. baumannii* isolates from a Spanish hospital over 12 years showed that 60% of CRAB isolates harbored *bla*$_{\text{OXA-58-like}}$ (Villalón *et al.,* 2015).

In 2009, a novel class D beta-lactamase with carbapenemase activity was identified in Brazil and was called OXA-143 (Higgins *et al.,* 2009). A variant of this enzyme, the OXA-182

was detected among 12 CRAB isolates obtained from various Korean hospitals (Kim *et al.*, 2010). Two other variants of OXA-143 were detected in Brazil and Honduras and are OXA-231 and OXA-253, respectively (Zander *et al.*, 2014). OXA-253 was also identified in another clinical *A. baumannii* isolate from Brazil (Girlich *et al.*, 2014). This family of OXAs does not seem to be widely disseminated nor highly prevalent, to date. Nevertheless, one *A. baumannii* isolate was positive for OXA-143-like in a study that screened for the presence of *A. baumannii* among environmental and foodstuff samples in Lebanon (Rafei *et al.*, 2015a). Another novel family of OXAs has been identified in a study that investigated 10 *A. baumannii* strains from USA and Mexico. This family includes the chromosomal or plasmid-borne OXA-235, OXA-236, and OXA-237 carbapenemases (Higgins *et al.*, 2013). However, no further reports regarding the prevalence of these enzymes among *A. baumannii* isolates seem to exist. OXA-48-like is another carbapenem hydrolyzing family whose prevalence is mainly reported among *Enterobacteriaceae*. However, one *A. baumannii* isolate obtained from a fecal sample of a nursing home resident in Portugal was shown to be positive for this enzyme (Nowak and Paluchowska, 2016). Nevertheless, the prevalence of this enzyme among *A. baumannii* isolates seems to be very limited. OXAs, as presented above, are widely disseminated all over the globe and pose a real threat to the continued use of carbapenems in the treatment of *A. baumannii* infections. Moreover, their associations with mobile genetic elements facilitates their transfer in between clones and further complicates the global antibiotic resistance problem that we are facing nowadays. The distribution of oxacillinases across the different countries in the world are shown in Table 3.

Table 3. Distribution of Acquired Oxacillinases across the Globe.

OXA-23-like	OXA-24-like	OXA-58-like	OXA-143-like	OXA-235-like	OXA-48
Algeria	Algeria	Algeria	Brazil	Mexico	Portugal
Australia	Bahrain	China	Honduras	USA	
Bahrain	Belgium	Croatia	Lebanon		
Belgium	Brazil	Egypt	South Korea		
Brazil	Bulgaria	Finland			
Canada	China	France			
China	Colombia	Germany			
Croatia	Croatia	Greece			
Egypt	Egypt	Italy			
Finland	Finland	Lebanon			
France	France	Serbia			
Germany	Iran	Singapore			
Greece	Japan	Spain			
Iran	Kuwait	Taiwan			
Iraq	Lebanon	Tunisia			
Italy	Lithuania	Turkey			
Japan	Oman	USA			
Kuwait	Poland				
Latvia	Portugal				
Lebanon	Qatar				
Libya	Saudi Arabia				
Madagascar	Serbia				
New Caledonia	Singapore				
Nigeria	South Korea				
Oman	Spain				
Poland	Sweden				
Portugal	Taiwan				
Qatar	Turkey				
Réunion island	UAE				
Saudi Arabia	USA				
Scotland					
Senegal					
Serbia					
South Africa					
South Korea					
Spain					
Sweden					
Tahiti					
Thailand					
Turkey					
UAE					
USA					
Vietnam					

4.2.2.1.4. *In-vitro* Detection of Beta-Lactamases

Due to the large dissemination and importance on clinical outcome of beta-lactamases, numerous laboratory techniques have been developed for their detection. The following section will discuss the different methods available for the detection of these enzymes, with emphasis on the detection of carbapenemases. The presence of ESBL among clinical isolates could be detected by the double disk synergy test. In this test, the “keyhole effect” resulting from the increase of the inhibition zone of ceftazidime, cefotaxime, and/or aztreonam disks after the

action of clavulanic acid indicates the presence of ESBL (Uzunović *et al.,* 2016). Over-expression of AmpC could also be phenotypically detected by the use of boronic acid or cloxacillin as inhibitors of this enzyme. An increase in the inhibition zone of ceftazidime or cefoxitin disks after the addition of these agents is indicative of AmpC over-production (Reuland *et al.,* 2014). "Blunting" of the inhibition zones of ceftazidime and/or cefotaxime while in proximity of a cefoxitin disk could also be indicative of over-expression of AmpC (Bhattacharjee *et al.,* 2008). Several phenotypic tests have been developed for the detection of the different types of carbapenemases. These include a screening for carbapenemases performed by the modified Hodge test. In this test, the visualization of a "clover leaf" in the inhibition zone of carbapenem disks where the tested isolate is streaked indicates carbapenemase production (Ouertani *et al.,* 2016). KPCs could be phenotypically detected by the increase of the inhibition zones of imipenem and/or meropenem disks after the addition of phenylboronic acid (PBA), its chelator. Similarly, MBLs could be detected by the addition of their chelator, ethylenediaminetetraacetic (EDTA) to carbapenem disks (Tsakris *et al.,* 2010). Another test for the phenotypic detection of MBLs is the use of double disk synergy tests and E-test MBL strips (bioMeriéux, France) that utilize the chelating property of EDTA (Yan *et al.,* 2004). Moreover, a four-fold increase in the MIC as detected by broth dilution methods or E-test strips after the addition of NaCl to the medium could be indicative of OXA production (Pournaras *et al.,* 2006).

Biochemical tests have also been developed for the detection of carbapenemases among *A. baumannii* isolates. These tests include the CarbAcineto NP test (bioMeriéux, France) which detects a change in pH resulting from the carbapenemase activity of the tested isolate (Dortet *et al.,* 2014). Another colorimetric test that detects changes in pH after carbapenem hydrolysis is the Rapid CARB Blue Kit (Rosco Diagnostica, Denmark). This test is able to phenotypically detect the presence of several carbapenemases directly from bacterial cultures (Pires *et al.,* 2013). Ultra violet spectrophotometry for the measurement of the breakdown of imipenem when incubated with sonicated overnight bacterial cultures could also be used for the detection of carbapenemases. However, this technique did not show adequate detection of NDM and OXAs (Bonnin *et al.,* 2012). The detection of the breakdown of imipenem after the identification of the carbapenemases and their metabolites using MALDI-TOF MS could also be used to detect carbapenem hydrolyzing enzymes. This method, though requiring expensive equipment, was shown to be rapid and highly effective at detecting carbapenemases (Kempf *et al.,* 2012a).

Despite the several phenotypic and biochemical tests developed for the detection of carbapenemases, molecular methods remain the golden standard. Numerous singleplex PCRs (for the detection of single genes) and multiplex PCRs (for the detection of several genes at once) have been developed for the detection of all known carbapenemase genes (Nowak and Paluchowska, 2016). Another molecular technique that could also be applied for the detection of carbapenemases is Real-Time, or quantitative qPCR. In this technique, DNA dyes are applied to reaction mixtures similar to those of PCRs and are used for the detection and quantification of the amplified gene after each cycle of amplification. One study has shown the effective detection and quantification of almost all carbapenemase genes identified in *A. baumannii* in just two assays (Pasanen *et al.,* 2014). The Loop-Mediated Isothermal Amplification (LAMP) assay is another technique that could be applied for the detection of carbapenemases. This technique is similar to PCR but does not require a change in temperatures for each amplification step. Additionally, a polymerase with high replication and strand displacement activities is used (Nagamine *et al.,* 2002). In this technique, six different primers targeting eight different regions of the target gene are incubated with the other needed reagents for around an hour under constant temperature conditions. The amplification of the target gene would then occur in a specific and intense manner, to the point where amplification could be detected by the naked eye through visualizing turbidity in the tube. Amplicons could also be detected by spectrophotometric measurements, gel electrophoresis and even in real time if DNA dyes are added to the mixture (Solanki *et al.*, 2013). DNA microarrays that depend on the hybridization of DNA from a nucleic acid sample to oligonucleotide probes fixed on a solid surface is another technique employed for detecting carbapenemases. The presence of a large number of genes could be simultaneously, rapidly, and accurately tested for using this technique (Dally *et al.,* 2013). Finally, Next-Generation Sequencing (NGS) is a technique that could provide a wealth of information regarding the bacterial isolates. NGS not only could detect carbapenemase genes, but it also reveals the entire genetic makeup of the cell and is being increasingly used in the epidemiological investigation of health care-associated outbreaks (Kanamori *et al.,* 2015). The choice of which of these techniques to apply will always have to depend on the aim, availability of funds and equipment, and the required accuracy, speed, and efficiency of the study.

4.2.2.2. Aminoglycoside Modifying Enzymes

Aminoglycoside Modifying Enzymes (AMEs) convey resistance to aminoglycosides through the enzymatic modification of the amino or hydroxyl groups of these antimicrobial agents. This would result in a poor binding between aminoglycosides and ribosomes and would allow the survival of bacterial isolates despite their presence in the cell (Vakulenko, and Mobashery, 2003). AMEs found in *A. baumannii* include the Aminoglycoside Acetyltransferases (AAC), Phosphoryltransferases (APH), and Adenyltransferases (ANT) (Zavascki *et al.,* 2010). The genes that code for these enzyme include *aac(6')-Ih, aac(3)-Ia, aac(3)-IIa, aac(6')-Ib, aph(3')-Ia,* and *aph(3')-VI* that could be incorporated into mobile genetic elements and co-exist with each other (Nowak *et al.,* 2014). Studies reporting the frequency of AMEs among *A. baumannii* clinical isolates are, relatively, not very common. 97% of the amikacin-resistant *A. baumannii* isolates obtained from 75 military and civilian patients in a medical center in USA were positive for the APH gene *aphA6*. Moreover, the ANT genes *aadA1* and *aadB* and the AAC genes *aacC1* and *aacC2* were detected in 39%, 48%, 56%, and 5% of these isolates, respectively (Hujer *et al.,* 2006). A study that investigated 12 *A. baumannii* strains that are representatives of a clone circulating in Europe, in addition to four strains obtained from Belgium, showed that all these isolates harbored the *aphA6* and *aadB* genes (Huys *et al.,* 2005). Another study investigating 106 MDR *A. baumannii* isolates obtained from several European countries showed that 84% had more than one gene coding for AMEs. Seventy six of these isolates were positive for *aphA1*, 55 for *aphA6*, 68 for *aadA1*, 31 for *aadB*, 68 for *aacC1*, 7 for *aacC2*, and 3 for *aacC4* (Nemec *et al.,* 2004). In South Korea, out of 56 *Acinetobacter* spp. isolates, *aac(6')-Ib* was detected in 52, *aph(3')-Ia* in 31, and *aph(3')-VI* in 12 isolates (Sung *et al.* 2011). Another study showed that 8 *A. baumannii* isolates of veterinary origin pertaining to the same clone were all found to have the *aadA1*, *aacC1*, and *aacC2* genes (Endimiani *et al.,* 2011).

A study from Algeria investigating 71 *A. baumannii* clinical isolates showed that *aph(3')-VI* was present in 50.7%, *aadA* in 63.4%, *ant(2")-I* in 14.1%, *aac(3)-Ia* in 91.1%, and *aac(6')-Ib* in 4.2% of the isolates (Bakour *et al.,* 2013). In Iran, one study showed that more than 60% of 60 *A. baumannii* isolates harbored both *aphA6* and *aacC1*, 41.7% harbored *aadA1*, 3.3% harbored *aadB*, and 21.7% harbored *aphA6, aadA1*, and *aacC1* (Moniri *et al.,* 2010). Another study from Iran showed that the most prevalent AME gene was *aph(3')-VIa* where it was detected in 90.6% of 75 *A. baumannii* clinical isolates (Aghazadeh *et al.,* 2013). A study

from Venezuela showed that three out of seven amikacin resistant *A. baumannii* isolates had the *aph(3')-VIa* gene (Salazar De Vegas *et al.*, 2007). An early study from Spain showed that all three types of AMEs were detected among 54 *A. baumannii* isolates. Moreover, the AME coded by *aph(3')-VI* was the most prevalent where it was detected in 28% of the isolates (Vila *et al.*, 1993). Another study from northern Spain showed a predominance of the *aph(3')-VI* gene among amikacin-resistant *A. baumannii* isolates. Moreover, the activity of the ANT(2") and AAC(6')-Ib was detected among 70 *A. baumannii* clinical isolates in this study (Gallego and Towner, 2001). A study in Australia between 2000 and 2010 determined the presence of *aphA1b*, *aphA6*, *aadB*, and *aacC1* co-existing in different combinations in all the aminoglycoside-resistant *A. baumannii* isolates obtained (Nigro *et al.,* 2011). Nevertheless, several studies have concluded that there isn't always a correlation between the detection of these genes and the susceptibility profile of the isolate (Aghazadeh *et al.,* 2013; Salazar De Vegas *et al.,* 2007; Nemec *et al.,* 2004; and Nowak *et al.,* 2014). This could explain the scarcity of studies investigating the prevalence of these isolates in *A. baumannii*.

4.3. Resistance to Colistin

The wide dissemination of CRAB isolates all over the world and the lack of development of new antimicrobial agents have forced clinicians to revert to the use of colistin, despite its nephrotoxic effects (Cai *et al.*, 2012). Colistin has shown very good activity against MDR *A. baumannii* isolates, further encouraging its use in the treatment of these organisms (Li *et al.*, 2006). The increased use of colistin as last-line therapy for infections with CRAB isolates led to the inevitable rise in resistance to this antimicrobial agent. Moreover, resistance to colistin among *A. baumannii* isolates is accompanied by a co-resistance to innate cationic antimicrobial compounds that are produced by the host (Napier *et al.*, 2013). The mechanisms of resistance to colistin are not yet fully understood but several different mutations in the *A. baumannii* genome have been shown to lead to colistin resistance. The common factor between all these mutations is the reduction of the net negative charge of the outer membrane which impedes the interaction between colistin and the bacterial cell (Olaitan *et al.*, 2014). Mutations in the first three genes that are involved in the synthesis of Lipid A have been found to lead to resistance to colistin. These genes are *lpxA*, *lpxC*, and *lpxD*, and mutations in them would result in the complete loss of LPS production and deprive colistin of its target (Moffat *et al.*, 2010). Moreover, transcriptomic analysis by high-throughput RNA sequencing of colistin-sensitive and colistin-resistant *A. baumannii* isolates showed the involvement of six genes in colistin

resistance. These genes are all involved in LPS biosynthesis or electrostatic changes in the bacterial cells. They include the three genes of the *pmr*CAB operon and genes coding for a poly-β-1,6-N-acetylglucosamine deacetylase, a glycosyltransferase, and a putative membrane protein. Moreover, all the colistin-resistant isolates in this study were found to have a modified structure of Lipid A mediated by the addition of phosphoethanolamine (Park *et al.,* 2015). Nevertheless, the most frequently reported cause of colistin resistance in *A. baumannii* is a wide array of mutations in the *pmrCAB* operon (Olaitan *et al.,* 2014).

The *pmrCAB* operon codes for a two-component response regulator and sensor kinase system called pmrA/B. These enzymes respond to environmental change in Fe^{3+} and Mg^{2+} levels and are involved in modifications of Lipid A through *pmrC*, which codes for a phosphoethanolamine transferase (Arroyo *et al.,* 2011). Mutations in *pmrB* are the most frequent mutations reported among clinical colistin-resistant *A. baumannii* isolates (Olaitan *et al.,* 2014). Specifically, a P233S mutation in pmrB is commonly reported to lead to colistin resistance (Pournaras *et al.,* 2014; Kim *et al.,* 2014; Beceiro *et al.,* 2011; and Adams *et al.,* 2009). This activating mutation in pmrB falls within the histidine kinase domain and is involved in dimerization (Durante-Mangoni *et al.,* 2015). Moreover, over-expression of *pmrA* and *pmrB* was found to lead to colistin resistance (Park *et al.,* 2011). The down-regulation of these genes was also found to reverse the modifications in Lipid A and colistin resistance (Harris *et al.,* 2014). This shows that the *pmr*CAB operon is a key factor in acquired resistance to colistin.

A. baumannii has been shown to be able to develop *in-vitro* resistance to colistin upon exposure to this antimicrobial agent (Tan *et al.,* 2007). Colistin resistance in *A. baumannii* is normally reported in isolated sporadic cases in regions all over the world (Cai et al., 2012). Surveillance studies indicate that, although *A. baumannii* isolates that are resistant to colistin are usually PDR, their incidence is relatively rare (Falagas and Bliziotis, 2007). However, this could be due to the fact that MIC determination by broth dilution techniques, which are considered as the golden standard for testing for this resistance, are not routinely applied in all laboratories (Hindler and Humphries, 2013). Nevertheless, infection with colistin-resistant PDR isolates is associated with high mortality rates and these isolates deserve special attention (Falagas *et al.,* 2008). The first colistin-resistant *A. baumannii* isolate was reported in the Czech Republic in 1999 (Cai *et al.,* 2012). In 2005, another colistin-resistant *A. baumannii* clinical isolate was reported from South Korea. However, after further investigation, 15 out of 16

studied isolates developed heteroresistance to colistin. Colistin heteroresistance was henceforth defined as the emergence of resistance through a sub-population from an otherwise susceptible population (Li *et al.,* 2006). Heteroresistance has usually a higher rate than that of resistance but its detection is laborious and requires special equipment. Therefore, detection of heteroresistance is not routinely performed in all laboratories (Cai *et al.,* 2012). One study investigating heteroresistance among clinical *A. baumannii* isolates showed that although all 75 isolates studied showed sensitivity to colistin, 14 of them showed heteroresistance to this antimicrobial agent (Herrera *et al.,* 2011). Another study from Argentina showed that 46.4% of 28 isolates obtained from ICU patients were heteroresistant to colistin (Rodriguez *et al.,* 2009). Yet another study from Argentina showed that 6 out of 14 *A. baumannii* isolates were heteroresistant to this antimicrobial agent (Rodriguez *et al.,* 2010). In Brazil, 26 out of 29 randomly selected CRAB isolates showed heteroresistance to polymyxin B (Barin *et al.,* 2013). Furthermore, a surveillance study from the western Pacific region showed that, of the 30 *A. baumannii* isolates obtained from various countries, one was resistant to colistin and seven showed heteroresistance (Yau *et al.,* 2009).

A study across two hospitals in South Korea showed that 18.1% of 214 clinical *A. baumannii* isolates were resistant to polymyxin B and 27.9% were resistant to colistin (Ko *et al.,* 2007). Another study in Iran showed that 14.2% of 91 *A. baumannii* isolates were resistant to colistin (Bahador *et al.,* 2013). In USA, half of 28 XDR *A. baumannii* isolates longitudinally recovered during colistin therapy were shown to be colistin-resistant (Lesho *et al.,* 2013). In Malaysia, 25.9% of clinical *A. baumannii* isolates collected from a tertiary care center were resistant to polymyxin B (Lean *et al.,* 2014). A surveillance study across European countries reported a rate of colistin resistance among *A. baumannii* isolates of 2.7% (Souli *et al.,* 2008). A study from Bulgaria investigating 18 *A. baumannii* clinical isolates showed that 3 of these isolates were resistant to colistin (Dobrewski *et al.,* 2006). Although rates of colistin resistance are reported to be low in most studies around the world, several Spanish studies have reported an alarmingly high rate of resistance to this antimicrobial agent. In one Spanish study, colistin resistance was detected in 19.1% of 115 *A. baumannii* isolates (Arroyo *et al.,* 2005). Another study from Spain over six years showed that 40.67% out of 150 *A. baumannii* isolates were resistant to colistin (Arroyo *et al.,* 2008). Additionally, an outbreak affecting 12 patients in a tertiary care center in Spain that lasted for around one year was shown to be caused by colistin-resistant PDR *A. baumannii* strains (Valencia *et al.,* 2009). Finally, it is worth mentioning the recently discovered plasmid-borne *mcr-1* gene that conveys resistance to colistin. This gene

has been detected among a large number of Gram-negative organisms isolated from all over the world. It has even been detected through retrospective studies in isolates obtained in the 1980s (Skov and Monnet, 2016). However, to date, this gene has not yet been identified in *A. baumannii* (Thi Khanh Nhu *et al.*, 2016).

5. GLOBAL EPIDEMIOLOGY OF RESISTANT *Acinetobacter baumannii*

Advances in molecular biology have allowed the investigation of the clonality of MDR *A. baumannii* isolates and their tracking all throughout the world. The strengths and weaknesses of some of the most commonly used molecular techniques in clonality analysis, as well as the global dissemination of MDR *A. baumannii* clones, will be discussed in the following sections.

5.1. Common Molecular Tools Used for Epidemiological Studies

These molecular tools used for epidemiological studies range from unspecific amplification of the different segments of the DNA to whole-genome sequencing. Random Amplified Polymorphic DNA (RAPD) analysis is a fast molecular tool that could be used in order to determine clonality. This technique has been developed in the early 1990s and has been extensively used since then in the differentiation of bacterial clones (Sharma *et al.*, 2002). In this PCR-based technique, random 10-mer primers are used that selectively amplify different DNA segments depending on their ability to anneal to- and trigger the amplification of- each fraction of the chromosome (Williams *et al.*, 1990). Low annealing temperatures are usually used in this technique and the amplified segments would then be run on agarose gels and visualized. The Single Nucleotide Polymorphisms (SNPs) present in the chromosome will result in the amplification of a unique clone-specific pattern (Hsueh *et al.*, 1998). This technique has proven to be a powerful typing method that is fast and cheap to implement. It produces specific patterns of amplicons that could allow for the determination of clonality without the need of prior knowledge of the nucleotide sequence. However, the results heavily depend on the type of primers used and inter-laboratory comparison of results is not possible without the physical exchange of the strains' DNA (Mbawna *et al.*, 2006).

Another technique that allows for the detection of clonality, with a higher discriminatory power than RAPD, is Pulsed Field Gel Electrophoresis (PFGE) (Mbawna *et al.*, 2006). In this technique, the whole genome of the tested strain is digested by a RE which generates a unique DNA fingerprint for each clone. The DNA segments are then run on an

agarose gel using a Contour-Clamped Homogeneous Electric Field (CHEF) apparatus which generates alternating currents in different directions. This would result in a high resolution image of DNA bands of different sizes that could be analyzed by imaging software. Then, specific patterns obtained are assigned to a specific clone (Seifert *et al.*, 2005). PFGE has several advantages that include a high rate of reproducibility, a high discriminatory power, and not requiring prior knowledge of the genomic sequences. However, it is a laborious and time-consuming technique and interpretation of the patterns may vary according to the person performing the experiment. Moreover, inter-laboratory comparison of results is often not possible without the physical exchange of the bacterial isolates (Kjeldsen *et al.*, 2015).

In 2007, a simple and fast method for the detection of clonality was developed by Turton *et al.,* (2007). This method exploits the SNPs present in three housekeeping genes in the *A. baumannii* genome, allowing for the differential amplification of the different alleles present in different clones. Two sets of primers divided among two multiplex PCRs were developed that were targeted at the intrinsic *OmpA*, *CsuE*, and *bla*$_{\text{OXA-51-like}}$ genes. The primers were designed in such a manner that they would amplify different combinations of alleles in the different multiplex PCRs and would generate a specific pattern of amplified genes. Once the pattern has been associated with a certain clone through further investigation, other laboratories could allocate their isolates to one of these clones and determine the international clonality of their strains by the use of these PCRs (Turton *et al.*, 2007). Fourteen different amplification patterns have been allocated to International Clones (ICs), the most widely disseminated of which are IC I, IC II, and IC III. The patterns of groups 1, 2, and 3 identified using this method were found to pertain to IC II, IC I, and IC III, respectively (Karah *et al.*, 2012). This technique is fast, simple, and does not require complicated machinery nor expensive material. Moreover, it allows for the comparison of the isolates on a world-wide scale, in addition to the local determination of clonality. Nevertheless, since it targets only three genes, it does not provide a high discriminatory power for subdivisions among clones. Additionally, not all the clones could be detected by the identified patterns and characterization of new patterns needs to be done before being able to rely on their multiplex PCR profile (Martins *et al.*, 2016).

Multi-Locus Sequence Typing (MLST) is yet another technique used in the identification of clonality that produces data that is comparable on a world-wide scale (Hamouda *et al.*, 2010). This technique is based on the comparison of the sequences of seven

housekeeping genes that, in turn, generate a profile that allows for the determination of clonality. The generated profiles could then be uploaded to online databases, allowing for the comparison and determination of clonality on a local and international level (Karah *et al.,* 2012). Two MLST schemes have been developed for *A. baumannii*. The first is called the Oxford, or Bartual, scheme and is based on the sequencing of *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD* (Bartual *et al.,* 2005). The second scheme is called the Pasteur scheme and relies on the sequencing of *gltA*, *recA*, *cpn60*, *fusA*, *pyrG*, *rpoB,* and *rplB* (Diancourt *et al.,* 2010). Both schemes were shown to yield comparable results. However, the Oxford scheme has been shown to have a higher resolution than the Pasteur scheme (Grosso *et al.,* 2011). The determined clones are often referred to as Sequence Types (STs) and from here onwards, the superscript "^{O}" will be used to indicate that the MLST data was obtained using the Oxford scheme and "^{P}" will indicate the use of the Pasteur scheme. Moreover, Clonal Complexes (CCs) is an MLST-based nomenclature used to describe a group of STs that share the same sequences of 5 or 6 out of the 7 housekeeping genes (Karah *et al.,* 2012). MLST is highly advantageous in terms of exchangeability of data on a world-wide scale and relative ease of performing it. However, sequencing services may not be readily available in all laboratories, the two different schemes may result in some confusion when comparing the data, and its resolution is limited to the seven genes. Moreover, using this method as a basic epidemiological tool could be time consuming as compared to other methods (Pobeiga *et al.,* 2013).

One high-throughput alternative to MLST is Whole-Genome Sequencing (WGS). This technique provides a complete record of the isolates' genome and allows the tracking of all the genetic changes taking place. WGS is being used in epidemiological studies and provides highly accurate information that could be the definitive form of clonality analysis (Harris *et al.,* 2010). WGS is undoubtedly a very powerful analytical tool that has numerous advantages that include the comparability of data worldwide, the wealth of information provided, and the accuracy of the data. However, this method is very expensive to be implicated in routine laboratory testing and requires access to next generation sequencers, which are not readily available to all laboratories. Moreover, the assembly algorithms used, as well as the annotation pipelines chosen, could result in discrepancies of reported data between different laboratories. Finally, the prolonged times needed for the assembly and analysis of the vast amount of data obtained from genomes could be time consuming and overwhelming if a large number of isolates is to be analyzed (Kwong *et al.,* 2015).

5.2. Worldwide Dissemination of Multi-Drug Resistant *A. baumannii* Clones

There are several MDR *A. baumannii* clones that are more widely disseminated than others. International Clones I, II, and III are the most commonly reported from all over the world (Karah *et al.*, 2012). These clones were previously called European clones since they were first identified in European countries. However, their increased detection in countries all around the world resulted in the change of their name to International Clones (Diancourt *et al.*, 2010). Although these three ICs are the most widely disseminated, other international clones have also been shown to be prevalent in certain regions. Studies have shown that most *A. baumannii* infections caused all over the world are caused by a few ancestrally related clones. One such study investigating the clonality of 496 *A. baumannii* isolates from all over the world showed that 283 non-sporadic strains pertained to only 26 clones. Of the 26 clones, 18 were disseminated in more than one continent, 6 were restricted to Europe, and 2 restricted to Asia (Karah *et al.*, 2012). This shows the success of certain clones to persist and disseminate cross vast regions.

In the aforementioned study, $CC92^{B}/CC2^{P}$ was found to be by far the most widely disseminated clonal complex. It was detected in 34 countries spread across the five continents (Karah *et al.*, 2012). This clonal complex is very similar to IC II and STs within this complex are considered as pertaining to this IC (Hamouda *et al.*, 2010). IC II is implicated in numerous MDR *A. baumannii* outbreaks and is commonly reported to harbor a wide array of resistance mechanisms, which include production of OXA-23-like, OXA-24-like, and/or OXA-58-like. Additionally, though some carbapenem-sensitive isolates belonging to IC II have been reported, strains pertaining to this clone are almost always MDR (Karah *et al.*, 2012). Different pulsotypes, as detected by PFGE, could be found among MDR isolates pertaining to IC II. This is probably due to the different selection pressures in different regions (Nemec *et al.*, 2004). Additionally, PDR *A. baumannii* isolates resistant to colistin (Park *et al.*, 2010) and to colistin and polymyxin B (Park *et al.*, 2009) were reported to pertain to this clone.

An intercontinental study showed that MDR *A. baumannii* isolates harboring $bla_{\text{OXA-23-like}}$ obtained from three different continents pertained to IC II and that these isolates had similar PFGE patterns (Mugnier *et al.*, 2010). In another study, *A. baumannii* isolates pertaining to IC II were detected in Singapore, China, Portugal, Spain, Belgium, Italy, Greece, United Kingdom, and USA (Zarrilli *et al.*, 2013). IC II was found to be widely disseminated in China,

where it was detected in both carbapenem-resistant and carbapenem-sensitive isolates. 86.8% of the CRAB isolates obtained from 16 different Chinese cities pertained to this clone and were positive for OXA-23-like. However, not all the strains belonging to IC II had the same pulsotype (Fu *et al.,* 2010). Another study from China showed that 72 CRAB isolates belonged to this clone but had at least three different pulsotypes (Ho *et al.,* 2010). In western China, 36 representative *A. baumannii* isolates obtained from ten hospitals were all found to belong to IC II (He *et al.,* 2011). A study from Japan investigating 79 *A. baumannii* isolates collected from 2001 to 2012 also showed a predominance of $ST2^{P}$ (Suzuki *et al.,* 2014). Another study from this country showed that eight *A. baumannii* isolates obtained between 2004 and 2013 had a similar pulsotype and pertained to $ST2^{P}$ (Yamada *et al.,* 2016). Moreover, an MBL-positive *A. baumannii* isolate obtained from Taiwan was found to pertain to this clone (Zarrilli *et al.,* 2013). In South Korea, a large outbreak of OXA-23-like-positive CRAB strains pertaining to $CC92^{B}$ was reported in 2008 (Lee *et al.,* 2011). A study in the Miami area in USA showed that 14% of 144 CRAB isolates collected from 1994 to 2011 pertained to $ST2^{P}$ (Munoz-Price *et al.,* 2013). Moreover, 12 *A. baumannii* isolates pertaining to IC II were responsible for an outbreak that took place in Los Angeles (Warner *et al.,* 2016). Additionally, though this clone is not pre-dominant in Brazil, it was detected for a limited time in this country (Martins *et al.,* 2013).

A study from Kenya showed that 8 out of 16 selected CRAB isolates pertained to IC II (Revathi *et al.,* 2013). In South Africa, the presence of IC II among CRAB isolates was detected although it wasn't the pre-dominant clone in that country (Lowings *et al.,* 2015). IC II was also detected among 15 *A. baumannii* isolates that were representative of 117 CRAB strains obtained from Saudi Arabia, Oman, Qatar, United Arab Emirates, Bahrain, and Kuwait (Zowawi *et al.,* 2015). In the Kurdistan region in Iraq, IC II was found to be predominant among ACB isolates (Ganjo *et al.,* 2016). In Iran, 36 out of 62 *A. baumannii* isolated pertained to IC II (Bahador *et al.,* 2015) and 22 of 98 *A. baumannii* isolates were found to belong to IC II in Turkey (Metan *et al.,* 2013). A study that involved several Mediterranean countries showed that isolates pertaining to IC II were identified from two hospitals in Italy and three hospitals from Greece (Giannouli *et al.,* 2009). Another study investigating 35 MDR *A. baumannii* isolates from Mediterranean countries showed that IC II is present in Italy, Greece, and Lebanon (Di Popolo *et al.,* 2011). In Lebanon, a study investigating 42 *A. baumannii* isolates collected from different hospitals showed a predominance of IC II, where it was detected in 34 of these isolates (Rafei *et al.,* 2014a). Another study from northern Lebanon analyzing 57 randomly selected *A. baumannii* isolates also showed that IC II is the most

prevalent (Rafei *et al.,* 2014b). Yet another Lebanese study investigating isolates obtained from livestock animals showed that one out of five CRAB isolates pertained to IC II (Al Bayssari *et al.,* 2015). Interestingly, a study from Switzerland also showed the prevalence of this clone among *A. baumannii* isolates obtained from pets and horses (Endimiani *et al.,* 2011).

IC II has also been reported to be widely disseminated in Europe. Similarly to what was reported in China, a study from Norway showed that isolates pertaining to this clone had several discreet pulsotypes (Karah *et al.,* 2011a). Sixteen out of 28 CRAB isolates obtained over a one year period pertained to IC II in Sweden (Karah *et al.,* 2016). In Greece, CRAB isolates pertaining to IC II have been reported on several occasions (Zarrilli *et al.,* 2013). IC II was also shown to be the predominant clone in a study that investigated 26 distinct outbreaks in 24 hospitals spread across Sweden, Poland, Germany, and Turkey (Tomaschek *et al.,* 2016). In Croatia, 19% of 150 *A. baumannii* isolates pertained to IC II, all of which were carbapenem-resistant (Vranić-Ladavac *et al.,* 2014). An outbreak caused by 30 *A. baumannii* isolates, most of which pertained to IC II, was reported between 2008 and 2009 in Latvia (Saule *et al.,* 2013). Moreover, investigation of 28 CRAB isolates obtained from Serbia showed that 67.68% of them pertained to IC II (Novovic *et al.,* 2015). In Italy, a multi-center study showed that 95.6% of MDR *A. baumannii* isolates obtained from ICUs between 2005 and 2009 pertained to IC II. Moreover, these strains showed genetic similarity to the OXA-58-like positive strain that previously caused outbreaks across several ICUs in Rome (D'Arezzo *et al.,* 2011). Another study from Italy showed that, although all 30 *A. baumannii* isolates investigated pertained to IC II, there seems to be a shift from harboring *bla*$_{\text{OXA-58-like}}$ to harboring *bla*$_{\text{OXA-23-like}}$ among those clonally related strains (Minandri *et al.,* 2012). More recently, an outbreak caused by a strain pertaining to IC II was also reported from northeast Italy (Milan *et al.,* 2016). A study across 15 German medical centers showed that only 32% of 140 *A. baumannii* isolates belonged to ICs, 34 of which pertained to IC II (Schleicher *et al.,* 2013). Another study from Germany showed that the majority of CRAB isolates collected over four years pertained to IC II, although they were of different sequence types (Rieber *et al.,* 2016). In Portugal, ST92^{B} and closely related single-locus variants, all pertaining to IC II, were found to be endemic in the country (Manageiro *et al.,* 2012). Another study from southern Poland also showed the high prevalence of IC II where it was detected in 79 out of 125 *A. baumannii* isolates (Chmielarczyk *et al.,* 2016). In Spain, an OXA-23-like positive strain belonging to IC II was responsible for an outbreak in Barcelona that affected 17 patients and lasted from October 2010 to May 2011 (Mosqueda *et al.,* 2013). Another study from Spain investigating 405 ACB isolates showed

that 60% of these isolates belonged to IC II (Villalón *et al.,* 2015). Moreover, comparison of *A. baumannii* isolates over a ten-year period in Spain showed that IC II is still the predominant clone nowadays (Garnacho-Montero *et al.,* 2016).

Another widely disseminated clonal complex that was detected in 31 countries all over the world is $CC109^{B}/CC1^{P}$ (Karah *et al.,* 2012). This complex was found to be closely related to IC I and isolates within it are considered as such (Evans *et al.,* 2008). MDR *A. baumannii* isolates pertaining to this clone harboring *bla*$_{\text{VIM-4}}$, *bla*$_{\text{OXA-23-like}}$, and/or *bla*$_{\text{OXA-58-like}}$ have been reported from different studies around the world (Karah *et al.,* 2012). CRAB isolates belonging to IC I were detected from Greece, Germany, Belgium, United Kingdom, France, Czech Republic, Turkey, Algeria, Iraq, South Korea, China, India, Brazil, USA, Bangladesh, Singapore, and Taiwan (Zarrilli *et al.,* 2013). Another study that investigated *A. baumannii* isolates from all around the world identified isolates pertaining to IC I from India, Greece, Iraq, Czech Republic, Romania, Norway, Sweden, France, Germany, USA, and Canada (Karah *et al.,* 2015). In Japan, although a predominance of IC II was shown, three *A. baumannii* strains collected over eleven years pertained to IC I (Suzuki *et al.,* 2014). IC I was also detected in USA among soldiers that were wounded during the conflicts in Iraq (Huang *et al.,* 2012). Twenty clonally related *A. baumannii* isolates pertaining to IC I were also responsible for an outbreak in Los Angeles, USA (Warner *et al.,* 2016). IC I is also reported to be one of the predominant clones in Brazil (Martins *et al.,* 2016), certain regions of Argentina (Stietz *et al.,* 2013), and South Africa (Lowings *et al.,* 2015). This clone was also detected in 29% of 62 *A. baumannii* strains in Iran (Bahador *et al.,* 2015) and 18 of 98 *A. baumannii* strains in Turkey (Metan *et al.,* 2013). Additionally, in one study involving several Mediterranean countries, strains belonging to IC I were identified from Greece, Italy, and Lebanon (Giannouli *et al.,* 2009). In another Mediterranean study, IC I was present among CRAB isolates obtained from Italy and Greece (Di Popolo *et al.,* 2011). In Lebanon, seventeen *A. baumannii* isolates pertaining to IC I and positive for OXA-58-like were responsible for an outbreak in a Lebanese hospital (Zarrilli *et al.,* 2008). Moreover, *A. baumannii* isolates pertaining to IC I were detected in 12 out of 57 isolates obtained from north Lebanon (Rafei *et al.,* 2014b).

Six strains pertaining to IC I were involved in outbreaks as reported by a study investigating 78 outbreak-related strains from Germany, Sweden, Poland, and Turkey (Tomaschek *et al.,* 2016). In Croatia, although a predominance of IC II was shown among CRAB isolates, IC I was highly prevalent among 185 *A. baumannii* isolates, without

necessarily being carbapenem-resistant (Vranić-Ladavac *et al.,* 2014). In Serbia, only one out of 28 CRAB isolates pertained to IC I (Novovic *et al.,* 2015) whereas a study from a Belgian burn center reported that ten *A. baumannii* strains obtained from patients wounded in North Africa were XDR and pertained to IC I (De Vos *et al.,* 2016). In a study from Germany, 6 out of 45 isolates that were grouped into clones belonged to IC I (Schleicher *et al.,* 2013). Moreover, this clone was detected among *A. baumannii* isolated from pets and horses in a study from Switzerland (Endimiani *et al.,* 2011). ST-81^{P}, which is closely related to IC I and is considered as such, was detected in northern Spain in 10.2% of 729 *A. baumannii* isolates collected from 12 Spanish hospitals. Nevertheless, this clone was only detected between 1999 and 2002 and seems to disappear in subsequent years, as reported by this eleven-year study (Villalón *et al.,* 2011).

CC187^{B}/CC3^{P} is another widely disseminated clonal complex that is considered as IC III. This clone has been detected in several European countries such as France, Germany, Netherlands, Italy, Spain, and Belgium, in addition to USA and Lebanon (Karah *et al.,* 2012). MDR *A. baumannii* isolates pertaining to this clone have been reported to harbor *bla*$_{\text{OXA-58-like}}$ (Diancourt *et al.,* 2010). In USA, this clone was detected among wounded soldiers returning from Iraq (Huang *et al.,* 2012). IC III was also detected among representative CRAB isolates obtained from Saudi Arabia, United Arab Emirates, Qatar, Oman, Kuwait, and Bahrain (Zowawi *et al.,* 2015). A clone pertaining to IC III, which was negative for all tested carbapenemases, was also identified in Beirut, Lebanon (Giannouli *et al.,* 2009). In another study in that country, one sporadic isolate detected in a Lebanese hospital was found to belong to IC III (Zarrilli *et al.,* 2008). Yet another study from Lebanon showed that CRAB isolates obtained from four patients pertained to this clone (Di Popolo *et al.,* 2011). Sporadic *A. baumannii* isolates pertaining to IC III were also detected from north Lebanon in two separate studies (Rafei *et al.,* 2014a; Rafei *et al.,* 2014b). In Serbia, seven CRAB isolates obtained over two years were found to belong to IC III (Novovic *et al.,* 2015) while in Spain 26.7% of 405 ACB isolates pertained to IC III (Villalón *et al.,* 2015). Another study that lasted for 11 years across 19 Spanish hospitals showed that 5.1% of 729 *A. baumannii* isolates belonged to IC III (Villalón *et al.,* 2011). This clone, though detected in various countries around the world, was shown to have a limited intra-clonal diversity that could indicate a relatively short-term existence (Karah *et al.,* 2012).

ST25^{P} is another well-established international clone that has been reported for around 25 years (Di Nocera *et al.,* 2011). MDR *A. baumannii* isolates pertaining to ST25^{P} have been reported to be positive for OXA-23-like, OXA-24-like, and/or OXA-58-like (Bonnin *et al.,* 2011c). One study showed that ST25^{P} is present among *A. baumannii* isolates originating from Thailand, Colombia, Argentina, USA, Iraq, United Arab Emirates, Serbia, Sweden, Netherlands, Greece, and Italy (Karah *et al.,* 2015). Isolates pertaining to ST25^{P} were also reported from Spain, Italy, Croatia, Taiwan, South Korea, China, and Brazil in another study (Zarrilli *et al.,* 2013). Yet another study showed the presence of this clone in Turkey, Greece, Italy, Netherlands, Singapore, and USA (Karah *et al.,* 2012). A Mediterranean study also showed that this clone is present in Italy, Greece, and Turkey (Di Popolo *et al.,* 2011). ST25^{P} was also detected among two outbreak-related *A. baumannii* strains in a study that spanned across 24 hospitals located in Germany, Poland, Turkey, and Sweden (Tomaschek *et al.,* 2016). Moreover, a recent study in Sweden showed that 7 out of 28 CRAB isolates pertained to this sequence type (Karah *et al.,* 2016). ST25^{P} was also found to be widely disseminated in Brazil and is considered as one of the major clones circulating in that country (Martins *et al.,* 2016). Additionally, investigation of 16 CRAB isolates in east Africa reported the presence of this ST among these isolates (Revathi *et al.,* 2013). Another study across several Lebanese hospitals showed that 3 out of 42 two *A. baumannii* isolates pertained to ST25^{P} (Rafei *et al.,* 2014a). A study from north Lebanon also showed the presence of this clone among six randomly selected *A. baumannii* isolates (Rafei *et al.,* 2014b).

A less widely disseminated clonal complex is CC131^{B}/ST79^{P}. *A. baumannii* isolates harboring *bla*$_{\text{OXA-23-like}}$, and/or *bla*$_{\text{OXA-58-like}}$ have been reported within this clonal complex (Villalón *et al.,* 2011). A study from USA showed that ST79^{P} represented 76% of *A. baumannii* isolates collected over eighteen years, making it the most prevalent in that country (Munoz-Price *et al.,* 2013). Moreover, this clone was also found to be pre-dominant in Brazil (Martins *et al.,* 2016) and Argentina (Stietz *et al.,* 2013). ST79^{P} was also described in Spain and represented 13.6% of 729 *A. baumannii* isolates (Villalón *et al.,* 2011). CC104^{B}/CC15^{P} is yet another international clonal complex that was detected in 9 countries spread across Europe and South America. MDR isolates of this clone were reported to harbor *armA*, *bla*$_{\text{OXA-23-like}}$, and/or *bla*$_{\text{OXA-58-like}}$ (Grosso *et al.,* 2011). An eleven-year study from Spain showed that 1.7% of 729 *A. baumannii* isolates pertained to this clonal complex (Villalón *et al.,* 2011). Moreover, 6.7% of 405 ACB isolates were found to pertain to this sequence type in another study from Spain (Villalón *et al.,* 2015). *A. baumannii* isolates pertaining to ST15^{P} are reported to be among the

major clones disseminated in Brazil (Martins *et al.,* 2016) and the second most prevalent in Argentina (Stietz *et al.,* 2013). In Turkey, this clone also seems to be predominant where 41 of 98 *A. baumannii* isolates were found to pertain to it (Metan *et al.,* 2013). One isolate of this clone was also reported in Norway from an imported case from Pakistan (Karah *et al.,* 2011a).

CC20^{B} is another an international clone which harbors MDR *A. baumannii* isolates producing OXA-23-like and ArmA (Fu *et al.,* 2010). Isolates pertaining to ST20^{P} were detected in USA (Karah *et al.,* 2015) and in one CRAB isolate obtained from livestock animals in Lebanon (Al Bayssari *et al.,* 2015). *A. baumannii* isolates pertaining to yet another international clone called CC110^{B} were detected in Korea, Argentina, and USA. The isolate obtained from Korea was also resistant to colistin (Park *et al.,* 2010). This clone was also detected among CRAB isolates obtained from Italy and Lebanon (Di Popolo *et al.,* 2011). CC32^{P} is another clone that was detected in several European countries that include Denmark, Sweden, Portugal and Spain (Karah *et al.,* 2012). In Spain, 1.7% of 729 *A. baumannii* isolates pertained to this clone (Villalón *et al.,* 2011). Moreover, one CRAB isolate pertaining to this clone was found to be positive for IMP-5 in a study from Portugal (Da Silva *et al.,* 2010). Additionally, this clone was detected in Italy, North Korea, Japan, Singapore, China, India, and Brazil (Zarrilli *et al.,* 2013).

Some MDR *A. baumannii* strains pertaining to STs that are limited in geographical distribution have caused noteworthy outbreaks and have important clinical significance. One such strain is the *bla*$_{\text{OXA-58-like}}$ harboring *A. baumannii* strain belonging to ST78^{P} that caused an outbreak lasting for two years in Italy (Giannouli *et al.,* 2010). Another strain belonging to this ST was found to be positive for CTX-M and OXA-24-like and was prevalent in the Miami area in USA. ST229^{P} is another clone that was detected in USA and represented 10% of 144 CRAB isolates (Munoz-Price *et al.,* 2013). ST96^{B} is another clinically significant strain that caused an outbreak in Honk Kong and was positive for OXA-23-like (Ho *et al.,* 2010). Moreover, an *A. baumannii* strain pertaining to ST56^{B} caused an outbreak that started in 2006 and lasted for more than 30 months. This outbreak is among the largest to be reported in the literature where it affected 337 patients in a hospital in Madrid, Spain (Acosta *et al.,* 2011). Moreover, this clone was found to be among the predominant clones in a study that assessed the changes in *A. baumannii* infections over a ten-year period in Spain (Garnacho-Montero *et al.,* 2016). Another MDR clone that has shown its ability to be transmitted across several Spanish hospitals is ST80^{P}. In one study, this clone was detected in 6.7% of 405 ACB isolates collected over 12

years from a Spanish hospital (Villalón *et al.*, 2015). In another study, this clone was detected in 20.3% of 729 epidemic strains isolated from 12 Spanish hospitals over an 11-year period (Villalón *et al.*, 2011). The ST94^{P} clone is a bit more disseminated across countries and was detected among isolates obtained from India, Iraq, and USA (Karah *et al.*, 2015). Moreover, a CRAB isolate pertaining to ST103^{P} that was isolated from a patient transferred from Egypt to Germany was found to be positive for NDM-2 (Kaase *et al.*, 2011). Other clinically significant MDR *A. baumannii* clones include ST115^{B}, ST125^{B}, and ST134^{B}, which are positive for OXA-23-like, and the CTX-M-15-positive ST117^{P} and ST118^{P} (Karah *et al.*, 2012). As is presented above, numerous MDR *A. baumannii* clones were able to disseminate into wide geographical regions and persist for prolonged periods of time. This could be due to an interplay between the genetic plasticity of this organism (Antunes *et al.*, 2014) and the different selective pressures exerted on it (Nemec *et al.*, 2004). These factors give rise to these successful clones that are capable of wide dissemination and prolonged persistence. Additionally, the international dissemination of these clones could be enhanced by the increase in international travel and the interchange of patients on a global scale (Peleg *et al.*, 2008). The global spread of clinically important clones are demonstrated in Figure 6.

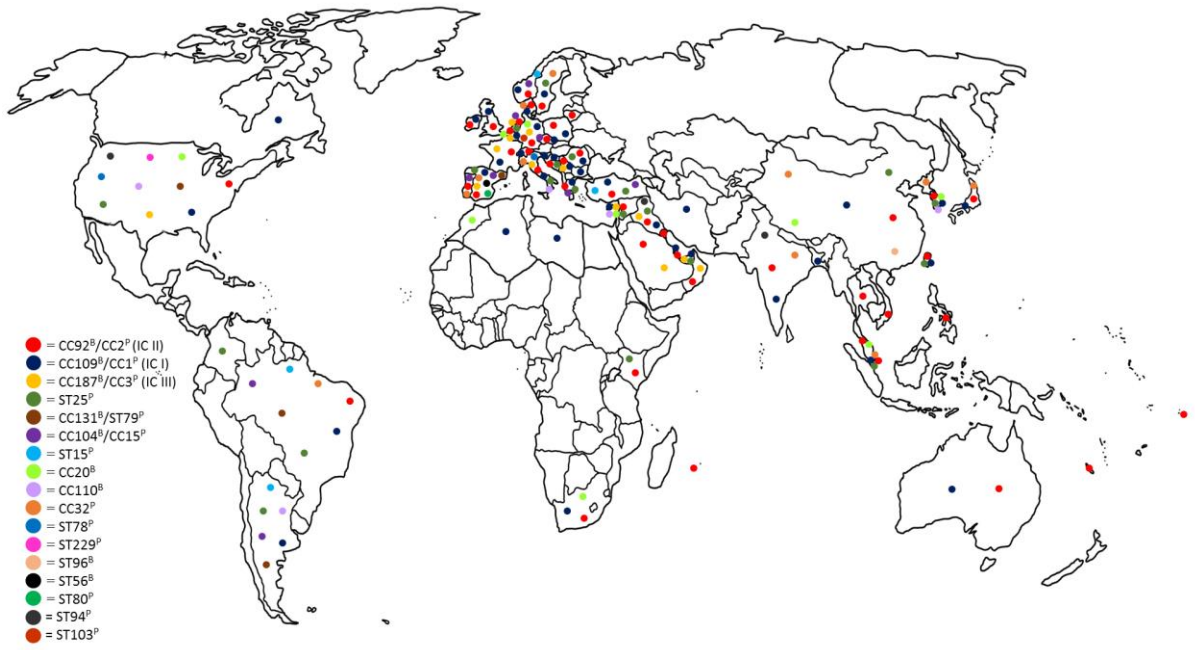


Figure 6. Global dissemination of clinically important clones. The Dots within each country are not meant for geographical accuracy but are a graphical representation of which clones were reported from that country.

5.3. Current Global Rates of Resistant *Acinetobacter baumannii*

MDR, XDR, and even PDR *Acinetobacter baumannii* isolates are disseminated all around the globe, regardless of their ancestral lineage. In a recent worldwide survey investigating 1,235 *A. baumannii* isolates obtained from integumentary sources from 2010 to 2014, the rate of MDR isolates was shown to be 43%. Moreover, this rate was shown to increase from 29% in 2011 to 56% in 2014. More specifically, resistance to meropenem increased over these four years from 41% to 63%. The highest rates of resistance were reported from Latin America (63%) and the Middle East (62%) whereas the lowest rate (33%) was reported from North America (Tärnberg *et al.,* 2016). Another study that evaluated 1,011 clinical *A. baumannii* isolates obtained from intra-abdominal and urinary tract infections from 48 countries between 2013 and 2014 also showed a regional variation in MDR rates. The highest MDR rate exceeded 93% and was detected in Europe and the Middle East, whereas the lowest rate (47%) was also detected in North America. MDR rates in Asia, Africa, and Latin America ranged from 77% to 87%. Moreover, this study showed that susceptibility rates to imipenem in Europe and the Middle East was below 11% (Lob *et al.*, 2016). Yet another global surveillance study that included 1,312 clinical *A. baumannii* isolates obtained from USA, Latin America, Asia Pacific, Europe, and the Mediterranean regions showed that 81.6% of these isolates were MDR (Flamm *et al.,* 2016).

In USA, 50% of combatants injured in conflicts abroad were found to be infected by MDR organisms, of which *A. baumannii* was very notable, especially among those returning from Iraq (Yun and Murray, 2016). Another study from a hospital in Los Angeles detected 21 MDR *A. baumannii* isolates over one year, only eight of which were sensitive to imipenem and two to meropenem (El-Shazly, *et al.,* 2015). In Miami, a study that spanned from 1994 to 2011 showed that the rate of carbapenem resistance was increasing and that the overall rate of carbapenem non-susceptibility among 4,484 *A. baumannii* isolates was 18.9% (Munoz-Price *et al.,* 2013). In Brazil, 118 clinical *A. baumannii* isolates collected between 2010 and 2013 from patients hospitalized for a minimum of 72 hours were resistant to carbapenems (Cortivo *et al.,* 2015). A review study evaluating reports of MDR *A. baumannii* isolates in Latin America from 2002 to 2013 showed that resistance rates to carbapenems ranged from 37% to 92%, depending on the country. This review also showed a high increase in carbapenem non-susceptibility in this region between the years 2006 and 2009. Moreover, countries such as Nicaragua, Bolivia, Dominican Republic, El Salvador, and Honduras showed generally lower

rates of resistance. On the other hand, Brazil, Argentina, Colombia, Guatemala, Cuba, Panama, Paraguay and Mexico showed generally higher rates of resistance (Labarca *et al.,* 2016).

In South Korea, 92 MDR *A. baumannii* isolates were collected over a five-year period from one hospital. All these isolates were then shown to be positive for OXA-23-like (Sung *et al.,* 2016). A Chinese national survey of bloodstream infections showed that the overall prevalence of XDR *A. baumannii* isolates over one year was 13.7%. Of note, 97.4% of these isolates were resistant to imipenem (Xu *et al.,* 2016). In Japan, 49 MDR *A. baumannii* isolates were obtained from 12 hospitals distributed all across the country between July and December 2012 (Tada *et al.,* 2014). A study from 2004 to 2011 covering 33 medical centers spread across Middle Eastern and African countries showed that 51.3% and 33.2% of 664 *A. baumannii* isolates were resistant to imipenem and meropenem, respectively (Kanj *et al.,* 2014). Another study evaluating 411 ACB isolates from 14 European and Mediterranean countries showed that the resistance rate to doripenem was 58.9% (Castanheira, *et al.,* 2014). A study that investigated 35 representative *A. baumannii* isolates obtained from 28 outbreaks that took place in Greece, Turkey, Italy, and Lebanon showed that all these isolates were MDR (Di Popolo *et al.,* 2011). In South Africa, 86% of 94 *A. baumannii* isolates obtained from different clinical samples over three months were resistant to carbapenems and 90% were resistant to fourth generation cephalosporins (Lowings *et al.,* 2015). In a one-year study from Libya, 20% of organisms that caused 79 cases of device-associated nosocomial infections were *A. baumannii*, and 56.3% of those isolates were MDR (Zorgani *et al.,* 2015).

In Lebanon, an early study investigating 36 *Acinetobacter* spp. isolates obtained in a 10 month period showed that only one of these isolates was resistant to imipenem (Matar *et al.,* 1992). For many years after this study, reports on MDR *A. baumannii* isolates on a national level were limited to a few scattered studies analyzing individual outbreaks (Salem *et al.,* 2013). Recently, however, the global increased interest in MDR *A. baumannii* has triggered several reports from Lebanon on these isolates. One study showed an increase in incidence of MDR *A. baumannii* isolates from 73.5% in 2005 to 77.7% in 2009 among in- and out- patients in a Lebanese hospital. Susceptibility to imipenem was shown to decrease from 92.3% in 2006 to 30% in 2009 in that same study (Hamouche and Sarkis, 2012). Moreover, susceptibility to imipenem in a study from a tertiary care center in Beirut was 65% among 64 consecutive clinical *Acinetobacter* spp. samples (Araj and Ibrahim, 2008). Another study analyzing 42 *A. baumannii* isolates obtained between 2009 and 2012 from different Lebanese hospitals showed

that 31 of these isolates were resistant to carbapenems (Rafei *et al.,* 2014a). Additionally, 88% of 724 *A baumannii* isolates obtained from 9 Lebanese hospitals during 2012 were also shown to be resistant to imipenem (Hammoudi *et al.,* 2015a). Another multi-center study conducted in north Lebanon, where there is a concentration of Syrian refugees, showed that of the 116 isolates collected over two years, 70 were resistant to carbapenems (Rafei *et al.,* 2015b). Moreover, a study involving two hospitals from north Lebanon showed the presence of NDM-1 and OXA-72 among two *Acinetobacter pittii* isolates (Al Atrouni *et al.,* 2016b). A retrospective study involving 257 patients between 2012 and 2013 in a Lebanese tertiary care center showed that the rate of XDR *A. baumannii* was 15.6% (Moghnieh *et al.,* 2016). Finally, a multi-center study across Lebanon and Jordan from 2011 to 2013 showed a disturbing rate of resistance among *A. baumannii* isolates were susceptibility to various antimicrobial agents ranged from 4% to 27% (Hayajneh *et al.,* 2015).

One study that spanned 24 hospitals across Turkey, Germany, Sweden, and Poland over six years showed that 84 out of 298 clinical *A. baumannii* isolates were resistant to carbapenems (Tomaschek *et al.,* 2016). Another study conducted in 39 centers spread across Italy, France, Germany, Spain, and the UK showed that susceptibility of *A. baumannii* was below 90% in Italy, Spain, and the UK (Rodloff *et al.,* 2008). Moreover, CRAB isolates detected from two medical centers in a Turkish study were shown to increase in frequency from 20% to 60% over six years (Gur *et al.,* 2008). In Germany, 70 *A. baumannii* isolates that were resistant to carbapenems were collected over four years from a diagnostic laboratory (Rieber *et al.,* 2016). A comparative study from that same country showed that the rate of CRAB isolates across 15 medical centers decreased from 96% in 2005 to 76% in 2009 (Schleicher *et al.,* 2013). A study from southern Poland showed that 80.8% of 125 clinical *A. baumannii* isolates obtained over a year were XDR (Chmielarczyk *et al.,* 2016). Another study that spanned across 22 Italian hospitals showed an increase in the incidence of CRAB isolates from 120 in 2004-2005 to 190 in 2008-2009 (Mezzatesta *et al.,* 2012). In Hungary, CRAB isolates from one hospital were shown to dramatically increase in incidence from 6.2% in 2000 to 73% in 2011 (Mózes *et al.,* 2014). A study from London showed a relatively low rate of CRAB isolates. Nevertheless, their incidence increased from 9.1% of 11 isolates in 2011 to 31.2% of 16 in 2013 (Hughes *et al.,* 2016).

The rate of MDR *A. baumannii* isolates in Spain is notoriously higher than other European countries (Tomaschek *et al.,* 2016). Surveillance programs in this country have

shown that worrisome rates of MDR pathogens in the ICU still pose an unresolved problem. This, in turn, has recently triggered the launching of national programs aimed at tackling this problem (Montero *et al.,* 2015). The issue in this country is further compounded by the lack of uniformity in applying infection control measures in the hospitals (Garcia-Ortega *et al.*, 2011). One study across 25 Spanish hospitals showed that 43% of *A. baumannii* isolated from 203 patients were resistant to carbapenems (Cisneros *et al.,* 2005). Moreover, in a similar study, phenotypic heterogeneous resistance to imipenem was found to be 20% and that of meropenem was 24% (Fernández Cuenca *et al.,* 2012). A retrospective survey over six years, in which 246 Spanish hospitals per year participated, showed that the average rate of CRAB isolates was 34.5% and that in ICUs it was 43.8% (Asensio *et al.,* 2008). Additionally, nineteen PDR *A. baumannii* isolates that are resistant to all antimicrobial agents, including colistin, where collected during an outbreak from a Spanish hospital over around one year (Valencia *et al.,* 2009). In another study from a Spanish tertiary care center, 61 non-duplicate *A. baumannii* clinical isolates collected over six years were PDR (Arroyo *et al.,* 2009). Moreover, in a study that investigated representative samples of 729 epidemic *A. baumannii* isolates obtained from 19 Spanish hospitals over ten years showed that they were all MDR (Villalón *et al.,* 2013). Another study that investigated the mechanisms of resistance of clinical *A. baumannii* isolates showed the presence of 101 CRAB isolates from a total of 493 isolates obtained from two Spanish multi-center studies (Mosqueda *et al.,* 2014). A cohort study that compared *A. baumannii* isolates obtained ten years apart from several Spanish hospitals showed that the rate of susceptibility to imipenem dropped from 33.8% in 2000 to 17.3% in 2010 (Villar *et al.,* 2014). Moreover, three sequential outbreaks caused by MDR *A. baumannii* isolates were reported from a Spanish hospital. The first outbreak was in 2009 and it affected 38 ICU patients. The second and third outbreaks were between 2010 and 2011 and they affected 9 and 11 patients, respectively (Alvargonzalez *et al.,* 2014). Finally, analysis of 405 *A. baumannii* isolates collected over eleven years from a Spanish tertiary care hospital showed that 64.7% of these isolates were susceptible to carbapenems. 231 of the total isolates obtained formed 15 clonally related groups that were all XDR (Villalón *et al.,* 2015).

6. RELATIONSHIP BETWEEN VIRULENCE AND RESISTANCE

The key factor that causes success of certain clones in terms of dissemination and persistence is their ability to out-compete other strains in the highly stressful environment that is the human body. This environment acts as a bottleneck that allows only the clones that have

a certain balance of virulence factors and resistance mechanisms to survive (Martínez and Baquero, 2002). Moreover, different virulence factors were shown to be expressed by different MDR *A. baumannii* clones, suggesting an interplay between virulence and resistance (Antunes *et al.,* 2011). The differential expression of virulence was also shown to affect mortality with different levels (Antunes *et al.,* 2014). The relationship between bacterial virulence, antibiotic resistance, and fitness will be discussed in this final section.

6.1. Effects of Resistance on Bacterial Fitness and Virulence

The common factor between bacterial mechanisms of resistance and those of virulence is that they are both needed for the bacterium's survival. Mechanisms of resistance are crucial for overcoming the antimicrobial threat whereas mechanisms of virulence are necessary for overcoming the host defense systems (Beceiro *et al.,* 2013). Genes coding virulence and resistance could both be disseminated through horizontal gene transfer between organisms, while sometimes being co-selected for (Burrus and Waldor, 2004). Both these factors have an effect on bacterial fitness and the ability of bacteria to grow and divide. The interaction between resistance, virulence, and fitness seems to be complex and highly dependent on an intricate balance that would allow certain clones to survive. Moreover, this relationship seems to be dependent on the species being studied and associations made in one species may not hold true for another (Beceiro *et al.,* 2013).

The involvement of certain cellular components in various metabolic processes is bound to result in an interplay between them. One such example is the involvement of porins in the uptake of nutrients needed by bacterial cells (Nikaido, 2003), resistance to antimicrobial agents (Sugawara and Nikaido, 2012), formation of biofilms (Gaddy *et al.,* 2009), and the induction of cell death (Peleg *et al.,* 2008). Down-regulation of porins resulting from antibiotic pressure could have an effect on the other mechanisms in which these molecules are involved. This was indeed shown to be the case in a PDR *A. baumannii* isolate having the CarO and OprD-like porins downregulated. These porins were downregulated in order to convey resistance to carbapenems. However, this also resulted in slower growth and attenuated virulence of the clone (Fernandez-Cuenca *et al.,* 2011). Production of biofilms is another example of the interaction between virulence and resistance. Biofilms are considered as virulence factors that protect bacteria from eradication and lead to their persistence. However, biofilms also have a role in antimicrobial resistance since the sequestered bacterial cells will

be protected from the action of antibiotics (Patel, 2005). In addition, two-component systems that respond to environmental changes are also implicated in the regulation of both resistance and virulence. These systems could act as global regulators that would affect the bacterium's metabolism, virulence, and antimicrobial resistance at the same time (Yeung *et al.,* 2011). One such example in *A. baumannii* is the response to high NaCl concentrations in the environment through the increased production of efflux pumps and the release of outer membrane proteins. The increase in efflux pumps would in turn lead to resistance to several antimicrobial agents while the release of outer membrane proteins would contribute to an increased virulence (Hood *et al.,* 2010).

Resistance to antimicrobial agents has been reported to have varying effects on virulence and bacterial fitness that depends on the mechanism, organism, and even the specific strain being investigated (Beceiro *et al.,* 2013). A study investigating changes in pmrA/B in *Salmonella enterica* with reduced susceptibility to colistin showed that mutations in *pmrA* resulted in a reduced *in-vitro* fitness, although *in-vivo* growth rates were not affected (Sun *et al.,* 2009). Another study in *Escherichia coli* showed that the acquisition of certain beta-lactamases, such as OXA-10 and OXA-24 incurred a fitness cost on the strains and resulted in reduced virulence by inducing changes in the peptidoglycan layer (Fernández *et al.,* 2012). Over-expression of AmpC in *Salmonella enterica* was also shown to result in reduced fitness, although no changes in the peptidoglycan layer were detected (Morosini *et al.,* 2000). Acquisition of resistance to vancomycin in *Staphylococcus aureus* through the *vanA* operon, which results in alteration of the target of this antimicrobial agent, was also shown to incur a great fitness cost on the bacterial cell (Foucault *et al.,* 2009). Other resistance mechanisms seem to not have any effect on bacterial virulence nor fitness. For instance, high level resistance to aminoglycosides mediated by methyltransferases seem to not have an effect on bacterial fitness in *E. coli* (Gutierrez *et al.,* 2012). The acquisition of CTX-M-1, TEM-1, and CTX-M-32 in *E. coli* (Dubois *et al.,* 2009; Fernández *et al.,* 2012) and IMP in *P. aeruginosa* (Aoki *et al.,* 2004) also seem to not affect the virulence of these organisms. Nevertheless, the effect on virulence and fitness resulting from the acquisition of beta-lactamases has not been vigorously investigated and therefore no global conclusions could be made (Beceiro *et al,* 2013).

Although it is generally thought that resistance to antimicrobial agents incurs a biological cost, some studies have shown increased fitness and virulence of highly resistant bacteria. Several reasons have been attributed to this phenomenon. One reason is the activation

of the SOS system as a result of the action of antimicrobial agents. This response would in turn accelerate the spread and exchange of genetic material, which could harbor genes of resistance and virulence, among the bacterial population. As a result, certain strains that have simultaneously acquired several mechanisms of resistance and virulence could emerge (Ubeda *et al.,* 2007). Another mechanism described in *E. coli* which results in resistant and virulent strains is the accumulation of alarmones which leads to increased biofilm formation and tolerance to beta-lactams (Joseleau-Petit *et al.,* 1994). Finally, mobile genetic elements, such as plasmids, could carry genes coding for virulence and resistance at the same time. The acquisition of such elements by bacterial organisms could result in highly resistant and virulent strains (Woodford *et al.,* 2009). One example of highly MDR and virulent organisms is *E. coli* strain ST131 which was found to express a wide array of virulence factors in addition to being positive for NDM-1 (Peirano *et al.,* 2011). Another example is the so called "Liverpool Epidemic Strain", a MDR *P. aeruginosa* strain which has enhanced virulence. This strain was isolated from cystic fibrosis patients and was found to be resistant to beta-lactams and aminoglycosides through the up-regulation of efflux pumps. The over-expressed efflux pumps also enhanced the spreading of quorum-sensing molecules that activate several virulence mechanism and resulted in an overall enhanced virulence of the strain (Maeda *et al.,* 2012).

Numerous resistance mechanisms were found to incur a biological cost on the bacterial cell. However, some strains were found to be able to compensate for this cost and persist, even in the absence of antibiotic stress (Andersson and Hughes, 2011). Three major mechanisms have been proposed as to how bacteria compensate for their resistance. The first is through the direct restoration of efficiency of a certain molecule through mutations that revert its negative effect on fitness. The second is the replacement of the function that was affected by another similar function in the cellular metabolic processes. And the third is the reduction in the need for a specific function that was affected by resistance (Andersson and Hughes, 2010). These compensatory mechanisms could not only lead to the persistence of highly resistant and virulent strains, but to actually select for them through the elimination of competing organisms (Beceiro *et al.,* 2013). The threat of MDR and virulent strains is so great that some researchers have proposed the restriction of the use of antimicrobial agents in an attempt to eradicate these organisms (Andersson and Levin, 1999). This, along with the fact that the development of new antimicrobial agents has almost halted since the 1990s, has led researchers to investigating molecules that could have anti-virulence effects (Livermore, 2011). This research is aimed at targeting virulence mechanisms of bacteria while administering antibiotic therapy in an attempt

to decrease the selective pressure and prevent the emergence of resistance (Rasko and Sperandio, 2010). Along these lines, rifampin, an antimicrobial agent that has shown little to no effect on *A. baumannii* in terms of inhibition of growth, was able to attenuate the cellular damage mediated by MDR and PDR *A. baumannii* strains (Smani *et al.,* 2011). Proanthocyanidins are another class of molecules that has been successfully used in the clinical practice. These molecules act by binding to the filaments or fimbriae of *E. coli* and therefore prevent successful adherence and recurring urinary tract infections (Lavigne *et al.,* 2011). Development of new anti-virulence molecules to be used alongside antimicrobial agents could be a very promising strategy to combat MDR and/or virulent organisms in the future.

6.2. Biological Cost of Resistance in *Acinetobacter baumannii*

Several investigations have aimed at determining the biological cost exerted by specific resistance mechanisms on *A. baumannii*. One such investigation was mentioned in the previous section where the down-regulation of the CarO and OprD-like porins results in slower growth and attenuated virulence of the *A. baumannii* strain (Fernandez-Cuenca et al., 2011). Resistance to quinolones in *A. baumannii* through mutations in the topoisomerase and DNA gyrase genes was also shown to result in reduced fitness and virulence (Smani *et al.,* 2012). Another study investigating the effect of the loss of multi-drug efflux pumps on virulence in *A. baumannii* showed that the loss of these pumps would result in increased antibiotic susceptibility. Additionally, the gained susceptibility was accompanied by a loss of virulence as demonstrated in a mouse infection model. These findings indicate that increased antimicrobial resistance is associated with increased virulence in these strains (Roux *et al.,* 2015).

Although resistance to beta-lactams among *A. baumannii* isolates is of great importance, very few studies have investigated the impact of this resistance on virulence and fitness (Beceiro *et al.,* 2013). One study investigating the effect of the acquisition of PER-1 in *A. baumannii* showed a positive correlation between the acquisition of this enzyme and cell adhesion (Sechi *et al.,* 2004). The penicillin binding protein PBP 7-8, although shown to mediate resistance to beta-lactams in *P. aeruginosa*, was not concretely correlated with resistance to these antimicrobial agents in *A. baumannii* (Cayô *et al.,* 2011). Nevertheless, it was shown to play a role in the maintenance of cell morphology and survival of *A. baumannii* in infection models. Moreover, alterations in this molecule was found to have a negative effect on virulence in this organism (Russo *et al.,* 2009). The MDR *A. baumannii* strain pertaining to

ST56[B], which caused one of the largest reported outbreaks, was notorious for its heightened ability of causing invasive infections. This strain was positive for OXA-24-like and additionally harbored a plasmid coding for the TonB-dependent receptor, which is involved in iron uptake, and the toxin septicolysin (Acosta *et al.,* 2011). This shows that MDR *A. baumannii* strains could also maintain their ability to be highly virulent. Another study investigating the relationship between the clonality of *A. baumannii* and virulence showed that twitching motility is common among MDR isolates pertaining to IC I and strong biofilm formers. Moreover, swarming motility was associated with isolates that did not pertain to any international clone (Eijkelkamp *et al.,* 2011b). Recently, a fatal outbreak in USA, associated with the death of the majority of infected patients, was shown to pertain to a clone that is distinct from the three widely disseminated ICs but similar to clones isolated in California, Germany, and Czech Republic. This clone was MDR and showed enhanced virulence in terms of iron metabolism, protein secretion, and glycosylation (Jones *et al.,* 2015).

The effect of colistin resistance on the biological cost in *A. baumannii* has been addressed by several studies. The two main mechanisms of colistin resistance that involve alterations in LPS and Lipid A were shown to have different effects on the bacterial cell. This is mainly due to the involvement of these molecules in several biological processes that include pyrogenicity, toxicity, mitogenicity, and the induction of pro-inflammatory responses in the host (Beceiro *et al.,* 2013). Though not without exceptions, colistin resistance in *A. baumannii* was generally shown to greatly reduce fitness (Lesho *et al.,* 2013). Induction of the complete loss of lipid A through *in-vitro* mutations in the *lpx* genes was shown to lead to colistin resistance in addition to an alteration in the antibiotic resistance profile. This alteration includes a gained susceptibility to cefepime (Moffat *et al.,* 2010). One clinical case demonstrated that an *A. baumannii* isolate that acquired resistance to colistin during therapy also gained susceptibility to cefepime and sulbactam (López-Rojas *et al.,* 2011). Further investigation of this isolate showed that it had impaired ability to compete with a colistin sensitive isolate as demonstrated by competition assays. Moreover, lower *in-vivo* fitness and virulence was detected for this isolate (López-Rojas *et al.,* 2013). Another case report from France showed that a colistin-resistant isolate had reduced *in-vivo* fitness, lost its infectivity, and had attenuated virulence (Rolain *et al.,* 2011). Yet another clinical isolate resistant to colistin was shown to have impaired virulence and fitness as investigated by a rat pneumonia model (Hraiech *et al.,* 2013).

Resistance to colistin through mutations in the *pmr*CAB operon have also been shown to result in a fitness cost and impaired virulence (Olaitan *et al,* 2014). Specifically, the P233S mutation in PmrB was shown to result in decreased invasiveness and growth retardation (Pournaras *et al.*, 2014). Conversely, another study investigating colistin resistant *A. baumannii* isolates with this same mutation reported that it had no effect on the bacterium in terms of fitness and virulence (Durante-Mangoni *et al.*, 2015). Yet another study comparing the biological cost of colistin resistance between the two main described mechanisms of colistin resistance showed that resistance to this antibimicrobial agent through mutations in *pmr*CAB had very modest effects on fitness. On the other hand, resistance due to the loss of LPS after mutations in the *lpx* genes was found to incur a much greater biological cost on the bacterial cell (Beceiro *et al.,* 2014). Another similar study showed that when colistin resistance is acquired through mutations in the *lpx* genes, a loss of virulence accompanied with susceptibility to other antimicrobial agents is observed. This study also showed that when resistance is acquired through mutations in *pmrB,* the mutants retained similar levels of virulence as compared to their parental strains (Wand *et al.,* 2015).

The plethora of resistance and virulence mechanisms found among *A. baumannii* strains makes this bacterium a threat to be reckoned with. The wide dissemination of MDR clones, especially those showing enhanced virulence, cannot be ignored. Though numerous studies have addressed this problematic organism, there is still a large amount of information that needs to be gathered if we are to one day be able to eradicate this organism from the clinical setting.

VI. OBJECTIVES AND JUSTIFICATION

OBJECTIVES AND JUSTIFICATION

Acinetobacter baumannii is a nosocomial opportunistic pathogen that is renowned for its high capacity to develop resistance, produce virulence determinants, and persist in hospitals for prolonged periods of time. Several highly successful MDR clones have disseminated on a global scale and are associated with particular mechanisms of resistance. The wide dissemination of carbapenem resistant clones that render the use of this potent class of antimicrobial agents obsolete, is especially important. This led clinicians to revert to the use of colistin, despite its nephrotoxicity, and to the subsequent rise of colistin-resistant strains. Highly resistant *A. baumannii* clones have a detrimental effect on the survival of critically ill patients, especially those admitted to the intensive care units. Information about their dissemination in particular regions could be crucial for implementing adequate empirical therapies, which would have a great effect on patient outcome. In addition, numerous virulence factors are associated with clinical *A. baumannii* isolates but are found to be differentially expressed among different isolates. Associations between the acquisition of resistance, especially through beta-lactamases, and their effect on virulence are poorly investigated in *A. baumannii*. For this reason, the knowledge of resistance rates and virulence profiles could positively affect treatment outcomes and will help in controlling the dissemination of MDR *A. baumannii* clones.

The aim of this Doctoral Thesis is the epidemiological, phenotypic, and genotypic characterization of clinical *A. baumannii* isolates in two Mediterranean countries in which the spread of MDR strains is notoriously high. Additionally, the interplay between certain mechanisms of resistance and the expression of specific virulence factors will be investigated. Furthermore, investigations on mechanisms of colistin resistance for two strains that developed resistance to this antimicrobial agent during therapy will be performed. Additionally, biofilm formation patterns for selected isolates with different antimicrobial susceptibility profiles will be investigated. This will provide clinicians, infection control specialists, and researchers with information that could be very valuable in combating this highly successful pathogen and prevent the further emergence of antimicrobial resistance. In order to achieve the aim of the study, the following specific objectives have been developed, to:

- Collect clinical *A. baumannii* isolates from two hospitals, one of which is Hospital Universitario-La Paz, located in Madrid, Spain, and the other is Saint Georges Hospital-University Medical Center in Beirut, Lebanon.
- Determine the identity of the isolates using both phenotypic and genotypic methods that include biochemical identification using 20NE API strips and the amplification of the intrinsic *bla*$_{\text{OXA-51-like}}$ gene.
- Obtain the antibiotic susceptibility profiles of the isolates according to the CLSI guidelines.
- Detect the presence of beta-lactamase genes by PCR.
- Determine the clonality of the isolates using RAPD analysis, PFGE, and multiplex PCRs that indicate their global lineage.
- Detect the presence of selected genes involved in virulence by PCR.
- Phenotypically detect hemolysis, biofilm formation, siderophore production, surface motility, and proteolytic activity of the isolates.
- Perform statistical analyses in order to determine associations between clonality, specific mechanisms of resistance, and the virulence factors that were tested for.
- Analyze the mechanisms of colistin resistance in two sets of isolates where resistance was acquired during therapy by sequencing the *pmr*CAB operon and whole-genome sequencing.
- Determine the effect of colistin resistance on the aforementioned virulence factors.
- Investigate the biofilm formation patterns of selected isolates with varying profiles through growth on steel coupons followed by confocal laser scanning microscopy.

VII. RESULTS

1. Phenotypic and Genotypic Characterization of *Acinetobacter baumannii* Strains Isolated from a Spanish Hospital

1.1 Abstract

Acinetobacter baumannii is a nosocomial pathogen that is showing increasing rates of carbapenem resistance. Multi-Drug Resistant (MDR) International Clones (ICs), associated with the production of oxacillinases, are being reported globally. This organism also harbors numerous virulence factors. In this study, we aim at characterizing *A. baumannii* isolated from a Spanish hospital over a 5 year period in terms of antimicrobial susceptibility, clonality, carbapenemase genes harbored, and virulence factors expressed. Fifty nine clinical bloodstream isolates were obtained from 2009 until 2013. Antimicrobial Susceptibility Testing was performed according to the CLSI guidelines. PFGE and tri-locus PCR typing were then performed in order to determine local and international clonality. PCRs for the detection of *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24-like}}$, *bla*$_{\text{OXA-58-like}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM}}$, and *bla*$_{\text{KPC}}$ were also performed. Production of hemolysis, biofilms, siderophores, surface motility, and proteolysis were determined phenotypically. Doubling times for selected strains were also calculated. Finally, statistical analysis for detecting associations between the tested factors was conducted. Carbapenem non-susceptibility was as high as 84.75%, suggesting the immediate need for intervention. PFGE showed the distribution of the majority of the isolates among 7 clusters. Although all three ICs were detected, IC II was predominant at 71.19%. *bla*$_{\text{OXA-24-like}}$ was the most prevalent carbapenemase (62.71%), followed by *bla*$_{\text{OXA-58-like}}$ (13.56%), and *bla*$_{\text{OXA-23-like}}$ (11.86%). Strains pertaining to IC II and those harboring *bla*$_{\text{OXA-24-like}}$, were positively associated with α-hemolysis, production of strong biofilms, fast doubling times, and siderophore production. Harboring *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-58-like}}$ was associated with reduced virulence, suggesting a lesser toll exerted by these genes on the cell as compared to *bla*$_{\text{OXA-24-like}}$. Moreover, weak proteolytic activity was associated with slower doubling times ($p<0.05$). These associations suggest that detection of clonality by two multiplex PCRs could be a predictive factor for determining the level of virulence of these isolates. An alarmingly high rate of carbapenem non-susceptibility has been detected in this study. There was a predominance of IC II and *bla*$_{\text{OXA-24-like}}$ and those factors were associated with heightened virulence whereas strains belonging to IC I and IC III were shown to have attenuated virulence. This association could be exploited for modifying treatment regimens and for improving on infection control protocols.

1.1. Resumen

Acinetobacter baumannii es un patógeno nosocomial que muestra tasas crecientes de resistencia a carbapenemas a nivel mundial. Se han descrito cepas con resistencia a múltiples clases de antibióticos, que pertenecen a clones internacionales y que llevan oxacilinasas. En este estudio, nuestro objetivo es la caracterización de cepas de *A. baumannii* obtenidas de un hospital español durante 5 años en términos de antibiorresistencia, clonalidad, y factores de virulencia. Cincuenta y nueve cepas se aislaron de la sangre de pacientes desde 2009 hasta 2013 en el HU-LP. Se hicieron las pruebas de PFGE para el análisis de clonalidad local y PCR multiplex para tres genes "housekeeping" para el análisis de clonalidad internacional. La detección de $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-48}}$, bla_{NDM}, y bla_{KPC} se llevó a cabo mediante PCR. Se analizaron fenotípicamente la hemólisis, formación de biofilms, producción de sideróforos, motilidad de superficie, y actividad proteolítica. Posteriormente, se determinaron los tiempos de generación de cepas seleccionadas y se hizo un análisis estadístico para determinar posibles relaciones entre todos estos factores. El 84,75% de las cepas analizadas no eran susceptibles a carbapenemas, sugiriendo una necesidad inmediata de intervención. El análisis del PFGE resultó en la distribución de la mayoría de las cepas en 7 grupos. Se detectaron los tres clones internacionales más comunes, siendo el clon internacional II el predominante, representando el 71,19% de las cepas. El gen codificando una carbapenemasa que se encontró con más frecuencia fue $bla_{\text{OXA-24-like}}$ (62,71%), seguida por $bla_{\text{OXA-58-like}}$ (13,56%), y $bla_{\text{OXA-23-like}}$ (11,86%). Las cepas que pertenecían al clon internacional II y las cepas que llevaban $bla_{\text{OXA-24-like}}$, se asociaron positivamente con hemólisis, producción de biofilms densos, tiempos de generación más rápidos, y producción de sideróforos. Los genes $bla_{\text{OXA-23-like}}$ y $bla_{\text{OXA-58-like}}$ se asociaron con reducción de la virulencia, sugiriendo que estas enzimas pueden ser menos exigentes para las cepas en comparación con $bla_{\text{OXA-24-like}}$. Además, la baja actividad proteolítica estaba asociada con tiempos de generación más lentos ($p<0,05$). El clon internacional II y $bla_{\text{OXA-24-like}}$ fueron predominantes entre estas cepas y se asociaron con una virulencia más alta en comparación con los otros clones y oxacilinasas. Se pueden utilizar estas asociaciones entre clonalidad, presencia de carbapenemasas y virulencia para mejorar el tratamiento y los protocolos de control de la infección.

1.2. Introduction

Acinetobacter baumannii is an opportunistic nosocomial pathogen that has caused severe outbreaks all around the world (Fishbain and Peleg, 2010). Secondary infections with *A. baumannii* could cause ventilator-associated pneumonia, burn wound infections, prosthetic-related infections, and bacteremia (Gordon and Wareham, 2010). Infections with Multi-Drug Resistant (MDR) *A. baumannii* isolates and improper empricial treatments increase mortality rates among critically ill patients (Ñamendys-Silva *et al.*, 2015). In addition to the wide array of intrinsic mechanisms of antimicrobial resistance found in *A. baumannii*, this bacterium has a heightened ability of acquiring resistance (Peleg *et al.*, 2008).

Carbapenems have long been the treatment of choice for MDR *A. baumannii*. However, rates of carbapenem resistance are increasing worldwide (Tärnberg *et al.*, 2016). Mortality rates associated with Carbapenem Resistant *Acinetobacter baumannii* (CRAB) isolates could reach as high as 33% (Lemos *et al.*, 2014). Numerous mechanisms of carbapenem resistance were identified in *A. baumannii*. However, Oxacillinases (OXAs), mainly OXA-23-like, OXA-24-like, and OXA-58-like, are the most common cause of resistance to these antimicrobial agents (Nowak and Paluchowska, 2016). Moreover, a few globally disseminated International Clones (ICs), that include IC I, IC II, and IC III, were found to be the most successful clones causing outbreaks on a global scale (Karah *et al.*, 2012). CRAB isolates from all three ICs are being reported from all around the world (Perez *et al.*, 2010; Tomaschek *et al.*, 2016). In Spain, a notoriously high incidence of CRAB isolates, with a predominance of IC II, is found (Villalón *et al.*, 2011; Villalón *et al.*, 2015). One study showed that imipenem resistance among *A. baumannii* isolates from Spain increased from 74.2% in 2000 to 89.2% in 2010 (Villar *et al.*, 2014). A CRAB incidence rate of 43% among patients in Spanish Intensive Care Units (ICUs) was reported from another study (Cisneros *et al.*, 2005). Moreover, although $bla_{\text{OXA-23}}$ was reported in some strains (Merino *et al.*, 2014), $bla_{\text{OXA-24}}$ was found to be the predominant OXA in Spain (Tena *et al.*, 2013).

The pathogenicity of *A. baumannii* is not yet fully understood. However, several factors have been associated with its virulence (Peleg *et al.*, 2012). One such factor is OmpA which is able to bind to epithelial cells and induce eukaryotic cell death (Howard *et al.*, 2012). Moreover, CsuE, which is part of a chaperon-usher pili assembly system, is also involved in biofilm formation in this organism (Tomaras *et al.*, 2003). Additionally, despite its

classification as a non-motile organism, certain *A. baumannii* strains have shown twitching surface motility (Clemmer *et al.*, 2011). Hemolysis on Sheep Blood Agar (SBA), exoprotease activity, and siderophore production were also reported among *A. baumannii* strains (Antunes *et al.*, 2011). A complex relationship seems to exist between virulence and antimicrobial resistance. One study demonstrated reduced virulence after the acquisition of carbapenem resistance through non-enzymatic means (Fernández-Cuenca *et al.*, 2011). Another study showed that the acquisition of the PER-1 beta-lactamase actually resulted in increased virulence (Sechi *et al.*, 2004). Nevertheless, despite the great importance of OXAs in clinical *A. baumannii* isolates, the effect of these enzymes on virulence is not vigorously investigated (Beceiro *et al.*, 2013).

In this study, we aim at characterizing *A. baumannii* nosocomial isolates in terms of antimicrobial resistance, virulence determinants, prevalence of carbapenemases, and clonal relatedness. We also aim at investigating any association between these factors that could have an impact on treatment of CRAB infections, in addition to the implementation of successful infection control measures.

1.3. Materials and Methods

1.3.1. Bacterial Strains

Fifty nine consecutive non-repetitive bloodstream isolates were collected from different wards of Hospital Universitario-La Paz (HU-LP), Madrid, Spain in a time period spanning from January 2009 until March 2013. Identification was done using Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH) as previously described (Romero-Gomez *et al.*, 2012), in addition to the detection of the *bla*$_{\text{OXA-51-like}}$ gene by PCR (Tourton *et al.*, 2006). The strains were stored in Luria-Bertani (LB) Broth with 20% glycerol at -20°C until used. *Pseudomonas fluorescens* strain B52 (Richardson and Te Whaiti, 1978) was used as a positive control for surface motility, siderophore production, and determination of proteolytic activity. This bacterium was incubated at 30°C instead of 37°C whenever it was used.

1.3.2. Antimicrobial Susceptibility Testing

Minimum Inhibitory Concentrations (MICs) were determined using Vitek2 with AST-N-245 cards (BioMérieux Mercy l'Etoile, France). The antimicrobial agents used were piperacillin (4-128 µg/mL), piperacillin/tazobactam (4/4-128/4 µg/mL), ticarcillin (4-128 µg/mL), ampicillin/sulbactam (2/2-32/16 µg/mL), cefepime (1-64 µg/mL), ceftazidime (1-64 µg/mL), meropenem (0.25-16 µg/mL), imipenem (0.25-16 µg/mL), colistin (0.5-16 µg/mL), amikacin (2-64 µg/mL), gentamicin (1-16 µg/mL), tobramycin (1-16 µg/mL), minocycline (1-16 µg/mL), levofloxacin (0.12-8 µg/mL), ciprofloxacin (0.25-4 µg/mL), and trimethoprim/sulfamethoxazole (1/19-16/304 µg/mL). E-tests (BioMérieux Mercy l'Etoile, France) were performed for verification of colistin resistance. The MIC cutoff values were implemented according to the Clinical and Laboratory Standards Institute (CLSI, 2014) guidelines and the results were accordingly reported as resistant, intermediately resistance, or susceptible.

1.3.3. Polymerase Chain Reactions

PCRs were performed for the detection of the *OmpA*, *CsuE*, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24-like}}$, *bla*$_{\text{OXA-58-like}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM}}$, and *bla*$_{\text{KPC}}$ genes. The primers used, as well as the annealing temperatures for each primer pair, are found in Supplementary Table S1. Positive controls were obtained for each gene from the clinical microbiology laboratory of HU-LP. One third of all visualized bands were chosen randomly and sent to Secugen S. L. (Madrid, Spain) for sequencing in order to verify the amplicon sequence.

In order to determine the global lineage of the strains, two multiplex PCRs proposed by Turton *et al.* (2007) that target specific alleles of the *OmpA*, *CsuE*, and *bla*$_{\text{OXA-51-like}}$ genes were performed. The primers for each Multiplex PCR are listed in Supplementary Table S2. Using a classification scheme summarized by Karah *et al.* (2012), the strains were classified as belonging to one out of 15 identified groups based on the combination of the different alleles amplified. Group 1 of this scheme corresponds to IC II, Group 2 to IC I, and Group 3 to IC III (Karah *et al.*, 2012).

1.3.4. Pulsed Field Gel Electrophoresis

Pulsed Field Gel Electrophoresis (PFGE) was performed using the ApaI restriction enzyme as previously described (Seifert *et al.*, 2005). Restriction fragments were separated in 1% agarose gels using the Contour-Clamped Homogeneous Electric Field (CHEF) apparatus (Bio-Rad, Munich, Germany). The visualized fragments were analyzed using the Bionumerix software version 6.6 (Applied Maths, St-Martens-Latem, Belgium) and the dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with 1.5% tolerance and 1.5% optimization. 80% similarity was considered as the cutoff value for considering the generated pattern as pertaining to the same cluster.

1.3.5. Surface Motility

In order to detect surface motility, a single colony was grown overnight in LB at 37°C. The following day, 1µL was plated on 0.3% LB-Agar (Difco, BD, USA) plates and incubated at 37°C for 14 hours. The motility rate was calculated as the diameter of the resulting circular pattern over time (Clemmer *et al.*, 2011). This, and all subsequent experiments were performed in independent triplicates.

1.3.6. Biofilm Formation

In order to detect biofilms, inoculation of 1mL LB broth in polystyrene tubes with one isolated colony was performed. The tubes were then incubated overnight at 37°C, stained with 1% crystal violet for 10 minutes, washed with water, and left to dry (Tomaras *et al.*, 2003). Strong ring formations indicated by a heavy colorization at the liquid-air interface were reported as "++" while weak ring formations indicated by a very thin colorized ring were reported as "+". The absence of any colorization in the test tube was recorded as "-".

1.3.7. Hemolytic Activity

In order to detect hemolytic activity, LB broth was inoculated with a single colony from the tested strain and incubated at 37°C overnight. The suspension was then adjusted to 10^6 CFU/mL and 10µL was plated on 5% Sheep Blood Agar (SBA) (Biomérieux Mercy L'Etoile, France). The plates were incubated at 37°C and observed for 6 days (Taybali *et al.*, 2012).

1.3.8. Proteolytic Activity

Proteolytic activity of the tested strains was determined as previously described (Antunes *et al.*, 2011). One colony was incubated overnight in Trypticase Soy Broth Dyalisate overnight at 37°C with shaking at 200 rpm. The bacterial suspension was then centrifuged at 4000×g for 10 minutes and the supernatant was filter sterilized. 500µL of 1mg/mL Azoalbumin solution dissolved in 50mM Tris-HCl (pH=7.7) was then incubated with 500µL of the supernatant at 37°C for 24 hours with gentle shaking. Then, trichloroacetic acid was added to each tube with a final concentration of 13% and the tubes were incubated at -20°C for 20 minutes. This was followed by centrifugation at 15,000×g for 10 minutes and measurement of the OD_{440} of the supernatant. U/L values were calculated as previously described (Ronca-Testoni, 1983) where one U was defined as the amount of enzyme needed to degrade one micromole of Azoalbumin.

1.3.9. Siderophore Production

For the determination of siderophore production, one colony was used to inoculate 5mL of the PMS_7-Ca medium. The suspension was then incubated for 72 hours with shaking at 200 rpm. Then, the suspensions were centrifuged at 4000×g for 10 minutes and the supernatant was filter sterilized. The Chrome Azurol Solution (CAS) was also prepared as described by Louden *et al.* (2011). 1 mL of this solution was incubated with 1 mL of the filter sterilized supernatant for one hour and the OD_{630} was measured. Un-inoculated PMS_7-Ca and CAS were used as a reference measurement and a 10% difference between the sample and the reference was considered as positive (Machuca *et al.*, 2003).

1.3.10. Growth Curves

Doubling times were calculated for representative strains from each cluster with varying susceptibility profiles. 500µL from bacterial cultures grown overnight at 37°C in LB broth were diluted in 50mL fresh LB broth. The suspension was then incubated at 37°C with shaking at 200 rpm for 8 hours and the OD_{600} was measured, in duplicate, each hour (Hitachi, U-1900 Spectrophotometer, Tokyo, Japan). Doubling times were calculated from two measurements that fall within the exponential phase (Hall *et al.*, 2014).

1.3.11. Statistical Analysis

All statistical analyses were performed using the SPSS program, version 17.0 (SPSS 111 Inc., Chicago, USA). The chi squared test and Fisher's exact test (two sided) were used in order to analyze the qualitative data. Normal distribution of the quantitative data was tested for using the Shapiro-Wilk test. One-way ANOVA and the student t-tests were performed for the comparison of normally distributed data while the Kruskal-Wallis and the Mann-Whitney tests were used for the comparison of non-normally distributed data. P values of less than 0.05 were considered as statistically significant for all the statistical tests performed.

1.4. Results

1.4.1. Distribution of Bacterial Isolates

A total of 59 bloodstream *A. baumannii* isolates were obtained from HU-LP. Thirty (50.85%) of the isolates were collected in 2009, nine (15.25%) in 2010, five (8.47%) in 2011, eleven (18.65%) in 2012, and four (6.78%) in 2013. Twenty six (44.07%) of these isolates were obtained from the ICU, 22 (37.29%) from the Critical Care Nursing Unit (CCNU), two (3.39%) from each of the Emergency (EM), Resuscitation (RE), and Internal Medicine (IM) wards, and one (1.69%) from each of the Nephrology (NE), Hematology/Oncology (HO), Cardio/Thoracic (CT), General Surgery (GS), and Neonatal (NEO) wards.

1.4.2. Antibiotic Susceptibility Profiles

Antibiotic susceptibility profiles were obtained by interpreting the Minimum Inhibitory Concentration (MIC) values according to CLSI guidelines (2014). Figure 1 shows the percentage of sensitivity, intermediate resistance, and resistance of the tested *A. baumannii* isolates. The MIC values are found in Supplementary table S3. More than 80% of the isolates were non-sensitive to ticarcillin, piperacillin, ampicillin/sulbactam, piperacillin/tazobactam, ceftazidime, ciprofloxacin, levofloxacin, and trimethoprim/sulfamethoxazole. 84.75% of the isolates were non-susceptible to carbapenems, 79.66% to cefepime and 16.95% to minocycline. Non-susceptibility to aminoglycosides ranged from 18.64% for amikacin to 54.24% for gentamycin. Only two isolates (3.39%) were resistant to colistin.

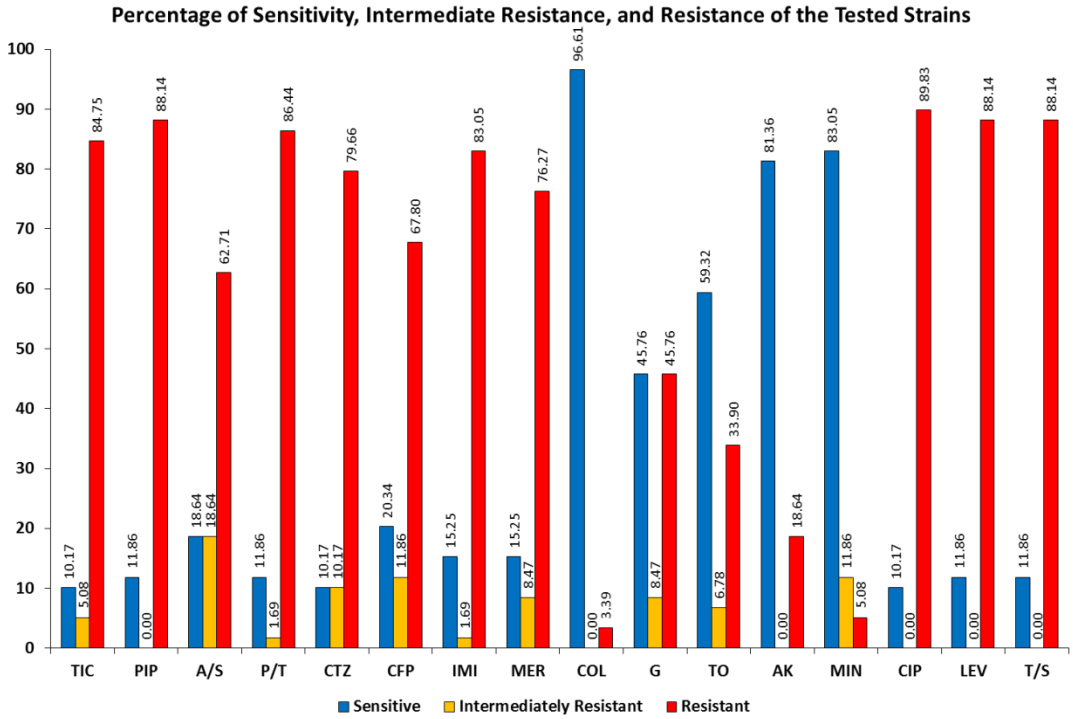


Figure 1. Percentage of sensitive (blue), intermediately resistant (yellow), and resistant (red) isolates to the different antimicrobial agents. "TIC" stands for ticarcillin, "PIP" for piperacillin, "A/S" for ampicillin/sulbactam, "P/T" for piperacillin/tazobactam, "CTZ" for ceftazidime, "CFP" for cefepime, "IMI" for imipenem, "MER" for meropenem, "COL" for colistin, "G" for gentamicin, "TO" for tobramycin, "AK" for amikacin, "MIN" for minocycline, "CIP" for ciprofloxacin, "LEV" levofloxacin, and "T/S" for trimethoprim/sulfamethoxazole.

1.4.3. Detection of Carbapenemases and Virulence Genes

The intrinsic *bla*$_{\text{OXA-51-like}}$ gene was detected in 56 (94.92%) of the isolates whereas *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM}}$, and *bla*$_{\text{KPC}}$ were not detected in any isolate. *bla*$_{\text{OXA-24-like}}$ was the most prevalent carbapenemase gene where it was detected in 37 (62.71%) isolates, followed by *bla*$_{\text{OXA-58-like}}$ in 8 (13.56%) isolates, and *bla*$_{\text{OXA-23-like}}$ in 7 (11.86%) isolates. Isolate 50 harbored both *bla*$_{\text{OXA-24-like}}$ and *bla*$_{\text{OXA-58-like}}$. All the isolates were positive for *OmpA* while 57 strains (96.61%) were positive for *CsuE*.

1.4.4. Clusters, Clones and Carbapenemases

PFGE was performed in order to determine the local clonality of the isolates. Tri-locus multiplex PCRs were then performed in order to determine the global lineage of the strains. Table 1 shows the dendrogram, isolation ward, isolation date, detected carbapenemases, as well as the ICs that the isolates pertained to. The isolates were spread across seven distinct clusters and five isolates did not belong to any cluster. All the isolates were found to pertain to a particular IC or group according to the scheme summarized by Karah *et al.* (2012), except for seven. Of these seven isolates, *bla*$_{\text{OXA-66}}$ was amplified from the first multiplex and *OmpA* from

the second for isolates 12 and 13, *OmpA* from multiplex 1 and *bla*$_{\text{OXA-69}}$ from multiplex 2 for isolate 57, only *CsuE* from multiplex 1 for isolate 59, and, despite several attempts, no genes were amplified from any multiplex for isolates 16, 32, and 46. Of the isolates that did not pertain to any IC, only isolates 13, 57, and 32 pertained to a particular cluster by PFGE (Table 1).

Three out of four isolates from Cluster 1 (C1) pertained to IC III and harbored *bla*$_{\text{OXA-24-like}}$. Two of the isolates from Cluster 2 (C2) had a slightly different pulsotype from the other four in that cluster, pertained to IC II, and had *bla*$_{\text{OXA-24-like}}$. The other four isolates in C2 pertained to IC I and harbored the *bla*$_{\text{OXA-58-like}}$ gene. All the isolates from this cluster were isolated in 2009 and none was detected in subsequent years. Cluster 3 (C3) is formed of two carbapenem sensitive isolates that do not pertain to any IC. Four out of five of the isolates of Cluster 4 (C4) pertained to IC II and were resistant to carbapenems. The fifth isolate had a slightly different pulsotype and harbored *bla*$_{\text{OXA-23-like}}$ instead of the *bla*$_{\text{OXA-24-like}}$ gene harbored by the others in this cluster. Cluster 5 (C5) included two carbapenem sensitive isolates with one pertaining to IC II and the other to a less disseminated clone designated as Group 14 (Karah *et al.*, 2012).

The largest cluster identified, containing 29 isolates, was Cluster 6 (C6). All the isolates in this cluster were carbapenem resistant except for isolate 53 which was intermediately resistant and isolates 26 and 38 which were sensitive. None of the carbapenem sensitive isolates harbored any of the tested carbapenemase genes whereas all of the carbapenem non-sensitive clones of C6 harbored *bla*$_{\text{OXA-24-like}}$ with the exception of isolates 41 and 45 that harbored *bla*$_{\text{OXA-58-like}}$ instead, and isolate 60 which harbored none of the tested carbapenemase genes. In addition to *bla*$_{\text{OXA-24-like}}$, isolate 50 harbored *bla*$_{\text{OXA-58-like}}$. The majority of isolates were obtained from the ICU but this clone was also detected in several other hospital wards (Table 1). Moreover, isolates pertaining to this cluster were detected in 2009, 2010, 2012, and 2013 demonstrating its persistence in the hospital. All the isolates from this cluster belonged to IC II except for isolate 58 which belonged to IC III and 41 which belonged to IC I.

Table 1. Results of the dendrogram obtained from PFGE in addition to which International Clone the tested isolated belonged to, which ward they were isolated from, the date of isolation, susceptibility to carbapenems, and the detected carbapenemases. The red line on the dendrogram represents the 80% cutoff in similarity. "Carb R." stands for carbapenem resistance, "C1 till C7" stand for Cluster 1 to Cluster 7, "IC" stands for international clone, "G" stands for group, and "I" stands for intermediate resistance. The wards are as follows: "CCNU" is the Critical Care Nursing Unit, "ICU" is the Intensive Care Unit, "EM" is Emergencies, "RE" is Resuscitation, "IM" is Internal Medicine, "NE" is nephrology, "HO" is Hematology/Oncology, "CT" is Cardio/Thoracic, "GS" is General Surgery, and "NEO" is Neonatal.

Dendrogram	Epidemiological Data				Resistance			
	Cluster	Clone	Ward	Isolation Date	Carb. R	OXA-23	OXA-24	OXA-58
16	-	-	NE	17/12/2012	-	-	-	-
56	C1	IC III	CCNU	10/6/2009	+	-	+	-
15	C1	IC III	ICU	12/11/2012	+	-	+	-
14	C1	IC III	CCNU	10/10/2012	+	-	+	-
13	C1	-	ICU	26/9/2012	+	-	+	-
59	-	-	IM	18/5/2010	-	-	-	-
12	-	-	CCNU	20/8/2012	+	-	+	-
49	C2	IC II	ICU	1/10/2009	+	-	+	-
37	C2	IC II	CCNU	19/9/2009	+	-	+	-
42	C2	IC I	CCNU	15/12/2009	+	-	-	+
36	C2	IC I	CCNU	25/9/2009	+	-	-	+
35	C2	IC I	CCNU	6/10/2009	+	-	-	+
34	C2	IC I	CCNU	7/12/2009	+	-	-	+
57	C3	-	ICU	25/11/2009	-	-	-	-
32	C3	-	CT	24/9/2009	-	-	-	-
5	C4	IC II	ICU	21/12/2011	+	+	-	-
6	C4	IC II	CCNU	29/12/2011	+	-	+	-
4	C4	IC II	ICU	10/1/2012	+	-	+	-
3	C4	IC II	CCNU	22/6/2011	+	-	+	-
1	C4	IC II	CCNU	12/1/2011	+	-	+	-
31	C5	G 14	CCNU	29/12/2009	-	-	-	-
30	C5	IC II	HO	14/4/2009	-	-	-	-
27	C6	IC II	ICU	25/5/2009	+	-	+	-
26	C6	IC II	ICU	4/5/2010	-	-	-	-
58	C6	IC III	CCNU	24/6/2009	+	-	+	-
23	C6	IC II	CCNU	22/3/2013	+	-	+	-
22	C6	IC II	CCNU	20/3/2013	+	-	+	-
21	C6	IC II	EM	4/2/2013	+	-	+	-
33	C6	IC II	RE	31/3/2009	+	-	+	-
62	C6	IC II	ICU	9/1/2009	+	-	+	-
60	C6	IC II	ICU	28/4/2010	+	-	-	-
55	C6	IC II	ICU	14/1/2009	+	-	+	-
54	C6	IC II	ICU	9/1/2009	+	-	+	-
53	C6	IC II	EM	3/11/2009	I	-	+	-
52	C6	IC II	ICU	21/1/2010	+	-	+	-
51	C6	IC II	ICU	16/12/2009	+	-	+	-
50	C6	IC II	ICU	27/8/2009	+	-	+	+
38	C6	IC II	ICU	3/11/2009	-	-	-	-
29	C6	IC II	RE	16/2/2009	+	-	+	-
61	C6	IC II	ICU	7/1/2009	+	-	+	-
28	C6	IC II	ICU	25/2/2010	+	-	+	-
44	C6	IC II	IM	13/10/2009	+	-	+	-
41	C6	IC I	CCNU	9/12/2009	+	-	-	+
25	C6	IC II	CCNU	23/6/2009	+	-	+	-
45	C6	IC II	CCNU	27/1/2010	+	-	-	+
43	C6	IC II	ICU	21/5/2009	+	-	+	-
48	C6	IC II	ICU	9/6/2010	+	-	+	-
47	C6	IC II	ICU	28/9/2010	+	-	+	-
39	C6	IC II	GS	9/2/2010	+	-	+	-
24	C6	IC II	CCNU	16/9/2009	+	-	+	-
17	C6	IC II	ICU	20/11/2012	+	-	+	-
46	-	-	NEO	9/9/2009	-	-	-	-
40	-	IC II	ICU	6/11/2009	+	-	+	-
20	C7	IC II	CCNU	9/1/2013	+	+	-	-
10	C7	IC II	CCNU	8/5/2012	+	+	-	-
11	C7	IC II	ICU	21/12/2011	+	+	-	-
9	C7	IC II	CCNU	17/4/2012	+	+	-	-
8	C7	IC II	ICU	15/3/2012	+	+	-	-
7	C7	IC II	ICU	10/1/2012	+	+	-	-

The isolates of Cluster 7 (C7) harbored *bla*$_{\text{OXA-23-like}}$, belonged to IC II, and were detected in 2011, 2012, and 2013. Of the five isolates that did not belong to any cluster, only isolate 40 pertained to IC II whereas the rest did not belong to any IC. This isolate had 79% similarity with C7 but was not considered as part of the cluster because the cutoff was set at 80%. Isolates 12 and 40 were resistant to carbapenems and harbored the *bla*$_{\text{OXA-24-like}}$ gene. Additionally, isolate 12 was resistant to colistin. In total, 5 (8.47%) isolates pertained to IC I, 42 (71.19%) to IC II, 4 (6.78%) to IC III, 8 (13.56%) didn't belong to any IC, and one to Group 14. Statistical analysis showed a statistically significant association between IC I and IC II on one hand, and *bla*$_{\text{OXA-24-like}}$ and *bla*$_{\text{OXA-58-like}}$ on the other ($p<0.05$). IC III wasn't statistically associated with the production of any oxacillinase in particular.

1.4.5. Determination of the Virulence Factors and Association with Clonality and Carbapenemases

The results of the virulence factors tested for, which are biofilm formation, hemolysis, siderophore production, surface motility, and proteolytic activity are presented in Table 2. In addition, the ICs, clusters they belong to, and the doubling times for selected isolates are shown in that table. No statistical association was detected when the virulence factors were compared one to another. However, IC I was negatively associated with α-hemolysis on SBA, siderophore production, and strong biofilm formation ($p<0.05$). IC II on the other hand was positively associated with these three factors ($p<0.05$). Nevertheless, all the isolates of C7, which pertained to IC II but had *bla*$_{\text{OXA-23-like}}$, were negative for α-hemolysis on SBA. IC III was negatively associated with α-hemolysis on SBA and production of strong biofilms ($p<0.05$).

In terms of carbapenemase genes, harboring *bla*$_{\text{OXA-23-like}}$ was negatively associated with α-hemolysis on SBA while harboring *bla*$_{\text{OXA-24-like}}$ was positively associated with α-hemolysis and siderophore production ($p<0.05$). Harboring *bla*$_{\text{OXA-58-like}}$ was negatively associated with siderophore production and α-hemolysis ($p<0.05$). Proteolytic activity was not associated with any of the other factors ($p<0.05$). The highest level of proteolytic activity (48.26 ± 11.41 U/L) was detected for the carbapenem resistant isolate 57. This isolate was negative for α-hemolysis on SBA, a strong biofilm former, produced siderophores, and had the highest motility rate (0.616 ± 0.12 μm/sec). The carbapenem resistant, *bla*$_{\text{OXA-24-like}}$ harboring, α-hemolytic, siderophore producing, isolate 40 also had high proteolytic activity (47.67 ± 13.68 U/L). However, it showed a low level of motility (0.047 ± 0.021 μm/sec). Isolates 13 and 62

showed the lowest proteolytic activity (9.24 ± 2.06 and 9.24 ± 1.37 U/L, respectively). Both were α-hemolytic, had comparable motility rates, were resistant to carbapenems, harbored *bla*$_{\text{OXA-24-like}}$, and produced siderophores. The only difference between them was that isolate 13 was a weak biofilm former whereas isolate 62 was a strong biofilm former.

In terms of motility, only isolates 20, 25, 26, 27, 31, and 57 showed a circular diffusion pattern on 0.3% LB-Agar. The only common factor found among these isolates was that they were all strong biofilm formers and that the two that were selected for the calculation of doubling times (20 and 26) showed a relatively fast doubling time (0.342 ± 0.077 and 0.350 ± 0.033 hours, respectively). The slowest doubling time was detected for isolate 12 (0.666 ± 0.037 hours) which was resistant to colistin and carbapenems and harbored *bla*$_{\text{OXA-24-like}}$. This isolate showed α-hemolysis on SBA and was a strong biofilm former but was negative for siderophore production. The two closest isolates to it were isolates 41 and 35 which showed doubling times of 0.651 ± 0.026 and 0.613 ± 0.082 hours, respectively. Both harbored the *bla*$_{\text{OXA-58-like}}$ gene and were negative for α-hemolysis on SBA and siderophore production. Isolate 35 was a strong biofilm former whereas isolate 41 was a weak biofilm former. The fastest doubling time was detected for the other colistin resistant isolate, isolate 45 (0.324 ± 0.027 hours). Interestingly, this isolate harbored *bla*$_{\text{OXA-58-like}}$ in addition to being a strong biofilm former, being positive for α-hemolysis on SBA and siderophore production, and having a high proteolytic activity (42.9 ± 7.74 U/L). Finally, isolates pertaining to IC I were associated with slower doubling times whereas those of IC II were associated with faster doubling times ($p<0.005$).

Table 2. Results of the virulence factors experiments for the tested strains, in addition to which cluster and international clone they belong to. "C1 till C7" stand for Cluster 1 to Cluster 7, "IC" stands for international clone, "G" stands for group, and "ND" stands for not determined. In the column of hemolysis, "α" designates the type of hemolysis detected and "D1 to D3" designates the day in which hemolysis was first detected. The averages and standard deviations presented in the last three columns are a result of 3 independent experiments.

Strain	Cluster	Clone	Hemolysis	Biofilm	Siderophores	Motility (μm/sec)	Proteolytic Activity (U/L)	Doubling Time (hours)
16	-	-	-	++	+	0.057 ± 0.006	25.92 ± 3.10	ND
56	C1	IC III	-	+	+	0.030 ± 0.013	20.85 ± 8.01	0.386 ± 0.041
15	C1	IC III	-	+	+	0.034 ± 0.000	30.09 ± 1.86	0.559 ± 0.127
14	C1	IC III	-	+	+	0.044 ± 0.010	25.92 ± 1.79	ND
13	C1	-	α - D3	+	+	0.044 ± 0.010	9.24 ± 2.06	0.461 ± 0.031
59	-	-	α - D2	++	+	0.044 ± 0.006	35.15 ± 3.14	0.519 ± 0.079
12	-	-	α - D3	++	-	0.024 ± 0.010	12.51 ± 3.58	0.666 ± 0.037
49	C2	IC II	α - D2	+	+	0.045 ± 0.008	23.54 ± 7.81	0.401 ± 0.023
37	C2	IC II	α - D2	++	+	0.024 ± 0.014	23.24 ± 5.44	0.382 ± 0.018
42	C2	IC I	-	+	-	0.027 ± 0.011	35.45 ± 3.14	ND
36	C2	IC I	-	+	-	0.014 ± 0.010	19.96 ± 2.87	ND
35	C2	IC I	-	++	-	0.031 ± 0.037	17.88 ± 3.22	0.613 ± 0.082
34	C2	IC I	-	++	-	0.014 ± 0.010	14.60 ± 6.34	ND
57	C3	-	-	++	+	0.616 ± 0.120	48.26 ± 11.41	ND
32	C3	-	-	++	-	0.027 ± 0.006	17.88 ± 1.55	0.577 ± 0.053
5	C4	IC II	-	++	+	0.034 ± 0.010	27.71 ± 3.10	0.436 ± 0.040
6	C4	IC II	α - D1	++	-	0.054 ± 0.010	29.20 ± 10.28	ND
4	C4	IC II	α - D2	++	-	0.070 ± 0.011	34.26 ± 2.58	ND
3	C4	IC II	α - D2	++	+	0.077 ± 0.015	26.81 ± 2.36	0.449 ± 0.042
1	C4	IC II	α - D2	++	-	0.080 ± 0.011	39.62 ± 8.68	ND
31	C5	G 14	-	++	+	0.116 ± 0.047	12.21 ± 5.08	ND
30	C5	IC II	-	++	+	0.044 ± 0.006	28.60 ± 7.09	0.384 ± 0.038
27	C6	IC II	α - D1	++	-	0.143 ± 0.060	27.41 ± 2.87	ND
26	C6	IC II	α - D2	++	+	0.116 ± 0.083	35.15 ± 4.41	0.342 ± 0.077
58	C6	IC III	-	++	-	0.025 ± 0.008	30.39 ± 9.67	ND
23	C6	IC II	-	++	-	0.038 ± 0.005	22.34 ± 3.90	ND
22	C6	IC II	-	++	+	0.021 ± 0.006	31.28 ± 1.55	0.340 ± 0.069
21	C6	IC II	-	++	+	0.040 ± 0.030	34.86 ± 2.36	ND
33	C6	IC II	α - D1	++	+	0.032 ± 0.013	14.30 ± 3.90	ND
62	C6	IC II	α - D2	++	+	0.035 ± 0.018	9.24 ± 1.37	ND
60	C6	IC II	α - D2	++	-	0.070 ± 0.006	17.88 ± 0.89	ND
55	C6	IC II	α - D1	++	+	0.057 ± 0.015	31.58 ± 19.18	ND
54	C6	IC II	α - D2	++	-	0.060 ± 0.015	24.73 ± 3.14	ND
53	C6	IC II	α - D1	++	+	0.040 ± 0.006	25.03 ± 5.58	0.428 ± 0.017
52	C6	IC II	α - D2	++	+	0.056 ± 0.016	24.13 ± 4.98	0.436 ± 0.088
51	C6	IC II	α - D2	++	+	0.050 ± 0.006	27.41 ± 3.38	ND
50	C6	IC II	α - D2	++	+	0.047 ± 0.006	24.43 ± 2.58	ND
38	C6	IC II	-	++	-	0.024 ± 0.000	28.90 ± 4.03	0.482 ± 0.049
29	C6	IC II	-	++	+	0.060 ± 0.011	23.24 ± 0.89	ND
61	C6	IC II	α - D2	++	+	0.067 ± 0.006	13.70 ± 7.22	ND
28	C6	IC II	α - D2	++	+	0.057 ± 0.006	24.73 ± 3.14	ND
44	C6	IC II	α - D2	++	+	0.063 ± 0.010	29.49 ± 4.47	ND
41	C6	IC I	-	+	-	0.021 ± 0.006	18.47 ± 1.37	0.651 ± 0.026
25	C6	IC II	α - D1	++	+	0.116 ± 0.066	28.00 ± 2.73	ND
45	C6	IC II	α - D2	++	+	0.057 ± 0.015	42.90 ± 7.74	0.324 ± 0.027
43	C6	IC II	α - D2	++	+	0.057 ± 0.006	31.88 ± 10.66	ND
48	C6	IC II	α - D2	++	+	0.055 ± 0.010	21.15 ± 1.86	ND
47	C6	IC II	α - D2	++	+	0.047 ± 0.006	25.03 ± 4.64	ND
39	C6	IC II	α - D2	++	+	0.044 ± 0.017	29.20 ± 7.71	ND
24	C6	IC II	α - D1	++	+	0.063 ± 0.010	33.37 ± 8.30	ND
17	C6	IC II	α - D1	++	+	0.060 ± 0.021	23.54 ± 2.06	0.462 ± 0.016
46	-	-	-	++	-	0.052 ± 0.018	24.73 ± 4.22	ND
40	-	IC II	α - D2	++	+	0.047 ± 0.021	47.67 ± 13.68	ND
20	C7	IC II	-	++	-	0.178 ± 0.253	23.24 ± 3.10	0.350 ± 0.033
10	C7	IC II	-	++	+	0.047 ± 0.011	28.00 ± 7.60	ND
11	C7	IC II	-	++	+	0.031 ± 0.020	29.49 ± 6.26	ND
9	C7	IC II	-	++	+	0.030 ± 0.006	29.20 ± 7.60	0.379 ± 0.019
8	C7	IC II	-	++	+	0.040 ± 0.020	36.35 ± 7.22	ND
7	C7	IC II	-	++	+	0.030 ± 0.006	37.84 ± 1.86	ND

1.5. Discussion

This study shows the phenotypic and genotypic characterization of 59 *A. baumannii* isolates collected over five years from a Spanish hospital. It shows the predominance of the MDR IC II in this hospital and its prolonged persistence, which falls in agreement with national and international epidemiological data (Karah *et al.*, 2012; Villalón *et al.*, 2015). The largest cluster of isolates (C6), mostly pertaining to IC II, caused a large outbreak in 2009 that persisted to 2013 and spread to various hospital wards (Table 1). This reflects the reported success of this clone to persist in hospitals, whether on abiotic surfaces or in the patients' gut flora (Andersson *et al.*, 2011), and cause repeated infections over prolonged periods of time. IC I and IC III were also detected in various clusters but at a lower incidence (Table 1). The prevalence of these three ICs is very similar to that reported by Villalón *et al.*, (2011) which investigated the dissemination of *A. baumannii* clones in Spain over 11 years. This shows a degree of uniformity in terms of distribution of these clones in the country.

A previous study demonstrated that clinical isolates, even those very successful at causing nosocomial outbreaks, have varying profiles of virulence (Antunes *et al.*, 2011). This is in agreement with our results where the virulence profiles of individual isolates varied greatly. Nevertheless, some associations were detected that could help in predicting the degree of virulence of a certain isolate. Our data show an association between clones pertaining to ICs I and III and attenuated virulence as compared to those pertaining to IC II ($P<0.005$). This association could be useful for clinicians in terms of adjusting treatment regimens based on the expected degree of virulence and the severity of the illness of the patient. Moreover, this association could be exploited by infection control specialists through the adaptation of eradication protocols to specific clones.

Most of the isolates (58.85%) in this study were collected in 2009. In general, few infections were caused by *A. baumannii* in subsequent years. This could be an indication of the implementation of good infection control protocols at this hospital. In terms of carbapenem non-susceptibility, an alarmingly high rate of 84.75% over 5 years was detected. This high rate is similar to that reported by Perez *et al.*, (2010) with the difference being the predominance of $bla_{\text{OXA-24-like}}$ in our study as opposed to the predominance of $bla_{\text{OXA-23-like}}$ in the other (Perez *et al.*, 2010). This rate, however, is much higher than that reported for other European countries (Tomaschek *et al.*, 2016) revealing a worrisome situation in this country.

Even though *bla*$_{\text{OXA-23-like}}$ is the most disseminated oxacillinase among *A. baumannii* isolates worldwide, *bla*$_{\text{OXA-24-like}}$ seems to be more prevalent in Spain (Tena *et al.*, 2013). This was reflected in our study where *bla*$_{\text{OXA-24-like}}$ was present in 62.71% of the isolates whereas *bla*$_{\text{OXA-23-like}}$ was only detected in 11.86% of the isolates. Harboring *bla*$_{\text{OXA-24-like}}$ was associated with increased virulence as compared to harboring *bla*$_{\text{OXA-23-like}}$ or *bla*$_{\text{OXA-58-like}}$ in this study ($p<0.05$). This association could also be exploited in a similar manner to that of IC II in treatment regimens and infection control protocols. Taken together, AST, two multiplex PCRs for the detection of clonalilty, and one multiplex PCR for the detection of oxacillinases could provide a fast, cheap, and non-laborious method that could give clinicians and infection control specialists valuable tools in predicting the virulence of an isolate and allow them to make appropriate decisions accordingly.

Some PFGE clusters contained isolates pertaining to different ICs, probably owing to the higher discriminatory power of this technique to differentiate between clones as compared to tri-locus PCR typing. This observation is demonstrated in C6 where two isolates pertained to ICs I and III, respectively, instead of IC II. This cluster also harbored two IC II isolates that were sensitive to carbapenems and one CRAB isolate with no carbapenemase gene detected, suggesting the acquisition of resistance through means that were not tested for in this study. Carbapenem-susceptible *A. baumannii* isolates pertaining to IC II have been previously reported (Diancourt *et al.*, 2010) and this could be due to the loss of resistance resulting from the lack of antibiotic pressure over a period of time (Andersson *et al.*, 2010). Nevertheless, for the most part, the strains in this cluster shared similar virulence profiles, furthering the observation that clonality could be indicative of the degree of virulence.

Most isolates in this study remain susceptible to amikacin and colistin. Susceptibility to the latter antibiotic is especially important due to the international trend to use colistin in the treatment of MDR *A. baumannii* infections (Chen *et al.*, 2015). Colistin resistance among *A. baumannii* has been previously reported in Spain (López-Rojas *et al.*, 2013), though at low incidence. Two colistin resistant isolates were encountered in this study and they were isolates 12, which did not belong to any cluster nor IC, and 45, which was part of C6 and pertained to IC II. Interestingly, the former showed the slowest doubling time while the latter showed the fastest. Both showed α-hemolysis, although isolate 12 was slower in the development of hemolysis. Both were also relatively non-motile. Isolate 12 harbored *bla*$_{\text{OXA-24-like}}$ and did not produce siderophores while isolate 45 harbored *bla*$_{\text{OXA-58-like}}$, had a greater exoprotease

production, and produced siderophores. Isolate 45 only remained susceptible to amikacin and minocycline whereas isolate 12 was susceptible to ampicillin/sulbactam, amikacin, netilmycin, and minocycline. Further genetic characterization of these isolates could help in understanding the different mechanisms in play that made isolate 45 highly virulent, highly resistant, and with a very fast doubling time. Additionally, it is worth mentioning that the isolate pertaining to Group 14 was sensitive to carbapenems as opposed to its counterpart which was first detected in Romanian hospitals and was carbapenem-resistant and harbored *bla*$_{\text{OXA-58-like}}$ (Bonin *et al.*, 2011).

Doubling times are clearly affected by much more factors than are in the scope of this study since they are affected by the entire metabolism of the cell. Therefore, the doubling times were calculated for a few isolates with varying profiles and, when no pattern was detected, determination of doubling times for all the isolates was discontinued. Nevertheless, the doubling times calculated showed an association between slower doubling times and weak proteolytic activity ($p<0.05$) suggesting a direct interaction between these two factors that could be investigated in the future. Finally, it is worth mentioning that the highly susceptible sporadic isolate 57 showed the highest motility rate and proteolytic activity, produced strong biofilms, and was positive for siderophore production. Although the interplay between resistance and virulence seems to be a highly complex one, this observation could suggest that its lack of resistance could be attributing to its increased virulence. Performing a similar study on more sporadic isolates, and isolates from different origins and clonality could reveal further clinically important associations and help better understand the interaction between antimicrobial resistance and virulence.

1.6. Conclusions

In conclusion, a very high level of carbapenem non-susceptibility was detected in this Spanish hospital over a period of five years. IC II and *bla*$_{\text{OXA-24-like}}$ were predominant in this hospital although other ICs and OXAs were detected. IC II and *bla*$_{\text{OXA-24-like}}$ were also associated with increased virulence as compared to other ICs and OXAs. This association could be exploited by clinicians and infection control specialists in order to improve treatment outcomes, especially since determination of clonality and presence of *bla*$_{\text{OXA-24-like}}$ could be performed rapidly. Future investigations using larger pools of isolates with different clonal

lineages could shed light on these associations and help in better understand the complex interactions that affect virulence and antibiotic resistance.

Table S1. Primers used with their respective annealing temperatures.

Primer	Sequence	Annealing Temperature	Reference
OmpA-F OmpA-R	5'-CAATTGTTATCTCTGGAG-3' 5'-ACCTTGAGTAGACAAACGA-3'	50˚C	Turton *et al.*, 2007
CsuE-F CsuE-R	5'-ATGCATGTTCTCTGGACTGATGTTGAC-3' 5'-CGACTTGTACCGTGACCGTATCTTGATAAG-3'	65˚C	Turton *et al.*, 2007
Oxa-51-like-F Oxa-51-like-R	5'-ATGAACATTAAAGCACTC-3' 5'-CTATAAAATACCTAATTGTTC-3'	46˚C	Turton *et al.*, 2007
Oxa-23-like-F Oxa-23-like-R	5'-GATCGGATTGGAGAACCAGA-3' 5'-ATTTCTGACCGCATTTCCAT-3'	53˚C	Mostachio *et al.*, 2009
Oxa-24-like-F Oxa-24-like-R	5'-GGTTAGTTGGCCCCCTTAAA-3' 5'-AGTTGAGCGAAAAGGGGATT-3'	53˚C	Mostachio *et al.*, 2009
Oxa-58-like-F Oxa-58-like-R	5'-AAGTATTGGGGCTTGTGCTG-3' 5'-CCCCTCTGCGCTCTACATAC-3'	53˚C	Mostachio *et al.*, 2009
KPC-F KPC-R	5'-CGTCTAGTTCTGCTGTCTTG-3' 5'-CTTGTCATCCTTGTTAGGCG-3'	52˚C	Poirel *et al.*, 2011
NDM-F NDM-R	5'-GGTTTGGCGATCTGGTTTTC-3' 5'-CGGAATGGCTCATCACGATC-3'	52˚C	Poirel *et al.*, 2011
Oxa-48-F Oxa-48-R	5'-GCGTGGTTAAGGATGAACAC-3' 5'-CATCAAGTTCAACCCAACCG-3'	52˚C	Poirel *et al.*, 2011

Table S2. Primers used in the identification of the global lineages of the strains.

Multiplex 1		
Primer	**Sequence**	**Amplicon Size (bp)**
Group1ompAF306 Group1and2ompAR660	5'-GATGGCGTAAATCGTGGTA-3' 5'-CAACTTTAGCGATTTCTGG-3'	355
Group1csuEF Group1csuER	5'-CTTTAGCAAACATGACCTACC-3' 5'-TACACCCGGGTTAATCGT-3'	702
Gp1OXA66F89 Gp1OXA66R647	5'-GCGCTTCAAAATCTGATGTA-3' 5'-GCGTATATTTTGTTTCCATTC-3'	559
Multiplex 2		
Group2ompAF378 Group1and2ompAR660	5'-GACCTTTCTTATCACAACGA-3' 5'-CAACTTTAGCGATTTCTGG-3'	343
Group2csuEF Group2csuER	5'-GGCGAACATGACCTATTT-3' 5'-CTTCATGGCTCGTTGGTT-3'	580
Gp2OXA69F169 Gp2OXA69R330	5'-CATCAAGGTCAAACTCAA-3' 5'-TAGCCTTTTTTCCCCATC-3'	162

Table S3. Minimum Inhibitory Concentrations (MICs) of the tested antimicrobial agents along with the interpretation according to CLSI guidelines. “R” stands for Resistant, “I” stands for Intermediately Resistant, and “S” stands for susceptible. “TIC” stands for ticarcillin, “PIP” for piperacillin, “A/S” for ampicillin/sulbactam, “P/T” for piperacillin/tazobactam, “CTZ” for ceftazidime, “CFP” for cefepime, “IMI” for imipenem, “MER” for meropenem, “COL” for colistin, “G” for gentamicin, “TO” for tobramycin, “AK” for amikacin, “MIN” for minocycline, “CIP” for ciprofloxacin, “LEV” levofloxacin, and “T/S” for trimethoprim/sulfamethoxazole.

	TIC		PIP		A/S		P/T		CTZ		CFP		IMI		MER		COL		G		TO		AK		MIN		CIP		LEV		T/S	
Strain	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S
1	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
3	>64	R	>64	R	16	I	>64/4	R	>32	R	16	I	>8	R	>8	R	≤0.5	S	≤1	S	≤1	S	≤2	S	2	S	>2	R	>4	R	>4/76	R
4	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	8	I	≤1	S	≤2	S	2	S	>2	R	>4	R	>4/76	R
5	>64	R	>64	R	16	I	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	>8	R	>32	R	8	I	>2	R	>4	R	>4/76	R
6	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	8	I	≤1	S	≤2	S	2	S	>2	R	>4	R	>4/76	R
7	>64	R	>64	R	16	I	>64/4	R	>32	R	>32	R	>8	R	>8	R	2	S	>8	R	>8	R	>32	R	8	I	>2	R	>4	R	>4/76	R
8	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	>8	R	>32	R	8	I	>2	R	>4	R	>4/76	R
9	>64	R	>64	R	16	I	>64/4	R	>32	R	32	R	>8	R	>8	R	2	S	>8	R	>8	R	>32	R	8	I	>2	R	>4	R	>4/76	R
10	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	2	S	>8	R	>8	R	>32	R	8	I	>2	R	>4	R	>4/76	R
11	>64	R	>64	R	16	I	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	>8	R	>32	R	8	I	>2	R	>4	R	>4/76	R
12	64	I	>64	R	4	S	>64/4	R	16	I	32	R	>8	R	>8	R	>8	R	>8	R	>8	R	8	S	≤1	S	>2	R	>4	R	>4/76	R
13	>64	R	>64	R	16	I	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	>8	R	16	S	≤1	S	>2	R	>4	R	>4/76	R
14	>64	R	>64	R	16	I	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	>8	R	16	S	≤1	S	>2	R	>4	R	>4/76	R
15	>64	R	>64	R	16	I	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	>8	R	16	S	≤1	S	>2	R	>4	R	>4/76	R
16	32	I	16	S	≤2/1	S	8	S	16	I	2	S	≤1	S	1	S	≤0.5	S	≤1	S	≤1	S	≤2	S	≤1	S	>2	R	1	S	≤2/38	S
17	>64	R	>64	R	16	I	>64/4	R	32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	>8	R	>32	R	≤1	S	>2	R	>4	R	>4/76	R
20	>64	R	>64	R	16	I	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	>8	R	>32	R	8	I	>2	R	>4	R	>4/76	R
21	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	2	S	>32	R	>8	R	>2	R	>4	R	>4/76	R
22	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	2	S	>32	R	>8	R	>2	R	>4	R	>4/76	R
23	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	>8	R	>32	R	>8	R	>2	R	>4	R	>4/76	R
24	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
25	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
26	32	I	>64	R	4	S	>64/4	R	>32	R	16	I	≤1	S	1	S	1	S	≤1	S	≤1	S	≤2	S	2	S	>2	R	>4	R	>4/76	R
27	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
28	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	2	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
29	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
30	>64	R	>64	R	4	S	64	I	16	I	16	I	2	S	2	S	≤0.5	S	>8	R	8	I	4	S	≤1	S	>2	R	>4	R	>4/76	R
31	16	S	16	S	4	S	16	S	4	S	2	S	≤1	S	1	S	≤0.5	S	≤1	S	≤1	S	≤2	S	≤1	S	≤0.25	S	≤0.12	S	≤2/38	S
32	≤8	S	16	S	≤2/1	S	≤4/4	S	4	S	2	S	≤1	S	≤0.25	S	≤0.5	S	≤1	S	≤1	S	≤2	S	≤1	S	≤0.25	S	≤0.12	S	≤2/38	S
33	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	8	I	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
34	>64	R	>64	R	>16/8	R	>64/4	R	32	R	16	I	>8	R	>8	R	≤0.5	S	>8	R	>8	R	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
35	>64	R	>64	R	>16/8	R	>64/4	R	32	R	8	S	>8	R	8	I	≤0.5	S	>8	R	>8	R	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
36	>64	R	>64	R	>16/8	R	>64/4	R	32	R	16	I	>8	R	8	I	≤0.5	S	>8	R	>8	R	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
37	>64	R	>64	R	>16/8	R	>64/4	R	32	R	8	S	>8	R	8	I	≤0.5	S	>8	R	>8	R	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
38	≤8	S	8	S	≤2/1	S	≤4/4	S	4	S	2	S	≤1	S	≤0.25	S	≤0.5	S	≤1	S	≤1	S	≤2	S	≤1	S	≤0.25	S	≤0.12	S	≤2/38	S
39	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
40	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
41	>64	R	>64	R	>16/8	R	>64/4	R	32	R	8	S	>8	R	>8	R	≤0.5	S	>8	R	>8	R	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
42	>64	R	>64	R	>16/8	R	>64/4	R	32	R	8	S	>8	R	>8	R	≤0.5	S	>8	R	>8	R	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
43	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
44	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
45	>64	R	>64	R	>16/8	R	>64/4	R	16	I	8	S	>8	R	4	I	>8	R	>8	R	8	I	≤2	S	2	S	>2	R	>4	R	>4/76	R
46	16	S	16	S	4	S	≤4/4	S	4	S	2	S	≤1	S	≤0.25	S	≤0.5	S	≤1	S	≤1	S	≤2	S	≤1	S	≤0.25	S	≤0.12	S	≤2/38	S
47	>64	R	>64	R	16	I	>64/4	R	>32	R	16	I	>8	R	>8	R	≤0.5	S	≤1	S	≤1	S	≤2	S	2	S	>2	R	>4	R	>4/76	R
48	>64	R	>64	R	>16/8	R	>64/4	R	16	I	32	R	>8	R	>8	R	2	S	8	I	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
49	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	8	I	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
50	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	2	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
51	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
52	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
53	>64	R	>64	R	4	S	>64/4	R	16	I	16	I	8	I	8	I	≤0.5	S	>8	R	>8	R	16	S	≤1	S	>2	R	>4	R	>4/76	R
54	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
55	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
56	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	8	I	8	S	≤1	S	>2	R	>4	R	>4/76	R
57	8	S	16	S	≤2/1	S	≤4/4	S	4	S	2	S	≤1	S	≤0.25	S	≤0.5	S	≤1	S	≤1	S	≤2	S	≤1	S	≤0.25	S	≤0.12	S	≤2/38	S
58	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	>8	R	8	I	8	S	≤1	S	>2	R	>4	R	>4/76	R
59	≤8	S	8	S	≤2/1	S	≤4/4	S	2	S	2	S	≤1	S	≤0.25	S	≤0.5	S	≤1	S	≤1	S	≤2	S	≤1	S	≤0.25	S	≤0.12	S	≤2/38	S
60	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
61	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R
62	>64	R	>64	R	>16/8	R	>64/4	R	>32	R	>32	R	>8	R	>8	R	≤0.5	S	4	S	≤1	S	≤2	S	≤1	S	>2	R	>4	R	>4/76	R

2. Phenotypic and Genotypic Characterization of *Acinetobacter baumannii* Strains Isolated from a Lebanese Hospital

2.1 Abstract

Acinetobacter baumannii is a nosocomial pathogen that usually affects critically ill patients. High mortality rates have been associated with MDR *A. baumannii* infections. Carbapenem resistance among these isolates is increasing worldwide and is associated with certain International Clones (ICs) and oxacillinases (OXAs). Moreover, this organism possesses a wide range of virulence factors, whose expression is not yet fully understood. In this study, clinical *A. baumannii* isolates are characterized in terms of antibiotic resistance, mechanisms of carbapenem resistance, clonality, and virulence. *A. baumannii* clinical isolates (n=90) where obtained from a tertiary care center in Beirut, Lebanon. API 20NE strips in addition to the amplification of *bla*$_{\text{OXA-51-like}}$ was used for identification. Antibiotic susceptibility testing by disk diffusion was then performed in addition to PCRs for the detection of the most commonly disseminated carbapenemases. Clonality was determined by tri-locus PCR typing and doubling times were determined for isolates with varying susceptibility profiles. Biofilm formation, hemolysis, siderophore production, proteolytic activity, and surface motility was then determined for all the isolates. Statistical analysis was then performed for the determination of associations. 81 (90%) of the isolates were resistant to carbapenems. These high rates are similar to other multi-center studies in the country suggesting the need of intervention on a national level. 76 (93.8%) of the isolates harbored *bla*$_{\text{OXA-23-like}}$, including two that also harbored *bla*$_{\text{OXA-24-like}}$. 80 (88.9%) isolates pertained to ICII and three other international clones were detected, showing the wide dissemination of clones into geographically distinct locations. Virulence profiles were highly diverse and no specific pattern was observed. Nevertheless, an association between motility, siderophore production, and biofilm formation was detected ($p<0.05$). A very high rate of carbapenem resistance was detected, showing the need for immediate intervention. IC II and OXA-23-like were the most disseminated, reflecting their international dissemination. No specific associations were made between virulence and resistance, but instead associations among certain virulence factors were found. Investigating a more clonally diverse pool of isolates could help in the determination of associations between virulence and resistance.

2.1. Resumen

Acinetobacter baumannii es un patógeno nosocomial que afecta a las personas críticamente enfermas. Altas tasas de mortalidad se han asociado con infecciones producidas por cepas de *A. baumannii* resistentes a múltiples antibióticos. La resistencia a carbapenemas en estas cepas ha aumentado a nivel mundial y se han asociado con algunos clones internacionales y presencia de oxacilinasas. *A. baumannii* muestra una gran amplitud de factores de virulencia pero su manera de expresarlos todavía se encuentra en estudio. En nuestro trabajo, se aislaron cepas clínicas de *A. baumannii* y se caracterizaron en base a su antibiorresistencia, mecanismos de resistencia a carbapenemas, clonalidad, y virulencia. Noventa cepas de *A. baumannii* se aislaron de un Hospital Universitario situado en Beirut, Líbano, y se identificaron utilizando tiras API 20NE y amplificación de *bla*$_{\text{OXA-51}}$ mediante PCR. Posteriormente, se realizaron las pruebas de sensibilidad a los antibióticos por difusión en disco, y PCR para la detección de las carbapenemasas más frecuentes. Se determinó la clonalidad por amplificación diferencial de genes "housekeeping" mediante dos PCRs multiplex. Posteriormente, se determinó la producción de biofilms, hemólisis, y sideróforos, así como la actividad proteolítica, movilidad en la superficie, y el tiempo de generación para ciertas cepas seleccionadas. A continuación, se realizó un análisis estadístico para determinar las posibles asociaciones entre estos factores. 81 cepas (90%) resultaron resistentes a carbapenemas. Este alto porcentaje es similar al porcentaje de resistencia obtenido en otros estudios en este país, sugiriendo la gran necesidad de intervención a nivel nacional. 76 de las cepas resistentes a carbapenemas (93,8%) fueron positivas para el gen *bla*$_{\text{OXA-23}}$, incluyendo dos que además contenían *bla*$_{\text{OXA-24}}$. 80 cepas (88.9%) pertenecieron al clon internacional II, mientras que el resto pertenecieron a otros tres clones internacionales. Los perfiles de virulencia variaron mucho entre las cepas y no se detectó un patrón específico entre los mismos. Sin embargo, se detectó una asociación entre movilidad, producción de sideróforos, y formación de biofilms ($p<0.05$). En conclusión, existe un alto porcentaje de resistencia a carbapenemas en *A. baumannii* en este hospital libanés. El clon internacional II y OXA-23 fueron los más frecuentes entre las cepas analizadas. No se encontraron correlaciones estadísticas significativas entre resistencia antibiótica, clonalidad, y virulencia en estas cepas, lo que sugiere que la asociación entre estos factores puede ser un hallazgo local y no internacional. Sin embargo, se encontraron asociaciones entre ciertos factores de virulencia, cuyas investigaciones futuras pueden ayudar a entender mejor los mecanismos patogénicos en *A. baumannii*.

2.2. Introduction

Acinetobacter baumannii is a nosocomial pathogen that could cause severe secondary infections among critically ill patients (Gordon and Wareham, 2010). This organism has a wide range of intrinsic resistance mechanisms and a heightened ability to acquire resistance to a broad range of antimicrobial agents (Peleg *et al.,* 2012). Mortality rates among critically ill patients infected with Multi-Drug Resistant (MDR) *A. baumannii* are high, especially when improper empirical treatments are given (Ñamendys-Silva *et al.,* 2015).

Carbapenems have been the treatment of choice for treating critically ill patients with MDR *A. baumannii* infections (Breilh *et al.,* 2013). However, the increasing rates of Carbapenem Resistant *Acinetobacter baumannii* (CRAB) isolates (Tärnberg *et al.,* 2016) have limited their efficacy and increased mortality rates among infected patients (Lemos *et al.,* 2014). Oxacillinases (OXAs) are the most commonly identified mechanism of carbapenem resistance among *A. baumannii* isolates. These OXAs include OXA-23-like, OXA-24-like, and OXA-58-like (Nowak and Paluchowska, 2016). A few globally disseminated International Clones (ICs) have been found to cause most CRAB infections worldwide and have been associated with the presence of these OXAs (Karah *et al.,* 2012).

A global surveillance program showed that the Mediterranean regions harbored the highest rate of MDR *A. baumannii* isolates (Flamm *et al.,* 2016). Moreover, IC II was found to be widely disseminated among these countries (Di Popolo *et al.,* 2011). This clone was also found to be largely disseminated in Lebanon (Rafei *et al.,* 2014a). Moreover, CRAB isolates were found to increase in prevalence in this country throughout the past decade (Hamouche and Sarkis, 2012). In 2012, 88% of 724 *A. baumannii* isolates recovered from various Lebanese hospitals were found to be resistant to imipenem (Hammoudi *et al.,* 2015a). Although OXA-24-like (Hammoudi *et al.,* 2015b) and OXA-58-like (Zarrilli *et al.,* 2008) have been detected in this country, OXA-23-like seems to be the most prevalent among CRAB isolates (Rafei *et al.,* 2015b).

In addition to the various mechanisms of resistance detected among *A. baumannii* isolates, this organism can deferentially express various virulence factors. These factors include biofilm formation, surface motility, hemolysis on blood agars, siderophore production, and exoprotease activity (Antunes *et al.,* 2011). The relationship between virulence and

antimicrobial resistance seems to be a highly complex one that is still not completely understood (Peleg *et al.*, 2012). Importantly, the effect of harboring OXAs on virulence in *A. baumannii* is not well defined (Beceiro *et al.*, 2013).

The aim of this study is to characterize *A. baumannii* isolates obtained from a Lebanese hospital in terms of antibiotic susceptibility, carbapenemases harbored, clonality, and virulence determinants. Moreover, the relationship between virulence and carbapenem resistance will be explored. This would help in providing clinicians and infection control specialists with crucial data that would allow for the development of successful empirical treatments and infection control measures. Moreover, our findings could open the way for the exploitation of the interplay between virulence and resistance in the clinical setting.

2.3. Materials and Methods

2.3.1. Bacterial Strains

Ninety five non-repetitive *Acinetobacter* spp. isolates were obtained from Saint George University – University Medical Center (SGH-UMC) over a period extending from June 2013 until August 2014. The strains were identified using 20NE API strips (BioMérieux, France) in addition to the detection of the *bla*$_{\text{OXA-51-like}}$ gene by PCR (Turton *et al.*, 2006). The isolates were then stored at -20°C in Luria-Bertani (LB) Broth supplemented with 20% glycerol until used.

2.3.2. Antimicrobial Susceptibility Testing

The Kirby Bauer disk diffusion method was performed according to the CLSI guidelines (2014) in order to determine the Antibiotic Susceptibility Profiles (ASTs) of the isolates. The results were interpreted according to the cutoff values of the CLSI document M100-S24 (CLSI, 2014). Antimicrobial agents tested for were cefotaxime (30µg), ceftazidime (30µg), cefepime (30µg), piperacillin/tazobactam (100µg/10µg), meropenem (10µg), imipenem (10µg), trimethoprim/sulfamethoxazole (1.25µg/23.75µg), ciprofloxacin (5µg), gentamycin (10µg), and colistin (10µg). In order to verify resistance to colistin, E-tests (BioMérieux Mercy l'Etoile, France) and minimum inhibitory concentrations by broth microdilution were performed for isolates that showed narrow inhibition zones around the colistin disks.

2.3.3. Polymerase Chain Reactions

PCRs for the detection of *bla*$_{\text{OXA-51-like}}$ (Turton *et al.,* 2006); *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24-like}}$, and *bla*$_{\text{OXA-58-like}}$ (Mostachio *et al.,* 2009); and *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM}}$, and *bla*$_{\text{KPC}}$ (Poirel *et al.,* 2011) as previously described by the respective authors were performed. Random amplicons were sequenced in order to verify the accuracy of the amplified genetic product. International lineage was determined using tri-locus PCR typing as described by Turton *et al.* (2007). The table summarized by Karah *et al.,* (2012) was used in order to assign the international clonality of the tested isolates.

2.3.4. Growth Curves

In order to calculate the doubling time for selected isolates with varying antibiotic susceptibility profiles, 1:100 dilutions from overnight cultures liquid cultures in fresh LB broth were performed. The fresh suspensions were then incubated at 37°C with shaking at 200 rpm for 8 hours. Each hour, the OD_{600} was measured and the doubling times were calculated from the resulting curve as previously described (Hall *et al.,* 2014). The growth curves and all the following experiments were performed in triplicates.

2.3.5. Biofilm Formation

Biofilm formation was detected in polystyrene tubes after staining with crystal violet as previously described (Tomaras *et al.,* 2003). Briefly, 1mL of inoculated LB broth was incubated overnight at 37°C and then washed and stained with 1% crystal violet for 10 minutes. The dye was then rinsed away and biofilms were visualized and graded as "++" if heavy stains were observed and as "+" if faint stains were observed.

2.3.6. Hemolytic Activity

Hemolytic activity on blood agar plates were tested for by inoculating 10µL of a bacterial suspension adjusted to 10^6 CFU/mL in the center of these plates. The plates were then incubated for 6 days at 37°C and observed daily (Taybali *et al.,* 2012).

2.3.7. Siderophore Production

Siderophore production in a liquid medium using the Chrome Azurol Solution (CAS) was performed as previously described (Louden *et al.*, 2011). 5mL of the PMS_7-Ca medium was inoculated and incubated for 72 hours. The suspension was then centrifuged at 4000×g for 10 minutes and 1mL of the filter-sterilized supernatant was incubated 1:1 with the CAS. The OD_{630} was then measured and a 10% difference between the sample and un-inoculated PMS_7-Ca with CAS was considered as positive (Machuca *et al.*, 2003).

2.3.8. Surface Motility

Surface motility was detected by inoculating 1µL of the bacterial suspension on freshly prepared 0.3% LB-Agar (Difco, BD, USA) plates. The plates were then incubated at 37°C for 14 hours and the diameter of the circular diffusion pattern was measured. Motility rates were reported as the diameter of diffusion divided by incubation time (Clemmer *et al.*, 2011).

2.3.9. Proteolytic Activity

Proteolytic activity was determined by inoculating 5mL of Trypticase Soy Broth Dyalisate with one colony and incubating it overnight at 37°C with shaking at 200 rpm. The following day, the suspension was centrifuged at 4,000×g for 10 minutes and 500µL of the filter-sterilized supernatant was incubated with 500µL of 1mg/mL Azoalbumin dissolved in 50mM Tris-HCl (pH=7.7). This preparation was incubated at 37°C for 24 hours and 13% trichloroacetic acid was then added. The tubes were incubated at -20°C for 20 minutes and centrifuged at 15,000×g for 10 minutes. The OD_{440} of the supernatant was then measured and U/L values were calculated where one U was the amount of enzyme needed to degrade one micromole of Azoalbumin (Antunes *et al.*, 2011; Ronca-Testoni, 1983)

2.3.10. Statistical Analysis

Normality of the data, when applicable, was tested for using the Shapiro-Wilk test. One-way ANOVA and student t-tests were performed for normally distributed data while the Kruskal-Wallis and Mann-Whitney tests were performed for non-normally distributed data. Qualitative data was analyzed using the chi squared and the two-sided Fisher's exact tests. P values of less than 0.05 were considered as statistically significant and all tests were performed using the SPSS program, version 17.0 (SPSS 111 Inc., Chicago, USA).

2.4. Results

2.4.1. Bacterial Isolates

In total, 95 *Acinetobacter* spp. isolates were collected. Of these isolates, three were *Acinetobacter haemolyticus*, one was *Acinetobacter junii*/*johnsonii*, and one was *Acinetobacter radioresistens*/*lwoffii* as identified by the API strips. The rest of the isolates (n= 90) were *Acinetobacter baumannii* as identified by API and the amplification of *bla*$_{\text{OXA-51-like}}$ (Turton *et al.*, 2006). Thirty five (38.9%) *A. baumannii* isolates were collected in 2013 while fifty five (61.1%) were collected in 2014. Fifty eight (64.5%) of the *A. baumannii* isolates were collected from sputum, 17 (18.9%) from pus, 9 (10%) from urine, and 4 (4.4%) from blood samples. Two (2.2%) isolates were collected from catheters.

2.4.2. Antibiotic Susceptibility

Antibiotic susceptibility profiles were obtained after testing by the Kirby Bauer disk diffusion method according to CLSI guidelines (2014). Eighty one (90%) of the *A. baumannii* isolates were resistant to both meropenem and imipenem. Only one isolate was resistant to colistin. Susceptibility to other antimicrobial agents tested for was very low and did not exceed 14.4% (Table 1). Complete antibiotic susceptibility profiles are found in Supplementary Table S1.

Table 1. Antibiotic susceptibility profiles for 90 *A. baumannii* isolates collected over a one-year period. CTX stands for cefotaxime, CAZ for ceftazidime, FEP for cefepime, TZP for piperacillin/tazobactam, MEM for meropenem, IMP for imipenem, SXT for trimethoprim/sulfamethoxazole, CIP for ciprofloxacin, GT for gentamycin, and COL for colistin.

	Antimicrobial Agent									
	CTX		**CAZ**		**FEP**		**TZP**		**MEM**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Resistant	79	87.8	78	86.7	58	64.4	81	90	81	90
Intermediate	9	10	1	1.1	19	21.1	2	2.2	0	0
Sensitive	2	2.2	11	12.2	13	14.4	7	7.8	9	10
	Antimicrobial Agent									
	IMP		**SXT**		**CIP**		**GT**		**COL**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Resistant	81	90	83	92.2	84	93.3	77	85.6	1	1.1
Intermediate	0	0	0	0	0	0	0	0	0	0
Sensitive	9	10	7	7.8	6	6.7	13	14.4	89	98.9

2.4.3. Dissemination of Carbapenemases and International Clones

PCRs for the detection of the most common carbapenemases among *A. baumannii* were performed in addition to tri-locus PCR typing which determines international clonality. *bla*$_{\text{OXA-51-like}}$ was amplified from all the isolates identified as *A. baumannii* using the API strips. Of the 81 *A. baumannii* isolates that showed resistance to carbapenems, 76 (93.8%) harbored *bla*$_{\text{OXA-23-like}}$. Two of these isolates additionally harbored *bla*$_{\text{OXA-24-like}}$. These are isolates 42 and 49. None of the other carbapenemases tested for were detected. Seven isolates were resistant to carbapenems but did not harbor any of the tested carbapenemases (Isolates 54, 59, 68, 72, 83, 84, and 85). Two isolates were sensitive to carbapenems but harbored *bla*$_{\text{OXA-23-like}}$ (Isolates 17 and 87).

Eighty *A. baumannii* isolates (88.9%) pertained to IC II, whereas six (6.7%) pertained to group 4 (Isolates 11, 25, 39, 62, 82, and 91), one (1.1%) to group 10 (Isolate 56), and two (2.2%) to group 14 (Isolates 42 and 72), as summarized by Karah *et al.,* (2012) (Table 2). One *A. baumannii* isolate showed a pattern that did not pertain to any IC where *CsuE* and *bla*$_{\text{OXA-66}}$ were amplified from the first multiplex, and the other allele of *CsuE* was also amplified from the second multiplex.

2.4.4. Virulence Determinants in Relation with Clonality and Carbapenem Susceptibility

The result of the various virulence determinants, in addition to international clonality and carbapenem resistance, are shown in Table 2. Seventy seven (85.6%) of the *A. baumannii* isolates showed strong biofilm formations while 10 (11.1%) showed weak formations and 3 (3.3%) showed no biofilm formation. Forty two (46.7%) of the *A. baumannii* isolates showed α-hemolysis on blood agar plates while one isolate showed β-hemolysis. Seventy two (80%) *A. baumannii* isolates showed a diffusion pattern indicating surface motility on 0.3% LB-Agar while 52 (57.8%) isolates were positive for siderophore production. Proteolytic activity ranged from 4.4 ± 1.63 U/L to 61.97 ± 12.65 U/L and the doubling times for selected isolates ranged from 0.262 ± 0.021 to 0.653 ± 0.049 hours.

Table 2. Virulence determinants of 90 *A. baumannii* isolates in addition to international clonality and carbapenem resistance. IC stands for international clone, Carb R. for carbapenem resistance, Bio for biofilm production, Hemo for hemolysis where the type of hemolysis and the day (D1-D3) on which it was first observed was recorded, Sidero for siderophore production, Proteo for proteolytic activity, and DT for doubling times. ND stands for Not Determined.

Isolate	IC	Carb R.	Bio	Hemo	Sidero	Motility (µm/sec)	Proteo (U/L)	DT (hours)
2	2	+	+	-	+	0.095 ± 0.017	7.66 ± 1.81	ND
3	2	+	++	-	+	0.083 ± 0.025	25.32 ± 4.22	ND
4	2	+	++	-	+	0.125 ± 0.013	28.9 ± 6.34	0.594 ± 0.036
5	2	+	++	β / D1	+	0.238 ± 0.126	61.97 ± 12.65	ND
7	2	+	++	α / D3	+	0.098 ± 0.033	23.68 ± 10.74	ND
8	2	+	+	α / D2	+	0.105 ± 0.035	20.56 ± 7.32	0.471 ± 0.067
10	2	+	++	α / D2	+	0.277 ± 0.156	5.59 ± 3.68	ND
11	4	-	++	-	+	0.061 ± 0.020	21.15 ± 2.06	ND
12	2	+	++	-	+	0.090 ± 0.031	3.33 ± 1.61	ND
13	2	+	+	α / D3	+	0.844 ± 0.131	7.47 ± 2.98	0.403 ± 0.056
14	2	+	++	α / D2	+	0.089 ± 0.027	26.22 ± 1.86	ND
15	2	+	++	-	-	0.094 ± 0.027	32.28 ± 5.44	0.539 ± 0.063
16	2	+	++	α / D3	+	0.181 ± 0.092	23.48 ± 1.96	ND
17	2	-	++	-	+	0.381 ± 0.043	16 ± 1.39	0.283 ± 0.031
18	-	-	++	-	+	0.411 ± 0.001	22.47 ± 3.39	ND
19	2	+	++	-	+	0.193 ± 0.028	23.24 ± 4.1	ND
20	2	+	++	α / D2	+	0.549 ± 0.091	30.69 ± 6.94	ND
21	2	+	++	-	+	0.748 ± 0.132	27.26 ± 0.63	0.377 ± 0.031
22	2	-	+	-	+	0.067 ± 0.027	5.98 ± 5.45	0.310 ± 0.063
23	2	+	++	α / D2	+	0.071 ± 0.026	24.71 ± 3.38	ND
24	2	+	++	-	+	0.267 ± 0.035	5.26 ± 1.42	ND
25	4	+	++	α / D2	+	0.435 ± 0.055	21.45 ± 3.9	ND
26	2	+	++	-	+	0.466 ± 0.065	8.72 ± 0.3	ND
27	2	+	++	-	+	0.377 ± 0.048	18.32 ± 0.63	ND
28	2	+	++	α / D2	-	0.462 ± 0.082	10.53 ± 4.43	0.653 ± 0.049
29	2	+	++	-	+	0.457 ± 0.061	32.27 ± 10.28	ND
30	2	+	++	-	+	0.302 ± 0.041	7.08 ± 3.31	ND
31	2	+	++	-	+	0.387 ± 0.019	27.71 ± 3.9	ND
32	2	+	++	-	-	0.511 ± 0.041	23.77 ± 1.77	ND
33	2	+	++	α / D2	+	0.649 ± 0.112	19.04 ± 2.15	ND
34	2	+	++	-	+	0.416 ± 0.049	23.24 ± 9.42	ND
35	2	+	++	-	+	0.402 ± 0.040	26.51 ± 1.86	ND
36	2	+	++	α / D2	+	0.716 ± 0.102	7.49 ± 4.58	ND
37	2	+	++	-	+	0.488 ± 0.031	23.24 ± 4.47	ND
38	2	+	++	-	+	0.426 ± 0.063	10.59 ± 3.46	ND
39	4	+	-	α / D3	-	0.112 ± 0.059	6.9 ± 2.4	ND
40	2	+	++	-	+	0.458 ± 0.045	6.68 ± 1.93	ND
41	2	+	++	α / D2	-	0.382 ± 0.041	9.09 ± 2.32	0.401 ± 0.030
42	14	+	++	-	+	0.38 ± 0.016	20.85 ± 4.92	ND
43	2	+	+	α / D3	+	0.516 ± 0.048	21.15 ± 7.6	ND
44	2	+	++	-	-	0.541 ± 0.046	19.48 ± 2.78	0.467 ± 0.039
45	2	+	++	-	+	0.484 ± 0.036	4.72 ± 2.17	ND
46	2	+	++	-	+	0.416 ± 0.049	11.05 ± 4.5	ND
47	2	+	++	α / D2	+	0.519 ± 0.043	15.94 ± 6.88	ND
48	2	+	++	α / D2	+	0.433 ± 0.052	11.65 ± 4.2	ND
49	2	+	++	-	+	0.291 ± 0.043	11.72 ± 4.99	0.343 ± 0.120
51	2	+	++	-	+	0.367 ± 0.034	24.43 ± 2.73	ND
52	2	+	++	α / D2	+	0.431 ± 0.027	9.43 ± 3.22	ND
53	2	+	++	α / D2	+	0.386 ± 0.007	11.4 ± 4.09	ND
54	2	+	++	-	-	0.328 ± 0.036	16.22 ± 5.1	ND
55	2	+	++	-	+	0.336 ± 0.035	30.47 ± 10	ND
56	10	-	++	α / D3	+	0.533 ± 0.057	11.64 ± 2.02	ND
57	2	+	+	α / D2	-	0.357 ± 0.053	25.32 ± 2.73	ND
58	2	+	-	α / D1	-	0.16 ± 0.017	24.49 ± 4.55	ND
59	2	+	++	α / D2	-	0.361 ± 0.048	13.81 ± 4.49	ND
60	2	+	++	-	-	0.406 ± 0.035	8.37 ± 2.92	ND
61	2	+	++	α / D3	+	0.355 ± 0.054	21.61 ± 0.24	ND
62	4	-	++	α / D2	-	0.381 ± 0.043	7.3 ± 0.21	ND
63	2	+	++	α / D1	-	0.160 ± 0.015	4.4 ± 1.63	ND
64	2	-	+	α / D1	-	0.208 ± 0.034	10.6 ± 1.45	0.339 ± 0.065

65	2	+	++	α / D2	-	0.417 ± 0.033	7.38 ± 3.81	ND
66	2	+	++	α / D2	-	0.451 ± 0.029	27.41 ± 2.87	0.369 ± 0.021
67	2	+	++	α / D2	+	0.517 ± 0.06	22.94 ± 2.87	ND
68	2	+	++	-	-	0.504 ± 0.042	22.64 ± 7.44	ND
69	2	+	++	-	-	0.526 ± 0.028	28.3 ± 2.1	0.385 ± 0.056
70	2	+	++	α / D2	-	0.549 ± 0.085	22.05 ± 6.94	ND
71	2	+	++	-	-	0.474 ± 0.045	26.81 ± 3.1	ND
72	14	+	++	α / D3	+	0.326 ± 0.036	22.34 ± 2.36	ND
73	2	+	++	α / D1	-	0.59 ± 0.055	11.08 ± 5.56	ND
74	2	+	++	α / D2	-	0.476 ± 0.076	9.17 ± 0.93	ND
75	2	+	++	α / D2	-	0.361 ± 0.024	8.45 ± 3.1	0.328 ± 0.076
76	2	+	++	α / D3	+	0.406 ± 0.035	27.41 ± 4.03	0.311 ± 0.021
77	2	+	++	-	+	0.251 ± 0.035	12.02 ± 1.96	ND
78	2	+	++	-	-	0.610 ± 0.088	12.94 ± 2.61	0.262 ± 0.021
79	2	+	++	-	+	0.555 ± 0.082	11.13 ± 0.69	ND
80	2	+	+	α / D2	-	0.607 ± 0.101	22.94 ± 2.87	ND
81	2	+	++	α / D3	-	0.491 ± 0.077	26.81 ± 0.89	ND
82	4	+	-	-	-	0.161 ± 0.016	8.28 ± 0.33	ND
83	2	+	+	-	-	0.479 ± 0.029	20.85 ± 4.5	ND
84	2	+	++	-	-	0.484 ± 0.036	3.21 ± 3.04	ND
85	2	+	++	-	-	0.833 ± 0.133	8.49 ± 2.85	ND
86	2	+	++	-	-	0.407 ± 0.047	6.54 ± 4.7	ND
87	2	-	++	-	+	0.383 ± 0.025	13.9 ± 4.48	ND
89	2	+	++	-	-	0.717 ± 0.103	21.81 ± 8.09	ND
90	2	+	++	α / D2	-	0.833 ± 0.104	23.56 ± 10.31	ND
91	4	+	+	α / D3	-	0.253 ± 0.026	25.14 ± 2.31	ND
92	2	+	++	-	-	0.716 ± 0.124	6.04 ± 3.49	ND
93	2	+	++	α / D3	-	0.769 ± 0.111	24.55 ± 9.35	ND
94	2	-	++	α / D2	-	2.605 ± 0.461	26.37 ± 4.42	ND
95	2	+	++	-	-	0.809 ± 0.113	23.65 ± 8.61	0.414 ± 0.025

No general pattern was observed between the doubling times and antibiotic susceptibility profiles. The slowest doubling time was determined for isolate 28 which was carbapenem-resistant, α-hemolytic, produced strong biofilms, showed a motility pattern, harbored *bla*$_{\text{OXA-23-like}}$, and pertained to IC II. Nevertheless, other isolates with similar profiles showed faster doubling times (Table 2). One such example is isolate 78 which incidentally had the fastest doubling time. The colistin-resistant isolate 75 showed a similar antibiotic susceptibility pattern and virulence profile to the aforementioned two isolates and had a relatively fast doubling time of 0.328 ± 0.076 hours.

Isolate 94 showed the highest motility rate (2.605 ± 0.461μm/sec), produced strong biofilms, showed hemolysis on blood agars, and had a relatively high proteolytic activity (26.37 ± 4.42 U/L). This isolate was susceptible to carbapenems and pertained to IC II. Isolate 5 was the only isolate that showed β-hemolysis and it had the highest proteolytic activity. It also showed a motility diffusion pattern, produced strong biofilms and siderophores, pertained to IC II, and harbored *bla*$_{\text{OXA-23-like}}$. Isolates 2, 3, 4, 11, 12, and 82 showed very modest to no motility diffusion patterns and were negative for hemolysis. Isolate 4 also had a slow doubling time (0.594 ± 0.036 hours). However, not all isolates that had similar motility rates shared similarities with the rest of the virulence profile of these isolates.

Two of the isolates that pertained to group 4 were susceptible to carbapenems and four out of six isolates of this group were negative for siderophore production. Moreover, two other isolates of this group were negative for biofilm formation. Four of the six isolates pertaining to this group harbored *bla*$_{\text{OXA-23-like}}$ and were resistant to carbapenems. One isolate pertaining to group four was susceptible to carbapenems and showed elevated levels of virulence determinants. Both isolates pertaining to Group 14 had similar profiles but one was negative for *bla*$_{\text{OXA-23-like}}$ and positive for hemolysis whereas the other harbored *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-24-like}}$ but was negative for hemolysis. Isolate 18, which did not pertain to any of the international clones by tri-locus sequence typing, was susceptible to carbapenems and had similar virulence profiles as isolates pertaining to IC II. Both isolates that harbored *bla*$_{\text{OXA-24-like}}$ in addition to *bla*$_{\text{OXA-23-like}}$ were negative for hemolysis and had similar virulence profiles.

2.4.5. Associations between Virulence and Resistance

IC II was positively associated with both carbapenem resistance and harboring *bla*$_{\text{OXA-23-like}}$ ($p<0.01$). All the isolates that did not produce biofilms were also negative for siderophore production. The isolates that showed moderate motility diffusion patterns were associated with strong biofilm formation ($p<0.01$) while those that were either highly motile or non-motile showed a positive association with siderophore production ($p<0.05$). No other statistical association was made.

2.5. Discussion

In this study, *A. baumannii* isolates obtained from a major tertiary care center in Beirut, Lebanon were characterized in terms of antibiotic susceptibility, clonality, and virulence determinants. An extremely high rate of carbapenem resistance (90%) was detected among the *A. baumannii* isolates. This rate, however, is very similar to that reported from a nation-wide study (Hammoudi *et al.*, 2015a) where the prevalence of CRAB isolates was 88%. These findings suggest an immediate need for the implementation of effective infection control measures and antibiotic stewardship programs in Lebanese hospitals. This need is even more urgent due to the high resistance rates of these isolates to other antimicrobial agents that were tested for in this study (Table 1). The low rate of resistance to colistin among these isolates hold a viable alternative for treatment. Nevertheless, its nephrotoxic effects (Bergen *et al.*, 2012) and the ability of *A. baumannii* to develop resistance towards this antimicrobial agent during therapy (Valencia *et al.*, 2009) limits its effectiveness.

In accordance with other studies performed in Lebanon, IC II was, by far, the most prevalent clone among the *A. baumannii* isolates (Rafei *et al.*, 2014a), and OXA-23-like the most disseminated (Rafei *et al.*, 2015b). These findings are also similar to those reported from other Mediterranean countries (Di Popolo *et al.*, 2011). Interestingly, although an outbreak caused by *bla*$_{\text{OXA-58-like}}$ harboring CRAB isolates was reported from SGH-UMC a few years ago (Zarrilli *et al.*, 2008), this carbapenemase was not detected among our isolates. This could be an indication of the successful eradication of the clone that caused the outbreak at the time of that study.

The presence of *bla*$_{\text{OXA-23-like}}$ in two carbapenem-sensitive isolates suggest that either the expression of this gene in these isolates is very modest or that it harbors a mutation that renders it ineffective. Further characterization of these isolates by sequencing the genetic environment of *bla*$_{\text{OXA-23-like}}$ and preforming RT-PCRs could help in better understanding why no carbapenem resistance was detected in these isolates. The seven CRAB isolates in which no carbapenemase was detected could be expressing resistance through carbapenemases that were not tested for in this study and/or through alteration of membrane permeability and efflux pump over-expression (Peleg *et al.*, 2008).

Ten CRAB isolates pertaining to PCR group 4 were first identified in a study investigating *A. baumannii* isolates from several European countries (Towner *et al.*, 2008). In our study, two out of the six isolates pertaining to this group were susceptible to carbapenems. Similarly, the isolate pertaining to group 10 was sensitive to carbapenems as opposed to the detection of carbapenem resistance among isolates pertaining to this group where it was first identified in Portugal (Grosso *et al.*, 2008). The two isolates pertaining to group 14 were both resistant to carbapenems. One of them did not harbor any of the tested carbapenemases while the other had both OXA-23-like and OXA-24-like. This group was first identified in a study from Romanian hospitals and the isolate pertaining to this group harbored *bla*$_{\text{OXA-58-like}}$ (Bonnin *et al.*, 2011). The diversity of profiles between the isolates pertaining to these groups, in addition to the diversity seen among isolates pertaining to IC II, reflect the plasticity of the *A. baumannii* genome (Antunes *et al.*, 2014). Moreover, the presence of these clones, in addition to the presence of the globally disseminated IC II (Karah *et al.*, 2012), demonstrate the global expansion of *A. baumannii* clones that are present across wide geographical areas.

A study by Antunes *et al.,* (2011) showed that different *A. baumannii* clinical isolates are able to display different virulence profiles. This was indeed shown to be the case in our study where no specific pattern of virulence was associated with a specific clone (Table 2). The *A. baumannii* isolates investigated had a high degree of variability in terms of virulence profiles. Moreover, no associations between antibiotic susceptibility profiles and doubling times were detected. These findings suggest that each isolate should be treated as a unique case and no general assumptions could be made based on clonality and AST. This also shows that the relationship between virulence and antibiotic resistance is indeed a complex one and warrants further investigation (Peleg *et al.,* 2012). Nevertheless, the low diversity of clones among the tested isolates could be obscuring associations between clonality and virulence, since some isolates within a group showed similar virulence profiles but their numbers might have been too few for the detection of statistical associations. Further investigating these associations using larger and more clonally diversified pools could shed further light on the matter.

Finally, while comparing virulence factors one to another, an association between motility on one hand, and biofilm formation and siderophore production on the other, was determined. The relationship between motility and strong biofilm formation has been previously reported among MDR *A. baumannii* isolates (Eijkelkamp *et al.,* 2011). Moreover, the association detected between motility and siderophore production is not surprising since the former factor was associated with biofilm production while the latter allows for iron acquisition that is crucial for biofilm formation (Gentile *et al.,* 2014). These associations reveal a highly complex interplay between the different virulence determinants in *A. baumannii*, especially those that are multi-factorial.

2.6. Conclusions

In conclusion, a very high rate of carbapenem resistance was detected among clinical *A. baumannii* isolates obtained from a Lebanese tertiary care center. IC II was the most prevalent clone and OXA-23-like was the most prevalent carbapenemase. The isolates showed highly varied virulence profiles that were not associated with any specific clone or oxacillinase gene. However, associations between motility, biofilm formation, and siderophore production have been found. Increasing the diversity of the pool of isolates could reveal associations

between clonality and virulence that could allow for the prediction of pathogenicity of a clinical *A. baumannii* isolate.

Supplementary Table S1. Antibiotic susceptibility profiles as interpreted according to the CLSI guidelines (2014). R stands for resistant, S for Susceptible, I for Intermediate resistance, and mm for the diameter of the inhibition zone in millimeters. CTX stands for cefotaxime, CAZ for ceftazidime, FEP for cefepime, TZP for piperacillin/tazobactam, MEM for meropenem, IMP for imipenem, SXT for trimethoprim/sulfamethoxazole, CIP for ciprofloxacin, GT for gentamycin, and COL for colistin. The * indicates that the determination of susceptibility to colistin was performed using the broth microdilution method.

	Antibiotic Susceptibility Testing																			**Carbapenemases**	
	CTX		**CAZ**		**FEP**		**TZP**		**MEM**		**IMP**		**SXT**		**CIP**		**GT**		**COL***		
Isolate	**mm**	**S-I-R**	**mm**	**S-I-R**	**mm**	**S-I-R**	**mm**	**S-I-R**	**mm**	**S-I-R**	**mm**	**S-I-R**	**mm**	**S-I-R**	**mm**	**S-I-R**	**mm**	**S-I-R**	**mm**	**S-I-R**	**OXA-23-like**	**OXA-24-like**
2	6	R	6	R	10	R	6	R	6	R	6	R	6	R	6	R	6	R	12	S	+	-
3	6	R	6	R	6	R	6	R	6	R	8	R	6	R	6	R	6	R	12	S	+	-
4	6	R	6	R	10	R	6	R	6	R	6	R	6	R	6	R	6	R	12	S	+	-
5	6	R	6	R	15	I	9	R	12	R	15	R	6	R	6	R	6	R	13	S	+	-
7	6	R	6	R	10	R	6	R	6	R	6	R	6	R	6	R	15	S	18	S	+	-
8	6	R	6	R	12	R	8	R	6	R	6	R	6	R	6	R	6	R	11	S	+	-
10	15	I	19	S	27	S	17	R	11	R	17	R	20	S	22	S	20	S	11	S	+	-
11	17	I	20	S	21	S	21	S	21	S	26	S	14	S	21	S	17	S	12	S	-	-
12	6	R	6	R	11	R	6	R	6	R	8	R	6	R	6	R	6	R	11	S	+	-
13	6	R	6	R	12	R	6	R	6	R	6	R	6	R	6	R	6	R	11	S	+	-
14	6	R	6	R	10	R	6	R	6	R	10	R	6	R	6	R	6	R	13	S	+	-
15	6	R	6	R	6	R	6	R	6	R	6	R	6	R	6	R	6	R	12	S	+	-
16	6	R	6	R	11	R	7	R	6	R	6	R	6	R	6	R	6	R	13	S	+	-
17	13	R	17	I	19	S	18	I	18	S	24	S	6	R	6	R	6	R	11	S	+	-
18	16	I	19	S	20	S	20	S	23	S	28	S	6	R	6	R	6	R	13	S	-	-
19	6	R	6	R	12	R	9	R	10	R	11	R	6	R	6	R	6	R	11	S	+	-
20	6	R	6	R	14	R	6	R	10	R	10	R	6	R	6	R	6	R	13	S	+	-
21	6	R	6	R	12	R	8	R	8	R	11	R	6	R	6	R	6	R	12	S	+	-
22	16	I	18	S	21	S	19	I	21	S	28	S	20	S	24	S	17	S	13	S	-	-
23	6	R	6	R	13	R	12	R	8	R	8	R	6	R	6	R	6	R	13	S	+	-
24	6	R	6	R	13	R	6	R	12	R	12	R	6	R	6	R	6	R	13	S	+	-
25	6	R	6	R	14	R	13	R	6	R	12	R	6	R	6	R	6	R	12	S	+	-
26	6	R	6	R	13	R	10	R	6	R	11	R	6	R	6	R	6	R	12	S	+	-
27	6	R	6	R	11	R	6	R	6	R	6	R	6	R	6	R	6	R	12	S	+	-
28	6	R	6	R	10	R	6	R	6	R	6	R	6	R	6	R	6	R	14	S	+	-
29	6	R	6	R	11	R	9	R	6	R	9	R	6	R	6	R	6	R	13	S	+	-
30	6	R	6	R	6	R	6	R	6	R	6	R	6	R	6	R	6	R	13	S	+	-
31	6	R	6	R	9	R	6	R	6	R	6	R	6	R	6	R	6	R	15	S	+	-
32	6	R	6	R	11	R	6	R	6	R	9	R	6	R	6	R	6	R	11	S	+	-
33	6	R	6	R	10	R	6	R	6	R	6	R	6	R	6	R	6	R	12	S	+	-
34	6	R	6	R	16	I	6	R	11	R	13	R	6	R	6	R	6	R	12	S	+	-
35	6	R	6	R	14	R	6	R	6	R	6	R	6	R	6	R	6	R	12	S	+	-
36	6	R	6	R	14	R	9	R	8	R	13	R	6	R	6	R	6	R	13	S	+	-
37	6	R	6	R	13	R	6	R	6	R	10	R	6	R	6	R	6	R	11	S	+	-
38	6	R	6	R	14	R	9	R	10	R	6	R	6	R	6	R	6	R	13	S	+	-
39	6	R	6	R	13	R	6	R	6	R	6	R	6	R	6	R	15	S	10	S	+	-
40	6	R	6	R	10	R	6	R	6	R	8	R	6	R	6	R	6	R	12	S	+	-
41	6	R	6	R	13	R	9	R	6	R	10	R	6	R	6	R	6	R	13	S	+	-
42	15	I	23	S	25	S	13	R	6	R	6	R	6	R	6	R	6	R	16	S	+	+
43	6	R	6	R	14	R	9	R	14	R	15	R	6	R	6	R	6	R	12	S	+	-
44	6	R	6	R	14	R	11	R	10	R	13	R	6	R	6	R	6	R	13	S	+	-

45	6	R	6	R	6	R	6	R	6	R	12	R	6	R	6	R	6	R	14	S	+	-
46	6	R	6	R	15	I	6	R	8	R	13	R	6	R	6	R	6	R	11	S	+	-
47	6	R	6	R	16	I	12	R	10	R	13	R	6	R	6	R	6	R	12	S	+	-
48	6	R	6	R	14	R	10	R	11	R	12	R	6	R	6	R	6	R	12	S	+	-
49	16	I	21	S	23	S	15	R	6	R	10	R	6	R	6	R	6	R	13	S	+	+
51	6	R	6	R	17	I	10	R	6	R	12	R	6	R	6	R	6	R	12	S	+	-
52	6	R	6	R	11	R	8	R	9	R	11	R	13	R	6	R	6	R	14	S	+	-
53	6	R	6	R	14	R	12	R	11	R	13	R	6	R	6	R	6	R	12	S	+	-
54	6	R	6	R	11	R	8	R	10	R	13	R	6	R	6	R	6	R	13	S	-	-
55	6	R	6	R	12	R	8	R	8	R	10	R	6	R	6	R	6	R	11	S	+	-
56	17	I	20	S	23	S	21	S	23	S	36	S	20	S	28	S	19	S	11	S	-	-
57	6	R	6	R	16	I	12	R	12	R	13	R	6	R	6	R	6	R	12	S	+	-
58	6	R	6	R	16	I	11	R	11	R	16	R	6	R	6	R	16	S	10	S	+	-
59	6	R	6	R	15	I	6	R	6	R	10	R	6	R	6	R	6	R	10	S	-	-
60	6	R	6	R	14	R	13	R	10	R	12	R	6	R	6	R	6	R	14	S	+	-
61	6	R	6	R	15	I	12	R	12	R	13	R	6	R	6	R	6	R	12	S	+	-
62	6	R	10	R	18	S	11	R	23	S	30	S	6	R	6	R	6	R	12	S	-	-
63	6	R	6	R	12	R	10	R	13	R	16	R	6	R	6	R	6	R	13	S	+	-
64	18	I	24	S	26	S	22	S	27	S	30	S	22	S	6	R	23	S	13	S	-	-
65	6	R	6	R	11	R	10	R	9	R	12	R	6	R	6	R	6	R	12	S	+	-
66	6	R	6	R	14	R	11	R	12	R	13	R	6	R	6	R	6	R	13	S	+	-
67	6	R	6	R	11	R	6	R	6	R	11	R	6	R	6	R	6	R	13	S	+	-
68	6	R	6	R	8	R	6	R	6	R	10	R	6	R	6	R	6	R	13	S	-	-
69	6	R	6	R	15	I	12	R	6	R	10	R	6	R	6	R	18	S	14	S	+	-
70	6	R	6	R	12	R	10	R	8	R	12	R	6	R	6	R	6	R	12	S	+	-
71	6	R	6	R	15	I	10	R	11	R	11	R	6	R	6	R	6	R	13	S	+	-
72	18	I	22	S	24	S	15	R	6	R	6	R	6	R	6	R	6	R	14	S	-	-
73	6	R	6	R	13	R	9	R	6	R	9	R	6	R	6	R	6	R	12	S	+	-
74	6	R	6	R	12	R	6	R	10	R	11	R	6	R	6	R	6	R	14	S	+	-
75	6	R	6	R	15	I	11	R	10	R	14	R	10	R	6	R	6	R	6	R	+	-
76	6	R	6	R	15	I	11	R	11	R	13	R	6	R	6	R	6	R	12	S	+	-
77	6	R	6	R	13	R	8	R	8	R	12	R	6	R	6	R	6	R	12	S	+	-
78	6	R	6	R	15	I	8	R	6	R	12	R	6	R	6	R	6	R	13	S	+	-
79	6	R	6	R	15	I	7	R	7	R	12	R	6	R	6	R	6	R	13	S	+	-
80	6	R	6	R	17	I	16	R	10	R	13	R	6	R	6	R	6	R	12	S	+	-
81	6	R	6	R	15	I	12	R	11	R	12	R	6	R	6	R	6	R	12	S	+	-
82	6	R	6	R	11	R	6	R	10	R	11	R	6	R	6	R	15	S	12	S	+	-
83	6	R	6	R	15	I	9	R	6	R	14	R	6	R	6	R	6	R	12	S	-	-
84	6	R	6	R	14	R	7	R	6	R	11	R	6	R	6	R	6	R	13	S	-	-
85	6	R	6	R	12	R	9	R	9	R	11	R	6	R	6	R	6	R	12	S	-	-
86	6	R	6	R	14	R	6	R	11	R	13	R	6	R	6	R	6	R	14	S	+	-
87	20	S	24	S	27	S	24	S	30	S	38	S	16	S	27	S	21	S	12	S	+	-
89	6	R	6	R	9	R	6	R	6	R	6	R	6	R	6	R	6	R	11	S	+	-
90	6	R	6	R	10	R	6	R	6	R	6	R	6	R	6	R	6	R	12	S	+	-
91	6	R	6	R	13	R	6	R	6	R	12	R	6	R	6	R	18	S	11	S	+	-
92	6	R	6	R	15	I	6	R	11	R	11	R	6	R	6	R	6	R	15	S	+	-
93	6	R	6	R	8	R	6	R	6	R	7	R	6	R	6	R	6	R	13	S	+	-
94	20	S	22	S	24	S	22	S	28	S	32	S	27	S	26	S	22	S	15	S	-	-
95	6	R	6	R	11	R	6	R	6	R	10	R	6	R	6	R	6	R	15	S	+	-

3. Genomic and Phenotypic Characterization of two Colistin Resistant *Acinetobacter baumannii* Clinical Isolates in Comparison to their Sensitive Counterparts

3.1 Abstract

Acinetobacter baumannii is a concerning nosocomial pathogen worldwide. Multi-drug resistance among *A. baumannii* isolates led to use of colistin, in turn resulting in colistin-resistant isolates. In this study, the genetic and phenotypic profiles of two *A. baumannii* isolates that acquired resistance during therapy from two different patients are investigated. Two *A. baumannii* isolates where obtained from Patient 1 (C071 and C440) and three from Patient 2 (C080, C314, and C428). Colistin resistance was determined using Vitek-2 and E-test. Clonality was determined by RAPD analysis and global-lineage multiplex PCRs. The *pmrCAB* operon was sequenced and common carbapenemases were screened for by PCR. Doubling times, hemolysis, surface motility, biofilm formation, siderophore production, and proteolytic activity were then determined phenotypically. Finally, whole-genome sequencing was performed for all the isolates. C440 and C428 were resistant to colistin and clonally identical to their sensitive counterparts. The sole cause of colistin resistance was traced to the previously described P233S mutation in *pmrB* of C440 and a novel ΔIle19 mutation in *pmrB* of C428. $bla_{\text{OXA-58-like}}$ and $bla_{GES\text{-}5}$ from the strains of Patients 1 and 2, respectively were also detected. C440 had attenuated proteolytic activity and increased siderophore production as compared to C071 whereas no difference in *in-vitro* virulence determinants was detected between C080, C314, and C428. One common and one novel mutations were encountered in *pmrB* from two distinct *A. baumannii* colistin resistant isolates. These mutations caused colistin resistance during therapy in two distinct clones and only one of them had altered *in-vitro* virulence.

3.1. Resumen

Acinetobacter baumannii es un preocupante patógeno nosocomial. Los elevados niveles de resistencia a varios antibióticos han conducido al uso de colistina en la clínica, lo que a su vez, ha producido el desarrollo de resistencia frente a este antibiótico en cepas clínicas de *A. baumannii*. En este estudio se investigan los perfiles genéticos y fenotípicos de dos cepas de *A. baumannii* resistentes a colistina, aisladas de dos pacientes y se comparan con las cepas sensibles. Dos cepas fueron tomadas de un primer paciente (C071 y C440) y tres cepas de un segundo paciente (C080, C314, y C428). Se determinó la susceptibilidad a colistina mediante Vitek-2 y E-test. La clonalidad se determinó mediante las técnicas de RAPD y PCR multiplex para tres genes "housekeeping". A continuación, se secuenció el operón *pmr*CAB y se amplificaron las carbapenemasas por PCR. Posteriormente, se determinaron los tiempos de generación, formación de biofilms, producción de sideróforos, movilidad en superficie, hemólisis, y actividad proteolítica. Finalmente, se secuenció el genoma completo de las cinco cepas. Las cepas C440 y C428 resultaron resistentes a colistina y fueron idénticas a las cepas sensibles obtenidas del paciente respectivo. La única causa de resistencia a colistina fue una mutación de P233S en PmrB de la cepa C440. Esta mutación es bien conocida entre cepas clínicas de *A. baumannii* resistentes a colistina. La segunda mutación encontrada en PmrB, en la otra cepa resistente a colistina (C428), fue ΔIle19. Esta mutación ha sido un descubrimiento original que no había sido publicado anteriormente. El gen *bla*$_{\text{OXA-58-like}}$ fue detectado en las cepas C071 y C440 y *bla*$_{\text{GES-5}}$ se amplificó por primera vez en *A. baumannii* a partir de las cepas C080, C314, C428. La cepa C440 presentó menor actividad proteolítica que la cepa C071 y fue positiva para la producción de sideróforos, mientras que C071 fue negativa. Esto puede indicar que la mutación P233S en PmrB afecta a la virulencia en *A. baumannii*, por lo que es necesario un estudio más profundo para saber exactamente el mecanismo de la interrelación de ambos procesos. No se encontraron diferencias de virulencia entre las cepas C080, C314, y C428. En conclusión, dos mutaciones en PmrB, de las cuales una es nueva, son las responsables de la resistencia a colistina en *A. baumannii* durante el tratamiento con este antibiótico. La mutación P233S afecta a la virulencia de *A. baumannii*, mientras que la mutación ΔIle19 parece no afectar. La investigación en profundidad de los mecanismos de virulencia y resistencia a colistina en estas cepas puede conducir a una mejor compresión de los procesos de intercambio de información entre virulencia y antibiorresistencia.

3.2. Introduction

Acinetobacter baumannii is a versatile nosocomial pathogen that has a heightened ability to adapt to its environment and acquire resistance to antimicrobial agents. This organism commonly infects critically ill patients and is implicated in ventilator associated pneumonia, wound infections, bacteremia, meningitis, and urinary tract infections (Antunes *et al.,* 2014). Multi Drug Resistant (MDR) *A. baumannii* isolates are becoming a common finding all over the world (Peleg *et al.,* 2012). The high prevalence of carbapenem resistance among MDR *A. baumannii* isolates has forced clinicians to use colistin, despite its nephrotoxic effects. This, in turn, led to an increase in the rate of colistin resistance among *A. baumannii* isolates (Napier *et al.,* 2013).

Colistin is a cationic peptide that acts upon Gram-negative bacteria through the disruption of the negatively charged outer membrane (Vaara *et al.,* 1985). Several different mutations that result in the reduction of the net negative charge of the outer membrane lead to colistin resistance. Mutations in the *pmrCAB* operon, that is involved in Lipid A modifications in response to environmental stimuli, have been most commonly implicated with colistin resistance in *A. baumannii* (Olaitan *et al.,* 2014). Moreover, mutations in genes involved in lipid A biosynthesis (*lpxA, lpxC,* and *lpxD*) also cause colistin resistance (Beceiro *et al.,* 2014). Finally, the plasmid mediated *mcr-1* gene has been recently associated with colistin resistance. This gene was only recently discovered but retrospective studies showed that it is widely disseminated among Gram-negative bacteria (Skoc and Monnet, 2016).

Colistin resistance among *A. baumannii* clinical isolates has been sporadically reported from countries all over the world (Cai *et al.,* 2012). Although most studies report rates below 7%, a study from Spain reported a resistance rate of 40.7% (Arroyo *et al.,* 2009). Moreover, a colistin resistant *A. baumannii* strain caused a pan-drug resistant nosocomial outbreak in a Spanish university hospital in 2009 (Valencia *et al.,* 2009). These reports highlight the potential of *A. baumannii* to cause threatening outbreaks.

Pathogenicity of *A. baumannii* is not yet fully understood but has been linked to several virulence factors. These include its ability to produce biofilms, hemolysis, siderophores, proteolysis, and twitching motility (Antunes *et al.,* 2014). Some studies suggest a negative correlation between colistin resistance and virulence in *A. baumannii* (Lopez-Rojas *et al.,* 2011;

Rolain *et al.*, 2011). Nevertheless, the exact effect of colistin resistance on virulence is still not very well defined (Pogue *et al.*, 2015).

In this study, we aim at investigating the mechanisms of colistin resistance in *A. baumannii* and its subsequent effect on virulence. To that end, genotypic and phenotypic characterization of two clonally distinct strains that developed colistin resistance during treatment was performed.

3.3. Materials and Methods

3.3.1. Bacterial Strains

Five strains were isolated from two different patients from Hospital Universitario La Paz, Madrid, Spain. Identification of the strains and antibiotic susceptibility testing using AST-N-245 cards were performed using the Vitek2 system (BioMérieux Mercy l'Etoile, France) (Granzer *et al.*, 2016). The breakpoint criteria were established in accordance with Clinical and Laboratory Standards Institute guidelines (M100-S20) (CLSI, 2011). Resistance to colistin was confirmed using E-test strips (BioMérieux Mercy L'Etoile, France). The strains were stored in brain heart infusion broth (Oxoid, UK) with 15% glycerol at -80°C until used.

3.3.2. Determination of Clonality

Clonality of the isolates was initially tested for by Random Amplified Polymorphic DNA (RAPD) analysis (Hsueh *et al.*, 1998). The primers used were OPA-2 (5'-TGCCGAGCTG-3'), OPA-18 (5'-AGGTGACCGT-3'), and OPA-12 (5'-TCGGCGATAG-3'). The PCR conditions were an initial denaturation at 94°C followed by 45 cycles of 94°C for 1 minute, 37°C for 1 minute, and 72°C for 2 minutes; with a final extension at 72°C for 2 minutes. Then, in order to determine the global lineages of the strains, two multiplex PCRs described by Turton *et al.* (2007) targeting different alleles of the intrinsic *OmpA*, *CsuE*, and *bla*$_{\text{OXA-51-like}}$ genes were performed.

3.3.3. Sequencing of the *pmrCAB* Operon

In order to sequence the *pmrCAB* operon, DNA was extracted using a commercial kit (Qiagen, Netherlands). The primers described by Adams *et al.* (2009) were used in order to amplify various segments of the *pmrCAB* operon under standard PCR conditions. Additional

primers were designed in this study in order to fill the gaps in the sequences obtained (Supplementary Table 1). Primers G2F, G2R, G3F, and G3R where designed to also include an insertion sequence between *pmr*A and *pmr*B that was discovered in the two strains isolated from Patient 1. PCR products were purified using a kit (Qiagen, Netherlands) and sent to Secugen S. L. (Madrid, Spain) for sequencing. The online tool "Oligo Calc" (http://www.basic.northwestern.edu/biotools/oligocalc.html) was used in order to check for the integrity of the primers designed in this study. The BioEdit software version 7.2.5 (Ibis Biosciences), Serial Cloner software version 2.6.1 (Serial Basics), and the NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) tools were used in order to analyze the sequences, construct the operon, and detect mutations.

3.3.4. Polymerase Chain Reactions

PCRs were performed in order to detect the *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24-like}}$, *bla*$_{\text{OXA-58-like}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM}}$, and *bla*$_{\text{KPC}}$ genes. The primer sequences, annealing temperatures, and expected amplicon sizes are published by Tourton *et al.* (2007), for *bla*$_{\text{OXA-51-like}}$, Mostachio *et al.* (2009) for the subsequent three genes, and Poirel *et al.* (2011) for the final three genes. Previously verified and sequenced template DNAs for each of the respective genes was obtained from the hospital's strain library for use as positive controls.

3.3.5. Full Genome Sequencing

In order to rule out any other mutation that might be affecting colistin resistance, full genome sequencing of all five isolates was performed. DNA was extracted using a kit (Qiagen, Netherlands) from an overnight culture concentrated by centrifugation at 8,000×g for two minutes. It was then quantified using the Qubit fluorimeter and the Quant-it kit (Invitrogen, USA). Purified DNA was sent to Eurofins Genomics (GmbH) and 2x125 paired-end reads were obtained in a HiSeq 2500 Illumina system using the HiSeq SBS Kit, v4. Contigs were assembled using the Geneious assembler within the Geneious package, version 9.1.5. The MLST (version 1.8), Resfinder (version 2.1) and Plasmidfinder (version 1.3) services available at the website of the Center of Genomic Epidemiology (http://www.genomicepidemiology.org) were used in order to determine the sequence types of the strains, acquired resistance genes, and plasmids present, respectively. The Geneious software was used for comparison of whole genomes and the Rapid Annotation using Subsystem Technology (RAST) (http://rast.nmpdr.org/rast.cgi) tool (version 2.0) was used for annotation. Genome sequences

for C071, C440, C080, C314, and C428 were uploaded to Genbank with accession numbers MEDG00000000, MEDH00000000, MDTR00000000, MDTT00000000, and MDTS00000000, respectively. The sequences were then automatically annotated with the NCBI Prokaryotic Genome Annotation Pipeline.

3.3.6. Growth Curves

In order to determine doubling times, one colony was suspended in Luria-Bertani (LB) Broth and was incubated at 37°C overnight. The following day, a 1:100 dilution in 50mL LB broth was made and the suspension was incubated at 37°C with shaking at 200 rpm for 8 hours. Each hour, the OD_{600} was spectrophotometrically measured and the doubling times were calculated as previously described (Hall *et al.*, 2014). Three independent experiments were performed for this, and all subsequent experiments.

3.3.7. Hemolysis

Hemolysis was tested for by inoculating 10µL of a suspension adjusted to 10^3CFU/mL on 5% Sheep Blood Agar (SBA) (Biomérieux Mercy L'Etoile, France). The plates were then incubated at 37°C and hemolytic activity was monitored for 6 days (Antunes *et al.*, 2011).

3.3.8. Biofilm Formation

Biofilm formation was determined by inoculating 10µL from an overnight culture into 1mL LB broth in 12x75mm polystyrene tubes followed by an overnight incubation at 37°C. The bacterial suspension was then aspirated and the tube rinsed with water and stained with 1% crystal violet for 10 minutes. Visualization of a ring at the air-liquid interface after washing the dye was indicative of biofilm formation (Toamaras *et al.*, 2003).

3.3.9. Motility

Surface motility was tested by plating 1µL of an overnight culture on 0.3% LB-Agar (Difco, BD, USA). The plates were subsequently incubated at 37°C and the diameter of the distance travelled was measured at 14 hours (Antunes *et al.*, 2011). The motility rate is presented as this diameter divided by time. *Pseudomonas fluorescens* strain B52 was used as a positive control for this experiment, in addition to the two following ones. The incubation temperature for this strain was 30°C.

3.3.10. Proteolytic Activity

Proteolytic activity was measured via the Azoalbumin assay. One colony from each isolate was incubated in Trypticase Soy Broth Dyalisate overnight at 37°C with shaking at 200 rpm. The suspension was then centrifuged at 4,000xg for 10 minutes and 500µL of the filter-sterilized supernatant was incubated with 500µL of a 1mg/mL Azoalbumin solution (in 50mM Tris-HCl, pH=7.7), at 37°C for 24 hours. Trichloroacetic Acid with a final concentration of 13% was then added and the tubes were placed at -20°C for 20 minutes. The tubes were then centrifuged at 15,000xg for 10 minutes and the OD_{440} of the supernatant was measured (Antunes *et al.,* 2011). Results were presented in U/L where one U was defined as the amount of enzyme needed to degrade one micromole of Azoalbumin.

3.3.11. Siderophore Production

Detection of siderophores was performed using the liquid Chrome Azurol S (CAS) assay. One colony was used to inoculate the PMS_7-Ca medium and was incubated at 37°C for 72 hours with shaking at 200 rpm. The suspension was then centrifuged at 4000xg and 1mL of the filter sterilized supernatant was incubated with 1mL of the CAS solution for 1 hour. A blank was prepared using sterile PMS_7-Ca and CAS solution. The OD_{630} was then measured and a 10% difference between the blank and the sample was considered as positive (Louden *et al.,* 2011).

3.4. Results

3.4.1. Patient History

Patient 1 had non-Hodgkin lymphoma complicated with graft versus host disease and was admitted to the Burns Unit with epidermolysis progressing to 100% of the body surface. The patient required assisted ventilation which was later complicated by pneumonia, septic shock, and multiple organ failure. On the first day of admission, treatment was started with meropenem 2g/8h, tigecycline 100mg/12h, and liposomal amphotericin B 300mg/24h. Bronchial aspirate and blood samples were obtained and *A. baumannii* (C071) was identified in both samples. The patient was switched to colistin with a loading dose of 4.5 MU IV and a maintenance dose of 3 MU IV per 8 hours while retaining the tigecycline treatment. A second isolate from the same patient (C440) was obtained from blood on the 16^{th} day and was found

to be resistant to colistin. It was only susceptible to amikacin and minocycline (Table 1). The patient died on day 18.

Table 1. Antibiotic susceptibility testing of the five *A. baumannii* isolates. "MIC" represents Minimum Inhibitory Concentration expressed in µg/mL, "R" represents Resistant, "I" represents Intermediate resistance, and "S" represents Susceptible. "*" indicates that the results were verified by the E-test.

Antimicrobial Agent	Patient 1				Patient 2					
	C071		C440		C080		C314		C428	
	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S	MIC	R-I-S
Ampicillin/Sulbactam	>16/8	R	>16/8	R	>16/8	R	>16/8	R	>16/8	R
Ticarcillin	>64	R	>64	R	>64	R	>64	R	>64	R
Piperacillin /Tazobactam	>64/4	R	>64/4	R	>64/4	R	>64/4	R	>64/4	R
Ceftazidime	16	I	16	I	>16	R	>16	R	>16	R
Cefepime	8	I	8	I	>16	R	>16	R	>16	R
Imipenem	>8	R	>8	R	>8	R	>8	R	>8	R
Meropenem	>8	R	8	I	>8	R	>8	R	>8	R
Ciprofloxacin	>2	R	>2	R	>2	R	>2	R	>2	R
Levofloxacin	>4	R	>4	R	>4	R	>4	R	>4	R
Gentamicin	>8	R	>8	R	>8	R	>8	R	8	I
Tobramycin	>8	R	>8	R	>8	R	>8	R	>8	R
Amikacin	≤2	S	≤2	S	≤2	S	≤2	S	≤2	S
Colistin*	≤0.5	S	8	R	≤0.5	S	≤0.5	S	12	R
Minocycline	≤1	S	2	S	≤1	S	≤1	S	≤1	S
Tigecycline	≤0.5	-	4	-	1	-	2	-	2	-
Trimethoprim/Sulfamethoxazole	>4/76	R	>4/76	R	>4/76	R	>4/76	R	>4/76	R

Patient 2 was a diabetic patient with kidney failure that required dialysis and renal transplant five years before admission. The patient was admitted to the Intensive Care Unit presenting with paraventricular brain haemorrhage and subarachnoid hematoma that required external ventricular drainage. On the first day of admission, the patient was started on meropenem 2g/8h and linezolid 600mg/8h IV. A Cerebrospinal Fluid (CSF) sample was taken wherein *A. baumannii* (C080) was isolated. The patient was switched to colistin with a loading dose of 4.5 MU IV and a maintenance dose of 3 MU IV per 8 hours on the 3rd day. A second *A. baumannii* isolate (C314) was obtained from the CSF on the 8th day which remained susceptible to colistin. In the 12th day, a third isolate (C428) was obtained that was resistant to all beta-lactams, fluoroquinolones, trimethoprim-sulfamethoxazole, and colistin (Table 1). The intraventricular catheter was changed on the 13th day and the following culture of a CSF sample

was sterile. The patient recovered and was discharged on the 37th day (Table 1). Figure 1 shows the timeline of treatment and strain isolation of each patient.

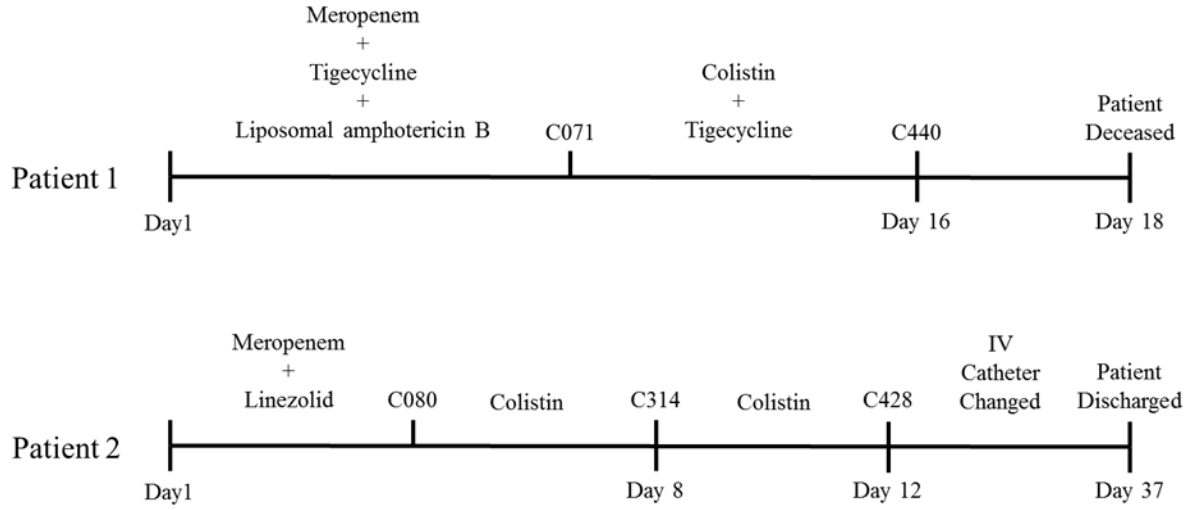


Figure 1. Timeline of treatment and strain isolation of each patient.

3.4.2. Sequencing of the *pmrCAB* Operon

The *pmrCAB* operon of the five strains was amplified by PCR and sequenced. Both the sensitive (C071) and resistant (C440) strains obtained from Patient 1 had an *IS*Aba1 sequence inserted between *pmr*A and *pmr*B in the opposite sense as compared to the rest of the operon. This sequence was lacking from all three strains isolated from Patient 2 (C080, C314, and C428). Strain C440 had a C to T change at nucleotide 697 of the *pmr*B gene as compared to C071 that resulted in a P233S mutation. As for the strains obtained from Patient 2, deletion of the nucleotides 55-57 in *pmr*B, resulting in the deletion of Isoleucine at position 19 of the protein, was detected in the colistin resistant strain C428 as compared to C080 and C314.

3.4.3. Detection of Carbapenemases by PCR

PCRs for the detection of the intrinsic and acquired carbapenemases were performed. *bla*$_{\text{OXA-51-like}}$ was amplified from all 5 isolates. In addition, *bla*$_{\text{OXA-58-like}}$ was detected in C071 and C440. None of the other tested genes were amplified.

3.4.4. Clonality Analysis

In order to determine the clonality of the strains, RAPD analysis and global lineage multiplex PCRs were performed. RAPD analysis showed that the two strains isolated from

Patient 1 were identical to each other and distinct from those isolated from Patient 2. The isolates obtained from Patient 2 were also identical to each other. The multiplex PCRs showed that C071 and C440 pertained to International Clone (IC) IC I while C080, C314, and C428 pertained to IC II.

3.4.5. Whole-Genome Sequencing

The Multi-Locus Sequence Typing (MLST) tool showed that strains C071 and C440 pertained to sequence type (ST) ST81 using the Pasteur scheme (Diancourt *et al.,* 2010) while C080, C314, and C428 pertained to ST2. Table 2 shows the antibiotic resistance genes identified by ResFinder. The aminoglycoside resistance genes *aac(6')Ib-cr* and *aadB,* as well as the sulphonamide resistance gene *sul1* were detected in all strains. *bla*$_{\text{OXA-58-like}}$ was detected in strains C071 and C440 while *bla*$_{\text{GES-5}}$ was detected in strains C080, C314, and C428. The *bla*$_{\text{OXA-69}}$ and *bla*$_{\text{OXA-66}}$ genes, which belong to the *bla*$_{\text{OXA-51-like}}$ family (Turton *et al.,* 2007), were also detected. Moreover, the Ser83Leu in *gyr*A and Ser80Leu in *par*C that are responsible for fluoroquinolone resistance were detected in the genome of all five strains (Warner *et al.,* 2016). Other than the mutations previously described in the *pmrCAB* operon, no significant mutations that affect colistin resistance, nor the *mcr-1* gene were detected.

Table 2. Resistance genes as detected in the different strains by the ResFinder service.

Patient 1 (Strains C071 and C440)			**Patient 2 (Strains C080, C314, and C428)**		
Gene	**Function**	**% Identity**	**Gene**	**Function**	**% Identity**
aacA4	Aminoglycoside resistance	100.00	*aadA2*	Aminoglycoside resistance	100.00
aadB	Aminoglycoside resistance	98.88	*aadB*	Aminoglycoside resistance	100.00
aph(3')-Ic	Aminoglycoside resistance	100.00	*strB*	Aminoglycoside resistance	99.04
aac(6')Ib-cr	Aminoglycoside resistance	99.61	*aac(6')Ib-cr*	Aminoglycoside resistance	99.42
sul1	Sulphonamide resistance	100.00	*sul1*	Sulphonamide resistance	100.00
bla$_{OXA-69}$	beta–lactamase	100.00	*sul2*	Sulphonamide resistance	100.00
bla$_{OXA-58}$	beta–lactamase	100.00	*bla*$_{OXA-66}$	beta–lactamase	100.00
			bla$_{GES-5}$	beta–lactamase	100.00
			cmlA1	Phenicol resistance	99.60
			dfrA7	Trimethoprim resistance	100.00

Plasmidfinder and Genbank searches showed that C071 and C440 harbor two small plasmids. The first was around 8 Kb long and did not harbor any resistance gene. It had high

similarity with plasmid pAC12 (Genbank accession number: CP007550). The second plasmid was around 6Kb long and was identical to plasmid pRAY* (Genbank accession number: KU869529) which harbors the *aadB* aminoglycoside resistance gene cassette. C080, C314, and C428 harbored a large plasmid of around 75 Kb that was similar to plasmid p1AB5075 (Genbank accession number: CP008707.1) but was missing a segment to be identical to that plasmid. Almost all the resistance genes detected in these three strains were in this plasmid.

Several genes associated with virulence in *A. baumannii* were detected in all five isolates. They include genes coding for the type IV pili and fimbriae assembly systems, in addition to *pil*A, *pil*B, and *pil*T, all of which are involved in twitching motility (Harding *et al.*, 2013). The *pga*A, *pga*B, *pga*C, and *pga*D genes involved in biofilm formation (Choi *et al.*, 2009) were also detected. Genes coding for the assembly of the siderophore Achromobactin as well as its receptor were detected in all isolates. C080, C314, and C428 additionally had two genes that are part of the operon coding for the assembly of the siderophore Anthrachelin. Finally, genes coding for several hemolysins in addition to the phospholipases C and D have been found in all five strains.

3.4.6. Generation times and *in-vitro* Virulence

Generation times and phenotypic detection of virulence factors were determined for all five strains. Generation times seem not to be affected by resistance to colistin when the colistin resistant strains were compared to their sensitive counterparts. Strain C314 showed a slightly reduced motility rate as compared to C080 and C428. Nevertheless, none of the tested strains showed any diffusion pattern on the agar surface and should be considered as negative as compared to other studies (Antunes *et al.*, 2011). C080, C314, and C428 showed α-hemolysis on SBA that started on day two whereas C071 and C440 showed no hemolysis on SBA. All the tested strains were able to develop biofilms in polystyrene tubes. Strain C440 was the only strain that was positive for *in-vitro* production of siderophores and had almost half the proteolytic activity as compared to C071 (Table 3).

Table 3. Generation times (in minutes), motility, hemolysis, biofilm formation, siderophore production, and proteolytic activity of the five tested strains. "D" stands for the day in which hemolysis was observed and "α" and "β" refer to the type of hemolysis observed.

Patient 1	**Generation Time (min)**	**Motility (µm/s)**	**Hemolysis**	**Biofilms**	**Siderophores**	**Proteolytic Activity (U/L)**
C071	39.3 ± 7.98	0.019 ± 0.007	-	+	-	30.69 ± 5.82
C440	44.7 ± 5.1	0.021 ± 0.011	-	+	+	15.49 ± 2.73
Patient 2	**Generation Time (min)**	**Motility (µm/s)**	**Hemolysis**	**Biofilms**	**Siderophores**	**Proteolytic Activity (U/L)**
C080	30 ± 4.68	0.066 ± 0.009	α since D2	+	-	29.89 ± 3.83
C314	24.42 ± 0.6	0.044 ± 0.008	α since D2	+	-	35.45 ± 5.39
C428	27.78 ± 4.74	0.052 ± 0.007	α since D2	+	-	25.03 ± 7.64

3.5. Discussion

The *pmr*CAB operon contains the two-component response regulator and sensor kinase pmrA/B that responds to changes in Fe^{3+} and Mg^{2+} levels in the environment. This system is also involved in lipid A modifications through the phosphoethanolamine transferase coded by *pmr*C (Arroyo *et al.*, 2011). Several mutations in *pmr*CAB, especially in *pmr*B, lead to colistin resistance in *A. baumannii* (Olaitan *et al.*, 2014; Adams *et al.*, 2009). In our study, two distinct *A. baumannii* isolates have been shown to acquire resistance to colistin through mutations in *pmr*B. The lack of other significant mutations in their genomes and of *mcr-1* demonstrates that these mutations are solely responsible for this resistance. The P233S mutation in *pmr*B of C440 is commonly reported among colistin resistant clinical isolates (Olaitan *et al.*, 2014; Adams *et al.*, 2009; Arroyo *et al.*, 2011; Pournaras *et al.*, 2014; Durante-Mangoni *et al.*, 2015). It is an activating mutation located within the histidine kinase domain of PmrB and is involved in dimerization (Durante-Mangoni *et al.*, 2015). The other mutation detected was the ΔIle19 deletion in *pmr*B of C428. This mutation is positioned in the center of the first trans-membrane helix of PmrB and, to the best of our knowledge, has not been reported elsewhere. It is possible that this mutation causes PmrB to be permanently activated which in turn could lead to colistin resistance in a manner similar to the hyperactive kinase mechanism (Arroyo *et al.*, 2011). Further characterization regarding the effect of this mutation on the proteins coded by *pmr*CAB could help better understand the mechanisms of colistin resistance. *IS*Aba1 was present within *pmr*CAB of both the sensitive and resistant strains obtained from Patient 1 indicating no direct effect on colistin resistance.

Acinetobacter baumannii is notorious for its ability to acquire DNA and adapt its genetic expression depending on a wide range of factors (Antunes *et al.*, 2014). Different

strains were shown to be able to adapt differently to the same stress conditions through mechanisms that are not yet fully understood (Antunes *et al.*, 2011). This was demonstrated in our study where different virulence profiles were obtained for the different *A. baumannii* strains. This becomes especially evident in the lack of motility and siderophore production in C080, C314, and C428, despite the presence of the required genes. Plasticity of the *A. baumannii* regulation systems could also have led to the conflicting reports regarding the effect of colistin resistance on virulence. Some studies report reduced virulence after acquisition of colistin resistance (Olaitan *et al.*, 2014; Lesho *et al.*, 2013), especially after mutations involving the *lpx*A, *lpx*C, or *lpx*D genes (Beceiro *et al.*, 2014). As for the P233S mutation, one study reports that it causes reduced virulence (Pournaras *et al.*, 2014) whereas another reports it to not affect virulence (Durante-Mangoni *et al.*, 2015). In our study, the colistin resistant strain C440 harboring the P233S mutation showed an increase in siderophore activity and almost half of the proteolytic activity of C071 (Table 3), despite having the rest of the genome identical. Since *pmr*B is involved in iron regulation, this mutation could have resulted in alterations in iron regulation, which in turn led to an over-production of siderophores, allowing for their phenotypic detection. Moreover, proteolytic activity in C440 seems to be negatively affected. These findings suggest an interplay between the mechanisms governing these virulence factors and colistin resistance in this strain that could be due to the detected mutations, the strain's genetic background, or some other factors. Further investigation into this matter could help better understand the interplay between colistin resistance and virulence in *A. baumannii*. The other colistin strain (C428) does not seem to have a difference in the tested virulence factors as compared to its sensitive counterparts.

Siderophore assembly and recovery systems in *A. baumannii* are still not fully characterized but the presence of such systems, similar to those of Achromobactin, have been identified in this bacterium (Penwell *et al.*, 2015). Such systems have been identified in all the tested isolates. In addition, the *asb*A and *asb*B genes were detected in C080, C314, and C428. These genes are part of a putative siderophore biosynthesis operon that was first described in *Bacillus anthracis* and belongs to the same group as that of Achromobactin (Challis *et al.*, 2005). However, siderophore production was not phenotypically detected in these isolates possibly indicating a low level expression of these genes.

C071 and C440 pertained to the MDR ST81 which was encountered in 10.2% of 729 *A. baumannii* isolates obtained from various Spanish hospitals in an 11-year study (Villalon *et*

al., 2011). It is different from IC I (also known as ST1) by a single allele that is not tested for by the multiplex PCRs used in this study, explaining why it was reported as pertaining to this clone. Carbapenem resistance in C071 and C440 is a result of the *bla*$_{\text{OXA-58-like}}$ gene they harbor that is commonly reported among *A. baumannii* (Mostachio *et al.,* 2009). C080, C314, and C428 were found to harbor *bla*$_{\text{GES-5}}$ and they belong to the widely spread MDR sequence type ST2, also known as IC II. *bla*$_{\text{GES-5}}$ has been reported in several other species (Bonnin *et al.,* 2011), but, to the extent of our knowledge, was never reported in *A. baumannii*. Nevertheless, the *bla*$_{\text{GES-5}}$ genes detected by Resfnder in these strains differed by only one nucleotide from some sequences of *bla*$_{\text{GES-14}}$ deposited in Genbank. The ability of strains from different widely disseminated MDR lineages to develop resistance to colistin presents a great threat on the continued use of this antibiotic and highlights the potential of pan-drug resistant *A. baumannii* outbreaks.

3.6. Conclusions

In conclusion, the previously reported P233S and the novel ΔIle19 mutations in *pmrB* were identified as sole genetic mutations that lead to colistin resistance in two distinct isolates pertaining to widely disseminated international clones. The former mutation resulted in a change in the virulence profile of the colistin resistant isolate whereas the latter did not produce any change. In addition to colistin resistance, one set of isolates harbored *bla*$_{\text{OXA-58-like}}$ and the other set harbored *bla*$_{\text{GES-5}}$. Further investigations into the interlocking mechanisms involved in colistin resistance and virulence could lead the way for the development of antimicrobial agents that could attenuate one or the other, if not both.

Supplementary Table S1. Primers used for the amplification and sequencing of the *pmrCAB* operon.

Primer	Sequence (5' to 3')	Reference	Primer	Sequence (5' to 3')	Reference
pmrBAC_L	GCGAGGAGCACATTTCCTAA	Adams *et al.*, 2009	G1F	CCTGATGCGGGGTGTAC	This study
pmrBAC_R	TGTAGTCACTCACGATGCTGAA	Adams *et al.*, 2009	G1R	CGAACACCTGTGACTGC	This study
pmrBAC_L2	TTAAAGTTACATCTTGCTTTGCC	Adams *et al.*, 2009	G2F	GGTGCCCCAAATCAGTCG	This study
pmrBAC_L2c	GGCAAAGCAAGATGTAACTTTAA	Adams *et al.*, 2009	G2R	CTCTCTGTCTGCGAACAC	This study
pmrBAC_R2	TCGATGAAATTCTAGATACTCAAATG	Adams *et al.*, 2009	G3F	AGCGCTTCTTTGCAGGTC	This study
pmrBAC_L3	CCCAAATATCGATAAACAGATCTTC	Adams *et al.*, 2009	G3R	GAGCGAAGCTGGGTAAAG	This study
pmrBAC_R3	TTGAAGCAGATCCGTCAAAG	Adams *et al.*, 2009	G4F	TACTCCACTACGGCGTAG	This study
pmrBAC_L4	TGCACCCAAATTTAAACCATC	Adams *et al.*, 2009	G4R	GTTTGAGGTGGAATGGGTC	This study
pmrBAC_R4	CCGACTTGTGATACGAATGC	Adams *et al.*, 2009	G5F	GTGTTTGTTGGCTCGGTG	This study
pmrBAC_L5	TCATTTGGCTTAATACATGGTCTG	Adams *et al.*, 2009	G5R	CGTGCGGGTTACCAAGTG	This study
pmrBAC_R5	GGATGATTTCACCGCAAAATAG	Adams *et al.*, 2009	G6F	GGCACGTGCCGTTTCACC	This study
pmrBAC_R5c	CTATTTTGCGGTGAAATCATCC	Adams *et al.*, 2009	G6R	GTAGTCACTCACGATGCTG	This study

4. Different Patterns and Kinetics of Biofilms Produced by *Acinetobacter baumannii* clinical isolates with Different Antibiotic Susceptibility Profiles

4.1 Abstract

Acinetobacter baumannii is an organism that has been implicated in several nosocomial infections. Biofilm production in this species is key for its persistence for prolonged periods of time in hospitals. In this study, the biofilm formation patterns and rates among clinical *A. baumannii* isolates are investigated in light of different antibiotic susceptibility patterns. Our aim is to investigate a possible link between antimicrobial susceptibility and specific biofilm formation patterns that would help clinicians and infection control specialist combat this pathogen. Biofilms were cultured on steel coupons immersed in Brain Heart Infusion broth at 37 °C for 48 hours. The attached viable cells were counted after 5, 24 and 48 hours. Confocal Laser Scanning Microscopy (CLSM) images were then obtained for two isolates that produced a brown pigment while being cultured and two others that did not show pigmentation. The images were analyzed using image analysis software and biofilm volume and substratum coverage were estimated. This study, though preliminary, shows a possible link between aminoglycoside susceptibility and quick rates of biofilm formation. It also shows a link between carbapenem resistance and thicker, more uniform surface coverage of the produced biofilms. Since the investigated isolates were relatively few in number, future studies involving larger pools of isolates could help consolidate the findings of this study.

4.1. Resumen

Acinetobacter baumannii es un organismo implicado en infecciones nosocomiales. Su capacidad de producir biofilms es clave en su capacidad de persistir durante periodos prolongados de tiempo en el ambiente hospitalario. En este estudio se investigan los patrones y velocidad de formación de biofilms de cepas clínicas de *A. baumannii* con diferentes perfiles de resistencia a antibióticos. Nuestro objetivo es determinar una posible asociación entre resistencia a determinados antibióticos y cierto patrón de formación de biofilms para ayudar a la comunidad médica y los especialistas en el control de infecciones para combatir este patógeno. Se cultivaron doce cepas clínicas de *A. baumannii* en soportes de acero inoxidables, inmersos en caldo de BHI durante 48 horas a 37 °C. Las células bacterianas adjuntas a los soportes se contaron tras 5, 24, y 48 horas de incubación. A continuación, se analizaron las imágenes obtenidas por Microscopía Confocal Láser de Barrido para dos cepas que formaron pigmentos durante el cultivo y dos que no los formaron. El volumen del biofilm y la cobertura de sustrato fueron analizados utilizando un programa informático. Nuestros resultados preliminares muestran una relación entre las tasas rápidas de formación de biofilms y la susceptibilidad a aminoglicósidos. Además, se observó una relación entre la resistencia a carbapenemas y la formación densa de biofilms con cobertura uniforme del sustrato. A pesar de que el número de cepas investigadas no es suficiente para elaborar conclusiones globales, investigaciones futuras que incluyan un mayor número de cepas en el estudio, podrían consolidar los resultados de este trabajo.

4.2. Introduction

Acinetobacter spp. are Gram-negative bacteria that could act as bioremediation agents, oil extraction aids, and even as antibiotic-resistant nosocomial pathogens. They possess an outstanding chemical ability in degrading xenobiotic compounds that include alkanes, herbicides, and pharmaceuticals (Doughari *et al.,* 2011; Touchon *et al.,* 2014). In addition to its intrinsic resistance to numerous antibiotics, *A. baumannii* can easily acquire resistance to various antimicrobials by over-expressing efflux pumps, down-regulating porins, producing antibiotic-degrading enzymes, and/or modifying the target of antibiotics (Tenover *et al.,* 2006; Antunes *et al.,* 2014). These resistance mechanisms have resulted in considering *A. baumannii* as one of the most dangerous nosocomial pathogens, implicated in ventilator-associated pneumonia, as well as bloodstream-, burn-, wound- and catheter-related infections. Antimicrobial agent resistance among *A. baumannii* clinical isolates are very frequent and mortality rates caused by infections with these resistant strains could be as high as 60% (Peleg *et al.,* 2008, McConnell *et al.,* 2013; Doi *et al.,* 2015).

The ability of the clinical isolates of *A. baumannii* to produce biofilms at different rates and patterns is often reported (Rodriguez-Baño *et al.,* 2008; Lee *et al.,* 2008; Longo *et al.,* 2014). Regulation of the formation mechanisms of biofilms, including the involvement of quorum sensing molecules, is progressively being characterized (Gaddy *et al.,* 2009; Rumbo-Feal *et al.,* 2013; Liou *et al.,* 2014). Several genes, including *Csu*E and *Omp*A have been found to be involved in biofilm formation (Choi *et al.,* 2009). Biofilms provide bacteria with protection against hazards that range from antimicrobial agents to stress conditions, such as desiccation. They have also been found to assist in the development of antibiotic recalcitrance by different mechanisms that mainly involve the facilitation of the exchange of genetic elements that contain resistance genes (Doi *et al.,* 2015; Ciofu *et al.,* 2011; Lebeaux *et al.,* 2014; Olsen *et al.,* 2014). Moreover, biofilms provide diffusion limitations due to the charged macromolecular mesh of their matrix and act as protective barriers for the cells embedded within. Moreover, the slowly dividing cells at the deep layers of the biofilm are scarcely susceptible to antimicrobial agents that hinder cell division. In addition, due to high cell densities within the biofilms, antimicrobial exposure give rise to small subpopulations of persister cells that survive the antimicrobial treatment and that will eventually repopulate the biofilm (Bhargava *et al.,* 2014).

Antibiotic resistance and biofilm formation both depend on genetic diversity and genetic expression within the individual strains. Understanding the connections among these phenomena has great potential to understand the persistence of *A. baumannii* in the hospital environment and its colonization of medical equipment (Badmasti *et al*., 2015). In this study, 12 clinical isolates, with different antimicrobial susceptibility profiles, chosen from a set of isolates collected from Hospital Universitario – La Paz, were investigated in terms of the kinetics and structure of biofilm formation on abiotic surfaces. The patterns obtained were then analyzed in light of the antibiotic susceptibility profiles, presence of *Csu*E and *Omp*A, and production of siderophores and pigments. The association between clinical findings and the ability to attach and form biofilms on abiotic surfaces is clinically important, especially from an infection control perspective, where special care could be taken for strains that are expected to have certain patterns to biofilm formation based on antibiotic susceptibility profiles. This could lead to avoiding the persistence of these isolates for prolonged periods of time in the hospital.

4.3. Materials and Methods

4.3.1. Bacterial Strains

Acinetobacter baumannii bloodstream isolates were obtained from patients with different conditions and primary sites of infection. *Pseudomonas fluorescens* B52, originally isolated from cold bulk raw milk (Richardson and Te Whaiti, 1978), was used as a control strain.

The Bactec™ (Becton Dickinson, Franklin Lakes, NJ, US) automated system was used in order to process blood cultures. The isolates were identified using Matrix Assisted Laser Desorption-Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH). Positive blood cultures were centrifuged at 140×g for 5 minutes. The supernatant was then centrifuged at 16,000×g for 10 minutes and the supernatant was discarded. The pellet was washed with 1 mL deionized water and a solution containing 300 µL water and 900 µL absolute ethanol was added. The suspension was centrifuged at 29,000×g for 2 minutes, the supernatant discarded, and the pellet re-suspended in 20 µL of 70% (v/v) formic acid. Then, 20 µL of acetonitrile was added to the mixture and the solution was centrifuged at 29,000×g for 1 minute. 1µL of the supernatant was transferred unto a steel target plate and gently mixed with 1µL α-cyano-4-hydroxy-cinnamic acid matrix solution in an organic solvent containing 50%

acetonitrile and 2.5% trifluoroacetic acid. The plates were allowed to air dry and mass spectra were obtained using a Microflex LT Mass Spectrometer (Burker Daltonik, GmbH) (Romero-Gómez *et al.*, 2012). The spectra were compared to reference libraries provided by the manufacturer (Reference library 3.0.10) in the MALDI-BIOTYPER 2.0 software (Bruker Daltonik, GmbH).

The strains were stored at -20°C in Tryptone Soy Broth (TSB, Oxoid) supplemented with 15% glycerol until used. Pre-inocula were obtained after overnight incubation at 37ºC in Brain Heart Infusion broth (BHI, Oxoid). Cells were harvested by centrifugation at 4000×g for 10 min and washed twice in sterile BHI. Their OD_{600} was then adjusted so as to obtain 10^3 CFU/mL of each strain after inoculation.

4.3.2. Biofilm Experimental System

Biofilms were cultured in BHI broth at 37ºC on disposable 24-well microtiter plates (Thermo Fisher Scientific) with 10x10mm 304 Stainless Steel (SS) coupons as substratum surfaces placed in each well. Before use, coupons were gently swabbed with a postsurgical toothbrush and soap solution, rinsed with distilled water, and autoclaved while inside glass Petri dishes. In each well, one sterile coupon was immersed into 1mL of bacterial suspension. The whole system was wrapped in aluminum foil and a tray filled with water was placed under the microplate in order to avoid evaporation. Only the upper side of the SS coupon was considered for quantification of attached biofilm forming cells whereas the lower side was marked and remained downwards all along the assay.

4.3.3. Cell Recovery and Counting

For cell recovery and counting, at 5, 12, and 24 hours after incubation, the surface of the steel coupon was repeatedly scraped in several directions in order to recover as much of the attached cells as possible. The cells were then transferred into a tube containing 1.5 mL peptone water and vigorously stirred using a vortex so as to break up the cell aggregates. Serial dilutions in peptone water was then done and 10µL from each tube were plated on Tryptone Soy Agar (TSA, Oxoid). The TSA plates were then incubated at 37ºC and viable cells were counted after 24 hours. Two SS coupons for each time point were taken per strain and the entire experiment was independently repeated three times.

4.3.4. Siderophore Determination in CAS Solution

While performing the experiment, some strains appeared to be pigmented while others did not. Therefore, differential expression of siderophore production was suspected and siderophores were tested for. In order to detect siderophore production, the 12 *A. baumannii* clinical isolates, in addition to *P. fluorescens* strain B52 were cultured in an iron free mineral medium (PMS_7-Ca) that contains, per liter: 10.7g of N,N-bis-(2-hydroxymetyl)-2-aminoethanesulfonic acid (BES), 11g of sodium pyruvate, 0.86g of dibasic potassium phosphate, 0.65g of ammonium chloride, and 0.2g of magnesium sulphate. The solution was then adjusted to pH 7.0, autoclaved, and supplemented with 0.111 g/L of filter-sterilized calcium chloride. After incubation in this medium, the cells were harvested by centrifugation at 4,000 × g for 10 min, washed twice with the same medium, and diluted in order to reach an initial concentration of 10^3 CFU/mL after inoculation. Cultures were carried out for 24h, at 37ºC for *A. baumannii* strains, and at 21ºC for *P. fluorescens* B52.

For siderophore detection, the Chrome Azurol S (CAS) assay was used (Schwyn and Neilands, 1987). In order to prepare the CAS solution, three solutions were prepared. 0.06g CAS powder (Sigma Aldrich) were dissolved in 50 mL ultra-pure water and labeled Solution 1. Solution 2 consisted of 0.0027g $FeCl_3$-$6H_2O$ dissolved in 10 mM HCl. 0.073 g of hexadecyl trimethyl ammonium bromide (HDTMA) were dissolved in 40 mL ultra-pure water in yet another solution labelled Solution 3. Then, Solution 1 was mixed with 9 ml of Solution 2 and then mixed with Solution 3, resulting in a mixture with an intense blue color. The mixture was stored in a plastic container and protected from light until used. For siderophore detection, 1 mL of cell-free supernatant resulting from growing the strain in PMS_7-Ca was mixed with 1 mL of the CAS solution. A negative control was prepared with 1mL of sterile PMS_7-Ca medium instead of the culture's supernatant and *P. fluorescens* B52's supernatant was used as a positive control for siderophore production. A blue to green change in color of the CAS solution was indicative of the presence of siderophores (Louden *et al.*, 2011).

4.3.5. Antibiotic Susceptibility Testing

The Minimum Inhibitory Concentrations (MICs) were determined by broth microdilution using the automated Vitek2 system with AST-N-245 cards (bioMérieux, Marcy l'Etoile, France). The results were interpreted according to contemporary Clinical and Laboratory Standards Institute (CLSI) standards. The results were reported as "R" if the strain

had an MIC value higher than the cutoff value for resistance, “I” if it the MIC was between the cutoff values of resistance and susceptibility, and “S” if the MIC value was below the cutoff value for susceptibility for each antibiotic. The ranges of the antimicrobial agents tested for were: Ticarcillin (4-128µg/mL), piperacillin (4-128µg/mL), ampicillin/sulbactam (2/2-32/16µg/mL), piperacillin/tazobactam (4/4-128/4µg/mL), ceftazidime (1-64µg/mL), cefepime (1-64µg/mL), imipenem (0.25-16µg/mL), meropenem (0.25-16µg/mL), colistin (0.5-16µg/mL), gentamicin (1-16µg/mL), tobramycin (1-16µg/mL), amikacin (2-64µg/mL), minocycline (1-16µg/mL), ciprofloxacin (0.25-4µg/mL), levofloxacin (0.12-8µg/mL), and trimethoprim/ sulfamethoxazole (1/19-16/304µg/mL).

4.3.6. Polymerase Chain Reaction

DNA was extracted from the tested strains according to manufacturer’s instructions using a commercial kit (Qiagen, Netherlands). Polymerase Chain Reaction (PCR) in order to detect the presence of the *Omp*A and *Csu*E genes was then performed. The PCR mix contained 1X PCR Buffer with 1.5mM $MgCl_2$, 12.5pmol of each primer, 200µM dNTPs, and 1 U Taq polymerase. The primers used for the *Omp*A gene were: 5’CAATTGTTATCTCTGGAG3’ and 5’ACCTTGAGTAGACAAACGA3’. The primers for the *Csu*E gene were 5’ATGCATGTTCTCTGGACTGATGTTGAC3’ and 5’CGACTTGTACCGTGACCG-TATCTTGATAAG3’. PCR conditions were 94°C for 3 minutes, followed by 35 cycles of 94°C for 45 seconds, 50°C and 65°C for *OmpA* and *CsuE* respectively for 45 seconds, 72°C for 1 minute, and a final extension step at 72°C for 5 minutes (Turton *et al.,* 2007). PCR products were then run on 1.5% agarose gels and visualized using a gel documentation system (BioRad, Germany).

4.3.7. Confocal Laser Scanning Microscopy (CLSM)

For CLSM observations, biofilms developed on SS coupons were rinsed with sterile 0.9% NaCl and then stained with Syto 13 (S7575, Life Technologies) after 24 hours of incubation at 37°C. This stain labels all the bacteria present. Calcofluor White (18909, Fluka), a non-specific fluorochrome that binds to cellulose, chitin, and other polysaccharides commonly present in the biofilm matrix was then added. The green color observed in CLSM images corresponds to bacterial cells, whereas the blue color corresponds to Extracellular Polymeric Substances (EPS). CLSM images of the SS coupons were obtained using a Fluoview® FV 1200 Laser Scanning Confocal Microscope (Olympus) with an oil immersion

60X objective lens. Three- dimensional projections (Maximun Intensity Projection, MIP) were reconstructed from z-stacks using IMARIS® 7.7 software (Bitplane AG, Zurich, Switzerland). The whole image was then segmented into channels and analyzed to obtain the total volume occupied by cells (green) and EPS (blue). Biofilm ratios were then calculated using the MeasurementPro module of the IMARIS software.

4.3.8. Statistical Analysis

Three independent experiments for biofilm attachment on steel coupons were performed and two coupons were sampled at each time point (in total, n=6). One-way ANOVA using the STATGRAPHICS PLUS 5.0 software (Statistical Graphics Corporation, Rockville, Md., USA) was performed in order to check for statistical associations. A multiple range test was also performed in order to check if there were differences among strains in terms of biofilm formation rates. Mean comparisons were carried out to determine significant differences at a 95% confidence level ($p < 0.05$).

4.4. Results

4.4.1. Antibiotic Susceptibility and Characteristics of the Strains

All the strains were bloodstream isolates and were deliberately chosen for their heterogeneity in antibiotic susceptibility and clinical history. Their origin, according to the patient's unit assignment and/or treatment, is shown in Table 1. Five isolates were obtained from the Intensive Care Unit (ICU) and four from the Burn Unit. Strain 30 was obtained from Hematology/Oncology and strain 59 from Internal Medicine. Strain 20 also was from Internal Medicine, but the patient was not previously admitted to the ICU. Two thirds of the isolates were from patients previously exposed to mechanical ventilation and catheterization. All the patients, except those from whom isolates 9, 20, and 38 were obtained, had undergone antibiotic treatment in the 15 days prior to isolation of the strain.

Table 1. Clinical data of the patients harboring the isolates. Risk factors that may contribute to biofilm formation ("MV= Mechanical Ventilation; CVC= Central Venous Catheterization, and UC= Urethral Catheterization), previous antibiotic treatment (15 days prior to isolation), primary infection prior to bacteremia, underlying disease and clinical outcome of the patient.

Strain	Risk Factors	Previous Antimicrobial Treatment	Disease	Primary infection	Patient Outcome
3	MV CVC UC	Carbapenems	2nd degree burns on 70% of body surface	Unknown	Recovered
9	None	None	Rapidly progressive glomerulonephritis	Urinary Tract Infection	Recovered
12	MV CVC UC	Carbapenems Colistin Linezolid	Flame burn on 26% of the body surface	Respiratory Infection	Died
15	MV CVC UC	Carbapenems Colistin Linezolid	Amputation of left leg	Respiratory Infection and Soft Tissue Infection	Recovered
20	None	None	Multifactorial chronic anemia Septic shock	Soft Tissue Infection	Recovered
26	MV CVC	Carbapenems Linezolid	Bilateral eosinophilic Pneumonia	Respiratory Infection	Recovered
30	None	Carbapenems Vancomycin	Severe combined immunodeficiency Bone marrow transplant	Unknown	Recovered
35	MV CVC UC	Carbapenem Colistin Linezolid	Flame burn on 70% of the body surface	Respiratory Infection	Died
38	MV CVC UC	None	Intracranial hematoma	Respiratory Infection	Recovered
45	MV CVC UC	Carbapenems Colistin Tigecycline	Non-Hodgkin lymphoma Allogeneic transplant	Respiratory Infection	Died
52	MV CVC UC	Carbapenems Colistin Vancomycin	Acute lymphoblastic leukemia Bone marrow transplant	Respiratory Infection	Died
59	None	Piperacillin/ Tazobactam	Crohn's Disease	Unknown	Recovered

According to AST data (Table 2), the only fully susceptible strains were 38 and 59. The rest of the strains were all resistant to beta-lactams and quinolones and eight of the twelve strains were resistant to carbapenems. Strains 12 and 45 were additionally resistant to colistin. All strains tested here were still relatively susceptible to some aminoglycosides and tetracyclins. The exceptions were strains 9 and 20 that were resistant to all aminoglycosides and had an intermediate resistance to tetracyclins. According to the definitions proposed by Magiorakos *et al.* (2011), strains 9, 12, 15, 20, 35 and 45 would be classified as Extensively Drug Resistant (XDR) and strains 3, 26, 30 and 52 as Multi Drug Resistant (MDR) while strains 38 and 59 remain susceptible.

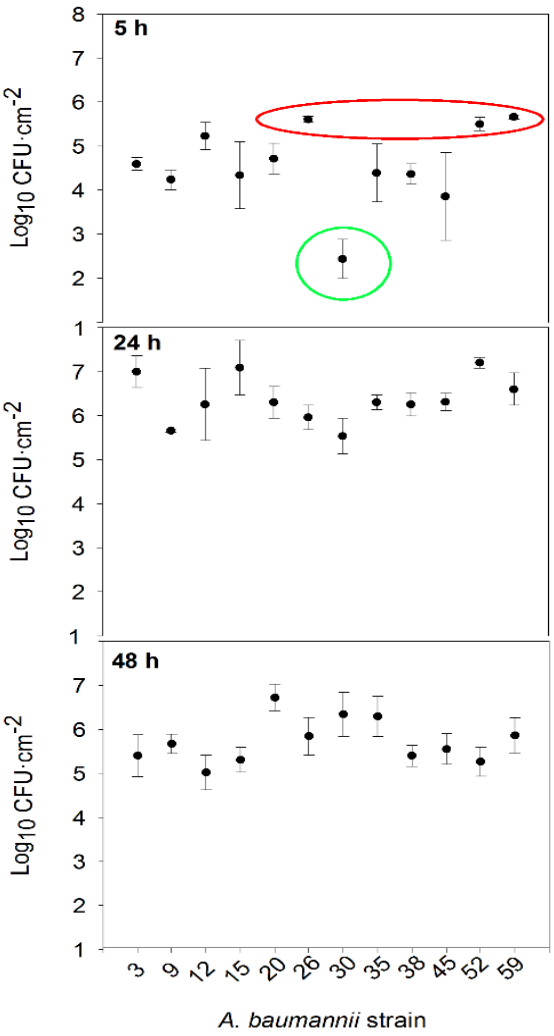
Table 2. Resistance of the *A. baumannii* strains to antimicrobials agents. “MIC” is the Minimum Inhibitory Concentration acquired in μg mL−1. "R" stands for Resistant, "I" stands for Intermediate resistance, and "S" stands for Susceptible, as interpreted according to the CLSI guidelines. "TIC" stands for Ticarcillin, "PIP" for Piperacillin, "A/S" for Ampicillin/Sulbactam, "P/T" for Piperacillin/Tazobactam, "CTZ" for Ceftazidime, "CFP" for Cefepime, "IMI" for Imipenem, "MER" for Meropenem, "COL" for Colistin, "G" for Gentamycin, "TO" for Tobramycin, "AK" for Amikacin", "MIN" for Minocycline, "CIP" for Ciprofloxacin, "LEV" for Levofloxacin, and "T/S" for Trimethoprim/Sulfamethoxazole".

Strain		Antimicrobial Agents														
		Beta-Lactams								Polymyxin	Aminoglycosides			Tetracyclin	Fluoroquinolones		Folic Acid Synthesis Inhibitors
		TIC	PIP	A/S	P/T	CTZ	CFP	IMI	MER	COL	G	TO	AK	MIN	CIP	LEV	T/S
3	MIC	>64	>64	16/8	>64/4	>32	16	>8	>8	≤0.5	≤1	≤1	≤2	2	>2	>4	>4/76
	S-I-R	R	R	I	R	R	I	R	R	S	S	S	S	S	R	R	R
9	MIC	>64	>64	16/8	>64/4	>32	32	>8	>8	≤0.5	>8	>8	>32	8	>2	>4	>4/76
	S-I-R	R	R	I	R	R	R	R	R	S	R	R	R	I	R	R	R
12	MIC	64	>64	4/2	>64/4	16	32	>8	>8	16	>8	>8	8	≤1	>2	>4	>4/76
	S-I-R	I	R	S	R	I	R	R	R	R	R	R	S	S	R	R	R
15	MIC	>64	>64	16/8	>64/4	>32	>32	>8	>8	≤0.5	>8	>8	16	≤1	>2	>4	>4/76
	S-I-R	R	R	I	R	R	R	R	R	S	R	R	S	S	R	R	R
20	MIC	>64	>64	16/8	>64/4	>32	>32	>8	>8	≤0.5	>8	>8	>32	8	>2	>4	>4/76
	S-I-R	R	R	I	R	R	R	R	R	S	R	R	R	I	R	R	R
26	MIC	32	>64	4/2	>64/4	>32	16	≤1	1	≤0.5	≤1	≤1	≤2	2	>2	>4	>4/76
	S-I-R	I	R	S	R	R	I	S	S	S	S	S	S	S	R	R	R
30	MIC	>64	>64	4	64/4	16	16	2	2	≤0.5	>8	8	4	≤1	>2	>4	>4/76
	S-I-R	R	R	S	I	I	I	S	S	S	R	I	S	S	R	R	R
35	MIC	>64	>64	>16/8	>64/4	>16	8	>8	8	≤0.5	>8	>8	≤2	≤1	>2	>4	>4/76
	S-I-R	R	R	R	R	R	I	R	I	S	R	R	S	S	R	R	R
38	MIC	≤8	≤8	≤2/1	≤4/2	4	2	≤1	≤0.25	≤0.5	≤1	>8	≤2	≤1	≤0.25	≤0.12	≤2/38
	S-I-R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
45	MIC	>64	>64	>16	>64/4	>32	8	>8	8	8	>8	8	≤2	2	>2	>4	>4/76
	S-I-R	R	R	R	R	R	I	R	I	R	R	I	S	S	R	R	R
52	MIC	>64	>64	>16/8	>64/4	>32	>32	>8	>8	≤0.5	4	≤1	≤2	≤1	>2	>4	>4/76
	S-I-R	R	R	I	R	R	R	R	R	S	S	S	S	S	R	R	R
59	MIC	≤8	≤8	≤2/1	≤4/2	2	2	≤1	1	≤0.5	≤1	≤1	≤2	≤1	≤0.25	≤0.12	≤2/38
	S-I-R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

4.4.2. Patterns of Biofilm Formation among the Different Strains

The strains were tested for their biofilm formation ability on steel coupons at 5, 24, and 48 hours. As shown in Figure 1, all of the strains were able to attach to SS coupons at 37ºC, though with different development kinetics. The strains were classified into three different groups according to the density of attached cells per surface unit after 5 hours of incubation (Figure 1a). Group 1 (circled in red) includes strains 26, 52 and 59 and were designed as quick biofilm formers. These strains had counts of more than 5 Log CFU/cm^2 at this time point. Group 3 (circled in green) represented a single slow biofilm-forming isolate, isolate 30, which attained just around 2 Log CFU/cm^2 after 5h. The rest of the strains, having intermediate attached cell densities, constituted Group 3.

Figure 1. Attached cell density of each *A. baumannii* strain, after 5 h (A), 24h (B) and 48h (C) incubation. Dots represent the average of three independent experiments (n=6) where two coupons were sampled for each time point in each experiment, while the bars represent the standard deviation.



After 24h incubation (Figure 1b) most strains attained similar counts of viable biofilm forming cells. After 48h (Figure 1c), dispersal caused a decrease in the number of attached cells. Attached cell densities continued to increase between 24 to 48 hours for only two out of the twelve tested strains (strains 20 and 30). In terms of the presence of the biofilm-related genes, all the strains were positive for *Omp*A and all but strains 12 and 59 were positive for *Csu*E.

4.4.3. Pigment Production, Siderophores, and Biofilm Structure

While testing for the rate of biofilm formation, strains 3, 26, 45 and 52 were noted to produce a brown pigment while the others presented a neutral hue (Figure 2). All the strains, except for strain 30, were positive for siderophore production. Confocal Laser Scanning Microscopy (CLSM) images of 24-hour biofilms of two pigment producing strains (52 and 45) and two non-pigmented strains (30 and 38) were then obtained in order to see if a relationship exists between pigmentation and biofilm structure. The images were then analyzed in order to estimate substratum surface coverage and biofilm thickness and volume (Table 3). Pigmented strains showed thicker biofilm structures than non-pigmented ones and covered almost the whole surface of the available substratum (Figure 3). The biofilms of strain 45 had the highest biovolume value after 24 hours and both pigmented strains gave rise to relatively homogeneous biofilms with wide surface coverage. On the other hand, the non-pigmented strains, gave rise to biofilms with small volumes that did not homogeneously cover the substratum surface. Strain 38 had a heterogeneous biofilm mostly consisting of scattered colonies unable to cover the whole surface. Strain 30 had a light and thin homogeneous coating and had a higher proportion of matrix to cells than the others (Table 3).

Table 3. Structural parameters of *Acinetobacter baumannii* biofilms (n=2).

***A. baumannii* strain**	**Biofilm thickness (μm)**	**Biovolume (μm^3)**	**Biovolume distribution (%)**	
			Cells	**Matrix**
38	6	$1.6*10^3$	90	10
30	2	$3.3*10^3$	51	49
45	9	$112*10^3$	83	17
52	9	$12.3*10^3$	73	27

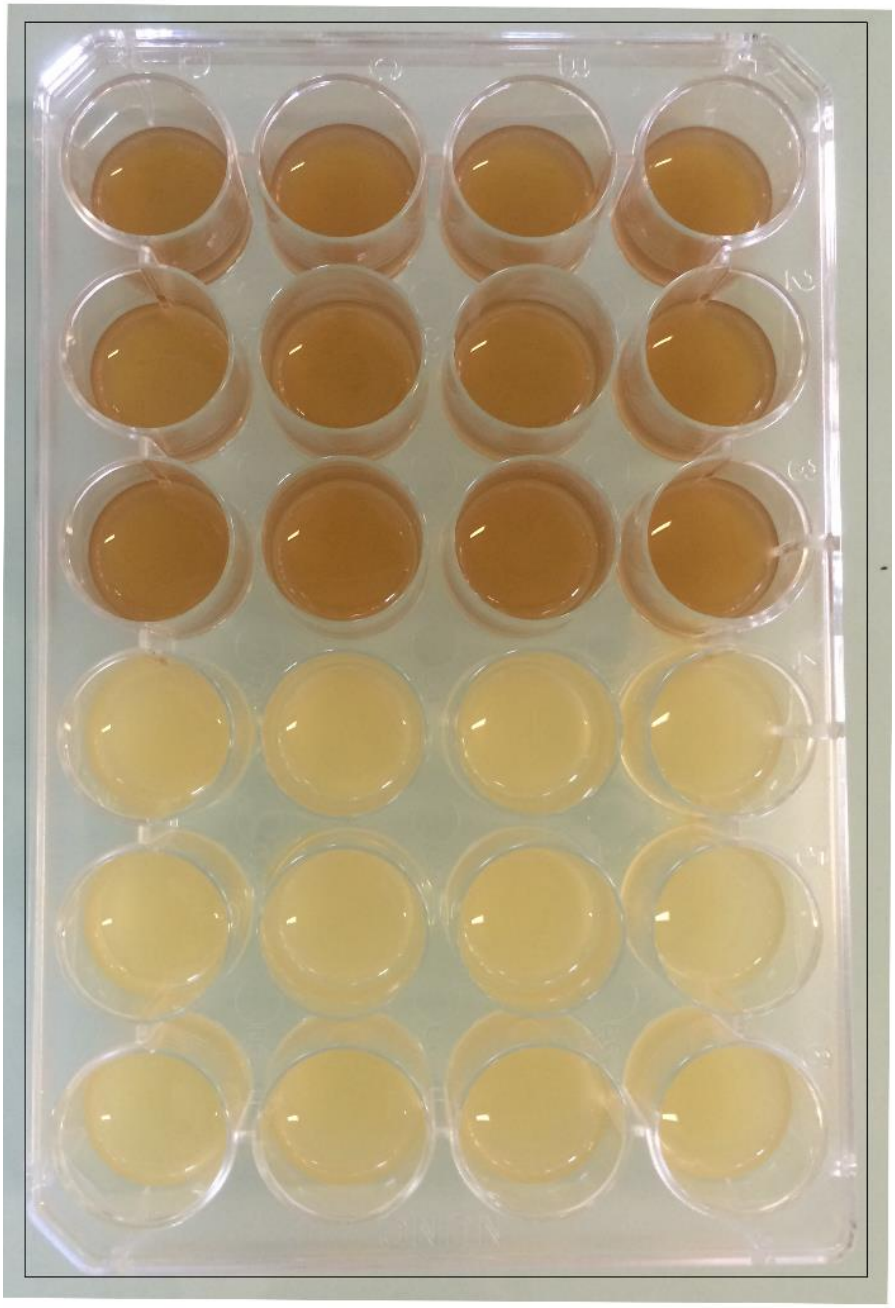


Figure 2. Pigment production during incubation of strain 52 (above) and 38 (below).

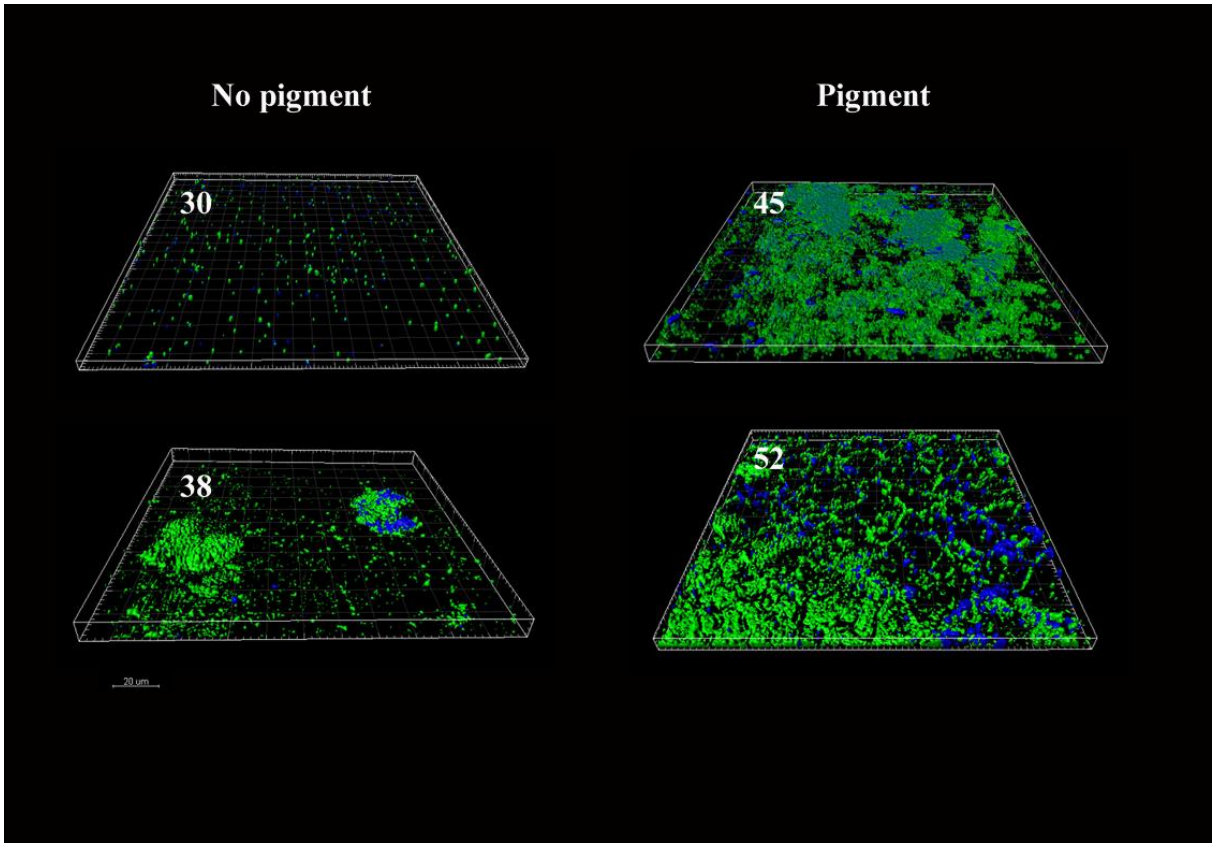


Figure 3. CLSM images (zenithal view) of 24h-biofilms formed by *A. baumannii* strains pigment (+): 45 and 52 and pigment (-): 30 and 38. Cells were in green and EPS in blue.

4.5. Discussion

The twelve clinical isolates included in this study were obtained from different origins in an attempt to assess the effect of origin on biofilm formation patterns. Nevertheless, since most *A. baumannii* infections occur in the ICU, most of the isolates where from the ICU and were from patients that were exposed to mechanical ventilation and catheterization. Both these factors are considered risk factors for biofilm formation and subsequent *A. baumannii* infection (McConnell *et al.*, 2013), making the fact that all the isolates produced biofilms, an expected one. No direct relationship between the source of the isolate and the rate of biofilm formation seems to exist. The XDR and MDR profiles obtained is also not surprising due to the vast array of intrinsic resistance mechanisms in *A. baumannii* and its outstanding ability to acquire resistance (Peleg *et al.*, 2008). The most concerning of the observed resistances is resistance towards carbapenems since, in addition to being globally reported at increasing rates, it is associated with increased mortality (Lemos *et al.*, 2014). Additionally, the two colistin resistant strains are troubling from a clinical perspective since this antibiotic is one of the few available alternatives for dealing with carbapenem resistant *A. baumannii* isolates (Fishbain and Peleg, 2010). Even more concerning, the pigmented isolates that were resistant to carbapenems with

one being also resistant to colistin showed dense and voluminous biofilms by CLSM. How much of the extensive antibiotic resistance exhibited by these strains could be due to the protection provided by these biofilms, is hard to determine at this point. This is mainly due to the lack of information regarding whether the biofilm was forming while the antimicrobial susceptibility profile was being tested for. The non-pigmented carbapenem sensitive strains produced biofilms with low densities and the colonies within were dispersed and did not cover the entire SS coupon. This pattern usually results from separate adhered cells that could later rapidly divide and give rise to cell stacks.

Different rates of biofilm formation on abiotic surfaces has been previously shown among clinical *A. baumannii* strains (Lee *et al.*, 2008; McQueary *et al.*, 2011; Orsinger-Jacobsen *et al.*, 2013). Additionally, the ability to form biofilms was reported to be differential among *A. baumannii* clinical isolates (De Breij *et al.*, 2010). However, in general, a positive relationship seems to exist between biofilm formation and resistance to antimicrobial agents (Rao *et al.*, 2008; Gurung *et al.*, 2013). The variations in the rates of biofilm formation among different strains could be a result of the differential regulation of the genes involved in biofilm formation or quorum sensing among the different isolates. This is further consolidated by the fact that all the strains were positive for *Omp*A and most of them for *Csu*E, which are key genes involved in biofilm formation, but showed different patterns of biofilm formation. One of the strains that was negative for *CsuE*, strain 59, was obtained from a Crohn's disease patient. Studies have shown that these patients usually select for strongly adherent microbial strains (Chassaing *et al.*, 2015). Therefore, further investigating the mechanisms of biofilm formation in this strain could help further our understanding of this complex phenomenon. Moreover, despite the crucial involvement of *Csu*E in biofilm formation, studies have shown that some *A. baumannii* strains are still able to attach to epithelial cells and form biofilms despite the absence of this gene (Gaddy *et al.*, 2009; De Breij *et al.*, 2009). Determining how exactly these strains are able to achieve this could shed new light on alternative mechanisms of biofilm formation in *A. baumannii*.

Quick biofilm formation patterns are presumably due to earlier adherence to the surface, which is a bottleneck step for attached cell division and surface colonization. The speed of attachment and subsequent biofilm formation on abiotic surfaces reflect the bacterium's ability to quickly adhere unto surfaces in the hospital setting and form biofilms. This, in turn, leads to a longer persistence of *A. baumannii* and a possible source of repeated infections and outbreaks.

The different rates of biofilm formation after 5 hours of incubation observed in our study reflects the different abilities possessed by the different strains to overcome this bottleneck step. Interestingly, the strains of group 1 were all susceptible to aminoglycosides and only strain 52 was resistant to carbapenems. Though aminoglycoside-susceptible isolates are found in other groups, this could suggest a link between susceptibility to this class of antimicrobial agents and a fast rate of biofilm formation. Sub-inhibitory concentrations of aminoglycosides have also been reported to induce biofilm formation in *Pseudomonas aeruginosa* and *Escherichia coli* that is inhibited by GTP (Hoffman *et al.,* 2005). It was postulated that biofilm formation could act as a defensive mechanism against antibiotic exposure, and whose molecular basis is linked to alterations in the level of c-di-GMP. In *A. baumannii*, previous aminoglycoside use was associated with biofilm forming isolates formation (Rodriguez-Baño *et al.* 2008), whereas, unlike what we observed in our study, treatment in an intensive care unit and isolation from respiratory samples were associated with lack of biofilm formation (Rodriguez-Baño *et al.* 2008). However, our small sample size does not allow for the generalization of our results. A correlation between biofilm formation and use of levofloxacin was also reported by He *et al.* (2015). Moreover, these authors reported a correlation between biofilm induction and upregulation of the transcription of the gene encoding the adeG efflux pump. They suggested that a link exists between low doses of antimicrobial agents and infection by biofilm forming *A. baumannii*. These studies demonstrate the link between biofilm formation and antimicrobial resistance. Though this is demonstrated in our study, further investigations on larger pools of isolates could help determine if the relationship between aminoglycoside susceptibility and fast biofilm formation is related to *A. baumannii* in general or is an observation that is only applicable to these particular strains.

The only slow biofilm former was obtained from an oncology immunodeficient patient not previously exposed to catheterization. It was sensitive to carbapenems, resistant to gentamycin and moderately resistant to tobramycin. However, though these factors may be indicative of a predisposition of such a profile to be slow in forming biofilms, no general conclusions could be drawn since it was the only case. Nevertheless, this strain continued growing after 24 hours and eventually showed higher densities of biofilm forming cells. Also of note, all the XDR isolates pertained to Group 2 and had similar rates of biofilm formation, possibly suggesting a tradeoff in terms of energy allocation between this rate and the mechanisms of resistance. Combined, our preliminary information could be useful in terms of

infection control were specialists could expect the rate of biofilm formation by certain strains based on their antibiotic susceptibility and act accordingly.

4.6. Conclusions

Though our conclusions could only be preliminary due to the low number of strains involved, several conclusions could be drawn. First, the tested *A. baumannii* strains showed fast, moderate, and slow biofilm forming rates on steel coupons after 5 hours of growth. The fast biofilm forming group seems to be associated with aminoglycoside susceptibility. Second, pigmented XDR strains produced more homogenous and voluminous biofilms, suggesting an interplay between the density of the formed biofilms and resistance to carbapenems and/or colistin. This also highlights the importance of biofilm forming abilities among MDR *A. baumannii* strains that could result in their persistence in the hospital for prolonged periods of time. Third, there was no association between pigmentation and siderophore production was found but rather that pigmented strains produced more voluminous and homogeneous biofilms. Fourth, all the tested strains were positive for *Omp*A and all except two were positive for *Csu*E. Further investigation into the mechanisms of biofilm formation for the strains lacking *Csu*E could be of interest for future studies. Finally, investigation of a broader set of clinical isolates is need in order to shed a clearer light on the interplay between the various clinical and laboratory findings and biofilm formation patterns.

VIII. DISCUSSION

1. Prevalence of Carbapenem Non-Sensitive Isolates

Characterization of clinical *A. baumannii* isolates in terms of antimicrobial susceptibility showed an alarmingly high rate of carbapenem resistance (84.75%) in HU-LP. The high prevalence of CRAB isolates seems to be a common finding in Spain where several studies have reported similar rates (Perez *et al.,* 2010; Villar *et al.,* 2014; Rodloff *et al.,* 2008). These rates are much higher than those reported from most other European countries, such as Germany and the UK (Tomaschek *et al.,* 2016; Hughes *et al.,* 2016), but somewhat similar to reports from Italy, Greece, and Poland (Rodloff *et al.,* 2008; Chmielarczyk *et al.,* 2016). This high rate raises an alarm that requires direct intervention. This alarm has actually reached the Spanish government where it officially launched the "Zero Resistance" project, aiming at reducing the incidence of MDR organisms through improving prescription, prevention of cross-contamination, and eradication of reservoirs (Montero *et al.,* 2015). The high CRAB incidence rate could partly be due to the heightened ability of *A. baumannii* in persisting through adherence to biotic and abiotic surfaces and/or through the presence of CRAB isolates in the patient's gut flora (Gordon *et al.,* 2010). In addition, a study has shown a general lack of uniformity in applying infection control measures in Spanish hospitals (Garcia-Ortega *et al.,* 2011). Taken together, these factors could have been contributing to the persistence of CRAB isolates in this hospital and could also result in repeated infections, and a subsequent increase in the rate of CRAB infections. The fact that most infections over a five-year period in this hospital were produced by a handful of clones further supports this hypothesis. Nevertheless, the reduced incidence of CRAB isolates during the latter years of the study is a positive indicator of the implementation of adequate infection control measures.

OXA-23-like is the most widely disseminated OXA in the world (Karah *et al.,* 2012). In Spain, though CRAB isolates harboring *bla*$_{\text{OXA-23-like}}$ were reported to cause outbreaks (Mosqueda *et al.,* 2013; Merino *et al.,* 2014), *bla*$_{\text{OXA-24-like}}$ remains the most prevalent OXA (Villalón *et al.,* 2013; Acosta *et al.,* 2011; Tena *et al.,* 2013). This was reflected in this study were OXA-24-like was present in 62.71% of the isolates whereas OXA-23-like was only detected in 11.86%. In fact, OXA-24 was first discovered in Spain (Bou *et al.,* 2000c) and has remained highly prevalent in the Iberian Peninsula ever since (Manageiro *et al.,* 2012; Mosqueda *et al.,* 2014; Villalón *et al.,* 2015). This carbapenemase has been detected in several CRAB outbreaks, including in neighboring France (Barnaud *et al.,* 2010), but its predominance seems to be restricted to the Iberian Peninsula. This could actually prove to be advantageous

due to the positive associations that were made with hemolysis and siderophore production, in a sense that it would help clinicians expect a high degree of virulence while dealing with CRAB infections in Spain. This would allow them to tailor their treatment approaches accordingly and could result in an increased chance of survival for the patient. This association could also be advantageous in other countries that also have this carbapenemase highly disseminated, such as Serbia (Novovic *et al.,* 2015).

In Lebanon, the rate of carbapenem non-susceptible isolates was even higher than that of Spain (90%) and the most disseminated OXA among these isolates was OXA-23-like. Our findings fall in line with other studies performed in this country, where it was detected in 97.89% of 142 CRAB isolates obtained over one year (Hamoudi *et al.,* 2015a). Though even higher rates have been reported worldwide, such as in China, where CRAB isolates were reported to 97.4% in one study (Xu *et al.,* 2016), this rate is much higher than the average rate of CRAB among Middle Eastern and African countries, where it was reported to be 51.3% (Kanj *et al.,* 2014). Due to legal and logistical problems, it was not possible to transfer the bacterial strains isolated in Lebanon to Spain in order to perform the PFGE and MALDI TOFF MS experiments. Access to facilities that contained the appropriate machines for performing these experiments was also not possible in Lebanon. Therefore, the identification was performed in a different manner for the Lebanese set of isolates and clonality by PFGE was not possible in order to determine whether similar clones were causing these infections. Nevertheless, the rate of carbapenem resistance detected reveals the need for immediate intervention on national and local levels through the implementation of successful infection control protocols and antibiotic stewardship programs. Government interference on a level similar to that that is being performed in Spain is highly encouraged in Lebanon in an attempt to decrease these very high rates of carbapenem resistance. In accordance with our findings, the Middle Eastern region is generally reported to have higher rates of carbapenem resistance as compared to the rest of the world (Tärnberg *et al.,* 2016). Global studies have reported susceptibility rates to carbapenems some countries in this region to be lower than 11% (Lob *et al.,* 2016). Moreover, this region generally has higher CRAB incidence rates than the rest of the Mediterranean region (Kanj *et al.,* 2014; Castanheira, *et al.,* 2014). This could be due to the lack of proper implementation of infection protocols and proper antibiotic prescription in Middle Eastern countries. However, further confirmation through surveillance studies and questionnaires are needed before confirming this hypothesis.

There were some isolates from both sets that were resistant to carbapenems but did not have a positive result for any of the tested carbapenemase genes. These isolates could have acquired the resistance through the presence of carbapenemases that were not tested for, over-expression of *bla*$_{\text{OXA-51-like}}$, down-regulation of outer membrane porins, and/or up-regulation of multi-drug efflux pumps (Peleg *et al.,* 2008). Further investigation is needed before determining the cause of carbapenem resistance among these isolates. Moreover, though *bla*$_{\text{OXA-51-like}}$ was detected in the vast majority of isolates, a few isolates were negative for this gene. This is not uncommon since a subset of *A. baumannii* isolates testing negative for this gene have been previously described (Turton *et al.,* 2006). Interestingly, two isolates from the Lebanese set of isolates tested positive for *bla*$_{\text{OXA-23-like}}$ but were sensitive to carbapenems. This could suggest the presence of a mutation in this gene that make it less effective, or in its genetic environment that caused it to be under-expressed. Sequencing of this gene and its genetic environment could help shed further light on the matter. However, due to limited accessibility to sequencing services in Lebanon, such investigations were not possible.

The AST data collected from both Spain and Lebanon also show very high rates of cross-resistance to other antimicrobial agents among CRAB isolates. This could be due to the co-acquisition of resistance determinants on mobile genetic elements (Fournier *et al.,* 2006). Moreover, the plasticity of the *A. baumannii* genome allows for the incorporation of a wide range of genetic elements, allowing it to acquire resistance to a wide range of antimicrobial agents at the same time (Antunes *et al.,* 2014). Despite the very high rates of resistance detected among both sets of isolates, most of them remain susceptible to colistin. This is especially important since the use of this antimicrobial agent was re-emerged in the treatment of MDR organisms (Cai *et al.,* 2012). However, colistin has a nephrotoxic effect (Bergen *et al.,* 2012) and *A. baumannii* isolates have shown an ability to develop resistant to this antimicrobial agent during therapy (Valencia *et al.,* 2009). Moreover, resistance to colistin is usually accompanied by co-resistance to the innate cationic antimicrobials produced by the host (Napier *et al.,* 2013). Nevertheless, the high susceptibility rates to this antimicrobial agent make it a viable option when no other alternatives for the treatment of infections caused by CRAB isolates exist. Finally, since the use of antibiotics is one of the main driving factors for the development of resistance (He *et al*., 2015), the proper use of these agents through antibiotic stewardship programs might be the best option for safeguarding them for future use. This should also be accompanied by rigorous implementation of infection control protocols in order to eliminate

the existing reservoirs and prevent the re-emergence of CRAB clones that cause repeated infections.

2. Prevalence of International Clones

Clonality analysis revealed that IC II is by far the most predominant clone among the isolates obtained from both Lebanon and Spain. This finding is in agreement with international reports that indicate that IC II is the most globally disseminated clone (Karah *et al.,* 2012). Moreover, it is in agreement with local data in Lebanon (Rafei *et al.,* 2014a), Spain (Villalón *et al.,* 2015), and throughout the Mediterranean region (Di Popolo *et al.,* 2011). Additionally, the percentages of prevalence of each IC among the Spanish set of isolates are very similar to another study conducted in Spain. In our study, the percentages were 8.47% for IC I, 71.19% for IC II, and 6.78% for IC III as opposed to 10.2% for IC I, 47.5% for IC II, and 5.1% for IC III in the other study (Villalon et al., 2011). The global success of IC II could be partially attributed to the ability of isolates pertaining to this IC to harbor all three most commonly disseminated OXAs (Karah *et al.,* 2012). This would allow these isolates to incorporate any of the aforementioned carbapenemases and gain a competitive edge on other carbapenem-sensitive clones, or clones that might not have this kind of genetic flexibility. The selective pressure produced by the extensive use of carbapenems also helps in the elimination of competitive organisms and aid in the persistence of these clones, and their eventual dissemination (Nemec *et al.,* 2004). In addition, these clones could cross international borders and continents due to the global interchange of patients and the increase in international travel (Peleg *et al.,* 2008). Finally, the lack of proper infection control protocols could allow for these clones to persist in the hospital and cause repeated infections. They could even persist in the gut flora (Gordon *et al.,* 2010) and eventually use the host as a transport to far-away destinations.

PFGE analysis showed that isolates showing different pulsotypes could pertain to the same IC, as shown in the Spanish set of isolates. This is due to the higher resolution provided by PFGE as compared to the detection of clonality by tri-locus PCR typing. While the latter technique capitalizes on difference in three housekeeping genes, the former takes differences in the entire genome into account. Nevertheless, the grouping of several isolates with different pulsotypes into the same international clone demonstrates the plasticity of the isolates pertaining to this clone. This observation unfortunately could not be verified for the Lebanese

set of isolates due to the difficulties that were mentioned in the first section of the discussion. PFGE analysis for the Spanish set of isolates also showed the ability of a single clone to persist for prolonged periods of time (Cluster 6). In regard to this cluster, isolates 26 and 38 pertained to IC II but were susceptible to carbapenems. Isolate 38 was susceptible to almost all tested antimicrobial agents while isolate 26 was resistant to most beta-lactams, ciprofloxacin, levofloxacin, and trimethoprim/sulfamethoxazole. This finding, though rare, is not the first of its kind since isolates pertaining to all three ICs have been reported elsewhere to be susceptible to carbapenems and to no have MDR profiles (Diancourt *et al.*, 2010). One hypothesis that could explain this susceptibility is that these strains may have been "hiding" in the hospital environment for so long so that they have lost their resistance genes due to lack of antibiotic pressure. Those that have lost the plasmid encoding the carbapenemases might have then outcompeted their resistant counterparts in the population and caused these infections (Andersson *et al.*, 2011; Andersson *et al.*, 2010).

PFGE analysis of the Spanish set of isolates also showed the dissemination of clones of the same cluster of pulsotypes in several hospital wards. This observation has been previously reported where, though *A. baumannii* infections are most commonly reported from the ICU (Perez *et al.*, 2010), they have been detected in other hospital wards as well (Rodriguez-Baño *et al.*, 2005). Additionally, a study has reported the co-existence of sporadic and epidemic clones at the same time in different settings, including the hospital (Abbo *et al.*, 2005). This indeed was found to be true in our dataset where several isolates from both the Spanish and Lebanese set of isolates seem to be sporadic and did not pertain to any IC nor did they share a common pulsotype with any of the other isolates. Of the isolates that did not belong to any cluster, only isolates 12, 13, and 40 from the Spanish set of isolates were resistant to carbapenems. All other isolates did not pertain to any IC and were susceptible to most tested antimicrobial agents. This suggests that most, but not all, sporadic infections do not cause a great threat in terms of treatment. Performing MLST for these strains could help in determining their international clonality and whether they are truly sporadic or they belong to a clone that could not be detected by tri-locus sequence typing.

Among the Spanish set of isolates, ICs III and I were shown to cause several infections, as was evident from the first and second PFGE clusters, respectively. However, these infections were much less frequent and were confined to a one-year period as compared to those caused by IC II. All the isolates of IC III expressed OXA-24-like and all those of IC I expressed OXA-

58-like. Nevertheless, the isolates of IC II expressed all three oxacillinases tested for in different combinations. These findings are similar to those of the Lebanese set of isolates where IC II was by far predominant and caused the vast majority of infections over one year. However, in contrast to the isolates of the Spanish set, the majority of the Lebanese set of isolates expressed OXA-23-like. The prevalence of different OXAs among isolates of IC II suggest that these OXAs are not directly related to clonality, but rather to availability in the surrounding environment. This is further supported by the fact that these OXAs could be bourn on plasmids and could be horizontally exchanged by bacterial organisms (Corvec *et al.,* 2007). Moreover, the data could also suggest the superior ability of IC II to persist and cause repeated infections in the hospital. *In-vitro* competition assays could be performed in the future in order to verify this anecdotal data, where isolates pertaining to IC II could be placed in competition with isolates pertaining to other ICs, including IC I and III.

Isolates that pertained to tri-locus PCR groups 4, 10, and 14 were also detected in this study. These clones are not as globally disseminated as the ICs I, II, and III, but were reported to be MDR when they were first discovered (Karah *et al.,* 2012). Nevertheless, not all the isolates that were part of these groups where MDR in our study. Isolate 31 from the Spanish set of isolates, which pertained to G14, was sensitive to carbapenems as opposed to the carbapenem-resistant isolate that was first described within this group from a Romanian hospital (Bonin *et al.,* 2011). Similarly, two isolates from the Lebanese set pertaining to G4 and the isolate pertaining to G10 were sensitive to carbapenems as opposed to the CRAB isolates pertaining to this group that were described in several European countries (Towner *et al.,* 2008; Grosso *et al.,* 2008). Although the relatively low prevalence of these isolates does not allow for a global conclusion, these isolate demonstrate the high plasticity of *A. baumannii* isolates in producing different profiles, even when pertaining to the same clone.

3. Relationship between Virulence and Resistance

The virulence profiles obtained in this study for both sets of isolates were highly variable. Differentially expressing virulence factors was previously shown between *A. baumannii* isolates with different ancestral clonality (Antunes *et al.,* 2011). In our study, this difference was also evident among isolates with the same clonal lineage, and even among isolates having the same pulsotypes. This further highlights the high adaptability and plasticity of *A. baumannii* and could be the reason behind its clinical success as a pathogen. No statistical

association between the different virulence factors tested for in the Spanish set of isolates was detected. However, an association was detected among the Lebanese set of isolates between moderate motility and strong biofilm formation, and also between highly motile and non-motile isolates on one hand and siderophore production on the other ($p<0.05$). Moreover, all the isolates in this set that were negative for biofilm formation were also negative for siderophore production. The association between biofilm formation and motility has been described elsewhere (Eijkelkamp *et al.,* 2011). Nevertheless, the lack of association between the tested virulence factors among the Spanish set of isolates suggests further mechanisms at play that are resulting in the differential expression of virulence determinants among the different isolates. Since most virulence factors tested for in this study are multi-factorial, direct correlations between different factors were not be possible at the species-level, but should be considered on an individual level.

In terms of statistical associations between virulence factors and ICs, associations between IC II on one hand and hemolysis, siderophore production, and strong biofilm formation ($p<0.05$) on the other were detected among the Spanish set of isolates. In comparison, isolates pertaining to ICs I and III were associated with reduced virulence. Similarly, harboring $bla_{\text{OXA-24-like}}$ was associated with hemolysis and production of siderophores while $bla_{\text{OXA-23-like}}$ was negatively associated with hemolysis ($p<0.05$). These associations, however, were absent from the isolates of the Lebanese set. The virulence profiles generated for these latter isolates were highly variable and variation in clonality was not as prominent as that of the Spanish set. This could have contributed to the lack of detection of associations among these isolates. Another likely explanation is the effect of the different environments from which the isolates were obtained. A link between antibiotic consumption and virulence has been previously described (He *et al.,* 2015). This link could be due to the presence of common factors in the mechanisms of antibiotic resistance and virulence where an interplay between these phenomena could take place (Yeung *et al.,* 2011). The difference in the hospital protocols between the Spanish and Lebanese hospitals could result in different antibiotic prescription regimens that, in turn, expose the strains to different antimicrobial agents. This could result in the differential adaptation of the individual isolates to the different antimicrobial agents they get exposed to. Moreover, the ability of *A. baumannii* to horizontally acquire genes for both resistance and virulence (Burrus, and Waldor, 2004) could result in the acquisition of different genes in different countries, based on the availability of genetic material

in the bacteria's environment. All these factors combined could have resulted in the lack of associations in one country while encountering associations in another.

In lines of what was mentioned in the previous paragraph, sub-populations within the Spanish set of isolates seem to deviate from the detected associations. This was evident in Cluster 7 where all the isolates of this cluster pertained to IC II but none was positive for hemolysis. Moreover, very few strains from the Spanish set showed surface motility as compared to the Lebanese strains, even among the isolates pertaining to IC II. Moreover, the Spanish set of isolates on average had a slightly higher average proteolytic activity (26.6 ± 8.4 U/L) as compared to the Lebanese isolates (17.7 ± 9.5 U/L). Since the materials were purchased from the same companies and the experiments performed by the same person, the difference in these factors could be attributed to the strains themselves. These findings further demonstrate the "locality" of the virulence to be expected and the need to perform a local study before being able to predict virulence in the clinical setting.

Among the Spanish set of isolates, 69.5% produced siderophores, 84.4% produced strong biofilms, and 54.2% showed hemolysis. Among the Lebanese isolates, 57.8% of the isolates produced siderophores, 85.6% produced strong biofilms, and 47.8% showed hemolysis. Though a larger percentage of isolates seem to produce siderophores and hemolysis among the Spanish isolates, these percentages seem to not be very different between both populations. Of note, in both populations, some isolates that were MDR, XDR, and even PDR (such as isolate 12 from the Spanish set of isolates) showed highly virulent profiles. This further demonstrates that resistance and virulence could be acquired simultaneously (Burrus, and Waldor, 2004) and that, based on our findings, acquiring one does not necessarily diminish the chances of acquiring the other. The doubling times that were tested for both populations showed similar ranges (0.324 ± 0.027 to 0.666 ± 0.037 hours for the Spanish isolates and 0.262 ± 0.021 to 0.653 ± 0.049 hours for the Lebanese isolates). Also, in both populations, no associations between these times and particular profiles were made. Since doubling times are affected by the entire metabolic processes of the bacterial cell, such an observation is not surprising. Moreover, since no clear association was detected among the selected isolates, and due to the laborious and time consuming nature of the experiment, determining the doubling times for the entire set of isolates was abandoned.

Based on our findings, a global conclusion regarding a link between virulence on one hand, and clonality or presence of certain OXAs on other, is not recommended. Rather, local associations within a hospital or a precise geographical region need to be determined before being used in the prediction of virulence of a certain clone. These local associations could be vital if anti-virulence drugs are to be developed and assessed for their usefulness, since they may result in different effects based on the different virulence profiles of the isolates. After determining the local associations, a clinician could predict the usefulness of such agents based on the expected virulence of the isolate and thus restrain from using them in case the prediction is unfavorable. This also means that the results of clinical or *in-vitro* trials of anti-virulence drugs will be highly influenced by the local virulence epidemiology and care should be taken before interpreting such results. Moreover, if, after local investigation, low virulence of a particular clone is expected, the use of less aggressive antibiotic treatment approaches, and ultimately the safeguarding of crucial antimicrobial agents, would be possible. Finally, performing this study on a much larger scale that includes more countries and hospitals could result in global conclusions of associations that may have been missed in our study due to the relatively small populations investigated.

4. Mechanisms of Colistin Resistance in Two Sets of Clinical Isolates

Two sets of isolates were obtained from two distinct patients undergoing colistin therapy in HU-LP, Madrid, Spain. Isolates C071 and C440 were obtained from Patient 1 whereas isolates C080, C314, and C428 were obtained from Patient 2. Isolates C440 and C428 from patients 1 and 2, respectively were resistant to colistin. These isolates were not part of the investigated Spanish set of isolates since they were recovered at an earlier time and kept in the hospital's freezer until the time of this study. Clonality analysis showed that the respective isolates obtained from the same patient are clonally identical, while sequencing of the *pmr*CAB operon showed specific mutations in *pmr*B in the colistin resistant isolates that were absent from the colistin sensitive isolates. Full genome sequencing then showed the lack of other significant mutations in the genomes of the colistin resistant mutants as compared to their sensitive counterparts. Moreover, no difference in the tested virulence factors was detected among the isolates from Patient 2, while decreased proteolytic activity and increased siderophore production was detected in isolate C440, as compared to C071.

Two mechanisms have been proposed as a mode of action for colistin. The first mechanism consists of targeting the negatively charged LPS molecules found on the bacterial cell's outer membrane. This subsequently disrupts the stability of the membrane by displacing the divalent cations that bridge LPS molecules together and enables self-promoted uptake across the membrane (Arroyo *et al.*, 2011). The second mechanism is inducing rapid killing through the activation of a hydroxyl radical death pathway in the bacterial cell (Sampson *et al.*, 2012). These mechanisms often work together in order to induce the rapid killing of Gram-negative bacterial cells. The *pmr*CAB operon contains the two-component response regulator and sensor kinase pmrA/B that responds to environmental changes, and is involved in lipid A modifications through *pmr*C (Gunn, 2008; Arroyo *et al.*, 2011). In *Salmonella* spp., the pmrA/B system was found to be involved in the modification of Lipid A, the LPS anchor, by the addition of phosphoethanolamine and this, in turn, conveys resistance to colistin. It is also thought that the aminoarabinose biosynthetic pathways in *Salmonella* spp. play a role in this resistance (Murray *et al.*, 2007). Similar mechanisms have been described in *Pseudomonas* spp. (Moskowitz *et al.*, 2004). However, the aminoarabinose biosynthetic pathways were not found to play a role in colistin resistance in *Acinetobacter* spp. since they lack the target enzymes for this mechanism. This is unlike mutations in the *pmr*CAB operon which are thought to convey high level colistin resistance in this organism (Olaitan *et al.*, 2014).

One study showed that down-regulating the expression of the *pmr*CAB operon could revert colistin resistance (Harris *et al.*, 2014). Another recent study showed that six genes, that include *pmr*A, *pmr*B, and *pmr*C, and other genes involved in lipopolysaccharide synthesis or electrostatic changes in the bacterial cell membrane, are linked to colistin resistance (Park *et al.*, 2015). Deletion of *pmr*B was found to revert resistance to colistin, making this gene essential for this mechanism in *A. baumannii* (Adams *et al.*, 2009). Although mutations in the *pmr*CAB operon, and especially in *pmr*B, have been reported to cause resistance to colistin, a study in 2011 showed that increased expression in the pmrAB sensor kinase system could lead to colistin resistance without any mutations (Park *et al.*, 2011).

The *pmr*B gene is found to be the most commonly mutated gene among colistin resistant *A. baumannii* strains (Olaitan *et al.*, 2014). Our study has shown that two different mutations in two distinct *A. baumannii* strains originating from two separate patients occurred in *pmr*B and caused resistance to colistin. The P233S mutation that is located within *pmr*B of the resistant strain taken from Patient 1 has been reported in several other clinical isolates

(Pournaras *et al.*, 2014; Beceiro *et al.*, 2011; Adams *et al.*, 2009; Kim *et al.*, 2014; Arroyo *et al.*, 2011; Durante-Mangoni *et al.*, 2015). This mutation is located in the histidine kinase domain of PmrB and is thought to be involved in dimerization (Arroyo *et al.*, 2011). It has been defined as an activating mutation (Durante-Mangoni *et al.*, 2015). Both the resistant and sensitive strains isolated from Patient 1 contained the insertion sequence between *pmr*B and *pmr*A, suggesting that this sequence does not affect colistin resistance nor susceptibility. The deletion of Isoleucine at position 19 found in *pmr*B of the colistin resistant strain obtained from Patient 2 has not been reported elsewhere, to the best of our knowledge. This mutation is positioned in the center of the first trans-membrane helix of PmrB. It is possible that this mutation causes PmrB to be permanently activated, which in turn could lead to colistin resistance in a manner similar to the hyperactive kinase mechanism reported elsewhere (Arroyo *et al.*, 2011). Further characterization of the effect of this mutation is required before a definite conclusion regarding how it affects colistin resistance could help better understand the mechanisms of resistance to this antimicrobial agent. No differences in the *lpx*A, *lpx*C, and *lpx*D genes, as well as the genes reported by Park *et al.* (2015), were detected in the resistant strains as compared to their sensitive counterparts. Moreover, *mcr-1* was not present among the colistin-resistant isolates, thus ruling out a plasmidic origin for colistin resistance in C440 and C428. The only other mutation that resulted in an amino acid change was the Ala1648Val mutation in aconitate hydratase in strain C428. This enzyme is involved in the tricarboxylic acid cycle (Pirog *et al.*, 2003) and is unlikely to have a role in mediating colistin resistance. This leads us to conclude that the reported mutations in the *pmr*CAB operon are causing colistin resistance in these strains. Moreover, this study presents a novel clinically significant mutation that allows *A. baumannii* isolates to acquire resistance to colistin during treatment.

The sequence type ST-81^{P} identified according to the Pasteur scheme in isolates C071 and C440 has been previously encountered in 10.2% of 729 A. baumannii isolates obtained from various Spanish hospitals. This sequence type was found to be different from the international clone ST-1^{P} (also known as IC I) in a single allele, justifying the result that these strains pertained to IC I as tested by the multiplex PCRs. Strains belonging to this clone were previously encountered in northeastern Spain from 1992 to 2002 and were all found to be sensitive to colistin. This ST was not detected after 2002 in this 11 year-long study (Villalon *et al.*, 2011). This clone seems to have gone undetected since then only to re-appear several years later in Madrid, as is detected in our study. Resistance to carbapenems in isolates C071 and C440 is due to the presence of the *bla*$_{\text{OXA-58-like}}$ gene which codes for a carbapenem

hydrolyzing oxacillinase (Mostachio *et al.*, 2009). Isolates C080, C314, and C428 belong to the widely spread sequence type ST-2^{P} which is also commonly known as IC II (Karah *et al.*, 2012). In addition to being spread all over the world, some isolates belonging to this sequence type were shown to be resistant to colistin (Tan *et al.*, 2013). Although the gene *bla*$_{GES-5}$ was detected in several bacterial species, including *Pseudomonas aeruginosa* (Mataseje *et al.*, 2012), it hasn't been previously detected in *A. baumannii*, to the best of our knowledge. This gene coding for the carbapenemase hydrolyzing GES-5 (Bonnin *et al.*, 2011) has been detected in all three strains isolated from Patient 2. This not only explains the resistance to carbapenems among these strains, but could also be an important indication of inter-species exchange of resistance genes and warrants further investigation. Nevertheless, the *bla*$_{GES-5}$ genes detected in these strains differed by only one nucleotide from some sequences of *bla*$_{GES-14}$ deposited in Genbank. This latter gene is commonly reported to exist among *A. baumannii* isolates and is known to hydrolyze imipenem (Bogaerts *et al.*, 2010). Therefore, even though the detected gene had 100% identity with *bla*$_{GES-5}$ in Genbank, its extremely high similarity to *bla*$_{GES-14}$ requires further investigation into the similarities between the products of these two genes before reaching a definite conclusion on the matter.

In terms of the effect of colistin resistance on virulence in *A. baumannii*, conflicting reports exist regarding the fitness cost and reduced virulence of colistin resistant *A. baumannii* isolates. Generally, it is thought that resistance to colistin incurs a fitness cost on the cell and causes decreased virulence (Olaitan *et al.*, 2014). One study shows that there is a markedly reduced fitness in colistin resistant *A. baumannii* strains as compared to the sensitive ones, with a few exceptions (Lesho *et al.*, 2013). In particular, the P233S mutation is thought to cause decreased invasiveness and growth retardation in *A. baumannii* (Pournaras *et al.*, 2014). On the other hand, mutations in the *pmr*CAB operon in *Salmonella* spp. has shown to produce a very low fitness cost (Sun *et al.*, 2009). A recent study surprisingly showed that XDR *A. baumannii* isolates could actually show enhanced virulence (Jones *et al.*, 2015). Durante-Mangoni *et al.* (2015) showed in another recent study that the P233S mutation in *pmr*B incurred no cost on the bacterial cell in terms of fitness nor virulence (Durante-Mangoni *et al.*, 2015). Finally, a study showed that colistin resistance due to mutations in the *pmr*CAB operon showed very modest decreases in virulence as opposed to mutations in the *lpx*A, *lpx*C, or *lpx*D genes that lead to total loss of the LPS and sensitivity to other antimicrobial agents (Beceiro *et al.*, 2014).

In our study, the mutations causing colistin resistance seem to not have any effect on the bacterial cell when the sensitive strains were compared to the resistant ones, with the exception of the decreased proteolytic activity of strain C440 and its production of siderophores. Genes involved in siderophore assembly and uptake were detected for all 5 isolates, but this was not represented in the phenotypic test. One explanation for the increased production of siderophores, allowing for their phenotypic detection, in C440 is the involvement of PmrB in iron acquisition (Arroyo *et al.*, 2011). The P233S mutation might be affecting PmrB in a way that decreases intake of iron, resulting in the increased production of siderophores in order to compensate for low intracellular iron levels. This could in turn have had an effect on the reduced proteolytic activity detected in this isolate where less energy is expended on exoproteases in order to increase siderophore expression. RT-PCR analyses on the genes involved in the production of both siderophores and exoproteases could help shed further light on the matter.

These results demonstrate the ability of clones originating from different ICs to develop resistance to colistin independently from one another, suggesting that the unique driving factor for the development of this resistance is the selective pressure exerted by colistin itself. Based on this, it is recommended that close monitoring of the patients and the strain infecting them be performed during colistin therapy through frequent isolations and susceptibility testing. The early discovery of colistin-resistant isolates could be a main factor in the prevention of the spread of PDR clones through the sequestration of the patient. It could also contribute to better treatment outcome through switching the therapy to other antimicrobial agents or through using combination therapies as soon as resistance is detected.

5. Biofilm Formation Patterns among *Acinetobacter baumannii* Isolates

Twelve isolates from the Spanish set of strains were chosen, having different pulsotypes and antibiotic susceptibility profiles in order to further investigate the relationship between antibiotic resistance and biofilm formation. The objective of this investigation is to test if the rate of biofilm formation could be predicted by the antibiotic susceptibility profile alone, so as to provide clinicians and infection control specialists with quick insight regarding this factor. Eight of those isolates were resistant to carbapenems. Isolates 12 and 45 were additionally resistant to colistin. All these isolates produced biofilms on steel coupons, but at varying rates. However, all the isolates included in this study showed reduced attached cell densities after 48

hours, caused by dispersal. Eight of the patients from which these isolates were obtained were previously exposed to mechanical ventilation and catheterization, both of which are recognized as risk factors for the development of biofilms (McConnell *et al.,* 2013). Moreover, all the patients, except for three, had previous antibiotic therapy, another factor associated with biofilm formation (Rodriguez-Baño *et al.,* 2008). These associations could be the first piece of useful information, where specialists could expect isolates from similar backgrounds to form biofilms and act accordingly.

Three distinct groups with different rates of biofilm formation were identified. Isolates 26, 52, and 59 were under the "fast biofilm formers" group; isolate 30 was alone in the "slow biofilm former" group; while the rest of the isolates where in the "intermediate biofilm formers" group. Interestingly, all three isolates that were fast biofilm formers were susceptible to aminoglycosides. Nevertheless, isolates 3 and 38 also were susceptible to aminoglycosides but pertained to the intermediate group. This observation is based on a small sample and a general conclusion could not be made based only on these isolates but it could open the way for further investigation. Though our study was more directed towards carbapenem resistance due to the globally increasing rates of resistance to these antimicrobial agents and their clinical importance (Tärnberg *et al.,* 2016), future studies could be directed towards isolates with differing aminoglycoside resistance patterns. The only isolate that pertained to the slow biofilm former group was obtained from a patient that was not previously exposed to catheterization. This isolate was also sensitive to carbapenems but since it was the only case, and because other isolates with similar profiles pertained to other groups, no specific associations could be made with slow rates of biofilm formation. However, this strain continued to increase its cell density on steel coupons until it reached the same cell densities as the other isolates at 48 hours. This indicates that, even at a slow rates, *A. baumannii* isolates are able to eventually produce biofilms able to cover abiotic surfaces.

All the isolates of the intermediate group, expect for isolate 38, were resistant to carbapenems, and all the patients from which these isolates were recovered died, except for one. Moreover, this group contained two colistin resistant isolates. These findings could indicate a role for biofilm formation in mortality and is in disagreement with the findings of Rodriguez-Baño *et al.* (2008). In the latter study, 89 *A. baumannii* isolates were studied and negative associations between carbapenem resistance and mortality on one hand and biofilm formation on the other was detected. That study contained a much larger pool of isolates than

our study and therefore statistical correlation in that study is more representative. Nevertheless, our small pool of isolates show that this is not always the case and these associations should not be taken for granted while treating a patient. Moreover, as opposed to the other study, we studied biofilm formation over time, and quantified the attached cells, which could have made it possible to detect biofilms that were missed by the other study which employed a spectrophotometric technique in the detection of biofilms.

Our results are similar to other studies, in a sense that diverse biofilm formation patterns were observed after 24 hours of incubation. Lee *et al.,* (2008) showed a positive correlation between biofilm formation and the expression of the carbapenemase PER-1. Though PER-1 was not screened for in our set of isolates due to the lack of a positive control, a relationship between biofilm formation and carbapenem resistance seems to exit. This relationship was evident by the CLSM analysis performed after 24 hours of growth on steel coupons where carbapenem resistant isolates had denser biofilms than carbapenem sensitive ones. De Breij *et al.,* (2010) showed that biofilm formation patterns differed between the different *A. baumannii* ICs. Though there were only one representative from each of ICs I and III included in our study, this observation was not true for our isolates. The difference in biofilm pattern rather seems to be related to another factor that we still haven't pinpointed. Involvement of quorum sensing molecules could be this factor affecting the differential expression of biofilms (Niu *et al.,* 2008), but this warrants further investigation. Our results are also similar to the results reported by Rao *et al.* (2008) and Gurung *et al.* (2013) were a relationship between MDR profiles and biofilm formation was detected. Our results indeed show that isolates with MDR and XDR profiles, though variable in their patterns, were able to show intermediate to fast rates of biofilm formation patterns. This also falls in agreement with a study performed by Orsinger-Jacobsen *et al.* (2013) were differing CLSM profiles were obtained for 13 isolates grown on steel coupons. Moreover, this group showed that the bacterial cells grown on steel coupons were significantly more resistant to disinfectants than their planktonic counterparts. All of this indicates that our preliminary study falls in agreement with other similar studies and open the way for a future large-scale investigations that could determine associations between specific antibiotic resistance profiles and biofilm formation patterns.

All the isolates were positive for *Omp*A and all except for isolates 12 and 59 were positive for *Csu*E. It has been shown that Crohn's disease patients have altered normal flora, allowing for the selection of strongly adherent strains (Chassaing *et al.,* 2015). This was indeed

shown to be the case in our study where isolate 59 was obtained from a Crohn's disease patient and pertained to the fast biofilm former group although it lacked *Csu*E. Further investigation regarding the exact mechanisms of biofilm formation in this isolate, and in isolate 12, could help better understand the complex cellular interactions that result in biofilm formation in *A. baumannii*. Moreover, the negative result for *Csu*E for these isolates could be due to them being sporadic, and therefore harboring an allele for this gene that was not detected by the primers we used.

Isolates 3, 26, 45, and 52 produced a brown pigment that was evident at the time of performing the experiment. Pigment formation does not seem to affect the rate of biofilm formation since these isolates were spread across different groups of biofilm formation rates. Testing for siderophores among this population did not show a statistical association with pigment formation, thus ruling it out as the cause of pigmentation. However, since pigment formation has been associated with virulence in a study in Argentina (Vilacoba *et al.*, 2013), two pigment-forming and two non-pigment-forming isolates were chosen for CLSM analysis in order to further investigate the biofilm structure of these isolates. The two carbapenem resistant pigmented isolates (45 and 52) produced denser biofilms as compared to the carbapenem sensitive non-pigmented ones (30 and 38). This result could be indicative that the density of the biofilm could be related to pigmentation. Performing this analysis on larger populations could further clarify the matter. This result could also be an indicator of an interplay between carbapenem resistance and dense biofilm formation patterns. This information is particularly useful for infection control specialists where they can implement aggressive approaches while eradicating biofilms caused by CRAB isolates. Moreover, future research assessing the different molecules being evaluated for their effectiveness in eradicating biofilms, such as some essential oils (Kavanaugh and Ribbeck, 2012) and dynamic nanoparticle platforms (Wu *et al.*, 2009), could be evaluated against pathogens with different biofilm formation patterns in order to check for a relationship between the usefulness of such molecules and specific patterns.

No statistical significance was detected between the biofilm forming groups and any of the tested virulence factors, carbapenemases, clonality, and doubling times ($p<0.05$). The resulting profiles of the isolates included in this study were highly variable one from another. Moreover, specific factors looked upon individually do not seem to pertain to any biofilm group in particular, but rather similar profiles could be found in distinct groups. Due to the time-

consuming and labor-intensive nature of these experiments, performing this study on the entire set of isolates was not possible. However, such an investigation could prove insightful in the future. Nevertheless, the preliminary associations between dense biofilms and carbapenem resistance show promise for future consolidation of this association and for gaining insight on how biofilms could act as both virulence and antibiotic resistance determinants.

In conclusion, high rates of CRAB isolates were detected in both HU-LP and SGH-UMC. There was a predominance of IC II in both hospitals but sporadic isolates, as well as isolates pertaining to other ICs, were able to produce XDR and MDR infections. *bla*$_{\text{OXA-24-like}}$ was predominant among isolates from HU-LP whereas *bla*$_{\text{OXA-23-like}}$ was predominant among the isolates of SGH-UMC. The virulence profiles obtained were highly variable, but an association between IC II and *bla*$_{\text{OXA-24-like}}$ on one hand, and increased virulence on the other, was detected among the HU-LP isolates. This association was not detected in the isolates of SGH-UMC, suggesting that these associations could only be used locally. Additionally, a novel mutation (ΔIle19) in PmrB was shown to lead to colistin resistance without affecting the virulence of the strain. Another mutation detected in this study (P233S in PmrB) also led to colistin resistance but had an effect on siderophore production and proteolytic activity. Finally, preliminary associations between aminoglycoside sensitivity and fast rates of biofilm formation; and between carbapenem resistance and dense biofilm formations, were detected in this study. The data presented in this Doctoral Thesis could be very valuable for clinicians, researchers, and infection control specialists in terms of understanding the complex mechanisms and relationships between virulence and antimicrobial resistance. This would, in turn, lead to better prescription regimens and infection control protocols that are based on certain expectation, resulting in safeguarding important antimicrobial agents for future use and in the eradication of MDR organisms from the hospital.

IX. CONCLUSIONS

The following conclusions have been achieved in this Doctoral Thesis:
Las conclusiones de esta Tesis Doctoral son las siguientes:

First: Carbapenem resistance among *A. baumannii* strains isolated from Hospital Universitario – La Paz (HU-LP) in Madrid (Spain) was 84.75%. These isolates showed very high rates of resistance to other antimicrobial agents where more than 80% of the isolates were non-sensitive to ticarcillin, piperacillin, ampicillin/sulbactam, piperacillin/tazobactam, ceftazidime, ciprofloxacin, levofloxacin, and trimethoprim/sulfamethoxazole. Additionally, 79.66% of the isolates were non-susceptible to cefepime and 16.95% to minocycline. Non-susceptibility to aminoglycosides ranged from 18.64% for amikacin to 54.24% for gentamycin. Only two isolates (3.39%) were resistant to colistin.

Primero: Se encuentra un elevado nivel de resistencia a carbapenemas (84,75%) en las cepas de *A. baumannii* aisladas del Hospital Universitario – La Paz (HU-LP) en Madrid (España). Estas cepas muestran además altos niveles de resistencia a otros antibióticos, siendo más del 80% de las cepas resistentes a ticarcilina, piperacilina, ampicilina/sulbactam, piperacilina/ tazobactam, ceftazidima, ciprofloxacino, levofloxacino, y trimetoprim/sulfametoxazol. Asimismo, el 79,66% de las cepas no resultaron susceptibles a cefepima y el 16,95% no fueron susceptibles a minociclina. El 18,64% y 54,24% de las cepas no fueron susceptibles a los aminoglicósidos, amikacina y gentamicina, respectivamente. Sólo dos cepas (3,39%) fueron resistentes a colistina.

Second: IC II was the most prevalent clone at 71.19% among the isolates analyzed from HU-LP. Additionally, 8.47% of these isolates pertained to IC I, 6.78% to IC III, 13.56% didn't belong to any IC, and one isolate pertained to group 14. These isolates were distributed among 7 clusters as defined by PFGE where Cluster 6 was the largest and five isolates were sporadic and did not pertain to any cluster.

Segundo: El clon internacional II fue el más frecuente, correspondiendo al 71,19% de las cepas analizadas del HU-LP. Además, el 8,47% de las cepas pertenecieron al clon internacional I, el 6,78% al clon internacional III, una cepa al grupo 14, y el 13,56% no perteneció a ningún clon internacional. Estas cepas fueron distribuidas entre 7 grupos definidos por PFGE, incluyendo el grupo 6 la mayoría de las cepas. Cinco de las cepas no pertenecieron a ningún grupo de PFGE.

Third: OXA-24-like was the most commonly disseminated carbapenemase among the HU-LP isolates at 62.71%, while OXA-23-like and OXA-58-like were detected at lower frequencies; 11.86% and 13.56%, respectively.

Tercero: La familia de oxacilinasa OXA-24 fue la más frecuente, en las cepas analizadas del HU-LP, detectándose en el 62,71% de éstas, mientras que las familias OXA-23 y OXA-58 se detectaron únicamente en el 11,86% y 13,56%, respectivamente.

Fourth: Isolates pertaining to IC II were associated with increased virulence as compared to isolates pertaining to ICs I and III. In particular, they were positively associated with hemolysis, siderophore production, and strong biofilm formation ($p<0.05$).

Cuarto: Las cepas del clon internacional II se asociaron con un nivel de virulencia más alto en comparación con las cepas de los clones internacionales I y III. Particularmente, se asociaron positivamente con hemólisis, producción de sideróforos, y formaciones intensas de biofilms ($p<0,05$).

Fifth: Presence of OXA-24-like was also associated with increased virulence as compared to the presence of OXA-23-like and OXA-58-like where harboring $bla_{\text{OXA-24-like}}$ was positively associated with α-hemolysis and siderophore production ($p<0.05$).

Quinto: La presencia de la familia de OXA-23 se asoció positivamente con un nivel de virulencia más alto, en comparación con su presencia en otras familias de oxacilinasas. La presencia de la familia OXA-23 se asoció con hemólisis y producción de sideróforos ($p<0,05$).

Sixth: The *A. baumannii* isolates obtained from Saint Georges Hospital – University Medical Center (SGH-UMC) in Beirut (Lebanon) showed even higher rates of carbapenem resistance: 90%. Susceptibility to other tested antimicrobial agents did not exceed 14.4% among these isolates, but only one isolate was resistant to colistin.

Sexto: Las cepas de *A. baumannii* aisladas del Hospital Saint Georges – University Medical Center (HSG-UMC) en Beirut mostraron aún mayores niveles de resistencia a carbapenemas (90%). La susceptibilidad de estas cepas a otros antibióticos fue inferior al 14,4%, pero sólo una cepa resultó resistente a colistina.

Seventh: Similarly to the set of isolates obtained from HU-LP, IC II was the most predominant clone at 88.9% among the isolates obtained from SGH-UMC, while 6.7% of the isolates pertained to group 4, 2.2% to group 14, 1.1% to group 10, and one isolate did not pertain to any defined clone.

Séptimo: Al igual que las cepas aisladas de HU-LP, el clon internacional II fue el más frecuente en las cepas aisladas del HSG-UMC, donde representó el 88,9% de las cepas. Entre las otras cepas estudiadas, el 6,7% pertenecieron al grupo 4, el 2,2% al grupo 14, el 1,1% al grupo 10, y una cepa no perteneció a ninguno grupo internacional.

Eighth: Unlike the data obtained from HU-LP, OXA-23-like was the most prevalent carbapenemase among the CRAB isolates obtained from SGH-UMC (93.8%), while OXA-24-like was only detected in two isolates and OXA-58-like was not detected.

Octavo: La familia de OXA-23 fue la más frecuente entre las cepas resistentes a carbapenemas (93,8%) aisladas en HSG-UMC, a diferencia de las cepas aisladas en HU-LP. Además, en las cepas analizadas, la familia de OXA-24 fue detectada en sólo dos cepas y la familia OXA-58 no fue detectada en ninguna.

Ninth: Among the Lebanese set of isolates, phenotypic associations between motility, siderophore production, and biofilm formation have been found ($p<0.05$).

Noveno: Se encontraron asociaciones fenotípicas positivas entre movilidad, producción de sideróforos, y formación de biofilms en las cepas del HSG-UMC ($p<0,05$).

Tenth: The isolates obtained from Lebanon failed to show the associations previously mentioned between clonality and virulence detected in the Spanish set of isolates. Therefore, the globalization of data, based on these results, could not be possible.

Décimo: No se encontraron asociaiones entre clonalidad y virulencia si comparamos las cepas del HU-LP y las del HSG-UMC. Esto significa que los datos obtenidos a partir de las cepas del HU-LP son locales y no se pueden considerar globales.

Eleventh: A novel mutation in the *pmr*CAB operon (Δ19Ile in PmrB) was pinpointed as the cause of colistin resistance among a set of *A. baumannii* isolates that developed resistance to colistin during therapy. Another mutation in that operon (P233S in PmrB) was also detected in another set of isolates that developed resistance to this antimicrobial agent.

Undécimo: Una mutación en el operón *pmr*CAB (Δ19Ile en PmrB) ha sido descrita por primera vez en nuestro estudio y es la responsable de causar resistencia a colistina en un set de cepas clínicas de *A. baumannii*. Otra mutación en este operón (P233S en PmrB) fue también descrita en otro set de cepas que desarrollan resistencia a colistina durante el tratamiento terapéutico.

Twelfth: The colistin resistant isolate that had the P233S mutation had an altered virulence profile in comparison to its sensitive counterpart, where is was positive for siderophore production and had reduced proteolytic activity. The ΔIle19 mutation did not seem to affect the virulence of the colistin resistant isolate as compared to its sensitive counterparts.

Duodécimo: La cepa con la mutación P233S resistente a colistina mostró un perfil de virulencia alterado en comparación con la cepa sensible, en cuanto a la producción de sideróforos y su inferior actividad proteolítica. Comparando la cepa con la mutación ΔIle19 con las cepas sensibles a colistina, se observó que la mutación no afectaba a la virulencia.

Thirteenth: *bla*$_{\text{GES-5}}$ was identified for the first time in *A. baumannii* in one of the two sets of isolates that developed colistin resistance during therapy isolated from HU-LP.

Decimotercero: El gen *bla*$_{\text{GES-5}}$ ha sido descrito por primera vez en *A. baumannii* en las cepas aisladas de uno de los pacientes del HU-LP.

Fourteenth: The studies performed on selected isolates from the Spanish set regarding biofilm formation showed an association between dense biofilm formation and carbapenem resistance.

Decimocuarto: Los estudios realizados con las cepas españolas en la formación de biofilms indican que hay una asociación entre formaciones densas de biofilms y resistencia a carbapenemas.

Fifteenth: Investigating the rates of biofilm formation also showed three distinct rates of biofilm formation, where three isolates were dubbed as fast biofilm formers, one as a slow biofilm former, and the others as intermediate biofilm formers.

Decimoquinto: La investigación de los patrones de formación de biofilms mostró que se encuentran tres patrones distintos. Tres cepas fueron formadoras rápidas de biofilms, una fue lenta, y las otras fueron intermedias.

Sixteenth: The isolates that were carbapenem resistant all had fast or moderate biofilm formation rates, and the three fast biofilm formers were susceptible to aminoglycosides.

Decimosexto: Todas las cepas resistentes a carbapenemas fueron formadoras rápidas o intermedias de biofilms y las que fueron rápidas fueron también susceptibles a aminoglicósidos.

Seventeenth: Some strains for which biofilm formation patterns were investigated showed pigmentation that was not associated with siderophore production. The pigmented isolates showed denser biofilms on steel coupons as assessed by CLSM, as opposed to the non-pigmented isolates.

Decimoséptimo: Algunas cepas en las que se estudiaron patrones de formación de biofilms mostraron una pigmentación no asociada a la formación de sideróforos. Las cepas pigmentadas dieron lugar a biofilms más densos bajo observación por microscopia confocal, mientras que las no pigmentadas crearon estructuras mucho más débiles.

X. BIBLIOGRAPHY

Abbo, A., Navon-Venezia, S., Hammer-Muntz, O., Krichali, T., Siegman-Igra, Y., and Carmeli, Y. (2005). Multidrug-resistant Acinetobacter baumannii. *Emerging Infectious Diseases, 11*(1), 22-29.

Acosta, J., Merino, M., Viedma, E., Poza, M., Sanz, F., Otero, J. R., Chaves, F., and Bou G. (2011). Multidrug-resistant Acinetobacter baumannii Harboring OXA-24 carbapenemase, Spain. *Emerging Infectious Diseases, 17*(6), 1064-1067.

Adams, M. D., Nickel, G. C., Bajaksouzian, S., Lavender, H., Murthy, A. R., Jacobs, M. R., and Bonomo, R. A. (2009). Resistance to colistin in Acinetobacter baumannii associated with mutations in the pmrAB two-component system. *Antimicrobial Agents and Chemotherapy, 53*(9), 3628-3634.

Adams-Haduch, J. M., Paterson, D. L., Sidjabat, H. E., Pasculle, A. W., Potoski, B. A., Muto, C. A., Harrison, L. H., and Doi, Y. (2008). Genetic basis of multidrug resistance in Acinetobacter baumannii clinical isolates at a tertiary medical center in Pennsylvania. *Antimicrobial Agents and Chemotherapy, 52*(11), 3837-3843.

Afzal-Shah, M., Woodford, N., and Livermore, D. M. (2001). Characterization of OXA-25, OXA-26, and OXA-27, molecular class D beta-lactamases associated with carbapenem resistance in clinical isolates of Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 45*(2), 583-588.

Aghazadeh, M., Rezaee, M. A., Nahaei, M. R., Mahdian, R., Pajand, O., Saffari, F., Hassan, M., and Hojabri, Z. (2013). Dissemination of aminoglycoside-modifying enzymes and 16S rRNA methylases among acinetobacter baumannii and Pseudomonas aeruginosa isolates. *Microbial Drug Resistance, Larchmont, N. Y., 19*(4), 282-288.

Ahmed-Bentley, J., Chandran, A. U., Joffe, A. M., French, D., Peirano, G., and Pitout, J. D. (2013). Gram-negative bacteria that produce carbapenemases causing death attributed to recent foreign hospitalization. *Antimicrobial Agents and Chemotherapy, 57*(7), 3085-3091.

Akers, K. S., Chaney, C., Barsoumian, A., Beckius, M., Zera, W., Yu, X., Guymon, C., Keen, E. F. 3rd, Robinson, B. J., Mende, K., and Murray, C. K. (2010). Aminoglycoside resistance and susceptibility testing errors in Acinetobacter baumannii-calcoaceticus complex. *Journal of Clinical Microbiology, 48*(4), 1132-1138.

Al Atrouni, A., Joly-Guillou, M. L., Hamze, M., and Kempf, M. (2016a). Reservoirs of Non-baumannii Acinetobacter Species. *Frontiers in Microbiology, 7*:49.

Al Atrouni, A., Joly-Guillou, M. L., Hamze, M., and Kempf, M. (2016b). Emergence of NDM-1 and OXA-72 producing Acinetobacter pittii clinical isolates in Lebanon. *New Microbes and New Infections, 12*(1), 43-44. doi: 10.1016/j.nmni.2016.04.005.

Al Bayssari, C., Dabboussi, F., Hamze, M., and Rolain, J. M. (2015). Emergence of carbapenemase-producing Pseudomonas aeruginosa and Acinetobacter baumannii in livestock animals in Lebanon. *The Journal of Antimicrobial Chemotherapy, 70*(3), 950-951.

Al-Agamy, M. H., Khalaf, N. G., Tawfick, M. M., Shibl, A. M., and El Kholy, A. (2014). Molecular characterization of carbapenem-insensitive Acinetobacter baumannii in Egypt. *International Journal of Infectious Diseases: IJID: Official Publication of the International Society of Infectious Diseases, 22*(1), 49-54. doi: 10.1016/j.ijid.2013.12.004.

Al-Hassan, L., El Mahallawy, H., and Amyes, S. G. (2013). First report of bla(PER-3) in Acinetobacter baumannii. *International Journal of Antimicrobial Agents, 41*(1), 93-94.

Al-Sweih, N. A., Al-Hubail, M., and Rotimi, V. O. (2012). Three distinct clones of carbapenem-resistant Acinetobacter baumannii with high diversity of carbapenemases isolated from patients in two hospitals in Kuwait. *Journal of Infection and Public Health, 5*(1), 102-108.

Alvargonzalez, J. J., Vindel, Hernando, A., Martín, M. D., Casas, C. M., Iglesias, J. O., Marín, M. F., Alvarez, M. L., Sanchez, V. B., and Marí, J. M. (2014). Sequential outbreaks in a Spanish hospital caused by multiresistant OXA-58-producing Acinetobacter baumannii ST92. *Journal of Medical Microbiology, 63*(Pt 8), 1093-1098.

Ambler, R. P. (1980). The structure of beta-lactamases. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 289*(1036), 321-331.

American Thoracic Society; Infectious Diseases Society of America. Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. *American Journal of Respiratory Care and Critical Care Medicine. 171*(4), 388-416.

Aminov, R. I. (2011). Horizontal gene exchange in environmental microbiota. *Frontiers in Microiology, 2*(1), 158. doi: 10.3389/fmicb.2011.00158.

Andersson, D. I., and Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews. Microbiology, 8*(4), 260-271.

Andersson, D. I., and Hughes, D. (2011). Persistence of antibiotic resistance in bacterial populations. *FEMS Microbiology Reviews, 35*(5), 901–911.

Andersson, D. I., and Levin, B. R. (1999). The biological cost of antibiotic resistance. *Current Opinion in Microbiology, 2*(5), 489-493.

Antunes, L. C., Imperi, F., Carattoli, A., and Visca, P. (2011). Deciphering the multifactorial nature of Acinetobacter baumannii pathogenicity. *PLoS One, 6*(8), e22674.

Antunes, L. C., Visca, P., and Towner, K. J. (2014) Acinetobacter baumannii: evolution of a global pathogen. *Pathogens and Disease, 71*(3) 292-30.

Aoki, S., Hirakata, Y., Kondoh, A., Gotoh, N., Yanagihara, K., Miyazaki, Y., Tomono, K., Yamada, Y., Kohno, S., and Kamihira, S. (2004). Virulence of metallo-beta-lactamase-producing Pseudomonas aeruginosa in vitro and in vivo. *Antimicrobial Agents and Chemotherapy, 48*(5), 1876-1878.

Araj, G. F., and Ibrahim, G. Y. (2008). Tigecycline in vitro activity against commonly encountered multidrug-resistant Gram-negative pathogens in a Middle Eastern country. *Diagnostic Microbiology and Infectious Disease, 62*(4), 411-415.

Arroyo, A. L., Garcia-Curiel, A., Pachon-Ibañez, M. E., Llanos, A. C., Ruiz, M., Pachon, J., and Aznar, J. (2005). Reliability of the E-test method for detection of colistin resistance in clinical isolates of Acinetobacter baumannii. *Journal of Clinical Microbiology, 43*(2), 903-905.

Arroyo, L. A., Herrera, C. M., Fernandez, L., Hankins, J. V., Trent, M. S., and Hancock, R. E. W. (2011). The pmrCAB operon mediates polymyxin resistance in Acinetobacter baumannii ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. *Antimicrobial Agents and Chemotherapy, 55*(8), 3743-3751.

Arroyo, L. A., Mateos, I., Gonzalez, V., and Aznar, J. (2008). In vitro activities of tigecycline, minocycline, and colistin-tigecycline combination against multi- and pan-drug-resistant clinical isolates of Acinetobacter baumannii group. *Antimicrobial Agents and Chemotherapy, 53*(3), 1295-1296.

Asensio, A., Cantón, R., Vaqué, J., Calbo-Torrecillas, F., Herruzo, R., Arribas, J. L., Sáenz, M. C. and Grupo de Trabajo EPINE. (2008). Prevalence of infection by carbapenem-resistant Acinetobacter baumannii in Spain (1999-2005). *Enfermedades Infecciosas y Microbiologia Clinica, 26*(4), 199-204.

Badmasti, F., Siadat, S. D., Bouzari, S., Ajdary, S., and Shancheraghi, F. (2015). Molecular detection of genes related to biofilm formation in multidrug-resistant Acinetobacter baumannii isolated from clinical settings. *Journal of Medical Microbiology, 64*(5) 538-543.

Bahador, A., Raoo An, R., Farshadzadeh, Z., Beitollahi, L., Khaledi, A., Rahimi, S., Mokhtaran, M., Mehrabi Tavana, A., and Esmaeili, D. (2015). The Prevalence of IS Aba 1 and IS Aba 4 in Acinetobacter baumannii Species of Different International Clone Lineages Among Patients With Burning in Tehran, Iran. *Jundishapur Journal of Microbiology, 8*(7), e17167. doi: 10.5812/jjm.

Bahador, A., Taheri, M., Pourakbari, B., Hashemizadeh, Z., Rostami, H., Mansoori, N., and Raoofian, R. (2013). Emergence of rifampicin, tigecycline, and colistin-resistant Acinetobacter baumannii in Iran; spreading of MDR strains of novel International Clone variants. *Microbial Drug Resistance (Larchmont, N. Y.), 19*(5), 397-406.

Bakour, S., Touati, A., Bachiri, T., Sahli, F., Tiouit, D., Naim, M., Azouaou, M., Rolain, J. M. (2014). First report of 16S rRNA methylase ArmA-producing Acinetobacter baumannii and rapid spread of metallo-β-lactamase NDM-1 in Algerian hospitals. *Journal of Infection and Chemotherapy: Official Journal of the Japan Society of Chemotherapy, 20*(11), 696-701.

Bakour, S., Touati, A., Sahli, F., Ameur, A. A., Haouchine, D., and Rolain, J. M. (2013). Antibiotic resistance determinants of multidrug-resistant Acinetobacter baumannii clinical isolates in Algeria. *Diagnostic Microbiology and Infectious Disease, 76*(4), 529-531.

Barin, J., Martins, A. F., Heineck, B. L., Barth, A. L., and Zavascki, A. P. (2013). Hetero- and adaptive resistance to polymyxin B in OXA-23-producing carbapenem-resistant Acinetobacter baumannii isolates. *Annals of Clinical Microbiology and Antimicrobials, 12*, 15. doi: 10.1186/1476-0711-12-15.

Barnaud, G., Zihoune, N., Ricard, J. D., Hippeaux, M. C., Eveillard, M., Dreyfuss, D., and Branger, C. (2010). Two sequential outbreaks caused by multidrug-resistant Acinetobacter baumannii isolates producing OXA-58 or OXA-72 oxacillinase in an intensive care unit in France. *The Journal of Hospital Infection, 76*(4), 358-360.

Bartual, S. G., Seifert, H., Hippler, C., Luzon, M. A., Wisplinghoff, H., and Rodriguez-Valera F. (2005). Development of a multilocus sequence typing scheme for characterization of clinical isolates of Acinetobacter baumannii. *Journal of Clinical Microbiology, 43*(9), 4382-4390.

Batirel, A., Balkan, I. I., Karabay, O., Agalar, C., Akalin, S., Alici, O., Alp, E., Altay, F. A., Altin, N., Arslan, F., Aslan, T., Bekiroglu, N., Cesur, S., Celik, A. D., Dogan, M., Durdu, B., Duygu, F., Engin, A., Engin, D. O., Gonen, I., Guclu, E., Guven, T., Hatipoglu, C. A., Hosoglu, S., Karahocagil, M. K., Kilic, A.U., Ormen, B., Ozdemir, D., Ozer, S., Oztoprak, N., Sezak, N., Turhan, V., Turker, N., and Yilmaz, H. (2014). Comparison of colistin-carbapenem, colistin-sulbactam, and colistin plus other antibacterial agents for the treatment of extremely drug-resistant Acinetobacter baumannii bloodstream infections. *European Journal of Clinical Microbiology and*

Infectious Diseases: Official Publication of the European Society of Clinical Microbiology, 33(8), 1311–1322.

Baumann, P. (1968). Isolation of Acinetobacter from soil and water. *Journal of Bacteriology, 96*(1), 39–42.

Baumann, P., Doudoroff, M., and Stanier, R. Y. (1968). A study of the Moraxella group. II. Oxidative-negative species (genus Acinetobacter). *Journal of Bacteriology, 95*(5), 1520–1541.

Beceiro, A., Llobet, E., Aranda, J., Bengoechea, J. A., Doumith, M., Hornsey, M., Dhanji, H., Chart, H., Bou, G. Livermore, D. M., and Woodford, N. (2011). Phosphoethanolamine modification of lipid A in colistin-resistant variants of Acinetobacter baumannii mediated by the pmrAB two-component regulatory system. *Antimicrobial Agents and Chemotherapy, 55*(7), 3370-3379.

Beceiro, A., Moreno, A., Fernandez, N., Vallejo, J. A., Aranda, J., Adler, B., Harper, M., Boyce, J. D., and Bou, G. (2014). Biological cost of different mechanisms of colistin resistance and their impact on virulence in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 58*(1), 518-526.

Beceiro, A., Tomás, M., and Bou, G. (2013). Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clinical Microbiology Reviews, 26*(2), 185-230.

Beijerinck, M. (1911). Pigmenten als oxydatieproducten gevormd door bacterien. *Versl. Koninklijke Akad. Wetensch. Amsterdam, 19*(1), 1092–1103.

Bergen, P. J., Landersdorfer, C. B., Zhang, Zhao, M., Lee, H. J., Nation, R. L., and Li, J. (2012). Pharmacokinetics and pharmacodynamics of 'old' polymyxins: what is new? *Diagnostic Microbiology and Infectious Disease, 74*(3), 213–223.

Bergogne-Bérézin, E., and Towner, K. J. (1996). Acinetobacter spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clinical Microbiology Reviews, 9*(2), 148–165.

Berlau, J., Aucken, H. M., Houang, E. and Pitt, T. L. (1999a). Isolation of Acinetobacter spp. including A. baumannii from vegetables: implications for hospital-acquired infections. *The Journal of Hospital Infection, 42*(3), 201–204.

Berlau, J., Aucken, H., Malnick, H. and Pitt, T. (1999b). Distribution of Acinetobacter species on skin of healthy humans. *European Journal of Clinical Microbiology and Infectious Diseases: Official Publication of the European Society of Clinical Microbiology, 18*(3), 179–183.

Bernards, A. T., Dijkshoorn, L., Van der Toorn, J., Bochner, B. R., and Van Boven. C. P. (1995). Phenotypic characterisation of Acinetobacter strains of 13 DNA-DNA hybridisation groups by means of the biolog system. *Journal of Medical Microbiology, 42*(2), 113–119.

Bernards, A. T., van der Toorn, J., van Boven, C. P., and Dijkshoorn, L. (1996). Evaluation of the ability of a commercial system to identify Acinetobacter genomic species. *European Journal of Clinical Microbiology and Infectious Diseases: Official Publication of the European Society of Clinical Microbiology, 15*(4), 303–308.

Bhargava, N., Sharma, P., and Capalash, N. (2014). Pyocyanin stimulates quorum sensing-mediated tolerance to oxidative stress and increases persister cell populations in Acinetobacter baumannii. *Infection and Immunity, 82*(8), 3417-3425.

Bhattacharjee, A., Anupurba, S., Gaur, A., and Sen, M. R. (2008). Prevalence of inducible AmpC β-lactamase-producing Pseudomonas aeruginosa in a tertiary care hospital in northern India. *Indian Journal of Medical Microbiology, 26*(1), 89-90.

Bogaerts, P., Naas, T., El Garch, F., Cuzon, G., Deplano, A., Delaire, T., Huang, T. D., Lissoir, B., Nordmann, P., and Glupczynski, Y. (2010). GES extended-spectrum β-lactamases in Acinetobacter baumannii isolates in Belgium. *Antimicrobial Agents and Chemotherapy, 54*(11), 4872-4878.

Bogaerts, P., Rezende de Castro, R., Roisin, S., Deplano, A., Huang, T. D., Hallin, M., Denis, O., and Glupczynski, Y. (2012). Emergence of NDM-1-producing Acinetobacter baumannii in Belgium. *The Journal of Antimicrobial Chemotherapy, 67*(6), 1552-1553.

Böhni, E. (1969). Chemotherapeutic activity of the combination of trimethoprim and sulphamethoxazole in infections of mice. *Postgraduate Medical Journal, 45*(Suppl.), 18-21.

Bonnin, R. A., Naas, T., Poirel, L., and Nordmann, P. (2012). Phenotypic, biochemical, and molecular techniques for detection of metallo-β-lactamase NDM in Acinetobacter baumannii. *Journal of Clinical Microbiology, 50*(4), 1419-1421.

Bonnin, R. A., Nordmann, P., Potron, A., Lecuyer, H., Zahar, J. R., and Poirel L. (2011b). Carbapenem-hydrolyzing GES-type extended-spectrum beta-lactamase in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 55*(1), 349-354.

Bonnin, R. A., Poirel, L., and Nordmann, P. (2014). New Delhi metallo-β-lactamase-producing Acinetobacter baumannii: a novel paradigm for spreading antibiotic resistance genes. *Future Microbiology, 9*(1), 33-41.

Bonnin, R. A., Poirel, L., Licker, M., and Nordmann, P. (2011c). Genetic diversity of carbapenem-hydrolysing β-lactamases in Acinetobacter baumannii from Romanian hospitals. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 17*(10), 1524-1528.

Bonnin, R. A., Poirel, L., Naas, T., Pirs, M., Seme, K., Schrenzel, J., and Nordmann, P. (2012). Dissemination of New Delhi metallo-β-lactamase-1-producing Acinetobacter baumannii in Europe. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 18*(9), E362-E365.

Bonnin, R. A., Potron, A., Poirel, L., Lecuyer, H., Neri, R., and Nordmann, P. (2011a). PER-7, an extended-spectrum beta-lactamase with increased activity toward broad-spectrum cephalosporins in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 55*(5), 2424-2427.

Bonnin, R. A., Rotimi, V. O., Al Hubail, M., Gasiorowski, E., Al Sweih, N., Nordmann, P., and Poirel, L. (2013). Wide dissemination of GES-type carbapenemases in Acinetobacter baumannii isolates in Kuwait. *Antimicrobial Agents and Chemotherapy, 57*(1), 183-188.

Bonomo, R. A., and Szabo, D. (2006). Mechanisms of multidrug resistance in Acinetobacter species and Pseudomonas aeruginosa. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 1*(Supp. 2), S49-S56.

Bou, G., and Martínez-Beltrán, J. (2000a). Cloning, nucleotide sequencing, and analysis of the gene encoding an AmpC β-lactamase in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 44*(2), 428-432.

Bou, G., Cerveró, G., Domínguez, M. A., Quereda, C., and Martínez-Beltrán, J. (2000b). Characterization of a nosocomial outbreak caused by a multiresistant Acinetobacter baumannii strain with a carbapenem-hydrolyzing enzyme: high-level carbapenem resistance in A. baumannii is not due solely to the presence of beta-lactamases. *Journal of Clinical Microbiology, 38*(9), 3299-3305.

Bou, G., Oliver, A., and Martínez-Beltrán J. (2000c). OXA-24, a novel class D beta-lactamase with carbapenemase activity in an Acinetobacter baumannii clinical strain. *Antimicrobial Agents and Chemotherapy, 44*(6), 1556-1561.

Bouchet, V., Huot, H., and Goldstein, R. (2008). Molecular basis of ribotyping. *Clinical Microbiology Reviews, 21*(2), 262-273.

Bouvet, P. J., and Grimont, P.A. (1986). Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov., and emended description of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *International Journal of Systemic Bacteriology*, *36*(1), 228–240.

Bouvet, P. J., and Jeanjean, S. (1989). Delineation of new proteolytic genomic species in the genus Acinetobacter. *Research in Microbiology, 140*(4-5), 291–299.

Bratu, S., Landman, D., Martin, D. A., Georgescu, C., and Quale J. (2008). Correlation of antimicrobial resistance with beta-lactamases, the OmpA-like porin, and efflux pumps in clinical isolates of Acinetobacter baumannii endemic to New York City. *Antimicrobial Agents and Chemotherapy, 52*(9), 2999-3005.

Breilh, D., Texier-Maugein, J., Allaouchiche, B., Saux, M. C., and Boselli, E. (2013). Carbapenems. *Journal of Chemotherapy (Florence, Italy), 25*(1), 1-17.

Brigante, G., Migliavacca, R., Bramati, S., Motta, E., Nucleo, E., Manenti, M., Migliorino, G., Pagani, L., Luzzaro, F., and Viganò, F. E. (2012). Emergence and spread of a multidrug-resistant Acinetobacter baumannii clone producing both the carbapenemase OXA-23 and the 16S rRNA methylase ArmA. *Journal of medical Microbial, 61*(Pt 5), 653-661.

Brisou, J., and Prevot. A. R. (1954). Studies on bacterial taxonomy. X. The revision of species under Achromobacter group. *Annales de L'Institut Pasteur (Paris), 86*(6), 722–728.

Burrus V., and Waldor, M. K. (2004). Shaping bacterial genomes with integrative and conjugative elements. *Research in Microbiology, 155*(5), 376-386.

Cai, Y., Chai, D., Wang, R., Liang, B., and Bai, N. (2012). Colistin resistance of Acinetobacter baumannii: clinical reports, mechanisms and antimicrobial strategies. *The Journal of Antimicrobial Chemotherapy, 67*(7), 1607-1615.

Camarena, L., Bruno, V., Euskirchen, G., Poggio, S., and Snyder, M. (2010). Molecular mechanisms of ethanol-induced pathogenesis revealed by RNA-sequencing. *PLoS Pathogens, 6*(4), e1000834.

Candel, F. J., Calvo, N., Head, J., Sánchez, A., Matesanz, M., Culebras, E., Barrientos, A., and Picazo, J. (2010). A combination of tigecycline, colistin, and meropenem against multidrug-resistant Acinetobacter baumannii bacteremia in a renal transplant recipient: pharmacodynamic and microbiological aspects. *Revista Española de Quimioterapia: Publicacion Official de la Sociedad Española de Quimioterapia, 23*(2), 103-108.

Carr, E. L., Kampfer, P., Patel, B. K., Gurtler, V., and Seviour, R. J. (2003). Seven novel species of Acinetobacter isolated from activated sludge. *International Journal of Systemic and Evolutionary Microbiology, 53*(Pt 4), 953–963.

Castanheira, M., Costello, S. E., Woosley, L. N., Deshpande, L. M., Davies, T. A., and Jones, R. N. (2014). Evaluation of clonality and carbapenem resistance mechanisms among Acinetobacter baumannii-Acinetobacter calcoaceticus complex and Enterobacteriaceae isolates collected in European and Mediterranean countries and detection of two novel β-lactamases, GES-22 and VIM-35. *Antimicrobial Agents and Chemotherapy, 58*(12), 7358-7366.

Cayô, R., Rodríguez, M. C., Espinal, P., Fernández-Cuenca, F., Ocampo-Sosa, A. A., Pascual, A., Ayala, J. A., Vila, J., and Martínez-Martínez, L. (2011). *Antimicrobial Agents and Chemotherapy, 55*(12), 5907-5913.

Celenza, G., Pellegrini, C., Caccamo, M., Segatore, B., Amicosante, G., and Perilli, M. (2006). Spread of bla(CTX-M-type) and bla(PER-2) beta-lactamase genes in clinical isolates from Bolivian hospitals. *The Journal of Antimicrobial Chemotherapy, 57*(5), 975-978.

Cerqueira, G. M., and Peleg, A. Y. (2011). Insights into Acinetobacter baumannii pathogenicity. *IUBMB Life, 63*(12), 1055-1060.

Cicek, A. C., Saral, A., Iraz, M., Ceylan, A., Duzgun, A. O., Peleg, A. Y., and Sandalli, C. (2014). OXA- and GES-type β-lactamases predominate in extensively drug-resistant Acinetobacter baumannii isolates from a Turkish University Hospital. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 20*(5), 410-415.

Cieslinski, J. M., Arend, L., Tuon, F. F., Silva, E. P., Ekermann, R. G., Dalla-Costa, L. M., Higgins, P. G., Seifert, H., and Pilonetto, M. (2013). Molecular epidemiology characterization of OXA-23 carbapenemase-producing Acinetobacter baumannii isolated from 8 Brazilian hospitals using repetitive sequence-based PCR. *Diagnostic Microbiology and Infectious Diseases, 77*(4), 337-340.

Ciofu, O., and Tolker-Nielsen, T. (2011). Antibiotic Tolerance and Resistance in Biofilms. In T. Bjarnsholt *et al.*, Biofilm Infections, *Springer Science+Business Media*, 215-229.

Cisneros, J. M., Rodríguez-Baño, J., Fernández-Cuenca, F., Ribera, A., Vila, J., Pascual, A., Martínez-Martínez, L., Bou, G., Pachón, J., and Spanish Group for Nosocomial Infection (GEIH) for the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC). (2005). Risk factors for the acquisition of imipenem-resistant Acinetobacter baumannii in Spain: A nationwide study. *Clinical Microbiology and Infection: the Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 11*(11), 874-879.

Cisneros, J. M., Reyes, M. J., Pachón, J., Becerril, B., Caballero, F. J., García-Garmendía, J. L., Ortiz, C., and Cobacho, A. R. (1996). Bacteremia due to Acinetobacter baumannii: epidemiology, clinical findings, and prognostic features. *Clinical Infectious Diseases: an Official Publication of the Infectious Diseases Society of America, 22*(6), 1026-1032.

Clinical and Laboratory Standards Institute. (2014). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. M100-S24, *34*(1), 62-64.

Connell, S. R., Tracz, D. M., Nierhaus, K. H., and Taylor, D. E. (2003). Ribosomal protection proteins and their mechanism of tetracycline resistance. *Antimicrobial Agents and Chemotherapy, 47*(12), 3675-3681.

Constantiniu, S., Romaniuc, A., Iancu S. L., Filimon, R., and Tarasi, I. (2004). Cultural and Biochemical characteristics of Acinetobacter spp. strains isolated from hospital units. *The Journal of Preventive Medicine, 12*(3-4): 35-42.

Cortivo, G. D., Gutberlet, A., Ferreira, J. A., Ferreira, L. E., Deglmann, R. C., Westphal, G. A., and França, P. H. (2015). Antimicrobial resistance profiles and oxacillinase genes in carbapenem-resistant Acinetobacter baumannii isolated from hospitalized patients in Santa Catarina, Brazil. *Revista de Sociedade Braseleira de Medicina Troical, 48*(6), 699-705.

Corvec, S., Poirel, L., Naas, T., Drugeon, H., and Nordmann, P. (2007). Genetics and expression of the carbapenem-hydrolyzing oxacillinase gene blaOXA-23 in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 51*(4), 1530-1533.

Coyne, S., Courvalin, P., and Périchon, B. (2011). Efflux-mediated antibiotic resistance in Acinetobacter spp. *Antimicrobial Agents and Chemotherapy, 55*(3), 947-953.

Culebras, E., González-Romo, F., Head, J., Gómez, M., Morales, G., and Picazo, J. J. (2010). Outbreak of Acinetobacter baumannii producing OXA-66 in a Spanish hospital: epidemiology and study of patient movements. *Microbial Drug Resistance (Larchmont, N. Y.), 16*(4), 309-315.

Chan, J. D., Graves, J. A., and Dellit, T. H. (2010). Antimicrobial treatment and clinical outcomes of carbapenem-resistant Acinetobacter baumannii ventilator-associated pneumonia. *Journal of Intensive Care Medicine, 25*(6), 343-348.

Chang, H. C., Wei, Y. F., Dijkshoorn, L., Vaneechoutte, M., Tang, C. T., and Chang, T. C. (2005). Species-level identification of isolates of the Acinetobacter calcoaceticus-Acinetobacter baumannii complex by sequence analysis of the 16S-23S rRNA gene spacer region. *Journal of Clinical Microbiology, 43*(4), 1632-1639.

Charfi-Kessis, K., Mansour, W., Ben Haj Khalifa, A., Mastouri, M., Nordmann, P., Aouni, M., and Poirel, L. (2014). Multidrug-resistant Acinetobacter baumannii strains carrying the bla(OxA-23) and the bla(GES-11) genes in a neonatology center in Tunisia. *Microbial Pathogenesis, 74*(1), 20-24.

Chassaing, B., Garénaux, E., Carriere, J., Rolhion, N., Guérardel, Y., Barnich, N., Bonnet, R., and Darfeuille-Michaud, A. (2015). Analysis of the σE regulon in Crohn's disease-associated Escherichia coli revealed involvement of the waaWVL operon in biofilm formation. *Journal of Bacteriology, 197*(8), 1451-1565.

Chatterjee, N., Bhattacharyya, K., and Dam, S. (2016). Acinetobacter baumannii: an emerging unique multi-drug resistant pathogen. *European Journal of Biomedical and Pharmaceutical Sciences, 3*(1), 117-130.

Chaulagain, B. P., Jang, S. J., Ahn, G. Y., Ryu, S. Y., Kim, D. M., Park, G., Kim, W. Y., Shin, J. H., Kook, J. K., Kang, S. H., Moon, D. S., and Park, Y. J. (2012). Molecular epidemiology of an outbreak of imipenem-resistant Acinetobacter baumannii carrying the ISAba1-bla(OXA-51-like) genes in a Korean hospital. *Japanese Journal of Infectious Diseases, 65*(2), 162-166.

Chen, Q., Li, X., Zhou, H., Jiang, Y., Chen, Y., Hua, X., Yu, Y. (2014). Decreased susceptibility to tigecycline in Acinetobacter baumannii mediated by a mutation in trm encoding SAM-dependent methyltransferase. *The Journal of Antimicrobial Chemotherapy, 69*(1), 72-76.

Chen, Y. P., Lu, P. L., and Kuo, C. M. (2015). An increasing trend of Carbapenems Resistance Acinetobacter baumannii (CRAB) and CRAB's co-resistance to ceftazidime,

gentamicin, cefepime, levofloxacin and amikacin in a Taiwan regional hospital. *Journal of Microbiology, Immunology, and Infection, 48*(2, S1), S126-S127.

Chen, Y., Zhou, Z., Jiang, Y., and Yu, Y. (2011). Emergence of NDM-1-producing Acinetobacter baumannii in China. *The Journal of Antimicrobial Chemotherapy, 66*(6), 1255-1259.

Chiu, C. H., Lee, H. Y., Tseng, L. Y., Chen, C. L., Chia, J. H., Su, L. H., Liu, S. Y. (2010). Mechanisms of resistance to ciprofloxacin, ampicillin/sulbactam and imipenem in Acinetobacter baumannii clinical isolates in Taiwan. *International Journal of Antimicrobial Agents, 35*(4), 382-386.

Chmielarczyk, A., Pilarczyk-Żurek, M., Kamińska, W., Pobiega, M., Romaniszyn, D., Ziółkowski, G., Wójkowska-Mach, J., and Bulanda, M. (2016). Molecular Epidemiology and Drug Resistance of Acinetobacter baumannii Isolated from Hospitals in Southern Poland: ICU as a Risk Factor for XDR Strains. *Microbial Drug Resistance (Larchmont, N. Y.), 22*(4), 328-335.

Cho, Y. J., Moon, D. C., Jin, J. S., Choi, C. H., Lee, Y. C., and Lee, J. C. (2009). Genetic basis of resistance to aminoglycosides in Acinetobacter spp. and spread of armA in Acinetobacter baumannii sequence group 1 in Korean hospitals. *Diagnostic Microbiology and Infectious Diseases, 64*(2), 185-190.

Choi, A. H., Slamti, L., Avci, F. Y., Pier, G. B., and Maira-Litrán, T. (2009). The pgaABCD locus of Acinetobacter baumannii encodes the production of poly-beta-1-6-N-acetylglucosamine, which is critical for biofilm formation. *Journal of Bacteriology, 191*(19), 5953-5963.

Choi, C. H., Lee, E. Y., Lee, Y. C., Park, T. I., Kim, H. J., Hyun, S. H., Kim, S. A., Lee, S. K., and Lee, J. C. (2005). Outer membrane protein 38 of Acinetobacter baumannii localizes to the mitochondria and induces apoptosis of epithelial cells. *Cellular Microbiology, 7*(8), 1127-1138.

Chu, Y. W., Afzal-Shah, M., Houang, E. T., Palepou, M. I., Lyon, D. J., Woodford, N., and Livermore, D. M. (2001). IMP-4, a novel metallo-beta-lactamase from nosocomial Acinetobacter spp. collected in Hong Kong between 1994 and 1998. *Antimicrobial Agents and Chemotherapy*, *45*(3), 710-714.

Chu, Y. W., Leung, C. M., Houang, E. T., Ng, K. C., Leung, C. B., Leung, H. Y., and Cheng, A. F. (1999). Skin carriage of acinetobacters in Hong Kong. *Journal of Clinical Microbiology, 37*(9), 2962–2967.

Chuang, Y. C., Sheng, W. H., Li, S. Y., Lin, Y. C., Wang, J. T., Chen, Y. C., and Chang, . C. (2011). Influence of Genospecies of Acinetobacter baumannii Complex on Clinical Outcomes of Patients with Acinetobacter Bacteremia. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 52*(3), 352-360.

Da Silva, G. J., Mendonça, N., Batista, G., and Duarte, A. (2010). Sequence types of Portuguese carbapenem-resistant Acinetobacter baumannii isolates collected over 10 years. *The Journal of Antimicrobial Chemotherapy, 65*(10), 2254-2256.

Da Silva, G. J., Quinteira, S., Bértolo, E., Sousa, J. C., Gallego, L., Duarte, A., Peixe, L., and Portugese Resistance Study Group. (2004). Long-term dissemination of an OXA-40 carbapenemase-producing Acinetobacter baumannii clone in the Iberian Peninsula. *The Journal of Antimicrobial Chemotherapy, 54*(1), 255-258.

Da Silva, G.J., Correia, M., Vital, C., Ribeiro, G., Sousa, J. C., Leitão, R., Peixe, L., and Duarte, A. (2002). Molecular characterization of bla(IMP-5), a new integron-borne metallo-

beta-lactamase gene from an Acinetobacter baumannii nosocomial isolate in Portugal. *FEMS Microbiology Letters, 215*(1), 33-39.

Dalhoff, A., Janjic, N., and Echols, R. (2006). Redefining penems. *Biochemical Pharmacology, 71*(7), 1085–1095.

Dally, S., Lemuth, K., Kaase, M., Rupp, S., Knabbe, C., and Weile, J. (2013). DNA microarray for genotyping antibiotic resistance determinants in Acinetobacter baumannii clinical isolates. *Antimicrobial Agents and Chemotherapy, 57*(10), 4761-4768.

Damier-Piolle, L., Magnet, S., Brémont, S., Lambert, T., and Courvalin, P. (2008). AdeIJK, a resistance–nodulation–cell division pump effluxing multiple antibiotics in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 52*(2), 557–562.

D'Arezzo, S., Principe, L., Capone, A., Petrosillo, N., Petrucca, A., and Visca, P. (2011). Changing carbapenemase gene pattern in an epidemic multidrug-resistant Acinetobacter baumannii lineage causing multiple outbreaks in central Italy. *The Journal of Antimicrobial Chemotherapy, 66*(1), 54-61.

D'Costa, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H. N., and Wright, G. D. (2011). Antibiotic resistance is ancient. *Nature, 477*(7365), 457-461.

De Breij, A., Dijkshoorn, L., Lagendijk, E., van der Meer, J., Koster, A., Bloemberg, G., Wolterbeek, R., van den Broek, P., and Nibbering, P. (2010). Do biofilm formation and interactions with human cells explain the clinical success of Acinetobacter baumannii? *PLoS ONE, 5*(5), e10732. doi: 10.1371/journal.pone.0010732.

De Sá Cavalcanti, F. L., Almeida, A. C., Vilela, M. A., de Morais Junior, M. A., de Morais, M. M., and Leal-Balbino, T. C. (2013). Emergence of extensively drug-resistant OXA-72-producing Acinetobacter baumannii in Recife, Brazil: risk of clonal dissemination? *Diagnostic Microbiology and Infectious Diseases, 77*(3), 250-251.

De Vos, D., Pirnay, J. P., Bilocq, F., Jennes, S., Verbeken, G., Rose, T., Keersebilck, E., Bosmans, P., Pieters, T., Hing, M., Heuninckx, W., De Pauw, F., Soentjens, P., Merabishvili, M., Deschaght, P., Vaneechoutte, M., Bogaerts, P., Glupczynski, Y., Pot, B., van der Reijden, T. J., and Dijkshoorn, L. (2016). Molecular Epidemiology and Clinical Impact of Acinetobacter calcoaceticus-baumannii Complex in a Belgian Burn Wound Center. *PLoS One, 11*(5), e0156237. doi: 10.1371/journal.pone.0156237.

Decousser, J. W., Jansen, C., Nordmann, P., Emirian, A., Bonnin, R. A., Anais, L., Merle, J. C., and Poirel, L. (2013). Outbreak of NDM-1-producing Acinetobacter baumannii in France, January to May 2013. *Euro Surveillance: European Communicable Disease Bulletin, 18*(31), pii: 20547.

Derlon, N., Coufort-Saudejaud, C., Queinnec, I., and Paul, E. (2012). Growth limiting conditions and denitrification govern extent and frequency of volume detachment of biofilms. *Chemical Engineering Journal, 218*(1), 368-375.

Di Nocera, P. P., Rocco, F., Giannouli, M., Triassi, M., and Zarrilli, R. (2011). Genome organization of epidemic Acinetobacter baumannii strains. *BMC Microbiology, 11*(1), 224. doi: 10.1186/1471-2180-11-224.

Di Popolo, A., Giannouli, M., Triassi, M., Brisse, S., and Zarrilli R. (2011). Molecular epidemiological investigation of multidrug-resistant Acinetobacter baumannii strains in four Mediterranean countries with a multilocus sequence typing scheme. *Clinical Microbiology and Infection: the Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 17*(2), 197-201.

Diancourt, L., Passet, V., Nemec, A., Dijkshoorn, L., and Brisse, S. (2010). The population structure of Acinetobacter baumannii: expanding multiresistant clones from an ancestral susceptible genetic pool. *PLoS One, 5*(4): e10034.

Dijkshoorn, L., van Aken, E., Shunburne, L., van der Reijden, T. J., Bernards, A. T., Nemec, A., and Towner, K. J. (2005). Prevalence of Acinetobacter baumannii and other Acinetobacter spp. in faecal samples from non-hospitalised individuals. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 11*(4), 329–332.

Dobrewski, R., Savov, E., Bernards, A. T., van den Barselaar, M., Nordmann, P., van den Broek, P. J., and Dijkshoorn, L. (2006). Genotypic diversity and antibiotic susceptibility of Acinetobacter baumannii isolates in a Bulgarian hospital. *Clinical Microbiology and Infection, 12*(11), 1135-1137.

Doi, Y., Adams, J. M., Yamane, K., and Paterson, D. L. (2007a). Identification of 16S rRNA methylase-producing Acinetobacter baumannii clinical strains in North America. *Antimicrobial Agents and Chemotherapy, 51*(11), 4209-4211.

Doi, Y., and Arakawa, Y. (2007b). 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 45*(1), 88-94.

Doi, Y., Murray, G. L., and Peleg, A. Y. (2015). Acinetobacter baumannii: Evolution of Antimicrobial Resistance—Treatment Options. *Seminars in Respiratory and Critical Care Medicine, 36*(1) 85-98.

Dolzani, L., Tonin, E., Lagatolla, C., and Monti-Bragadin, C. (1995). Identification of Acinetobacter isolates in the A. calcoaceticus-A. baumannii complex by restriction analysis of the 16S-23S rRNA intergenic-spacer sequences. *Journal of Clinical Microbiology, 33*(5), 1108-1113.

Dortet, L., Poirel, L., Errera, C., and Nordmann, P. (2014). CarbAcineto NP test for rapid detection of carbapenemase-producing Acinetobacter spp. *Journal of Clinical Microbiology, 52*(7), 2359-2364.

Doughari, H. J., Ndakidemi, P. A., Human, I. S., and Benade, S. (2011). The ecology, biology and pathogenesis of Acinetobacter spp.: an overview. *Microbes and Environment, 26*(2), 101-112.

Drawz, S. M., and Bonomo, R. A. (2010). Three decades of beta-lactamase inhibitors. *Clinical Microbiology Reviews, 23*(1), 160–201.

Drzewiecki, A., Bulanda, M., Talaga, K., Sodo, A., & Adamski, P. (2012). Comparison of in vitro activity of doripenem, imipenem and meropenem against clinical isolates of enterobacteriaceae, pseudomonas and acinetobacter in southern poland. *Polski Przeglad Chirurgiczny, 84*(9), 449-453.

Dubois, D., Prasadarao, N. V., Mittal, R., Bret, L., Roujou-Gris, M., and Bonnet, R. (2009). CTX-M beta-lactamase production and virulence of Escherichia coli K1. *Emerging Infectious Diseases, 15*(12), 1988-1990.

Durante-Mangoni, E., Del Franco, M., Andini, R., Bernardo, M., Giannouli, M., and Zarrilli, R. (2015). Emergence of colistin resistance without loss of fitness and virulence after prolonged colistin administration in a patient with extensively drug-resistant Acinetobacter baumannii. *Diagnostic Microbiology and Infectious Disease, 82*(3), 222-226.

Durante-Mangoni, E., Grammatikos, A., Utili, R., and Falagas, M. E. (2009). Do we still need the aminoglycosides? *International Journal of Antimicrobial Agents, 33*(3), 201–5.

Ecker, J. A., Massire, C., Hall, T. A., Ranken, R., Pennella, T. T., Agasino Ivy, C., Blyn, L. B., Hofstadler, S. A., Endy, T. P., Scott, P. T., Lindler, L., Hamilton, T., Gaddy, C., Snow, K., Pe, M., Fishbain, J., Craft, D., Deye, G., Riddell, S., Milstrey, E., Petruccelli, B., Brisse, S., Harpin, V., Schink, A., Ecker, D. J., Sampath, R., and Eshoo, M. W. (2006). Identification of Acinetobacter species and genotyping of Acinetobacter baumannii by multilocus PCR and mass spectrometry. *Journal of Clinical Microbiology, 44*(8), 2921-2932.

Eijkelkamp, B. A., Hassan, K. A., Paulsen, I. T., and Brown, M. H. (2011a). Investigation of the human pathogen Acinetobacter baumannii under iron limiting conditions. *BMC Genomics, 12*, 26. doi: 10.1186/1471-2164-12-126.

Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Papadimitrious, M. S., Paulsen, I. T., and Brown, M. H. (2011b). Adherence and motility characteristics of clinical Acinetobacter baumannii isolates. *FEMS Microbiology Letters, 323*(1), 44-51.

Elander, R. P. (2003). Industrial production of beta-lactam antibiotics. *Applied Microbiology and Biotechnology*, *61*(5–6), 385–392.

El-Sayed-Ahmed, M. A., Amin, M. A., Tawakol, W. M., Loucif, L., Bakour, S., and Rolain, J. M. (2015). High prevalence of bla(NDM-1) carbapenemase-encoding gene and 16S rRNA armA methyltransferase gene among Acinetobacter baumannii clinical Isolates in Egypt. *Antimicrobial Agents and Chemotherapy, 59*(6), 3602-3605.

El-Shazly, S., Dashti, A., Vali, L., Bolaris, M., and Ibrahim, A. S. (2015). Molecular epidemiology and characterization of multiple drug-resistant (MDR) clinical isolates of Acinetobacter baumannii. *International Journal of Infectious Diseases: IJID: Official Publication of the International Society of Infectious Diseases, 41*(1), 42-49. doi: 10.1016/j.ijid.2015.10.016.

Endimiani, A., Hujer, K. M,. Hujer, A. M., Bertschy, I., Rossano, A., Koch, C., Gerber, V., Francey, T., Bonomo, R. A., and Perreten, V. (2011). Acinetobacter baumannii isolates from pets and horses in Switzerland: molecular characterization and clinical data. *The Journal of Antimicrobial Chemotherapy, 66*(10), 2248-2254.

Endimiani, A., Luzzaro, F., Migliavacca, R., Mantengoli, E., Hujer, A. M., Hujer, K. M., Pagani, L., Bonomo, R. A., Rossolini, G. M., and Toniolo, A. (2007). Spread in an Italian hospital of a clonal Acinetobacter baumannii strain producing the TEM-92 extended-spectrum beta-lactamase. *Antimicrobial Agents and Chemotherapy, 51*(6), 2211-2214.

Ermertcan, S., Hoşgör, M., Tünger, O., and Coşar, G. (2001). Investigation of synergism of meropenem and ciprofloxacin against Pseudomonas aeruginosa and Acinetobacter strains isolated from intensive unit infections. *Scandinavian Journal of Infectious Diseases, 33*(11), 818-821.

Espinal, P., Fugazza, G., López, Y., Kasma, M., Lerman, Y., Malhotra-Kumar, S., Goossens, H., Carmeli, Y., and Vila, J. (2011). Dissemination of an NDM-2-producing Acinetobacter baumannii clone in an Israeli rehabilitation center. *Antimicrobial Agents and Chemotherapy, 55*(11), 5396-5398.

Espinal, P., Macia, M. D., Roca, I., Gato, E., Ruiz, E., Fernandez-Cuenca, F., Oliver, A., Rodriguez-Bano, J., Bou, G., Tomas, M., and Vila, J. (2012). First report of an OXA-

23 carbapenemase-producing Acinetobacter baumannii clinical isolate related to Tn2006 in Spain. *Antimocrobial Agents and Chemotherapy, 57*(1), 589-591.

Espinal, P., Poirel, L., Carmeli, Y., Kaase, M., Pal, T., Nordmann, P., and Vila, J. (2013). Spread of NDM-2-producing Acinetobacter baumannii in the Middle East. *The Journal of Antimicrobial Chemotherapy, 68*(8), 1928-1930.

Evans, B. A., and Amyes, S. G. (2014). OXA β-lactamases. *Clinical Microbiology Reviews, 27*(2), 241-263.

Evans, B. A., Hamouda, A., Towner, K. J., and Amyes, S. G. (2008). OXA-51-like beta-lactamases and their association with particular epidemic lineages of Acinetobacter baumannii. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 14*(3), 268-275.

Falagas, M. E., and Bliziotis, I. A. (2007). Pandrug-resistant Gram-negative bacteria: the dawn of the post-antibiotic era? *International Journal of Antimicrobial Agents, 29*(6), 630-636.

Falagas, M. E., Rafailidis, P. I., Matthaiou, D. K., Virtzili, S., Nikita, D., and Michalopoulos, A. (2008). Pandrug-resistant Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter baumannii infections: characteristics and outcome in a series of 28 patients. *International Journal of Antimicrobial Agents, 32*(5), 450-454.

Falagas, M. E., Vardakas, K. Z., and Roussos, N. S. (2015). Trimethoprim/sulfamethoxazole for Acinetobacter spp.: A review of current microbiological and clinical evidence. *International Journal of Antimicrobial Agents, 46*(3), 231-241.

Farajnia, S., Azhari, F., Alikhani, M. Y., Hosseini, M. K., Peymani, A., and Sohrabi, N. (2013). Prevalence of PER and VEB Type Extended Spectrum Betalactamases among Multidrug Resistant Acinetobacter baumannii Isolates in North-West of Iran. *Iranian Journal of Basic Medical Sciences, 16*(6), 751-755.

Fernández Cuenca, F., Sánchez, C. M., Caballero-Moyano, F. J., Vila, J., Martínez-Martínez, L., Bou, G., Bano, J.R., and Pascual, A. (2012). Prevalence and analysis of microbiological factors associated with phenotypic heterogeneous resistance to carbapenems in Acinetobacter baumannii. *International Journal of Antimicrobial Agents, 39*(1), 471-477.

Fernández, A., Pérez, A., Ayala, J. A., Mallo, S., Rumbo-Feal, S., Tomás, M., Poza, M., and Bou, G. (2012). Expression of OXA-type and SFO-1 β-lactamases induces changes in peptidoglycan composition and affects bacterial fitness. *Antimicrobial Agents and Chemotherapy, 56*(4), 1877-1884.

Fernández-Cuenca, F., Smani, Y., Gómez-Sánchez, M. C., Docobo-Pérez, F., Caballero-Moyano, F. J., Domínguez-Herrera, J., Pascual, A., and Pachón, J. (2011). Attenuated virulence of a slow-growing pandrug-resistant Acinetobacter baumannii is associated with decreased expression of genes encoding the porins CarO and OprD-like. *International Journal of Antimicrobial Agents, 38*(6), 548-549.

Fernández-Cuenca, F., Tomás, M., Caballero-Moyano, F. J., Bou, G., Martínez-Martínez, L., Vila, J., Pachón, J., Cisneros, J. M., Rodríguez-Baño, J., Pascual, Á., and the Spanish Group of Nosocomial Infections (GEIH) from the Spanish Society of Clinical Microbiology and Infectious Diseases (SEIMC) and the Spanish Network for Research in Infectious Diseases (REIPI). (2015). Reduced susceptibility to biocides in Acinetobacter baumannii: association with resistance to antimicrobials, epidemiological behaviour, biological cost and effect on the expression of genes

encoding porins and efflux pumps. *The Journal of Antimicrobial Chemotherapy, 70*(12), 3222-3229.

Fishbain, J. and Peleg, A. Y. (2010). Treatment of Acinetobacter infections. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 51*(1), 79-84.

Fisher, J. F., Meroueh, S. O., and Mobashery, S. (2005). Bacterial Resistance to β-Lactam Antibiotics: Compelling Opportunism, Compelling Opportunity. *Chemical Reviews, 105*(2), 395–424.

Flamm, R. K., Castanheira, M., Streit, J. M., and Jones, R. N. (2016). Minocycline activity tested against Acinetobacter baumannii complex, Stenotrophomonas maltophilia, and Burkholderia cepacia species complex isolates from a global surveillance program (2013). *Diagnostic Microbiology and Infectious Diseases, 85*(3), 352-355.

Fouad, M., Attia, A. S., Tawakkol, W. M., and Hashem, A. M. (2013). Emergence of carbapenem-resistant Acinetobacter baumannii harboring the OXA-23 carbapenemase in intensive care units of Egyptian hospitals. *International Journal of Infectious Diseases: IJID: Official Publication of the International Society of Infectious Diseases, 17*(2), e1252-1254.

Foucault, M. L., Courvalin, P., and Grillot-Courvalin, C. (2009). Fitness cost of VanA-type vancomycin resistance in methicillin-resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy, 53*(6), 2354-2359.

Fournier, P. E., and Richet, H. (2006). The epidemiology and control of Acinetobacter baumannii in health care facilities. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 42*(5), 692– 699.

Fournier, P. E., Vallenet, D., Barbe, V., Audic, S., Ogata, H., Poirel, L., Richet, H., Robert, C., Mangenot, S., Abergel, C., Nordmann, P., Weissenbach, J., Raoult, D., and Claverie, J. M. (2006). Comparative genomics of multidrug resistance in Acinetobacter baumannii. *PLoS Genetics, 2*(1), e7.

Foxman, B. (2010). The epidemiology of urinary tract infection. *Nature Reviews. Urology, 7*(12), 653-660.

Francey, T., Gaschen, F., Nicolet, J. and Burnens, A. P. (2000). The role of Acinetobacter baumannii as a nosocomial pathogen for dogs and cats in an intensive care unit. *Journal of Veterinary Internal Medicine / American College of Veterinary Internal Medicine, 14*(2), 177–183.

Fu, Y., Zhou, J., Zhou, H., Yang, Q., Wei, Z., Yu, Y., and Li, L. (2010). Wide dissemination of OXA-23-producing carbapenem-resistant Acinetobacter baumannii clonal complex 22 in multiple cities of China. *The Journal of Antimicrobial Chemotherapy, 65*(4), 644-650.

Gaddy, J. A., Tomaras, A. P., and Actis, L. A. (2009). The Acinetobacter baumannii 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infection and Immunity, 77*(8), 3150-3160.

Gales, A. C., Tognim, M. C., Reis, A. O., Jones, R. N., and Sader, H. S. (2003). Emergence of an IMP-like metallo-enzyme in an Acinetobacter baumannii clinical strain from a Brazilian teaching hospital. *Diagnostic Microbiology and Infectious Diseases, 45*(1), 77-79.

Gallego, L., and Towner, K. J. (2001). Carriage of class 1 integrons and antibiotic resistance in clinical isolates of Acinetobacter baumannii from northern Spain. *Journal of Medical Microbiology, 50*(1), 71-77.

Ganjo, A. R., Maghdid, D. M., Mansoor, I. Y., Kok, D. J., Severin, J. A., Verbrugh, H. A., Kreft, D., Fatah, M. H., Alnakshabandi, A. A., Dlnya, A., Hammerum, A. M., Ng, K., and Goessens, W. (2016). OXA-Carbapenemases Present in Clinical Acinetobacter baumannii-calcoaceticus Complex Isolates from Patients in Kurdistan, Region, Iraq. *Microbial Drug Resistance,* Epub Ahead of Print.

García-Ortega, L., Arch, O., Pérez-Canosa, C., Lupión, C., González, C., Rodríguez-Baño, J., and Spanish Study Group of Nosocomial Infections (GEIH-SEIMC). (2011). Control measures for Acinetobacter baumannii: a survey of Spanish hospitals. *Enfermedades Infecciosas y Microbiologia Clinica, 29*(1), 36-38.

Garnacho-Montero, J., Gutiérrez-Pizarraya, A., Díaz-Martín, A., Cisneros-Herreros, J. M., Cano, M. E., Gato, E., Ruiz de Alegría, C., Fernández-Cuenca, F., Vila, J., Martínez-Martínez, L., Tomás-Carmona, M. D., Pascual, Á., Bou, G., Pachón-Diaz, J., and Rodríguez-Baño, J. (2016). Acinetobacter baumannii in critically ill patients: Molecular epidemiology, clinical features and predictors of mortality. *Enfermedades Infecciosas y Microbiologia Clinica,* pii: *S0213-005X*(15), 00456-5. doi: 10.1016/j.eimc.2015.11.018.

Gehrlein, M., Leying, H., Cullmann, W., Wendt, S., and Opferkuch, W. (1991). Imipenem resistance in Acinetobacter baumanii is due to altered penicillin-binding proteins. *Chemotherapy, 37*(6), 405-412.

Gentile, V., Frangipani, E., Bonchi, C., Minandri, F., Runci, F., and Visca, P. (2014). Iron and Acinetobacter baumannii Biofilm Formation. *Pathogens, 3*(3) 704-719.

Gerner-Smidt, P. (1992). Ribotyping of the Acinetobacter calcoaceticus-Acinetobacter baumannii complex. *Journal of Clinical Microbiology, 30*(10), 2680–2685.

Gerner-Smidt, P., Tjernberg, I., and Ursing, J. (1991). Reliability of phenotypic tests for identification of Acinetobacter species. *Journal of Clinical Microbiology, 29*(2), 277–282.

Ghazawi, A., Sonnevend, A., Bonnin, R. A., Poirel, L., Nordmann, P., Hashmey, R., Rizvi, T. A., B Hamadeh, M., and Pál, T. (2012). NDM-2 carbapenemase-producing Acinetobacter baumannii in the United Arab Emirates. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 18*(2), E34-E36.

Giannouli, M., Cuccurullo, S., Crivaro, V., Di Popolo, A., Bernardo, M., Tomasone, F., Amato, G., Brisse, S., Triassi, M., Utili, R., and Zarrilli, R. (2010). Molecular epidemiology of multidrug-resistant Acinetobacter baumannii in a tertiary care hospital in Naples, Italy, shows the emergence of a novel epidemic clone. *Journal of Clinical Microbiology, 48*(4), 1223-1230.

Giannouli, M., Tomasone, F., Agodi, A., Vahaboglu, H., Daoud, Z., Triassi, M., Tsakris, A., and Zarrilli, R. (2009). Molecular epidemiology of carbapenem-resistant Acinetobacter baumannii strains in intensive care units of multiple Mediterranean hospitals. *The Journal of Antimicrobial Chemotherapy, 63*(4), 828-830.

Gilbert, D. N. Aminoglycosides. In: Mandell, G. L., Bennett, J. E., Dolin, R., editors. Principles and practice of infectious diseases. 4th edition, New York, N.Y: Churchill Livingstone; 1995. pp. 279–306.

Girlich, D., Damaceno, Q. S., Oliveira, A. C., and Nordmann, P. (2014). OXA-253, a variant of the carbapenem-hydrolyzing class D β-lactamase OXA-143 in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 58*(5), 2976-2978.

Glew, R. H., Moellering, R. C. Jr., and Kunz, L. J. (1977). Infections with Acinetobacter calcoaceticus (Herellea vaginicola): clinical and laboratory studies. *Medicine (Baltimore), 56*(2), 79–97.

Goldberg, E., and Bishara, J. (2012). Contemporary unconventional clinical use of co-trimoxazole. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 18*(1), 8-17.

Goldstein, F. W., Labigne-Roussel, A., Gerbaud, G., Carlier, C., Collatz, E., and Courvalin, P. (1983). Transferable plasmid-mediated antibiotic resistance in Acinetobacter. *Plasmid, 10*(2), 138-147.

Goncalves-Pereira, J., and Povoa, P. (2011). Antibiotics in critically ill patients: A systematic review of the pharmacokinetics of beta-lactams. *Critical Care, 15*(5), R206. doi: 10.1186/cc10441; 10.1186/cc10441.

Gonzalez-Villoria, A. M. and Valverde-Garduno, V. (2016). Antibiotic-Resistant Acinetobacter baumannii Increasing Success Remains a Challenge as a Nosocomial Pathogen. *Journal of Pathogens, 2016*, 7318075.

Gordon, N. C. and Wareham, D. W. (2010). Multidrug-resistant acinetobacter baumannii: mechanisms of virulence and resistance. *International Journal of Antimicrobial Agents, 35*(3), 219-226.

Gounden, R., Bamford, C., van Zyl-Smit, R., Cohen, K., and Maartens, G. (2009). Safety and effectiveness of colistin compared with tobramycin for multidrug resistant Acinetobacter baumannii infections. *BMC Infectious Diseases,* 9:26. doi: 10.1186/1471-2334-9-26.

Grosso, F., Quinteira, S., and Peixe, L. (2011). Understanding the dynamics of imipenem-resistant Acinetobacter baumannii lineages within Portugal. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 17*(8), 1275-1279.

Güler, G., and Eraç, B. (2016). [Investigation of fluoroquinolone resistance mechanisms in clinical Acinetobacter baumannii isolates]. *Mikrobiyologi Bülteni, 50*(2), 278-286.

Gunn, J. S. (2008). The Salmonella PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. *Trends in Microbiology, 16*(6), 284–290.

Gur, D., Korten, V., Unal, S., Deshpande, L. M., and Castanheira, M. (2008). Increasing carbapenem resistance due to the clonal dissemination of oxacillinase (OXA-23 and OXA-58)-producing Acinetobacter baumannii: report from the Turkish SENTRY Program sites. *Journal of Medical Microbiology, 57*(Pt 12), 1529-1532.

Gurung, J., Khyriem, A. B., Banik, A., Lyngdoh, W. V., Choudhury, B., and Bhattacharyya, P. (2013). Association of biofilm production with multidrug resistance among clinical isolates of Acinetobacter baumannii and Pseudomonas aeruginosa from intensive care unit. *Indian Journal of Critical Care Medicine: Peer-Reviewed Official Publication of Indian Society of Critical Care Medicine, 17*(4), 214-218.

Gutierrez, B., Escudero, J. A., San Millan, A., Hidalgo, L., Carrilero, L., Ovejero, C. M., Santos-Lopez, A., Thomas-Lopez, D., and Gonzalez-Zorn, B. (2012). Fitness cost and

interference of Arm/Rmt aminoglycoside resistance with the RsmF housekeeping methyltransferases. *Antimicobial Agents and Chemetherapy, 56*(5), 2335-2341.

Hallal, A., Cohn, S. M., Namias, N., Habib, F., Baracco, G., Manning, R. J., Crookes, B., and Schulman, C. I. (2007). Aerosolized tobramycin in the treatment of ventilator-associated pneumonia: a pilot study. *Surgical Infections, 8*(1), 73-82.

Hammoudi, D., Moubareck, C. A., Hakime, N., Houmani, M., Barakat, A., Najjar, Z., Suleiman, M., Fayad, N., Sarraf, R., and Sarkis, D. K. (2015a). Spread of imipenem-resistant Acinetobacter baumannii co-expressing OXA-23 and GES-11 carbapenemases in Lebanon. *International Journal of Infectious Diseases: IJID: Official Publication of the International Society of Infectious Diseases, 36*(1), 56-61. doi: 10.1016/j.ijid.2015.05.015.

Hammoudi, D., Moubareck, C. A., Kanso, A., Nordmann, P., and Sarkis, D. K. (2015b). Surveillance of carbapenem non-susceptible gram negative strains and characterization of carbapenems of classes A, B and D in a Lebanese hospital. *The Lebanese Medical Journal, 63*(2), 66-73.

Hamouche, E., and Sarkis, D. K. (2012). [Evolution of susceptibility to antibiotics of Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Acinetobacter baumanii, in a University Hospital Center of Beirut between 2005 and 2009]. *Pathologie-Biologie (Paris), 60*(3), e15-20. doi: 10.1016/j.patbio.2011.03.011.

Hamouda, A., Evans, B. A., Towner, K. J., and Amyes, S. G. (2010). Characterization of epidemiologically unrelated Acinetobacter baumannii isolates from four continents by use of multilocus sequence typing, pulsed-field gel electrophoresis, and sequence-based typing of bla(OXA-51-like) genes. *Journal of Clinical Microbiology, 48*(7), 2476-2483.

Harris, S. R., Feil, E. J., Holden, M. T., Quail, M. A., Nickerson, E. K., Chantratita, N., Gardete, S., Tavares, A., Day, N., Lindsay, J. A., Edgeworth, J. D., de Lencastre, H., Parkhill, J., Peacock, S. J., and Bentley, S. D. (2010). Evolution of MRSA during hospital transmission and intercontinental spread. *Science (New York, N. Y.), 327*(5964), 469-474.

Harris, T. L., Worthington, R. J., Hittle, L. E., Zurawski, D. V., Ernst, R. L., and Melander, C. (2014). Small molecule downregulation of PmrAB reverses lipid A modification and breaks colistin resistance. *ACS Chemical Biology, 9*(1), 122-127.

Hawkey, P., and Finch, R. (2007). Tigecycline: in-vitro performance as a predictor of clinical efficacy. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 13*(4), 354-362.

Hayajneh, W. A., Hajj, A., Hulliel, F., Sarkis, D. K., Irani-Hakimeh, N., Kazan, L., and Badal, R. E. (2015). Susceptibility trends and molecular characterization of Gram-negative bacilli associated with urinary tract and intra-abdominal infections in Jordan and Lebanon: SMART 2011-2013. *International Journal of Infectious Diseases: IJID: Official Publication of the International Society of Infectious Diseases, 35*(1), 56-61. doi: 10.1016/j.ijid.2015.04.011.

He, C., Xie, Y., Fan, H., Kang, M., Tao, C., Zhang, R., Hu, Y., Chen, Z., and Wang, L. (2011). Spread of imipenem-resistant Acinetobacter baumannii of European clone II in Western China. *International Journal of Antimicrobial Agents, 38*(3), 257-260.

He, X., Lu, F., Yuan, F., Jiang, D., Zhao, P., Zhu, J., Cheng, H., Cao, J., and Lu G. (2015). Biofilm formation caused by clinical Acinetobacter baumannii isolates is associated

with overexpression of the AdeFGH efflux pump. *Antimicrobial Agents and Chemotherapy, 59*(8), 4817–4825.

Henriksen, S. D. (1973). Moraxella, Acinetobacter, and the Mimeae. *Bacteriological Reviews, 37*(4), 522–561.

Héritier, C., Dubouix, A., Poirel, L., Marty, N., and Nordmann, P. (2005b). A nosocomial outbreak of Acinetobacter baumannii isolates expressing the carbapenem-hydrolysing oxacillinase OXA-58. *The Journal of Antimicrobial Chemotherapy, 55*(1), 115-118.

Héritier, C., Poirel, L., and Nordmann, P. (2006). Cephalosporinase over-expression resulting from insertion of ISAba1 in Acinetobacter baumannii. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 12*(2), 123-130.

Héritier, C., Poirel, L., Fournier, P. E., Claverie, J. M., Raoult, D., and Nordmann P. (2005a). Characterization of the naturally occurring oxacillinase of Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 49*(10), 4174–4179.

Herrera, M. E., Mobilia, L. N., and Posse, G. R. (2011). [Comparative evaluation of the sensitivity of Acinetobacter to colistin, using the prediffusion and minimum inhibitory concentration methods: detection of heteroresistant isolates]. *Revista Argentina de Microbiologia, 43*(2), 115-119.

Hidron, A. I., Edwards, J. R., Patel, J., Horan, T. C., Sievert, D. M., Pollock, D. A., Fridkin, S. K., the National Healthcare Safety Network Team, and the Participating National Healthcare Safety Network Facilities. (2008). NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. *Infection Control and Hospital Epidemiology, 29*(11), 996-1101.

Higgins, P. G., Pérez-Llarena, F. J., Zander, E., Fernández, A., Bou, G., and Seifert, H. (2013). OXA-235, a novel class D β-lactamase involved in resistance to carbapenems in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 57*(5), 2121-2126.

Higgins, P. G., Poirel, L., Lehmann, M., Nordmann, P., and Seifert, H. (2009). OXA-143, a novel carbapenem-hydrolyzing class D beta-lactamase in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 53*(12), 5035-5038.

Hindler, J. A., and Humphries, R. M. (2013). Colistin MIC variability by method for contemporary clinical isolates of multidrug-resistant Gram-negative bacilli. *Journal of Clinical Microbiology, 51*(6), 1678-1684.

Ho, P. L., Ho, A. Y., Chow, K. H., Lai, E. L., Ching, P., and Seto, W. H. (2010). Epidemiology and clonality of multidrug-resistant Acinetobacter baumannii from a healthcare region in Hong Kong. *The Journal of Hospital Infection, 74*(4), 358-364.

Hong, S. B., Shin, K. S., Ha, J., and Han, K. (2013). Co-existence of blaOXA-23 and armA in multidrug-resistant Acinetobacter baumannii isolated from a hospital in South Korea. *Journal of Medical Microbiology, 62*(Pt 6), 8336-844.

Hood, M. I., Jacobs, A. C., Sayood, K., Dunman, P. M., and Skaar, E. P. (2010). Acinetobacter baumannii increases tolerance to antibiotics in response to monovalent cations. *Antimicrobial Agents and Chemotherapy, 54*(3), 1029-1041.

Hooper, D. C. Mode of action of fluoroquinolones. *Drugs, 58*(Supp.2), 6-10.

Horrevorts, A., Bergman, K., Kollee, L., Breuker, I., Tjernberg, I., and Dijkshoorn, L. (1995). Clinical and epidemiological investigations of Acinetobacter genomospecies 3 in a neonatal intensive care unit. *Journal of Clinical Microbiology, 33*(6), 1567–1572.

Houang, E. T., Chu, Y. W., Leung, C. M., Chu, J. K. Y., Berlau, K. C. Ng, and Cheng, A. F. (2001). Epidemiology and infection control implications of Acinetobacter spp. in Hong Kong. *Journal of Clinical Microbiology, 39*(1), 228–234.

Hraiech, S., Roch, A., Lepidi, H., Atieh, T., Audoly, G., Rolain, J. M., Raoult, D., Brunel, J. M., Papazian, L., and Brégeon, F. (2013). Impaired virulence and fitness of a colistin-resistant clinical isolate of Acinetobacter baumannii in a rat model of pneumonia. *Antimicrobial Agents and Chemotherapy, 57*(10), 5120-5121.

Hsueh, P. R., Teng, L. J., Yang, P. C., Chen, Y. C., Ho, S. W., and Luh, K. T. (1998). Persistence of a multidrug-resistant Pseudomonas aeruginosa clone in an intensive care burn unit. *Journal of Clinical Microbiology, 36*(5), 1347-1351.

Huang, L. Y., Chen, T. L., Lu, P. L., Tsai, C. A., Cho, W. L., Chang, F. Y., Fung, C. P., and Siu, L. K. (2008). Dissemination of multidrug-resistant, class 1 integron-carrying Acinetobacter baumannii isolates in Taiwan. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 14*(11), 1010-1019.

Huang, X. Z., Chahine, M. A., Frye, J. G., Cash, D. M., Lesho, E. P., Craft, D. W., Lindler, L. E., and Nikolich, M. P. (2012). Molecular analysis of imipenem-resistant Acinetobacter baumannii isolated from US service members wounded in Iraq, 2003-2008. *Epidemiology and Infection, 140*(12), 2302-2307.

Huang, Y. S., Wang, J. T., Sheng, W. H., Chuang, Y. C., and Chang, S. C. (2015). Comparative in vitro activity of sitafloxacin against bacteremic isolates of carbapenem resistant Acinetobacter baumannii complex. *Journal of Immunology, Microbiology, and Infection, 48*(5), 545-551.

Hughes, J., Goldenberg, S. D., Tosas, O., Edgeworth, J. D., and Otter, J. A. (2016). Recent emergence of carbapenem-resistant organisms in a low prevalence UK setting in London. Journal of Infection Prevention, 17(3), 130-134.

Hujer, K. M., Hamza, N. S., Hujer, A. M., Perez, F., Helfand, M. S., Bethel, C. R., Thomson, J. M., Anderson, V. E., Barlow, M., Rice, L. B., Tenover, F. C., and Bonomo, R. A. (2005). Identification of a new allelic variant of the Acinetobacter baumannii cephalosporinase, ADC-7 β-lactamase: defining a unique family of class C enzymes. *Antimicrobial Agents and Chemotherapy, 49*(7), 2941–2948.

Hujer, K. M., Hujer, A. M., Hulten, E. A., Bajaksouzian, S., Adams, J. M., Donskey, C. J., Ecker, D. J., Massire, C., Eshoo, M. W., Sampath, R., Thomson, J. M., Rather, P. N., Craft, D. W., Fishbain, J. T., Ewell, A. J., Jacobs, M. R., Paterson, D. L., and Bonomo R. A. (2006). Analysis of antibiotic resistance genes in multidrug-resistant Acinetobacter sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. *Antimicrobial Agents and Chemotherapy, 50*(12), 4114-4123.

Huys, G., Cnockaert, M., Nemec, A., Dijkshoorn, L., Brisse, S., Vaneechoutte, M., and Swings, J. (2005). Repetitive-DNA-element PCR fingerprinting and antibiotic resistance of pan-European multi-resistant Acinetobacter baumannii clone III strains. *Journal of Medical Microbiology, 54*(Pt 9), 851-856.

Inweregbu, K., Dave, J., and Pittard, A. (2005). Nosocomial infections. *Continuing Education in Anesthesia, Critical Care, and Pain, 5*(1), 14-17.

Jacobs, A. C., Hood, I., Boyd, K. L., Olson, P. D., Morrison, J. M., Carson, S., Sayood, K., Iwen, P. C., Skaar, E. P., and Dunman, P. M. (2010) Inactivation of phospholipase D diminishes Acinetobacter baumannii pathogenesis. *Infection and Immunity, 78*(5), 1952–1962.

Janssen, P., Maquelin, K., Coopman, R., Tjernberg, I., Bouvet, P., Kersters, K., and Dijkshoorn, L. (1997). Discrimination of Acinetobacter genomic species by AFLP fingerprinting. *International Journal of Systemic Bacteriology, 47*(4), 1179–1187.

Ji, S., Chen, Y., Ruan, Z., Fu, Y., Ji, J., Fu, Y., Wang, H., and Yu, Y. (2014). Prevalence of carbapenem-hydrolyzing class D β-lactamase genes in Acinetobacter spp. isolates in China. *European Journal of Clinical Microbiology and Infectious Diseases: Official Publication of the European Society of Clinical Microbiology, 33*(6), 989-997.

Jones, C. L, Clancy, M., Honnold, C., Singh, S., Snesrud, E., Onmus-Leone, F., McGann, P., Ong, A. C., Kwak, Y., Waterman, P., Zurawski, D. V., Clifford, R. J., and Lesho, E. (2015). Fatal outbreak of an emerging clone of extensively drug-resistant Acinetobacter baumannii with enhanced virulence. *Clinical Infectious Diseases: an Official Publication of the Infectious Diseases Society of America, 61*(2), 145-154.

Joseleau-Petit, D., Thévenet, D., and D'Ari, R. (1994). ppGpp concentration, growth without PBP2 activity, and growth-rate control in Escherichia coli. *Molecular Microbiology, 13*(5), 911-917.

Kaase, M., Nordmann, P., Wichelhaus, T. A., Gatermann, S. G., Bonnin, R. A., and Poirel, L. (2011). NDM-2 carbapenemase in Acinetobacter baumannii from Egypt. *The Journal of Antimicrobial Chemotherapy, 66*(6), 1260-1262.

Kanamori, H., Parobek, C. M., Weber, D. J., van Duin, D., Rutala, W. A., Cairns, B. A, and Juliano, J. J. (2015). Next-Generation Sequencing and Comparative Analysis of Sequential Outbreaks Caused by Multidrug-Resistant Acinetobacter baumannii at a Large Academic Burn Center. *Antimicrobial Agents and Chemotherapy, 60*(3), 1249-1257.

Kanj, S. S., Whitelaw, A., and Dowzicky, M. J. (2014). In vitro activity of tigecycline and comparators against Gram-positive and Gram-negative isolates collected from the Middle East and Africa between 2004 and 2011. *International Journal of Antimicrobial Agents, 43*(2), 170-178.

Kansakar, P., Dorji, D., Chongtrakool, P., Mingmongkolchai, S., Mokmake, B., and Dubbs, P. (2011). Local dissemination of multidrug-resistant Acinetobacter baumannii clones in a Thai hospital. *Microbial Drug Resistance (Larchmont, N. Y.), 17*(1), 109-119.

Karageorgopoulos, D. E., and Falagas, M. E. (2008). Current control and treatment of multidrug-resistant Acinetobacter baumannii infections. *The Lancet. Infectious Diseases, 8*(12), 751-762.

Karah, N., Dwibedi, C. K., Sjöström, K., Edquist, P., Johansson, A., Wai, S. N., and Uhlin, B. E. (2016). Novel Aminoglycoside Resistance Transposons and Transposon-Derived Circular Forms Detected in Carbapenem-Resistant Acinetobacter baumannii Clinical Isolates. *Antimicrobial Agents and Chemoterapy, 60*(3), 1801-1818.

Karah, N., Giske, C. G., Sundsfjord, A., and Samuelsen, Ø. (2011b). A diversity of OXA-carbapenemases and class 1 integrons among carbapenem-resistant Acinetobacter baumannii clinical isolates from Sweden belonging to different international clonal lineages. *Microbial Drug Resistance (Larchmont, N. Y.), 17*(4), 545-549.

Karah, N., Haldorsen, B., Hermansen, N. O., Tveten, Y., Ragnhildstveit, E., Skutlaberg, D. H., Tofteland, S., Sundsfjord, A., and Samuelsen, Ø. (2011a). Emergence of OXA-carbapenemase- and 16S rRNA methylase-producing international clones of Acinetobacter baumannii in Norway. *Journal of Medical Microbiology, 60*(Pt 4), 515-521.

Karah, N., Samuelsen, Ø., Zarrilli, R., Sahl, J. W., Wai, S. N., and Uhlin, B. E. (2015). CRISPR-cas subtype I-Fb in Acinetobacter baumannii: evolution and utilization for strain subtyping. *PLoS One, 10*(2), e0118205. doi: 10.1371/journal.pone.0118205.

Karah, N., Sundsfjord, A., Towner, K., and Samuelsen, Ø. (2012). Insights into the global molecular epidemiology of carbapenem non-susceptible clones of Acinetobacter baumannii. *Drug Resistance Updates: Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy, 15*(4), 237-247.

Karaiskos, I., Galani, L., Baziaka, F., and Giamarellou, H. (2013). Intraventricular and intrathecal colistin as the last therapeutic resort for the treatment of multidrug-resistant and extensively drug-resistant Acinetobacter baumannii ventriculitis and meningitis: a literature review. *International Journal of Antimicrobial Agents, 41*(6), 499–508.

Karlowsky, J., and Richter, S. (2015). Antimicrobial Susceptibility Testing Systems*, p 1274-1285. In Jorgensen, J., Pfaller, M., Carroll, K., Funke, G., Landry, M., Richter, S., and Warnock, D. (ed), Manual of Clinical Microbiology, Eleventh Edition. *ASM Press, Washington, DC*, doi: 10.1128/9781555817381.ch72.

Karthikeyan, K., Thirunarayan, M. A., and Krishnan, P. (2010). Coexistence of blaOXA-23 with blaNDM-1 and armA in clinical isolates of Acinetobacter baumannii from India. *The Journal of Antimicrobial Chemotherapy, 65*(10), 2253-2254.

Kavanaugh, N. L., and Ribbeck, K. (2012). Selected antimicrobial essential oils eradicate Pseudomonas spp. and Staphylococcus aureus biofilms. *Applied and Environmental Microbiology, 78*(11), 4057-4061.

Kempf, M., Bakour, S., Flaudrops, C., Berrazeg, M., Brunel, J. M., Drissi, M., Mesli, E., Touati, A., and Rolain, J. M. (2012a). Rapid detection of carbapenem resistance in Acinetobacter baumannii using matrix-assisted laser desorption ionization-time of flight mass spectrometry. *PLoS One, 7*(2), e31676. doi: 10.1371/journal.pone.0031676.

Kempf, M., Rolain, J. M., Azza, S., Diene, S., Joly-Guillou, M. L., Dubourg, G., Colson, P., Papazian, L., Richet, H., Fournier, P. E., Ribeiro, A., and Raoult, D. (2013). Investigation of Acinetobacter baumannii resistance to carbapenems in Marseille hospitals, south of France: a transition from an epidemic to an endemic situation. *APMIS: Acta Pathologica, Microbiologica, et Immunologica Scandinavica, 121*(1), 64-71.

Kempf, M., Rolain, J. M., Diatta, G., Azza, S., Samb, B., Mediannikov, O., Gassama Sow, A., Diene, S. M., Fenollar, F., and Raoult, D. (2012b). Carbapenem resistance and Acinetobacter baumannii in Senegal: the paradigm of a common phenomenon in natural reservoirs. *PLoS One, 7*(6), e39495. doi: 10.1371/journal.pone.0039495.

Keyik, S., Arslan, U., Türk Dağı, H., Seyhan, T., and Fındık, D. (2014). [Investigation of OXA type beta-lactamases and PFGE patterns in Acinetobacter baumannii strains resistant to carbapenems]. *Mikrobiyologi Bülteni, 48*(4), 556-565.

Kim, C. K., Lee, Y., Lee, H., Woo, G. J., Song, W., Kim, M. N., Lee, W. G., Jeong, S. H., Lee, K., and Chong, Y. (2010). Prevalence and diversity of carbapenemases among

imipenem-nonsusceptible Acinetobacter isolates in Korea: emergence of a novel OXA-182. *Diagnostic Microbiology and Infectious Diseases, 68*(4), 432-438.

Kim, N. H., Hwang, J. H., Song, K. H., Choe, P. G., Kim, E. S., Park, S. W., Kim, H. B., Kim, N. J., Park, W. B., and Oh, M. D. (2013). Tigecycline in carbapenem-resistant Acinetobacter baumannii bacteraemia: susceptibility and clinical outcome. *Scandinavian Journal of Infectious Diseases, 45*(4), 315– 319.

Kim, Y., Bae, I. K., Lee, H., Jeong, S. H., Yong, D., and Lee, K. (2014). In vivo emergence of colistin resistance in Acinetobacter baumannii clinical isolates of sequence type 357 during colistin treatment. *Diagnostic Microbiology and Infectious Disease, 79*(3), 362-366.

Kishii, K., Kikuchi, K., Yoshida, A., Okuzumi, K., Uetera, Y., Yasuhara, H., and Moriya, K. (2014). Antimicrobial susceptibility profile of Acinetobacter species isolated from blood cultures in two Japanese university hospitals. *Microbiology and Immunology, 58*(2), 142-146.

Kjeldsen, M. K., Torpdahl, M., Pedersen, K., and Nielsen, E. M. (2015). Development and comparison of a generic multiple-locus variable-number tandem repeat analysis with pulsed-field gel electrophoresis for typing of Salmonella enterica subsp. enterica. *Journal of Applied Microbiology, 119*(6), 1707-1717.

Ko, K. S., Suh, J. Y., Kwon, K. T., Jung, S. I., Park, K. H., Kang, C. I., Chung, D. R., Peck, K. R., and Song, J. H. (2007). High rates of resistance to colistin and polymyxin B in subgroups of Acinetobacter baumannii isolates from Korea. *The Journal of Antimicrobial Chemotherapy, 60*(5), 1163-1167.

Koh, T. H., Sng, L. H., Wang, G. C., Hsu, L. Y., and Zhao, Y. (2007). IMP-4 and OXA beta-lactamases in Acinetobacter baumannii from Singapore. *The Journal of Antimicrobial Chemotherapy, 59*(4), 627-632.

Kolayli, F., Gacar, G., Karadenizli, A., Sanic, A., Vahaboglu, H., and Study Group. (2005). PER-1 is still widespread in Turkish hospitals among Pseudomonas aeruginosa and Acinetobacter spp. *FEMS Microbiology Letters, 249*(2), 241-245.

Kong, B. H., Hanifah, Y. A., Yusof, M. Y., and Thong, K. L. (2011). Application of amplified ribosomal DNA restriction analysis in identification of Acinetobacter baumannii from a tertiary teaching hospital, Malaysia. *Tropical Biomedicine, 28*(3), 563-568.

Krizova, L., Bonnin, R. A., Nordmann, P., Nemec, A., and Poirel L. (2012). Characterization of a multidrug-resistant Acinetobacter baumannii strain carrying the blaNDM-1 and blaOXA-23 carbapenemase genes from the Czech Republic. *The Journal of Antimicrobial Chemotherapy, 67*(6), 1550-1552.

Kroeger, L. A., Hovde, L. B., Mitropoulos, I. F., Schafer, J. and Rotschafer, J. C. (2007). Colistin methanesulfonate against multidrug-resistant Acinetobacter baumannii in an in vitro pharmacodynamic model. *Antimicrobial Agents and Chemotherapy, 51*(9), 3431–3433.

Kwong, J. C., McCallum, N., Sintchenko, V., and Howden, B. P. (2015). Whole genome sequencing in clinical and public health microbiology. *Pathology, 47*(3), 199-210.

La Scola, B., and Raoult. D. (2004). Acinetobacter baumannii in human body louse. *Emerging Infectious Diseases, 10*(9), 1671–1673.

La Scola, B., Gundi, V. A. K. B., Khamis, A., and Raoult, D. (2006). Sequencing of the rpoB gene and flanking spacers for molecular identification of Acinetobacter species. *Journal of Clinical Microbiology*, *44*(3), 827-832.

Labarca, J. A., Salles, M. J., Seas, C., and Guzmán-Blanco, M. (2016). Carbapenem resistance in Pseudomonas aeruginosa and Acinetobacter baumannii in the nosocomial setting in Latin America. *Critical Reviews in Microbiology*, *42*(2), 276-292.

Lautrop, H. (1974). Bergey's manual of determinative bacteriology. Williams & Wilkins Co., Baltimore, MD.

Lavigne, J. P., Vitrac, X., Bernard, L., Bruyère, F., and Sotto, A. (2011). Propolis can potentialise the anti-adhesion activity of proanthocyanidins on uropathogenic Escherichia coli in the prevention of recurrent urinary tract infections. *BMC Research Notes*, *4*(1), 522. doi: 10.1186/1756-0500-4-522.

Lean, S. S., Suhaili, Z., Ismail, S., Rahman, N. I., Othman, N., Abdullah, F. H., Jusoh, Z., Yeo, C. C., and Thong, K. L. (2014). Prevalence and Genetic Characterization of Carbapenem- and Polymyxin-Resistant Acinetobacter baumannii Isolated from a Tertiary Hospital in Terengganu, Malaysia. *ISRN Microbiology, 2014*, 953417. doi: 10.1155/2014/953417.

Lebeaux D, Ghigo J-M & Beloin C (2014) Biofilm-Related Infections: Bridging the Gap between Clinical Management and Fundamental Aspects of Recalcitrance toward Antibiotics. Microbiol. Mol. Biol. Rev. 78 (3) 510-543.

Lee, H. W., Koh, Y. M., Kim, J., Lee, J. C., Lee, Y. C., Seol, S. Y., Cho, D. T., and Kim J. (2008). Capacity of multidrug-resistant clinical isolates of Acinetobacter baumannii to form biofilm and adhere to epithelial cell surfaces. *Clinical Microbiology and Infections: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *14*(1), 49–54.

Lee, K., Kim, M. N., Choi, T. Y., Cho, S. E., Lee, S., Whang, D. H., Yong, D., Chong, Y., Woodford, N., Livermore, D. M., and KONSAR Group. (2009). Wide dissemination of OXA-type carbapenemases in clinical Acinetobacter spp. isolates from South Korea. *International Journal of Antimicrobial Agents*, *33*(6), 520-524.

Lee, K., Lee, W. G., Uh, Y., Ha, G. Y., Cho, J., Chong, Y. and the Korean Nationwide Surveillance of Antimicrobial Resistance Group. (2003). VIM- and IMP-type metallo-beta-lactamase-producing Pseudomonas spp. and Acinetobacter spp. in Korean hospitals. *Emerging Infectious Diseases*, *9*(7), 868-871.

Lee, K., Yum, J. H., Yong, D., Lee, H. M., Kim, H. D., Docquier, J. D., Rossolini, G. M., and Chong, Y. (2005). Novel acquired metallo-beta-lactamase gene, bla(SIM-1), in a class 1 integron from Acinetobacter baumannii clinical isolates from Korea. *Antimicrobial Agents and Chemotherapy*, *49*(11), 4485-4491.

Lee, N. Y., Wang, C. L., Chuang, Y. C., Yu, W. L., Lee, H. C., Chang, C. M., Wang, L. R., and Ko, W. C. (2007). Combination carbapenem-sulbactam therapy for critically ill patients with multidrug-resistant Acinetobacter baumannii bacteremia: four case reports and an in vitro combination synergy study. *Pharmacotherapy*, *27*(11), 1506-1511.

Lee, Y., Lee, J., Jeong, S. H., Lee, J., Bae, I. K., and Lee, K. (2011). Carbapenem-non-susceptible Acinetobacter baumannii of sequence type 92 or its single-locus variants with a G428T substitution in zone 2 of the rpoB gene. *The Journal of Antimicrobial Chemotherapy*, *66*(1), 66-72.

Lee, Y., Yum, J. H., Kim, C. K., Yong, D., Jeon, E..H., Jeong, S. H., Ahn, J. Y., and Lee, K. (2010). Role of OXA-23 and AdeABC efflux pump for acquiring carbapenem resistance in an Acinetobacter baumannii strain carrying the blaOXA-66 gene. *Annals of Clinical and Laboratory Science, 40*(1), 43-48.

Leite, G. C., Oliveira, M. S., Perdigão-Neto, L. V., Rocha, C. K., Guimarães, T., Rizek, C., Levin, A. S., and Costa, S. F. (2016). Antimicrobial Combinations against Pan-Resistant Acinetobacter baumannii Isolates with Different Resistance Mechanisms. *PLoS One, 11*(3), e0151270. doi: 10.1371/journal.pone.0151270.

Lesho, E., Yoon, E. J.., McGann, P., Snesrud, E., Kwak, Y., Milillo, M., Onmus-Leone, F., Preston, L., St. Claire, K., Nikolich, M., Viscount, H., Wortmann, G., Zapor, M., Grillot-Courvalin, C., Courvalin, P., Clifford, R., and Waterman, P. E. (2013). Emergence of colistin resistance in extremely drug resistant Acinetobacter baumannii containing a novel pmrCAB operon during colistin therapy of wound infections. *The Journal of Infectious Diseases, 208*(7), 1142-1151.

Lessel, E. F. (1971). Subcommittee on nomenclature of Moraxella and allied bacteria. *International Journal of Systemic Bacteriology, 21*(1), 213–214.

Levy, J., Oshry, T., Rabinowitz, R., and Lifshitz, T. (2005). Acinetobacter corneal graft ulcer and endophthalmitis: report of two cases. *Canadian Journal of Ophthalmology, 40*(1), 79–82.

Li, J., Rayner, C. R., Nation, R. L., Owen, R. J., Spelman, D., Tan, K. E., and Liolios, L. (2006). Heteroresistance to colistin in multidrug-resistant Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 50*(9), 2946–2950.

Li, W., Atkinson, G. C., Thakor, N. S., Allas, U., Lu, C. C., Chan, K. Y., Tenson, T., Schulten, K., Wilson, K. S., Hauryliuk, V., and Frank, J. (2013). Mechanism of tetracycline resistance by ribosomal protection protein Tet(O). *Nature Communications, 4*(1), 1477. doi: 10.1038/ncomms2470.

Liakopoulos, A., Miriagou, V., Katsifas, E. A., Karagouni, A. D., Daikos, G. L., Tzouvelekis, L. S., and Petinaki, E. (2012). Identification of OXA-23-producing Acinetobacter baumannii in Greece, 2010 to 2011. *Euro Surveillance: European Communicable Disease Bulletin, 17*(11), pii: 20117.

Liou, G. F., Yoshizawa, S., Courvalin, P., and Galimand, M. (2006). Aminoglycoside resistance by ArmA-mediated ribosomal 16S methylation in human bacterial pathogens. *Journal of Molecular Biology, 359*(2), 358–364.

Liou, M. L., Soo, P. C., Ling, S. R., Kuo, H. Y., Tang, C. Y., and Kai-Chih Chang, K. C. (2014). The sensor kinase BfmS mediates virulence in Acinetobacter baumannii. *Journal of Microbiology, Immunology and Infection, 47*(4), 275-281.

Litake, G. M., Ghole, V. S., Niphadkar, K. B., and Joshi, S. G. (2009). PER-1-type extended-spectrum beta-lactamase-producing Acinetobacter baumannii clinical isolates from India. *International Journal of Antimicrobial Agents, 34*(4), 388-389.

Liu, X., Yin, H., Weng, C. X., and Cai, Y. (2016). Low-Frequency Ultrasound Enhances Antimicrobial Activity of Colistin-Vancomycin Combination against Pan-Resistant Biofilm of Acinetobacter baumannii. *Ultrasound in Medicine and Biology, 42*(8), 1968-1975.

Livermore, D. M. (2005). Tigecycline: what is it, and where should it be used? *Journal of Antimicrobial Chemotherapy, 56*(4), 611–614.

Livermore, D. M.; British Society for Antimicrobial Chemotherapy Working Party on The Urgent Need: Regenerating Antibacterial Drug Discovery and Development. (2011). Discovery research: the scientific challenge of finding new antibiotics. *The Journal of Antimicrobial Chemotherapy, 66*(9), 1941-1944.

Lob, S. H., Hoban, D. J., Sahm, D. F., and Badal, R. E. (2016). Regional differences and trends in antimicrobial susceptibility of Acinetobacter baumannii. *International Journal of Antimicrobial Agents, 74*(4), 317-323.

Loehfelm, T. W., Luke, N. R., and Campagnari, A. A. (2008). Identification and characterization of an Acinetobacter baumannii biofilm-associated protein. *Journal of Bacteriology, 190*(3), 1036–1044.

Lolans, K., Rice, T. W., Munoz-Price, L. S., and Quinn, J. P. (2006). Multicity outbreak of carbapenem-resistant Acinetobacter baumannii isolates producing the carbapenemase OXA-40. *Antimicrobial Agents and Chemotherapy, 50*(9), 2941-2945.

Longo, F., Vuotto, C., and Donelli, G. (2014). Biofilm formation in Acinetobacter baumannii. *The New Microbiologica, 37*(2), 119-127.

Loong, S. K., Khor, C. S., Jafar, F. L., and AbuBakar, S. (2016). Utility of 16S rDNA Sequencing for Identification of Rare Pathogenic Bacteria. *Journal of Clinical Laboratory Analysis,* doi: 10.1002/jcla.21980.

Lopes, B. S., Al-Hassan, L., and Amyes, S. G. (2012). ISAba825 controls the expression of the chromosomal bla(OXA-51-like) and the plasmid borne bla(OXA-58) gene in clinical isolates of Acinetobacter baumannii isolated from the USA. *Clinical Microbiology and Infection: the Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 18*(11), E446-E451.

López-Cortés, L. E., Cisneros, J. M., Fernández-Cuenca, F., Bou, G., Tomás, M., Garnacho-Montero, J., Pascual, A., Martínez-Martínez, L., Vila, J., Pachón, J., Rodríguez Baño, J. and the GEIH/REIPI-Ab2010 Group. (2014). Monotherapy versus combination therapy for sepsis due to multidrug-resistant Acinetobacter baumannii: analysis of a multicenter prospective cohort. *Journal of Antimicrobial Chemotherapy, 69*(11), 3119–3126.

López-Rojas, R., Jiménez-Mejías, M. E., Lepe, J. A., and Pachón J. (2011). Impaired virulence and in vivo fitness of colistin-resistant Acinetobacter baumannii. *The Journal of Infectious Diseases, 203*(4), 545-548.

López-Rojas, R., McConnell, M. J., Jiménez-Mejías, M. E., Domínguez-Herrera, J., Fernández-Cuenca, F., and Pachón, J. (2013). Colistin resistance in a clinical Acinetobacter baumannii strain appearing after colistin treatment: effect on virulence and bacterial fitness. *Antimicrobial Agents and Chemotherapy, 57*(9), 4587-4589.

Lowings, M., Ehlers, M. M., Dreyer, A. W., and Kock, M. M. (2015). High prevalence of oxacillinases in clinical multidrug-resistant Acinetobacter baumannii isolates from the Tshwane region, South Africa - an update. *BMC Infectious Diseases, 15*(1), 521. doi: 10.1186/s12879-015-1246-8.

Lu, J. J., Perng, C. L., Lee, S. Y., and Wan, C. C. (2000). Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. *Journal of Clinical Microbiology, 38*(6), 2076–2080.

Lu, P. L., Doumith, M., Livermore, D. M., Chen, T. P., and Woodford, N. (2009). Diversity of carbapenem resistance mechanisms in Acinetobacter baumannii from a Taiwan

hospital: spread of plasmid-borne OXA-72 carbapenemase. *The Journal of Antimicrobial Chemotherapy, 63*(4), 641-647.

Luke, N. R., Sauberan, S. L., Russo, T. A., Beanan, J. M., Olson, R., Loehfelm, T. W., Cox, A. D., St Michael, F., Vinogradov, E. V., and Campagnari, A. A. (2010) Identification and characterization of a glycosyltransferase involved in Acinetobacter baumannii lipopolysaccharide core biosynthesis. *Infection and Immunity, 78*(5), 2017–2023.

Maeda, T., García-Contreras, R., Pu, M., Sheng, L., Garcia, L. R., Tomás, M., and Wood, T. K. (2012). Quorum quenching quandary: resistance to antivirulence compounds. *The ISME Journal, 6*(3), 493-501.

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T., and Monnet, D. L. (2011). Multidrug resistant, extensively drug resistant, and pan drug resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection, 18*(3), 268-281.

Magnet, S., Courvalin, P., and Lambert, T. (2001). Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in Acinetobacter baumannii strain BM4454. *Antimicrobial Agents and Chemotherapy, 45*(12), 3375-3380.

Mammina, C., Bonura, C., Aleo, A., Calà, C., Caputo, G., Cataldo, M. C., Di Benedetto, A., Distefano, S., Fasciana, T., Labisi, M., Sodano, C., Palma, D. M., and Giammanco, A. (2011). Characterization of Acinetobacter baumannii from intensive care units and home care patients in Palermo, Italy. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 17*(11), E12-E15.

Manageiro, V., Jones-Dias, D., Ferreira, E., Louro, D., Antimicrobial Resistance Surveillance Program in Portugal, and Caniça, M. (2012). Genetic diversity and clonal evolution of carbapenem-resistant Acinetobacter baumannii isolates from Portugal and the dissemination of ST118. *International Journal of Antimicrobial Agents, 40*(5), 398-403.

Martínez, J. L., and Baquero, F. (2002). Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. *Clinical Microbiology Reviews, 15*(4), 647-679.

Martins, N., Dalla-Costa, L., Uehara, A. A., Riley, L. W., and Moreira, B. M. (2013). Emergence of Acinetobacter baumannii international clone II in Brazil: reflection of a global expansion. *Infection, Genetics, and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases, 20*(1), 378-380. doi: 10.1016/j.meegid.2013.09.028.

Martins, N., Picão, R. C., Cerqueira-Alves, M., Uehara, A., Barbosa, L. C., Riley, L. W., and Moreira, B. M. (2016). A new trilocus sequence-based multiplex-PCR to detect major Acinetobacter baumannii clones. *Infection, Genetics, and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases, 42*(1), 41-45. doi: 10.1016/j.meegid.2016.04.031.

Matar, G. M., Gay, E., Cooksey, R. C., Elliott, J. A., Heneine, W. M., Uwaydah, M. M., Matossian, R. M., and Tenover, F. C. (1992). Identification of an epidemic strain of Acinetobacter baumannii using electrophoretic typing methods. *European Journal of Epidemiology, 8*(1), 9-14.

Mataseje, L. F., Bryce, E., Roscoe, D., Boyd, D. A., Embree, J., Gravel, D., Katz, K., Kibsey, P., Kuhn, M., Mounchili, A., Simor, A., Taylor, G., Thomas, E., Turgeon, N., Mulvey, M. R., and Canadian Nosocomial Infection Surveillance Program (2012). Carbapenem-resistant gram negative bacilli in Canada 2009-10: results from the Canadian Nosocomial Infection Surveillance Program (CNISP). *The Journal of Antimicrobial Chemotherapy, 67*(6), 1359-1367.

Mbwana, J., Bölin, I., Lyamuya, E., Mhalu, F., and Lagergård, T. (2006). Molecular characterization of Haemophilus ducreyi isolates from different geographical locations. *Journal of Clinical Microbiology, 44*(1), 132-137.

McConnell, M. J., Actis, L., and Pachón, J. (2013). Acinetobacter baumannii: human infections, factors contributing to pathogenesis and animal models. *FEMS Microbiology Reviews, 37*(2), 130-155.

McDonald, L. C., Banerjee, S. N., and Jarvis, W. R. (1999). Seasonal variation of Acinetobacter infections: 1987–1996 Nosocomial Infections Surveillance System. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 29*(5), 1133– 1137.

Mehrad, B., Clark, N. M., Zhanel, G. G., and Lynch, J. P. 3rd. (2015). Antimicrobial resistance in hospital-acquired gram-negative bacterial infections. *Chest, 147*(5), 1413-1421.

Merino, M., Poza, M., Roca, I., Barba, M. J., Sousa, M. D., Vila, J., and Bou, G. (2014). Nosocomial outbreak of a multiresistant Acinetobacter baumannii expressing OXA-23 carbapenemase in Spain. *Microbial Drug Resistance (Larchmont, N. Y.), 20*(4), 259-263.

Metan, G., Sariguzel, F., Sumerkan, B., Reijden, Tv., and Dijkshoorn, L. (2013). Clonal diversity and high prevalence of OXA-58 among Acinetobacter baumannii isolates from blood cultures in a tertiary care centre in Turkey. *Infection, Genetics, and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases, 14*(1), 92-97. 10.1016/j.meegid.2012.11.003.

Mezzatesta, M. L., D'Andrea, M. M., Migliavacca, R., Giani, T., Gona, F., Nucleo, E., Fugazza, G., Pagani, L., Rossolini, G. M., and Stefani, S. (2012). Epidemiological characterization and distribution of carbapenem-resistant Acinetobacter baumannii clinical isolates in Italy. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 18*(2), 160-166.

Mihara, K., Tanabe, T., Yamakawa, Y., Funahashi, T., Nakao, H., Narimatsu, S., and Yamamoto, S. (2004). Identification and transcriptional organization of a gene cluster involved in biosynthesis and transport of acinetobactin, a siderophore produced by Acinetobacter baumannii ATCC 19606T. *Microbiology, 150*(Pt 8), 2587–2597.

Milan, A., Furlanis, L., Cian, F., Bressan, R., Luzzati, R., Lagatolla, C., Deiana, M. L., Knezevich, A., Tonin, E., and Dolzani, L. (2016). Epidemic Dissemination of a Carbapenem-Resistant Acinetobacter baumannii Clone Carrying armA Two Years After Its First Isolation in an Italian Hospital. *Microbial Drug Resistance*, Epub Ahead of Print.

Minandri, F., D'Arezzo, S., Antunes, L. C., Pourcel, C., Principe, L., Petrosillo, N., and Visca, P. (2012). Evidence of diversity among epidemiologically related carbapenemase-producing Acinetobacter baumannii strains belonging to international clonal lineage II. *Journal of Clinical Microbiology*, *50*(3), 590-597.

Mingeot-Leclercq, M. P., Glupczynski, Y., and Tulkens, P. M. (1999). Aminoglycosides: activity and resistance. *Antimicrobial Agents and Chemotherapy, 43*(4), 727–37.

Moffatt, J. H., Harper, M., Harrison, P., Hale, J. D. F., Vinogradov, E., Seemann, T., Henry, R., Crane, B., St. Michael, F., Cox, A. D., Adler, B., Nation, R. L., Li, J., and Boyce, J. D. (2010). Colistin resistance in Acinetobacter baumannii is mediated by complete loss of lipopolysaccharide production. *Antimicrobial Agents and Chemotherapy, 54*(12), 4971-4977.

Moghnieh, R., Siblani, L., Ghadban, D., El Mchad, H., Zeineddine, R., Abdallah, D., Ziade, F., Sinno, L., Kiwan, O., Kerbaj, F., and El Imad, Z. (2016). Extensively drug-resistant Acinetobacter baumannii in a Lebanese intensive care unit: risk factors for acquisition and determination of a colonization score. *The Journal of Hospital Infection, 92*(1), 47-53.

Mohamed, A. F., Karaiskos, I., Plachouras, D., Karvanen, M., Pontikis, K., Jansson, B., Papadomichelakis, E., Antoniadou, A., Giamarellou, H., Armaganidis, A., Cars, O., and Friberg, L. E. (2012). Application of a loading dose of colistin methanesulfonate in critically ill patients: population pharmacokinetics, protein binding, and prediction of bacterial kill. *Antimicrobial Agents and Chemotherapy, 56*(8), 4241–4249.

Moniri, R., Farahani, R. K., Shajari, G., Shirazi, M. N., and Ghasemi, A. (2010). Molecular epidemiology of aminoglycosides resistance in acinetobacter spp. With emergence of multidrug-resistant strains. *Iranian Journal of Public Health, 39*(2), 63-68.

Montealegre, M. C., Maya, J. J., Correa, A., Espinal, P., Mojica, M. F., Ruiz, S. J., Rosso, F., Vila, J., Quinn, J. P., and Villegas, M. V. (2012). First identification of OXA-72 carbapenemase from Acinetobacter pittii in Colombia. *Antimicrobial Agents and Chemotherapy, 56*(7), 3996-3998.

Montero, J. G., Lerma, F. Á., Galleymore, P. R., Martínez, M. P., Rocha, L. Á., Gaite, F. B., Rodríguez, J. Á., González, M. C., Moreno, I. F., Baño, J. R., Campos, J., Andrés, J. M., Varela, Y. A., Gay, C. R., García, M. S., and Scientific Expert Committee for Zero Resistance Project. (2015). Combatting resistance in intensive care: the multimodal approach of the Spanish ICU "Zero Resistance" program. *Critical Care (London, England), 19*(1), 114. doi: 10.1186/s13054-015-0800-5.

Monterrubio-Villar, J., González-Velasco, C., Valdezate-Ramos, S., Córdoba-López, A., Villalón-Panzano, P., and Saéz-Nieto, J. A. (2009). Outbreak of multiresistant Acinetobacter baumannii in a polyvalent intensive care unit: clinical, epidemiological analysis and PFGE-printing evolution. *European Journal of Microbiology and Infectious Diseases: Official Publication of the European Society of Clinical Microbiology, 28*(10), 1281-1284.

Moran, A. P. (2007). Lipopolysaccharide in bacterial chronic infection: insights from Helicobacter pylori lipopolysaccharide and lipid A. *International Journal of Medical Microbiology: IJMM, 297*(5), 307–319.

Morosini, M. I., Ayala, J. A., Baquero, F., Martínez, J. L., and Blázquez, J. (2000). Biological cost of AmpC production for Salmonella enterica serotype Typhimurium. *Antimicrobial Agents and Chemotherapy, 44*(11), 3137-3143.

Moskowitz, S. M., Ernst, R. K., and Miller, S. I. (2004). PmrAB, a two-component regulatory system of Pseudomonas aeruginosa that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. *Journal of Bacteriology, 186*(2), 575–579.

Mosqueda, N., Espinal, P., Cosgaya, C., Viota, S., Plasensia, V., Alvarez-Lerma, F., Montero, M., Gómez, J., Horcajada, J. P., Vila, J., and Roca, I. (2013). Globally expanding carbapenemase finally appears in Spain: nosocomial outbreak of acinetobacter baumannii producing plasmid-encoded OXA-23 in Barcelona, Spain. *Antimicrobial Agents and Chemotherapy, 57*(10), 5155-5157.

Mosqueda, N., Gato, E., Roca, I., López, M., de Alegría, C. R., Fernández Cuenca, F., Martínez-Martínez, L., Pachón, J., Cisneros, J. M., Rodríguez-Baño, J., Pascual, A., Vila, J., Bou, G., Tomás, M., GEIH-GEMARA (SEIMC), and REIPI. (2014). Characterization of plasmids carrying the blaOXA-24/40 carbapenemase gene and the genes encoding the AbkA/AbkB proteins of a toxin/antitoxin system. *The Journal of Antimicrobial Chemotherapy, 69*(10), 2629-2633.

Moubareck, C., Brémont, S., Conroy, M. C., Courvalin P., and Lambert, T. (2009). GES-11, a novel integron-associated GES variant in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 53*(8), 3579-3581.

Mózes, J., Ebrahimi, F., Gorácz, O., Miszti, C., and Kardos, G. (2014). Effect of carbapenem consumption patterns on the molecular epidemiology and carbapenem resistance of Acinetobacter baumannii. *Journal of Medical Microbiology, 63*(Pt 12), 1654-1662.

Mugnier, P. D., Poirel, L., Naas, T., and Nordmann, P. (2010). Worldwide dissemination of the blaOXA-23 carbapenemase gene of Acinetobacter baumannii. *Emerging Infectious Diseases, 16*(1), 35-40.

Munoz-Price, L. S., Arheart, K., Nordmann, P., Boulanger, A. E., Cleary, T., Alvarez, R., Pizano, L., Namias, N., Kett, D. H., and Poirel, L. (2013). Eighteen years of experience with Acinetobacter baumannii in a tertiary care hospital. *Critical Care Medicine, 41*(12), 2733-2742.

Murray, S. R., Ernst, R. K., Bermudes, D., Miller, S. I., and Low, K. B. (2007). pmrA confers pmrHFIJKL-dependent EGTA and polymyxin resistance on msbB Salmonella by decorating lipid A with phosphoethanolamine. *Journal of Bacteriology 189*(14), 5161–5169.

Mussi, M. A., Gaddy, J. A., Cabruja, M., Arivett, B. A., Viale, A. M., Rasia, R., and Actis, L. A. (2010). The opportunistic human pathogen Acinetobacter baumannii senses and responds to light. *Journal of Bacteriology, 192*(24), 6336-6345.

Mussi, M. A., Limansky, A. S., and Viale, A. M. (2006a). Acquisition of resistance to carbapenems in multidrug-resistant clinical strains of Acinetobacter baumannii: natural insertional inactivation of a gene encoding a member of a novel family of beta-barrel outer membrane proteins. *Antimicrobial Agents and Chemotherapy, 49*(4), 1432-1440.

Naas, T., Bogaerts, P., Bauraing, C., Degheldre, Y., Glupczynski, Y., and Nordmann, P. (2006b). Emergence of PER and VEB extended-spectrum beta-lactamases in Acinetobacter baumannii in Belgium. *The Journal of Antimicrobial Chemotherapy, 58*(1), 178-182.

Naas, T., Coignard, B., Carbonne, A., Blanckaert, K., Bajolet, O., Bernet, C., Verdeil, X., Astagneau, P., Desenclos, J. C., Nordmann, P. and the French Nosocomial Infection Early Warning Investigation and Surveillance Network. (2006). VEB-1 Extended-spectrum beta-lactamase-producing Acinetobacter baumannii, France. *Emerging Infectious Diseases, 12*(8), 1214-1222.

Naas, T., Cuzon, G., Villegas, M. V., Lartigue, M. F., Quinn, J. P., and Nordmann, P. (2008). Genetic structures at the origin of acquisition of the beta-lactamase bla KPC gene. *Antimicrobial Agents and Chemotherapy, 52*(4), 1257-1263.

Naas, T., Kernbaum, S., Allali, S., and Nordmann, P. (2007b). Multidrug-resistant Acinetobacter baumannii, Russia. *Emerging Infectious Diseases, 13*(4), 669-671.

Naas, T., Nordmann, P., and Heidt, A. (2007a). Intercountry transfer of PER-1 extended-spectrum beta-lactamase-producing Acinetobacter baumannii from Romania. *International Journal of Antimicrobial Agents, 29*(2), 226-228.

Nagamine, K., Hase, T., and Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes, 16*(3), 223-229.

Nagano, N., Nagano, Y., Cordevant, C., Shibata, N., and Arakawa, Y. (2004). Nosocomial transmission of CTX-M-2 beta-lactamase-producing Acinetobacter baumannii in a neurosurgery ward. *Journal of Clinical Microbiology, 42*(9), 3978-3984.

Naiemi, N. A., Duim, B., Savelkoul, P. H., Spanjaard, L., de Jonge, E., Bart, A., Vandenbroucke-Grauls, C. M., and de Jong, M. D. (2005). Widespread transfer of resistance genes between bacterial species in an intensive care unit: implications for hospital epidemiology. *Journal of Clinical Microbiology, 43*(9), 4862-4864.

Nakazawa, Y., Ii, R., Tamura, T., Hoshina, T., Tamura, K., Kawano, S., Kato, T., Sato, F., Horino, T., Yoshida, M., Hori, S., Sanui, M., Ishii, Y., and Tateda K. (2013). A case of NDM-1-producing Acinetobacter baumannii transferred from India to Japan. *Journal of Infection and Chemotherapy: Official Journal of the Japan Journal of Chemotherapy, 19*(2), 330-332.

Napier, B. A., Burd, E. M., Satola, S. W., Cagle, S. M., Ray, S. M., McGann, P., Pohl, J., Lesho, E. P., and Weiss, D. S. (2013). Clinical use of colistin induces cross-resistance to host antimicrobials in Acinetobacter baumannii. *MBio, 4*(3), e00021-13. doi: 10.1128/mBio.00021-13.

Nemec, A., De Baere, T., Tjernberg, I., Vaneechoutte, M., van der Reijden, T. J., and Dijkshoorn. L. (2001). Acinetobacter ursingii sp. nov. and Acinetobacter schindleri sp. nov., isolated from human clinical specimens. *International Journal of Systemic and Evolutionary Microbiology*. *51*(Pt 5), 1891–1899.

Nemec, A., Dijkshoorn, L., Cleenwerck, I., De Baere, T., Janssens, D., Van Der Reijden, T. J., Jezek, P., and Vaneechoutte, M. (2003). Acinetobacter parvus sp. nov., a small-colony-forming species isolated from human clinical specimens *International Journal of Systemic and Evolutionary Microbiology, 53*(Pt 5), 1563–1567.

Nemec, A., Dolzani, L., Brisse, S., van den Broek, P., and Dijkshoorn, L. (2004). Diversity of aminoglycoside-resistance genes and their association with class 1 integrons among strains of pan-European Acinetobacter baumannii clones. *Journal of Medical Microbiology, 53*(Pt 12), 1233-1240.

Nemec, A., Krizova, L., Maixnerova, M., Sedo, O., Brisse, S., and Higgins, P. G. (2015). Acinetobacter seifertii sp. nov., a member of the Acinetobacter calcoaceticus-Acinetobacter baumannii complex isolated from human clinical specimens. *International Journal of Systemic and Evolutionary Microbiology*, *65*(Pt 3), 934-942.

Nemec, A., Krizova, L., Maixnerova, M., van der Reijden, T. J. K., Deschaght, P., Passet, V., Vaneechoutte, M., Brisse, S. and Dijkshoorn, L. (2011). Genotypic and phenotypic characterization of the Acinetobacter calcoaceticus–Acinetobacter baumannii complex

with the proposal of Acinetobacter pittii sp. nov. (formerly Acinetobacter genomic species 3) and Acinetobacter nosocomialis sp. nov. (formerly Acinetobacter genomic species 13TU). *Research in Microbiology, 162*(4), 393–404.

Nemec, A., Musílek, M., Sedo, O., De Baere, T., Maixnerová, M., van der Reijden, T. J., Zdráhal, Z., Vaneechoutte, M., and Dijkshoorn, L. (2010). Acinetobacter bereziniae sp. nov. and Acinetobacter guillouiae sp. nov., to accommodate Acinetobacter genomic species 10 and 11, respectively. *International Journal of Systemic and Evolutionary Microbiology, 60*(Pt 4), 896-903.

Nemec, A., Radolfova-Krizova, L., Maixnerova, M., Vrestiakova, E., Jezek, P., and Sedo O. (2016). Taxonomy of haemolytic and/or proteolytic strains of the genus Acinetobacter with the proposal of Acinetobacter courvalinii sp. nov. (genomic species 14 sensu Bouvet & Jeanjean), Acinetobacter dispersus sp. nov. (genomic species 17), Acinetobacter modestus sp. nov., Acinetobacter proteolyticus sp. nov. and Acinetobacter vivianii sp. nov. *International Journal of Systemic and Evolutionary Microbiology, 66*(4), 1673-1685.

Ng, J., Gosbell, I. B., Kelly, J. A., Boyle, M. J., and Ferguson, J. K. (2006). Cure of multiresistant Acinetobacter baumannii central nervous system infections with intraventricular or intrathecal colistin: case series and literature review. *The Journal of Antimicrobial Chemotherapy, 58*(5), 1078-1081.

Ni, W., Li, Y., Guan, J., Zhao, J., Cui, J., Wang, R., and Liu, Y. (2016). Effects of Efflux Pump Inhibitors on Colistin Resistance in Multidrug-Resistant Gram-Negative Bacteria. *Antimicrobial Agents and Chemotherapy, 60*(5), 3215-3218.

Nigro, S. J., Post, V., and Hall, R. M. (2011). Aminoglycoside resistance in multiply antibiotic-resistant Acinetobacter baumannii belonging to global clone 2 from Australian hospitals. *The Journal of Antimicrobial Chemotherapy, 66*(7), 1504-1509.

Nikaido H. (2009). Multidrug resistance in bacteria. *Annual Review of Biochemistry, 78*(1), 119-146.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews: MMBR, 67*(4), 593-656.

Nishimura, Y., Ino, T., and Iizuka, H. (1988). Acinetobacter radioresistens sp. nov. isolated from cotton and soil. *International Journal of Systemic Bacteriology, 38*(1), 209–211.

Niu, C., Clemmer, K. M., Bonomo, R. A., and Rather, P. N. (2008) Isolation and characterization of an autoinducer synthase from Acinetobacter baumannii. *Journal of Bacteriology, 190*(9), 3386–3392.

Nordmann, P., Dortet, L., and Poirel, L. (2012). Carbapenem resistance in Enterobacteriaceae: here is the storm! *Trends in Molecular Medicine, 18*(5), 263-272.

Novovic, K., Mihajlovic, S., Vasiljevic, Z., Filipic, B., Begovic, J., and Jovcic, B. (2015). Carbapenem-resistant Acinetobacter baumannii from Serbia: revision of CarO classification. *PLoS One, 10*(3), e0122793. doi: 10.1371/journal.pone.0122793.

Nowak, P., and Paluchowska, P. (2016). Acinetobacter baumannii: biology and drug resistance - role of carbapenemases. *Folia Histochemica et Cytobiologica*, E-pub ahead of print. doi: 10.5603/FHC.a2016.0009.

Nowak, P., Paluchowska, P. M., and Budak, A. (2014). Co-occurrence of carbapenem and aminoglycoside resistance genes among multidrug-resistant clinical isolates of

Acinetobacter baumannii from Cracow, Poland. *Medical Science Monitor Basic Research, 20*(1), 9-14.

Nowak, P., Paluchowska, P., and Budak, A. (2012). Distribution of blaOXA genes among carbapenem-resistant Acinetobacter baumannii nosocomial strains in Poland. *The New Microbiologica, 35*(3), 317-325.

Nwadike, V. U., Ojide, C. K., and Kalu, E. I. (2014). Multidrug resistant acinetobacter infection and their antimicrobial susceptibility pattern in a nigerian tertiary hospital ICU. *African Journal of infectious Diseases, 8*(1), 14-18.

Obara, M., and Nakae, T. (1991). Mechanisms of resistance to β-lactam antibiotics in Acinetobacter calcoaceticus. *The Journal of Antimicrobial Chemotherapy, 28*(6), 791-800.

Olaitan, A. O., Berrazeg, M., Fagade, O. E., Adelowo, O. O., Alli, J. A., and Rolain, J. M. (2013). Emergence of multidrug-resistant Acinetobacter baumannii producing OXA-23 carbapenemase, Nigeria. *International Journal of Infectious Diseases, 17*(6), e469-e470.

Olaitan, A. O., Morand, S., and Rolain, J. M. (2014). Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Frontiers in Microbiology, 5*(1), 643. doi: 10.3389/fmicb.2014.00643.

Oliveira, M. S., Prado, G. V., Costa, S. F., Grinbaum, R. S., and Levin, A. S. (2008) Ampicillin/sulbactam compared with polymyxins for the treatment of infections caused by carbapenem-resistant Acinetobacter spp. *The Journal of Antimicrobial Chemotherapy, 61*(6), 1369–1375.

Oliver, A., Levin, B. R., Juan, C., Baquero, F., and Blazquez, J. (2004). Hypermutation and the preexistence of antibiotic-resistant pseudomonas aeruginosa mutants: Implications for susceptibility testing and treatment of chronic infections. *Antimicrobial Agents and Chemotherapy, 48*(11), 4226-4233.

Olsen, I. (2015) Biofilm-specific antibiotic tolerance and resistance. *European Journal of Clinical Microbiology and Infectious Diseases, 34*(5), 877-886.

Opazo, A., Sonnevend, A., Lopes, B., Hamouda, A., Ghazawi, A., Pal, T., and Amyes, S. G. (2012). Plasmid-encoded PER-7 β-lactamase responsible for ceftazidime resistance in Acinetobacter baumannii isolated in the United Arab Emirates. *The Journal of Antimicrobial Chemotherapy, 67*(7), 1619-1622.

Opazo, A., Vali, L., Al Obaid, K., Dashti, A. A., and Amyes, S. G. (2014). Novel genetic structure harbouring blaPER-1 in ceftazidime-resistant Acinetobacter baumannii isolated from Kuwait. *International Journal of Antimicrobial Agents, 43*(4), 383-384.

Orsinger-Jacobsen, S.J., Patel, S.S., Vellozzi, E.M., Gialanella, P., Nimrichter, L., Miranda, K., and Martinez, L.R. (2013). Use of a stainless steel washer platform to study Acinetobacter baumannii adhesion and biofilm formation on abiotic surfaces. *Microbiology 159*(Pt 12), 2594–2604.

O'Toole G. A., and Kolter, R. (1998). Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. *Molecular Biology, 30*(2), 295-304.

Ouertani, R., Limelette, A., Guillard, T., Brasme, L., Jridi, Y., Barguellil, F., El Salabi, A., de Champs, C., and Chouchani, C. (2016). First report of nosocomial infection caused by Klebsiella pneumoniae ST147 producing OXA-48 and VEB-8 β-lactamases in Tunisia. *Journal of Global Antimicrobial Resistance, 4*(1), 53-56.

Pachón-Ibáñez, M. E., Docobo-Pérez, F., López-Rojas, R., Domínguez-Herrera, J., Jiménez-Mejias, M. E., García-Curiel, A., Pichardo, C., Jiménez, L., and Pachón, J. (2010). Efficacy of rifampin and its combinations with imipenem, sulbactam, and colistin in experimental models of infection caused by imipenem-resistant Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 54*(3), 1165–1172.

Pajand, O., Rezaee, M. A., Nahaei, M. R., Mahdian, R., Aghazadeh, M., Soroush, M. H., Tabrizi, M. S., and Hojabri, Z. (2013). Study of the carbapenem resistance mechanisms in clinical isolates of Acinetobacter baumannii: comparison of burn and non-burn strains. *Burns: Journal of the International Society for Burn Injuries, 39*(7), 1414-1419.

Park, Y. K., Choi, J. Y., Shin, D., and Ko, K. S. (2011). Correlation between overexpression and amino acid substitution of the PmrAB locus and colistin resistance in Acinetobacter baumannii. *International Journal of Antimicrobial Agents, 37*(6), 525-530.

Park, Y. K., Lee, G. H., Baek, J. Y., Chung, D. R., Peck, K. R., Song, J. H., and Ko, K. S. (2010). A single clone of Acinetobacter baumannii, ST22, is responsible for high antimicrobial resistance rates of Acinetobacter spp. isolates that cause bacteremia and urinary tract infections in Korea. *Microbial Drug Resistance (Larchmont, N. Y.), 16*(2), 143-149.

Park, Y. K., Lee, J. Y., and Ko, K. S. (2015). Transcriptomic analysis of colistin-susceptible and colistin-resistant isolates identifies genes associated with colistin resistance in Acinetobacter baumannii. *Clinical Microbiology and Infection, 21*(8), 765e1-7. doi: 10.1016/j.cmi.2015.04.009.

Park, Y. K., Peck, K. R., Cheong, H. S., Chung, D. R., Song, J. H., and Ko, K. S. (2009). Extreme drug resistance in Acinetobacter baumannii infections in intensive care units, South Korea. *Emerging Infectious Diseases, 15*(8), 1325-1327.

Pasanen, T., Koskela, S., Mero, S., Tarkka, E., Tissari, P., Vaara, M., and Kirveskari, J. (2014). Rapid molecular characterization of Acinetobacter baumannii clones with rep-PCR and evaluation of carbapenemase genes by new multiplex PCR in Hospital District of Helsinki and Uusimaa. *PloS One, 9*(1), e85854. doi: 10.1371/journal.pone.0085854.

Pasterán, F., Rapoport, M., Petroni, A., Faccone, D., Corso, A., Galas, M., Vázquez, M., Procopio, A., Tokumoto, M., and Cagnoni, V. (2006). Emergence of PER-2 and VEB-1a in Acinetobacter baumannii Strains in the Americas. *Antimicrobial Agents and Chemotherapy, 50*(9), 3222-3224.

Patel, R. (2005). Biofilms and antimicrobial resistance. *Clinical Orthopaedics and Related Research, 437*(1), 41-47.

Peirano, G., Schreckenberger, P. C., and Pitout, J. D. (2011). Characteristics of NDM-1-producing Escherichia coli isolates that belong to the successful and virulent clone ST131. *Antimicrobial Agents and Chemotherapy, 55*(6), 2986-2988.

Peleg, A. Y., de Breij, A., Adams, M. D., Cerqueira, G. M., Mocali, S., Galardini, M., Nibbering, P. H., Earl, A. M., Ward, D. V., Paterson, D. L., Seifert, H., and Dijkshoorn L. (2012). The success of acinetobacter species; genetic, metabolic and virulence attributes. *PLoS One, 7*(10), e46984.

Peleg, A. Y., Franklin, C., Bell, J., and Spelman, D. W. (2004). Emergence of IMP-4 metallo-beta-lactamase in a clinical isolate from Australia. *The Journal of Antimicrobial Chemotherapy, 54*(3), 699-700.

Peleg, A. Y., Seifert, H., and Paterson, D. L. (2008). Acinetobacter baumannii: emergence of a successful pathogen. *Clinical Microbiology Reviews, 21*(3), 538-582.

Perez, F., Endimiani, A., Ray, A. J., Decker, B. K., Wallace, C. J., Hujer, K. M., Ecker, D. J., Adams, M. D., Toltzis, P., Dul, M. J., Windau, A., Bajaksouzian, S., Jacobs, M. R., Salata, R. A., and Bonomo, R. A. (2010). Carbapenem-resistant Acinetobacter baumannii and Klebsiella pneumoniae across a hospital system: impact of post-acute care facilities on dissemination. *Journal of Antimicrobial Chemotherapy, 65*(8), 1807-1818.

Perez, F., Hujer, A. M., Hujer, K. M., Decker, B. K., Rather, P. N., and Bonomo, R. A. (2007). Global challenge of multidrug-resistant Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 51*(10), 3471-3484.

Périchon, B., Goussard, S., Walewski, V., Krizova, L., Cerqueira, G., Murphy, C., Feldgarden, M., Wortman, J., Clermont, D. Nemec, A., and Courvalin, P. (2014). Identification of 50 class D b-lactamases and 65 Acinetobacter-derived cephalosporinases in Acinetobacter spp. *Antimicrobial Agents and Chemotherapy, 58*(2), 936–949.

Pfeifer, Y., Wilharm, G., Zander, E., Wichelhaus, T. A., Göttig, S., Hunfeld, K. P., Seifert, H., Witte, W., and Higgins, P. G. (2011). Molecular characterization of blaNDM-1 in an Acinetobacter baumannii strain isolated in Germany in 2007. *The Journal of Antimicrobial Chemotherapy, 66*(9), 1998-2001.

Phe, K., Lee, Y., McDaneld, P. M., Prasad, N., Yin, T., Figueroa, D. A., Musick, W. L., Cottreau, J. M., Hu, M., and Tam, V. H. (2014). In vitro assessment and multicenter cohort study of comparative nephrotoxicity rates associated with colistimethate versus polymyxin B therapy. *Antimicrobial Agents and Chemotherapy, 58*(5), 2740–2746.

Pillonetto, M., Arend, L., Vespero, E. C., Pelisson, M., Chagas, T. P., Carvalho-Assef, A. P, and Asensi, M. D. (2014). First report of NDM-1-producing Acinetobacter baumannii sequence type 25 in Brazil. *Antimicrobial Agents and Chemotherapy, 58*(12), 7592-7594.

Pires, J., Novais, A., and Peixe, L. (2013). Blue-carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. *Journal of Clinical Microbiology, 51*(12), 4281-4283.

Pirog, T. P., and Kuz'minskaya (2003). Some characteristics of central metabolism in Acinetobacter sp. grown on ethanol. *Microbiology, 72*(4), 408-413.

Pobiega, M., Wójkowska-Mach, J., and Heczko, P. B. (2013). Typing of Staphylococcus aureus in order to determine the spread of drug resistant strains inside and outside hospital environment. *Prezglad Epidemiologiczny, 67*(3), 435-438, 539-542.

Poirel, L., and Nordmann P. (2006). Carbapenem resistance in Acinetobacter baumannii: mechanisms and epidemiology. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 12*(9), 826-836.

Poirel, L., Bonnin, R. A., Boulanger, A., Schrenzel, J., Kaase, M., and Nordmann, P. (2012). Tn125-related acquisition of blaNDM-like genes in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 56*(2), 1087-1089.

Poirel, L., Mansour, W., Bouallegue, O., and Nordmann, P. (2008). Carbapenem-resistant Acinetobacter baumannii isolates from Tunisia producing the OXA-58-like carbapenem-hydrolyzing oxacillinase OXA-97. *Antimicrobial Agents and Chemotherapy, 52*(5), 1613-1617.

Poirel, L., Marqué, S., Héritier, C., Segonds, C., Chabanon, G., and Nordmann, P. (2005). OXA-58, a novel class D {beta}-lactamase involved in resistance to carbapenems in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 49*(1), 202-208.

Poirel, L., Menuteau, O., Agoli, N., Cattoen, C., and Nordmann, P. (2003). Outbreak of extended-spectrum beta-lactamase VEB-1-producing isolates of Acinetobacter baumannii in a French hospital. *Journal of Clinical Microbiology, 41*(8), 2542-2547.

Poirel, L., Mugnier, P. D., Toleman, M. A., Walsh, T. R., Rapoport, M. J., Petroni, A., and Nordmann, P. (2009). ISCR2, another vehicle for bla(VEB) gene acquisition. *Antimicrobial Agents and Chemotherapy, 53*(11), 4940-4943.

Poirel, L., Naas, T., and Nordmann, P. (2010). Diversity, epidemiology, and genetics of class D beta-lactamases. *Antimicrobial Agents and Chemotherapy, 54*(1), 24-38.

Poirel, L., Pitout, J. D., and Nordmann, P. (2007). Carbapenemases: molecular diversity and clinical consequences. *Future Microbiology, 2*(5), 501-512.

Poirel, L., Walsh, T. R., Cuviller, V., and Nordmann, P. (2011). Multiplex PCR for detection of acquired carbapenemase genes. Diagnostic Microbiology and Infectious Disease, 70(1), 119-123.

Pongpech, P., Amornnopparattanakul, S., Panapakdee, S., Fungwithaya, S., Nannha, P., Dhiraputra, C., and Leelarasamee, A. (2010). Antibacterial activity of carbapenem-based combinations againts multidrug-resistant Acinetobacter baumannii. *Journal of the Medical Association of Thailand, 93*(2), 161-171.

Poole, K. (2002). Outer membranes and efflux: the path to multidrug resistance in Gram-negative bacteria. *Current Pharmaceutical Biotechnology, 3*(2), 77-98.

Potron, A., Munoz-Price, L. S., Nordmann, P., Cleary, T., and Poirel, L. (2011). Genetic features of CTX-M-15-producing Acinetobacter baumannii from Haiti. *Antimicrobial Agents and Chemotherapy, 55*(22), 5946-5948.

Potron, A., Poirel, L., and Nordmann, P. (2015). Emerging broad-spectrum resistance in Pseudomonas aeruginosa and Acinetobacter baumannii: Mechanisms and epidemiology. *International Journal of Antimicrobial Agents, 45*(6), 568-585.

Potron, A., Poirel, L., Croizé, J., Chanteperdrix, V., and Nordmann, P. (2009). Genetic and biochemical characterization of the first extended-spectrum CARB-type beta-lactamase, RTG-4, from Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 53*(7), 3010-3016.

Pournaras S., Poulou, A., Dafopoulou, K., Chabane, Y. N., Kristo, I., Makris, D., Hardouin, J., Cosette, P., Tsakris, A., and Dé, E. (2014). Growth retardation, reduced invasiveness, and impaired colistin-mediated cell death associated with colistin resistance development in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 58*(2), 828-832.

Pournaras, S., Markogiannakis, A., Ikonomidis, A., Kondyli, L., Bethimouti, K., Maniatis, A. N., Legakis, N. J., and Tsakris, A. (2006). Outbreak of multiple clones of imipenem-resistant Acinetobacter baumannii isolates expressing OXA-58 carbapenemase in an intensive care unit. *The Journal of Antimicrobial Chemotherapy, 57*(3), 557-561.

Povilonis, J., Seputiene, V., Krasauskas, R., Juskaite, R., Miskinyte, M., Suziedelis, K., and Suziedeliene, E. (2012). Spread of carbapenem-resistant Acinetobacter baumannii carrying a plasmid with two genes encoding OXA-72 carbapenemase in Lithuanian hospitals. *The Journal of Antimicrobial Chemotherapy, 68*(5), 1000-1006.

Principe, L., D'Arezzo, S., Capone, A., Petrosillo, N., and Visca, P. (2009). In vitro activity of tigecycline in combination with various antimicrobials against multidrug resistant Acinetobacter baumannii. *Annals of Clinical Microbiology and Antimicrobials, 21*(1), 8-18.

Qian, Y., Wong, C. C., Lai, S. C., Lin, Z. H., Zheng, W. L., Zhao, H., Pan, K. H., Chen, S. J., Si, J. M. (2016). Klebsiella pneumoniae invasive liver abscess syndrome with purulent meningitis and septic shock: A case from mainland China. *World Journal of Gastronterology, 22*(9), 2861-2866.

Quale, J., Bratu, S., Landman, D., and Heddurshetti, R. (2003). Molecular epidemiology and mechanisms of carbapenem resistance in Acinetobacter baumannii endemic in New York City. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 37*(2), 214-220.

Raetz, C. R., and Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annual Review of Biochemistry, 71*(1), 635–700.

Rafailidis, P. I., Ioannidou, E. N., and Falagas, M. E. (2007) Ampicillin/sulbactam: current status in severe bacterial infections. *Drugs, 67*(13), 1829–1849.

Rafei, R., Dabboussi, F., Hamze, M., Eveillard, M., Lemarié, C., Gaultier, M. P., Mallat, H., Moghnieh, R., Husni-Samaha, R., Joly-Guillou, M. L., and Kempf, M. (2014a). Molecular analysis of Acinetobacter baumannii strains isolated in Lebanon using four different typing methods. *PLoS One, 9*(12), e115969. doi: 10.1371/journal.pone.0115969.

Rafei, R., Dabboussi, F., Hamze, M., Eveillard, M., Lemarié, C., Mallat, H., Rolain, J. M., Joly-Guillou, M. L., and Kempf, M. (2014b). First report of blaNDM-1-producing Acinetobacter baumannii isolated in Lebanon from civilians wounded during the Syrian war. *International Journal of infectious Diseases: IJID: Official Publication of the International Society for Infectious Diseases, 21*(1), 21-23. doi: 10.1016/j.ijid.2014.01.004.

Rafei, R., Hamze, M., Pailhoriès, H., Eveillard, M., Marsollier, L., Joly-Guillou, M. L., Dabboussi, F., and Kempf, M. (2015a). Extrahuman epidemiology of Acinetobacter baumannii in Lebanon. *Applied and Environmental Microbiology, 81*(7), 2359-2367.

Rafei, R., Pailhoriès, H., Hamze, M., Eveillard, M., Mallat, H., Dabboussi, F., Joly-Guillou, M. L., and Kempf, M. (2015b). Molecular epidemiology of Acinetobacter baumannii in different hospitals in Tripoli, Lebanon using bla(OXA-51-like) sequence based typing. *BMC Microbiology, 15*(1), 103. doi: 10.1186/s12866-015-0441-5.

Rao, R.S., Karthika, R.U., Singh, S.P., Shashikala, P., Kanungo, R., Jayachandran, S., and Prashanth, K. (2008). Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of Acinetobacter baumannii. *Indian Journal of Medical Microbiology, 26*(4), 333-7.

Rasko, D. A., and Sperandio, V. (2010). Anti-virulence strategies to combat bacteria-mediated disease. *Nature Reviews. Drug Discovery, 9*(2), 117-128.

Reinert, R. R., Low, D. E., Rossi, F., Zhang, X., Wattal, C., and Dowzicky, M. J. (2007). Antimicrobial susceptibility among organisms from the Asia/Pacific Rim, Europe and Latin and North America collected as part of TEST and the in vitro activity of tigecycline. *The Journal of Antimicrobial Chemotherapy, 60*(5), 1018-1029.

Reuland, E. A., Hays, J. P., de Jongh, D. M., Abdelrehim, E., Willemsen, I., Kluytmans, J. A., Savelkoul, P. H., Vandenbroucke-Grauls, C. M., and al Naiemi, N. (2014). Detection

and occurrence of plasmid-mediated AmpC in highly resistant gram-negative rods. *PLoS One, 9*(3), e91396. doi: 10.1371/journal.pone.0091396.

Revathi, G., Siu, L. K., Lu, P. L., and Huang, L. Y. (2013). First report of NDM-1-producing Acinetobacter baumannii in East Africa. *International Journal of infectious Diseases: IJID: Official Publication of the International Society for Infectious Diseases, 17*(12), e1255-12558. doi: 10.1016/j.ijid.2013.07.016.

Riccio, M. L., Franceschini, N., Boschi, L., Caravelli, B., Cornaglia, G., Fontana, R., Amicosante, G., Rossolini, G. M. (2000). Characterization of the metallo-beta-lactamase determinant of Acinetobacter baumannii AC-54/97 reveals the existence of bla(IMP) allelic variants carried by gene cassettes of different phylogeny. *Antimicrobial Agents and Chemotherapy, 44*(5), 1229-1235.

Rieber, H., Frontzek, A., and Pfeifer, Y. (2016). Molecular Investigation of Carbapenem-Resistant Acinetobacter spp. from Hospitals in North Rhine-Westphalia, Germany. *Microbial Drug Resistance (Larchmont, N. Y.),* Epub Ahead of Print. doi:10.1089/mdr.2016.0035.

Rizos, I., Tsiodras, S., Papathanasiou, S., Rigopoulos, A., Barbetseas, J., and Stefanadis. C. (2007). Prosthetic valve endocarditis due to Acinetobacter spp: a rare case and literature review. *The American Journal of the Medical Sciences, 333*(3), 197–199.

Robenshtok, E., Paul, M., Leibovici, L., Fraser, A., Pitlik, S., Ostfeld, I., Samra, Z., Perez, S., Lev, B., and Weinberger, M. (2006). The significance of Acinetobacter baumannii bacteraemia compared with Klebsiella pneumoniae bacteraemia: risk factors and outcomes. *The Journal of Hospital Infection, 64*(3), 282–287.

Robledo, I. E., Aquino, E. E., Santé, M. I., Santana, J. L., Otero, D. M., León, C. F., and Vázquez, G. J. (2010). Detection of KPC in Acinetobacter spp. in Puerto Rico. *Antimicrobial Agents and Chemotherapy, 54*(3), 1354-1357.

Rodloff, A. C., Leclercq, R., Debbia, E. A., Cantón, R., Oppenheim, B. A., and Dowzicky, M. J. (2008). Comparative analysis of antimicrobial susceptibility among organisms from France, Germany, Italy, Spain and the UK as part of the tigecycline evaluation and surveillance trial. *Clinical Microbiology and Infection: the Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 14*(4), 307-314.

Rodriguez, C. H., Bombicino, K., Granados, G., Nastro, M., Vay, C., and Famiglietti, A. (2009). Selection of colistin-resistant Acinetobacter baumannii isolates in postneurosurgical meningitis in an intensive care unit with high presence of heteroresistance to colistin. *Diagnostic Microbiology and Infectious Disease, 65*(2), 188-191.

Rodriguez, C. H., De Ambrosio, A., Bajuk, M., Spinozzi, M., Nastro, M., Bombicino, K., Radice, M., Gutkind, G., Vay, C., and Famiglietti, A. (2010). In vitro antimicrobials activity against endemic Acinetobacter baumannii multiresistant clones. *The Journal of Infection in Developing Countries, 4*(3), 188-191.

Rodriguez-Baño, J., Marti, S., Soto, S., Fernandez-Cuenca, F., Cisneros, J. M., Pachon, J., Pascual, A., Martinez-Martinez, L., McQueary, C., Actis, L. A., Vila, J., and the Spanish Group for the Study of Nosocomial Infections (GEIH). (2008). Biofilm formation in Acinetobacter baumannii: associated features and clinical implications. *Clinical Microbiology and Infections: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 14*(3): 276–278.

Rodríguez-Martínez, J. M., Nordmann, P., Ronco, E., and Poirel, L. (2010). Extended-spectrum cephalosporinase in Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 54*(8), 3484-3488.

Rohit, A., Maiti, B., Shenoy, S., and Karunasagar, I. (2016). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for rapid diagnosis of neonatal sepsis. *The Indian Journal of Medical Research, 143*(1), 72-78.

Rolain, J. M., Roch, A., Castanier, M., Papazian, L., and Raoult, D. (2011). Acinetobacter baumannii resistant to colistin with impaired virulence: a case report from France. *The Journal of Infectious Diseases, 204*(7), 1146-1147.

Romero-Gómez, M. P., Gómez-Gil, R., Paño-Pardo, J. R., and Mingorance, J. (2012). Identification and susceptibility testing of microorganism by direct inoculation from positive blood culture bottles by combining MALDI-TOF and Vitek-2 Compact is rapid and effective. *The Journal of Infection, 65*(6), 513-520.

Rosenfeld, N., Bouchier, C., Courvalin, P., and Périchon, B. (2012). Expression of the resistance-nodulation-cell division pump AdeIJK in Acinetobacter baumannii is regulated by AdeN, a TetR-type regulator. *Antimicrobial Agents and Chemotherapy, 56*(5), 2504-2510.

Rossau, R., van Landschoot., A., Gillis, M., and de Ley, J. (1991). Taxonomy of Moraxellaceae fam. nov., a new bacterial family to accomodate the genera Moraxella, Acinetobacter, and Psychrobacter and related organisms. *International Journal of Systemic Bacteriology, 41*(1), 310–319.

Roux, D., Danilchanka, O., Guillard, T., Cattoir, V., Aschard, H., Fu, Y., Angoulvant, F., Messika, J., Ricard, J. D., Mekalanos, J. J., Lory, S., Pier, G. B., and Skurnik, D. (2015). Fitness cost of antibiotic susceptibility during bacterial infection. *Science Translational Medicine, 7*(297), 297ra114. doi: 10.1126/scitranslmed.aab1621.

Ruiz, M., Marti, S., Fernandez-Cuenca, F., Pascual, A., and Vila, J. (2007). High prevalence of carbapenem-hydrolysing oxacillinases in epidemiologically related and unrelated Acinetobacter baumannii clinical isolates in Spain. *Clinical Microbiology and Infection: the Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 13*(12), 1192-1198.

Rumbo, C., Gato, E., López, M., Ruiz de Alegría, C., Fernández-Cuenca, F., Martínez-Martínez, L., Vila, J., Pachón, J., Cisneros, J. M., Rodríguez-Baño, J., Pascual, A., Bou, G., Tomás, M., Spanish Group of Nosocomial Infections and Mechanisms of Action and Resistance to Antimicrobials (GEIH-GEMARA), Spanish Society of Clinical Microbiology and Infectious Diseases (SEIMC), and Spanish Network for Research in Infectious Diseases(REIPI). (2013). Contribution of efflux pumps, porins, and β-lactamases to multidrug resistance in clinical isolates of Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 57*(11), 5247–5257.

Russo, T. A., Luke, N. R., Beanan, J. M., Olson, R., Sauberan, S. L., MacDonald, U., Schultz, L. W., Umland, T. C., and Campagnari, A. A. (2010). The K1 Capsular Polysaccharide of Acinetobacter baumannii Strain 307-0294 is a Major Virulence Factor. *Infection and Immunity, 78*(9), 3993-4000.

Russo, T. A., MacDonald, U., Beanan, J. M., Olson, R., MacDonald, I. J., Sauberan, S. L., Luke, N. R., Schultz, L. W., and Umland, T. C. (2009). Penicillin-binding protein 7/8 contributes to the survival of Acinetobacter baumannii in vitro and in vivo. *The Journal of Infectious Diseases, 199*(4), 513-521.

Salazar De Vegas, E. Z., Nieves, B., Ruiz, M., Ruíz, J., Vila, J., María, A., and Elsa, V. (2007). Molecular epidemiology and characterization of resistance mechanisms to various antimicrobial agents in Acinetobacter baumannii isolated in Mérida, Venezuela. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research, 13*(4), BR89-94.

Salem, S. E., Dahdouh, E., and Daoud, Z. (2013). Resistance of Gram-Negative Bacilli in Lebanon. *ISRN Infectious Diseases, 2013*, 6 pages. http://dx.doi.org/10.5402/2013/759208.

Salimizand, H., Noori, N., Meshkat, Z., Ghazvini, K., and Amel, S. J. (2015). Prevalence of Acinetobacter baumannii harboring ISAba1/bla OXA-23-like family in a burn center. *Burns: Journal of the International Society for Burn Injuries, 41*(5), 1100-1106.

Sampson, T. R., Liu, X., Schroeder, M. R., Kraft, S. K., Burd, E. M., and Weiss, D. S. (2012). Rapid killing of Acinetobacter baumannii by polymyxins is mediated by a hydroxyl radical death pathway. *Antimicrobial Agents and Chemotherapy, 56*(11), 5642-5649.

Santajit S., and Indrawattana, N. (2016). Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. *BioMed Research International, 2016*, 2475067. doi: 10.1155/2016/2475067.

Sarı, A.N., Biçmen, M., and Gülay, Z. (2013). The first report on the outbreak of OXA-24/40-like carbapenemase-producing Acinetobacter baumannii in Turkey. *Japanese Journal of Infectious Diseases, 66*(5), 439-432.

Saule, M., Samuelsen, Ø., Dumpis, U., Sundsfjord, A., Karlsone, A., Balode, A., Miklasevics, E., and Karah, N. (2013). Dissemination of a carbapenem-resistant Acinetobacter baumannii strain belonging to international clone II/sequence type 2 and harboring a novel AbaR4-like resistance island in Latvia. *Antimicrobial Agents and Chemotherapy, 57*(2), 1069-1072.

Scaife, W., Young, H. K., Paton, R. H., and Amyes, S. G. (1995). Transferable imipenem-resistance in Acinetobacter species from a clinical source. *The Journal of Antimicrobial Chemotherapy, 36*(3), 585-586.

Schleicher, X., Higgins, P. G., Wisplinghoff, H., Körber-Irrgang, B., Kresken, M., and Seifert, H. (2013). Molecular epidemiology of Acinetobacter baumannii and Acinetobacter nosocomialis in Germany over a 5-year period (2005-2009). *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 19*(8), 737-742.

Sechi, L. A., Karadenizli, A., Deriu, A., Zanetti, S., Kolayli, F., Balikci, E., and Vahaboglu, H. (2004). PER-1 type beta-lactamase production in Acinetobacter baumannii is related to cell adhesion. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research, 10*(6), BR180-4.

Seifert, H., Baginski, R. , Schulze, A. and Pulverer, G. (1993). The distribution of Acinetobacter species in clinical culture materials. *Zentralblatt für Bakteriologie: International Journal of Medical Microbiology, 279*(4), 544–552.

Seifert, H., Dijkshoorn, L. , Gerner-Smidt, P., Pelzer, N., Tjernberg, I., and Vaneechoutte, M. (1997). Distribution of Acinetobacter species on human skin: comparison of phenotypic and genotypic identification methods. *Journal of Clinical Microbiology, 35*(11), 2819–2825.

Seifert, H., Dolzani, L., Bressan, R., van der Reijden, T., van Strijen, B., Stefanik, D., Heersma, H., and Dijkshoorn, L. (2005). Standardization and interlaboratory reproducibility

assessment of pulsed-field gel electrophoresis-generated fingerprints of Acinetobacter baumannii. *Journal of Clinical Microbiology, 43*(9), 4329-4335.

Seifert, H., Strate, A. , Schulze, A. and Pulverer, G. (1994). Bacteremia due to Acinetobacter species other than Acinetobacter baumannii. *Infection*, *22*(6), 379–385.

Sen, B., and Joshi, S. G. (2016). Studies on Acinetobacter baumannii involving multiple mechanisms of carbapenem resistance. *Journal of Applied Microbiology, 120*(3), 619-629.

Shakil, S, and Khan, A. U. (2010). Detection of CTX-M-15-producing and carbapenem-resistant Acinetobacter baumannii strains from urine from an Indian hospital. *Journal of Chemotherapy (Florence, Italy), 22*(5), 324-327.

Sharma, A., Kaur, R., Ganguly, N. K., Singh, P. D,. and Chakraborti, A. (2002). Subtype distribution of Haemophilus influenzae isolates from north India. *Journal of Medical Microbiology, 51*(5), 299-404.

Sherertz, R. J., and Sullivan, M. L. (1985). An outbreak of infections with Acinetobacter calcoaceticus in burn patients: contamination of patients' mattresses. *The Journal of Infectious Diseases, 151*(2), 252-258.

Shibata, N., Doi, Y., Yamane, K., Yagi, T., Kurokawa, H., Shibayama, K., Kato, H., Kai, K., and Arakawa, Y. (2003). PCR typing of genetic determinants for metallo-beta-lactamases and integrases carried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron. *Journal of Clinical Microbiology, 41*(12), 5407-5413.

Shorr, A. F., Zilberberg, M. D., Micek, S. T., and Kollef, M. H. (2014). Predictors of hospital mortality among septic ICU patients with Acinetobacter spp. bacteremia: a cohort study. *BMC Infectious Diseases, 14*(1), 572. doi: 10.1186/s12879-014-0572-6.

Sirijatuphat, R., and Thamlikitkul, V. (2014). Colistin versus colistin plus fosfo-mycin for treatment of carbapenem-resistant Acinetobacter baumannii infections: A preliminary study. *Antimicrobial Agents and Chemotherapy, 58*(9), 5598–5601.

Siroy, A., Molle, V., Lemaître-Guillier, C., Vallenet, D., Pestel-Caron, M., Cozzone, A. J., Jouenne, T., and Dé, E. (2005). Channel formation by CarO, the carbapenem resistance-associated outer membrane protein of Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 49*(12), 4876-4883.

Skerman, V. B. D., McGowan, V., Sneath, and P. H. A. (1980). Approved list of bacterial names. *International Journal of Systemic Bacteriology*, *30*(1), 225–420.

Skov, R. L., and Monnet, D. L. (2016). Plasmid-mediated colistin resistance (mcr-1 gene): three months later, the story unfolds. *Euro Surveillance: European Communicable Disease Bulletin, 21*(9). doi: 10.2807/1560-7917.ES.2016.21.9.30155.

Smani, Y., Domínguez-Herrera, J., and Pachón, J. (2011). Rifampin protects human lung epithelial cells against cytotoxicity induced by clinical multi and pandrug-resistant Acinetobacter baumannii. *The Journal of Infectious Diseases, 203*(8), 1110-1119.

Smani, Y., López-Rojas, R., Domínguez-Herrera, J., Docobo-Pérez, F., Martí, S., Vila, J., and Pachón, J. (2012). In vitro and in vivo reduced fitness and virulence in ciprofloxacin-resistant Acinetobacter baumannii. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 18*(1), E1-4. doi: 10.1111/j.1469-0691.2011.03695.x.

Sohrabi, N., Farajnia, S., Akhi, M. T., Nahaei, M. R., Naghili, B., Peymani, A., Amiri, Z., Rezaee, M. A., and Saeedi, N. (2012). Prevalence of OXA-type β-lactamases among

Acinetobacter baumannii isolates from Northwest of Iran. *Microbial Drug Resistance (Larchmont, N. Y.), 18*(4), 285-289.

Solanki, R., Vanjari, L., Ede, N., Gungi, A., Soory, A., and Vemu, L. (2013). Evaluation of LAMP assay using phenotypic tests and conventional PCR for detection of blaNDM-1 and blaKPC genes among carbapenem-resistant clinical Gram-negative isolates. *Journal of Medical Microbiology, 62*(Pt 10), 1540-1544.

Souli, M., Galani, I., and Giamarellou, H. (2008). Emergence of extensively drug-resistant and pandrug-resistant Gram-negative bacilli in Europe. *Euro Surveillance: European Communicable Disease Bulletin, 13*(47), pii: 19045.

Stewart, P. S., and Franklin, M. J. (2008). Physiological heterogeneity in biofilms. *Nature Reviews. Microbiology, 6*(3), 199-210.

Stietz, M. S., Ramírez, M. S., Vilacoba, E., Merkier, A. K., Limansky, A. S., Centrón, D., and Catalano, M. (2013). Acinetobacter baumannii extensively drug resistant lineages in Buenos Aires hospitals differ from the international clones I-III. *Infection, Genetics, and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases, 14*(1), 294-301. doi: 10.1016/j.meegid.2012.12.020.

Stirland, R. M., Hillier, V. F. and Steyger M. G. (1969). Analysis of hospital bacteriological data. *Journal of Clinical Pathology. Supplement (College of Pathologists), 3*(1), 82–86.

Strateva, T., Markova, B., Marteva-Proevska, Y., Ivanova, D., and Mitov, I. (2012). Widespread dissemination of multidrug-resistant Acinetobacter baumannii producing OXA-23 carbapenemase and ArmA 16S ribosomal RNA methylase in a Bulgarian university hospital. *The Brazilian Journal of Infectious Diseases: An Official Publication of the Brazilian Society of Infectious Diseases, 16*(3), 307-310.

Strateva, T., Todorova, A., Ouzounova-Raykova, V., and Mitov, I. (2008). Emergence of a PER-1 extended-spectrum beta-lactamase-producing Acinetobacter baumannii clinical isolate in Bulgaria. *Journal of Chemotherapy (Florence, Italy), 20*(3), 391-392.

Strauchman, M., and Morningstar, M. W. (2012). Fluoroquinolone toxicity symptoms in a patient presenting with low back pain. *Clinics and Practice: An International Journal of Medical Care Reports, 2*(4), e87, doi: 10.4081/cp.2012.e87.

Su, X. Z., Chen, J., Mizushima, T., Kuroda, T., and Tsuchiya, T. (2005). AbeM, an H+ coupled Acinetobacter baumannii multidrug efflux pump belonging to the MATE family of transporters. *Antimicrobial Agents and Chemotherapy, 49*(10), 4362–4364.

Sugawara, E., and Nikaido, H. (2012). OmpA is the principal nonspecific slow porin of Acinetobacter baumannii.

Sun, C., Hao, J., Dou, M., and Gong, Y. (2015). Mutant prevention concentrations of levofloxacin, pazufloxacin and ciprofloxacin for A. baumannii and mutations in gyrA and parC genes. *The Journal of Antibiotics, 68*(5), 313-317.

Sun, S., Negrea, A., Rhen, M., and Andersson, D. I. (2009). Genetic analysis of colistin resistance in Salmonella enterica serovar Typhimurium. *Antimicrobial Agents and Chemotherapy, 53*(60), 2298-2305.

Sung, J. Y., Koo, S. H., Kim, S., and Kwon, G. C. (2016). Persistence of multidrug-resistant Acinetobacter baumannii isolates harboring blaOXA-23 and bap for 5 years. *Journal of Microbiology and Biotechnology*, Epub Ahead of Print. doi: 10.4014/jmb.1604.04049.

Sung, J. Y., Kwon, K. C., Cho, H. H., and Koo, S. H. (2011). Antimicrobial resistance determinants in imipenem-nonsusceptible Acinetobacter calcoaceticus-baumannii complex isolated in Daejeon, Korea. *The Korean Journal of Laboratory Medicine, 31*(4), 265-270.

Suto, M. J., Domagala, J. M., Roland, G. E., Mailloux, G. B., and Cohen, M. A. (1992). Fluoroquinolones: relationships between structural variations, mammalian cell cytotoxicity, and antimicrobial activity. *Journal of Medicinal Chemistry, 35*(25), 4745–4750.

Suzuki, M., Hosoba, E., Matsui, M., and Arakawa, Y. (2014). New PCR-based open reading frame typing method for easy, rapid, and reliable identification of Acinetobacter baumannii international epidemic clones without performing multilocus sequence typing. *Journal of Clinical Microbiology, 52*(8), 2925-2932.

Szabó, D., Szentandrássy, J., Juhász, Z., Katona, K., Nagy, K., and Rókusz, L. (2008). Imported PER-1 producing Pseudomonas aeruginosa, PER-1 producing Acinetobacter baumanii and VIM-2-producing Pseudomonas aeruginosa strains in Hungary. *Annals of Clinical Microbiology and Antimicrobials, 30*(1), 7:12. doi: 10.1186/1476-0711-7-12.

Tada, T., Miyoshi-Akiyama, T., Kato, Y., Ohmagari, N., Takeshita, N., Hung, N. V., Phuong, D. M., Thu, T. A., Binh, N. G., Anh, N. Q., Nga, T. T., Truong, P. H., Xuan, P. T., Thu le, T. A., Son, N. T., and Kirikae, T. (2013). Emergence of 16S rRNA methylase-producing Acinetobacter baumannii and Pseudomonas aeruginosa isolates in hospitals in Vietnam. *BMC Infectious Diseases, 30*(1), 13:251. doi: 10.1186/1471-2334-13-251.

Tada, T., Miyoshi-Akiyama, T., Shimada, K., Shimojima, M., and Kirikae, T. (2014). Dissemination of 16S rRNA methylase ArmA-producing acinetobacter baumannii and emergence of OXA-72 carbapenemase coproducers in Japan. *Antimicrobial Agents and Chemotherapy, 58*(5), 2916-2920.

Tan, C. H., Li, J., and Nation, R. L. (2007). Activity of colistin against heteroresistant Acinetobacter baumannii and emergence of resistance in an in vitro pharmacokinetic/pharmacodynamic model. *Antimicrobial Agents and Chemotherapy, 51*(9), 3413-3415.

Tan, S. Y., Chua, S. L., Liu, Y., Hoiby, N., Andersen, L. P., Givskov, M., Song, Z., and Yang L. (2013). Comparative genomic analysis of rapid evolution of an extreme-drug-resistant Acinetobacter baumannii clone. *Genome Biology and Evolution, 5*(5), 807-818.

Tängdén, T. (2014). Combination antibiotic therapy for multidrug-resistant Gram-negative bacteria. *Upsala Journal of Medical Sciences, 119*(2), 149-153.

Tärnberg, M., Nilsson, L. E., and Dowzicky, M. J. (2016). Antimicrobial activity against a global collection of skin and skin structure pathogens: Results from the Tigecycline Evaluation and Surveillance Trial (T.E.S.T.), 2010-2014. *International Journal of Infectious Diseases: IJID: Official Publication of the International Society of Infectious Diseases, S1201-9712*(16), 31101-8. doi: 10.1016/j.ijid.2016.06.016.

Tena, D., Martínez, N. M., Oteo, J., Sáez, D., Vindel, A., Azañedo, M. L., Sánchez, L., Espinosa, A., Cobos, J., Sánchez, R., Otero, I., and Bisquert, J. (2013). Outbreak of multiresistant OXA-24- and OXA-51-producing Acinetobacter baumannii in an internal medicine ward. *Japanese Journal of Infectious Diseases, 66*(4), 323-326.

Tenover, F. C. (2006). Mechanisms of antimicrobial resistance in bacteria. *The American Journal of Medicine, 119*(6), S3-S10.

Thi Khanh Nhu, N., Riordan, D. W., Do Hoang Nhu, T., Thanh, D. P., Thwaites, G., Huong Lan, N. P., Wren, B. W., Baker, S., and Stabler, R. A. (2016). *Scientific Reports, 6*(1), 28291. doi: 10.1038/srep28291.

Tian, G. B., Adams-Haduch, J. M., Pasculle, A. W., Quinn, J. P., Wang, H. N., and Doi, Y. (2011a). Identification of diverse OXA-40 group carbapenemases, including a novel variant, OXA-160, from Acinetobacter baumannii in Pennsylvania. *Antimicrobial Agents and Chemotherapy, 55*(1), 429-432.

Tian, G. B., Adams-Haduch, J. M., Taracila, M., Bonomo, R. A., Wang, H. N., and Doi, Y. (2011b). Extended-spectrum AmpC cephalosporinase in Acinetobacter baumannii: ADC-56 confers resistance to cefepime. *Antimicrobial Agents and Chemotherapy, 55*(10), 4922-4925.

Tjernberg, I., and Ursing, J. (1989). Clinical strains of Acinetobacter classified by DNA-DNA hybridization. *APMIS: Acta Pathologica Microbiologica, et Immunologica Scandinavica, 97*(7), 595–605.

Todorova, B., Velinov, T., Ivanov, I., Dobreva, E., and Kantardjiev, T. (2014). First detection of OXA-24 carbapenemase-producing Acinetobacter baumannii isolates in Bulgaria. *World Journal of Microbiology and Biotechnology, 30*(4), 1427-1430.

Tomaras, A. P., Dorsey, C. W., Edelmann, R. E., and Actis, L. A. (2003). Attachment to and biofilm formation on abiotic surfaces by Acinetobacter baumannii: involvement of a novel chaperone-usher pili assembly system. *Microbiology, 149*(Pt 12), 3473–3484.

Tomaschek, F., Higgins, P. G., Stefanik, D., Wisplinghoff, H., and Seifert H. (2016). Head-to-Head Comparison of Two Multi-Locus Sequence Typing (MLST) Schemes for Characterization of Acinetobacter baumannii Outbreak and Sporadic Isolates. *PLoS One, 11*(4), e0153014.

Touati, M., Diene, S. M., Racherache, A., Dekhil, M., Djahoudi, A., and Rolain, J. M. (2012). Emergence of blaOXA-23 and blaOXA-58 carbapenemase-encoding genes in multidrug-resistant Acinetobacter baumannii isolates from University Hospital of Annaba, Algeria. *International Journal of Antimicrobial Agents, 40*(1), 89-91.

Touchon, M., Cury, J., Yoon, E. J., Krizova, L., Cerqueira, G. C., Murphy, C., Feldgarden, M., Wortman, J., Clermont, D., Lambert, T., Grillot-Courvalin, C., Nemec, A., Courvalin, P., and Rocha E. P. (2014). The genomic diversification of the whole Acinetobacter genus: origins, mechanisms, and consequences. *Genome Biology and Evolution, 6*(10), 2866–2882.

Towner, K. J., Levi, K., and Vlassiadi, M; ARPAC Steering Group. (2008). Genetic diversity of carbapenem-resistant isolates of Acinetobacter baumannii in Europe. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 14*(2), 161-167.

Tsakris, A., Ikonomidis, A., Pournaras, S., Tzouvelekis, L. S., Sofianou, D., Legakis, N. J., and Maniatis, A. N. (2006). VIM-1 metallo-beta-lactamase in Acinetobacter baumannii. *Emerging Infectious Diseases, 12*(6), 981-983.

Tsakris, A., Poulou, A., Pournaras, S., Voulgari, E., Vrioni, G., Themeli-Digalaki, K., Petropoulou, D., and Sofianou, D. (2010). A simple phenotypic method for the differentiation of metallo-beta-lactamases and class A KPC carbapenemases in Enterobacteriaceae clinical isolates. *The Journal of Antimicrobial Chemotherapy, 65*(8), 1664-1671.

Turton, J. F, Woodford, N., Glover, J., Yarde, S., Kaufmann, M. E., and Pitt, T. L. (2006a). Identification of Acinetobacter baumannii by detection of the blaOXA-51-like carbapenemase gene intrinsic to this species. *Journal of Clinical Microbiology, 44*(8), 2974–2976.

Turton, J. F., Gabriel, S. N., Valderrey, C., Kaufmann, M. E., and Pitt, T. L. (2007). Use of sequence-based typing and multiplex PCR to identify clonal lineages of outbreak strains of Acinetobacter baumannii. *Clinical Microbiology and Infections: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 13*(8), 807-815.

Turton, J. F., Ward, M. E., Woodford, N. Kaufmann, M. E., Pike, R., Livermore, D. M., and Pitt, T. L. (2006b).The role of ISAba1 in expression of OXA carbapenemase genes in Acinetobacter baumannii. *FEMS Microbiology Letters, 258*(1), 72–77.

Ubeda, C., Maiques, E., Tormo, M. A., Campoy, S., Lasa, I., Barbé, J., Novick, R. P., and Penadés, J. R. (2007). SaPI operon I is required for SaPI packaging and is controlled by LexA. *Molecular Microbiology, 65*(1), 41-50.

Uma Karthika, R., Srinivasa Rao, R., Sahoo, S., Shashikala, P., Kanungo, R., Jayachandran, S., and Prashanth, K. (2009). Phenotypic and genotypic assays for detecting the prevalence of metallo-beta-lactamases in clinical isolates of Acinetobacter baumannii from a South Indian tertiary care hospital. *Journal of Medical Microbiology, 58*(Pt 4), 430-435.

Uzunović, S., Ibrahimagić, A., Hodžić, D., and Bedenić, B. (2016). Molecular epidemiology and antimicrobial susceptibility of AmpC- and/or extended-spectrum (ESBL) ß-lactamaseproducing Proteus spp. clinical isolates in Zenica-Doboj Canton, Bosnia and Herzegovina. *Medicinski Glasnik: Official Publication of the Medical Association of Zenica-Doboj Canton, Bosnia and Herzegovina, 13*(2), E-pub ahead of print. doi: 10.17392/853-16.

Vaara, M., and Viljanen, P. (1985). Binding of polymyxin B nonapeptide to gram negative bacteria. *Antimicrobial Agents and Chemotherapy, 27*(4), 548-554.

Vakulenko, S. B., and Mobashery, S. (2003). Versatility of aminoglycosides and prospects for their future. *Clinical Microbiology Reviews, 16*(3), 430-450.

Valencia, R., Arroyo, L. A., Conde, M., et al. (2009). Nosocomial outbreak of infection with pan-drug resistant Acinetobacter baumannii in a tertiary care university hospital. *Infection Control and Hospital Epidemiology, 30*(3), 257-263.

van Diepen, S., Sligl, W. I., Washam, J. B., Gilchrist, I. C., Arora, R. C., and Katz, J. N. (2016). Prevention of Critical Care Complications in the Coronary Intensive Care Unit: Protocols, Bundles, and Insights From Intensive Care Studies. *The Canadian Journal of Cardiology, pii: S0828-282X(16)*30199-4. doi: 10.1016/j.cjca.2016.06.011.

Vaneechoutte, M., Devriese, L. A., Dijkshoorn, L., Lamote, B., Deprez, P., Verschraegen, G., and Haesebrouck. F. (2000). Acinetobacter baumannii-infected vascular catheters collected from horses in an equine clinic. *Journal of Clinical Microbiology, 38*(11), 4280–4281.

Vaneechoutte, M., Dijkshoorn, L., Tjernberg, I., Elaichouni, A., de Vos, P., Claeys, G., and Verschraegen, G. (1995). Identification of Acinetobacter genomic species by amplified ribosomal DNA restriction analysis. *Journal of Clinical Microbiology, 33*(1), 11–15.

Vila, J. (1999). Mechanisms of antimicrobial resistance in Acinetobacter baumannii. *Reviews in Medical Microbiology, 9*(1), 87-97.

Vila, J., Marcos, A., Marco, F., Abdalla, S., Vergara, Y., Reig, R., Gomez-Lus, R., and Jimenez de Anta, T. (1993). In vitro antimicrobial production of beta-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferase by and susceptibility of clinical isolates of Acinetobacter baumannii. *Antimicrobial Agents and Chemotherapy, 37*(1), 138-141.

Vila, J., Martí, S., and Sánchez-Céspedes, J. (2007). Porins, efflux pumps and multidrug resistance in Acinetobacter baumannii. *The Journal of Antimicrobial Chemotherapy, 59*(6), 1210-1215.

Vilacoba, E., Almuzara, M., Gulone, L., Rodriguez, R., Pallone, E., Bakai, R., and Ramírez, M. S. (2013). Outbreak of extensively drug-resistant Acinetobacter baumannii indigo-pigmented strains. *Journal of Clinical Microbiology, 51*(11), 3726-3730.

Villalón, P., Valdezate, S., Cabezas, T., Ortega, M., Garrido, N., Vindel, A., Medina-Pascual, M. J., and Saez-Nieto, J. A. (2015). Endemic and epidemic Acinetobacter baumannii clones: a twelve-year study in a tertiary care hospital. *BMC Microbiology,* 15:47. doi: 10.1186/s12866-015-0383-y.

Villalón, P., Valdezate, S., Medina-Pascual, M. J., Rubio, V., Vindel, A., and Saez-Nieto J. A. (2011). Clonal diversity of nosocomial epidemic Acinetobacter baumannii strains isolated in Spain. *Journal of Clinical Microbiology, 49*(3), 875-882.

Villalón, P., Valdezate, S., Medina-Pascual, M.J., Carrasco, G., Vindel, A., Saez-Nieto, J.A. (2013). Epidemiology of the Acinetobacter-derived cephalosporinase, carbapenem-hydrolysing oxacillinase and metallo-β-lactamase genes, and of common insertion sequences, in epidemic clones of Acinetobacter baumannii from Spain. *Journal of Antimicrobial Chemotherapy, 68*(3), 550-553.

Villar, M., Cano, M. E., Gato, E., Garnacho-Montero, J., Miguel Cisneros, J., Ruíz de Alegría, C., Fernández-Cuenca, F., Martínez-Martínez, L., Vila, J., Pascual, A., Tomás, M., Bou, G., Rodríguez-Baño, J., and GEIH/GEMARA/REIPI-Ab20101 Group. (2014). Epidemiologic and Clinical Impact of Acinetobacter baumannii Colonization and Infection: A Reappraisal. *Medicine (Baltimore), 93*(5), 202-210.

Vranić-Ladavac, M., Bedenić, B., Minandri, F., Ištok, M., Bošnjak, Z., Frančula-Zaninović, S., Ladavac, R., and Visca, P. (2014). Carbapenem resistance and acquired class D beta-lactamases in Acinetobacter baumannii from Croatia 2009-2010. *European Journal of Clinical Microbiology and Infectious Diseases: Official Publication of the European Society of Clinical Microbiology, 33*(3), 471-478.

Wand, M. E., Bock, L. J., Bonney, L. C., and Sutton, J. M. (2015). Retention of virulence following adaptation to colistin in Acinetobacter baumannii reflects the mechanism of resistance. *The Journal of Antimicrobial Chemotherapy, 70*(8), 2209-2216.

Wandersman, C., and Delepelaire, P. (2004). Bacterial iron sources: from siderophores to hemophores. *Annual Review of Microbiology, 58*(1), 611-647.

Wang, D., Ma, L., Wu, Z., Li, M., Li, X., Zhang, W., and Chen, K. (2015). Identification and characteristics of imipenem-resistant Acinetobacter baumannii in surgical wards in a Chinese university hospital. *Infectious Diseases (London, England), 47*(3), 182-186.

Wang, H., Guo, P., Sun, H., Wang, H., Yang, Q., Chen, M., Xu, Y., and Zhu, Y. (2007). Molecular epidemiology of clinical isolates of carbapenem-resistant Acinetobacter spp. from Chinese hospitals. *Antimicrobial Agents and Chemotherapy, 51*(11), 4022-4028.

Warner, W. A., Kuang, S. N., Hernandez, R., Chong, M. C., Ewing, P. J., Fleischer, J., Meng, J., Chu, S., Terashita, D., English, L., Chen, W., and Xu, H. H. (2016). Molecular

characterization and antimicrobial susceptibility of Acinetobacter baumannii isolates obtained from two hospital outbreaks in Los Angeles County, California, USA. *BMC Infectious Diseases, 16*(1), 194. doi: 10.1186/s12879-016-1526-y.

Weldhagen, G. F., Poirel, L., and Nordmann, P. (2003). Ambler class A extended-spectrum beta-lactamases in Pseudomonas aeruginosa: novel developments and clinical impact. *Antimicrobial Agents and Chemotherapy, 47*(8), 2385-2392.

Werneck, J. S., Picão, R. C., Carvalhaes, C. G., Cardoso, J. P., and Gales, A. C. (2011). OXA-72-producing Acinetobacter baumannii in Brazil: a case report. *The Journal of Antimicrobial Chemotherapy, 66*(2), 452-454.

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research, 18*(22), 6531-6535.

Wood, G. C., Hanes, S. D., Boucher, B. A., Croce, M. A., and Fabian, T. C. (2003). Tetracyclines for treating multidrug-resistant Acinetobacter baumannii ventilator-associated pneumonia. *Intensive Care Medicine, 29*(11), 2072-2076.

Wood, G. C., Hanes, S. D., Croce, M. A., Fabian, T. C., and Boucher, B. A. (2002). Comparison of ampicillin-sulbactam and imipenem-cilastatin for the treatment of acinetobacter ventilator-associated pneumonia. *Clinical Infectious Diseases, 34*(11), 1425–1430.

Woodford, N., Carattoli, A., Karisik, E., Underwood, A., Ellington, M. J., and Livermore, D. M. (2009). Complete nucleotide sequences of plasmids pEK204, pEK499, and pEK516, encoding CTX-M enzymes in three major Escherichia coli lineages from the United Kingdom, all belonging to the international O25:H4-ST131 clone. *Antimicrobial Agents and Chemotherapy, 53*(10), 4472-4482.

Wormser, G. P., Keusch, G. T., and Heel, R. C. (1982). Co-trimoxazole (trimethoprim-sulfamethoxazole): an updated review of its antibacterial activity and clinical efficacy. *Drugs, 24*(6), 459-518.

Wu, J., Xu, H., Tang, W., Kopelman, R., Philbert, M. A., and Xi, C. (2009). Eradication of bacteria in suspension and biofilms using methylene blue-loaded dynamic nanoplatforms. *Antimicrobial Agents and Chemotherapy, 53*(7), 3042-3048.

Xia, J., Gao, J., and Tang, W. (2016). Nosocomial infection and its molecular mechanisms of antibiotic resistance. *Bioscience Trends, 10*(1), 14-21.

Xu, A., Zheng, B., Xu, Y. C., Huang, Z. G., Zhong, N. S., and Zhuo, C. (2016). National epidemiology of carbapenem-resistant and extensively drug-resistant Gram-negative bacteria isolated from blood samples in China in 2013. *Clinical Microbiology and Infection: the Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 22*Supp. 1:S1-8

Yamada, Y., and Suwabe, A. (2013). Diverse carbapenem-resistance mechanisms in 16S rRNA methylase-producing Acinetobacter baumannii. *Journal of Medical Microbiology, 62*(Pt 4), 618-622.

Yamada, Y., Endo, K., Sawase, K., Anetai, M., Narita, K., Hatakeyama, Y., Ishifuji, K., Kurota, M., and Suwabe, A. (2016). Rapid species identification and epidemiological analysis of carbapenem-resistant Acinetobacter spp. by a PCR-based open reading frame typing method. *Journal of Medical Microbiology,* Epub Ahead of Print, doi: 10.1099/jmm.0.000314.

Yamamoto, M., Nagao, M., Matsumura, Y., Matsushima, A., Ito, Y., Takakura, S., and Ichiyama, S. (2011). Interspecies dissemination of a novel class 1 integron carrying blaIMP-19 among Acinetobacter species in Japan. *The Journal of Antimicrobial Chemotherapy, 66*(11), 2480-2483.

Yan, J. J., Wu, J. J., Tsai, S. H., and Chuang, C. L. (2004). Comparison of the double-disk, combined disk, and Etest methods for detecting metallo-beta-lactamases in gram-negative bacilli. *Diagnostic Microbiology and Infectious Disease, 49*(1), 5-11.

Yang, H., Huang, L., Barnie, P. A., Su, Z., Mi, Z., Chen, J., Aparna, V., Kumar, D., and Xu, H. (2015). Characterization and distribution of drug resistance associated β-lactamase, membrane porin and efflux pump genes in MDR A. baumannii isolated from Zhenjiang, China. *International Journal of Clinical and Experimental Medicine, 8*(9), 15393-15402.

Yapa, W. S. S., Li, J., Patel, K., Wilson, J. W., Dooley, M. J., George, J., Clark, D., Poole, S., Williams, E., Porter, C. J., Nation, R., L., and McIntosh, M. P. (2014). Pulmonary and systemic pharmacokinetics of inhaled and intravenous colistin methanesulfonate in cystic fibrosis patients: targeting advantage of inhalational administration. *Antimicrobial Agents and Chemotherapy, 58*(5), 2570-2579.

Yau, W., Owen, R. J., Poudyal, A., Bell, J. M., Turnidge, J. D., Yu, H. H., Nation, R. L., and Li, J. (2009). Colistin hetero-resistance in multidrug-resistant Acinetobacter baumannii clinical isolates from the Western Pacific region in the SENTRY antimicrobial surveillance programme. *The Journal of Infection, 58*(2), 138-144.

Yeung, A. T., Bains, M., and Hancock, R. E. (2011). The sensor kinase CbrA is a global regulator that modulates metabolism, virulence, and antibiotic resistance in Pseudomonas aeruginosa. *Journal of Bacteriology, 193*(4), 918-931.

Yong, D., Shin, J. H., Kim, S., Lim, Y., Yum, J. H., Lee, K., Chong, Y., and Bauernfeind, A. (2003). High prevalence of PER-1 extended-spectrum beta-lactamase-producing Acinetobacter spp. in Korea. *Antimicrobial Agents and Chemotherapy, 47*(5), 1749-1751.

Yoon, E. J., Balloy, V., Fiette, L., Chignard, M., Courvalin, P., and Grillot-Courvalin, C. (2016). Contribution of the Ade Resistance-Nodulation-Cell Division-Type Efflux Pumps to Fitness and Pathogenesis of Acinetobacter baumannii. *mBio, 7*(3), e00697-16. doi: 10.1128/mBio.00697-16.

Yoon, E. J., Courvalin, P., and Grillot-Courvalin, C. (2013). RND-type efflux pumps in multidrug-resistant clinical isolates of Acinetobacter baumannii: major role for AdeABC overexpression and AdeRS mutations. *Antimicrobial Agents and Chemotherapy, 57*(7), 2989-2995.

Yoon, E. J., Chabane, Y. N., Goussard, S., Snesrud, E., Courvalin, P., Dé, E., and Grillot-Courvalin, C. (2015). Contribution of resistance-nodulation-cell division efflux systems to antibiotic resistance and biofilm formation in Acinetobacter baumannii. *mBio, 6*(1), e00697-16. doi:.10.1128/mBio.00309-15.

Yun, H. C., and Murray, C. K. (2016). Infection Prevention in the Deployed Environment. *U. S. Army Medical Deployment Journal, 2-16*(1), 114-118.

Zander, E., Bonnin, R. A., Seifert, H., and Higgins, P. G. (2014). Characterization of blaOXA-143 variants in Acinetobacter baumannii and Acinetobacter pittii. *Antimicrobial Agents and Chemotherapy, 58*(5), 2704-2708.

Zarrilli, R., Pournaras, S., Giannouli, M., and Tsakris, A. (2013). Global evolution of multidrug-resistant Acinetobacter baumannii clonal lineages. *International Journal of Antimicrobial Agents, 41*(1), 11–19.

Zarrilli, R., Vitale, D., Di Popolo, A., Bagattini, M., Daoud, Z., Khan, A. U., Afif, C., and Triassi, M. (2008). A plasmid-borne blaOXA-58 gene confers imipenem resistance to Acinetobacter baumannii isolates from a Lebanese hospital. *Antimicrobial Agents and Chemotherapy, 52*(11), 4115-4120.

Zavascki, A. P., Carvalhaes, C. G., Picão, R. C., and Gales, A. C. (2010). Multidrug-resistant Pseudomonas aeruginosa and Acinetobacter baumannii: resistance mechanisms and implications for therapy. *Expert Review of Anti0Infective Therapy*, *8*(1), 71-93.

Zhang, J. P., Zhu, W., Tian, S. F., Chu, Y. Z., and Chen, B. Y. (2010). Molecular characteristics and resistant mechanisms of imipenem-resistant Acinetobacter baumannii isolates in Shenyang, China. *Journal of Microbiology (Seoul, Korea), 48*(5), 689-694.

Zhou, H., Du, X. X., Yang, Q., Zhou, J. Y., Yu, Y. S., and Li, L. J. (2009). [Study on carbapenemase and 16S rRNA methylase of imipenem-resistant Acinetobacter baumannii]. *Zhongua Liu Xing Bing Xue Za Zhi, 30*(3), 269-272.

Zilberberg, M. D., Shorr, A. F., Micek, S. T., Vazquez-Guillamet, C., and Kollef, M. H. (2014). Multi-drug resistance, inappropriate initial antibiotic therapy and mortality in Gram-negative severe sepsis and septic shock: a retrospective cohort study. *Critical Care (London, England), 18*(6), 596. doi: 10.1186/s13054-014-0596-8.

Zorgani, A., Abofayed, A., Glia, A., Albarbar, A., and Hanish, S. (2015). Prevalence of Device-associated Nosocomial Infections Caused By Gram-negative Bacteria in a Trauma Intensive Care Unit in Libya. *Oman Medical Journal*, *30*(4), 270-275.

Zowawi, H., M., Sartor, A. L., Sidjabat, H. E., Balkhy, H. H., Walsh, T.R., Al Johani, S. M., Al Jindan, R. Y., Alfaresi, M., Ibrahim, E., Al-Jardani, A., Al Salman, J., Dashti, A. A., Johani, K., and Paterson, D. L. (2015). Molecular epidemiology of carbapenem-resistant Acinetobacter baumannii isolates in the Gulf Cooperation Council States: dominance of OXA-23-type producers. *Journal of Clinical Microbiology, 53*(3), 896-903.