# UNIVERSIDAD COMPLUTENSE DE MADRID
## FACULTAD DE VETERINARIA
### DEPARTAMENTO DE SANIDAD ANIMAL



# TESIS DOCTORAL

## Detección y caracterización de *Campylobacter* procedentes de animales, alimentos y agua residual

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR


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**UNIVERSIDAD COMPLUTENSE MADRID**

Facultad de Veterinaria
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Centro de Vigilancia Sanitaria Veterinaria (VISAVET)
Servicio de Zoonosis de Transmisión Alimentaria y Resistencia a Antimicrobianos

TESIS DOCTORAL

# Detección y caracterización de *Campylobacter* procedentes de animales, alimentos y agua residual

Memoria para optar al grado de Doctor con Mención Europea presentada por

**María Ugarte Ruiz**

Bajo la dirección de los doctores: Lucas Domínguez Rodríguez, M. Concepción Porrero Calonge y Trudy M. Wassenaar

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**CERTIFICAN:**

Que la tesis doctoral que lleva por título **"Detección y caracterización de *Campylobacter* procedentes de animales, alimentos y agua residual"** ha sido realizada por la licenciada en Veterinaria Dña. María Ugarte Ruiz en el Centro de Vigilancia Sanitaria Veterinaria (VISAVET) de la Universidad Complutense bajo nuestra dirección, y estimamos que reúne los requisitos exigidos para optar al título de Doctor con Mención Europea por la Universidad Complutense.

Madrid, a 1 de junio de 2015

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## PROYECTOS, CONTRATOS Y BECAS DE INVESTIGACIÓN

El trabajo de tesis doctoral que se presenta a continuación se ha llevado a cabo gracias a los siguientes proyectos, contratos y becas de investigación:

- Beca de Formación del Profesorado Universitario (FPU) del Ministerio de Educación, Cultura y Deporte (AP2009-1747, convocatoria 2009).

- Proyectos del Ministerio de Economía y Competitividad:
  - Impacto Sanitario entre animales domésticos, hombre y fauna salvaje/silvestre. Subproyecto: Caracterización de bacterias zoonósicas aisladas a partir de animales salvajes, silvestres y domésticos (FAU2008-00021-C01).
  - *Campylobacter*: detección y caracterización molecular. Análisis genotípico de virulencia y posibles estrategias para su control (AGL2009-07550).
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# ÍNDICE

# 1. RESUMEN

Las especies termófilas de *Campylobacter*, especialmente *C. jejuni* y *C. coli*, han sido reconocidas como importantes patógenos gastrointestinales desde finales de la década de los 70. En general, son microorganismos comensales del intestino de los animales que suelen dar lugar a patologías de distinta gravedad en el hombre que van desde procesos asintomáticos, dolor abdominal o diarrea, hasta neuropatías graves como el Síndrome de Guillain-Barré o su variante, el síndrome de Miller Fisher.

Actualmente, la campilobacteriosis es la principal zoonosis de transmisión alimentaria en la Unión Europea, asociada al consumo y manipulación de alimentos de origen animal, en especial carne de ave. También se ha descrito la existencia de otras vías de transmisión como el consumo de agua contaminada y el contacto con animales (domésticos o salvajes) o con ambientes contaminados. Debido a su fragilidad y sus requerimientos específicos, se trata de un microorganismo de cultivo "fastidioso".

En el presente trabajo de tesis doctoral se ha estudiado la presencia de *Campylobacter* termófilos en muestras obtenidas en animales y alimentos en distintas fases de la cadena alimentaria (contenido cecal y piel de cuello de pollo, así como pechuga de pollo fresca envasada), en efluentes urbanos de una planta de tratamiento de aguas residuales y en heces de animales de vida libre (domésticos y salvajes) ubicados en un entorno geográfico compartido. La finalidad del estudio fue comparar diferentes protocolos de detección de *Campylobacter* y obtener información sobre su epidemiología. Adicionalmente, se propuso el estudio de la presencia de un sistema de secreción de proteínas (sistema de secreción tipo VI o SST6), relacionado con la patogenicidad y supervivencia de algunas cepas de *Campylobacter*.

Los resultados obtenidos mediante cultivo descritos en los trabajos de investigación, implican la presencia de *Campylobacter* termófilos en prácticamente la totalidad de las matrices analizadas, con la excepción de la cabra montés. La proporción de muestras positivas varió en función de la matriz analizada, siendo mayor en muestras de piel de cuello (35/38; 92%) y contenido cecal (34/38; 90%), seguida de pechuga de pollo fresca envasada (29/38; 76%), efluentes urbanos (32/50; 64%), jabalí (22/150; 15%) y ganado vacuno criado en extensivo (7/55; 13%). La proporción de positivos también varió en función de los distintos protocolos de detección utilizados,

funcionando mejor la siembra directa en muestras de contenido cecal (34/38, 90%) y piel de cuello (31/38; 82%), y el enriquecimiento en muestras de pechuga de pollo fresca envasada (26/38; 68%) y efluentes urbanos (19/50; 38%). Por otro lado, la PCR en tiempo real confirmó la presencia de ADN de *C. jejuni* y/o *C. coli* en un elevado porcentaje de muestras en todos los estudios: contenido cecal (37/38; 97%), piel de cuello (38/38; 100%), pechuga de pollo fresca envasada (37/38; 97%) y efluentes urbanos (50/50; 100%). Los resultados obtenidos podrían interpretarse como el máximo teórico de muestras positivas, teniendo en cuenta que la PCR no distingue entre células vivas, muertas o ADN libre. Además, esta técnica permitió la cuantificación de *Campylobacter* termófilos en las muestras de forma rápida, lo cual facilitaría la elección del protocolo de cultivo más adecuado en función de la carga bacteriana.

En cuanto a las especies detectadas, la distribución de las mismas varió en función de la matriz analizada, siendo mayor la proporción de aislados de *C. coli* en muestras de contenido cecal (55/82; 67%), pechuga de pollo (27/48; 56%) y efluentes urbanos (53/77; 69%), mientras que en piel de cuello la proporción de *C. jejuni* fue mayor (64/103; 62%). Además, la proporción de especies detectadas se vio influenciada significativamente por el protocolo de detección empleado, observándose que el enriquecimiento favoreció la detección de *C. coli* en muestras de la cadena alimentaria y efluentes urbanos, mientras que la siembra directa obtuvo una mayor proporción de muestras positivas a *C. jejuni*. De esta manera, la utilización de diversos protocolos de detección de *Campylobacter* favorecería la identificación de las distintas especies presentes en la muestra.

En el estudio llevado a cabo en animales de vida libre, se detectó *C. jejuni* en jabalí y ganado vacuno criado en extensivo (1/150; 0,7% *vs* 5/55; 9%), aunque *C. coli* sólo se identificó en muestras de jabalí (3/150; 2%). También se observó *C. lanienae* en ambos grupos (12% en jabalí *vs* 2% en ganado vacuno criado en extensivo), siendo la especie predominante en el jabalí. Estos resultados indicarían que podría existir un flujo potencial de *Campylobacter* entre jabalí y ganado bovino criado en extensivo. En nuestra zona de estudio, la cabra montés no desempeña ningún papel en la epidemiología de esta bacteria.

Por otro lado, la caracterización genética de los aislados de la cadena alimentaria indica que la matriz con mayor riqueza de genotipos es la piel de cuello. En base a los genotipos obtenidos, los aislados de *Campylobacter* encontrados en las muestras de

pechuga y piel de cuello podrían proceder tanto del mismo animal y/o lote, como de contaminaciones cruzadas con cepas de animales y/o lotes diferentes en el matadero. Asimismo, la riqueza de genotipos encontrada fue disminuyendo al final de la cadena de producción, viéndose influenciada por el protocolo de cultivo empleado. De este modo, la elección de un determinado protocolo de cultivo microbiológico podría ser especialmente relevante en estudios epidemiológicos comparados.

En lo referente a la caracterización de la resistencia a antimicrobianos en efluentes urbanos, se identificó una mayor proporción de resistencias en aislados de *C. coli* que en aislados de *C. jejuni*, siendo significativa la diferencia encontrada para estreptomicina y tetraciclina ($p<0,001$). El hecho de que los patrones de resistencia detectados sean similares a los descritos en aislados humanos, sugiere la posibilidad de utilizar esta matriz como un buen indicador del nivel de resistencias en cepas de *Campylobacter* de la comunidad.

Por último, se detectaron los trece genes que conforman el SST6 en el 14% (9/63) de los aislados de *C. jejuni* analizados. Se identificaron aislados positivos en todas las matrices de la cadena alimentaria (contenido cecal, piel de cuello y pechuga de pollo fresca envasada), aunque ninguno de los aislados de efluentes urbanos fue positivo. Nuestros resultados indican que la presencia del gen *hcp* (*hemolysin coregulated protein*), utilizado como indicador de la presencia de SST6, no implica la presencia del sistema completo, ya que algunas cepas poseían dicho gen y carecían del resto de genes propios del sistema (2/63; 3%). Así, el gen *hcp* no debería ser utilizado como marcador único para determinar la presencia del SST6 completo.

# 2. SUMMARY

Thermophilic *Campylobacter* species, in particular *C. jejuni* and *C. coli*, have been recognized as important gastrointestinal pathogens since the late 70s. Generally, they are commensal microorganisms of the animal intestine. However, these bacteria often result in pathological processes in the human gut, varying in severity from subclinical colonization, abdominal pain or diarrhea to severe neuropathies like Guillain-Barré syndrome or its variant, Miller Fisher syndrome.

Currently, campylobacteriosis is recognized as the most frequent food-borne zoonoses in the European Union, associated with meat handling and consumption, especially poultry meat. In addition, there are alternative ways of transmission, such as consumption of contaminated water and contact with animals (both domestic and wild) or polluted environments. Due to its fragility and specific growth requirements, it is considered a "fastidious-growth" microorganism.

In the present doctoral thesis, the presence of thermophilic *Campylobacter* in samples from foods and animals taken at different stages of the food chain (chicken ceacal content, chicken neck skin and packed fresh chicken breast) was investigated. In addition, urban effluents from a sewage water treatment plant, and faeces from free-living animals (domestic and wild) located in a shared geographical environment, were included in the analyses. The purpose of the study was to compare different protocols for *Campylobacter* detection and to obtain information of its epidemiology. Additionally, the study of the presence of a protein secretion system (type VI secretion system or T6SS), related to pathogenicity and survival of some strains of *Campylobacter*, was performed.

The results obtained by microbiological culture indicated presence of thermophilic *Campylobacter* in almost all matrices analyzed, with the exception of the Iberian ibex. The proportion of positive samples varied depending on the matrix analyzed, with higher incidence in neck skin samples (35/38; 92%) and ceacal content (34/38; 90%), followed by packed fresh chicken breast (29/38; 76%), urban effluents (32/50; 64%), wild boar (22/150; 15%) and free-ranging cattle (7/55; 13%). Moreover, the proportion of positive samples varied depending on the various protocols that were used. Direct plating obtained the best results for ceacal content (34/38, 90%) and neck skin (31/38;

82%), while enrichment resulted in more positive samples for freshly packed chicken breast (26/38; 68%) and urban effluents (19/50; 38%). Real-time PCR confirmed the presence of *C. jejuni* and/or *C. coli* DNA in a high percentage of samples for all matrices: ceacal contents (37/38; 97%), neck skin (38/38, 100%), freshly packed chicken breast (37/38; 97%) and urban effluents (50/50, 100%). These results could be considered as the theoretical maximum of positive samples, acknowledging that PCR does not distinguish between presence of live, dead bacterial cells or free DNA. Additionally, this technique offered quantitative estimates on the amount of thermophilic *Campylobacter* DNA in each sample, which could be useful as a guide to choose the most suitable detection protocol that corresponded with the identified bacterial load.

The distribution of the detected species varied depending on the matrix analyzed. A higher proportion of *C. coli* isolates was found in ceacal content (55/82; 67%), chicken breast (27/48; 56%) and urban effluents (53/77; 69%), while a higher proportion of *C. jejuni* was observed in neck skin samples (64/103; 62%). Furthermore, it was discovered that the proportion of species detected was significantly influenced by the detection protocol used, since enrichment favored the detection of *C. coli* in samples of the food chain and urban effluents, while direct plating achieved a higher proportion of samples positive to *C. jejuni*. Thus, the use of various protocols for *Campylobacter* detection could favor the identification of the different species present in the sample.

In the study conducted in free-living animals, *C. jejuni* was detected in wild boar and free-ranging cattle (1/150; 0.7% *vs* 5/55; 9%), whereas *C. coli* was only identified in wild boar samples (3/150, 2%). Besides, *C. lanienae* was observed in both groups (12% in wild boar *vs* 2% in free-ranging cattle), being the predominant species in wild boar. These results indicate that there may be a potential exchange of *Campylobacter* populations between wild boar and free-ranging cattle. In our study area, it seems that Iberian ibex does not play a role in the epidemiology of these bacteria.

The genetic characteristics of the isolates obtained from the food chain were determined. The results indicated that the bacterial population isolated from neck skin had the greatest richness of genotypes. Based on the genotypes obtained, it was concluded that *Campylobacter* isolates found in samples of breast and neck skin may have resulted from both contamination from the same animal and/or batch (autogenous contamination), as well as from cross contamination with strains coming

from different animal batches at the slaughterhouse. Moreover, the richness of genotypes found tended to decline towards the end of the production chain. These findings were influenced by the culture protocol used, which means that the choice of a particular microbiological culture protocol could have important implications when comparing epidemiological studies.

The antimicrobial resistance of the obtained isolates was determined, and the outcome of this resulted in a higher proportion of resistance in *C. coli* than in *C. jejuni* isolated from urban effluents, although the difference found was only significant for streptomycin and tetracycline ($p<0.001$). The fact that the resistance patterns detected in urban effluents are similar to those described in human isolates, suggests the possibility of using this matrix as a reliable indicator of the level of resistance in *Campylobacter* isolates present in the community.

A final analysis concentrated on the thirteen genes that make up the T6SS; these were detected in 14% (9/63) of *C. jejuni* isolates tested. Positive isolates to this mechanism were identified in all matrices of the food chain (ceacal content, neck skin and freshly packed chicken breast), although none of urban effluent isolates was found positive. Our results indicate that the presence of the *hcp* gene (coding for hemolysin coregulated protein), cannot be used as a marker of the presence of T6SS, since some strains that contained this gene lacked other genes of T6SS (2/63; 3%). Consequently, *hcp* gene should not been applied as the sole indicator for the presence of a complete T6SS.

# 3. INTRODUCCIÓN

## 3.1. *Campylobacter*

### 3.1.1. Antecedentes históricos

La primera observación de *Campylobacter* tuvo lugar en 1886 [1], aunque no fue hasta 1906 cuando se aisló a partir de la mucosa uterina de una oveja gestante [2]. El mismo organismo también fue aislado en 1919 en abortos bovinos en Estados Unidos (EEUU), denominándose *Vibrio fetus* [3]. Años más tarde, este microorganismo se relacionó con casos de disentería en terneros y cerdos, describiéndose dos nuevas especies: *Vibrio jejuni* y *Vibrio coli*, respectivamente [1]. En 1942, se publicó un estudio en el que se advirtió la asociación entre la presencia de *V. jejuni* y un brote de gastroenteritis en varios centros penitenciarios del estado de Illinois, en EEUU [4].

En la década de los 50, se hizo una clara distinción entre los vibrios clásicos (*V. fetus*), relacionados con problemas reproductivos, y aquellos cuya temperatura de crecimiento era cercana a los 42°C (*V. jejuni* y *V. coli*), asociados a procesos gastrointestinales [5]. La creación del género *Campylobacter* tuvo lugar en 1963 gracias a Sebald y Véron, que propusieron la separación de estos microorganismos del género *Vibrio*, a pesar de su semejanza morfológica [6]. En 1968, se aisló *Campylobacter jejuni* a partir de muestras de sangre y heces de un paciente con fiebre y diarrea, siendo reconocido oficialmente como patógeno entérico para el hombre en la década de los 70 [1].

Actualmente, dentro de la familia *Campylobacteraceae,* se incluyen tres géneros: *Campylobacter*, *Arcobacter* y *Sulfurospirillum*. Tanto los microorganismos del género *Campylobacter* como los del género *Arcobacter*, incluido previamente en el género *Campylobacter* por su semejanza fenotípica, han sido reconocidos como patógenos para el hombre y los animales. Sin embargo, las bacterias del género *Sulfurospirillum* han sido aisladas de suelo y aguas contaminadas pero no han sido relacionadas ni con personas ni con animales [6].

### 3.1.2. Características generales

*Campylobacter* es un microorganismo Gram negativo, incluido en el orden *Campylobacterales*, familia *Campylobacteraceae*, género *Campylobacter*. Se trata de

bacterias de pequeño tamaño (0,2-0,8 µm de grosor y 0,5-5 µm de longitud). Generalmente son oxidasa positivas, a excepción de *Campylobacter gracilis* [7]. Tienen forma de "S" o espiral en fase de crecimiento logarítmico, pero pueden adquirir forma cocoide en cultivos prolongados o cuando están expuestos a altas concentraciones de oxígeno [8]. Muestran un movimiento rápido y característico en forma de sacacorchos gracias a la presencia de un flagelo, el cual puede aparecer en uno o ambos extremos [9]. Existen algunas excepciones como algunas cepas inmóviles de las especies *C. gracilis* y *Campylobacter hominis* o con múltiples flagelos como miembros de la especie *Campylobacter showae* [6].

### 3.1.3. Especies incluidas

Actualmente existen 25 especies y 11 subespecies dentro del género *Campylobacter* (www.bacterio.net). En los últimos años se ha descrito la importancia que algunas tienen como patógenos para humanos y animales, reconociéndolas en algunos casos como emergentes [10]. Entre las especies más destacables del género se incluyen *Campylobacter jejuni, Campylobacter coli, Campylobacter lari, Campylobacter upsaliensis, Campylobacter concisus, Campylobacter ureolyticus o Campylobacter fetus* [11, 12].

Sin embargo, *C. jejuni* (*C. jejuni* subsp. *jejuni*) y *C. coli* son las que más frecuentemente se asocian con casos de campilobacteriosis en humanos (especialmente *C. jejuni*). A nivel europeo, la campilobacteriosis es la zoonosis de transmisión alimentaria más frecuente debido al consumo de alimentos de origen animal [11], siendo las aves de corral su principal hospedador [13]. Por este motivo, el presente trabajo se ha basado fundamentalmente en el estudio de estas dos especies.

### 3.1.4. Características relacionadas con el crecimiento

*Campylobacter* es una bacteria de crecimiento lento (hasta 96h), habiéndose observado diferencias en la velocidad de crecimiento en función de la especie [10]. Son organismos muy sensibles al oxígeno y los radicales libres, por lo que son consideradas bacterias esencialmente microaerofílicas (85% $N_2$, 3-5% $CO_2$, 5-10% $O_2$) [7]. Sin embargo, se han descrito algunas cepas capaces de crecer aeróbica y anaeróbicamente, o incluso que requieren una atmósfera concreta (enriquecida con hidrógeno o ácido fórmico) [6].

En relación con la temperatura, la mayoría de las especies del género son denominadas termófilas [13]. No obstante, no existe uniformidad en el uso de este término y algunos autores califican a estas bacterias como termo-tolerantes, ya que no presentan crecimiento a temperaturas elevadas (55°C o superiores) [14]. Normalmente, la mayoría de las especies crecen de forma óptima a 41,5°C, aunque también pueden hacerlo a temperaturas inferiores como 37°C, pero no por debajo de los 30°C [7]. Existen excepciones como algunas cepas de *C. fetus,* que no suelen ser capaces de crecer a 42°C pero sí lo hacen a temperaturas inferiores (incluso menores de 30°C) [6]. En general, cuando se emplee el término "termófilos" se hará referencia a las especies *C. jejuni* y *C. coli.*

### 3.1.5. Características relacionadas con la supervivencia

*Campylobacter* es una bacteria relativamente sensible a diversos factores de estrés ambiental como temperatura, desecación, radiación ultravioleta, altas concentraciones de cloruro de sodio o bajos niveles de pH [15]. Asimismo, el crecimiento de *Campylobacter* fuera del hospedador es limitado, como consecuencia de su carácter termófilo y microerofílico [13, 16, 17]. Sin embargo, es capaz de persistir en el ambiente y en los alimentos, considerándose una de las bacterias más ubicuas de la cadena alimentaria [15]. Normalmente, la supervivencia de *Campylobacter* se ve favorecida a bajas temperaturas, en ausencia de luz y a bajas concentraciones de oxígeno, aunque la presencia de organismos competidores dificulta su aislamiento [8, 18].

Se ha sugerido que algunos genes de respuesta al estrés propios de cada cepa pueden influir en la supervivencia bacteriana ante condiciones ambientales desfavorables [19]. Igualmente, en situaciones de estrés ambiental o condiciones de crecimiento potencialmente letales, *Campylobacter* entra en un estado de célula viable pero no cultivable (VPNC) [20]. Éste se ha definido como un estado de inactividad en el cual el crecimiento bacteriano en los medios de cultivo frecuentemente usados cesa, mientras que la bacteria se mantiene viable con una actividad metabólica mínima [21].

### 3.1.6. Detección y caracterización

El cultivo bacteriológico de *Campylobacter* puede ser un reto debido a la fragilidad y los requerimientos específicos de estos organismos para crecer en condiciones de laboratorio, habiendo sido descritos como microorganismos de "cultivo fastidioso" [22]. Generalmente, las metodologías descritas para el cultivo de la bacteria dependen

de la naturaleza de la muestra que se pretende analizar, siendo necesario mantener un equilibrio entre la inhibición de microorganismos competidores y la recuperación específica de *Campylobacter* hasta límites detectables [23]. No obstante, en las últimas décadas se ha dedicado mucho esfuerzo para mejorar los protocolos de detección de este microorganismo y facilitar el aislamiento rutinario del mismo [13].

Debido a las dificultades descritas en el cultivo tradicional, en la actualidad también es común el uso de otras técnicas más rápidas que proporcionan nuevas oportunidades para la detección de *Campylobacter* [24]. Por ejemplo, la PCR en tiempo real ha supuesto una mejora en la identificación del patógeno en muestras de diversos orígenes [24-28], ya que permite la detección de *Campylobacter* spp. de forma más rápida y sensible [29]. Sin embargo, aunque es posible cuantificar la cantidad de bacteria presente en la muestra, no diferencia si se trata de bacterias vivas, muertas, VPNC o ADN libre [30].

En lo que respecta a la caracterización genética de *Campylobacter*, una de las técnicas más utilizadas en la actualidad está basada en la secuenciación de la región variable del gen de la flagelina, *fla*A (*flaA-SVR*, *short variable region*). La secuencia obtenida se contrasta con la información depositada en una base de datos pública denominada PubMLST (http://pubmlst.org/campylobacter/) para obtener un perfil numérico [31]. Otra técnica ampliamente utilizada es el MLST (*Multilocus Sequence Typing*), la cual se basa en la secuenciación de un fragmento de siete genes conservados que permite la obtención de un perfil alélico denominado secuencia tipo (*Sequence Type* o ST) [32].

### 3.2. Patogénesis de las infecciones por *Campylobacter*

Las características de la infección (síntomas, severidad y duración) producida por *Campylobacter* termófilos dependen de la virulencia del aislado y de la respuesta inmune del hospedador [6, 7]. La diversidad genética y fenotípica que existe entre cepas y la habilidad de la bacteria para regular la expresión de sus genes, podría explicar la diferencia en patogenicidad de estos microorganismos [33]. A continuación se presenta un resumen no exhaustivo de algunos de los factores que pueden contribuir a la patogenicidad de *Campylobacter* en las especies termófilas.

### 3.2.1. Flagelo

La motilidad de *Campylobacter* se produce mediante flagelos polares que pueden aparecer en un extremo o en ambos. Se ha observado que el flagelo es esencial para contrarrestar los movimientos peristálticos del intestino favoreciendo la colonización, además de para acceder y atravesar la mucosa del epitelio intestinal. Así, el flagelo desempeña un papel activo en el proceso de invasión de las células epiteliales y se ha relacionado con la supervivencia bacteriana a lo largo del tracto gastrointestinal [6, 7].

### 3.2.2. Adhesinas

*Campylobacter* puede sobrevivir libre en la mucosa intestinal o adherirse a las células del epitelio intestinal. Tras la adhesión, se puede llevar a cabo la invasión de las células, lo cual conlleva un daño celular y la consecuente pérdida de funcionalidad y diarrea [6]. Existen diversos factores que están relacionados con la adherencia e invasión bacteriana, destacando el papel de las adhesinas. En *C. jejuni* se han descrito diversas adhesinas como la PEB1 (*binding periplasmic protein*), la JlpA (*jejuni lipoprotein A*), la CapA (*putative autotransporter*), la PorA (*major outer membrane protein*, MOMP) o la proteína de superficie CadF (*Campylobacter adhesion to fibronectin*), esta última también descrita en *C. coli* [33-35].

### 3.2.3. Cápsula y lipopolisacárido/lipoligosacárido

La cápsula de *C. jejuni* presenta una estructura hiper-variable, dentro de la misma cepa y entre cepas, y ha sido relacionada con la adhesión, invasión y virulencia bacteriana [35]. Además, *C. jejuni* es capaz de producir lipopolisacárido (LPS), lipoligosácarido (LOS) o ambos y, al igual que la cápsula, tienen una estructura altamente variable. Estos componentes se han involucrado con la adhesión e invasión celular y la evasión de la respuesta inmune del hospedador [35]. La estructura del LOS de algunas cepas de *C. jejuni* puede presentar mimetismo molecular con gangliósidos neuronales, lo cual se ha asociado con el desarrollo del síndrome de Guillain-Barré y Miller-Fisher (ver apartado 3.3) por la reacción cruzada de los anticuerpos producidos [14, 35]. También se ha comprobado que el LOS/LPS bacteriano, debido a la presencia del lípido A, tiene actividad endotóxica como ocurre en el resto de bacterias Gram negativas [35].

### 3.2.4. Sistema de secreción de proteínas

Se ha observado que *C. jejuni* posee diferentes sistemas de secreción de proteínas. El sistema de secreción flagelar tipo III (SST3 o *flagellar export apparatus*) está localizado en el flagelo. Este sistema es capaz de facilitar la secreción de proteínas no flagelares como las proteínas Cia o FlaC, las cuales se ha sugerido que podrían desempeñar un papel relacionado con la virulencia de la bacteria [33-35]. Recientemente se ha descrito la presencia de otro sistema de secreción denominado sistema de secreción tipo VI o SST6 (**Figura 1a y 1b**). En el caso de *C. coli*, se han identificado mediante secuenciación masiva algunos genes putativos que podrían codificar proteínas de este sistema, aunque no se ha identificado su papel en relación a la patogenicidad [12]. En *C. jejuni* se ha sugerido que el SST6 contribuye a la patogénesis de la bacteria mediante la liberación de macromoléculas al medio, que pueden ser tóxicas para otras bacterias y para las células eucariotas, como los glóbulos rojos. Asimismo, se ha relacionado la presencia de este sistema con la supervivencia bacteriana en presencia de sales biliares y deoxicolato y con mecanismos de colonización, adherencia e invasión celular [36, 37].

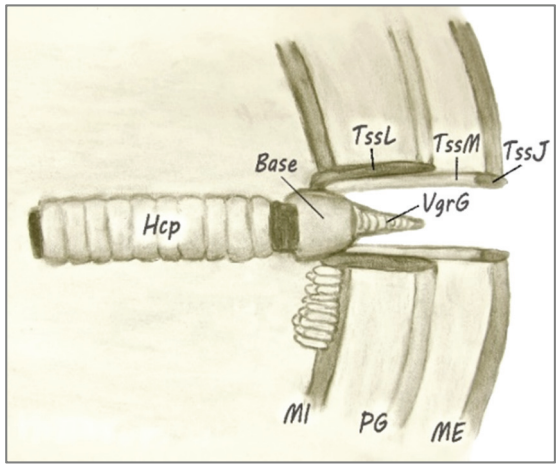


**Figura 1a.** Representación esquemática de los componentes del sistema de secreción tipo VI inactivo. Las tres proteínas de membrana (TssJ, TssL, TssM) forman un complejo en contacto con el peptidoglicano (PG) que permite la unión de la estructura tubular, formada por las proteínas Hcp (*hemolysin coregulated protein*) y VgrG (*valine-glycine repeat protein G*), a la membrana externa (ME). Asimismo, esta estructura tubular está conectada a la membrana interna (MI) gracias a una plataforma que sirve de base (*baseplate-like*).

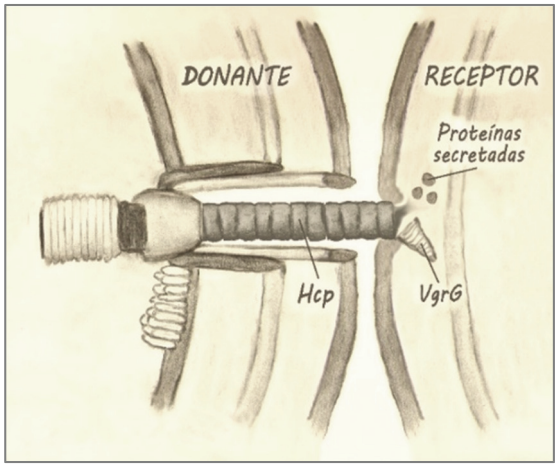


**Figura 1b.** Representación esquemática del sistema de secreción tipo VI activo. La activación del sistema supone la liberación de proteínas efectoras a la célula diana o al medio a través del tubo formado por hexámeros de Hcp.

### 3.2.5. Toxina CDT

*Campylobacter* es capaz de producir una holotoxina denominada CDT (*cytolethal distending toxin*). Esta toxina está formada por tres subunidades, codificadas por los genes *cdt*A, *cdt*B, *cdt*C, necesarias para que la toxina sea activa. El principal efecto que produce es la muerte de las células eucariotas, probablemente al impedir la mitosis o inducir daño en el ADN [38].

## 3.3. Aspectos clínicos de la infección

El proceso infeccioso ocasionado por las bacterias incluidas en el género *Campylobacter* se denomina campilobacteriosis. La mayoría de las investigaciones se han dirigido al estudio de *C. jejuni*, especialmente en el área de las complicaciones extra-intestinales asociadas. No obstante, el proceso clínico producido por *C. jejuni* y *C. coli* es muy similar, siendo su sintomatología indistinguible [39]. De esta manera, ambas especies se tratan de forma conjunta en este epígrafe.

Las infecciones ocasionadas por *Campylobacter* termófilos en el hombre suelen producir malestar, fiebre, dolor abdominal intenso, náuseas y, ocasionalmente vómitos (15% de los casos) [39]. Los pacientes presentan diarrea que puede ser leve, severa, mucosa y frecuentemente sanguinolenta, que suele prolongarse 3-4 días [40].

Se trata de una enfermedad auto-limitante cuya duración suele ser menor a una semana [39].

Sin embargo, tras la infección por *Campylobacter* en personas, pueden aparecer complicaciones (**Tabla 1**) tales como trastornos reumatológicos (artritis reactiva) o neuropatías periféricas (síndrome de Guillain-Barré o su variante, el síndrome de Miller Fisher). En la actualidad, el síndrome de Guillain-Barré es una de las complicaciones más severas de la campilobacteriosis y la forma más común de parálisis fláccida que afecta al sistema nervioso periférico en los países occidentales [41]. Se ha descrito que una de cada 1.000 infecciones por *C. jejuni* tiene como consecuencia la aparición de esta complicación [42]. En el caso del síndrome de Miller Fisher, caracterizado por oftalmoplejia y ataxia, hasta el 20% de los casos están asociados con una infección previa por *C. jejuni* [43]. Otra complicación severa es la bacteriemia, que suele producirse únicamente en el 1% de los pacientes, normalmente individuos inmunocomprometidos, mujeres embarazadas o ancianos [39]. Según datos de la EFSA (*European Food Safety Authority*), la severidad en términos de letalidad (*case-fatality rate*) suele ser baja teniendo en cuenta el elevado número de casos de campilobacteriosis, estando en el 0,05% en la Unión Europea (UE) en 2013 [11].

A continuación se presenta una tabla resumen (Tabla 1) de las principales complicaciones descritas tras la infección por *Campylobacter* termófilos [6, 39, 40].

**Tabla 1.** Complicaciones asociadas a la infección por *C. jejuni* y *C. coli*.

| Intestinales | Sistema hepatobiliar | Sistema renal y urinario | Otras | Tardías |
|---|---|---|---|---|
| Apendicitis | Hepatitis | Nefritis | Bacteriemia | Síndrome de Guillain-Barré |
| Megacolon tóxico | Colecistitis | Prostatitis | Miocarditis | Síndrome de Miller Fisher |
| Hemorragia intestinal | Pancreatitis | Cistitis | Rotura esplénica | Artritis reactiva |
| Absceso perirrectal | | | Urticaria/eritema nodoso | |
| Peritonitis | | | Abortos | |
| EII* post-infección | | | Sepsis neonatal | |

*EII: enfermedad inflamatoria intestinal.

En los animales, la presencia de *Campylobacter* suele cursar de forma subclínica, aunque se ha observado diarrea en animales jóvenes como perros, gatos y terneros [6, 44], hepatitis en aves como gallinas y avestruces [45] y abortos en rumiantes [46].

Por otro lado, también hay otras especies del género *Campylobacter* distintas de *C. jejuni* y *C. coli* que son capaces de ocasionar diarrea en el hombre como es el caso de *C. lari*, *C. upsaliensis*, *C. concisus* o *C. fetus* subsp. *fetus* [12, 47]. Además, esta última especie puede ocasionar infecciones sistémicas en personas, especialmente en individuos inmunocomprometidos [47, 48], y abortos esporádicos en ovejas y vacas. Finalmente, *C. fetus* subsp. *veneralis* causa problemas reproductivos como infertilidad, disminución de las tasas de preñez o abortos en el ganado bovino (campilobacteriosis genital bovina) [49].

Por último, algunas especies de la familia *Campylobacteraceae*, como el género *Arcobacter* (*Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter skirrowii*), son consideradas patógenas ya que pueden ocasionar diarrea acuosa y otros síntomas gastrointestinales en el hombre, así como abortos, mastitis y diarrea en animales domésticos [6, 50].

### 3.4. Resistencia a antimicrobianos

Generalmente, el tratamiento de las infecciones producidas por *Campylobacter* termófilos está basado en la aplicación de antimicrobianos únicamente en casos concretos. Este tipo de situaciones incluyen pacientes en los que la infección lleve asociada complicaciones graves, en infecciones sistémicas o como medida de control en grupos de alto riesgo [1, 9, 51]. Actualmente, en los casos en los que este tratamiento sea necesario, las quinolonas y, especialmente los macrólidos, son los antimicrobianos más utilizados [48, 52], aunque en casos de bacteriemia o infección sistémica, es habitual la utilización de aminoglucósidos por vía intravenosa [53].

La aparición de cepas de *Campylobacter* resistentes a antimicrobianos dificulta el tratamiento de los pacientes y supone así un importante problema de Salud Pública [53, 54]. Además, se ha descrito que los pacientes infectados con cepas resistentes a quinolonas o eritromicina sufren un proceso más severo (mayor duración, enfermedad sistémica o muerte) que los pacientes infectados con cepas sensibles [55], aunque se desconoce si estos problemas son resultado de la resistencia a antimicrobianos o de otros factores de virulencia asociados a estas cepas resistentes [54]. De hecho, otros autores han concluido que las infecciones con cepas resistentes a fluoroquinolonas

no ocasionan un proceso más largo y severo que las ocasionadas por cepas sensibles [56].

*Campylobacter* posee mecanismos de resistencia frente a antimicrobianos que son inherentes a la bacteria, mientras que otros han sido adquiridos mediante mutaciones o transferencia genética horizontal. Algunos de los mecanismos más frecuentes descritos en este microorganismo son la síntesis de enzimas modificadoras o inactivadoras, la alteración o protección del lugar de unión del antimicrobiano, la expulsión del antimicrobiano fuera de la célula mediante bombas de eflujo o la reducción de la permeabilidad de la bacteria [57, 58]. En la **Tabla 2** se resumen los principales mecanismos de resistencia de *C. jejuni* y *C. coli* frente a los grupos antimicrobianos empleados con mayor frecuencia en clínica [52, 53, 58-60].

**Tabla 2**. Principales mecanismos de resistencia frente a antimicrobianos en *C. jejuni* y *C. coli*.

| Grupo antimicrobiano | Antimicrobiano | Principal mecanismo de resistencia | Otros mecanismos |
|---|---|---|---|
| Quinolonas | Ciprofloxacina, ácido nalidíxico | Mutación en la subunidad *gyr*A de la enzima ADN girasa | Bomba de eflujo *CmeABC* |
| Macrólidos | Eritromicina | Mutación en el gen 23S rRNA o en las proteínas ribosómicas L4 o L22<br>Metilación en el gen 23S rRNA | Bomba de eflujo *CmeABC* |
| Aminoglucósidos | Estreptomicina, gentamicina | Enzimas modificadoras de antimicrobiano (fosfotransferasas, adenililtransferasas o acetiltransferasas) | |

Las cepas de *Campylobacter* termófilos resistentes a antimicrobianos pueden aparecer tanto en animales como en personas [54]. Actualmente, se llevan a cabo programas de vigilancia sobre los niveles de resistencia encontrados en animales de abasto, alimentos y personas, siendo variables los resultados en función del país [48]. Estos programas de vigilancia permiten comparar la evolución de los perfiles de resistencia a antimicrobianos a lo largo del tiempo, así como la detección de nuevos patrones de resistencia. En 1997, se creó en España la Red VAV (Red de Vigilancia de Resistencias a Antimicrobianos en Bacterias de Origen Veterinario) con el objetivo de proporcionar información sobre los niveles de resistencia a antimicrobianos en las bacterias presentes en animales sanos, animales enfermos y alimentos de origen animal. El

programa se basa fundamentalmente en el estudio de las bacterias productoras de zoonosis transmitidas por alimentos, así como de los microorganismos indicadores de resistencia a antimicrobianos [61]. Además, desde el año 2004, los datos obtenidos por esta Red son incluidos en el Informe Anual de Resistencias Antimicrobianas de la UE publicado por la EFSA [48].

En el caso de las quinolonas, en las últimas dos décadas se ha observado un incremento rápido de los porcentajes de resistencia a ciprofloxacina en cepas de *Campylobacter* en todo el mundo. Según datos de la EFSA, el 54,6% de las cepas de *C. jejuni* y el 66,6% de *C. coli* procedentes de casos clínicos en personas en la UE, fueron resistentes a ciprofloxacina [48]. Algunos estudios han relacionado este incremento en las resistencias con el uso de estos antimicrobianos en animales de abasto, especialmente en la industria avícola [1, 62, 63]. En la UE, los elevados niveles de resistencia a ciprofloxacina en *Campylobacter* procedentes de muestras de pollos de aptitud cárnica (*broiler*) y, especialmente de carne (53% *C. jejuni* y 76,2% *C. coli*), se han convertido en un serio problema de Salud Pública, ya que se estima que hasta el 30% de las infecciones por *Campylobacter* en humanos se atribuyen al manejo y/o consumo de este tipo de muestras/alimentos [48].

Si nos fijamos en la resistencia a eritromicina, los porcentajes detectados en aislados clínicos en la UE son bajos para *C. jejuni* (1,5%) y moderadamente altos para *C. coli* (13,4%) [48]. En los últimos años se ha descrito un incremento de la resistencia a eritromicina, especialmente en cepas animales de *C. coli*, en algunas regiones del mundo. Por ejemplo, en Estados Unidos se han registrado porcentajes de resistencia a eritromicina superiores al 40% en cepas de *C. coli* procedentes de pavos y cerdos [59] y del 56,7% en muestras de pollo en Letonia [64]. No obstante, los porcentajes de resistencia simultánea a ciprofloxacina y eritromicina (co-resistencia) en cepas clínicas humanas se mantienen bajos en la UE (1,7% *C. jejuni* y 4,1% *C. coli*) [48].

Por último, la resistencia frente a gentamicina se mantiene moderada en la UE para cepas clínicas de *C. coli* (11,1%) y baja para *C. jejuni* (0,6%) [48].

### 3.5. Epidemiología

El estudio de la epidemiología de *Campylobacter* termófilos es complejo, ya que hay un gran número de casos esporádicos de campilobacteriosis y múltiples causas de infección a partir de organismos con una elevada diversidad genética [32]. A

continuación se exponen algunas de las características epidemiológicas relacionadas con la infección de *Campylobacter*.

### 3.5.1. Estacionalidad

Una característica importante de la epidemiología de *Campylobacter* termófilos es su marcada estacionalidad [65]. En Europa, fundamentalmente en los países que tienen un clima templado, suele aparecer una mayor incidencia de casos a finales de primavera y comienzos de verano [13, 66]. Sin embargo, la causa de dicho fenómeno no ha sido claramente identificada y diferentes aspectos han sido relacionados con la estacionalidad. Así, el aumento de la temperatura ambiental [44] podría suponer un incremento en la población de moscas (vectores), que aumentarían las posibilidades de contacto con el microorganismo [65]. También se han relacionado los cambios en los hábitos alimentarios durante esta época, siendo más frecuente el consumo de alimentos crudos y/o poco cocinados (barbacoas, picnics). Igualmente, los hábitos de ocio se modifican y las actividades recreativas ligadas al agua son más habituales [44, 66].

### 3.5.2. Distribución de los casos

La campilobacteriosis es una enfermedad de carácter esporádico y, por lo tanto, la aparición de brotes suele ser poco frecuente [8]. Generalmente, son pequeños y de carácter familiar, siendo la identificación microbiológica de la fuente de contaminación complicada. En ocasiones, el alimento ha sido completamente consumido o, tras un almacenamiento prolongado, la recuperación de la bacteria puede resultar difícil debido a la exposición al oxígeno y la desecación [18, 67]. Según la EFSA, el 8% de los brotes transmitidos por alimentos en 2013 (414 casos) fueron producidos por *Campylobacter* termófilos, siendo la carne de pollo la fuente más común de infección [11].

### 3.5.3. Incidencia

La incidencia de campilobacteriosis varía entre países industrializados y países en vías de desarrollo. En el primer escenario, personas de todas las edades pueden desarrollar la enfermedad, siendo la gran mayoría de los infectados sintomáticos. Los dos grupos en los que la incidencia suele ser superior son jóvenes y adultos de entre 15-44 años y los niños menores de cuatro años [1, 9, 44]. También se ha observado que la incidencia de la infección es generalmente más alta en hombres que en mujeres, lo cual podría asociarse con la diferencia de hábitos alimenticios entre los dos grupos

[67]. Por el contrario, en los países en vías de desarrollo los pacientes son frecuentemente asintomáticos y la enfermedad es considerada esencialmente pediátrica, siendo los niños menores de 2 años los más afectados [6].

### 3.5.4. Prevalencia

A nivel mundial las especies de *Campylobacter* termófilos son reconocidas como la principal causa bacteriana de enteritis [7]. En los países industrializados, *C. jejuni* produce el 80-90% de las infecciones y *C. coli* el 5-10% [67], aunque se estima que la frecuencia de *C. coli* podría ser superior a los datos publicados [68, 69]. Según los últimos datos de la EFSA, en 2013 se confirmaron 214.779 casos de campilobacteriosis en personas en la UE, siendo la tasa de notificación de 64,8 casos por cada 100.000 habitantes [11]. Generalmente, los datos varían entre países, e incluso entre regiones dentro de un mismo país. Estas discrepancias se deben, probablemente, a las diferencias en los sistemas de producción de alimentos, los hábitos de consumo o incluso, el sistema de diagnóstico y de notificación [44]. Frecuentemente, el número de casos no confirmados también es importante, estimándose que la incidencia real de campilobacteriosis humana en Europa es de aproximadamente 9 millones de casos [70]. Además, el alto número de infecciones relacionadas con esta bacteria conlleva un coste económico considerable derivado de los gastos médicos y de productividad [7]. A pesar de que los síntomas no suelen ser graves (ver apartado 3.3), la proporción de casos que requieren hospitalización es elevada, habiéndose estimado en un 43,6% para el año 2013 en la UE [11, 42]. Según la EFSA, el coste derivado de los casos declarados de campilobacteriosis en la UE es de 2.400 millones de euros al año [71].

### 3.5.5. Principales hospedadores

Aunque las especies termófilas de *Campylobacter* han sido descritas incluso en insectos [72], estos microorganismos colonizan mayoritariamente el tracto intestinal de animales homeotermos, tanto domésticos como de vida libre, siendo la mayor parte de ellos hospedadores asintomáticos [13]. En general, se ha observado que el intestino de aves, especialmente aves de corral, es un ambiente favorable para estas especies [73].

#### 3.5.5.1. Animales domésticos

Las aves de corral, incluidos los *broiler*, las gallinas ponedoras, los pavos o los patos, son los principales portadores asintomáticos de *Campylobacter* termófilos, habiéndose observado cargas superiores a $10^8$ Unidades Formadoras de Colonias

(UFC)/g en el ciego [70]. También se ha descrito la presencia de *Campylobacter* termófilos en otras especies como cerdos y rumiantes [74-76], aunque la carga bacteriana presente en sus heces es inferior a la observada en aves (alrededor de $10^2$ UFC/g) [18]. Asimismo, los perros y los gatos han sido reconocidos como hospedadores asintomáticos de *Campylobacter* termófilos [44].

A continuación se presenta una tabla con datos de prevalencia de *Campylobacter* termófilos en distintos estudios llevados a cabo en animales domésticos. Se puede observar la elevada variabilidad de resultados en función del estudio, el método de detección, la especie animal muestreada o la localización geográfica, entre otros (**Tabla 3**).

**Tabla 3.** Estudios de detección de *Campylobacter* termófilos en animales domésticos.

| Referencia | Año | Localización | Animal | Muestreo | Detección de *Campylobacter* (%) |
|---|---|---|---|---|---|
| [77] | 2014 | Vietnam | Pollo/Pato/Cerdo | Granja | 32/24/54 |
| [78] | 2014 | Portugal | Pollo cría intensiva | Matadero | 79 |
| [79] | 2014 | EEUU | Pavo | Granja/ Matadero | 56 |
| [74] | 2014 | Grecia | Oveja/Cabra | Matadero | 34/8 |
| [80] | 2014 | España | Pollo cría intensiva | Granja | 38 |
| [81] | 2013 | Polonia | Perro/Gato | Doméstico/ Refugios | 4/10 |
| [75] | 2013 | Alemania | Cerdo engorde | Granja | 38 |
| [82] | 2013 | Malasia | Pato | Granja | 12 |
| [83] | 2012 | Nueva Zelanda | Pollo granja familiar | Granja | 58 |
| [84] | 2012 | Reino Unido | Pollo cría orgánica | Matadero | 94 |

| Referencia | Año | Localización | Animal | Muestreo | Detección de *Campylobacter* (%) |
|---|---|---|---|---|---|
| [85] | 2011 | Reino Unido | Pato | Granja | 93-100 |
| [86] | 2010 | Francia | Vacuno carne y leche | Matadero | 16 |
| [87] | 2010 | Suecia | Pollo cría intensiva | Matadero | 47 |
| [88] | 2009 | Irlanda | Pollo cría intensiva | Granja | 82 |
| [89] | 2007 | EEUU | Vacuno leche | Matadero | 51 |

3.5.5.2. Animales salvajes

Aunque los animales salvajes son portadores y tienen un papel potencial en la transmisión de *Campylobacter* termófilos [90-93], su importancia relativa es menor que la de los animales domésticos [94-96]. En general, en animales salvajes las prevalencias varían en función de la ecología de cada especie, incluyendo hábitos alimenticios, coprofagia, patrones de migración, hábitat, cría o periodo de muda [90, 93, 95]. Además, en estudios realizados en este tipo de animales, se han aislado otras especies de *Campylobacter* distintos de *C. jejuni* y *C. coli* como *Campylobacter lanienae, C. lari, Campylobacter hyointestinalis* subsp. *hyointestinalis, Campylobacter sputorum* o *C. fetus* [97-102], cuya significación clínica no es del todo conocida.

A continuación se presenta una tabla con un resumen de diferentes estudios de prevalencia de *Campylobacter* termófilos en animales salvajes llevados a cabo en los últimos años (**Tabla 4**).

**Tabla 4.** Estudios de detección de *Campylobacter* termófilos en animales salvajes.

| Referencia | Año | Localización | Animal | Detección de *C. jejuni* (%) | Detección de *C. coli* (%) |
|---|---|---|---|---|---|
| [97] | 2014 | España | Ciervo/Jabalí | 0/2 | 2/6 |
| [103] | 2014 | Italia | Codorniz común | 9 | 21 |
| [91] | 2014 | España | Buitre leonado | 1 | 0 |
| [90] | 2013 | España | Aves acuáticas | 1 | 12 |
| [98] | 2013 | España | Jabalí | 0 | 0 |
| [100] | 2012 | EEUU | Cerdo salvaje | 20 | 3 |
| [92] | 2012 | EEUU | Aves silvestres (granjas) | 4 | 1 |
| [104] | 2009 | Reino Unido | Gorrión europeo | 31 | 1 |
| [105] | 2009 | Italia | Ardilla roja | 8 | 0 |
| [95] | 2009 | Reino Unido | Aves silvestres | 1 | 0 |
| [106] | 2007 | Rep. Checa | Faisán silvestre | 17 | 1 |
| [107] | 2005 | Noruega | Corzo | 3 | 0 |
| [102] | 2003 | Suecia | Alce/Corzo/ Liebre | 1/3/0 | 0/0/1 |
| [93] | 2002 | Suecia | Aves migratorias | 5 | 1 |

### 3.5.6. Vías de transmisión

#### 3.5.6.1. Transmisión alimentaria

La campilobacteriosis es reconocida como una zoonosis en la cual la vía alimentaria es la principal forma de transmisión de la bacteria desde los animales al hombre [108]. A nivel mundial, *Campylobacter* ha sido descrita como la principal causa de

enfermedad diarreica de carácter bacteriano transmitida a través de los alimentos [7]. De hecho, la infección en personas se asocia fundamentalmente con el consumo de alimentos de origen animal contaminados, en especial productos de origen aviar [18, 44].

3.5.6.1.1. Carne de pollo

En la actualidad, los estudios epidemiológicos revelan que existe una asociación significativa entre la infección en humanos y el consumo de carne de ave cruda o poco cocinada, especialmente pollo, ya que es la carne más consumida [13, 32]. Según datos de la EFSA, el 31,4% de las muestras de carne fresca de *broiler* de la UE fueron positivas a *Campylobacter* termófilos en 2013 [11].

En la granja, la difusión de *Campylobacter* se produce rápidamente debido, entre otros factores, al comportamiento de coprofagia habitual en las aves, la contaminación del agua de bebida, de la cama o mediante vectores (como insectos, roedores o incluso el personal de la granja) [17, 80]. La colonización de los animales tiene lugar a partir de las 2-3 semanas de vida [109]. Cuanto mayor es la tasa de contaminación de los animales, mayor es el riesgo de contaminación de los productos obtenidos tras su procesado en el matadero; aunque la mayor fuente de contaminación procede del tracto intestinal, los animales también pueden presentar la bacteria en la piel o las plumas [18]. Así, animales procedentes de lotes no infectados pueden contaminarse en el matadero, especialmente en las etapas de aturdimiento, desplumado, evisceración y refrigeración [44, 110].

Los niveles de *Campylobacter* termófilos en productos frescos varían desde menos de $10^2$ hasta $10^6$ UFC en la superficie de las canales, dependiendo de los estudios y la metodología empleada [18].

3.5.6.1.2. Otros alimentos

La carne de ternera o cerdo también puede estar implicada en la transmisión alimentaria de *Campylobacter* termófilos [11], aunque normalmente en este tipo de productos la presencia de *Campylobacter* es infrecuente y la tasa de contaminación es baja [111]. Asimismo, se ha identificado la presencia de *Campylobacter* en canales de avestruz, jabalí o venado [112, 113]. Existen otros productos de origen animal como la leche cruda de vaca o cabra o los huevos que se han asociado con casos de campilobacteriosis [71]; mientras que la leche se ha relacionado con la aparición de

brotes, los huevos no son un vehículo frecuente para la bacteria debido a su alta sensibilidad a la desecación [18]. Otros alimentos como verduras, frutas o champiñones pueden contener *Campylobacter* siendo la carga observada baja [114, 115], principalmente debido a su contaminación ocasional por la aplicación de enmiendas orgánicas, contacto con aves, animales salvajes o agua superficial no tratada o por contaminaciones cruzadas con otros alimentos [18].

En relación con otras especies de *Campylobacter*, se ha observado que los moluscos y los crustáceos pueden presentar *C. lari* si se encuentran próximos a focos de contaminación como vertidos de depuradora, deshechos de granja o en zonas con intenso contacto con aves acuáticas como gaviotas [44, 115].

#### 3.5.6.2. Transmisión a través del agua

El consumo de agua de bebida contaminada ha sido reconocido como una vía de transmisión de *Campylobacter* termófilos a personas y animales domésticos, aunque de menor importancia que el consumo de alimentos de origen animal [8]. A pesar de que la infección por *Campylobacter* no suele ocasionar grandes brotes, se han descrito algunos asociados al consumo de agua contaminada en diversos países [116].

La presencia de la bacteria en el agua se debe normalmente a la contaminación fecal por aves salvajes o animales domésticos de vida libre, así como por el vertido de deshechos derivados de la agricultura [17]. Asimismo, se ha identificado que el vertido de aguas de depuradora procedentes de centros urbanos, es una de las principales fuentes de contaminación del agua [116].

#### 3.5.6.3. Otras vías de transmisión

La transmisión de *Campylobacter* termófilos también se puede producir por contacto directo con animales o personas infectadas [70]. La transmisión por contacto directo con animales es generalmente profesional, ya que el riesgo suele aumentar en personas con mayor exposición, especialmente durante el manejo de los animales y el procesado de la canal/carne [44, 112]. También puede producirse a nivel doméstico por contacto directo con animales de compañía como perros y gatos [17, 44]. La transmisión perinatal e interpersonal es infrecuente, siendo esta última normalmente descrita en niños [1, 51].

El medio ambiente también puede participar de forma activa en la transmisión de estas especies patógenas [44, 117]. Se ha descrito que la bacteria puede permanecer

en el suelo, la arena de playa y, especialmente en ambientes acuáticos, como el agua dulce o marina [14, 17, 95, 118, 119]. El baño en aguas recreativas como agua de mar, de río, de lago o de piscina ha sido identificado como un factor de riesgo para la infección por *Campylobacter* [119-121]. Además, la contaminación fecal del ambiente (pastos, granjas y particularmente aguas superficiales), supone una posible fuente de contaminación para el hombre y los animales [17, 122]. Dicha contaminación varía de forma estacional, como consecuencia de la carga bacteriana de los animales portadores y los vertidos procedentes de la actividad humana y ganadera (estiércol), y por la influencia de los factores ambientales que pueden condicionar su supervivencia en el medio ambiente [118].

Asimismo, se ha descrito que las aves salvajes pueden actuar como vectores de *Campylobacter* a larga distancia, fundamentalmente si se trata de aves migratorias [103, 123]. Además, la concentración de animales de vida libre en el entorno urbano (gorriones, palomas, gaviotas, ardillas o jabalíes), facilitaría el intercambio de patógenos como consecuencia del contacto directo con las defecaciones de estos animales en zonas frecuentadas por personas o animales como parques públicos, zonas verdes, fuentes o piscinas [124-126].

# 4. OBJETIVOS

Las especies termófilas de *Campylobacter* pueden sobrevivir a lo largo de la cadena de producción de alimentos, siendo los de origen animal la principal fuente de infección para el hombre. Además, estos microorganismos son capaces de persistir en medios acuáticos, como aguas residuales, suponiendo una posible fuente de contaminación para el medio ambiente y, de forma indirecta, para el hombre y los animales. Por otro lado, también colonizan el tracto intestinal de animales de vida libre, por lo que podría existir un flujo bacteriano potencial entre animales salvajes, domésticos y medio ambiente, especialmente en hábitats compartidos.

En el presente trabajo de tesis doctoral se estableció la necesidad de analizar la presencia de *Campylobacter* termófilos i) en muestras tomadas en diferentes fases de la cadena alimentaria (contenido cecal, piel de cuello y pechuga de pollo fresca envasada), como reflejo de las bacterias presentes en los animales de abasto, ii) en efluentes urbanos de una planta de tratamiento de aguas residuales, como muestra representativa de la población humana, y iii) en animales de vida libre (salvajes y domésticos), para evaluar el posible intercambio de *Campylobacter* en un entorno natural con bajo impacto humano.

Ante la dificultad de aislar *Campylobacter* mediante técnicas tradicionales y la diversidad de resultados descritos, se propuso llevar a cabo una comparación de diferentes protocolos de detección de esta bacteria. Asimismo, se estableció la necesidad de caracterizar los aislados obtenidos en las diferentes matrices con el fin de obtener información sobre la epidemiología del microorganismo. Adicionalmente, se planteó el estudio de la presencia de un sistema de secreción de proteínas recientemente descrito (SST6), relacionado con la patogenicidad y supervivencia de *Campylobacter*.

De esta manera, esta tesis ha sido organizada siguiendo los objetivos que se detallan a continuación:

### 4.1. Objetivo I. Detección y aislamiento de *Campylobacter* termófilos en diferentes matrices: comparación de métodos de detección

La dificultad en la detección de *Campylobacter* termófilos, así como la variedad de resultados descritos, nos llevó a evaluar diferentes protocolos de cultivo microbiológico y de detección molecular en muestras tomadas en diferentes fases de la cadena alimentaria (contenido cecal, piel de cuello y pechuga de pollo fresca envasada) y efluentes urbanos de una planta de tratamiento de aguas residuales. El trabajo realizado ha permitido obtener las siguientes publicaciones:

- Evaluación de métodos para la detección de *Campylobacter* termófilos en la cadena alimentaria, publicado en 2012 (Ugarte-Ruiz M., *et al*. 2012. "Evaluation of four protocols for the detection and isolation of thermophilic *Campylobacter* from different matrices". *Journal of Applied Microbiology* 113: 200-208).

- Evaluación de métodos para la detección de *Campylobacter* e identificación del perfil de resistencia a antimicrobianos en efluentes urbanos, publicado en 2015 (Ugarte-Ruiz M., *et al*. 2015. "Method comparison for enhanced recovery, isolation and quantitative detection of *C. jejuni* and *C. coli* from wastewater effluent samples". *International Journal of Environmental Research and Public Health* 12: 2749-2764).

### 4.2. Objetivo II. Caracterización de *Campylobacter* termófilos a lo largo de la cadena alimentaria

Con el objeto de llevar a cabo un estudio epidemiológico y evaluar la influencia del método de cultivo en la detección de *Campylobacter* termófilos en las distintas etapas de la cadena alimentaria, se llevó a cabo la caracterización genética de los aislados obtenidos. Como consecuencia del análisis realizado se obtuvo la siguiente publicación:

- Efecto del protocolo de aislamiento en la detección y caracterización genética de *Campylobacter* termófilos en la cadena alimentaria, publicado en 2013 (Ugarte-Ruiz M., *et al*. 2013. "The effect of different isolation protocols on detection and molecular characterization of *Campylobacter* from poultry". *Letters in Applied Microbiology* 57: 427-435).

### 4.3. Objetivo III. Análisis de la presencia del sistema de secreción tipo VI en aislados de *Campylobacter jejuni*

Recientemente se ha descrito la presencia de un nuevo sistema de secreción de proteínas (sistema de secreción tipo VI), relacionado con la patogenicidad y supervivencia de algunas cepas de *C. jejuni.* Debido a la falta de conocimiento sobre la presencia de este mecanismo en cepas de *C. jejuni* en España, nos propusimos la evaluación del mismo en aislados obtenidos en el estudio I y II (contenido cecal, piel de cuello, pechuga de pollo fresca envasada y efluentes urbanos). Este objetivo ha dado lugar a la siguiente publicación:

- Prevalencia del sistema de secreción tipo VI en cepas de *Campylobacter jejuni* procedentes de España, publicado en 2014 (Ugarte-Ruiz M., *et al*. 2014. "Prevalence of Type VI secretion system in Spanish *Campylobacter jejuni* isolates". *Zoonoses and Public Health* doi: 10.1111/zph.12176).

### 4.4. Objetivo IV. Detección de *Campylobacter* en muestras de animales de vida libre (domésticos y salvajes)

El papel que la fauna salvaje juega en la epidemiología de *Campylobacter* termófilos no ha sido completamente identificado. Por lo tanto, se decidió llevar a cabo un análisis sobre la presencia de *Campylobacter* termófilos en animales de vida libre (domésticos y salvajes) en un entorno geográfico natural compartido con bajo impacto humano. Esta investigación ha sido recogida en el siguiente trabajo de investigación:

- Identificación e importancia de la transmisión de *Campylobacter* spp. entre animales salvajes y domésticos de vida libre, publicado en 2014 (Navarro-Gonzalez N., and Ugarte-Ruiz M., *et al*. 2014. "*Campylobacter* shared between free-ranging cattle and sympatric wild ungulates in a natural environment (NE Spain)". *Ecohealth* 11: 333-342).

Además, como parte de este trabajo de tesis, se ha completado la escritura de la sección del capítulo de un libro centrado en la perspectiva europea sobre la relevancia que los animales salvajes pueden tener en la circulación de *Campylobacter* spp. entre animales y personas:

- Perspectiva europea sobre la transmisión de *Campylobacter* spp. entre animales salvajes, domésticos y personas, en fase de revisión (Navarro-Gonzalez N., Ugarte-Ruiz M., *et al*. 2015 "A European perspective on the transmission of food-borne pathogens at the wildlife-livestock-human interface". Dentro del libro *Food Safety Risks from wildlife: Challenges in Agriculture, Conservation and Public Health. Springer*).

# 5. OBJECTIVES

Thermophilic *Campylobacter* species can survive along the food chain, with foods of animal origin as the main source of human infection. In addition, these microorganisms are able to persist in aquatic environments, such as wastewater, considered a possible source of environmental contamination, which could have an impact in humans and animals. Moreover, they can also colonize the intestinal tract of free-living warm-blooded animals, so there might be a potential bacterial flow between wild and domestic animals, as well as the environment, especially in shared habitats.

In this doctoral thesis, we have analyzed the presence of thermophilic *Campylobacter* i) in chicken samples taken at different stages of the food chain (ceacal content, neck skin and fresh chicken breast already packed), reflecting the bacteria present in food animals, ii) in urban effluents of a wastewater treatment plant, as a representative sample of the population present in an urban environment, and iii) in free-living animals (wild and domestic), to assess the possible exchange of *Campylobacter* in a natural environment with low human impact.

Given the difficulty of detecting *Campylobacter* using traditional culture techniques and the diversity of results reported, a comparison of different protocols for detection and isolation of this species was performed. In addition, in order to obtain information about the epidemiology of the microorganism, the isolates obtained in the different matrices were characterized. Moreover, the presence of a recently described protein secretion system (type VI secretion system) was investigated, since this secretion system is related to survival and pathogenicity of *Campylobacter*.

Thus, this thesis is structured according to the objectives described below:

## 5.1. Objective I. Detection and isolation of thermophilic *Campylobacter* from different matrices: comparison of detection protocols

The difficulty of thermophilic *Campylobacter* detection and the wide range of results reported, led us to evaluate different microbiological and molecular detection protocols in samples taken at different stages of the food chain (ceacal contents, neck

skin and fresh chicken breast already packed) and urban effluents from a wastewater treatment plant. The work resulted in the following publications:

- Evaluation of four protocols for the detection and isolation of thermophilic *Campylobacter* from different matrices, published in 2012 (Ugarte-Ruiz M., *et al*. 2012. *Journal of Applied Microbiology* 113: 200-208).

- Method comparison for enhanced recovery, isolation and quantitative detection of *C. jejuni* and *C. coli* from wastewater effluent samples, published in 2015 (Ugarte-Ruiz M., *et al*. 2015. *International Journal of Environmental Research and Public Health* 12: 2749-2764).

### 5.2. Objective II. Characterization of thermophilic *Campylobacter* from the food chain

In order to carry out an epidemiological survey and to evaluate the influence of culture methods in thermophilic *Campylobacter* detection at various stages of the food chain, a genetic characterization of the isolates obtained was carried out. This analysis resulted in the following publication:

- The effect of different isolation protocols on detection and molecular characterization of *Campylobacter* from poultry, published in 2013 (Ugarte-Ruiz M., *et al*. 2013. *Letters in Applied Microbiology* 57: 427-435).

### 5.3. Objective III. Analysis of the presence of type VI secretion system in *C. jejuni* isolates

Recently, the presence of a new protein secretion system (type VI secretion system) related to pathogenicity and survival of particular strains of *C. jejuni* was described. Due to the lack of knowledge about the presence of this mechanism in strains of *C. jejuni* circulating in Spain, we evaluated presence of this secretion system in strains obtained from the studies described under Objective I (isolated from chicken ceacal contents, neck skin and fresh chicken breast already packed as well as from urban effluents). This work was described in the following publication:

- Prevalence of Type VI secretion system in Spanish *Campylobacter jejuni* isolates, published in 2014 (Ugarte-Ruiz M., *et al*. 2014. *Zoonoses and Public Health* doi: 10.1111/zph.12176).

### 5.4. Objective IV. Detection of *Campylobacter* from free-living animals (domestic and wild animals)

The role of wildlife in the epidemiology of thermophilic *Campylobacter* has not been fully identified. Therefore, an analysis of the presence of thermophilic *Campylobacter* was performed in free-living animals (both domestic and wild animals) in a shared natural geographical area with low human impact. This research has given rise to the following article:

- *Campylobacter* shared between free-ranging cattle and sympatric wild ungulates in a natural environment (NE Spain), published in 2014 (Navarro-Gonzalez N., and Ugarte-Ruiz M., *et al*. 2014. *Ecohealth* 11: 333-342).

As a further output, a chapter was contributed to a book that focused on the European perspective of the relevance that wild animals might have on the dissemination of *Campylobacter* spp. between animals and humans:

- A European perspective on the transmission of food-borne pathogens at the wildlife-livestock-human interface. In *Food Safety Risks from wildlife: Challenges in Agriculture, Conservation and Public Health. Springer,* to be published in 2015 (Navarro-Gonzalez N., Ugarte-Ruiz M., *et al*. 2015).

# 6. TRABAJOS PUBLICADOS

## 6.1. Estudio I. Evaluación de métodos para la detección de *Campylobacter* termófilos en la cadena alimentaria

Numerosos estudios han confirmado que la prevalencia de *Campylobacter* termófilos en el tracto intestinal de aves de corral es especialmente elevada [7, 73]. Frecuentemente, esta bacteria contamina las canales, siendo capaz de sobrevivir a lo largo de la cadena de producción de alimentos [8, 109]. No obstante, el cultivo de *Campylobacter* es complejo como consecuencia de su sensibilidad a factores ambientales como el oxígeno, por sus requerimientos nutritivos o por la presencia de otras bacterias competidoras [22, 23]. Dadas las dificultades descritas para el aislamiento de este microorganismo en el laboratorio, se propuso una evaluación de métodos de detección.

Para identificar el método más adecuado de detección de *Campylobacter* termófilos (*C. coli* y *C. jejuni*) en muestras tomadas en diferentes fases de la cadena alimentaria, se compararon diferentes protocolos. Se tomaron muestras de contenido cecal (n=38) y piel de cuello (n=38) en matadero, así como muestras de pechuga de pollo fresca envasada en matadero y punto de venta (n=38) durante los años 2010 y 2011. Se evaluaron seis métodos de cultivo diferentes empleando siembra directa o enriquecimiento con Bolton (ISO 10272:2006) o Preston, seguido del cultivo en mCCDA (*modified charcoal cefoperazone deoxycholate agar*) o CFA (*Campyfood agar*). Además, todas las muestras fueron analizadas y cuantificadas mediante un método de detección molecular (PCR en tiempo real cuantitativa, qPCR).

La siembra directa en CFA obtuvo el mayor número de positivos sobre la totalidad de las muestras analizadas de contenido cecal (33/38; 87%) y piel de cuello (29/38; 76%), mientras el enriquecimiento en caldo Preston seguido de la incubación en mCCDA identificó el mayor número de muestras positivas en pechuga de pollo (20/38; 53%). Si se comparan estos resultados de pechuga con los identificados usando caldo Bolton (9/38; 24%), recomendado en la norma ISO 10272:2006, la diferencia observada fue estadísticamente significativa ($p<0,05$). Finalmente, la qPCR identificó una mayor proporción de muestras positivas (98,2%) que el cultivo microbiológico (22,8-60,5%, en función del protocolo), interpretándose estos resultados como el máximo teórico

de muestras positivas, dado que esta técnica permite la detección de células muertas y VPNC, además de células vivas o ADN libre.

Así, en muestras muy contaminadas como el contenido cecal y la piel de cuello, la siembra directa fue el protocolo que permitió detectar un mayor número de muestras positivas. Sin embargo, en muestras con menor carga de *Campylobacter* termófilos o incluso con cepas potencialmente dañadas (como por ejemplo la pechuga de pollo), el uso de una etapa de enriquecimiento previa fue más efectivo, especialmente con caldo Preston.

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ORIGINAL ARTICLE

# Evaluation of four protocols for the detection and isolation of thermophilic *Campylobacter* from different matrices

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**Keywords**
*Campylobacter*, detection methods, enrichment, matrices, real-time PCR, quantification.

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## Abstract

**Aims:** To identify the optimal method for detection of thermophilic *Campylobacter* at various stages in the food chain, three culture-dependent (direct plating, Bolton and Preston enrichment) and one molecular method (qPCR) were compared for three matrices: poultry faeces ($n$ = 38), neck skin ($n$ = 38) and packed fresh meat ($n$ = 38).

**Methods and Results:** Direct plating was compared to enrichment with either Bolton broth (ISO 10272:2006-1) or Preston broth, followed by culture on two selective agars: modified charcoal cefoperazone desoxycholate agar (mCCDA) and Campyfood agar (CFA). Direct plating on CFA provided the highest number of positive samples for faeces and neck skin samples. Enrichment of meat samples in Preston followed by plating on mCCDA gave significantly higher number of positives than the recommended ISO method. Real-time qPCR yielded the highest number of positive samples.

**Conclusion:** Direct plating on CFA is optimal for *Campylobacter* isolation from highly contaminated samples such as faeces or neck skin. When enrichment is required for less-contaminated samples such as poultry meat, Preston broth is the best choice. The maximum of detectable cells predicted by qPCR is a sensitive and powerful evaluation tool.

**Significance and impact of the study:** The recommended ISO protocol had the least sensitivity, and application of this method could result in underreporting. We detected a high prevalence of *Campylobacter* on packed meat to be distributed, which suggests this is still a significant risk for consumers.

## Introduction

*Campylobacter* infections pose a serious public health problem; the incidence of campylobacteriosis has progressively increased in developed countries, and the pathogen is now considered the leading cause of bacterial gastroenteritis throughout the world (Humphrey *et al.* 2007; FAO/WHO, 2009). Thermophilic *Campylobacter jejuni* and *Campylobacter coli* are the most frequently isolated species in foodborne zoonoses in humans (EFSA Journal, 2011). *Campylobacter* can establish itself as a subclinical infection in humans, but frequently causes a range of clinical symptoms varying from self-limited, mild diarrhoea to severe inflammatory bloody diarrhoea. Occasionally, acute or long-term and potentially serious complications occur such as septicaemia, irritable bowel syndrome, reactive arthritis or autoimmune neuropathies (Guillain-Barré and Miller Fisher Syndrome) (Godschalk *et al.* 2004; Leonard *et al.* 2004; Takahashi *et al.* 2005; Humphrey *et al.* 2007). Large outbreaks are uncommon, and the vast majority of human campylobacteriosis cases are sporadic; they most likely result from handling or consumption of raw or undercooked contaminated meat products. Other foodstuffs, untreated drinking water and

milk have also been associated with the illness, but poultry products are considered the major source of infection (Pebody *et al.* 1997; Altekruse *et al.* 1999; Pires *et al.* 2010).

Bacteriological culture of *Campylobacter* spp. can be a challenge, owing to the fragility of these organisms. The use of a selective medium is recommended for the recovery from stool and faeces; for samples with low bacterial numbers, filtration or enrichment steps are typically added to improve recovery (Hu and Kuo 2011). Direct plating on selective agar media is common practice for *Campylobacter* isolation from several matrices (drinking water, environmental (dust) or intestinal samples), but an ideal single method for the entire range of samples requiring testing has not been developed (Baylis *et al.* 2000; Engberg *et al.* 2000; Musgrove *et al.* 2001; Commission Decision 2007/516/EC). In 2006, the International Organization for Standardization (ISO) standard method for detection of *Campylobacter* spp. in food recommended enrichment using Bolton broth, followed by culture on selective modified charcoal cefoperazone desoxycholate agar (mCCDA) and one other alternative agar plate (ISO, 2006).

For our study, which covered various matrices, we compared the results of traditional culturing methods and a real-time quantitative PCR assay, in an attempt to combine optimal sensitivity with short isolation and confirmation time. We evaluated three different procedures for *Campylobacter* isolation: direct plating on selective media [mCCDA or Campyfood Agar (CFA)], four combinations of enrichment and plating media (Bolton or Preston enrichment, combined with mCCDA or CFA plates) and molecular detection by real-time PCR (qPCR). The evaluation was performed on naturally contaminated broiler faeces, neck skin and poultry meat samples.

## Materials and methods

### Samples

A total of 114 chicken samples were tested from April 2010 to February 2011 consisting of neck skin ($n = 38$), breast meat ($n = 38$) and faecal samples ($n = 38$). From individual batches of birds, intestines ($n = 380$, ten homogenized caecum contents per sample), neck skin ($n = 38$) and packaged breast meat specimens ($n = 28$) were obtained at the slaughterhouse after evisceration (caecum), immediately after chilling (neck skin) and at the end of the processing line (meat). In addition, independent breast meat packages ($n = 10$) were sampled at retail. All samples were kept refrigerated during transport to the laboratory, and culture was performed immediately after reception. In addition, 300 mg of each fresh sample was stored at −40°C for subsequent DNA extraction and qPCR.

### Method 1: direct plating onto selective medium (mCCDA and CFA)

For direct plating of stool samples, a swab was dipped into the sample and streaked onto selective plates. For neck skin and meat samples, a surface of approximately 5 $cm^2$ was swabbed. All swabs were directly streaked onto *Campylobacter* blood-free selective medium (mCCDA, modified charcoal cefoperazone desoxycholate agar, CM739; Oxoid, Basingstoke, UK) and onto ready-to-use, chromogenic-like CFA plates (Campyfood agar; Ref 43471, bioMérieux, Marcy l'Etoile, France). Following incubation at 42°C for 48 h under microaerobic conditions (Genbag microaerobic atmosphere generator, Ref 45532, bioMérieux), the plates were examined. Up to five colonies with *Campylobacter*-typical morphology (according to the manufacturer's instruction) were cultured onto blood agar plates (bioMérieux) at 37°C for 48 h in a microaerobic atmosphere for further identification using conventional PCR. If more than one colony morphology was observed, representative colonies of these were picked. A sample was considered positive if at least one colony was confirmed by PCR.

### Method 2: ISO 10272:2006-1 using enrichment with Bolton broth

The recommended ISO 10272:2006-1 protocol included enrichment in Bolton broth (CM0983; Oxoid) supplemented with antibiotic supplement (SR0183) and 5% lysed horse blood (SR0048) (both from Oxoid). One gram of neck skin was aseptically transferred to a 10-ml sterile screwcap bottle, and 9 ml Bolton broth was added. Meat samples (25 g taken from the surface) were transferred to sterile stomacher bags with filter and pouch and mixed with 225 ml Bolton broth, while 10 g of fresh faeces was mixed in stomacher bags with 90 ml Bolton broth. These were incubated with a Genbox atmosphere generator. Enrichment was performed for 4–6 h at 37°C followed by 48 h at 42°C, after which 200 $\mu$l was cultured for 48 h on the two selective agar plates (mCCDA and CFA) as described above.

### Method 3: enrichment method using Preston broth

The third tested procedure was based on a previous recommendation described in ISO 10272:1995-1 (ISO, 1995, now withdrawn) and included enrichment using Preston broth (Nutrient broth No. 2, CM0067; Oxoid) that was prepared according to the manufacturer's instructions

and supplemented with 5% lysed horse blood (SR0048) and antibiotic (SR0204 and SR0232E; Oxoid). The enrichment step with Preston broth was performed at 42°C for 48 h according to Corry *et al.* 1995 (though ISO 10272:1995-1 recommended 18 h), and all further steps were performed as described in Method 2.

### Identification of suspected *Campylobacter* colonies

Suspected *Campylobacter* colonies were picked and subcultured onto blood agar plates (bioMérieux) by microaerobic incubation at 37°C for 48 h. DNA was liberated by boiling a colony, suspended in 600 μl of sterile double distilled water, for 10 min.

Conventional multiplex PCR was used for simultaneous identification of the genus *Campylobacter* and the differentiation between *Camp. jejuni* and *Camp. coli*. All primers were designed by Oligo 6.0 software (Molecular Biology Insights, Cascade, CO, USA). For genus identification, a primer set specific for the 16S rRNA gene of all *Campylobacter* spp. was designed based on 79 sequences (14 *Camp. jejuni*, 13 *Camp. coli*, 47 *Campylobacter* spp. and five from other genera). Primer 16s1 (5′-GGATGACACTTTTCGGAGC) combined with degenerated primer 16s2 (5′-TTDGYATTYCSGCTTCGAGT) produced a 1039-bp amplicon. Their specificity was verified using 30 strains of *Campylobacter* spp. that had been speciated based on biochemical characterization as well as PCR identification (Mateo *et al.* 2005). For identification of *Camp. coli*, species-specific primers targeting *ceu*E (enterochelin uptake periplasmic-binding protein gene) were designed, based on 30 different *Camp. jejuni* and *Camp. coli ceuE* sequences. The primers COL1 (5′-ACTTTCCATGCCCTAAGAC) and COL2 (5′-TCCACCTATACTAGGCTTGTC) produced a 102-bp amplicon for *Camp. coli* only. These primers were verified using 24 *Camp. jejuni* and *Camp. coli* strains that had been unambiguously speciated, while 20 strains of other *Campylobacter* species did not produce an amplicon. The strains used for verification included *Camp. jejuni* ATCC 33560 and *Camp. coli* CRL C 2·2, (2007) that were obtained from the EU Reference Laboratory of Antimicrobial Resistance (Technical University of Denmark, Lyngby, Denmark). For the identification of *Camp. jejuni,* the *hip*O gene (hippurate hydrolase) was chosen, and for primer selection, 40 different sequences of *Camp. jejuni* were compared. A 130-bp amplicon was obtained using JEJ1 (5′-CTCCTATGCTTACAACTGCTG) and JEJ2 (5′-GGTGGTCATGGAAGTGCT) whose specificity was verified as above. Furthermore, positive controls were included using DNA from *Camp. jejuni* strain ATCC 33560 and *Camp. coli* strain CRL C 2.2, and a negative control contained all reagents except DNA. PCR amplification was performed in 20 μl containing 1·8 μl of lysed cell supernatant, 10 μl of a PCR master mix (kit Qiagen Multiplex PCR; Hilden, Germany) and 0·19 μmol $l^{-1}$ of each primer (Invitrogen, Life Technologies, Paisley, UK). The amplification was performed in a Thermal Cycler (C1000; Bio-Rad Laboratories, Hercules, CA, USA) with denaturation for 15 min at 95°C, 35 cycles with 30 s at 95°C, 90 s at 56°C and 1 min at 72°C and a final 10-min extension at 72°C. Amplicons were detected by gel electrophoresis using 2% agarose gels containing 10 mg $ml^{-1}$ SYBR green stain (Invitrogen, Life Technologies) for 40 min at 400 mA. A DNA molecular weight marker (100-bp low ladder; Biotools, B&M Labs, Madrid, Spain) was included for reference. Bands were visualized under UV light, and gel images were taken with a UV Bio-Rad Molecular Imager (Bio-Rad).

A sample was considered confirmed if the genus-specific amplicon as well as either a *Camp. coli* or a *Campy. jejuni*-specific amplicon was obtained from a colony.

### Method 4: molecular detection (multiplex real-time PCR)

DNA was extracted from 300 mg of neck skin or meat using the QIAamp DNA Mini Kit 50 (Qiagen) and from 300 mg stool using the QIAamp DNA Stool Mini Kit 50 (Qiagen). Extracted DNA (eluated in 130 μl) was subjected to an in-house multiplex real-time PCR assay using the *ceu*E and *hip*O amplification primers as mentioned above, to detect and differentiate both species in a single reaction. Fluorophore-linked probes were added for detection of the amplicons: *Camp. jejuni*-specific *hipO* amplicon was detected using probe HEX-5′-AGATCCTATTTATGCTGCTTCTTTRC-BHQ, and the *Camp. coli*-specific *cueE* amplicon was detected with probe FAM-5′-ATAAAGTTGCAGGAGTTCCAGCTAAA-BHQ. The specificity of these hydrolysis probes was confirmed using the set of 24 *Camp. jejuni* and *Camp. coli*, as well as 20 strains of other *Campylobacter* species, described above. All reactions were carried out in triplicate with inclusion of a negative template control as well as positive controls. For generation of a standard curve, 1 ng DNA of *Camp. jejuni* ATCC 33560 was mixed with 1 ng DNA of *Camp. coli* CRL C 2.2, and ten-fold serial dilutions were produced up to $10^{-4}$ (range, $5{\cdot}649 \times 10^{5}$–$5{\cdot}649 \times 10^{1}$ DNA copies). When tested, a $10^{-5}$ dilution of this standard mixture frequently remained negative. All standard dilutions and samples were performed in triplicate. The simultaneous detection and quantification of *Camp. jejuni* and *Camp. coli* allowed detection of contamination by more than one *Campylobacter* species.

The multiplex PCR was performed using an iCycler thermal cycler (Bio-Rad Laboratories). Reactions (final

volume 25 $\mu$l) contained 5 $\mu$l of template DNA, 12·5 $\mu$l of QuantiTect Multiplex PCR No ROX Mastermix (QIAGEN), 0·4 $\mu$mol $l^{-1}$ of each amplification primer and 0·25 $\mu$mol $l^{-1}$ of each probe. The thermal cycle protocol included initial denaturation at 95°C for 15 min, followed by 40 cycles (94°C for 1 min, 56°C for 1 min) and a final extension at 72°C for 10 min. Fluorescence of FAM and HEX was measured at their respective wavelengths during the annealing step of each cycle. An internal amplification control was included in the form of a construct of 111 bp of foreign sequence (derived from *Oncorhynchus mykiss viperin* NCBI accession number: NM_001124253.1) flanked by the *Camp. jejuni*-specific *hipO* primers.

### Data analysis

Data were analysed using SPSS (19.0 IBM, Chicago, Il, USA). Significance of differences ($P < 0·05$) between proportions of positive samples obtained with the different protocols was assessed using chi-squared and Fisher's exact test depending on sample size. Quantitative results of qPCR were transformed to base-10 log values. Correlations between *Camp. coli* and *Camp. jejuni* results were evaluated using Spearman's rank correlation coefficient.

## Results

The results of the four detection methods, regardless of the *Campylobacter* species detected, are summarized in Table 1. Irrespective of the sample type, between 22·8% and 60·5% were found positive by culture-dependent methods 1–3, depending on the method, while qPCR detected 98·2% of positive samples. Direct plating was more sensitive than the two enrichment-dependent methods, and this difference was highly significant when compared with Bolton enrichment ($P < 0·001$; there was no significant difference between direct plating and Preston enrichment for all samples combined). When enrichment was included, Preston broth performed better than Bolton, and this difference was highly significant ($P < 0·001$). The ISO standard protocol performed worst for all three sample types. However, the alternative methods performed differently depending on the type of sample matrix. For faeces and neck skin samples, direct plating resulted in the highest numbers of positives, although the difference between direct plating and Preston enrichment was not statistically significant for neck skin samples. In contrast, for meat samples, enrichment with Preston broth was superior to the other two methods ($P = 0·001$, between Preston and Bolton enrichment; $P < 0·001$, between Preston enrichment and direct plating).

Table 1 also summarizes the number of samples found positive with any combination of enrichment broth and culture plates. Although this resulted in the same total of 69 positive samples as direct plating did, the number of positive faecal samples obtained with enrichment was lower, while that of positive meat samples was higher than what was obtained by direct plating. The poor performance of enrichment of stool samples was most likely due to competing intestinal microbiota, while the Preston enrichment improved detection of *Campylobacter* in meat, probably reflecting the lower initial bacterial load of these samples. Of the 69 samples that were positive by enrichment with at least one of the two tested broths, only 21 (30%) tested positive using both broths. Quantitative PCR was performed on all samples, and this was the most sensitive method tested. As expected, all samples found positive by culture were also positive by qPCR. The results obtained with qPCR were interpreted to reflect a theoretical maximum of positive samples, and they could be an overestimate; culture-negative but qPCR-positive samples might be due to detection of noncultivable and dead cells. Taking the qPCR as the theoretical maximum (100%) of detection, the results of the other methods were expressed as a fraction of this to visualize their respective performance (Fig. 1).

All tested culture-dependent methods included, as a final step, incubation on both mCCDA and CFA agar

**Table 1** Results obtained from 114 samples of neck skin ($n$ = 38), faeces ($n$ = 38) and chicken meat ($n$ = 38) using direct plating, enrichment protocols (Bolton, Preston or both broths) and molecular detection of *Campylobacter*

| Method | Faeces ($n$ = 38) (%) | Neck skin ($n$ = 38) (%) | Meat ($n$ = 38) (%) | Total ($n$ = 114) (%) |
|---|---|---|---|---|
| 1. Direct plating* | 34 (90) | 31 (82) | 4 (11) | 69 (61) |
| 2. Bolton enrichment* (ISO 10272-1:2006) | 7 (18) | 10 (26) | 9 (24) | 26 (23) |
| 3. Preston enrichment* (ISO 10272:1995-1) | 12 (32) | 28 (74) | 24 (63) | 64 (56) |
| 4. Enrichment (Bolton + Preston)† | 14 (37) | 29 (76) | 26 (68) | 69 (61) |
| 5. Quantitative PCR | 37 (97) | 38 (100) | 37 (97) | 112 (98) |

*Considered positive when at least one confirmed colony was present on either selective agar [mCCDA or Campyfood agar (CFA)].
†Considered positive when at least one enrichment broth (Bolton or Preston) resulted in a confirmed colony on either selective agar (mCCDA or CFA).

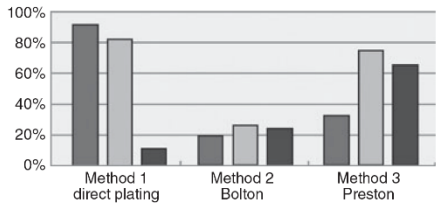


**Figure 1** Comparison of the three detection methods (direct plating, Bolton enrichment ISO 10272-1:2006 and Preston enrichment ISO 10272:1995-1) expressed as a fraction of the qPCR results, which were taken as 100% (representing the theoretical maximum of detecting live, dead and viable, non-culturable cells), for the 114 samples tested. (■) Faeces; (▨) Neck Skin; (■) Meat.

plates, and performance of these two selective media is compared in Table 2. Direct plating on CFA produced more positives than direct plating on mCCDA, for all matrices except for meat; for that matrix, direct plating was not as sensitive as enrichment, and there was no difference between the two agars tested. The difference between direct plating of faecal samples onto CFA or mCCDA was not statistically significant, but these selective media only moderately agreed for faecal samples (Kappa value, 0·350) so that samples detected positive by one could be missed by the other. Following enrichment in Bolton broth, CFA plates recovered more positives than mCCDA plates, for all matrices. Only for meat samples did enrichment in Preston, combined with selective culture on mCCDA, perform better than the other methods (Table 2). Nevertheless, Method 3 (Bolton enrichment) followed by CFA plating identified two meat samples as positive that were missed by all other culture methods.

The cultured *Campylobacter* isolates were speciated using multiplex PCR. A single colony per morphology and protocol was investigated, unless the selective culture plate had resulted in multiple morphologies, in which case these were separately tested. A total of 82 colonies (53 *Camp. coli*, 27 *Camp. jejuni* and two samples containing both species) were derived from faeces, 103 (39 *Camp. coli*, 63 *Camp. jejuni* and one sample with both species) from neck skin and 48 (27 *Camp. coli* and 21 *Camp. jejuni*) from meat samples. This resulted in 119 *Camp. coli* and 111 *Camp. jejuni* isolates and three mixed samples (Table 3). Culture-independent qPCR results, however, identified 11 samples as contaminated solely by *Camp. coli*, 16 samples exclusively by *Camp. jejuni* and 85 samples produced amplicons for both species of a total of 112 positives (Table 3). These data suggest that the culture-dependent speciation of single colonies might have underestimated the true diversity of the bacterial

**Table 2** Results obtained from neck skin, faeces and chicken meat samples with the two types of selective agar plates (mCCDA or CFA), with or without enrichment (Bolton or Preston)

| Culture method | Plate | Faeces (percentage of mCCDA + CFA)* | Neck skin (percentage of mCCDA + CFA)* | Meat (percentage of mCCDA + CFA)* | Total (percentage of mCCDA + CFA)* |
|---|---|---|---|---|---|
| Direct plating | mCCDA | 26 (76) | 23 (74) | 2 (50) | 51 (73) |
| | CFA | 33 (97) | 29 (94) | 2 (50) | 64 (92) |
| Enrichment | | | | | |
| Bolton | mCCDA | 2 (29) | 1 (10) | 2 (22) | 5 (19) |
| | CFA | 7 (100) | 9 (90) | 8 (89) | 24 (92) |
| Preston | mCCDA | 2 (17) | 17 (60) | 20 (83) | 39 (61) |
| | CFA | 12 (100) | 24 (86) | 14 (58) | 50 (78) |

*For absolute values of the readings taken on mCCDA + CFA plates combined, see Table 1.

**Table 3** Obtained *Campylobacter* species from 114 samples of neck skin, faeces and chicken meat using all tested culture protocols (direct plating, Bolton and Preston enrichment) as well as molecular detection

| Species | *Campylobacter coli* | | *Campylobacter jejuni* | | *Campylobacter coli* + *Campylobacter jejuni* | |
|---|---|---|---|---|---|---|
| Protocol/samples | Culture | qPCR | Culture | qPCR | Culture | qPCR |
| Faeces | 53 | 2 | 27 | 5 | 2 | 30 |
| Neck skin | 39 | 1 | 63 | 4 | 1 | 33 |
| Meat | 27 | 8 | 21 | 7 | 0 | 22 |
| Total | 119 | 11 | 111 | 16 | 3 | 85 |

**Table 4** Quantitative data obtained by qPCR

| Sample | No. of copies (log10) *Campylobacter coli* | No. of copies (log10) *Campylobacter jejuni* |
|---|---|---|
| Faeces | | |
| Mean | 4·91 | 5·56 |
| Median | 5·25 | 5·92 |
| Maximum | 8·82 | 9·47 |
| Range | 0–2 copies (log10): 7 samples | 0–2 copies (log10): 6 samples |
| | 3–5 copies (log10): 17 samples | 3–5 copies (log10): 13 samples |
| | 6–8 copies (log10): 14 samples | 6–8 copies (log10): 18* samples |
| Neck skin | | |
| Mean | 2·50 | 3·39 |
| Median | 2·77 | 3·16 |
| Maximum | 5·06 | 6·23 |
| Range | 0–2 copies (log10): 22 samples | 0–2 copies (log10): 16 samples |
| | 3–5 copies (log10): 16 samples | 3–5 copies (log10): 20 samples |
| | 6–8 copies (log10): 0 samples | 6–8 copies (log10): 2 samples |
| Meat | | |
| Mean | 2·10 | 2·56 |
| Median | 2·55 | 2·88 |
| Maximum | 3·65 | 6·31 |
| Range | 0–2 copies (log10): 30 samples | 0–2 copies (log10): 21 samples |
| | 3–5 copies (log10): 8 samples | 3–5 copies (log10): 15 samples |
| | 6–8 copies (log10): 0 samples | 6–8 copies (log10): 2 samples |

*One additional sample contained more than 9 log(10) copies of *Camp. jejuni*.

population, because the majority of samples (85 of 112) turned out to contain DNA for both *Camp. jejuni* and *Camp. coli*.

The proportion of positive samples for each species detected by qPCR did not differ significantly; the obtained quantitative data for all samples are summarized in Table 4. The highest amount of *Campylobacter* DNA was detected in faecal samples, for both species. Higher mean and median values were observed for *Camp. jejuni* than for *Camp. coli*, for all three types of samples. For those samples found positive of both species, the quantity of one species is weakly correlated (Spearman's rho = 0·410) with that of the other. In the box-and-whiskers plot of Fig. 2, the base-10 log values were related to

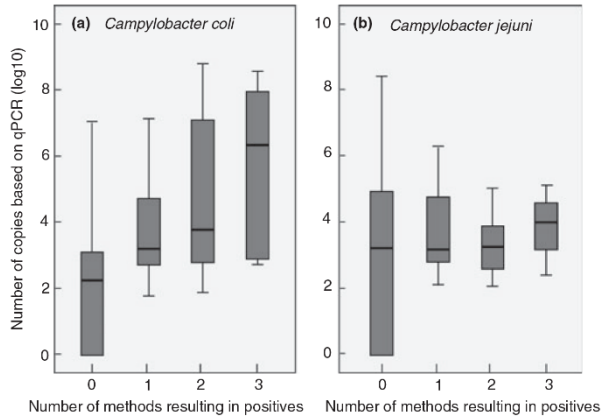


**Figure 2** Box-and-whisker plot relating the quantitative base 10 log value from qPCR for *Campylobacter coli* (a) and *Campylobacter jejuni* (b) to the number of methods (direct plating, Bolton enrichment ISO 10272-1:2006 and Preston enrichment ISO 10272:1995-1) found positive.

the number of culture-dependent protocols that were able to detect each species. Comparing the results for the two species shows that, for *Camp. coli*, more methods detect this species as the bacterial load per sample increased. This was not observed for *Camp. jejuni*.

## Discussion

Culture-dependent detection of *Campylobacter* from food sources has improved much since the introduction of selective agar media and enrichment broths. Selective media contain antibiotics to suppress the growth of competing organisms. Typically, cefoperazone, cycloheximide, trimethoprim, rifampicin, vancomycin or polymyxin B are used in various combinations. The most common culture methods make use of blood-based, antibiotic-containing media, such as Skirrow's, Butzler's and Campy-BAP media (Baylis *et al.* 2000; Granato *et al.* 2010). The blood-free, charcoal-containing selective medium mCCDA is typically used for cultural recovery from stool specimens (Aspinall *et al.* 1993; Corry *et al.* 1995; Engberg *et al.* 2000).

Broth enrichment is essential when low numbers of (damaged) *Campylobacter* are present in the sample, and the most commonly used enrichment media are Preston, Bolton or *Campylobacter* enrichment broth. These differences in methodology can potentially skew results when the complete food chain is being investigated. Bolton broth is currently the medium recommended by the US Food and Drug Administration, the International Standard Organization (International Organization for Standardization (ISO) (2006) and the Nordic Committee of Food Analysis (Habib *et al.* 2011). It is realized that the enrichment step has to compromise between selectivity and the inhibition of competitor organisms, together with the recovery and growth of the target organism to detectable levels (Baylis *et al.* 2000).

When analysing large numbers of samples, the workload should be minimized, and avoidance of duplication of selective agar, or omission of an enrichment step, might be an attractive choice, even accepting a possible consequential lesser sensitivity. In the present study, the fast, simple and cheap method of direct plating was shown to yield the best isolation efficiency for detection of *Campylobacter* in faeces and neck skin samples. For these matrices, enrichment hampered effective detection, especially for faecal samples. Although the difference between mCCDA and CFA was not statistically significant, we recommend the latter type of selective plates, because colony identification is easier on CFA plates than on mCCDA. According to Kiess *et al.* (2010), direct plating significantly increased isolation of *Campylobacter* from litter samples when compared with *Campylobacter* enrichment broth (CEB). Musgrove *et al.* (2001) observed a decrease of 36·7% in the detection of *Campylobacter* spp. in caecal samples caused by enrichment when compared with the direct plating procedure. Omitting the enrichment could reduce sensitivity for neck skin samples, as suggested by our findings: four samples were negative by direct plating that showed up positive after enrichment with Preston. However, the reverse was true as well, and in total, more positive neck skin samples were detected by direct plating than by enrichment.

Typically, *Campylobacter* is present on food at much lower levels than in faecal samples, so that for meat samples, an enrichment step is necessary. Food samples typically contain injured and dead cells as a result of exposure to heating, chilling, freezing or others detrimental conditions related to food processing and storage (Rosenquist *et al.* 2006). In an early study, Bolton broth was found to be the best compromise between inhibition of competing microflora and growth of *Campylobacter*, when compared with Preston or CEB (Baylis *et al.* 2000). However, our results identified that for meat samples, Preston broth and subsequent plating on mCCDA resulted in a significantly higher recovery of *Campylobacter* than the current ISO 10272:2006-1 ($P = 0{\cdot}001$). To increase sensitivity, after Preston enrichment of meat samples, both mCCDA and CFA plates should be used, as the concordance of the two selective media was low (Kappa value, 0·273).

On the basis of our results for all the matrices tested here, Preston enrichment (which contains rifampicin and polymixin) would be better for *Campylobacter* isolation than Bolton broth (containing cefoperazone). Recovery of *Campylobacter* using Bolton broth is influenced by the choice of the subsequent plating agar, and our data produced better results for Bolton combined with CFA than with mCCDA. According to Jasson *et al.* (2009), Bolton broth allowed growth of extended spectrum beta-lactamase *Escherichia coli* present in poultry meat and these bacteria can mask the growth of *Campylobacter*, leading to false-negative results. Overgrowth by *Pseudomonas* spp., which are also frequently present in food stuffs (Baylis *et al.* 2000), is another problem, attributable to the absence of polymixin and rifampicin in Bolton broth. A revision of ISO 10272 Part 1 and Part 2:2006 is in progress by the EURL (The European Union Reference Laboratory for *Campylobacter*. National Veterinary Institute, SVA, Uppsala, Sweden). Proficiency tests showed that Preston broth was superior to Bolton broth for samples with high background flora of multiresistant *E. coli*, but for samples with low numbers of *Campylobacter* or samples containing *Campylobacter lari*, Bolton broth seemed to be a better alternative (Olsson Engvall *et al.* 2011).

A culture-independent approach based on DNA amplification (qPCR) has several advantages over classical

bacteriology for *Campylobacter* detection, notably a faster performance combined with a lower detection limit. Moreover, PCR will detect viable but not cultivable cells, for which it is unknown whether they provide a risk for consumers (Nogva *et al.* 2000; Humphrey *et al.* 2007). Real-time PCR yields highly sensitive and specific results while avoiding manipulation of PCR products after amplification, thereby reducing the risk of cross-contamination; it can be used for rapid quantitative screening of samples (Debretsion *et al.* 2007; Botteldoorn *et al.* 2008; Melero *et al.* 2011). However, phenotypic expression of certain properties cannot be tested, and, without cultures, additional information such as subtyping or antimicrobial resistance testing cannot be obtained. A potential disadvantage of PCR-dependent techniques is that they may overestimate the number of pathogens present in a matrix, as dead cells will also be detected. Therefore, qPCR results can be considered the theoretical maximum of detectable micro-organisms, accepting that this may be an overestimate as molecular detection also reports the presence of dead cells. We used qPCR-positive results as a maximum value to correlate culture-dependent results. Interestingly, for *Camp. coli*, we observed a relationship between the qPCR quantitative values and the number of protocols at which each sample yielded positive, but this was not the case for *Camp. jejuni*. Further studies are needed to confirm this result and to investigate the reason for this difference.

In summary, direct plating on CFA selective agar resulted in optimal *Campylobacter* isolation for highly contaminated samples such as faeces and neck skin, while enrichment in Preston broth offers reliable recovery from matrices containing low levels of (damaged) organisms. The internationally recommended ISO method is not the best choice for detection of *Campylobacter* spp. in the food chain.

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## 6.2. Estudio II. Evaluación de métodos para la detección de *Campylobacter* e identificación del perfil de resistencia a antimicrobianos en efluentes urbanos

Las especies termófilas de *Campylobacter* son capaces de mantenerse de forma prolongada en muestras de agua, habiéndose identificado en efluentes urbanos [117]. No obstante, el aislamiento del microorganismo en este tipo de muestras usando métodos tradicionales (como la normativa ISO 17995: 2005) es laborioso y suele tener una baja sensibilidad [116]. El sobre-crecimiento de otras bacterias o la tendencia de *Campylobacter* a entrar en un estado de VPNC, dificultan el aislamiento en el laboratorio [8].

Con el objeto de comparar diferentes protocolos de aislamiento de *Campylobacter* termófilos en aguas residuales, procedimos a analizar 50 muestras de efluentes urbanos obtenidos en una planta depuradora. Las muestras estaban constituidas por el agua residual de diversos colectores urbanos que fueron analizadas tras la separación de solutos groseros durante el periodo comprendido entre 2010 y 2013. En este trabajo se evaluaron diferentes protocolos para el aislamiento de *Campylobacter* termófilos, usando varias combinaciones de medios selectivos (con o sin enriquecimiento) e incluyendo una etapa de filtrado en una selección de muestras. Para esta comparación se incluyeron tres medios selectivos (mCCDA, CFA y CASA) y tres caldos de enriquecimiento (Bolton, Preston y CFB, *Campyfood broth*), así como qPCR. Además, para caracterizar las cepas obtenidas, se analizó el perfil de resistencia a antimicrobianos mediante microdilución en caldo.

Los resultados mostraron que todas las muestras contenían ADN de *Campylobacter* según los datos de la qPCR, sin embargo, sólo el 64% (32/50) de las muestras fueron positivas al aislamiento. La qPCR fue un método sensible para la detección de ADN, cuyos resultados fueron interpretados como el máximo teórico de muestras positivas ya que detecta células vivas, muertas, VPNC o incluso ADN libre. El enriquecimiento obtuvo un mayor número de muestras positivas (19/50; 38%) que la siembra directa (14/50; 28%), aunque esta diferencia no fue significativa ($p>0,05$). Comparando los resultados de los caldos de enriquecimiento, el caldo Preston (18/50; 36%) consiguió más muestras positivas que el Bolton (2/50; 4%) o el CFB (0/50; 0%), siendo la diferencia significativa ($p<0,001$). Además, el filtrado y/o el enriquecimiento, especialmente con caldo Preston, fueron capaces de detectar muestras positivas no reconocidas utilizando siembra directa. Por otro lado, la recuperación de

*Campylobacter* utilizando siembra directa fue mayor ($p<0,05$) empleando agar CASA (10/24; 42%) que empleando mCCDA (0/50; 0%) o CFA (5/50; 10%). El aislamiento de *C. coli* fue más frecuente tras el uso de una etapa de enriquecimiento, fundamentalmente Preston seguido de agar CASA (8/16; 50%), mientras que la recuperación de *C. jejuni* se vio favorecida por el uso de siembra directa con agar CASA (11/12; 92%).

El análisis de resistencia a antimicrobianos realizado (n=62 aislados), reveló que las cepas de *C. coli* presentaban una menor susceptibilidad, detectándose resistencia a todos los antimicrobianos analizados (ciprofloxacina, ácido nalidíxico, tetraciclina, eritromicina, estreptomicina y gentamicina), comparado con los aislados de *C. jejuni,* que sólo presentaban resistencia a ciprofloxacina, ácido nalidíxico y tetraciclina. En general, aislados pertenecientes a una misma muestra presentaron diferentes perfiles de resistencia a antimicrobianos.

Nuestros resultados mostraron que el método de aislamiento determina en parte los resultados obtenidos y que el uso de diferentes protocolos incrementa las posibilidades de detectar diferentes especies en la misma muestra, así como la diversidad de los aislados obtenidos. El hecho de que los patrones de resistencia detectados fueran similares a los descritos para la especie humana y dado el origen de la muestra, nuestros datos apuntan a la posibilidad de emplear los estudios de *Campylobacter* en agua residual como un indicador de los *Campylobacter* termófilos presentes en el entorno urbano.

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*Article*

# Method Comparison for Enhanced Recovery, Isolation and Qualitative Detection of *C. jejuni* and *C. coli* from Wastewater Effluent Samples

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**Abstract:** Seeking a sensitive protocol, culture-dependent methods were compared to detect thermophilic *Campylobacter* species in untreated urban effluents. We evaluated various combinations of selective media, with and without an enrichment steps, as well as an extra filtration step. Culture-independent real-time quantitative PCR was also included and all detected isolates underwent antimicrobial susceptibility testing. All tested water samples contained *Campylobacter* DNA, but only 64% were positive after culture. Although enrichment using Preston broth resulted in better recovery of potentially stressed *Campylobacter* than Bolton or Campyfood broth (CFB), there was no significant increase in efficiency compared to direct plating. The type of selective agar media used, on the other hand, had a significant effect, with CASA plates performing better than mCCDA or CFA ones. Inclusion of an enrichment step increased the ratio of *C. coli vs. C. jejuni* being isolated. Resistances against all antimicrobials tested were observed in *C. coli*, but fewer instances of resistance were found in *C. jejuni* isolates. Whether this difference was the result of selection during the enrichment step could not be determined. The presence of

*Campylobacter* in urban effluents can be considered as a valuable proxy for *Campylobacter* populations present in urban environments.

**Keywords:** *Campylobacter*; urban effluents; isolation protocols; antimicrobial resistance

## 1. Introduction

*Campylobacter* is one of the most common causes of food-borne bacterial disease in humans worldwide. In the European Union, the most commonly species detected associated with campylobacteriosis are *C. jejuni*, followed by *C. coli* [1,2]. These thermophilic species asymptomatically colonize the intestinal mucosa of a wide variety of birds and mammals, including food-producing animals [3,4], which explains their frequent encounter as zoonotic, food-borne pathogens. The observed increase in the proportion of *Campylobacter* isolates with reduced susceptibility to antimicrobials, particularly against quinolones and macrolides, is a cause of concern [5,6] as this could have serious potential consequences for public health [7,8].

Despite the fact that poultry products are considered the major source of *Campylobacter* infection, multiple other sources exist, including untreated drinking water [1,5]. *C. jejuni* and *C. coli* (collectively described here as *Campylobacter*) are often present in aquatic environments, which may provide a reservoir, or even a direct source, of *Campylobacter* infection [9]. Multiple outbreaks associated with drinking water have been described [10–12]. Recreation in surface waters or ingestion of raw fruits or vegetables that have been in contact with contaminated water pose recognized risks [13,14]. Waterborne *Campylobacter* spp. is assumed to originate from animal faeces, agricultural leaks or wastewater contamination. The organism can indeed be recovered from untreated wastewater [9,15–18]. However, the detection methods are rather insensitive and elaborate.

Efforts are ongoing to improve the methodology for detection and isolation of *Campylobacter*, with attempts to reduce isolation or confirmation t ime, increase sensitivity, and standardize procedures; although, it is unlikely that any single method would be optimal for all kind of samples, irrespective of their origin [19–21]. For water samples, isolation of *Campylobacter* is rarely successful, as the methodology is not sufficiently sensitive [22,23]. It should be noted that the media typically used for isolation were originally developed to be applied to stool and other clinical samples, which contain vastly higher numbers of viable *Campylobacter* cells than are present in environmental samples [12,24].

An enrichment step is often necessary to recover low numbers or potentially damaged cells, as is typically the case with wastewater samples. However, background bacteria also proliferate during enrichment culture, and often grow at a rate faster than *Campylobacter*. *Campylobacter* enrichment media typically contain oxygen quenching agents to neutralize the adverse effects of toxic oxygen species, as well as selective agents such as cefoperazone, polymyxin B, rifampicin or trimethoprim to reduce background flora [19,25]. These latter supplements may actually reduce the isolation of damaged *Campylobacter* cells, since particular strains or *Campylobacter* species can be sensitive to these antibiotics [22,24].

To gain insights into the presence of *Campylobacter* in urban effluents, we initiated a study to compare the performance of different culture-dependent methods for its detection in urban effluent samples obtained from a wastewater treatment plant (WWTP). In view of the difficulty with detection

of *Campylobacter* in water samples that typically contain a highly variable background flora, we decided to compare real samples, rather than perform a standardized comparison with artificially spiked samples. Effluent wastewater samples were also tested with real time quantitative PCR (qPCR) and the strains obtained were characterized by antimicrobial susceptibility testing. All the samples were found positive using qPCR, while culture alone recovered *Campylobacter* in 64% of the samples. Direct plating on CASA agar was a fast and reliable method for *Campylobacter* isolation and performed better than the recommended mCCDA plates. Addition of a filtration step or enrichment in Preston media could improve the chance of recovering *Campylobacter*, however, the method of choice influences the recovered species (*C. jejuni vs. C. coli*).

## 2. Material and Methods

### *2.1. Sampling Collection*

Fifty samples of untreated urban effluents were taken from November 2010 to November 2013 from a wastewater treatment plant collecting water from a city in the center of Spain. All samples were collected in sterile containers and transported to the laboratory where culture was performed immediately after reception. In addition, an aliquot of each fresh sample was stored at 4 °C for subsequent DNA extraction and qPCR.

### *2.2. Culture-Dependent Detection by Direct Plating and Enrichment*

Three selective plates were compared by direct culture: blood-free modified charcoal Cefoperazone Deoxycholate agar (mCCDA, PO5091A, Oxoid, Basingstoke, UK), chromogenic-like Campyfood agar (CFA, Ref 43471, bioMérieux, Marcy l´Etoile, France) and selective chromogenic medium CASA (AEB520270, AES Chemunex, Marcy l´Etoile, France). These selective plates were also combined with an enrichment step using one of three enrichment broths in all possible combinations (Table 1): Bolton, Preston (both from Oxoid) and Campyfood broth (CFB, bioMérieux).

For direct plating, a swab was dipped into the homogeneous sample (25 mL) and streaked onto selective plates. Following incubation at 42 °C for 48 h under microaerobic atmosphere (Campygen, Oxoid), the plates were examined. Regarding enrichment, Bolton broth (CM0983, Oxoid) was added antibiotic supplement (SR0183, Oxoid) and 5% lysed horse blood (SR0048, Oxoid); while Preston broth (Nutrient broth N° 2, CM0067, Oxoid) was supplemented with 5% lysed horse blood (SR0048, Oxoid) and antibiotic (SR0204 and SR0232E, Oxoid). CFB broth (Ref 42643, bioMérieux) was bought commercially and ready-to-use. A volume of 25 ml of urban effluents samples were transferred to sterile stomacher bags with filter and pouch and mixed with 225 mL of each enrichment broth. These were incubated with a Genbox atmosphere generator (bioMérieux). Enrichment was performed for 4–6 h at 37 °C followed by 48 h at 42 °C for Bolton broth and 48 h at 42 °C for Preston broth and CFB. After this incubation step, 200 µL were cultured for 48 h on the selective agar plates (mCCDA, CFA, CASA) as described above.

In this analysis, the volume of urban effluents tested was 25 mL for each protocol. In addition, 27 water samples were pre-filtrated before culturing onto selective agar or enrichment broths as recommended in ISO 17995:2005 [26]. For this purpose, 25 mL of water was passed through filters of

0.22 µm (Nalgene, Thermo Fisher Scientific, Waltham, MA, USA), after which the membrane was either directly added to the enrichment broth or streaked out onto a selective plate.

All colonies with a *Campylobacter*-typical morphology (according to the manufacturer's instruction for each plate type) were cultured onto blood agar plates (bioMérieux) at 37 °C for 48 h in microaerobic atmosphere (Campygen, Oxoid). For further identification, conventional PCR to amplify a gene specific for *C. jejuni*, one for *C. coli*, and a genus-specific 16S rRNA fragment was used, as previously described [27]. When more than one colony morphology was observed, representative colonies of different morphologies were picked. A sample was considered positive if at least one colony was confirmed by PCR as *C. coli*, *C. jejuni* or *Campylobacter* spp.

### *2.3. Culture-Independent Detection by Quantitative Real-Time PCR* (*qPCR*)

Culture-independent qPCR was carried out to confirm presence or absence of *Campylobacter* DNA in each water sample. Before DNA extraction, five aliquots containing 1.5 mL each of urban effluent were concentrated by centrifugation (15,500 g for 12 min). After removal of the supernatant, a further 1.5 mL of the original sample was added and centrifugation was repeated. The five precipitates thus obtained were resuspended each in 80 µL of the original supernatant and then combined. DNA was extracted from this concentrated suspension using a QIAamp DNA stool mini kit 50 (Qiagen, Hilden, Germany) and subjected to an in-house multiplex qPCR assay as previously described [27]. Reactions (final volume 25 µL) contained 5 µL of template DNA, 12.5 µL of QuantiTect Multiplex PCR No ROX Mastermix (Qiagen), 0.4 µM of each amplification primer, and 0.25 µM of each probe. The thermal cycle protocol included initial denaturation at 95 °C for 15 min, followed by 40 cycles (94 °C for 1 min, 56 °C for 1 min) and a final extension at 72 °C for 10 min. In order to generate a standard curve **(**Figure 1), 1 ng DNA of *C. jejuni* ATCC 33560 was mixed with 1 ng DNA of *C. coli* CRL C 2.2, and ten-fold serial dilutions were produced up to $10^{-4}$ (range: $5.649 \times 10^5$ to $5.649 \times 10^1$ DNA copies). All standard dilutions and samples were performed in triplicate. Fluorophore-linked probes and primers sequences used were:

*C. jejuni*
HEX-5′-AGATCCTATTTATGCTGCTTCTTTRC-BHQ
JEJ1 (5′ -GGTGGTCATGGAAGTGCT)
JEJ2 (5′-CTCCTATGCTTACAACTGCTG)
*C. coli*
FAM-5′-ATAAAGTTGCAGGAGTTCCAGCTAAA-BHQ
COL1 (5′-ACTTTCCATGCCCTAAGAC)
COL2 (5′-TCCACCTATACTAGGCTTGTC)

### *2.4. Identification of Isolates Using 16S rRNA and MALDI-TOF*

Isolates that were negative for the species-specific *C. jejuni* and *C. coli* PCR but positive for the *Campylobacter* genus PCR ($n = 4$) were further characterized by 16S rRNA PCR. Amplicons produced with universal 16S rRNA primers (described in Baliarda *et al.* [28]), were purified using the QIAquick PCR Purification kit (Qiagen) and externally sequenced (Stabvida, Lisbon, Portugal). All 16S rRNA

sequences were compared to GenBank entries [29] and homologies to most closely related sequences were determined using ClustalW [30]. In addition, calculations of pair-wise 16S rRNA gene sequence similarities were achieved using the EzTaxon server [31].

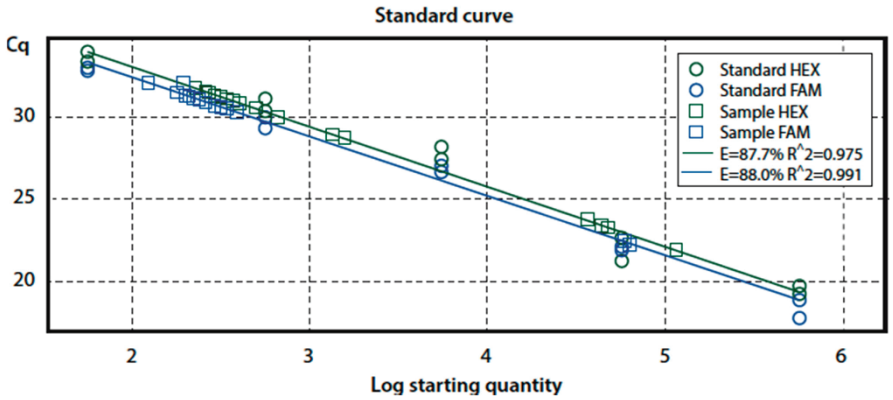


**Figure 1.** Linear regression curve of qPCR including the standards along with unknown sewage samples.

A selection of 18 isolates was further characterized using MALDI-TOF/TOF mass spectrom*etry*. The bacteria were directly spotted onto a polished steel target plate using a saturated solution of α-HCCA (Bruker Daltonics, Bremen, Germany) dissolved in 50% acetonitrile, 47.5% water and 2.5% trifluoroacetic acid (Fluka, Sigma-Aldrich, St. Louis, MO, USA). Mass spectra acquisition and analysis was performed on a Bruker UltraFlextrem platform (Bruker Daltonics) using MALDI Biotyper™ 3.0 software (Bruker Daltonics) using default settings.

*2.5. Antimicrobial Susceptibility Testing*

Antimicrobial susceptibility testing was performed using broth microdilution towards ciprofloxacin, erythromycin, gentamicin, tetracycline, streptomycin and nalidixic acid (Table 2). Isolates were grown on blood agar plates (bioMérieux) and incubated for 48 h at 37 °C at microaerophilic conditions (Oxoid). Collected cultures were added to 11 mL of cation-adjusted Mueller-Hinton broth with TES (TREK Diagnostics Systems, Waltham, MA, USA), standardized to 0.5 McFarland turbidity, and supplemented with 600 µL of lysed horse blood freshly prepared in house from defibrinated horse blood (Oxoid). This mix was distributed onto EUCAMP2 microdilution plates (TREK Diagnostics Systems) and incubated under microaerophilic conditions for 48 h at 37 °C. *C. jejuni* strain ATCC 33560 was used as a quality control. The interpretation of the quantitative data was performed as described by the European Committee of Antimicrobial Susceptibility Testing, EUCAST [32].

*2.6. Data Analysis*

Data were analyzed using SPSS (19.0 IBM, Armonk, NY, USA). Statistical significance of differences ($p$-value < 0.05) was assessed by Fisher's exact test using the WinPepi software (11.25 version, Abramson [33]). For multiple comparisons, Holm correction was applied with R Software (R Development Core Team 3.0.2, 2013). Confidence Intervals (CI) at 95% were calculated for a subset of 24 samples that were tested by all methods, using the online tool developed by WinEpi [34].

For the analysis of MALDI-TOF results, the scores obtained were contrast-based as per the manufacturer's recommendations. An identification log (score) value ranging from 0 to 3 indicated the pattern-matching extent according to the specifications of the Biotyper system. Score values of 0 to 1.699 generally indicated no reliable identification; score values of 1.7 to 1.999 indicated probable genus identification; score values of 2.0 to 2.299 indicated secure genus identification and probable species identification; and score values of 2.300 to 3.000 indicated highly probable species identification.

## 3. Results

*3.1. Comparison of Campylobacter Detection by All Tested Methods*

Irrespective of which protocol was used for isolation, 32 of 50 samples (64%) were found positive by culture-dependent methods, while qPCR detected *Campylobacter* DNA in all of the samples. The results of the culture-dependent detection methods, regardless of the *Campylobacter* species detected, are summarized in Table 1.

**Table 1.** Detection of *Campylobacter* spp. by culture-dependent methods.

| Method | | Nr. of Positive Samples/n° Samples Tested (all Plates) | Per Selective Agar Plate | | |
|---|---|---|---|---|---|
| | | | mCCDA | CFA | CASA [a] |
| Direct plating | | 14/50 | 0/50 | 5/50 | 10/24 |
| Enrichment (all broths) | | 19/50 | 10/50 | 12/50 | 10/24 |
| Per enrichment broth | Bolton | 2/50 | 0/50 | 0/50 | 2/24 |
| | Preston | 18/50 | 10/50 | 12/50 | 9/24 |
| | CFB | 0/50 | 0/50 | 0/50 | 0/24 |
| Filtration [c], direct plating | | 9/27 | 0/27 | 0/27 | 9/24 |
| Filtration [c], enrichment | | 8/27 | 0/27 | 3 [b]/27 | 6 [b]/24 |

[a] Only 24 samples were incubated on CASA plates; [b] Enrichment broth was Preston in all positive cases; [c] Filtration was added as an extra step in 27 samples.

For the investigated samples, direct plating on CASA was the best-performing isolation method, followed by Preston enrichment combined with CASA agar plates. Although the difference between these two methods was not statistically significant, direct plating was faster, simpler and cheaper than the method involving enrichment. When resources are limited or large numbers of samples need to be analysed, direct plating on CASA plates would therefore be preferable. For optimal coverage, however, filtering and Preston enrichment could be added as extra steps prior to CASA plating, separately and in combination.

### *3.2. Congruity of All Culture-Dependent Methods*

Table 1 includes nine protocols that did not yield any positive samples. Excluding these, there remained 12 protocols that resulted in positive findings, and the congruity of these was assessed for the 24 samples that had been checked using all these methods. Of these, 21 samples were positive by one or more method, whereby culture on CASA plates (with and without combined Preston enrichment) recovered *Campylobacter* in 17 cases (81%; CI 95%: 64–98). This is the highest number of positives obtained by any combination of two methods (for comparison: CASA direct plating only identified 10 and Preston with CASA produced nine positive samples). One of the 21 water samples was positive by six methods and two by five. At the other extreme, 10 samples were positive by one protocol only, and in every one of these 10 cases, the final culture had been performed on CASA plates, though in seven cases in combination with enrichment, filtering, or both. When combining all protocols with CASA plates as the final step, not one sample was reported negative that was positively cultured on an alternative plate media, which again identifies CASA plates as superior to CFA or mCCDA. For optimal coverage, filtering could be added, which added two exclusively positive sample using direct plating, Preston enrichment (adding three exclusively positive sample), and Preston combined with filtering, adding one exclusively positive when combined with CASA plating.

### *3.3. Campylobacter Species Isolated from Effluent Water*

All obtained *Campylobacter* isolates were identified individually using multiplex PCR. A total of 77 colonies were thus speciated, resulting in 53 *C. coli*, 20 *C. jejuni* and four non-*jejuni*, non-*coli* that were subsequently identified as *Arcobacter butzleri* (see below). For 18 samples, more than one colony was analysed separately. Twelve of these samples were found to contain a mixture of either *C. jejuni*, *C. coli* or *Arcobacter butzleri*. A higher proportion of samples was positive for *C. coli* than for *C. jejuni* and this difference was significant ($p < 0.05$). Considering only the subset of 24 samples tested using all protocols, from 16 of these *C. coli* was cultivated. Direct plating on CASA produced *C. coli* in two samples (12%; CI 95%: 0%–29%), adding filtration increased this to 6 (37%; CI 95%: 14%–61%) and the combination of Preston enrichment with CASA resulted in 8 *C. coli* positive samples (50%; CI 95%: 25%–74%). The other *C. coli* isolates were obtained with the alternative enrichment broths. *C. jejuni*, on the other hand, was more often detected by direct plating, in particular on CASA agar (with and without filtering), which obtained 11/12 (92%; CI 95%: 76%–100%) of the detected *C. jejuni* positive samples. The only other *C. jejuni* positive sample was derived from a CASA plate after Preston enrichment. If optimal recovery of both species were attempted with minimal experimental investment, direct plating on CASA with and without Preston enrichment would detect 10/16 *C. coli* (62%; CI 95%: 39%–86%) and 9/12 *C. jejuni* (75%; CI 95%: 50%–99%).

The culture-independent qPCR found one sample positive for *C. coli* exclusively, while DNA of both species was detected in all other samples. Most samples contained *Campylobacter* DNA in the range of $10^3$ to $10^4$ copies/mL, and there was no correlation between this detected DNA concentration and the number of culture-dependent methods reporting presence of viable cells.

Four isolates were obtained from four different water samples that were negative for the PCR probes specific for *C. jejuni* and *C. coli* genes nevertheless produced a genus-specific 16S amplicon.

Their complete 16S rRNA gene was amplified and sequenced for further identification. Comparative sequence analysis to sequences stored in GenBank revealed the highest similarity to the type strain *Arcobacter butzleri* NCTC 49616 and *A. butzleri* strain RM4018 [35,36] with homologies ranging between 98.7%–100%. The novel sequences were submitted to the European Nucleotide Archive with accession numbers LN811434, LN811435, LN811436, and LN811437 (*Arcobacter butzleri* partial 16S rRNA gene, from isolates ZTA11/01227CPF, ZTA11/00338CPF, ZTA11/00429CPF, and ZTA13/02375CASA_FILTRO, respectively).

### *3.4. Identification of Isolates Using MALDI-TOF*

The four presumed *A. butzleri* isolates were further characterized by MALDI-TOF, and for comparison 14 *Campylobacter* (six *C. jejuni* and eight *C. coli*) isolates were also included. The MALDI-TOF results confirmed the speciation by PCR in all *C. coli* and *C. jejuni* cases and 16S rRNA PCR in all *Arcobacter butzleri* isolates. Score values in the range of 2.083–2.281 (three isolates) and 2.363 (one isolate) were interpreted as *A. butzleri* species identification. There was no correlation between sequence similarity to the *A. butzleri* type strain NCTC 49616 and MALDI-TOF scores; for instance, the isolate whose amplicon produced the lowest similarity (98.71%) produced a score value of 2.259. The score values of *C. coli* and *C. jejuni* isolates ranged between 2.303–2.428 in most cases, but for four isolates that produced score values between 2.039 and 2.218.

### *3.5. Results of Antimicrobial Susceptibility Testing*

Sixty two of the 77 isolates obtained (51 C. *coli* and 11 *C. jejuni*) were analysed using susceptibility tests against six antimicrobials. The highest proportion of antimicrobial resistance was observed towards ciprofloxacin, to which 90% (CI 95%: 82%–98%) of all tested isolates found resistant, with no difference between *C. jejuni* and *C. coli.* Similar resistances were found for nalidixic acid, which is not surprising, as both might be the result of a common resistance mechanism. Lower percentages were found for tetracycline (84%; CI 95%: 75%–93% of total), with significant differences between the species (94%; CI 95%: 88%–100% of *C. coli* and 36%; CI 95%: 8%–65% of *C. jejuni*). Lower incidence still was found for gentamicin and erythromycin resistance in *C. coli*, (10%; CI 95%: 2%–18% and 16%; CI 95%: 6%–26% of the tested isolates, respectively), while all *C. jejuni* isolates were susceptible to these two antimicrobials. The higher incidence of resistance in *C. coli* compared to *C. jejuni* was statistically significant for streptomycin and tetracycline only ($p < 0.001$). In the majority of the cases (7/8; 87%, CI 95%: 65%–100%) when a sample produced more than one isolate, the antimicrobial resistance pattern found for these isolates was different. The distribution found in MIC are shown in Table 2.

## 4. Discussion

The detection of *Campylobacter* in urban effluent water can be used as a global parameter to estimate the presence of *Campylobacter* in an urban environment, provided detection methods are sufficiently sensitive. When we compared various culture-dependent detection protocols, we found 64% of water samples being positive, whereas *Campylobacter* DNA was detectable in all water

samples. Currently, the culture-dependent methods applied in water quality monitoring can be arduous and are rarely successful [12]. Conversely, molecular detection methods based on DNA amplification such as qPCR are faster and more sensitive for *Campylobacter* detection [37], however, the DNA detected may be from viable, non-cultivable or dead cells as well as from circulating DNA [12,37]. It is well documented that *Campylobacter* spp. may enter a viable but non-culturable state when exposed to adverse conditions, which can decrease the recoverability on laboratory media [38,39]. Indeed, despite qPCR positive results obtained, it was impossible to recover cultivable bacteria from 36% of the samples of our analysis.

**Table 2.** Antimicrobial susceptibility of *C.coli* and *C.jejuni* isolates from urban effluents samples.

| Antimicrobial Agents | Species | MIC Range (mg/L) | ECOFF (mg/L) [a] | Number of Isolates with a MIC (mg/L) of: | | | | | | | | | | | Number of Resistant Strains (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | |
| Gentamicin | *C.coli* | 0.12–16 | 2 | | 11 | 34 | 1 | | | | | 5 | | | 5 (10) |
| | *C.jejuni* | | 2 | 6 | 4 | 1 | | | | | | | | | 0 (0) |
| Ciprofloxacin | *C.coli* | 0.06–4 | 0.5 | 1 | 3 | 1 | | | | 46 | | | | | 46 (90) |
| | *C.jejuni* | | 0.5 | 1 | | | | | 1 | 9 | | | | | 10 (91) |
| Tetracycline | *C.coli* | 0.25–16 | 2 | | | | 2 | 1 | | | | 48 | | | 48 (94) |
| | *C.jejuni* | | 1 | | 3 | 3 | 1 | | | 1 | | 3 | | | 4 (36) |
| Erythromicin | *C.coli* | 0.5–32 | 8 | | | 2 | 23 | 17 | 1 | | | 3 | 5 | | 8 (16) |
| | *C.jejuni* | | 4 | | | 9 | 1 | 1 | | | | | | | 0 (0) |
| Nalidixic Acid | *C.coli* | 2–64 | 16 | | | | | | | 4 | 1 | | 2 | 44 | 46 (90) |
| | *C.jejuni* | | 16 | | | | | | 1 | | | | 4 | 6 | 10 (91) |
| Streptomycin | *C.coli* | 1–16 | 4 | | | | 6 | 10 | 2 | 1 | 1 | 31 | | | 33 (65) |
| | *C.jejuni* | | 4 | | | | 10 | 1 | | | | | | | 0 (0) |

[a] EUCAST: *C.coli and C.jejuni* data from the EUCAST MIC distribution website last accessed 6 June 2014.

Enrichment can, at least in theory, enhance the growth of damaged and injured cells [22], while filtration may improve recovery of low numbers of cells. For water samples, the ISO 17995: 2005 standard recommends the use of membrane filtration [26], followed by parallel enrichment with Bolton and Preston broth. However, in our study, filtration, enrichment, or both, did not significantly improve the detection of *Campylobacter* in samples of urban effluents. That enrichment does not improve recovery from water samples was already reported by Rosef *et al.* [40]. However, in contrast to previously published observations that direct plating of water samples would be suboptimal [41]; we obtained satisfying results by direct plating on CASA plates. The highest numbers of positive samples with a combination of any two methods would be obtained by direct plating on CASA together with Preston enrichment followed by CASA agar.

We deliberately performed this study with real wastewater samples, instead of a comparison of methods with spiked samples containing a known, artificially added number of viable *Campylobacter* cells. The latter approach would not have captured the difficulty with recovering injured organisms, or the complication that different water samples contain different types and quantities of background flora, as elaborately discussed by Jokinen *et al.* [24]. Injured *Campylobacter* cells also make a comparison of qPCR results and culture results difficult, since the first detects DNA from dead as well as live cells. The distinction between these two can be made by addition of propidium monoazide

(PMA) [42] or ethidium monoazide (EMA) [43] to the PCR, which avoids amplification of DNA from dead cells. However, for *Campylobacter*, conflicting results have been described for such assays. Whereas Josefsen *et al.* reported such a distinction to be successful for chicken carcass rinses [37]; Pacholewicz *et al.* [44] described insufficient PMA effectivity for carcass samples containing more than $10^4$ dead cells. That work demonstrated conflicting results between PMA-qPCR and culture methods, suggesting that qPCR combined with PMA did not fully reduce the signal from dead cells in naturally contaminated samples. Likewise, in another study, EMA-qPCR failed to detect viable cells correctly in spiked water samples [45]. In view of these disappointing results, we did not perform qPCR in presence of these chelating agents.

Nearly all samples (49/50 or 98%) contained *C. coli* and *C. jejuni* DNA in approximately the same quantity as determined by qPCR. Nevertheless, particular culture methods favoured the recovery of one species over another. *C.coli* was most often isolated after enrichment using Preston, followed by CASA plates, while direct plating on CASA agar recovered mostly *C. jejuni.* Various studies have shown that the method used for detection may influence both the yield and the genetic diversity of the population under study [9,17,46–48] but we point out that the ratio of species detected is also influenced by the method of detection. It has been reported that diversity in *Campylobacter* species may be affected by their survival characteristics under environmental conditions. Korhonen and Martikainen [49] observed that *C. jejuni* survived longer in cultivable form than *C. coli* in lake water. Moreover, Thomas *et al.* [50] described that *C. jejuni* is the species most frequently identified from surface waters, commonly associated with sewage water discharges. Likewise, Meinersmann *et al.* [51] as well as Khan *et al.* [52] detected a higher proportion of *C. jejuni* than *C. coli* from river water samples. Conversely, it was suggested that *C. coli* is possibly surviving better than *C. jejuni* under environmental circumstances [53]. Our results suggest that the use of different protocols improves the chance to detect both species from the same sample, thereby assessing the microbial diversity more accurately. Antimicrobial susceptibility data also support this hypothesis, as the majority of the strains derived from the same sample offered a different antimicrobial resistance profile.

Despite of similar amounts of DNA being present for both *C. jejuni* and *C. coli* in each water sample analyzed, we were not always able to cultivate both species. These results would indicate that microbiological methods might have underestimated the real diversity of the sample. There is also a growing number of *Campylobacter* species, other than *C. coli* or *C. jejuni*, as well as related genera, being recognized as emerging human and animal pathogens. The prevalence of these, such as *A. butzleri*, is probably unknown because the detection methods used can favor the recovery of some species and these emergent bacteria are not identified to the species level [20,54].

It has been proposed that aquatic environments are involved in the dispersion and evolution of antimicrobial resistance in bacteria, and wastewater may play a particularly important role in these processes [55]. This was considered a possibility for methicillin resistant *Staphylococccus aureus* [56]. Denis *et al.* [57] observed that the majority of *C. coli* and *C. jejuni* obtained from contaminated river waters in France were susceptible to ciprofloxacin, erythromycin, tetracycline, gentamicin and streptomycin. However, our data show that the majority of *C. coli* and *C. jejuni* isolates were resistant towards quinolones and, to a lesser extent, to tetracycline. A smaller proportion of *C. coli* but none of the *C. jejuni* isolates exhibited resistance against streptomycin, erythromycin and gentamicin. Similar difference between these two species were recently reported for poultry isolates from Spain [58] and

from human clinical isolates [59]. We therefore assume that our results can be taken as a proxy for the bacterial population found in an urban environment, although we cannot completely rule out that the enrichment step that favoured *C. coli* may have selected for resistance as well, as our data were inconclusive on this point.

Finally, four strains considered as *Campylobacter* spp. by PCR were subsequently identified as *Arcobacter butzleri*. All these strains were isolated using direct plating protocols. *Arcobacter* spp. has been described as closely related and phenotypically similar to *Campylobacter*, and has been isolated from various water samples, including untreated sewage [60,61]. There is currently no standardized protocol for *Arcobacter* isolation; thus, conventional culturing methods for *Campylobacter* detection have been used to identify *Arcobacter* [20,62]. *Arcobacter* has been isolated from wastewater samples before [62] and this organism receives increasing attention as a potential cause of human illness [63]. It has been reported that the genus *Arcobacter* has become increasingly important in recent years because its members have been associated with human illness and fecal contamination by humans and animals [62]. At present, *A. butzleri, A. cryaerophilus* and *A. skirrowii* are considered the most common species of the genus as emergent enteropathogens and potential zoonotic agents [61,64].

## 5. Conclusions

In conclusion, the effectiveness of recovery of *Campylobacter* from effluent waters by culture depends on the method of isolation. Direct plating on CASA agar provides a quick, simple and reliable method and performed better than the recommended mCCDA plates. Addition of a filtration step or enrichment in Preston could improve the chance of recovering *Campylobacter*, however, the method of choice influences the recovered species (*C. jejuni vs. C. coli*).

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## Author Contributions

María Concepción Porrero, Lucas Domínguez and María Ugarte Ruiz conceived and designed the study; experimental work and statistical analysis was mainly performed by María Ugarte Ruiz with assistance from Diego Florez-Cuadrado and Trudy Wassenaar assisted in preparation of the manuscript and in analysing the data. All authors contributed to editing the manuscript.

**Conflicts of Interest**

The authors declare no conflict of interest.

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### 6.3. Estudio III. Efecto del protocolo de aislamiento en la detección y caracterización genética de *Campylobacter* termófilos en la cadena alimentaria

Los estudios de caracterización genética de *Campylobacter* termófilos son especialmente útiles para determinar cuáles son los aislados que, de forma más predominante o persistente, pueden llegar a contaminar los alimentos, permitiendo evaluar posibles vías de transmisión [19]. Por otro lado, se ha observado que el método de aislamiento influye tanto en la recuperación de muestras positivas como en la distribución de las especies de *Campylobacter* [127, 128], de manera que el estudio de las poblaciones de esta bacteria, obtenidas por diferentes protocolos microbiológicos, permitiría comparar el efecto del método de cultivo en los genotipos detectados.

Así, procedimos a realizar un estudio de los aislados de *Campylobacter* termófilos obtenidos a partir de diversas fases de la cadena alimentaria mediante diferentes protocolos de aislamiento (capítulo 5.1). Se analizaron 296 cepas procedentes de muestras de pollo (n=98), incluyendo aislados de contenido cecal (n=97 aislados de 34 muestras), piel de cuello (n=133 aislados de 35 muestras) y pechuga de pollo fresca envasada (n=66 aislados de 29 muestras). Para cubrir la máxima diversidad de cepas posible, en este estudio se diferenciaron dos subgrupos, A y B:

- El subgrupo A (n=226 aislados de 76 muestras) incluía muestras procedentes de 28 lotes de animales muestreados en diferentes fases de la cadena alimentaria en el matadero (contenido cecal, piel de cuello y pechuga de pollo fresca envasada).

- El subgrupo B (n=70 aislados de 22 muestras) estaba compuesto por las mismas matrices, pero las muestras pertenecían a lotes de producción no relacionados y fueron obtenidas en matadero (contenido cecal y piel de cuello) y en supermercados locales (pechuga de pollo fresca envasada). En total se incluyeron muestras de diez lotes de contenido cecal, cuatro lotes de piel de cuello y diez lotes de pechuga de pollo envasada.

Para llevar a cabo la caracterización genética, se utilizó el tipado de la región variable del gen de la flagelina y se analizó la riqueza de genotipos (fla-gt), entendida como el número de genotipos de *fla*A distintos detectados. Este dato fue corregido

matemáticamente para la categoría (matriz o protocolo de aislamiento) con el menor número de aislados.

Analizando los datos del subgrupo A y el B de forma conjunta, se identificaron 51 genotipos distintos (33 en contenido cecal, 41 en piel de cuello y 24 en pechuga de pollo). El genotipo más frecuente fue el *fla*A 30 (21 aislados), aunque únicamente fue identificado en nueve muestras de las 98 analizadas (9/98; 9%). Considerando los genotipos por subgrupos, 31 fueron detectados solamente en el subgrupo A, ocho en el subgrupo B y 12 fueron compartidos entre ambos subgrupos. Los resultados reflejan la elevada variabilidad genética obtenida en la población de *Campylobacter*, así como la presencia de genotipos (n=12) compartidos entre ambos grupos (n=59 aislados del subgrupo A y n=52 aislados del subgrupo B), lo que podría estar relacionado con un mayor grado de diseminación de los mismos.

En el subgrupo A se detectaron en total 43 genotipos (28 en contenido cecal, 39 en piel de cuello y 18 en pechuga de pollo). La fla-gt fue mayor en las cepas de piel de cuello (29; IC 95%, 25–33) y contenido cecal (24; IC 95%, 21–26), que en aislados de pechuga (18; IC 95%, 18–18). Además, analizando los resultados para una misma muestra, el mayor número de genotipos diferentes fue encontrado en piel de cuello (de uno a cinco *fla*A diferentes/muestra), mientras que las muestras de contenido cecal y pechuga de pollo presentaban entre uno y dos genotipos distintos. Al examinar los aislados procedentes del mismo lote de producción, al menos uno de los genotipos detectados en pechuga fue también detectado en contenido cecal en trece de los 22 lotes positivos en las tres matrices (13/22; 59%). En tres de los lotes (3/22; 14%), los genotipos de los aislados de pechuga eran compartidos con los de piel de cuello. Sin embargo, en seis lotes (6/22; 27%), los genotipos observados en pechuga fueron completamente distintos a los aislados obtenidos en piel de cuello o contenido cecal.

Si analizamos los resultados obtenidos en el subgrupo B, el número de genotipos detectados fue 20 (7 en contenido cecal, 5 en piel de cuello y 10 en pechuga de pollo). En este caso, la riqueza de genotipos fue mayor en aislados de pechuga de pollo envasada (10; IC 95%, 10–10) y contenido cecal (6; IC 95%, 4–7) que en aislados de piel de cuello (5; IC 95%, 3–5), lo que podría estar relacionado con que en el caso de la piel de cuello las diez muestras analizadas pertenecían a cuatro lotes, mientras que en contenido cecal y pechuga de pollo las diez muestras se correspondían con diez lotes distintos. Comparando los datos por matriz para una misma muestra, el mayor número de genotipos diferentes se recuperó a partir de piel de cuello y pechuga de

pollo (de uno a tres *fla*A diferentes/muestra), mientras que las muestras de contenido cecal presentaban entre uno y dos genotipos distintos. Además, se identificaron genotipos compartidos para una misma matriz en el subgrupo B, tanto en contenido cecal (*fla*A 34) como en piel de cuello (*fla*A 9), pero no en el caso de las muestras de pechuga de pollo.

En general, la riqueza de genotipos disminuyó aplicando enriquecimiento, tanto caldo Bolton como Preston, comparado con la siembra directa para una misma muestra, lo que se explica por la propia selección de genotipos esperada al hacer enriquecimiento en caldo de cultivo. Por otro lado, la utilización de placas (con o sin enriquecimiento) de CFA, incrementó la riqueza de genotipos en comparación con el uso de placas de mCCDA.

Con el fin de comparar los datos de *fla*A con MLST, una selección de aislados basada en los distintos genotipos de *fla*A obtenidos en las diferentes matrices (n=112), fue caracterizada por MLST. Los resultados de ambas técnicas presentaron un grado equivalente de discriminación, ya que para los 112 aislados caracterizados, observamos 43 genotipos diferentes con *fla*A y 45 con MLST.

Nuestros resultados mostraron una gran variabilidad de genotipos, confirmando que la riqueza de los mismos disminuye al final de la cadena de producción de alimentos, siendo mayor en piel de cuello cuando analizamos muestras de lotes de producción relacionados. En el caso de la pechuga, destaca que los genotipos detectados son en algunos casos no identificados en el resto de matrices. Así, las cepas de *Campylobacter* identificadas en las muestras de pollo podrían proceder tanto de contaminaciones con cepas del mismo animal y/o lote, como de contaminaciones cruzadas con cepas de animales y/o lotes diferentes a lo largo del procesado. Además, la riqueza de genotipos se vio influenciada por el protocolo de cultivo microbiológico utilizado, variable que debería ser considerada en estudios epidemiológicos al facilitar la detección de la diversidad presente en las muestras.

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ORIGINAL ARTICLE

# The effect of different isolation protocols on detection and molecular characterization of *Campylobacter* from poultry

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**Significance and Impact of Study:** The tracing of *Campylobacter* through the food chain remains important to control campylobacteriosis in humans. Our study points out that the isolation method used affects the genotypes obtained, and this should be considered as a variant when comparing the results of surveillance studies.

**Keywords**
*fla*A typing, food chain, genotypes, isolation method, molecular typing, thermophilic *Campylobacter*.

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## Abstract

We determined whether different methods to isolate *Campylobacter* (including the ISO standard 10272:2006-1) affected the genotypes detectable from poultry, at three points during slaughter: caecal content, neck skin and meat. Carcasses from 28 independent flocks were thus sampled (subset A). In addition, ten neck skin samples from four flocks, ten caecal samples from ten different flocks and ten unrelated meat samples obtained from local supermarkets were collected (subset B). *Campylobacter* was isolated using eight different protocols: with and without enrichment using Bolton broth, Preston broth or Campyfood broth (CFB), followed by culture on either modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) or Campyfood agar (CFA). All obtained isolates were genotyped for *flaA*-SVR, and over half of the isolates were also typed by MLST. The strain richness, as a measure of number of detected fla-genotypes, obtained from subset A neck skin and caecal samples was higher than that of meat samples. In half of the cases, within a flock, at least one identical fla-genotype was obtained at all three slaughter stages, suggestive of autologous contamination of carcasses. Enrichment reduced the observed richness of isolates, while CFA plates increased richness compared to mCCDA plates, irrespective of inclusion of an enrichment step. Because the isolation protocol used influences both the yield and the fla-genotype richness obtained from poultry, this variable should be taken into account when different studies are being compared.

## Introduction

Monitoring of the poultry meat production process for occurrence of thermophilic *Campylobacter* (notably, *Camp. jejuni* and *Camp. coli*) remains an important mitigation to avoid this zoonotic pathogen from entering the food chain (Bull *et al.* 2006; Humphrey *et al.* 2007; Lin 2009; Newell *et al.* 2011; EFSA journal 2012; van Gerwe 2012). In addition to determining contamination at various stages of the meat processing chain (recording positive or negative findings), quantitative data are needed to determine how many bacteria are present at various stages of production (Miwa *et al.* 2003; Potturi-Venkata *et al.* 2007a; Rantsiou *et al.* 2010). The obtained *Campylobacter* isolates are often further characterized in order to determine the diversity of the bacterial population at a given

stage of the meat production process, for instance to investigate if particular isolates are dominant or persistent in the production line (e.g. Newell *et al.* 2001; Wang *et al.* 2011; Colles and Maiden 2012; Melero *et al.* 2012). Unfortunately, such studies are elaborate to perform, face a number of experimental challenges, and their results are often difficult to interpret or generalize. Genotyping studies to determine the fate of diverse bacterial populations during meat production are elaborate, as a large number of samples need to be genotyped; they are challenging, as different sample types require different isolation techniques that can potentially bias the outcome, and they are difficult to interpret when several genotyping methods are combined. Finally, the outcome of particular studies may not be applicable to other facilities where production conditions and their selective forces may be different.

Genetic diversity in a mixed population is the result of two factors: the richness and evenness of its components. Richness expresses how many different components are present, while evenness describes the relative distribution of these components. Because sampling, by definition, is incomplete, the true richness and evenness of a population must be estimated statistically. Some estimated diversity indices, such as Simpson's Index of Diversity (SID), confound these two factors into one single variable, which reduces the available information on the population in question. Here, we concentrate on diversity in terms of richness within a population.

There are two practical approaches of assessing the diversity of *Campylobacter* populations in the poultry meat production chain. In one approach, birds from a single flock, supposedly all being colonized by the same strain or strains of *Campylobacter*, are followed as they enter the slaughterhouse (if samples were not already taken on the farm) until their meat leaves the premises, whereby crucial steps in the production line are being sampled. In order to investigate possible fluctuations in the bacterial populations during processing, large numbers of samples per sampling point need to be investigated, both qualitatively and quantitatively. As a consequence, only few such investigations have been performed, and it is difficult to judge how representative these studies are for other flocks or other slaughterhouses (Hanning *et al.* 2010; Kudirkiene *et al.* 2012). The other approach is to follow multiple flocks, preferably from different farms so that they are colonized by different populations, at the cost of fewer samples being taken per production batch and per sampling point. This approach can investigate multiple populations, but the lack of 'deep sampling' per flock produces more superficial findings, so that population shifts per sample step within a batch may remain hidden (Hunter *et al.* 2009; Damjanova *et al.* 2011; Elvers *et al.* 2011; Habib *et al.* 2012).

Both approaches are labour-intensive and require high numbers of samples. Moreover, the use of enrichment media, often required to determine the presence of *Campylobacter* on meat (Corry *et al.* 1995; ISO 2006; Oyarzabal *et al.* 2013), may influence the outcome of the detected population. As reported in several studies, the recovery of *Campylobacter* may vary depending on the isolation protocol used. The use of enrichment broth not only limits the quantitative validity of the data, but may also affect strains which are found as dominant in case mixed populations are present (Jasson *et al.* 2009; Habib *et al.* 2011; Vidal *et al.* 2012; Chon *et al.* 2013; Hayashi *et al.* 2013). Even without enrichment, different isolation protocols may have an effect on the strains that are being detected, which introduces further variation as to which populations are determined (Newell *et al.* 2001; Potturi-Venkata *et al.* 2007b; Williams *et al.* 2012).

In this study, we compared the outcome of various isolation methods for detection of *Campylobacter* in the slaughterhouse, with or without enrichment. Multiple flocks, originating from different farms that were processed in a single abattoir, were sampled at three production stages. Because we attempted to obtain representatives of the predominant population in each of these steps, only single colonies were investigated from single samples, unless different morphologies were visible, in which case representative colonies were being picked. Because each sample was analysed by each of the eight investigated culture methods, multiple isolates per sample were frequently obtained. All isolates were genotyped by *flaA* polymorphism in order to identify strains, and a subset was being typed by MLST for confirmation.

## Results and discussion

Of the 28 flocks that belonged to subset A sampled in the slaughterhouse at three stages of meat processing (caecal content, neck skin and meat before packaging), 22 were found positive for *Campylobacter* at all three stages, irrespective of which method of detection resulted in a positive finding. In four sampled flocks, meat samples were negative, whereas caecal content and neck skin were positive. In one flock, only caecal content was positive and in one flock only neck skin was positive.

In total, 296 *Campylobacter* isolates were recovered. Of these, 46% were *Camp. jejuni* and 54% were *Camp. coli*. The frequency of recovered *Camp. coli* was highest in caecal content (69%), followed by meat (56%) while only 41% of the neck skin isolates were *Camp. coli*.

All obtained *Campylobacter* isolates were characterized for their *flaA*-SVR genotype (fla-gt), resulting in 51 different genotypes. The number of different genotypes detected in individual positive samples ranged from one

to two for meat and caecal samples, and from one to five for neck skin samples. In 18 of the 22 positive subset A meat samples, only one genotype could be detected; for caecal samples, this was the case in 14 of 27 samples. The total numbers of different fla-gts obtained per sample type are summarized in Table 1, and richness of fla-gt was calculated for each type after correcting for the variation in numbers of positives per sample type. The results show that the fla-gt richness for subset A samples is higher (with a 95% confidence) for caecal and in particular neck skin samples than for meat samples. According to Hunter *et al.* (2009), the genetic diversity of *Campylobacter* decreased as carcasses proceeded through processing. Contrary to our results, Hanning *et al.* (2010) found an increase in diversity indices along the farm to carcass continuum.

When the subset B results from unrelated caecal, neck skin and meat samples are added, the difference in fla-gt richness between sample types no longer holds, although neck skin still contained more diverse genotypes than meat samples (Table 1). Of note in this context is that neck skin resulted in more *Camp. jejuni* than the other two sample types.

Table 1 also summarizes the findings for subset B samples separately. The ten flocks sampled for caecal content in this subset were all raised at different farms, but as they were all processed in one abattoir on a single day, their analysis could provide insights into the variation of strains entering a slaughterhouse on a given day. Seven caecal samples were found positive for *Campylobacter*, and two independent flocks happened to be colonized by the same genotype. The ten neck skin samples of subset B were derived from four flocks only (not related to the flocks resulting in caecal samples of subset B), three of which turned out to be positive, which resulted in 8 positive samples. One genotype that was found as the only detectable gt in neck skin samples of the second flock of subset B was also found in the third flock analysed, which could indicate flock-to-flock cross-contamination within the processing line; that third flock produced two further genotypes that had not been encountered yet on that day. Of ten meat batches purchased at five supermarkets seven were found positive, and there were no shared genotypes between these unrelated meat batches.

A comparison of the obtained fla-genotypes between the three sample types is most meaningful for isolates that were obtained within a flock, as this would reveal whether the fla-gt found on meat matched that originally present in the caecal content of birds belonging to that flock. Therefore, this analysis was restricted to subset A. Of the 22 investigated flocks that were positive for all three sample points, the fla-gt found on a meat sample of a given flock matched at least one of the gts obtained from caecal content from that flock in 12 cases; these included two flocks where all obtained isolates from three-point sampling resulted in one and the same fla-gt. In three flocks, the fla-gt of meat isolates matched at least one type also found on neck skin, but not on caecal content of birds belonging to that flock, while in six cases, the fla-gt of meat isolates was completely different from those found

**Table 1** The number of *Campylobacter*-positive samples per matrix and the obtained number of different *flaA*-SVR genotypes, irrespective of the isolation method

| | Subset A samples (from 28 flocks) | | |
|---|---|---|---|
| | Caecal content | Neck skin | Meat |
| Total number of positives (%) | 27 (96) | 27 (96) | 22 (79) |
| Total number of different fla-gts | 28 | 39 | 18 |
| Richness of subset A* | 24 (CI 95%, 21 26) | 29 (CI 95%, 25 33) | 18 (CI 95%, 18 18) |
| | Subset B samples (unrelated samples) | | |
| | Caecal content (10 samples from 10 flocks) | Neck skin (10 samples from 4 flocks) | Meat (10 samples from 10 meat batches) |
| Total number of positives (%) | 7 (70) | 8 (80) | 7 (70) |
| Total number of different fla-gts | 7 | 5 | 10 |
| | All samples (subsets A plus B) | | |
| Total number of positives (%) | 34 (89) | 35 (92) | 29 (76) |
| Total number of different fla-gts | 33 | 24 | 41 |
| Total richness* | 28 (CI 95%, 24 31) | 31 (CI 95%, 27 35) | 24 (CI 95%, 24 24) |

*Richness of fla-gt was calculated using EcoSim software.

on neck skin or in caecal content. These results indicate that in approximately half of the cases, at least one of the strains present in the caecum was also found on the meat, suggesting autologous contamination. Newell *et al.* (2001) concluded that in some cases, the same subtypes were isolated from caeca and contaminated the end product as they were also observed in carcass washes. Several studies have shown that, apart from autologous contamination, cross-contamination between flocks is also common in poultry slaughterhouses, as *Campylobacter* is capable to survive the conditions that apply to the slaughter environment (Miwa *et al.* 2003; Potturi-Venkata *et al.* 2007a; Damjanova *et al.* 2011; Melero *et al.* 2012). Besides, Elvers *et al.* (2011) observed that the abattoir environment has a significant role in the contamination of carcasses from negative or not fully colonized flocks. From our analysis, we conclude that autologous and cross-contamination contribute equally to the bacterial load on meat.

We next assessed if the method of isolation potentially influenced the number of isolates recovered from a particular matrix. For this analysis, the two subsets A and B were combined, to cover the highest diversity of strains possible in this investigation. The total of 296 isolates, derived from 98 positive samples (34 caecal contents, 35 neck skin and 29 meat samples) were analysed. The results are summarized in Table 2, with the best-performing isolation methods shown on top. As previously reported (Ugarte-Ruiz *et al.* 2012), direct plating on Campyfood agar (CFA) (method A2) produces the highest numbers of positives for faecal or neck skin samples. The frequency of detected *Camp. coli* increased when enrichment was applied. Whereas direct plating resulted in 43 and 32% *Camp. coli* for modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) and CFA plates respectively, enrichment resulted in 53–75% *Camp. coli*, while all five colonies from method B2 were *Camp. coli*.

In terms of the number of genotypes detected, method A2 is also superior for caecal content, but neck skin samples resulted in more genotypes using method P2 (Preston enrichment followed by CFA selective plates). For meat samples, method P1 (Preston enrichment in combination with mCCDA plates) produced both the highest number of positive samples and the highest number of different genotypes.

Next, we assessed the effect of the different media used on the obtained fla-gt richness, regardless of the sample type. The first panel of Figure 1 shows the effect of enrichment. Two comparisons were performed, one in which Bolton (as the worst performing enrichment method, resulting in 31 isolates) was used as a reference against the three other methods; the other was restricted to direct plating and Preston enrichment, using the latter (101 isolates) as the reference. In both cases, a higher richness was obtained by direct plating. As the proportion of detected *Camp. coli* and *Camp. jejuni* varies between the isolation methods (in particular Preston enrichment produced relatively more *Camp. coli*), this difference could also affect the richness observed. We also calculated the Simpson's index of diversity (which is a combination of richness and evenness) with a 95% CI using the Jackknife method. This produced high diversity index values for all the protocols ranging between 0·948 and 0·970. This indicates that, as expected, genotype richness contributed more to the protocol differences than genotype evenness.

The second panel of Figure 1 assessed the effect of the two different selective plates, either combining all data, shown to the left, or restricted to Preston and direct plating (to the right). In both cases, higher richness was

**Table 2** The effect of isolation protocol on recovery and genotype diversity of *Campylobacter* isolated from different matrices (subsets A and B combined)

| | Caecal content | | Neck skin | | Meat | | Total per method | |
|---|---|---|---|---|---|---|---|---|
| Method | No. of isolates* | No. of different genotypes† | No. of isolates* | No. of diff. genotypes† | No. of isolates* | No. of diff. genotypes† | No. of isolates | No. of diff. genotypes |
| A2 | **38** | ***27*** | **32** | 20 | 2 | 2 | 72 | 39 |
| P2 | 14 | 10 | 27 | ***22*** | 15 | 13 | 56 | 30 |
| A1 | 26 | 20 | 25 | 15 | 2 | 2 | 53 | 28 |
| P1 | 2 | 2 | 18 | 14 | **25** | ***17*** | 45 | 25 |
| B2 | 7 | 6 | 10 | 6 | 9 | 8 | 26 | 16 |
| C1 | 3 | 3 | 10 | 10 | 7 | 5 | 20 | 14 |
| C2 | 5 | 5 | 10 | 10 | 4 | 4 | 19 | 15 |
| B1 | 2 | 2 | 1 | 1 | 2 | 2 | 5 | 4 |
| Total per sample type | 97 | 33 | 133 | 24 | 66 | 53 | 296 | 51 |

*The method producing the highest number of isolates for this matrix is given in bold.
†The method producing the highest number of genotypes for this matrix is given in bold, italics.

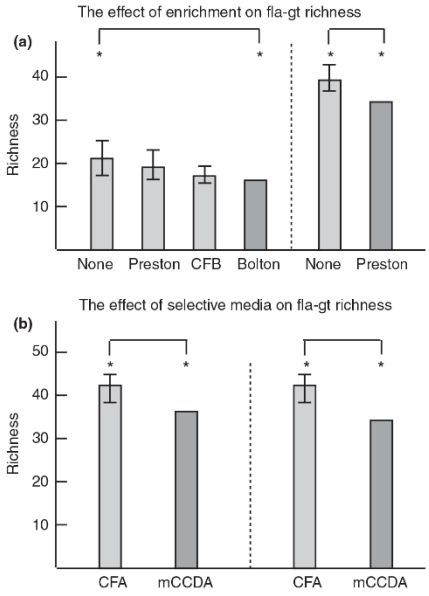


**Figure 1** The effect of enrichment and selective media on the richness of obtained genotypes. In Panel (a) results of methods A1 and A2 were combined (no enrichment), as were P1 + P2 (Preston), C1 + C2 (CFB) and B1 + B2 (Bolton). To the left, a comparison of all four methods is shown, while to the right, direct plating was compared to Preston only. The data used as reference are indicated by the darker shade. In Panel (b) to the left, results of A1, B1, C1 and P1 are combined to analyse all genotypes obtained with mCCDA selective plates, and these were compared to A2, B2, C2 and P2 (CFA). To the right, the data were restricted to direct plating and Preston. Differences with 95% CI are indicated by asterisks.

observed using CFA as selective plate. Malik-Kale *et al.* (2008) found that culturing *Camp. jejuni* in presence of the bile acid deoxycholate (which was present in our mCCDA plates) triggers its invasive potential by stimulating the expression of Cia proteins. As summarized by Newell *et al.* (2001), the method of recovery can influence the subtypes of strains observed, and these authors suggested that enrichment could preferentially select particular strains. Our study provides qualitative data of this effect and points out the influence of the media used during isolation.

In order to check if the grouping of genotypes based on the short variable region of *fla*A only was correct, we typed 112 isolates by MLST. These represented 43 different fla-gts from all sample types; they resulted in 42 different MLST sequence types (STs) (data not shown). The two methods did not always agree: only in 51 cases, two isolates that shared a fla-gt also had an identical ST. In 18 cases, two isolates with identical fla-gt produced a different ST, whereas in 19 cases, two isolates with identical ST produced different fla-gts. These results indicate that the degree of discrimination is comparable for these two methods. Although the combination of fla-gt and MLST results in enhanced discrimination power, this did not affect our conclusions. In the vast majority of cases (44 of 51) where concordance between the two genotyping methods was observed, the isolates were derived from the same batch or even from the same sample (e.g. when isolates resulted from different culture methods). Only in seven cases did unrelated isolates result in identical fla-gt and ST. It was assessed whether fla-gt would have under-estimated the genetic diversity by comparing isolates with the same fla-gt that in fact belonged to different STs. Pairs of isolates for which this applied were all from unrelated batches. The reverse was also true, in that isolates with identical ST but different fla-gt were obtained from unrelated batches (the results are summarized in the Table S1). Thus, the addition of MLST genotyping data did not alter the observations reported for *flaA* typing only.

Two factors influence the findings presented in Table 2 and Figure 1. First, the richness of fla-gts present in each sample type cannot be assumed to be constant – the bacterial population probably varied most for neck skin, both within and between samples. Second, the methods do not perform equally well with all sample types, so that using the optimal method per type introduces methodological variation that can introduce a bias in the genotypes that are detected. In addition, it has been suggested that the ability to adapt to environmental stress may be strain or genotype dependent and this may be genetically determined (Kudirkiene *et al.* 2012).

Table 3 summarizes the findings when two methods were compared for single samples, whereby the selective agar or the enrichment broth varied. Only those samples are considered for which evidence of diversity was obtained. For instance, in 23 cases direct plating of faeces or neck skin samples on CFA and mCCDA resulted in different genotypes, whereas in 11 cases, the results of the two plates corresponded, although other isolation methods detected diversity in genotypes for those samples. Similarly, the use of enrichment broth may change the composition of mixed populations, so that the ratio of common and less common cells may change and this may be broth dependent (Table 3). This was confirmed by SID comparison between the methods, which produced nonsignificant differences for the combined evenness and richness index, while the EcoSim analysis for richness only indicated differences with a 95% CI (data

**Table 3** Bias introduced by enrichment broth and selective agar plates on recovery of *Campylobacter* genotypes

| Comparison of methods* | Same outcome of genotypes was obtained | Different genotypes were detected |
|---|---|---|
| A1 and A2 (direct plating) | In 11 pairs, the diversity in a sample was incorrectly missed | In 23 pairs, the detected genotypes differed so that the outcome is affected by the selective medium |
| P1 with P2, B1 with B2, C1 with C2 (same enrichment broth, different selective agar) | In 17 pairs, the diversity in a sample was incorrectly missed | In 8 pairs, the detected genotypes differed so that the outcome is affected by the selective medium |
| P1 with C1 or B1, C2 with B2 or C2 (different enrichment broth, same selective agar) | In 14 pairs, the diversity in a sample was incorrectly missed | In 10 pairs, the detected genotypes differed so that the outcome is affected by the selective medium |

*Samples for which the two mentioned methods did not result in *Campylobacter* colonies were excluded, as were samples for which all genotypes detected by all positive methods were identical.

not shown). From the data presented in Table 3, we conclude that different enrichment broths or selective agar plates would miss some genotypes. The ISO recommendation to use two selective plates (mCCD and one alternative) increases the chance to identify diversity that would be missed if only one medium was used.

As described in the Material and Methods, colonies were picked from the selective plates based on their morphology; only when morphologically distinct colonies were visible on a single plate, more than one genotype could potentially be detected for one sample by a single method. We assessed retrospectively whether this strategy indeed resulted in detection of different genotypes. In 16 cases, two colonies picked from a single selective plate resulted in different genotypes, but in 11 cases, the two colonies produced the same genotype. Thus, differences in colony morphology were an indication for differences in genotypes more often than resulting in identical genotypes.

In conclusion, the protocol used to isolate *Campylobacter* from poultry influences both the yield and the fla-gt richness obtained. Williams *et al.* (2012) observed that isolates obtained with Preston and Bolton broth were less genetically diverse than those obtained with Exeter broth. The authors suggested a probable effect of the selective components of each broth or a broth-induced stress affecting recovering efficiency. This should be taken into account when different studies are being compared. An optimal method for isolation that can be applied to all three matrix types investigated here cannot be given. The use of direct plating is recommended for faecal samples, while enrichment in Preston works best for neck skin and meat samples. Other authors have shown that the effect of the isolation method on *Campylobacter* detection could vary depending on the level of bacterial contamination of the sample (Baylis *et al.* 2000; Williams *et al.* 2009; Olsson Engvall *et al.* 2011). In our setup, CFA selective plates resulted in detection of more diverse populations than mCCDA plates.

The method of detection can influence which genotypes are being detected. Consequently, the isolation method should be considered as a factor influencing the outcome of molecular surveillance studies.

## Material and methods

### Collecting poultry samples during the slaughter process and at retail

*Campylobacter jejuni* and *Camp. coli* were collected from poultry at the slaughterhouse and, in a limited number of cases, from chicken meat at retail in Spain. A total of 28 flocks were sampled at three time points during processing of carcasses, whereby each flock was sampled at a different day. Sampling was performed for caecal content directly after evisceration, neck skin immediately after chilling and skinless breast meat at the end of the processing line. This produced the samples collectively described here as subset A. In addition, to provide more insight into the distribution of strains within a processing plant in the course of the day, ten neck skin samples were obtained from four different flocks processed at one single day in one slaughterhouse. On another day ten caecal samples were obtained from ten different flocks in the same slaughterhouse. Finally, ten meat samples were obtained from ten unrelated batches of chicken breasts for sale at local supermarkets. Together these thirty samples comprised subset B.

### Isolation of *Campylobacter* from the poultry samples

For isolation and detection of thermophilic *Campylobacter* from the different poultry samples under investigation, eight different protocols were used. These included direct plating on selective agar plates for which either modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA, PO5091A, Oxoid, Basingstoke, UK) or Campyfood agar (CFA, Ref 43471, bioMérieux, Marcy l'Etoile, France) was used. For protocols that included an enrichment step, three broths were compared: Bolton [CM0983, Oxoid, supplemented with antibiotic supplement (SR0183) and

5% lysed horse blood (SR0048) (both from Oxoid)], Preston [Nutrient broth N° 2, CM0067, Oxoid, supplemented with 5% lysed horse blood (SR0048) and antibiotic (SR0204 and SR0232E; Oxoid)] or Campyfood broth (CFB, Ref 42643, bioMérieux, Marcy l'Etoile, France), again combined with either mCCDA or CFA selective plates. The combinations of media used in the eight isolation protocols are summarized in Table 4. Experimental details of methods A1 to P2 are described in Ugarte-Ruiz *et al.* (2012). Methods C1 and C2 are modifications of P1 and P2, respectively, with CFB used as selective broth rather than Preston. Colonies with a typical *Campylobacter* morphology were cultured onto blood agar plates and identified as *Camp. coli* or *Camp. jejuni* as previously described (Ugarte-Ruiz *et al.* 2012). If more than one colony morphology was observed, representative colonies of these were picked. A total of 296 strains from faeces ($n = 97$), neck skin ($n = 133$) and meat ($n = 66$) were analysed and characterized for this study.

### *fla*A typing

A confirmed *Campylobacter* colony was suspended in 600 μl sterile double distilled water and boiled for 10 min to lyse the cells. Cell lysates were centrifuged (600 ***g*** for 10 min), and supernatants were used for PCR. Conventional PCR was used for amplification of the short variable region (SVR) of *fla*A (Meinersmann *et al.* 2005) using the primer pair Fla1 (AAGTCCTGTTCCAACTG AAGT) and Fla2 (AATGGTAATGATGCTTTAGGTA). The primers were designed by Oligo 6.0 software (Molecular Biology Insights, Cascade, CO, USA) and were based on 27 different *Camp. jejuni* and *Camp. coli* *fla*A sequences. Their specificity was verified using 50 strains previously identified as *Camp. coli* and *Camp. jejuni*. PCR amplification of the 444-bp long product was performed in 40 μl containing 2·1 μl of lysed cell supernatant, 20 μl of a PCR master mix (QIAGEN Multiplex PCR kit; Qiagen, Hilden, Germany) and 0·19 μmol $l^{-1}$ of each primer (Invitrogen, Life Technologies, Paisley, UK). The amplification protocol started with denaturation for 15 min at 95°C, followed by 30 cycles with 30 s at 95°C, 90 s at 57°C and 1 min at 72°C, and a final 10 min extension at 72°C. PCR products detection was carried out as previously described (Ugarte-Ruiz *et al.* 2012).

**Table 4** Methods used for the isolation of *Campylobacter* from poultry matrices

| Method | Enrichment step | Selective agar plate* |
|---|---|---|
| A1 | None | mCCDA |
| A2 | None | CFA |
| B1 | Bolton, 37°C, 4 6 h + 42°C, 48 h | mCCDA |
| B2 | Bolton, 37°C, 4–6 h + 42°C, 48 h | CFA |
| P1 | Preston, 42°C, 48 h | mCCDA |
| P2 | Preston, 42°C, 48 h | CFA |
| C1 | CFB, 42°C, 48 h | mCCDA |
| C2 | CFB, 42°C, 48 h | CFA |

CFA, Campyfood agar; CFB, Campyfood broth; mCCDA, modified Charcoal Cefoperazone Deoxycholate Agar.
*All selective agar plates were incubated at 42°C for 48 h.

All *fla*A-derived amplicons were purified using the QIAquick PCR Purification kit (Qiagen) or the MinElute 96 UF PCR purification kit (Qiagen). The purified amplicons were sequenced by Secugen (Madrid, Spain) or Stabvida (Caparica, Portugal). Allele numbers were assigned by sequence comparisons against the existing *flaA*-SVR sequences deposited on the *Campylobacter* MLST database (http://pubmlst.org/campylobacter) sited at the University of Oxford.

### Multilocus sequence typing

Multilocus sequence typing of the seven housekeeping genes *asp*A (aspartase A), *gln*A (glutamine synthetase), *glt*A (citrate synthase), *gly*A (serine hydroxymethyltransferase), *pgm* (phosphoglucomutase), *tkt* (transketolase) and *unc*A (ATP synthase α subunit) was carried out following previously described methods (http://pubmlst.org/campylobacter/) with an annealing temperature of 56°C for all primers with the exception of 62° for *tkt*.

### Statistical analysis

The obtained diversity of genotypes was compared using their *fla*A alleles. Diversity is affected by strain richness as well as strain evenness. We concentrated on richness to assess which method or sample type reported the highest number of different genotypes. Because the detected richness (number of genotypes) depends on the number of samples being analysed (Hurlbert 1971), the observed richness in collections of different sampling size should not be directly compared. Instead, richness was calculated by rarefaction with a 95% confidence interval after correction for the number of isolates using Ecosim 7.72 software (Gotelli and Entsminger 2012). In each case, a specified number of individuals (the size of the smallest group within a comparison) are randomly taken from the larger one. The process is iterated 1000 times to generate a mean and a variance of fla-gt richness. We used the 95% CI of this estimate for comparison among groups of unequal sample size. The abundance of detected genotypes (evenness) in the sample was not assessed in detail, but in cases, a SID was calculated using the Online Tool for Quantitative Assessment of Classification Agreement

(Behringer *et al.* 2011), which produces an index that takes into account both richness and evenness.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1** fla-gt and available MLST sequence type of all isolates.

## 6.4. Estudio IV. Prevalencia del sistema de secreción tipo VI en cepas de *Campylobacter jejuni* procedentes de España

Debido a la alta frecuencia de infecciones alimentarias producidas por *C. jejuni*, se han destinado gran cantidad de recursos a la identificación de mecanismos implicados en la patogenicidad y la supervivencia de la bacteria en los últimos años. Recientemente se ha descrito un sistema de secreción de proteínas en cepas de *C. jejuni*, relacionado con la supervivencia bacteriana en presencia de sales biliares y deoxicolato y con mecanismos de colonización, adherencia e invasión celular denominado SST6 [36, 37].

Este trabajo se desarrolló con el objeto de conocer la presencia de este mecanismo de secreción en los aislados de *C. jejuni* de nuestra colección. El análisis se llevó a cabo mediante secuenciación masiva usando *Illumina MiSeq* en una selección al azar de 63 cepas de *C. jejuni* obtenidas en dos estudios previos (capítulos 5.1 y 5.2). Estas cepas procedían de la cadena alimentaria (n=17 de contenido cecal, n=23 de piel de cuello y n=19 de pechuga de pollo fresca envasada) y de efluentes urbanos (n=4).

Los datos obtenidos reflejaron que la proporción de cepas que contenía los 13 genes característicos del SST6 descritos para la cepa de referencia (*C. jejuni* 108) era del 14% (9/63). Por otro lado, el 81% (51/63) fueron consideradas negativas, ya que carecían de todos los genes propios del sistema, mientras que el 5% restante (3/63) poseían parte de los genes (faltando 1, 5 ó 10 respectivamente), siendo clasificadas igualmente como negativas. Las muestras positivas correspondían con cepas procedentes de contenido cecal (4/17; 24%), piel de cuello (3/23; 13%) y pechuga de pollo (2/19; 11%), sin embargo, ninguna de las cepas de efluentes urbanos fue positiva a ninguno de los 13 genes que componen el SST6. Aunque aparentemente hay una diferencia en la proporción de aislados de *C. jejuni* positivos al SST6 en función del origen de los aislados, el número de cepas analizadas de efluentes urbanos no permite establecer si existe una relación entre la matriz y la detección del SST6.

Asimismo, aunque la presencia del gen *hcp* había sido propuesta como un marcador de la presencia del SST6, nuestros datos mostraron que dicho marcador no implica la presencia del SST6 completo, ya que algunas cepas contenían dicho gen pero carecían de otros genes propios del sistema (2/63; 3%).

A pesar de que nuestros datos demuestran que el SST6 está presente en aislados de diferentes matrices en España, serían necesarios más estudios para determinar la

prevalencia de dicho mecanismo y, sobre todo, para identificar su papel específico en la patogenicidad o supervivencia de las cepas de *C. jejuni* positivas al mismo.

Zoonoses and Public Health

SHORT COMMUNICATION

# Prevalence of Type VI Secretion System in Spanish *Campylobacter jejuni* Isolates

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## Impacts

- Sixty three Spanish *C. jejuni* isolates (poultry and urban effluent) were investigated for presence of Type VI secretion system (T6SS) using whole-genome sequencing.
- The proportion of isolates harbouring all 13 T6SS ORFs was 14%.
- Further research would be necessary to determine the prevalence and importance of T6SS-positive *C. jejuni* strains.

**Keywords:**
*Campylobacter*; T6SS; virulence; Spain

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## Summary

Infections from *Campylobacter jejuni* pose a serious public health problem and are now considered the leading cause of foodborne bacterial gastroenteritis throughout the world. Sequencing of *C. jejuni* genomes has previously allowed a number of loci to be identified, which encode virulence factors that aid survival and pathogenicity. Recently, a Type VI secretion system (T6SS) consisting of 13 conserved genes was described in *C. jejuni* strains and recognised to promote pathogenicity and adaptation to the environment. In this study, we determined the presence of this T6SS in 63 Spanish *C. jejuni* isolates from the food chain and urban effluents using whole-genome sequencing. Our findings demonstrated that nine (14%) strains harboured the 13 ORFs found in prototype strain *C. jejuni* 108. Further studies will be necessary to determine the prevalence and importance of T6SS-positive *C. jejuni* strains.

## Introduction

Campylobacteriosis is the most frequently reported zoonotic diarrhoeal disease worldwide with 80–90% of infections being attributed to *Campylobacter jejuni* (Humphrey et al., 2007; Fitzgerald et al., 2008; Epps et al., 2013; EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2014). Transmission of *Campylobacter* occurs throughout the food chain, often through cross-contamination (Epps et al., 2013). Consumption of poultry, beef and pork products is the leading cause of human foodborne illness, with poultry estimated to account for 50–70% of human *Campylobacter* infections (Jorgensen et al., 2002; Humphrey et al., 2007; Epps et al., 2013).

Recently, *C. jejuni* strains isolated from South-East Asia have been shown to harbour novel type VI secretion system (T6SS). T6SS are able to promote pathogenicity, symbiotic relationships and a selective adaptation to environmental perturbations (Jani and Cotter, 2010; Lertpiriyapong et al., 2012). The *C. jejuni* T6SS was found to have pleiotropic effects ranging from virulence, influencing cell adhesion, cytotoxicity towards erythrocytes and colonisation of mice (Lertpiriyapong et al., 2012; Bleumink-Pluym et al., 2013; Harrison et al., 2014). Current structural models of T6SS consist of a bacteriophage-like structure and a cell envelope-spanning membrane-associated assembly that translocates protein effectors into different cell types (Cascales and Cambillau, 2012; Silverman et al., 2012). A loci containing 13 ORFs can be subdivided into three groups; group one genes *tssJ*, *tssL* and *tssM* encode for membrane-associated proteins; group two genes *tssB*, *tssC*, *tssD* (*hcp*), *tssE* and *tssI* (*vgrG*) encode for proteins with function related to tailed bacteriophage components; group three genes *tssA*, *tssF*, *tssG*, *tssH* (*tagH*) and *tssK* encode for proteins with unknown function (Silverman et al., 2012; Fritsch et al., 2013).

Bleumink-Pluym et al., 2013 showed that of 80 investigated strains (which were predominantly from Europe or USA), only 10% harboured a T6SS. More recently a study compared the presence of T6SS in *C. jejuni* strains from the UK and Vietnam (Harrison et al., 2014), where T6SS was present in 60.6% and 71.4% of humans and chicken isolates respectively in Vietnam. However, in the UK, *C. jejuni* strains harbouring a T6SS from humans and chickens were noted as being 2.6% and 3.9%, respectively (Harrison et al., 2014). Given the difference between strains harbouring T6SS and the potential impact regarding strain virulence, we investigated the identification of T6SS loci within 63 Spanish *C. jejuni* strains isolated from poultry and urban effluents. The strains were isolated from a range of sources including faeces, neck skin, chicken meat and urban effluents. For the identification of T6SS in these *C. jejuni* isolates, whole-genome sequencing was performed.

## Materials and Methods

### Sample collection

*C. jejuni* were collected from poultry at various slaughterhouses and chicken meat from retail markets in Spain from 2010 to 2011. *C . jejuni* was identified from neck skin immediately after chilling, skinless breast meat at the end of the processing line and faecal content directly after evisceration (Ugarte-Ruiz et al., 2012). In addition, *C. jejuni* was also isolated from urban effluents at a sewage water treatment plant from 2010 to 2014. For this study, 63 *C. jejuni* strains were investigated from the food chain which included 23 neck skin, 19 meat and 17 faecal isolates plus 4 isolates from urban effluents. Isolation and detection of *C. jejuni* was performed in Spain as described by Ugarte-Ruiz et al. (Ugarte-Ruiz et al., 2012). Genomic DNA (gDNA) was isolated using PureLink® Genomic DNA Mini - Life Technologies (Grand Island, NY, USA).

### Genome sequencing, assembly and annotation

Genome sequencing of all *C. jejuni* strains was performed using Illumina MiSeq 2 × 151 bp paired-end sequencing. Initial data quality was assessed in FastQC (Andrews, 2010). The sequencing reads were quality controlled using Trimmomatic (v0.32) ('leading' and 'trailing' setting of 3, a 'slidingwindow' setting of 4 : 20 and a 'minlength' of 36 nucleotides) (Bolger et al., 2014). Reads were mapped using BWA-MEM (v0.7.7-r441) against the genome sequence of T6SS-positive *C. jejuni* 414 (CM000855) (Li and Durbin, 2009). Assembly was performed using VelvetOptimiser (v2.2.5) using n50 optimization (Zerbino and Birney, 2008; Gladman and Seemann, 2012). Contigs were ordered against *C. jejuni* 414 using ABACAS (v1.3.1) (Assefa et al., 2009). Annotation of genomes was performed with RATT (Otto et al., 2011) using *C. jejuni* NCTC 11168 (AL111168), *C. jejuni* 414 (CM000855), *C. jejuni* RM1221 (CP000025), *C. coli* 76339 (HG326877), *C. coli* CVM N29710 (CP004066), *C. concisus* 13826 (CP000792), *C. fetus* 82-40 (CP000487), *C. jejuni* 81-176 (CP000538), *C. jejuni* M1 (CP001900) and *C. lari* RM2100 (CP000932). Genomes were visualised using Artemis and ACT software (Carver et al., 2012). T6SS ORFs were identified using BLAST (Altschul et al., 1990; Gish and States, 1993).

## Results

Using T6SS nucleotide and protein sequences from *C. jejuni* strain 108 (JX436460), the genomes of the 63 Spanish isolates were analysed to identify the presence of T6SS ORFs (Table 1). Our study identified 9 of 63 (14%) isolates harbouring all 13 T6SS ORFs. These strains were from faecal, neck skin and breast meat, whereas none of the isolates from urban effluents contained any T6SS ORFs (Table 1). A total of 51 of 63 (81%) strains did not include any T6SS ORFs and were considered as negative. Three isolates named as ZTA10/00846CPD PRESTON, ZTA10/02285CPF and ZTA10/02286CPF PRESTON (representing 5% of total sample number) did not contain the whole 13 T6SS repertoire, lacking 1, 5 and 10 ORFs respectively.

In addition to using the *C. jejuni* strain 108 T6SS ORF sequences to identify T6SS in the 63 Spanish isolates, we also used the T6SS from *C. jejuni* strain 414 (CM000855). The same T6SS ORFs were identified in the 63 Spanish isolates when using T6SS nucleotide and protein sequences from *C. jejuni* strain 414. The *C. jejuni* strain 414 genome was not annotated with a T6SS and so we used the *C. jejuni* strain 108 T6SS nucleotide and protein sequences to determined the location of the *C. jejuni* strain 414 T6SS ORFs (C414_000040085 (*tssD*), C414_000040087 (*tssM*), C414_000040089 (*tssH*), C414_000040090 (*tssL*), C414_000040091 (*tssK*), C414_000040092 (*tssJ*), C414_000040093 (*tssA*), C414_000040095 (*tssB*), C414_000040096 (*tssC*), C414_000040097 (*tssE*), C414_000040098 (*tssF*), C414_000040099 (*tssG*), C414_000040100 (*tssI*).

## Discussion

In this study, we found that the proportion of Spanish *C. jejuni* isolates containing all 13 T6SS ORFs was 14%, which is higher than data from previous studies that predominantly analysed strains from Europe and USA (Bleumink-Pluym et al., 2013; Harrison et al., 2014), but significantly below the rates in Vietnam (Harrison et al., 2014); noting that different sources and method for collection of samples have existed within the studies.

 

**Table 1.** T6SS from *C. jejuni* strain 108 with the respective amino acid size and matches identified in the Spanish isolates. Negative results are not shown

| | TssA | TssB | TssC | TssD | TssE | TssF | TssG | TssH | TssI | TssJ | TssK | TssL | TssM | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amino acids | 415 | 161 | 484 | 171 | 130 | 573 | 302 | 299 | 838 | 148 | 465 | 257 | 1175 | |
| Reference | TssA | TssB | TssC | TssD | TssE | TssF | TssG | TssH | TssI | TssJ | TssK | TssL | TssM | Sample source |
| ZTA10/00476CPD | 413 (99.5%) | 160 (99.4%) | 481 (99.4%) | 171 (100%) | 126 (96.9%) | 567 (99.0%) | 302 (100%) | 294 (98.3%) | 821 (98.0%) | 147 (99.3%) | 464 (99.8%) | 257 (100%) | 1169 (99.5%) | Faeces |
| ZTA10/00846CPD PRESTON | 400 (96.4%) | 54 (33.5%) | 310 (64.0%) | 149 (87.1%) | | 268 (46.8%) | 99 (32.8%) | 297 (99.3%) | 821 (98.0%) | 147 (99.3%) | 278 (59.8%) | 252 (98.1%) | 831 (70.7%) | Meat |
| ZTA10/00847*CPF PRESTON | 413 (99.5%) | 160 (99.4%) | 482 (99.6%) | 141 (82.5%) | 127 (97.7%) | 568 (99.1%) | 301 (99.7%) | 298 (99.7%) | 829 (98.9%) | 147 (99.3%) | 465 (100%) | 254 (98.8%) | 1111 (94.6%) | Meat |
| ZTA10/01736CPF PEQ | 412 (99.3%) | 159 (98.8%) | 482 (99.6%) | 171 (100%) | 127 (97.7%) | 568 (99.1%) | 302 (100%) | 297 (99.3%) | 832 (99.3%) | 146 (98.6%) | 465 (100%) | 257 (100%) | 1169 (99.5%) | Faeces |
| ZTA10/01876CPD | 413 (99.5%) | 160 (99.4%) | 482 (99.6%) | 171 (100%) | 127 (97.7%) | 569 (99.3%) | 302 (100%) | 297 (99.3%) | 820 (97.9%) | 147 (99.3%) | 465 (100%) | 254 (98.8%) | 1172 (99.7%) | Faeces |
| ZTA10/01877CPD | 413 (99.5%) | 160 (99.4%) | 482 (99.6%) | 171 (100%) | 127 (97.7%) | 569 (99.3%) | 302 (100%) | 297 (99.3%) | 820 (97.9%) | 147 (99.3%) | 465 (100%) | 254 (98.8%) | 1172 (99.7%) | Neck skin |
| ZTA10/01877CPFB | 413 (99.5%) | 160 (99.4%) | 482 (99.6%) | 171 (100%) | 127 (97.7%) | 568 (99.1%) | 301 (99.3%) | 298 (99.7%) | 820 (97.9%) | 147 (99.3%) | 465 (100%) | 254 (98.8%) | 1172 (99.7%) | Neck skin |
| ZTA10/02003CPFA | 412 (99.3%) | 160 (99.4%) | 480 (99.6%) | 171 (100%) | 127 (97.7%) | 569 (99.3%) | 302 (100%) | 294 (98.3%) | 818 (97.6%) | 147 (99.3%) | 465 (100%) | 254 (98.8%) | 1168 (99.4%) | Faeces |
| ZTA10/02285CPF | | 160 (99.4%) | 482 (99.6%) | 171 (100%) | 127 (97.7%) | 223 (38.9%) | | 159 (53.2%) | 799 (95.3%) | | | | 496 (42.2%) | Neck skin |
| ZTA10/02286CPF PRESTON | | | | | | | 211 (69.9%) | 187 (62.5%) | 827 (98.7%) | | | | | Meat |
| ZTA10/02655CPF | 412 (99.3%) | 160 (99.4%) | 481 (99.4%) | 171 (100%) | 126 (96.9%) | 569 (99.3%) | 301 (99.3%) | 297 (99.3%) | 824 (98.3%) | 147 (99.3%) | 464 (99.8%) | 257 (100%) | 1171 (99.7%) | Neck skin |
| ZTA11/00018CPD | 413 (99.5%) | 160 (99.4%) | 482 (99.6%) | 171 (100%) | 127 (97.7%) | 569 (99.3%) | 302 (100%) | 297 (99.3%) | 819 (97.7%) | 147 (99.3%) | 465 (100%) | 254 (98.8%) | 1172 (99.7%) | Meat |

The *hcp* gene (haemolysin coregulated protein; here denoted as *tssD*) has been noted as a key marker for the presence of T6SS and either forms a structural component similar to a cell puncturing device, or serves as a secreted effector protein that modulates host actin cytoskeleton rearrangement or cytokine production (Lertpiriyapong et al., 2012; Silverman et al., 2012; Zhou et al., 2012). Our analysis found that samples ZTA10/00846CPD PRESTON and ZTA10/02285CPF were missing one and five T6SS ORFs respectively; however, both contained the *hcp* gene. Furthermore, sample ZTA10/02286CPF PRESTON lacks 10 T6SS ORFs (including *hcp*). Thus, the *hcp* gene may not necessarily indicate the presence of a full T6SS loci (Harrison et al., 2014). We recommend whole-genome sequencing for investigating the presence of T6SS as isolates do not always contain the full repertoire of T6SS ORFs. Further research will be necessary to determine the prevalence and importance of T6SS-positive *C. jejuni* strains.

## Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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## 6.5. Estudio V. Identificación e importancia de la transmisión de *Campylobacter* entre animales salvajes y domésticos de vida libre

Existe una gran variedad de animales salvajes que pueden ser portadores de *Campylobacter* termófilos, suponiendo un riesgo potencial para el hombre y los animales de abasto [17]. Algunos autores han sugerido que el papel que los animales salvajes podrían tener en la transmisión de *Campylobacter* a animales domésticos es poco importante [129], mientras que otros sostienen que existe un flujo significativo de microorganismos entre ambos grupos, a pesar de que no haya sido claramente establecido el sentido del mismo [92, 95]. Dado que en determinadas áreas los animales salvajes pueden contactar con animales domésticos criados en extensivo, se planteó la necesidad de realizar este estudio.

Se analizaron muestras de heces de jabalí (n=150), de cabra montés (n=181), y de ganado vacuno de aptitud cárnica y lidia (n=55) en el Parque Natural de *Els Ports de Tortosa i Beseit*, localizado en el noreste de España. Las muestras recogidas fueron analizadas usando siembra directa en mCCDA (n=386) y una selección de las mismas (n=172) fue procesada en paralelo con dos caldos de enriquecimiento (Bolton y Preston) y cultivada posteriormente en dos medios selectivos (mCCDA y CFA).

Todas las muestras de cabra montés fueron negativas, mientras que en jabalí se aisló *C. coli* en dos animales (2/150; 1,3%) y *C. jejuni* en uno (1/150; 0,67%). *C. jejuni* también fue identificado en ganado vacuno (3/55; 5,45%), aunque en este caso ninguna muestra fue positiva a *C. coli.* Además de *C. coli* y *C. jejuni*, se detectó *C. lanienae*, tanto en jabalí como en vacuno. Esta especie fue la que se aisló con más frecuencia en el caso del jabalí (15/150; 10%) y, además, fue hallada en una vaca (1/55; 1,82%) que habitaba en el mismo barranco que jabalíes positivos a *C. lanienae.* Utilizando los datos de la siembra directa y el enriquecimiento de forma combinada, el porcentaje de muestras positivas a *C. lanienae* en jabalí (13/57; 22,81%) fue superior ($p<0,01$) al observado en el ganado vacuno (1/55; 1,81%). Por otro lado, la proporción de positivos a *C. jejuni* en el ganado doméstico (5/55; 9,09%) fue superior ($p=0,045$) a la observada en jabalí (0/150; 0%). Por último, se aisló una cepa de *C. fetus* subsp. *venerealis* en una muestra de vaca (1/55; 1,81%), microorganismo normalmente presente en el tracto genital del ganado bovino o en abortos.

A pesar de que el jabalí y el ganado vacuno parecen tener su propia especie predominante de *Campylobacter*, la presencia de *C. jejuni* y *C. lanienae* en ambos hospedadores podría indicar una transmisión de aislados de *Campylobacter* entre

ellos. Además, en nuestra zona de estudio, la cabra montés parecía no desempeñar un papel importante en la epidemiología de *Campylobacter*.

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*Original Contribution*

# *Campylobacter* Shared Between Free-Ranging Cattle and Sympatric Wild Ungulates in a Natural Environment (NE Spain)

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**Abstract:** *Campylobacter* infections are a public health concern and an increasingly common cause of food-borne zoonoses in the European Union. However, little is known about their spill-over from free-ranging livestock to sympatric wild ungulates, especially in regards to uncommon *Campylobacter* species. In this study, we aim to determine the prevalence of *C. coli*, *C. jejuni* and other *C.* spp. in game ungulates (wild boar *Sus scrofa* and Iberian ibex *Capra pyrenaica*) and free-ranging sympatric cattle in a National Game Reserve in NE Spain. Furthermore, we explore the extent to which *Campylobacter* species are shared among these co-habiting hosts. Faecal samples from Iberian ibex ($n = 181$) were negative for *C.* spp. By direct plating, two wild boars out of 150 were positive for *C. coli* (1.3%, 95% CI 0.16–4.73), and one was positive for *C. jejuni* (0.67%, 95% CI 0.02–3.66). The latter was predominant in cattle: 5.45% ($n = 55$, 95% CI 1.14–5.12), while *C. coli* was not isolated from this host. *C. lanienae* was the most frequent species in wild boar at 10% (95% CI 5.7–15.96), and one cow cohabiting with positive wild boars in the same canyon also carried *C. lanienae*. Four enrichment protocols (using Bolton or Preston broth combined with either mCCDA or CFA) were added for 172 samples (57 from wild boars, 55 cattle and 60 Iberian ibexes) to increase the number of isolates obtained allowing the detection of statistically significant differences. The prevalence of *C. lanienae* was statistically significantly higher in wild boar than in cattle ($P < 0.01$), but the prevalence of *C. jejuni* was higher in the latter ($P = 0.045$). These results suggest that wild boar and cattle carry their own predominant *Campylobacter* species, while Iberian ibex do not seem to play an important role in the epidemiology of *Campylobacter*. However, there is a potential spill-over of *C.* spp., and thus, further research is needed to elucidate the factors determining inter-species transmission.

**Keywords:** *Campylobacter*, Wildlife, Cattle, Inter-species transmission, Prevalence, *gyr*B, 16S rRNA

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## Introduction and Purpose

*Campylobacter* infections pose a serious public health problem as the principal agent causing bacterial gastroenteritis

throughout the world (Humphrey et al. 2007; FAO/WHO 2009; EFSA Panel on Biological Hazards (BIOHAZ) 2012). Untreated drinking water and unpasteurized milk have been associated with campylobacteriosis outbreaks, but poultry products are considered as the major source of sporadic infections (Olson et al. 2008; Pires et al. 2010). Several studies have implicated wildlife and water-borne sources in *Campylobacter* infection (Ethelberg et al. 2005; Karenlampi et al. 2007). However, information on the ecology of *Campylobacter* in wildlife is scarce; it is not believed to cause disease in wildlife and wildlife's role as a reservoir remains undetermined (Sippy et al. 2012). Research on the link between wildlife and *Campylobacter* in domestic animals has been assessed mainly in agricultural settings, and has especially focused on wild birds (Craven et al. 2000; Colles et al. 2008, 2011; Sippy et al. 2012), small mammals (Meerburg et al. 2006; Jones et al. 2007; Sippy et al. 2012) and insects (Hald et al. 2004). However, there is a gap of knowledge with regards to the potential spill-over from free-ranging or extensive livestock to co-habiting wild ungulates, and vice versa. In fact, *Campylobacter* transmission between wildlife and farm animals may be bidirectional (Sippy et al. 2012).

In some instances, *Campylobacter* prevalence in wildlife has been found to be low, but this strongly depends on the species sampled, its management and the geographic location. Wild boar in particular has been described as a carrier of *C. coli* (Wahlstrom et al. 2003) in Sweden and as a carrier of *C. jejuni* in the same country (Wahlstrom et al. 2003) and in Spain (Diaz-Sanchez et al. 2013). In addition, Atanassova et al. (2008) isolated *Campylobacter* from wild boar meat (2.1%) indicating its potential to enter the food chain. On the other hand, Wacheck et al. (2010) did not isolate *Campylobacter* from any of 153 wild boars in Switzerland.

Traditionally, thermophilic *C. jejuni* and *C. coli* are the most frequently isolated species in foodborne zoonoses in humans (EFSA Panel on Biological Hazards 2012); although a growing number of *Campylobacter* species have been recognised as emerging human and animal pathogens. In fact, their true prevalence is probably underestimated because the methods used for routine isolation favour the growth of some species over others, and these are not routinely identified to the species level (Man 2011). Moreover, there is a lack of information about the carriage of uncommon *Campylobacter* species in wild mammals (Lee et al. 2011). Consequently, further determination at the species level of both isolates from wildlife and livestock could shed light on the epidemiology of *Campylobacter* at the livestock–wildlife interface. Unfortunately, species-specific identification of many campylobacters is problematic because of their fastidious growth characteristics, along with the absence of suitable biochemical assays and the existence of atypical strains. Genotypic identification methods such as PCR targeting ribosomal genes are commonly used to identify several fastidious bacteria including *C.* spp. Moreover, Gorkiewicz et al. (2003) recommend 16S rRNA sequence analysis as an effective and rapid procedure for the unambiguous identification of the majority of campylobacters. Nevertheless, the lower levels of 16S rRNA variations found between some species make identification of strains to the species level difficult (Oporto and Hurtado 2011). Several studies have shown that an analysis based on the DNA gyrase B subunit gene (*gyr*B) sequence has a great degree of resolution, and is considered as a rapid and effective method for identifying bacterial species and for examining phylogenetic relationships (Yamamoto and Harayama 1996; Kawasaki et al. 2008).

In this study, we aim to (1) determine the prevalence of *Campylobacter* in large wild ungulates and free-ranging sympatric livestock; (2) identify the *Campylobacter* species carried by wildlife; and (3) understand the extent to which *Campylobacter* species are common, indicating a potential inter-species transmission. For this purpose, livestock, wild boar (*Sus scrofa*) and Iberian ibex (*Capra pyrenaica*) were sampled in a National Game Reserve in NE Spain. Both wild ungulates are important as popular game species and meat sources: around 3,000 Iberian ibexes and 160,000 wild boars are hunted each year in Spain (Ministerio de Agricultura, Alimentación y Medio Ambiente 2013). However, to our knowledge no previous study has assessed the presence of *Campylobacter* in either the Iberian ibex or in its closest relative, the Alpine ibex (*Capra ibex*). Like the domestic small ruminants (Schilling et al. 2012), the ibex may potentially serve as a silent *Campylobacter* carrier. Interestingly, the previous studies (Mentaberre et al. 2010; Navarro-Gonzalez et al. in press) described a low prevalence or absence of other zoonotic pathogens (*Salmonella enterica* and *Mycobacterium bovis*) in Iberian ibex in spite of a high prevalence in co-habiting wild boar and cattle.

## Methods

### Study Area

The study area is located within the National Game Reserve 'Els Ports de Tortosa i Beseit' (28,587.17 ha) in

northeastern Spain, part of the Natural Park of the same name. It is a calcareous mountain region with high orographic complexity that results in a rugged and abrupt terrain with numerous ravines and steep slopes. About 28% of the surface area is above 1,000 m.o.s.l., with the highest peak being Mont Caro (1,442 m). The predominant habitat is pine grove (39%) followed by oak grove (15%), and rivers account for 0.2% (see Navarro-Gonzalez et al. 2012 for further information on the habitat type). The most abundant wild ungulates are the Iberian ibex and the wild boar, which are exploited for hunting purposes. Wildlife and cattle share pastures in some of the canyons in the study area (see Fig. 1). The total surface of the sampled canyons is 6,791.46 ha.

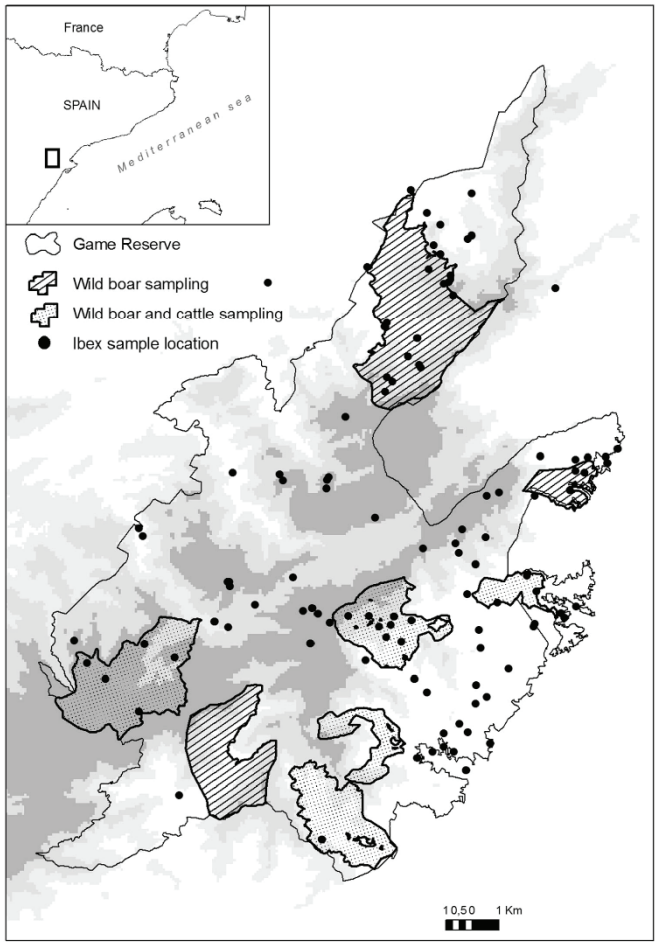


**Figure 1.** Map of the study area and location of the sampling sites.

## Animal Sampling

*Wild Boar*

One hundred and fifty individual faecal samples were obtained from hunter-harvested wild boars during the regular hunting seasons (October–January) of 2009–2010 and 2010–2011. The location of each hunting session was recorded. Faeces were collected directly from the rectum and stored in sterile containers. They were refrigerated and sent to the laboratory within 24 h. The culture was performed immediately after reception.

*Iberian Ibex*

One hundred and eighty-one Iberian ibexes were either hunter-harvested ($n$ = 151) or captured ($n$ = 30) during the regular hunting season (October–May) from March 2009 to January 2011, and faecal samples were obtained directly from the rectum. Due to characteristics of the hunting method, faecal samples were stored at −18°C until transfer to the laboratory. However, samples collected from captured animals were stored in refrigeration (4°C) and sent to the laboratory within 24 h after collection. The culture was performed immediately after reception.

*Free-Ranging Cattle*

Fifty-five samples from cattle were collected from February 2010 to February 2011 coinciding with the wild boar hunting season. The farming conditions in the National Game Reserve are free-ranging with supplemental feeding in the dry season (summer). The herds are small (5 herds and 380 head in total) and aimed at meat production with the exception of one herd bred for bullfighting. For security reasons, herd is fenced; but the enclosure is large in size (around 247 ha) to simulate free-ranging conditions. Cattle sampling was preferably performed on days that wild boars were also sampled. When livestock were located, animals were counted and observed until defecation. Then, faeces were collected and stored in a sterile container and refrigerated and sent to the laboratory within 24 h. The culture was performed immediately after reception.

## Microbiological Analyses

*Isolation of Campylobacter* from the Samples

For detection and identification of the bacterium to determine *Campylobacter* prevalence, direct plating on mCCDA selective agar plates (modified Charcoal Cefoperazone Deoxycholate Agar, Oxoid, Basingstoke, UK) was used for all the samples tested ($n$ = 386). Since we observed that this method yielded a small amount of isolates, we tried to increase the number of positive samples by incorporating alternative protocols; thus, 172 samples were subjected to the methods as shown in Table 1. These included an enrichment step using Bolton (supplemented with antibiotics and 5% lysed horse blood, Oxoid) or Preston broth (supplemented with antibiotics and 5% lysed horse blood, Oxoid). Both enrichment broths were combined with either mCCDA or CFA (Campyfood agar, bioMérieux, Marcy l'Etoile, France). These four enrichment protocols were performed on all 172 samples. The different treatment of the samples has been taken into account in calculating the prevalence of *Campylobacter*.

For direct plating of stool samples ($n$ = 386), a swab was dipped into the sample and streaked onto selective plates. They were incubated 48 h at 42°C under microaerobic conditions (Genbag microaerobic atmosphere generator, bioMérieux,).

For enrichment protocols ($n$ = 172, from which: $n_{\text{wild boar}}$ = 57, $n_{\text{cattle}}$ = 55 and $n_{\text{Iberian ibex}}$ = 60), 1 g of

**Table 1.** Methods used for the isolation of *Campylobacter* from faeces.

| Method | Enrichment step | Selective agar plate | Number of samples |
|---|---|---|---|
| Direct plating | None | mCCDA | 386 |
| B1 | Bolton, 37°C, 4–6 h + 42°C, 48 h | mCCDA | 172 |
| B2 | Bolton, 37°C, 4–6 h + 42°C, 48 h | CFA | 172 |
| P1 | Preston, 42°C, 48 h | mCCDA | 172 |
| P2 | Preston, 42°C, 48 h | CFA | 172 |

All selective agar plates were incubated at 42°C for 48 h.
*mCCDA* modified charcoal Cefoperazone deoxycholate agar, *CFA* Campyfood agar.

fresh faeces was aseptically transferred to a 10 ml sterile screw-cap bottle, and 9 ml of broth was added. Enrichment was performed for 4–6 h at 37°C followed by 48 h at 42°C for Bolton broth and 48 h at 42°C for Preston broth under microaerobic conditions. After this incubation period, 200 µl was cultured for 48 h on the two selective agar plates (mCCDA and CFA) as described above. Please see Ugarte-Ruiz et al. (2012, 2013) for further information on the performance of these protocols.

*Identification of Suspected Campylobacter* Colonies

Following incubation, the plates were examined, and colonies with *Campylobacter*-typical morphology (according to the manufacturer's instructions) were cultured onto blood agar plates (bioMérieux) at 37°C for 48 h in a microaerobic atmosphere. Conventional multiplex PCR, previously described in Ugarte-Ruiz et al. 2012, was used for simultaneous identification of the genus *Campylobacter* and the differentiation between *C. jejuni* and *C. coli*. DNA was liberated by boiling a colony, suspended in 600 µl of sterile double-distilled water, for 10 min.

*gyrB Analysis*

Cell lysates of the *Campylobacter* strains other than *C. coli* or C. *jejuni* ($n$ = 20, 18 from wild boar, 2 from cattle) were used for conventional PCR for amplification of the gene *gyrB* using the primer pair GyrB1 (AARCGYCCNGGHATGTATAT) and GyrB2 (CCDARNGCNGTDATCATATT). The primers were designed by Oligo 6.0 software (Molecular Biology Insights, Cascade, CO, USA). All *gyrB*-derived amplicons (1,352 bp) were purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany), and the PCR products were sequenced by Stabvida (Lisbon, Portugal).

*16S rRNA Analysis*

The 16S rRNA gene sequences of some *Campylobacter* other than *C. coli* or C. *Jejuni* ($n$ = 6) were determined by PCR amplification. The genomic DNA used was extracted and purified as previously described in Lawson et al. 1989. PCR-amplified products, derived from universal primers pA (5′-AGAGTTTGATCCTGGCTCAG; positions 8–27, *Escherichia coli* numbering) and pH* (5′-AAGGAGGTG ATCCAGCCGCA; positions 1,541–1,522), were purified and sequenced as described for *gyrB*.

**Statistical Analysis**

The prevalence and 95% CI of *Campylobacter* obtained by direct plating were calculated using the whole data set. Additionally, with the subset of data for which both enrichment and direct plating had been performed; the prevalence of each *Campylobacter* species was compared between host species with the Fisher test using Holm correction for multiple comparisons. The significance level was set at $\alpha = 0.05$. All statistical analyses were performed with R Software (R Development Core Team 3.0.2. 2013), including the 95% CI with package epiR (Stevenson et al. 2012).

All 16S rRNA and *gyrB* sequences were compared to the sequences of the most closely related species obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and homologies were calculated using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). In addition, calculations of pair-wise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (http://www.eztaxon.org/).

## Results

### Prevalence of *Campylobacter* spp.

Prevalence and 95% CI obtained by direct plating are shown in Table 2. No Iberian ibex was positive for *Campylobacter*.

Table 3 shows the prevalence of *Campylobacter* in the collection of samples for which both enrichment and direct plating had been performed. The prevalence of *C. jejuni* was higher in cattle than in wild boar ($P = 0.045$). Also, the prevalence of *C. lanienae* in wild boar was higher than in cattle ($P < 0.01$). When accounting for the different storage conditions of the Iberian ibex samples (frozen vs refrigerated), no statistically significant difference was found in the prevalence of *C. jejuni* or *C. lanienae* in this species.

*Identification of Campylobacter* Other Than *C. coli* and *C. jejuni*

We observed a high *gyrB* homology among wild boar sequences ranging between 96.6 and 100%, while homologies for cattle and wild boar strains ranged between 83.7 and 85.1% for one cattle strain and between 81.6 and 83.1% for the other (see Table S2 in the supplementary material).

**Table 2.** Prevalence and 95% confidence interval (CI) of *Campylobacter* in wild boar and sympatric cattle by direct plating.

| Host species (sample size) | *C. coli* (95% CI) | *C. jejuni* (95% CI) | *C. lanienae* (95% CI) |
|---|---|---|---|
| Wild boar (150) | 1.3% (0.16–4.73) | 0.67% (0.02–3.66) | 10% (5.7–15.96) |
| Cattle (55) | 0 (0–9.55) | 5.45% (1.14–5.12) | 1.82% (0.05–9.72) |
| Iberian ibex (151 frozen samples/30 fresh samples) | 0 (0–3.02)/0 (0–16.70) | 0 (0–3.02)/0 (0–16.70) | 0 (0–3.02)/0 (0–16.70) |

**Table 3.** Prevalence and 95% confidence interval (CI) of *Campylobacter* in wild boar, Iberian ibex and sympatric cattle by direct plating and enrichment.

| Host species (sample size) | *C. coli* (95% CI) | *C. jejuni* (95% CI) | *C. lanienae* (95% CI) | *C. fetus* (95% CI) |
|---|---|---|---|---|
| Wild boar (57) | 1.81% (0.05–9.72) | **0** (0–9.24) | **22.81%** (12.74–35.84) | 0 (0–9.24) |
| Cattle (55) | 0 (0–9.55) | **9.09%** (3.01–19.95) | **1.81%** (0.04–9.72) | 1.81% (0.04–9.72) |
| Iberian ibex (30 frozen samples/30 fresh samples) | 0/0 (0–16.70) | 0/0 (0–16.70) | 0/0 (0–16.70) | 0/0 (0–16.70) |

In bold, statistically significant differences.

**Table 4.** Results of 16S rRNA sequencing and homology searches.

| Isolate | Nucleotides sequenced (pb) | Nearest blast hit | % homology |
|---|---|---|---|
| 28-WB$^{wb}$ | 1,315 | *C. lanienae* strain S-K FAVW | 99.9 |
| 28-WB$^{wb}$ | 1,315 | *C. lanienae* NCTC 13004$^{T}$ | 99.5 |
| 210-WB$^{wb}$ | 1,306 | *C. lanienae* strain S-K FAVW | 99.9 |
| 210-WB$^{wb}$ | 1,306 | *C. lanienae*NCTC 13004$^{T}$ | 99.5 |
| 1061-WB$^{wb}$ | 1,310 | *C. lanienae* strain S-K FAVW | 99.9 |
| 1061-WB$^{wb}$ | 1,310 | *C. lanienae* NCTC 13004$^{T}$ | 99.5 |
| 1104-WB$^{wb}$ | 1,316 | *C. lanienae* strain S-K FAVW | 98.9 |
| 1104-WB$^{wb}$ | 1,316 | *C. lanienae* NCTC 13004$^{T}$ | 99.2 |
| 2078-C$^{c}$ | 1,337 | *C. lanienae* strain S-K FAVW | 98.8 |
| 2078-C$^{c}$ | 1,337 | *C. lanienae* NCTC 13004$^{T}$ | 98.4 |
| 145-C$^{c}$ | 1,325 | *C. fetus* subsp. *venerealis* NCTC 10354$^{T}$ | 100 |

*wb* wild boar strain, *c* cow strain.

Comparative 16S rRNA gene sequence analysis revealed a high similarity between wild boar and one cattle isolate, and *C. lanienae* strain S-K FAVW (Gorkiewicz et al. 2003). Table 4 shows that the 16S rRNA gene sequences also exhibited a high level of similarity with *C. lanienae* NCTC 13004$^{T}$. Furthermore, comparative 16S rRNA gene sequence analysis revealed 100% similarity between the last strain found in cattle and *C. fetus* subsp. *venerealis* NCTC 10354$^{T}$ (Table 4).

## Discussion

In wildlife, *Campylobacter* are not always determined to the species level since many of the *Campylobacter* species isolated are uncommon (Wahlstrom et al. 2003; Lee et al. 2011; Diaz-Sanchez et al. 2013). The identification of such isolates is important since the true prevalence of *Campylobacter* other than *C. jejuni* and *C. coli* is probably underestimated (Oporto and Hurtado 2011). In our case,

identification to the species level and sampling of co-habiting wildlife and livestock was crucial for better understanding the epidemiology and characteristics of *Campylobacter* in our study area. This procedure allowed us to discern that only *C. lanienae* was shared between cattle and wild boars from the same canyon in the study area. Three *C. lanienae* isolates came from animals (1 cow, 2 wild boars) that were sampled in the same location within a 1-week interval. From a herd of 30 cattle, nine animals were sampled and one was positive for *C. lanienae*. In the same canyon, three wild boars were hunted 6 days later and two were positive for *C. lanienae*. Comparative *gyrB* gene sequence analysis revealed 84–85% similarity between the *C. lanienae* strain from cattle and the *C. lanienae* from the wild boars sampled within a 1-week interval. However, the homology between *C. laniene* from these wild boars was high (99.5%). These findings suggest that there may be spill-over of *Campylobacter* from cattle to wild boar or *viceversa*. Nevertheless, this was the only point of space and time that *Campylobacter* was found in livestock and sympatric wildlife. These data are coincident with the results obtained for *M. bovis* and *S. enterica* in which an association between wild boar and cattle was observed (Mentaberre et al. 2010; Navarro-Gonzalez et al. 2012).

The frequency of *C. lanienae* in wild boars in our study area is surprising. This species has been detected previously in feral pigs from California (Jay-Russell et al. 2012), however, that study found *C. jejuni* to be the most frequent. Moreover, emerging campylobacters isolated from food animals are often strains of species typically associated with livestock, such as *C. lanienae* in cattle and swine (Inglis et al. 2005; Oporto and Hurtado 2011; Miller et al. 2012). In parallel, the importance of *C. fetus* has long been recognised in veterinary medicine. *C. fetus* subsp. *venerealis* is the causative agent of bovine genital campylobacteriosis, an infectious disease that leads to severe reproductive problems in cattle worldwide (Chaban et al. 2012; Iraola et al. 2012). Further studies are needed to confirm the importance of the detection of *C. fetus* subsp. *venerealis* in faeces due to its potential clinical and epidemiological importance in the area of study.

Despite the importance of the emerging *Campylobacter* species, thermophilic *C. jejuni* and *C. coli* are the most frequently isolated species in foodborne zoonoses in humans (EFSA Journal 2012), and our study also illustrates a potential risk of *Campylobacter* exposure for hunters during handling and processing of wild boar meat. *C. jejuni* was found in both cattle and wild boar, but these were not coincident in space.

In the literature, a substantial variability is found in the presence of *Campylobacter* in different species and locations. Kemper et al. (2006) found one of 2,500 reindeer positive for *C. hyointestinalis*, and Lillehaug et al. (2005) found only one of 324 wild cervids positive for *C. jejuni*. A broader survey of Swedish wildlife (Wahlstrom et al. 2003) found a great variability in the prevalence of thermophilic *Campylobacter*: it was low in moose, hare and roe deer, but considerable (>10%) in gulls, wild boar and Canada geese, and absent in red deer and fallow deer samples. Such results in the northern regions of Europe are suggested to be due to the climate conditions, which may be limiting for the survival of enteropathogens (Kemper et al. 2006), but the *Campylobacter* carrier status is not known for many wild species and habitats.

In central and southern Spain, Diaz-Sanchez et al. (2013) found no relation between *C.* spp. in large game animals and the presence of livestock in hunting estates. Despite certain habitat similarities with our study, these authors found a much higher prevalence of *Campylobacter* in wild boar (66%). This may be due to peculiarities in the hunting estates studied by those authors, such as estate fencing, high density of game species and possibly different farming conditions. Further research is needed in order to identify the factors responsible for such differences in the same host in a similar Mediterranean habitat.

The fact that no Iberian ibex was found to be positive for *Campylobacter* (in contrast to the wild boar results) suggests that ibex may not be an important reservoir of *Campylobacter* eligible to be grown. Although most Iberian ibex faeces had to be frozen and this can affect *Campylobacter* isolation, 30 samples processed without previous freezing were also negative for *C.* spp. Furthermore, the absence or low prevalence of other pathogens in ibex in this high-risk area suggests that it may be considered independent from the 'wild boar-cattle cycle', perhaps due to its feeding habits and altitudinal preferences. However, further research is needed, preferably with fresh faecal samples from Iberian ibex, to determine differences in the prevalence of *Campylobacter* in the wild and domestic hosts of our study area.

During the completion of this study, we faced challenges related to both obtaining samples and laboratory procedures. These factors may have introduced variation, leading to an underestimation of the prevalence that was

detected and described for each species in this work. Due to the fragility of bacteriological culture, accurate identification of these organisms can be problematic (Engberg et al. 2000; Musgrove et al. 2001; Davis and DiRita 2008). In addition, as described in previous studies, the protocol used to isolate *Campylobacter* from poultry influences both the yield, including the proportion of species identified, and genetic diversity obtained (Newell et al. 2001; Ugarte-Ruiz et al. 2013; Williams et al. 2012). As reported in Ahmed et al. (2013), the survival times of the bacterium may be influenced by the composition of the faeces or the mucus membrane in the intestine of birds. This may affect the isolation of the bacterium in the different species analysed. Additionally, a wide range of host animals can carry *C.* spp. asymptomatically and excrete it in their faeces. Whiley et al. (2013) described a variable density of *Campylobacter* in human and animal biosolids, and Rapp et al. (2012) reported a variable frequency of excretion and range of concentrations among different cows studied. It was thought that the genotypes present in the environment might also affect the excretion pattern of the cows. Moreover, Maridor et al. (2008) carried out experimental infections showing *Campylobacter* excretion and the intermittence in the excretion of the bacteria by pigs. With regards to the sampling, the remoteness of the study area and its steep terrain sometimes hampered hunting success and the location of the livestock herds. This problem frequently limits research on large wild mammals in mountainous habitats. Furthermore, seasonality, especially in temperate regions, may influence sampling as climate or migratory wild birds could affect the outcome of *Campylobacter* detection (Humphrey et al. 2007; Strachan et al. 2013).

## Conclusion

Wild boar and cattle appear to carry their own predominant *Campylobacter* species. *C. jejuni* (5.45%, 95% CI 1.14–5.12) is the most frequent species found in cattle and *C. lanienae* (10%, 95% CI 5.7–15.96) in wild boar. Iberian ibex do not seem to play an important role in the epidemiology of this microorganism in our study area. However, our findings show a potential spill-over of *C.* spp. including *C. jejuni, C. fetus* and particularly *C. lanienae*. Thus, further research is needed to quantify the occurrence of interspecies transmission between free-ranging cattle and sympatric wild boar especially for *C. fetus,* which induces a severe pathogenicity in domestic animals.

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### 6.6. Estudio VI. Perspectiva europea sobre la transmisión de *Campylobacter* spp. entre animales salvajes, domésticos y personas

El capítulo titulado "A European perspective on the transmission of food-borne pathogens at the wildlife-livestock-human interface", incluido dentro del libro "Food Safety Risks from wildlife: Challenges in Agriculture, Conservation and Public Health. Springer", describe la perspectiva europea sobre la importancia que los animales salvajes pueden tener en la circulación de microorganismos productores de zoonosis de transmisión alimentaria entre animales y personas. La sección del capítulo incluida en este trabajo de tesis contiene la información referida a *Campylobacter*, centrada en las vías de transmisión y en el papel que los animales de vida libre, especialmente las aves, tienen en la epidemiología de esta bacteria.

La principal vía de transmisión de *Campylobacter* termófilos es a través del consumo de alimentos de origen animal, en particular aves de corral [18]. Sin embargo, a pesar de que el consumo de carne de caza es menor que el consumo de carne de animales domésticos en Europa, la carne de especies cinegéticas también se ha relacionado con el desarrollo de campilobacteriosis [130], habiéndose observado la presencia de *Campylobacter* en carne de jabalí y corzo [112, 113]. Asimismo, el manejo de animales o cadáveres, sobre todo durante la caza, ha sido identificado como una fuente de infección de *Campylobacter* para el hombre [97, 112].

También se han definido otras vías de transmisión de *Campylobacter* spp. como el contacto con ambientes contaminados [44]. En este contexto, las heces de animales salvajes, fundamentalmente de aves, han sido reconocidas como una fuente de contaminación ambiental, tanto para animales domésticos como para personas [90, 92]. No obstante, se ha descrito que su papel podría ser limitado debido a la alta especificidad bacteria-hospedador encontrada en las cepas de aves de vida libre [94].

Respecto a los resultados sobre la recuperación de *Campylobacter* spp. a partir de animales salvajes, no hay estudios realmente comparables, ya que los datos varían en función de la época del año, la metodología utilizada, las especies analizadas, los patrones de migración o la localización geográfica, entre otros (introducción, tabla 4).

En definitiva, todos estos estudios reflejan la necesidad de contemplar los animales salvajes de vida libre en los estudios epidemiológicos de *Campylobacter* puesto que podrían participar de forma activa en la transmisión de esta zoonosis.

**Chapter 3**

**A European perspective on the transmission of food-borne pathogens at the wildlife-livestock-human interface**

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***Campylobacter* spp.**

Infections by *Campylobacter* pose a serious public health problem, as *Campylobacter* enteritis has been the most frequently reported zoonotic disease in humans in the EU since 2005, with 214,268 confirmed cases in 2012. *Campylobacter* infections in the EU human population has increased significantly in recent years (2008-2012), with a clear seasonal trend (EFSA, 2014). Thermophilic *Campylobacter*, specifically *C. jejuni* and *C. coli*, are the most common species associated with diarrheal disease in humans in the developed world, accounting for 80-90% and 5-10% of *Campylobacter* infections, respectively (Fitzgerald *et al*., 2008). Nevertheless, other *Campylobacter* spp. are also associated with gastrointestinal and extra-gastrointestinal infections in animals and humans. "Emerging" species, specifically *C. concisus* and *C. upsaliensis*, have been regularly isolated from patients with gastroenteritis in Europe. However, some *Campylobacter* species are uncommon and are not considered as emergent since they are newly identified or little is known about their pathogenic potential (Man, 2011). Frequently, the microbiological methodology used to isolate *Campylobacter* spp. can introduce culture bias thereby skewing the actual contribution of different species and sources. In the last decade, considerable effort has been devoted to improving the protocols for detecting this pathogen, as bacteriological culture of *Campylobacter* spp. can be a challenge due to the fragility of these microbes (Baylis *et al*., 2000; European Commission, 2007).

Transmission pathways for *Campylobacter* include direct and indirect contact with infected animals (normally healthy carriers), people, and the environment. The vast majority of human cases of *Campylobacter* enteritis are sporadic and linked to the food chain, resulting from handling or consumption of raw or undercooked contaminated poultry products. However, the prevalence of *Campylobacter* carriage by broilers has remained largely at the same level while the number of human cases in the EU increased considerably during the same time period. Consequently, it has been suggested that other risk factors not related to chicken are involved in the increasing incidence of *Campylobacter* enteritis in humans. Examples include meat products from other species, ingestion of untreated drinking water or milk, consumption of contaminated fruits, vegetables, fish or fishery products and, less frequently, recreational activities in aquatic environments. Although large outbreaks of *Campylobacter* enteritis are uncommon, some notable outbreaks have been reported within the EU, and typically involve broiler meat and unpasteurized milk (EFSA, 2014; Schönberg-Norio *et al*., 2004). Furthermore, waterborne transmission has been associated with *Campylobacter* outbreaks because of contamination from sewage or heavy rainfall (Pitkänen, 2013). Braeye *et al*. (2014) described a large community outbreak of gastroenteritis linked to the consumption of drinking water contaminated by river water. In addition, a substantial risk of zoonotic transmission could be associated with pets, livestock and wild animals, as they can play a role in the contamination of food products, or transmission through direct contact with an animal fecally shedding the pathogen. In this context, wild bird droppings have been recognized as a significant environmental source of *Campylobacter* spp. infection for humans and animals, as well as the consumption and handling of game-derived products. However, the number of wildlife species acting as reservoirs of *Campylobacter* spp. is still unknown (Humphrey *et al*., 2007; Epps *et al*., 2013; Waldenström *et al*., 2010).

Wild birds are considered, as with poultry, to be natural reservoirs of *Campylobacter* spp. Healthy wild birds can be a source for human or livestock infection, although their role is likely minor due to host specificity (Colles *et al*., 2008; Griekspoor *et al*., 2013). In Sweden, Waldenström *et al*. (2002) detected *C. jejuni*, *C. lari* and *C. coli* in a wide range of wild and migrating birds, suggesting a potential role as vectors in long-distance transmission to livestock or humans. In agreement with this finding, Wahlström *et al*. (2003) frequently detected *Campylobacter* spp. in Swedish wild

birds (Canada geese, *Branta canadensis* and seagulls, *Larus argentatus*, *L. canus* and *L. marinus*). In North-Western Italy, Robino *et al*. (2010) determined that hooded crows (*Corvus cornix*) were highly sensitive to *Campylobacter* infection. In Germany, studies by Atanassova *et al*. (1999) revealed that wild pheasants shed both *C. coli* and *C. jejuni* (25.9%). In Spain, free-living waterfowl were recognized as a source of *Campylobacter*, especially *C. coli* which was detected in birds and animals in an area densely populated with wild birds (Antilles *et al*., 2013). Other migrating birds, such as common quails (*Coturnix coturnix*), may also be environmental carriers serving as a source of infection for other birds, livestock and humans (Dipineto *et al*., 2014).In addition, a higher occurrence of the pathogen was observed in artificial environments compared with populations in natural environments. Specifically, Nebola *et al*. (2007) determined a higher prevalence of *Campylobacter* spp. in pheasants originating from farms with intensive production (70.2%) than in wild pheasants (27.5%) in the Czech Republic. Moreover, genetic diversity of *Campylobacter* strains isolated from farmed animals was greater than the diversity of strains isolated from wildlife, which might be due to close contact between animals or possibly contamination by farm workers. Díaz-Sánchez *et al*. (2012) highlighted the potential risk in Spain of *Campylobacter* transmission to natural populations of partridges from farmed and restocked red-legged partridges (*Alectoris rufa*).

Wahlström *et al*. (2003) detected *Campylobacter* in the majority of mammalian wildlife species analyzed in Sweden. The prevalence found was considerable (>10%) for wild boars; low for roe deer, mountain and European hares (*Lepus timidus* and *L. europeaus*) and moose (*Alces alces*), and absent for red deer and fallow deer (*Dama dama*). In Norway and Finland, Kemper *et al*. (2006) found one of 2,500 reindeer (*Rangifer tarandus*) was positive for *C. hyointestinalis* and Lillehaug *et al*. (2005) in a Norwegian study observed that only one of 324 wild cervids was positive for *C. jejuni*. In central and southern Spain, Díaz-Sánchez *et al*. (2013) determined there was no relation between *Campylobacter* spp. in large game animals and the presence of livestock in hunting estates despite the high prevalence of *Campylobacter* found in wild boars (66%). However, in northeastern Spain a lower prevalence of *Campylobacter* spp. was found in wild boars (10% *C. lanienae*, 1.3% *C. coli*), as well as a potential for cross-over from free-range livestock to wild boars, especially of uncommon *Campylobacter* species (Navarro-Gonzalez *et al*., 2014). Such

differences may be due to unique conditions in the hunting estates, such as estate fencing, high density of game species and different farming or management conditions that may also be affecting the presence and species of *Campylobacter*. In southern Spain, *Campylobacter* spp. was detected in wild artiodactyls (wild boar, red deer and mouflon), although the findings suggested only wild boars constituted an important reservoir of infection (Carbonero *et al*., 2014). Atanassova *et al*. (2008). and Paulsen *et al*. (2003) isolated *Campylobacter* from wild boar meat in Germany (2.1%) and roe deer meat (3%) respectively, indicating its potential to enter the food chain through these vehicles. In contrast, Wacheck *et al*. (2010) did not recover *Campylobacter* from any of 153 wild boar samples analyzed in Switzerland.

In general, it is difficult to understand all aspects of *Campylobacter* epidemiology due to its being a multi-host pathogen and variable prevalence. Furthermore, seasonality, especially in temperate regions, may affect the recovery of *Campylobacter* (EFSA, 2014; Humphrey *et al*. 2007; Strachan *et al*. 2013). In wildlife, the remoteness of the study areas and the difficulty of processing samples quickly may influence *Campylobacter* recovery, which tends to be laborious and delicate. In fact, the literature reveals there is substantial variability in the presence of the bacteria in different animal species and locations. Other factors such as diet, habitat preferences or migration patterns are likely very important variables in explaining the prevalence of *Campylobacter* species among host taxa (Griekspoor *et al*., 2013; Navarro-Gonzalez *et al*., 2014; Waldenström *et al*., 2010).

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# 7. DISCUSIÓN

*Campylobacter*, especialmente *C. jejuni* y *C. coli*, es una de las bacterias más frecuentemente asociadas con diarrea en personas [131]. La transmisión se produce por el consumo de alimentos de origen animal, fundamentalmente aves de corral [18, 32] y, con menor frecuencia, por el consumo de agua contaminada [8]. También existen otras vías de transmisión como el contacto con animales (domésticos o salvajes) o el contacto con ambientes contaminados [1, 44]. En el presente trabajo de tesis doctoral se ha analizado la presencia de *Campylobacter* en muestras tomadas en diferentes fases de la cadena alimentaria (contenido cecal, piel de cuello y pechuga de pollo fresca envasada), en efluentes urbanos y en animales de vida libre (domésticos y salvajes), analizando diferentes protocolos de detección y caracterizando los aislados mediante técnicas fenotípicas y genotípicas, con el fin de obtener información sobre la epidemiología de este microorganismo.

## 7.1. Detección de *Campylobacter* en las diferentes matrices

La frecuencia de detección de *Campylobacter* encontrada en las distintas matrices analizadas utilizando cultivo microbiológico (considerando el resultado de todos los protocolos) fue muy variable. En el primer estudio, centrado en muestras de *broiler* tomadas en diferentes fases de la cadena alimentaria (capítulo 5.1), la mayor proporción de positivos a *Campylobacter* termófilos correspondió con muestras de piel de cuello (35/38; 92%), seguido de contenido cecal (34/38; 90%) y pechuga de pollo fresca envasada (29/38; 76%). Estos resultados reflejan el alto grado de contaminación de las muestras de origen aviar. También se observaron altas prevalencias de *Campylobacter* spp. en contenido cecal y canales de pollo (piel) en un estudio global de prevalencia llevado a cabo en Francia a partir de muestras de matadero durante el año 2008 [110]. En dicho estudio se identificaron el 77,2% de lotes positivos a partir de contenido cecal y el 87,5% a partir de canales [110]. Por otro lado, los últimos datos del informe de fuentes y tendencias publicados por la EFSA indican que el 31,4% de la totalidad de muestras de carne de pollo fresca analizadas en 2013 (matadero, planta de procesado o punto de venta) fueron positivas a *Campylobacter* [11]. Asimismo, un estudio realizado en 2009 estimaba la prevalencia de *Campylobacter* en carne de pollo a la venta en torno al 60% [132].

En el caso del estudio llevado a cabo en efluentes urbanos (capítulo 5.2), la proporción de muestras positivas a *Campylobacter* mediante cultivo fue del 64% (32/50). En otro estudio realizado en España, el porcentaje de detección de *Campylobacter* termófilos en aguas residuales fue menor (3/10; 30%) [133], mientras que en Canadá el porcentaje de aguas residuales positivas a *Campylobacter* spp. fue del 10% [134] y del 25,6% [135], en función del estudio consultado. Así, la presencia de *Campylobacter* en efluentes urbanos (capítulo 5.2) podría estar reflejando una potencial vía de diseminación de la bacteria al medio ambiente y, de forma indirecta, al hombre y los animales.

Por último, en los animales de vida libre analizados (capítulo 5.5), el porcentaje de muestras positivas al aislamiento de *Campylobacter* spp. más elevado correspondió con muestras de jabalí (22/150; 15%), seguido del ganado vacuno criado en extensivo presente en la reserva (7/55; 13%), mientras que todas las muestras analizadas de cabra montés fueron negativas. En general, las prevalencias fueron muy inferiores a las detectadas en animales de abasto (*broiler*), lo que iría en línea con el mayor riesgo de adquirir campilobacteriosis tras el consumo de carne de pollo que tras el consumo de carne de especies cinegéticas [130]. Los resultados obtenidos en jabalíes en otros estudios realizados en España son muy heterogéneos (introducción, tabla 4). Por un lado, Díaz-Sánchez *et al.* [98] describieron una prevalencia de *Campylobacter* spp. del 66% en muestras de heces, mientras que Carbonero *et al.* [97] identificaron el 38,9% de las muestras de jabalíes positivas a *Campylobacter* spp., también en heces. En lo referente a la cabra montés, parece que esta especie no desempeña ningún papel en la ecología de la bacteria en nuestra zona de estudio (capítulo 5.5, tabla 2 y 3). Una posible explicación sería su preferencia por altitudes elevadas y sus hábitos alimenticios, lo cual reduciría la posibilidad de contacto con otros animales como jabalí o ganado criado en extensivo. Este hecho ya había sido reflejado en diversos estudios de cabra montés llevados a cabo en el mismo entorno geográfico frente a otros patógenos [136, 137].

Las diferencias observadas entre nuestros datos y otros estudios podrían deberse a las diferencias en el muestreo, la matriz analizada, la localización geográfica, la época del año y, especialmente, a los métodos de cultivo empleados. Al comparar los distintos protocolos de detección utilizados en cada matriz en nuestros trabajos, los resultados obtenidos fueron muy variables. Se ha visto que la siembra directa funciona mejor en muestras de contenido cecal (34/38, 90%) y piel de cuello (31/38; 82%), sin embargo, la diferencia entre los resultados obtenidos con enriquecimiento (14/38;

37% en contenido cecal y 29/38; 76% en piel de cuello) y siembra directa sólo fue significativa para las muestras de contenido cecal (p<0,001). Por otro lado, en muestras de pechuga de pollo fresca envasada, el enriquecimiento (26/38; 68%) aumentó la detección de *Campylobacter* termófilos (p<0,001) (capítulo 5.1, tabla 1). En el estudio de aguas residuales, aunque el enriquecimiento (19/50, 38%) obtuvo un mayor número de muestras positivas comparado con la siembra directa (14/50, 28%), la diferencia no fue significativa (capítulo 5.2, tabla 1). Desafortunadamente, en el estudio de detección de *Campylobacter* en animales salvajes (capítulo 5.5), aunque se utilizaron diferentes protocolos de aislamiento, el número de muestras positivas no permitió llevar a cabo un estudio comparado de los protocolos de detección aplicados.

Si comparamos los resultados de siembra directa en mCCDA y CFA, se ha descrito que el crecimiento de otras bacterias podría enmascarar la identificación y el recuento de colonias de *Campylobacter* [138]. Ahmed *et al*. [139] encontraron que el porcentaje de falsos-positivos (colonias sospechosas y no confirmadas como *Campylobacter* en muestras de pollo naturalmente contaminadas de carne y piel) fue superior utilizando CFA que mCCDA (13% *vs* 10%). Sin embargo, Habib *et al*. [140] identificaron una buena correlación entre los resultados obtenidos con mCCDA y CFA para la enumeración de *Campylobacter* a partir de muestras de carne de pollo, indicando también la mayor facilidad en la identificación de colonias con el medio cromogénico (CFA). En este sentido, si comparamos los resultados de mCCDA y CFA en el estudio realizado en muestras de contenido cecal o piel de cuello (capítulo 5.1, tabla 2), no se observaron diferencias significativas entre su uso en siembra directa. Asimismo, de forma equivalente a Habib *et al*. [140], las características del medio cromogénico (CFA) también permitieron diferenciar con mayor claridad las colonias sospechosas de ser *Campylobacter* por su color rojizo (capítulo 5.1).

En relación al caldo Bolton, Baylis *et al*. [23] determinaron que en muestras de alimentos crudos naturalmente contaminados (canales, carne e hígado de aves, ternera, cordero y cerdo), el caldo Bolton (66/100) favorecía el crecimiento de *Campylobacter* e inhibía las bacterias competidoras de forma más efectiva que el caldo Preston (53/100). Sin embargo en nuestro estudio (capítulo 5.1, tabla 1), el porcentaje de las muestras de pechuga de pollo que fueron positivas a *Campylobacter* termófilos fue mayor (p=0,001) empleando caldo Preston (24/38; 63%), en particular usando mCCDA como agar selectivo, en relación al uso del caldo Bolton (9/38; 24%), especialmente seguido de mCCDA. En línea con nuestros resultados, el aumento de

efectividad de Preston y mCCDA respecto al enriquecimiento con Bolton y mCCDA, también fue confirmado por Habib *et al.* [140]. Además, en nuestro estudio, la recuperación de *Campylobacter* termófilos a partir de pechuga de pollo usando caldo Bolton estuvo influido por el medio selectivo utilizado posteriormente (capítulo 5.1, tabla 2), incrementándose el porcentaje de las muestras positivas recuperadas con CFA (8/9; 89%) comparado con mCCDA (2/9; 22%). De forma equivalente, Habib *et al.* [140] también observaron una clara diferencia entre los resultados obtenidos con caldo Bolton seguido del cultivo en placas de mCCDA o CFA (30,61% *vs* 53,06%).

Así, en base a los resultados obtenidos en nuestro estudio (capítulo 5.1), en muestras de alimentos, una etapa previa de enriquecimiento favorece la recuperación de *Campylobacter*. Este hecho podría estar relacionado con que las muestras alimentarias presenten una carga contaminante que suele ser baja y que los *Campylobacter* presentes puedan estar en menor concentración o incluso dañados subletalmente como consecuencia de las técnicas de procesado utilizadas (congelado, refrigeración, calentamiento, salado, etc.) o factores intrínsecos al alimento (composición, pH, etc.) [18, 23]. Por otro lado, la siembra directa resultó más adecuada en muestras donde se estima una alta carga contaminante [22, 141, 142], como contenido cecal y piel de cuello (capítulo 5.1, tabla 1 y 4).

En el caso de las muestras de aguas residuales, se ha observado que la alta proporción de bacterias fecales puede enmascarar el crecimiento de *Campylobacter* tras una etapa de enriquecimiento [116]. Otros autores han descrito que una etapa de enriquecimiento favorece la recuperación de las células dañadas o estresadas en muestras de agua [143]. Sin embargo, Jokinen *et al.* [144] describieron que el enriquecimiento con caldo Bolton utilizando una etapa previa de filtración no aumentaba significativamente la recuperación de *Campylobacter* a partir de aguas superficiales (38,3% *vs* 36,7%), aunque disminuía la proporción de falsos positivos (97% *vs* 56%). En consonancia con estos trabajos, en las muestras de efluentes urbanos analizadas se comprobó que, aunque el filtrado y/o el enriquecimiento no aumentaban significativamente el porcentaje de detección de *Campylobacter*, eran capaces de detectar muestras positivas no identificadas utilizando siembra directa (capítulo 5.2, tabla 1).

A pesar de que se han visto grandes diferencias al aplicar técnicas de cultivo microbiológico en las diferentes matrices, los resultados obtenidos mediante qPCR han revelado un alto porcentaje de muestras positivas al aislamiento de ADN de

*Campylobacter* en todos los estudios, independientemente de la naturaleza de la muestra analizada. En el 97% (37/38) de las muestras de contenido cecal y pechuga de pollo y en el 100% de las muestras de piel de cuello (capítulo 5.1) y efluentes urbanos (capítulo 5.2), se confirmó la presencia de ADN de *C. jejuni* y/o *C. coli*, aunque no en todos los casos pudo aislarse la bacteria. No obstante, se observó que existía una relación entre los valores de cuantificación de *C. coli* en el estudio de la cadena alimentaria (capítulo 5.1) y el número de protocolos a los que la muestra resultaba positiva a esta especie, aunque esta relación no se observó para *C. jejuni* (capítulo 5.1, figura 2). Las diferencias observadas entre la qPCR y el cultivo tradicional podrían estar asociadas a los requerimientos de cultivo de *Campylobacter* y a la presencia de células muertas (ADN libre), subletalmente dañadas o VPNC, así como bacterias competidoras que podrían estar influyendo en la tasa de recuperación del microrganismo a partir de las muestras analizadas. De este modo, los resultados obtenidos con la qPCR podrían ser considerados como el máximo teórico de muestras positivas a *Campylobacter,* e incluso permitiría utilizar la técnica de PCR como método de *screening* para elegir el protocolo de cultivo más adecuado en función de la carga.

### 7.2. Especies de *Campylobacter* detectadas en las diferentes matrices

Se ha descrito que las características inherentes a cada cepa o especie de *Campylobacter*, que determinan su supervivencia bajo condiciones adversas, pueden influir en su recuperación en el laboratorio [19, 145, 146]. Asimismo, el método de cultivo empleado se ha relacionado con la distribución de las especies obtenidas. Por ejemplo, Williams *et al.* [128] describieron que el caldo de enriquecimiento, debido a los componentes selectivos que contiene, podría favorecer el crecimiento de algunos aislados o, incluso inducir estrés que alteraría las cepas obtenidas. En este sentido, Hakkinen *et al.* [147] describieron que el método de cultivo podría influir en la diversidad de especies detectadas en ganado bovino, ya que *C. coli* y *C. hyointestinalis* subsp. *hyointestinalis* no fueron identificadas mediante siembra directa pero sí usando una etapa de enriquecimiento.

Si consideramos los aislados obtenidos independientemente del protocolo de aislamiento, hubo diferencias en las especies detectadas en función de las matrices analizadas. Se obtuvo un mayor número de aislados de *C. coli* que de *C. jejuni* en muestras de contenido cecal (55/82; 67%) y pechuga de pollo (27/48; 56%), siendo significativa la diferencia encontrada entre las dos especies para el contenido cecal ($p<0,001$) (capítulo 5.1). Si la comparación se hace por muestra, la diferencia entre las

dos especies en el contenido cecal no es significativa mientras que el número de muestras de pechuga positivas a *C. jejuni* y *C. coli* es equivalente. Aunque en términos generales se ha descrito que *C. jejuni* es la especie más frecuentemente detectada en muestras de pollo [80] y *C. coli* se asocia normalmente al ganado porcino [24, 148], Ansari-Lari *et al.* [149] también identificaron a *C. coli* como la especie dominante en muestras de pollo en un estudio realizado en Irán. Además, se ha determinado que existen otras variables que podrían modificar la distribución de las especies como la localización geográfica o las características propias del estudio [146]. Por ejemplo, en un trabajo llevado a cabo en Canadá se detectó un 81% de muestras de carne de pollo positivas a *C. jejuni* y sólo un 17% a *C. coli*, mientras que en otro estudio sobre carne de pollo en el Reino Unido la proporción de *C. coli* fue más elevada (47,1%) [150]. No obstante, los casos de campilobacteriosis en humanos se asocian más frecuentemente con *C. jejuni*, que fue la especie más frecuentemente detectada en piel de cuello (64/103; 62%) (p=0,001) (capítulo 5.1, tabla 3). En esta especie se ha identificado una marcada estacionalidad [44, 48], aunque no hemos encontrado evidencias de la misma en el estudio de la cadena alimentaria (capítulo 5.1) ni en el estudio de efluentes urbanos (capítulo 5.2). Este hecho podría deberse al propio estudio, ya que el diseño del muestreo no se orientó para detectar cambios estacionales.

En muestras de efluentes urbanos (capítulo 5.2), se identificó un mayor número (p<0,001) de aislados de *C. coli* (53/77; 69%) que de *C. jejuni* (20/77; 26%). Estos datos concuerdan con los descritos por Moreno *et al.* [133] que aislaron *C. coli* en muestras de aguas residuales, siendo ésta la única especie identificada.

En general, observamos que el uso de métodos que incluyen una etapa de enriquecimiento, especialmente usando caldo Preston, favorecen la recuperación de *C. coli* (p<0,05) en muestras de la cadena alimentaria (48/58; 83%) (capítulo 5.3) y de efluentes urbanos (23/26; 88%) (capítulo 5.2). Por otro lado, el uso de siembra directa supone una mayor recuperación de *C. jejuni* (p<0,05), tanto en muestras de la cadena alimentaria (50/69; 72%) (capítulo 5.3) como en muestras de efluentes urbanos (11/14; 79%), especialmente utilizando agar CASA en el último caso (capítulo 5.2). Nuestros resultados indican que podría existir una selección de aislados en función de la ruta de aislamiento, ya que la proporción de especies detectadas se vio influenciada por el método de detección empleado.

La mayoría de las muestras de los estudios realizados (el 79% (30/38) de las muestras de contenido cecal, el 87% (33/38) de las de piel de cuello y el 58% (22/38) de las de

pechuga de pollo) fueron positivas a *C. coli* y *C. jejuni* de forma simultánea mediante qPCR (capítulo 5.1). No obstante, el cultivo tradicional sólo detectó *C. coli* y *C. jejuni* de forma conjunta en el 24% (9/38) de las muestras de contenido cecal, el 37% (14/38) de piel de cuello y el 8% (3/38) de las de pechuga de pollo (capítulo 5.1). Igualmente, en el estudio de efluentes urbanos (capítulo 5.2), la qPCR detectó ADN de las dos especies en el 98% (49/50) de las muestras, mientras que el cultivo bacteriológico sólo identificó ambas especies en el 18% (9/50). De forma similar, Melero *et al.* [24] detectaron 43 muestras positivas (89,53%) a *C. jejuni* mediante qPCR, mientras que únicamente seis muestras (de superficies) fueron positivas al aislamiento (12,50%) en una planta de procesado de pollo (carne, piel de cuello, mesas de procesado y guantes de los operarios). Asimismo, Rohonczy *et al.* [151] encontraron una baja correlación entre los resultados obtenidos mediante qPCR y cultivo tradicional (enriquecimiento y siembra directa) para la identificación de *C. jejuni* a partir de muestras de carne de pollo envasada; en este caso, la mitad de las muestras confirmadas mediante qPCR no fueron positivas utilizando cultivo. En consonancia con nuestros datos, estos estudios también sugieren que los métodos de cultivo microbiológico podrían llegar a subestimar la proporción de especies presentes en la muestra, siendo recomendable la utilización de varios protocolos de cultivo para estimar la diversidad real.

En nuestro trabajo llevado a cabo sobre animales de vida libre (capítulo 5.5), se detectó *C. jejuni* (1/150; 0,7%) y *C. coli* (3/150; 2%) en heces de jabalí, aunque otros autores han identificado tanto *C. coli* como *C. jejuni* en mayor proporción en esta especie animal (6,3% y 1,6%) [97], (4,54% y 3,03%) [102] y en cerdo salvaje (3,33% y 20%) [100]. En el caso del bovino, *C. jejuni* ha sido reconocida como la especie predominante de *Campylobacter*, especialmente en bovinos jóvenes [76, 86]. De forma equivalente en nuestra investigación, *C. jejuni* (5/55; 9%) fue la especie más frecuentemente identificada en el ganado bovino (capítulo 5.5). Asimismo, otros trabajos han detectado *C. lanienae* en heces de cerdos domésticos (2,22%), cerdos salvajes (20%), jabalí (26,98%) y rumiantes (57,14%), aunque en este último caso los resultados fueron determinados mediante PCR directa [97, 100, 152-154]. En nuestra investigación esta especie también fue identificada, de forma predominante en jabalí (18/150; 12%), y en una muestra en ganado vacuno (1/55; 2%). El aislado de *C. lanienae* identificado en vaca se obtuvo a partir de un animal de un rebaño de 30 individuos y, seis días más tarde, dos de los tres jabalíes muestreados en la misma zona fueron positivos. Así, aunque el jabalí y la vaca parecen tener su propia especie predominante (capítulo 5.5,

tabla 3), nuestros datos podrían estar reflejando un posible intercambio de *Campylobacter* entre ganado bovino y jabalí.

Finalmente, se han detectado otros microorganismos relacionados con el género *Campylobacter* en aguas residuales (capítulo 5.2) y en animales de vida libre (capítulo 5.5). Como se describe en el estudio realizado en efluentes urbanos (capítulo 5.2), se identificaron cuatro aislados de *Arcobacter butzleri* (4/50; 8%), microorganismo previamente descrito en muestras de aguas residuales en el sureste de España [133]. De hecho, *Arcobacter* es considerado como un patógeno emergente para el hombre, cuya principal vía de transmisión es el consumo de agua contaminada [50]. Aunque el hecho de que se aplicasen procedimientos de detección orientados al aislamiento de *Campylobacter* termófilos podría haber infravalorado la presencia real de *Arcobacter* en las muestras, nuestros datos reflejarían el posible papel del agua residual en la diseminación de este microorganismo. También se ha identificado *C. fetus* subsp. *venerealis* en una muestra de vaca, hecho que se considera como un hallazgo puntual (1/55; 2%) (capítulo 5.5), ya que se trata de una especie normalmente presente en el tracto genital del ganado bovino o en abortos [49].

## 7.3. Caracterización de los aislados obtenidos a partir de la cadena alimentaria y los efluentes urbanos

Los aislados de *Campylobacter* termófilos obtenidos a partir de muestras de diferentes fases de la cadena alimentaria (capítulo 5.1) y de los efluentes urbanos (capítulo 5.2), fueron caracterizados mediante técnicas fenotípicas y genotípicas, tal y como se detalla a continuación.

### 7.3.1. Caracterización genética de los aislados de la cadena alimentaria

Se ha descrito que la diversidad genética de los aislados de *Campylobacter* varía en función del método de cultivo empleado para su recuperación. Así, Williams *et al.* [128] observaron diferente perfil genético mediante campo pulsado en aislados procedentes de la misma muestra ambiental obtenida en granjas de pollos usando diferentes caldos de enriquecimiento (Preston, Exeter modificado, Bolton modificado). De igual forma, Newell *et al.* [19] describieron que la aplicación de una etapa de enriquecimiento podría seleccionar de forma preferente determinados aislados, ya que cepas aisladas mediante siembra directa no fueron identificadas mediante caldos de enriquecimiento en muestras de canales de pollo.

Para determinar la influencia que el método de cultivo podría tener sobre los genotipos de *Campylobacter* recuperados, así como determinar cuáles eran los aislados compartidos entre cada una de las fases de la cadena alimentaria, los *C. coli* y *C. jejuni* obtenidos en el primer estudio (capítulo 5.1) fueron caracterizados genéticamente. Las cepas analizadas (n=296 de 98 muestras) procedían del subgrupo A, que incluía lotes de producción analizados en tres fases diferentes en matadero (contenido cecal, piel de cuello y pechuga de pollo fresca envasada), y el subgrupo B, cuyas muestras no estaban relacionadas epidemiológicamente entre sí y que procedían de diez lotes de contenido cecal, cuatro lotes de piel de cuello y diez lotes de pechuga de pollo envasada. Así, se obtuvieron 97 aislados de contenido cecal (n=34 muestras), 133 de piel de cuello (n=35 muestras) y 66 de pechuga de pollo envasada (n=29 muestras) (capítulo 5.3). En todos los aislados se secuenció la región variable del gen de la flagelina (*fla*A), a partir de la cual se pudo determinar la riqueza de genotipos.

En general, el número de genotipos de *fla*A detectados en nuestro estudio (subgrupo A+B) fue elevado, ya que se identificaron 51 genotipos distintos (33 en contenido cecal, 41 en piel de cuello y 24 en pechuga de pollo). De hecho, el genotipo más repetido (*fla*A 30, con 21 aislados), únicamente fue detectado en nueve muestras de las 98 analizadas (9/98; 9%). En consonancia con estos datos, se ha descrito que normalmente la diversidad genética de *Campylobacter* es elevada [32, 145, 155]. De los genotipos obtenidos en nuestro estudio, 31 fueron identificados únicamente en el subgrupo A (n=167 aislados de 62 muestras), ocho solamente en el subgrupo B (n=18 aislados de 10 muestras) y 12 fueron compartidos entre ambos grupos (n=111 aislados de 53 muestras). La distribución de los genotipos compartidos entre ambos grupos (n=59 aislados de 35 muestras en el subgrupo A y n=52 aislados de 18 muestras en el subgrupo B), podría sugerir que se tratase de genotipos con mayor grado de diseminación en avicultura, habiendo sido descritos previamente en otro estudio llevado a cabo en canales de pollo en EEUU [156].

Considerando los datos por cada subgrupo, se encontraron 43 genotipos distintos (28 en contenido cecal, 39 en piel de cuello y 18 en pechuga de pollo) en el subgrupo A (n=226 aislados de 76 muestras) (**Tabla 5**). En el caso del subgrupo B (n=70 aislados de 22 muestras), se detectaron 20 genotipos diferentes (7 en contenido cecal, 5 en piel de cuello y 10 en pechuga de pollo) (capítulo 5.3., tabla 1) (**Tabla 6**). Calculando la proporción de genotipos diferentes detectados por muestras analizadas, los datos sugieren una mayor variabilidad genética del grupo B (0,91; 20/22) que del A (0,56;

43/76), es decir, mayor variabilidad en muestras obtenidas de lotes no relacionados epidemiológicamente.

**Tabla 5.** Distribución de los datos obtenidos mediante *fla*A y MLST en las muestras de la cadena alimentaria del subgrupo A.

| Muestra | Genotipo de *fla*A | Aislados/ *fla*A | Muestras/ *flaA* | Nº de lotes | Nº aislados (ST)* | Nº aislados (CC)* |
|---|---|---|---|---|---|---|
| Contenido cecal | 9 | 1 | 1 | 1 | ND | ND |
| | 10 | 1 | 1 | 1 | ST356 (1) | CC353 (1) |
| | 13 | 6 | 3 | 3 | ST1563 (1) | CC828 (1) |
| | 14 | 1 | 1 | 1 | ST1707 (1) | CC607 (1) |
| | 16 | 1 | 1 | 1 | ST367 (1) | CC257 (1) |
| | 18 | 1 | 1 | 1 | ND | ND |
| | 21 | 1 | 1 | 1 | ST51 (1) | CC443 (1) |
| | 23 | 2 | 1 | 1 | ST829 (1) | CC828 (1) |
| | 30 | 5 | 2 | 2 | ST5659 (1) ST828 (1) | CC828 (2) |
| | 32 | 1 | 1 | 1 | ST48 (1) | CC48 (1) |
| | 34 | 2 | 1 | 1 | ND | ND |
| | 36 | 4 | 3 | 3 | ST148 (1) | CC21 (1) |
| | 57 | 2 | 1 | 1 | ST52 (1) | CC52 (1) |
| | 66 | 6 | 3 | 3 | ST1750 (1) ST902 (1) | CC828 (2) |
| | 67 | 2 | 1 | 1 | ST47 (1) | CC21 (1) |
| | 78 | 1 | 1 | 1 | ST607 (1) | CC607 (1) |
| | 105 | 2 | 2 | 2 | ST883 (1) | CC21 (1) |
| | 239 | 1 | 1 | 1 | ST42 (1) | CC42 (1) |
| | 255 | 3 | 1 | 1 | ST827 (1) | CC828 (1) |
| | 260 | 4 | 2 | 2 | ST464 (1) | CC464 (1) |
| | 311 | 5 | 2 | 2 | ST5659 (2) | CC828 (2) |
| | 587 | 3 | 1 | 1 | ST2074 (1) | CC828 (1) |
| | 588 | 3 | 1 | 1 | ST2074 (1) | CC828 (1) |
| | 604 | 8 | 2 | 2 | ST827 (3) | CC828 (3) |
| | 866 | 1 | 1 | 1 | ST860 (1) | CC828 (1) |
| | M2 | 3 | 2 | 2 | ND | ND |
| | M5 | 1 | 1 | 1 | ND | ND |
| | M7 | 1 | 1 | 1 | ND | ND |

| Muestra | Genotipo de *flaA* | Aislados/ *flaA* | Muestras/ *flaA* | Nº de lotes | Nº aislados (ST)* | Nº aislados (CC)* |
|---|---|---|---|---|---|---|
| Piel de cuello | 5 | 2 | 1 | 1 | ST45 (1) | CC45 (1) |
| | 9 | 2 | 1 | 1 | ND | ND |
| | 10 | 2 | 2 | 2 | ND | ND |
| | 13 | 5 | 3 | 3 | ST854 (2) | CC828 (2) |
| | 14 | 3 | 1 | 1 | ST46 (1) | CC206 (1) |
| | 16 | 1 | 1 | 1 | ST367 (1) | CC257 (1) |
| | 18 | 2 | 1 | 1 | M4 | ND |
| | 21 | 3 | 3 | 3 | ST443 (1) | CC443 (1) |
| | 23 | 2 | 1 | 1 | ST829 (1) | CC828 (1) |
| | 24 | 1 | 1 | 1 | ST2123 (1) | CC362 (1) |
| | 30 | 5 | 3 | 3 | ST5659 (1) | CC828 (1) |
| | 32 | 3 | 2 | 2 | ST48 (1) | CC48 (1) |
| | 36 | 1 | 1 | 1 | ST148 (1) | CC21 (1) |
| | 37 | 4 | 2 | 2 | ST262 (1) | CC21 (1) |
| | 42 | 2 | 1 | 1 | ST61 (1) | CC61 (1) |
| | 49 | 4 | 2 | 2 | ND | ND |
| | 57 | 1 | 1 | 1 | ST52 (1) | CC52 (1) |
| | 66 | 5 | 3 | 3 | ST860 (1) | CC828 (1) |
| | 67 | 2 | 1 | 1 | ST47 (1) | CC21 (1) |
| | 78 | 2 | 2 | 2 | ST572 (1) | CC206 (1) |
| | 117 | 2 | 2 | 2 | ST7545 (1) | ST7545 (1)** |
| | 222 | 1 | 1 | 1 | ST7546 (1) | ST7546 (1)** |
| | 239 | 5 | 1 | 1 | ST42 (1) | CC42 (1) |
| | 255 | 7 | 2 | 2 | ST827 (2) | CC828 (2) |
| | 260 | 5 | 4 | 4 | ST464 (1) | CC464 (1) |
| | 267 | 3 | 2 | 2 | ST2077 (1) | CC828 (1) |
| | 293 | 1 | 1 | 1 | ST5659 (1) | CC828 (1) |
| | 311 | 3 | 2 | 2 | ST5659 (1)<br>ST2077 (1) | CC828 (2) |
| | 550 | 1 | 1 | 1 | ST49 (1) | CC48 (1) |
| | 553 | 1 | 1 | 1 | ND | ND |
| | 587 | 6 | 2 | 2 | ST2074 (2) | CC828 (2) |
| | 588 | 3 | 1 | 1 | ST2074 (1) | CC828 (1) |
| | 604 | 5 | 1 | 1 | ST827 (1) | CC828 (1) |

| Muestra | Genotipo de *fla*A | Aislados/ *fla*A | Muestras/ *flaA* | Nº de lotes | Nº aislados (ST)* | Nº aislados (CC)* |
|---|---|---|---|---|---|---|
| Piel de cuello | 726 | 1 | 1 | 1 | ND | ND |
| | 866 | 3 | 1 | 1 | ST860 (1) | CC828 (1) |
| | M2 | 2 | 1 | 1 | ND | ND |
| | M4 | 1 | 1 | 1 | ND | ND |
| | M6 | 1 | 1 | 1 | ND | ND |
| | M7 | 1 | 1 | 1 | ND | ND |
| Pechuga de pollo fresca envasada | 2 | 2 | 1 | 1 | ST45 (1) | CC45 (1) |
| | 13 | 5 | 2 | 2 | ST854 (3) | CC828 (3) |
| | 18 | 1 | 1 | 1 | ST354 (1) | CC354 (1) |
| | 30 | 11 | 4 | 4 | ST828 (2) ST5659 (3) | CC828 (5) |
| | 32 | 3 | 2 | 2 | ST48 (2) | CC48 (2) |
| | 34 | 2 | 2 | 2 | ND | ND |
| | 36 | 2 | 1 | 1 | ST2724 (1) | CC21 (1) |
| | 49 | 2 | 1 | 1 | ST122 (1) | CC206 (1) |
| | 66 | 1 | 1 | 1 | ST902 (1) | CC828 (1) |
| | 67 | 1 | 1 | 1 | ST47 (1) | CC21 (1) |
| | 239 | 3 | 2 | 2 | ST42 (2) | CC42 (2) |
| | 255 | 3 | 1 | 1 | ST827 (2) | CC828 (2) |
| | 260 | 1 | 1 | 1 | ST464 (1) | CC464 (1) |
| | 311 | 2 | 1 | 1 | ST5659 (1) | CC828 (1) |
| | 587 | 1 | 1 | 1 | ST2074 (1) | CC828 (1) |
| | 588 | 1 | 1 | 1 | ST2074 (1) | CC828 (1) |
| | 604 | 6 | 1 | 1 | ST827 (3) | CC828 (3) |
| | M2 | 3 | 1 | 1 | ND | ND |

ST: *Sequence Type*; CC: *Clonal Complex*; ND: no determinado.
*Sólo se incluyen los datos de los aislados que han sido analizados mediante MLST (n=112). Entre paréntesis se indica el nº de aislados.
**Sin CC asociado.

**Tabla 6.** Distribución de los datos obtenidos mediante *fla*A y MLST en las muestras de la cadena alimentaria del subgrupo B.

| Muestra | Genotipo de *fla*A | Aislados/ *fla*A | Muestras/ *flaA* | Nº de lotes | Nº aislados (ST)* | Nº aislados (CC)* |
|---|---|---|---|---|---|---|
| **Contenido cecal** | 2 | 1 | 1 | 1 | ND | ND |
| | 27 | 2 | 1 | 1 | ST7544 (1) | ST7544 (1)** |
| | 34 | 10 | 2 | 2 | ST3017 (5) | CC828 (5) |
| | 236 | 1 | 1 | 1 | ST1750 (1) | CC828 (1) |
| | 1348 | 8 | 1 | 1 | ST1750 (5) | CC828 (5) |
| | M1 | 1 | 1 | 1 | ND | ND |
| | M2 | 2 | 1 | 1 | ND | ND |
| **Piel de cuello** | 9 | 14 | 5 | 2 | ST21 (1) | CC21 (1) |
| | 10 | 5 | 3 | 1 | ST356 (1) | CC353 (1) |
| | 49 | 6 | 2 | 1 | ST227 (1) | CC206 (1) |
| | 162 | 2 | 2 | 1 | ST51 (1) | CC443 (1) |
| | 251 | 2 | 2 | 1 | ST51 (1) | CC443 (1) |
| **Pechuga de pollo fresca envasada** | 8 | 1 | 1 | 1 | ST45 (1) | CC45 (1) |
| | 9 | 1 | 1 | 1 | ST572 (1) | CC206 (1) |
| | 16 | 1 | 1 | 1 | ND | ND |
| | 21 | 3 | 1 | 1 | ST45 (1) | CC45 (1) |
| | 34 | 3 | 1 | 1 | ST904 (1) | CC607 (1) |
| | 36 | 1 | 1 | 1 | ST50 (1) | CC21 (1) |
| | 239 | 1 | 1 | 1 | ST583 (1) | CC45 (1) |
| | 260 | 1 | 1 | 1 | ND | ND |
| | 726 | 3 | 1 | 1 | ST825 (1) | CC828 (1) |
| | M3 | 1 | 1 | 1 | ND | ND |

ST: *Sequence Type*; CC: *Clonal Complex*; ND: no determinado.
*Sólo se incluyen los datos de los aislados que han sido analizados mediante MLST (n=112). Entre paréntesis se indica el nº de aislados.
**Sin CC asociado.

Si se comparan los datos por matriz para una misma muestra en el subgrupo A, en piel de cuello se encontró el mayor número de genotipos diferentes (de uno a cinco *fla*A diferentes/muestra), mientras que las muestras de contenido cecal y pechuga de pollo presentaban entre uno y dos genotipos distintos (**Tabla 7**). Así, estos resultados apuntan a que la mayor variedad de genotipos se encuentra en muestras de contenido

cecal y, especialmente piel de cuello, mientras que la menor variedad se asocia con las muestras de pechuga de pollo envasada.

**Tabla 7**. Distribución del número de muestras en función del número de genotipos diferentes por muestra (subgrupo A y subgrupo B).

| Subgrupo | Tipo de muestra | 1 gts* | 2 gts* | 3 gts* | 4 gts* | 5 gts* | Total |
|---|---|---|---|---|---|---|---|
| **Subgrupo A** | **Heces** | 14 | 13 | | | | 27 |
| | **Piel de cuello** | 6 | 15 | 3 | 1 | 2 | 27 |
| | **Pechuga** | 18 | 4 | | | | 22 |
| **Subgrupo B** | **Heces** | 6 | 1 | | | | 7 |
| | **Piel de cuello** | 4 | 2 | 2 | | | 8 |
| | **Pechuga** | 5 | 1 | 1 | | | 7 |

*gts: nº de genotipos de *fla*A identificados.

Contrastando el valor de la riqueza de genotipos, teniendo en cuenta únicamente al subgrupo A, los aislados de piel de cuello (29; IC 95%, 25–33) seguidos de los de contenido cecal (24; IC 95%, 21–26), obtuvieron una mayor riqueza de genotipos que los aislados de pechuga de pollo fresca envasada (18; IC 95%, 18–18) (capítulo 5.3, tabla 1). Nuestros datos indicarían que la riqueza de genotipos disminuye al final de la cadena de producción de alimentos, siendo menor en pechuga de pollo, tal y como han determinado otros autores [145, 156]. También se ha sugerido que la diferencia de resultados entre los aislados podría deberse al posible efecto selectivo de las propias técnicas de procesado utilizadas (exposición a oxígeno, refrigeración, congelación etc.) [145].

Dentro del subgrupo A, se obtuvieron 22 lotes en los que las tres matrices fueron positivas (22/28; 79%). Estos lotes se correspondieron con 196 aislados (n=66 muestras). En trece de los 22 lotes (13/22; 59%), se detectó al menos un genotipo en pechuga que también fue detectado en contenido cecal. De hecho, se identificó al menos un genotipo común en las tres matrices en 10 lotes (10/22; 45%). Asimismo, en tres casos (3/22; 14%), los genotipos encontrados en pechuga eran comunes con los identificados en piel de cuello (capítulo 5.3). Por otro lado, se identificaron seis lotes (6/22; 27%) en los que los genotipos de pechuga eran completamente distintos a los observados en piel de cuello o contenido cecal.

Si se comparan los datos por matriz para una misma muestra en el subgrupo B, en piel de cuello y pechuga de pollo se encontró el mayor número de genotipos

diferentes (de uno a tres *fla*A diferentes/muestra), mientras que las muestras de contenido cecal presentaban entre uno y dos genotipos distintos (Tabla 7). Considerando los valores de riqueza, los aislados de pechuga de pollo envasada (10; IC 95%, 10–10) y contenido cecal (6; IC 95%, 4–7), presentaron un valor más elevado que los aislados de piel de cuello (5; IC 95%, 3–5). Estos resultados podrían estar relacionados con la distribución de los lotes, ya que en el caso de la piel de cuello, las diez muestras analizadas pertenecían a cuatro lotes, mientras que en contenido cecal y pechuga de pollo, las diez muestras se correspondían con diez lotes distintos. Además, se identificó el mismo genotipo en dos lotes independientes de muestras de contenido cecal (*fla*A 34) y piel de cuello (*fla*A 9).

Se ha descrito que algunas cepas podrían ser más propensas a ser eliminadas durante el procesado en el matadero, mientras que otras tenderían a sobrevivir o prosperar a lo largo de la cadena alimentaria [19, 146, 156]. En este sentido, se ha observado que durante el procesado de las aves de corral, la contaminación es frecuente debido a la alta carga bacteriana que presentan los animales [18] y a la posibilidad de que se produzcan fugas de contenido intestinal que pueden llegar a contaminar las canales y la piel. Dicha contaminación puede producirse entre animales de un mismo lote o puede llegar a afectar a animales de distintos lotes [44, 110, 157]. Así, la alta variedad de genotipos observada en piel de cuello en lotes de producción relacionados (subgrupo A), podría indicar que esta matriz sería la más susceptible a contaminación cruzada en matadero, como ya había sido sugerido por otros autores [110, 158]. Además, la presencia de genotipos no compartidos con otras matrices en muestras de pechuga de pollo del mismo lote (subgrupo A) y genotipos comunes entre lotes independientes de muestras de contenido cecal y piel de cuello (subgrupo B), sugiere la posibilidad de que se deba a contaminaciones con cepas de *Campylobacter* procedentes de otros animales y/o lotes a lo largo de la cadena de producción de alimentos, pudiendo tratarse incluso de genotipos altamente diseminados. No obstante, a pesar de la utilización de diversas técnicas de cultivo microbiológico, también sería posible que algunos genotipos identificados en una matriz y no detectados en otra se deban a la sensibilidad de la propia técnica. Por último, la identificación de genotipos comunes entre matrices del mismo lote de producción (subgrupo A), apunta a la posibilidad de que la pechuga de pollo fuera contaminada también con las propias cepas de *Campylobacter,* más persistentes, presentes en el animal y/o lote. Este hecho había sido descrito como causa frecuente de contaminación de lotes positivos a *Campylobacter* por Elvers *et al*. [157].

Al analizar los datos de *fla*A en función del protocolo de cultivo microbiológico, se reconoció que tras la aplicación de una etapa de enriquecimiento (Bolton o Preston) se redujo la riqueza de genotipos de los aislados obtenidos en nuestro estudio (capítulo 5.3, tabla 2 y figura 1). Asimismo, al comparar los distintos caldos de enriquecimiento, en 10 muestras positivas (10/24; 42%) obtenidas con distinto caldo pero el mismo medio selectivo en placa, los genotipos identificados fueron diferentes (capítulo 5.3, tabla 3). Por otro lado, la utilización de placas de CFA (tanto en siembra directa como en enriquecimiento), supuso un incremento de la riqueza de genotipos, comparado con el uso de placas de mCCDA (capítulo 5.3, tabla 2 y figura 1). De esta manera, nuestros datos sugieren que la riqueza de genotipos se vio influida por el protocolo de cultivo microbiológico empleado, el cual parece capaz de seleccionar determinados aislados sobre otros, tal y como han descrito distintos autores [19, 128].

Comparando los agar utilizados, en 23 muestras (23/34; 68%) los genotipos obtenidos con siembra directa (mCCDA o CFA) no fueron coincidentes y en 8 ocasiones (8/25; 32%), empleando el mismo caldo de enriquecimiento pero distinto medio selectivo, los aislados tampoco fueron compartidos (capítulo 5.3, tabla 3). Estos resultados reflejan una mayor variabilidad obtenida a mayor número de medios en placa empleados, por lo que la utilización de varios protocolos de cultivo podría facilitar la detección de la diversidad real de las muestras contaminadas con más de un genotipo. De hecho, la norma ISO recomienda el uso de dos medios selectivos para el aislamiento de *Campylobacter* termófilos a partir de alimentos (mCCDA y otro medio con diferente composición) [140]. En este sentido, Newell *et al.* [19] también sugirieron que diversas técnicas de cultivo podrían ser necesarias para determinar los subtipos de *Campylobacter* presentes en una muestra, especialmente en poblaciones mixtas. Así, la elección de un determinado protocolo de cultivo microbiológico podría tener una gran relevancia, especialmente en estudios epidemiológicos comparados.

No obstante, los resultados obtenidos en nuestro estudio también podrían haberse visto influidos por la propia población bacteriana presente en cada muestra (según si es mixta o no y si tiene aislados mayoritarios), por azar (al elegir una colonia por morfología a partir de cada medio de cultivo) o por la selección que los medios de cultivo pueden hacer de determinadas especies, entre otros.

Por último, se aplicó la técnica de MLST en una selección de aislados (n=112 de 72 muestras) (Tablas 5 y 6). Dicha selección se basó en escoger al menos un genotipo de *fla*A distinto obtenido en las distintas matrices, incluyendo la mayor variedad posible

de protocolos de cultivo microbiológico. Comparando los mismos aislados, los datos de MLST nos permitieron identificar 45 ST, que se correspondieron con 15 complejos clonales (CC) distintos, mientras que el tipado de *fla*A detectó 43 genotipos diferentes. En 26 casos para el mismo valor de *fla*A se obtuvo más de un aislado, de los cuales 13 (50%) compartían también el mismo valor de MLST. De esta manera, aunque se pensó que la combinación de los datos de MLST y *fla*A podría mejorar el análisis, los datos de MLST no modificaron sustancialmente la interpretación epidemiológica obtenida únicamente con el tipado del gen *fla*A. De hecho, ha sido descrito que el tipado de la región variable del gen de la flagelina (*fla*A o *fla*B) tiene un índice de discriminación comparable al de la técnica de MLST para *Campylobacter* [32], siendo especialmente útil en estudios a corto plazo [159]. Además, se trata de una técnica más económica y que requiere un menor tiempo de ejecución que el MLST [159].

Según datos oficiales de la base de datos de *Campylobacter* (PubMLST), los complejos clonales más frecuentemente identificados son el CC21, el CC45, el CC48, el CC61, el CC206, el CC257 y el CC828. Generalmente, los complejos CC45 y CC257 han sido asociados a la industria avícola en Europa y Nueva Zelanda, aunque de forma generalizada los complejos CC21 y CC45 se han relacionado con numerosas fuentes animales y ambientales [160]. En nuestro estudio se recuperaron todos los complejos clonales considerados mayoritarios citados previamente, habiéndose identificado de forma más frecuente los complejos CC21 (10 muestras), CC45 (5 muestras), CC206 (5 muestras), CC48 (4 muestras) y CC443 (4 muestras). Cabe destacar el complejo clonal CC828, del que se obtuvieron 60 aislados (*C. coli*) a partir de las tres matrices analizadas (n=38 muestras). Se ha observado que se trata de uno de los genotipos mayoritarios en infecciones en personas ocasionadas por esta especie [161]. Además, utilizando esta técnica identificamos tres ST no descritos previamente, habiendo sido denominadas como ST7544, ST7545 y ST7546.

### 7.3.2. Análisis de la resistencia a antimicrobianos de los aislados de efluentes urbanos

En las muestras de efluentes urbanos se llevó a cabo una caracterización de los aislados obtenidos mediante el análisis de la susceptibilidad a antimicrobianos (capítulo 5.2, tabla 2).

Se ha descrito que existe una diferencia sustancial entre los niveles de resistencia a antimicrobianos en *C. jejuni* y *C. coli* [48]. En general, los aislados de *C. coli* de los efluentes urbanos fueron resistentes a todos los grupos antimicrobianos analizados,

siendo menor la proporción de resistencias en los aislados de *C. jejuni*; aunque esa diferencia sólo fue significativa para estreptomicina y tetraciclina (p<0,001) (capítulo 5.2, tabla 2). En consonancia con estos resultados, se ha descrito que las cepas de *C. coli* presentan resistencia a diversos antimicrobianos, especialmente macrólidos y fluoroquinolonas, más frecuentemente que los aislados de *C. jejuni.* Aunque no se conoce la causa de esta diferencia, una posible explicación sería la diferencia en la estabilidad a mutaciones o diferencias en la capacidad para intercambiar material genético entre distintas cepas [7, 54, 59].

Según datos de la EFSA obtenidos a partir de aislados de *Campylobacter* termófilos de casos clínicos en personas, la proporción de aislados resistentes a ciprofloxacina y tetraciclina fue elevada, mientras que el porcentaje de resistencia a eritromicina se mantuvo moderado-bajo [48]. Estos resultados son coincidentes con los obtenidos en nuestro estudio sobre efluentes urbanos (capítulo 5.2). Así, se detectó que la proporción de resistencias fue más alta para ciprofloxacina y ácido nalidíxico, seguido de tetraciclina, siendo menores los niveles de resistencia frente a gentamicina y eritromicina (capítulo 5.2, tabla 2). Datos muy similares a nuestro estudio también han sido confirmados en muestras de pollo en España y Letonia, donde se encontró que la mayor tasa de resistencia observada fue frente a quinolonas seguido de tetraciclina y, por último, estreptomicina y eritromicina [64, 162]. La concordancia entre los datos de muestras humanas y de pollo podría deberse a un origen común de los aislados, ya que se ha descrito que hasta el 80% de las campilobacteriosis se han asociado con este reservorio animal [70]. Igualmente, el hecho de que los patrones de resistencia detectados en nuestro estudio (capítulo 5.2) sean similares a los descritos para la especie humana, sugiere la posibilidad de emplear los estudios de *Campylobacter* en efluentes urbanos como un indicador de los *Campylobacter* termófilos presentes en el entorno urbano. Además, la presencia de *Campylobacter* resistentes a antimicrobianos en efluentes urbanos podría contribuir a la diseminación de resistencias descrita en ambientes acuáticos [163].

### 7.3.3. Análisis de la presencia del sistema de secreción tipo VI

Recientemente se ha identificado un sistema de secreción de proteínas en *C. jejuni* denominado sistema de secreción tipo VI [37]. Se ha relacionado con mecanismos de virulencia, simbiosis, colonización, interacciones entre bacterias y respuesta ante condiciones de estrés ambiental [36, 37]. Asimismo, se ha sugerido una posible correlación entre las cepas que contienen el SST6 y pacientes que presentan un cuadro

clínico más severo (septicemia, diarrea sanguinolenta etc.) [164]. En nuestro estudio se llevó a cabo una caracterización de una selección de aislados de *C. jejuni* (capítulo 5.4) procedentes de la cadena alimentaria (capítulo 5.1: contenido cecal, piel de cuello y pechuga de pollo fresca envasada) y efluentes urbanos (capítulo 5.2), para determinar la presencia de este nuevo sistema de secreción.

En base a los resultados obtenidos en nuestro trabajo, el 14% (9/63) de las cepas contenían los trece genes que conforman el sistema. Si se analizan los resultados en función del tipo de muestra, se obtuvieron aislados positivos al SST6 en las muestras animales, incluyendo contenido cecal (4/17; 24%), piel de cuello (3/23; 13%) y pechuga de pollo (2/19; 11%) (capítulo 5.4, tabla 1). En lo referente a las muestras de aguas residuales obtenidas a partir de vertidos urbanos, ninguna de las cepas fue positiva a alguno de los 13 genes que componen el SST6. De forma equivalente, Harrison *et al.* también detectaron el SST6 en muestras de carne de pollo, aunque de forma más frecuente en aislados de Vietnam (15/21; 71%) que en aislados procedentes del Reino Unido (1/28; 4%) [164]. Por otro lado, en un estudio en el que se analizaron secuencias de aislados de *C. jejuni* procedentes de animales y personas, mayoritariamente de Europa y EEUU (n=72), el porcentaje de los aislados que contenía el SST6 completo fue algo menor que el de nuestro trabajo, aproximadamente del 10%, detectándose el SST6 sólo en porcino en los aislados de animales (2/7; 29%) [36]. Otros autores han descrito que la prevalencia global encontrada, a partir del análisis de tres de los genes que conforman el SST6, fue del 4,6% (17/366) en muestras de diversos orígenes (cepas clínicas humanas, pollo, ganado, animales salvajes y agua residual de vertidos de granjas) en Pakistán [165]. En esta publicación, Siddiqui *et al.* [165] también localizaron el sistema de secreción en muestras de origen animal (17/332; 5%), incluyendo pollo (7/158; 4%), artiodáctilos (2/122; 2%), animales salvajes criados en cautividad (3/20; 15%) y aguas residuales de granjas (5/32; 16%).

Aunque este sistema se ha relacionado con casos clínicos de mayor severidad en el estudio de Harrison *et al.* [164] en Vietnam y con aislados clínicos humanos en Europa y EEUU (50/54; 93%) [36], Siddiqui *et al.* [165] observaron que la presencia del SST6 no estaba relacionado con casos de diarrea en humanos en Pakistán. En línea con estos datos, se ha descrito que el SST6 facilitaría la adhesión e invasión celular, así como la colonización efectiva del colon [37], por lo que, la presencia del SST6 podría estar asociada con cepas con un mayor poder de colonización que se corresponderían con portadores sanos. En nuestro caso, a pesar de la diferencia observada en las aguas residuales del entorno urbano, el bajo número de aislados analizados y la falta de

información sobre si se trata de muestras clínicas o de portadores sanos, no ha permitido establecer una conclusión precisa al respecto.

Por último, el gen *hcp* ha sido utilizado como un marcador de la presencia del SST6 completo en el estudio llevado a cabo por Harrison *et al*. [164]. Sin embargo, en nuestro estudio observamos que la detección de dicho marcador no implicaba la presencia del SST6 completo, ya que algunas cepas contenían dicho gen pero carecían de otros genes propios del sistema (2/63; 3%) (capítulo 5.4, tabla 1). De esta manera, sería preciso revisar la utilización de este gen como marcador único de la presencia del sistema de secreción en cepas de *Campylobacter*.

# 8. CONCLUSIONES

**1.** Para el aislamiento de *Campylobacter* en muestras con alta carga del mismo, la técnica de siembra directa es la que ofrece mejores resultados.

**2.** La utilización de medios de enriquecimiento o, incluso la concentración mediante filtración, favorecen el aislamiento en muestras con una baja carga o en las que *Campylobacter* pueda encontrarse dañado.

**3.** La cuantificación de *Campylobacter* mediante PCR en tiempo real utilizado como técnica de *screening* permite, no sólo detectar esta bacteria de forma rápida, sino incluso determinar el protocolo de cultivo más adecuado.

**4.** El número y tipo de especies y genotipos de *Campylobacter* detectados están condicionados significativamente por el método de cultivo empleado, lo que debería tenerse en cuenta en estudios epidemiológicos comparados.

**5.** El genotipado de los aislados procedentes de muestras de alimentos tomados en distintas fases de la cadena alimentaria, demuestra que las cepas de *Campylobacter* pueden proceder del propio animal y/o lote o del ambiente del matadero.

**6.** La similitud entre el patrón de resistencias descrito para cepas de *Campylobacter* humanas y el detectado por nosotros a partir de efluentes urbanos, sugiere que esta matriz podría ser un buen indicador para la vigilancia de las resistencias en cepas de *Campylobacter* comunitarias.

**7.** La detección del gen *hcp* no garantiza la existencia del resto de genes propios del sistema de secreción tipo VI, por lo que no debería ser utilizado como marcador único del mismo.

**8.** En el entorno natural estudiado, se ha identificado la existencia de especies de *Campylobacter* compartidas entre jabalí y ganado bovino criado en extensivo.

# 9. CONCLUSIONS

**1.** For the isolation of *Campylobacter* from samples with high loads, direct plating offers better results than a plating following enrichment.

**2.** The use of an enrichment step, with or without a concentration step by filtration, favors the isolation from samples with low load or where *Campylobacter* could be damaged.

**3.** Quantification of *Campylobacter* by real-time PCR can be used as a quick screening technique and could aid in choosing the most suitable culture protocol.

**4.** The number and type of *Campylobacter* species and genotypes detected are significantly conditioned by the method of isolation employed. The variation in outcome due to methodological choices should be considered in comparative studies.

**5.** The genotyping of isolates from food samples taken at different stages of the food chain demonstrates that the strains of *Campylobacter* may come from the animal itself and/or the same flock or from the slaughterhouse environment.

**6.** The similarity between the pattern of resistance described for human *Campylobacter* strains and the patterns observed in the obtained population in urban effluents, suggests that this matrix could be used as a reliable indicator for monitoring resistance in *Campylobacter* strains of the community.

**7.** Detection of gene *hcp* does not guarantee the existence of all the genes required to produce a type VI secretion system, so the gene should not be used as a single marker thereof.

**8.** In the natural environment studied, the existence of *Campylobacter* species shared between wild boar and free-ranging cattle have been identified.

# 10. OTROS TRABAJOS RELACIONADOS

A continuación se presenta el resumen de dos trabajos de colaboración relacionados con esta tesis:

- "Identification of the main quinolone resistance determinant in *Campylobacter jejuni* and *C. coli* by MAMA-DEG PCR", enviado para su publicación en 2015 (Hormeño L., Palomo G., Ugarte-Ruiz M. *et al*. 2015. *Journal of Antimicrobial Chemotherapy)*.

  Among zoonotic diseases, campilobacteriosis stands out as the major bacterial infection producing human gastroenteritis. Antimicrobial therapy, only recommended in critical cases, is challenged by resistance mechanisms that should be unambiguously detected for achievement of effective treatments. Quinolone (ciprofloxacin) resistance of *C. jejuni* and *C. coli*, the two main *Campylobacter* detected in humans, is conferred by the mutation *gyrA* C-257-T, which can be genotyped by several methods that require a previous identification of the pathogen species to circumvent the sequence polymorphism of the gene. A multiplex PCR, based on degenerated oligonucleotides, has been designed for unambiguous identification of the quinolone resistance determinant in *Campylobacter* spp. isolates. The method was verified with 249 *Campylobacter* strains isolated from humans (141 isolates) and the three most important animal sources for this zoonosis: poultry (34 isolates), swine (38 isolates) and cattle (36 isolates). High resistance to ciprofloxacin, with MIC above 4 µg/ml and frequently found among isolates from the different hosts, is linked to the mutant genotype predicted by MAMA-DEG PCR.

- "Three different adenylyl transferases confer streptomycin resistance in *Campylobacter",* en fase de preparación (Hormeño L., Ugarte-Ruiz M. *et al*. 2015).

  Thermophilic *Campylobacter* species *C. jeuni* and *C. coli* are actually recognize as the major bacteria responsible for food-borne gastroenteritis. Among the antibiotics effective against *Campylobacter*, macrolides and aminoglycosides,

susceptibility to streptomycin is still high, although different aminoglycoside O-nucleotidyltransferases (ANT) and mutations in the ribosomal protein (RPSL) have been described. Furthermore, genes encoding additional ANT enzymes were detected by analyzing the available genome drafts from *Campylobacter*. Nucleotide sequences for all these antimicrobial resistance determinants were screened among *C. jejuni* and *C. coli* isolates from human and animals, finding out that genes for three different ANT(6) enzymes, in addition to RPSL mutations, are linked to streptomycin resistance. Functionality of a new enzyme, distantly related and belonging to an uncharacterized ANT(6) sub-family, has been evidenced by expression in *Escherichia coli.* Streptomycin resistance determinants were differentially distributed among *C. jejuni* and *C. coli* isolates from different sources.

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