

**UNIVERSIDAD COMPLUTENSE DE MADRID**

FACULTAD DE VETERINARIA

DEPARTAMENTO DE MEDICINA Y CIRUGÍA ANIMAL



**TESIS DOCTORAL**

**Seroprevalence of equine infectious anemia, equine viral arteritis and equine herpesvirus-1/-4 in the Spanish purebred horse population in central Spain:**

**risk factors and association with reproductive problems**

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

**Fátima Cruz López**

Director

Richard Newton

**Madrid, 2014**



***SEROPREVALENCE OF EQUINE INFECTIOUS ANEMIA,  
EQUINE VIRAL ARTERITIS AND EQUINE  
HERPESVIRUS-1/-4 IN THE SPANISH PUREBRED  
HORSE POPULATION IN CENTRAL SPAIN.  
RISK FACTORS AND ASSOCIATION WITH  
REPRODUCTIVE PROBLEMS.***



Universidad Complutense de Madrid

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Madrid, 2014

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***SEROPREVALENCIA DE ANEMIA INFECCIOSA  
EQUINA, ARTERITIS VÍRICA EQUINA Y  
HERPESVIRUS EQUINO-1/-4 EN CABALLOS P.R.E.  
DE LA REGIÓN CENTRAL DE ESPAÑA.  
FACTORES DE RIESGO Y ASOCIACIÓN CON  
PROBLEMAS REPRODUCTIVOS.***

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**BAJO LA DIRECCIÓN DE LOS DOCTORES:**

**Paloma Forés Jackson, Joanne Ireland y Richard Newton**

**Madrid, Julio de 2014**



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Memoria de tesis presentada por:

Fátima Cruz López

Para la obtención del grado de Doctor.

VºBº Director:

Dra. Paloma Forés Jackson



Dra. Paloma Forés Jackson, Profesora Titular del Departamento de Medicina y Cirugía Animal, de la Facultad de Veterinaria de la Universidad Complutense de Madrid.

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Que el presente trabajo de investigación titulado: “Seroprevalence of Equine Infectious Anemia, Equine Viral Arteritis and Equine Herpesvirus-1/-4 in the Spanish Purebred horse population in central Spain. Risk factors and association with reproductive problems”, presentado por Fátima Cruz López para optar al grado de Doctor en Ciencias Veterinarias, ha sido realizado bajo mi supervisión, y hallándose concluido autorizo su presentación a fin de que sea juzgado por el tribunal correspondiente.

VºBº Director:

Dra. Paloma Forés Jackson



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That the present PhD Thesis with title: “Seroprevalence of Equine Infectious Anemia, Equine Viral Arteritis and Equine Herpesvirus-1/-4 in the Spanish Purebred horse population in central Spain. Risk factors and association with reproductive problems”, presented by the D.V.M. Fátima Cruz López in order to achieve the PhD grade, has been carried out under my supervision, and having concluded it I hereby authorise its presentation in order to be defended and judged by a committee of examiners.

VºBº Supervisor:

Dr. Joanne Ireland



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Dr. Richard Newton





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que, de un modo u otro, han  
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## *LIST OF ABBREVIATIONS AND ACRONYMS*

ABC: Avidin-biotin complex

AGID: Agar gel immuno-diffusion

AI: Artificial insemination

ANCCE: *Asociación Nacional de Criadores de Caballos Españoles*

AS: Apparent seroprevalence

ASOCAMAN: *Asociación de Castilla La Mancha de P.R.E.*

AUC: Area under the curve

BSK: Cold steppe climate

C.C.A.A.: *Comunidades Autónomas*

cELISA: competition ELISA

CEM: Contagious equine metritis

CESCALE: *Asociación de Criadores de Caballos P.R.E. de Castilla y León*

CF: Complement fixation

CFA: Temperate with a dry season and hot summer climate

CFB: Temperate with a dry season and temperate summer climate

CHAID: Chi-square automatic interaction detector

CI: Confidence interval

CPE: Cytopathic effect

CSA: Temperate with dry or hot summer climate

CSB: Temperate with dry or temperate summer climate

CSF: Cerebrospinal fluid

DGG: *Dirección General de Ganadería*

DNA: Deoxyribonucleic acid

DSB: Cold with temperate and dry summer climate

DSC: Cold with dry and fresh summer climate

EAV: Equine arteritis virus

EHV-1/-4: Equine herpesvirus-1 and/or -4

EI: Equine influenza

EIA: Equine infectious anaemia

EIAV: Equine Infectious Anaemia virus

ELISA: Enzyme-linked immunosorbent assay

EVA: Equine viral arteritis

Fab: Fragment antibodies

FENACE: *Federación de Asociaciones de Ganaderos de Caballo Español*

GDP: Gross Domestic Product

HBLB: Horserace Betting Levy Board

ICC: International Collating Centre

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IR: Interquartile range

MAGRAMA: Ministerio de Agricultura, Alimentación y Medio Ambiente

MAPA: Ministerio de Agricultura, Pesca y Alimentación

NC: Natural cover

NE: North-East

NSAID: Non-Steroidal Anti-inflammatory Drugs

NW: North-West

OD: Optical Density

OIE: World Organisation for Animal Health

OR: Odds ratio

P.R.E.: Pura Raza Española

PCR: Polymerase chain reaction

RK: Rabbit kidney

RNA: Ribonucleic acid

ROC: Receiver operating characteristic

Rpm: Revolutions per minute

RT-nPCR: Nested RT-PCR

RT-PCR: Reverse-transcriptase PCR

S/P: Sample-to-positive control

SD: Standard deviation

SE: Sensitivity

SE: South-East

SGSA: *Subdirección General de Sanidad Animal*

SN: Seroneutralization

SP: Spanish Purebred

SP: Specificity

SW: South-West

TCID<sub>50</sub>: 50% tissue culture infective dose

TMS: Trimethoprim and sulfadiazine

TS: True seroprevalence

UCCE: *Unión de Criadores de Caballos P.R.E.*

UK: United Kingdom

USA: United States of America

WNV: West Nile Virus

$\chi^2$ : Chi-squared



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Figure 16. Distribution by age group of the SP horses sampled in Central Spain (n=555).

Figure 17. Distribution by age group of the EHV-1/-4 unvaccinated SP horses sampled in Central Spain (n=334).

Figure 18. Distribution by breeding activity of the 555 SP horses sampled in Central Spain.

Figure 19. Distribution by breeding activity of the 334 EHV-1/-4 unvaccinated SP horses sampled in Central Spain.

Figure 20. History of reproductive problems within the active SP breeders sampled in Central Spain (n=348, 80 SP breeders with and 268 SP breeders without reproductive problems in the past 12 months).

Figure 21 History of reproductive problems within the active EHV-1/-4 unvaccinated SP breeders sampled in Central Spain (n=171, 30 SP breeders with and 141 SP breeders without reproductive problems in the past 12 months).

Figure 22. Distribution of horses vaccinated and unvaccinated against EHV-1/-4 in the sample (n=555, 334 unvaccinated and 221 vaccinated SP horses).

Figure 23. Distribution of the SP stud farms sampled in the central area of Spain.

Figure 24. Köppen-Geiger climatic map with the distribution and different climates of the SP stud farms sampled in this study.

Figure 25. Example of an EIA ELISA test carried out in this study.

Figure 26. Example of an EVA ELISA test carried out in this study.

Figure 27. Number of seropositive SP horses distributed by EVA log<sub>10</sub> titre (n=96).

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Figure 29. Distribution of EVA seropositive stud farms within the different climate areas in the area of study (n=35).

Figure 30. Example of an EHV-1/-4 ELISA test carried out in this study.

Figure 31. Percentage of SP horses seropositive to EHV-1, EHV-4 or both in the VN test (n=178, 90 SP horses seropositive to EHV-1 only, 80 SP horses seropositive to both EHV-1 and EHV-4 and 8 SP horses seropositive to EHV-4 only).

Figure 32. Percentages of the different wormers used in the 35 SP stud farms sampled in central Spain.

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Figure 34. ROC curve for the decision tree model for variables associated with seropositivity to EAV in the study population of 555 SP breeding horses in central Spain (AUC = 0.94).

Figure 35. Decision tree for variables associated with seropositivity to EHV-1/-4 in the 334 unvaccinated SP breeding horses sampled in central Spain.

Figure 36. ROC curve for the decision tree model for variables associated with seropositivity to EHV-1/-4 in the study population of SP breeding horses in central Spain (AUC = 0.79).

## *RESUMEN*

Los caballos de Pura Raza Española (PRE) representan el 85% de los caballos de raza pura en España, y casi el 100% de las exportaciones a Europa y América, las cuales tuvieron un valor de 58 millones de euros en 2012 (Deloitte, 2013). En España, la inmensa mayoría de caballos PRE se crían en explotaciones de particulares con un sistema semi-intensivo, donde cada caballo se trata de manera individual y supone una inversión importante de tiempo y dinero (MAPA, 2003).

Las enfermedades víricas equinas suponen un riesgo para las explotaciones de cría de caballos, debido a su rápida transmisión y a la inexistencia de un tratamiento específico para ellas. Entre las enfermedades víricas asociadas con problemas reproductivos y/o pérdidas económicas en las explotaciones de cría de caballos se encuentran la Anemia Infecciosa Equina (AIE), la Arteritis Vírica Equina (AVE) y los Herpesvirus equinos tipo 1 y tipo 4 (HVE-1/-4) (OIE, 2013d).

La AIE está presente en todo el mundo y se transmite mediante insectos mordedores (Issel and Foil, 1984), aunque puede ser transmitida verticalmente en el útero (Kemen and Coggins, 1972; Stein and Mott, 1942) y también iatrogénicamente (Williams *et al.*, 1981). La AIE puede producir enfermedad aguda con episodios de fiebre, trombocitopenia, anemia, abortos en yeguas y en ocasiones muerte; o bien enfermedad subclínica (OIE, 2013d). A pesar de que España fue declarada libre de AIE en 1983, la situación en algunos países europeos vecinos es preocupante, ya que en 2013 se detectaron brotes de enfermedad clínica en Bélgica y Alemania (OIE, 2014a; OIE, 2014b) y la seroprevalencia de AIE en Holanda es del 0.44% (de Boer *et al.*, 1979).

La AVE se transmite vía respiratoria o venérea (Timoney and McCollum, 1993), aunque la transmisión puede ocurrir también de manera vertical en útero (Bryans *et al.*, 1957a; Golnik, 1992; Vaala *et al.*, 1992) y a través de fómites (Guthrie *et al.*, 2003; Timoney and McCollum, 1993). Los signos clínicos más típicos de AVE son fiebre, leucopenia, edemas, la conjuntivitis, aborto en yeguas y neumonía intersticial en potros neonatos (del Piero *et al.*, 1997; Golnik *et al.*, 1981). Los sementales infectados con AVE son portadores asintomáticos y pueden excretar el

virus en semen (Neu *et al.*, 1992). En 1992 se confirmó el primer brote de AVE clínica en Barcelona, España (Monreal *et al.*, 1995). Más recientemente, en 2013, se han detectado brotes de AVE en Francia, Suiza y España (OIE, 2014b) y existen estudios de seroprevalencia que aportan datos que van de un 8.7% en Alemania (Herbst *et al.*, 1992) a un 55.1% en Polonia (Rola *et al.*, 2011).

Herpesvirus equino tipo 1 y tipo 4 producen enfermedad respiratoria, aborto, neumonía intersticial y mieloencefalopatía (HVE-1) en los caballos (Allen and Bryans, 1986; Allen, 1999; Bryans, 1988; Crabb and Studdert, 1995). La transmisión ocurre vía respiratoria, pero también a través del contacto con la placenta o las membranas fetales infectadas (Patel and Heldens, 2005). Alrededor del 80% de las infecciones por HVE-1 y HVE-4 son sucedidas por un estado de latencia; en situaciones de inmunocompromiso o estrés el virus puede reactivarse (Borchers *et al.*, 1999). El único estudio epidemiológico que evaluó la presencia de HVE-1/-4 en España data de 1992, y demostró una seroprevalencia del 4.9% mediante seroneutralización (Martin Otero, 1992). La presencia de brotes de HVE-1/-4 en países como Reino Unido, Francia, Suiza, Finlandia y España en 2013 (OIE, 2014b) y la publicación de estudios que demuestran datos de seroprevalencia de HVE-1/-4 del 76% en Holanda (de Boer *et al.*, 1979) o del 86.8% en Lituania (Liutkevicien, 2006) sugieren que HVE-1/-4 podría estar ampliamente distribuido en España.

Dada la ausencia reciente de estudios de seroprevalencia de AIE, AVE y HVE-1/-4 en España, así como la importancia de estas enfermedades en las explotaciones de cría de caballos y la relevancia de la cría de caballos PRE en la industria del caballo en nuestro país, los objetivos principales del presente estudio incluyeron la determinación de la seroprevalencia de AIE, AVE y HVE-1/-4 en la población de caballos de PRE en explotaciones de cría de la región central de España, así como la descripción de las características y las prácticas de manejo en relación a la prevención y control de enfermedades infecciosas equinas implementadas en estas explotaciones y por último la determinación de la presencia y la naturaleza de los problemas reproductivos existentes en la población de yeguas y sementales reproductores PRE de la región central de España.

Los objetivos secundarios del presente estudio incluían la identificación de potenciales factores de riesgo asociados con la exposición a las enfermedades de interés (AIE, AVE y HVE-1/-4) entre las características y medidas de manejo en relación a la prevención y el control de enfermedades infecciosas equinas en explotaciones de cría de PRE en la región central de España, y la evaluación de posibles asociaciones entre la exposición a las enfermedades de interés (AIE, AVE y HVE-1/-4) y la presencia de problemas reproductivos en yeguas y sementales PRE de esta región.

Entre Septiembre de 2011 y Noviembre de 2013, se obtuvieron 555 muestras de suero procedentes de caballos PRE residentes en 35 explotaciones de cría en la región central de España.

Tras el análisis de anticuerpos frente a AIE en las 555 muestras de suero mediante ELISA (ID Screen® Equine Infectious Anemia Double Antigen; IDVet Innovative diagnostics, Montpellier, Francia), la ausencia de caballos seropositivos a AIE demostró, con un nivel de confianza del 95%, que la población de caballos PRE en explotaciones de cría de la región central de España está libre de infección por AIE, a pesar de los recientes brotes de esta enfermedad en países europeos vecinos y de los frecuentes movimientos nacionales e internacionales de caballos en esta zona.

La seroprevalencia de AVE mediante seroneutralización en la población de este estudio fue del 17.3% (14.2% - 20.4%), demostrando la presencia actual y relevante de AVE en las explotaciones de cría de PRE de la región central de España; por tanto la AVE no debería ser considerada como una enfermedad esporádica en los caballos PRE en explotaciones de cría de esta región.

En la población de caballos no vacunados frente a HVE-1/-4 de este estudio, la seroprevalencia de HVE-1/-4 mediante seroneutralización fue del 53.3% (47.9% - 58.6%), confirmando una amplia distribución de HVE-1/-4 en la población de caballos PRE en explotaciones de cría de la región central de España.

Alrededor del 50% de las explotaciones de cría de PRE muestreadas en este estudio afirmaron realizar actividades equinas distintas adicionales a la cría de caballos PRE, salir con sus caballos PRE a ferias y competiciones y adquirir nuevos

caballos en los 12 meses previos a la toma de muestras, demostrando que los movimientos de caballos son frecuentes en las explotaciones de cría de PRE en la región central de España. La mayoría de las explotaciones de cría de caballos PRE tenían medidas básicas de prevención y control de enfermedades infecciosas equinas, tales como desparasitación (97% de las explotaciones), desinfección (83% de las explotaciones), medidas contra insectos (83% de las explotaciones) y medidas contra roedores (80% de las explotaciones). Sin embargo, casi la mitad de las explotaciones no presentaba medidas específicas de prevención y control, tales como la vacunación frente a HVE-1/-4 (ausente en el 46% de las explotaciones) o la separación de las yeguas reproductoras y los caballos jóvenes (ausente en el 49% de las explotaciones).

De las yeguas PRE muestreadas en este estudio, el 29% (23%-34%) habían presentado al menos un problema reproductivo en los 12 meses anteriores a la toma de la muestra. Entre estas yeguas, la pérdida de la gestación fue el problema reproductivo más frecuente (51%). La AVE debería considerarse al investigar la causa de problemas reproductivos en las yeguas PRE de la región central de España, ya que en nuestro estudio la presencia de anticuerpos frente a AVE estuvo asociada con la presencia de irregularidades en el ciclo/yeguas repetidoras ( $p=0.006$ ), abortos tardíos ( $p=0.006$ ) y problemas neonatales ( $p=0.006$ ).

De los sementales PRE muestreados en este estudio, solo el 7% (1.5%-11-7%) presentaron problemas reproductivos en los 12 meses anteriores a la toma de la muestra y, entre éstos, el 50% presentó falta de libido.

Para mantener la población de PRE en la región central de España libre de infección por AIE, deberían implementarse medidas contra insectos en todas las explotaciones, así como el análisis rutinario de AIE en los caballos importados, antes de la salida del país de origen y a su llegada a España.

De los caballos PRE muestreados en este estudio, aquellos residentes en explotaciones con una elevada densidad de población, o con un número elevado de visitas mensuales de personas, o donde existían frecuentes movimientos de caballos tenían mayores probabilidades de ser positivos a AVE; estas explotaciones deberían establecer una detección rápida y aislamiento de posibles portadores o caballos

infectados en fase aguda, así como análisis rutinario de AVE antes de introducir cualquier caballo a la explotación.

Los caballos de mayor edad, y aquellos residentes en explotaciones con un elevado número de visitas mensuales de personas, movimientos frecuentes de caballos y otras actividades equinas en la explotación tenían mayores probabilidades de ser positivos a HVE-1/-4; la implementación de medidas de bioseguridad, un esquema de vacunación frente a HVE-1/-4 y medidas específicas como separar las yeguas preñadas de otros caballos serían clave para reducir el riesgo de introducción de HVE-1/-4 en estas explotaciones.

En nuestra opinión, la información obtenida en el presente estudio debería animar a la Administración, las diferentes asociaciones de criadores y los veterinarios/as a perseguir la educación y concienciación de los dueños de explotaciones de cría equina acerca de la presencia, la prevención y el control de las enfermedades infecciosas equinas en las explotaciones de cría de PRE en España.



## SUMMARY

Spanish Purebred (SP) horses represent 85% of the pure breed horses in Spain and nearly 100% of exportations to Europe and America standing for more than 58 million euro in 2012 (Deloitte, 2013). The average SP horse in Spain is bred in privately owned stud farms with a semi-intensive system of exploitation, where each horse is individually treated and involves a major investment of time and money (MAPA, 2003).

Equine viral diseases pose a risk for a breeding stud farm due to their rapid transmission and the lack of specific treatment. Viral diseases associated with reproductive problems and/or economic losses in a breeding stud farm include Equine Infectious Anaemia (EIA), Equine Viral Arteritis (EVA), and Equine Herpesvirus-1/-4 (EHV-1/-4) (OIE, 2013d).

EIA is distributed worldwide and is transmitted mechanically by biting insects (Issel and Foil, 1984), although it may be passed from a mare to her foal in utero (Kemen and Coggins, 1972; Stein and Mott, 1942) and also iatrogenically (Williams *et al.*, 1981). The disease can be acute, causing febrile episodes, thrombocytopenia, anaemia, abortions in mares, and death; or it can also be subclinical (OIE, 2013d). Even though Spain was declared free from EIA infection in 1983, the situation in nearby European countries is of concern, with EIA outbreaks of clinical disease in Belgium and Germany in 2013 (OIE, 2014a; OIE, 2014b) and a seroprevalence estimate of 0.44% for EIA in the Netherlands (de Boer *et al.*, 1979).

EVA is transmitted via respiratory or venereal routes (Timoney and McCollum, 1993b), but also vertically in utero (Bryans *et al.*, 1957a; Golnik, 1992; Vaala *et al.*, 1992) and via fomites (Guthrie *et al.*, 2003; Timoney and McCollum, 1993b). The most typical clinical signs of EVA are fever, leukopenia, oedema, conjunctivitis, abortion in the mare, and fatal interstitial pneumonia in young foals (del Piero *et al.*, 1997; Golnik *et al.*, 1981). Stallions infected with the EAV are asymptomatic carriers of the virus and can shed the virus in semen (Neu *et al.*, 1992). In 1992 the first confirmed outbreak of EVA clinical disease was reported in Barcelona, Spain (Monreal *et al.*, 1995). Recently, EVA outbreaks of clinical disease

have been reported in France, Switzerland and Spain in 2013 (OIE, 2014b) and studies have shown EVA seroprevalences ranging from 8.7% in Germany (Herbst *et al.*, 1992) to 55.1% in Hucul horses in Poland (Rola *et al.*, 2011).

EHV-1 and EHV-4 produce respiratory disease, abortion, neonatal pneumonia, or myeloencephalopathy in horses (Allen and Bryans, 1986; Allen, 1999; Bryans, 1988; Crabb and Studdert, 1995). Transmission occurs via the respiratory route, but also through contact with the infected placentas and foetal membranes (Patel and Heldens, 2005). Around the 80% of primary infections by EHV-1 and EHV-4 are followed by a state of latency which can be reactivated with immunosuppression or stress (Borchers *et al.*, 1999). The only epidemiological study to evaluate the presence of EHV-1/-4 in Spain dates back to 1992 and showed a seroprevalence of 4.9% by seroneutralization (SN) (Martin Otero, 1992). The occurrence of EHV-1/-4 outbreaks of clinical disease in the UK, France, Switzerland, Finland and Spain in 2013 (OIE, 2014b) and studies showing EHV-1/-4 seroprevalence results of 76% in the Netherlands (de Boer *et al.*, 1979) or 86.8% in Lithuania (Liutkevicien, 2006) suggest that EHV-1/-4 could also be widespread in Spain.

Given the absence of recent studies of EIA, EVA and EHV-1/-4 seroprevalence in Spain, as well as the importance of these diseases for the equine breeding stud farms and the relevance of the breeding of SP horses for the Spanish equine industry, the main objectives of this study included the determination of the seroprevalence of EIA, EVA and EHV-1/-4 in the SP breeding population in the central area of Spain as well as the description of the characteristics and management practices with regards to the prevention and control of equine infectious diseases implemented in these stud farms and the determination of the occurrence and nature of the breeding problems in SP mares and stallions in the central area of Spain.

The secondary objectives of the study included the identification of any potential risk factors associated with the exposure to the infectious diseases of interest (EIA, EVA and EHV-1/-4) within the characteristics and management practices regarding the prevention and control of equine infectious diseases in SP stud farms in central Spain, and the evaluation of possible associations between the exposure to the infectious diseases of interest (EIA, EVA and EHV-1/-4) and the

occurrence of breeding problems in SP mares and stallions in the central area of Spain.

Between September 2011 and November 2013, serum samples were obtained from 555 SP horses residing on 35 different stud farms in the central region of Spain.

After testing the 555 serum samples for EIA antibodies by ELISA (ID Screen® Equine Infectious Anemia Double Antigen; IDVet Innovative diagnostics, Montpellier, France), the absence of any EIA seropositive animals provided 95% confidence that the SP breeding horse population in central Spain is free from EIA infection, despite the recent EIA outbreaks in nearby European countries and the frequent national and international horse movements in this area.

The seroprevalence of EVA in the population of this study by SN was 17.3% (14.2% - 20.4%), showing a relevant presence of EVA in the SP breeding horse population in central Spain; therefore, EVA should not be considered a sporadic disease in the SP breeding stud farms of this region.

In the population of horses unvaccinated against EHV-1/-4 of this study, the seroprevalence for EHV-1/-4 in this study by SN was deemed to be 53.3% (47.9% - 58.6%), demonstrating that EHV-1/-4 is widely spread in the SP breeding horse population in central Spain.

Nearly half of the SP stud farms sampled in this study (46%) reported having equine activities other than breeding in the premises, the same percentage (46%) reported outings to fairs and competitions with their horse and 54% acquired new horses in the past 12 months, demonstrating that horse movements are frequent in the SP stud farms in central Spain. Most of the SP stud farms sampled had basic measures of prevention and control of equine infectious diseases, such as a worming regime (present in 97% of stud farms), a disinfection regime (present in 83% of stud farms), measures against insects (present in 83% of stud farms) and rodents (present in 80% of the stud farms). However, nearly half of the SP stud farms showed an absence of specific measures, such as an EHV-1/-4 vaccination regime (absent in 46% of stud farms) and segregating breeding mares from young horses (absent in 49% of stud farms).

Of the SP breeding mares sampled in the study, 29% (23%-34%) were reported to have had at least one reproductive problem in the past 12 months and within these, gestation loss was the most frequent problem (51%). Within the SP mares with reproductive problems sampled, EVA seropositivity was significantly associated with cyclic irregularities/repeat breeding ( $p=0.006$ ), late abortions ( $p=0.006$ ) and problems with the neonate ( $p=0.006$ ).

Of the SP breeding stallions sampled in the study, only 7% (1.5%-11.7%) were reported to have reproductive problems during the past 12 months, and out of these horses, 50% showed a lack of libido.

In order to maintain the SP breeding horse population in central Spain free from EIA infection, measures against insects should be implemented in every stud farm and judicious biosecurity that includes routine EIA testing of the horses prior to importation into Spain and after their arrival should be established to prevent the introduction of subclinically EIA infected horses from Europe.

Within the SP horses of this study, those residing in stud farms where there was a high horse density of population, or had a large number of visits from people or frequent movements of horses were more likely to be EVA seropositive; these stud farms should establish good biosecurity measures in the premises as well as a prompt detection and isolation of possible carriers or acutely infected horses and routine EVA testing prior to the introduction of new horses.

Older SP horses, and those residing in stud farms with a large number of visits from people, frequent movements of horses and other equine activities in the premises were more likely to be EHV-1/-4 seropositive; the implementation of biosecurity measures, routine EHV-1/-4 vaccination and specific measures such as separating pregnant mares from other horses would be key in order to reduce the risk of the introduction of EHV-1/-4 into these stud farms.

In our opinion, the data obtained in the present study should encourage the Administration, the different Breeders' associations and veterinary surgeons to pursue the education and awareness of stud farm owners regarding the presence, prevention and control of equine infectious diseases in a breeding stud farm.



## *I. INTRODUCTION*



# *I. INTRODUCTION*

## I.1 EQUINE BREEDING IN SPAIN

### I.1.1 Brief historical perspective

Breeding based on the exploitation of the equine is one of the most ancient activities in Spain. The most ancient vestiges of the horse presence in Spain come from the Paleolithic period and appear in the shape of cave paintings that show the importance that equines had in the prehistoric systems of life.

The origin of the horse influence in the Spanish culture coincides with the blooming of the first big civilizations in the Peninsula. The Carthaginians incorporated horses in great numbers into their armies due to their great resistance and strength. Later on, the Romans promoted the horse as a means of transport in civil life and in warlike conflicts as a sign of distinction for kings and emperors.

During the Arabic expansion in Spain, Andalusian horses, which were admired by the Arabs, were included as part of a light cavalry in the invading army. In addition, important studs were created and horses were even exported to Constantinople, Baghdad and other big cities of the Islamic empire.

After the reconquest, low demand for horses with aptitudes for war together with an increasing role in agriculture made mules more important than horses, since they were more practical for the hard work. Nevertheless, at the end of the 15th century the monastery of the Carthusian was the cornerstone for breeding of the Andalusian horse. Over a period of three hundred years, the Carthusian monks established a stud that became one of the most famous and respected studs in the world.

With the French Invasion in the 19th century, the national equine population was greatly reduced. In addition, the crossings of Andalusian mares with other European breeds, such as the Norman horse, the Trakehner, the Holsteiner and the Hanoverian resulted in a serious genetic deterioration to the purebred Spanish

equine population. Consequently, in the middle of the 19<sup>th</sup> century, the veterinary schools of Zaragoza, Leon and Cordoba were created, and were dedicated to the care and the genetic improvement of the horse in Spain. In 1893 the War Ministry coordinated equine breeding in Spain; stallion studs were funded in Cordoba, Úbeda and Valladolid, followed by others in several Spanish cities.

In 1912 the Department for Equine Breeding created the first studbook in Spain in order to register the Arab, Thoroughbred and Anglo-Arab horses, naming the traditionally named Andalusian horses as “Spanish Purebred” (SP) horses. By the end of the 20<sup>th</sup> century, in 1972, the National Association for the Breeders of Spanish Purebred Horses (*Asociación Nacional de Criadores de Caballos Españoles* (ANCCE)) was created in Sevilla as a cornerstone for the breeding and genetic improvement of the SP horse in Spain.

## I.1.2 The Spanish Purebred Horse and its present situation

Although nowadays the SP horse is considered one of the most important breeds in the present Spanish equestrian industry, its origins are unclear.

Studies of mitochondrial deoxyribonucleic acid (DNA) carried out in the modern SP horse revealed that both breedlines in the SP breed (Andalusian and Carthusian), which originated from Iberian horses, also have an influence of the Bereber breed. Iberian horses located predominantly in the South of the Iberian Peninsula were crossed during the Arab invasion in Spain with Bereber horses originating in North Africa (Royo *et al.*, 2005). The influence of the Bereber breed, together with the work of conservation and recovery carried out by the Carthusian monks in the 15th century were probably the foundations of the present breed (Valera *et al.*, 2005).

Following the definition included in the Order APA/3319/2002, the SP horse is a eumetric, mesolinear horse with a subconcave to straight profile. It has appreciated elevations and extensions and a great ease for collection. Its character is defined as noble, docile and balanced. These qualities make the SP the most important pure breed in Spain.

The management of the Registry (or Stud-Book) is carried out by the Department of Equine Breeding (“Cría Caballar”). As of 31st December 2012, this department published an equine census with a number of 277,287 purebred equidae registered in Spain, of which 175,945 were SP horses (MAGRAMA, 2012). The census of SP horses by autonomous community in December 2012 in Spain, is shown in Table 1.

Table 1. Spanish Purebred Horse census by autonomous community in Spain (December 2012).

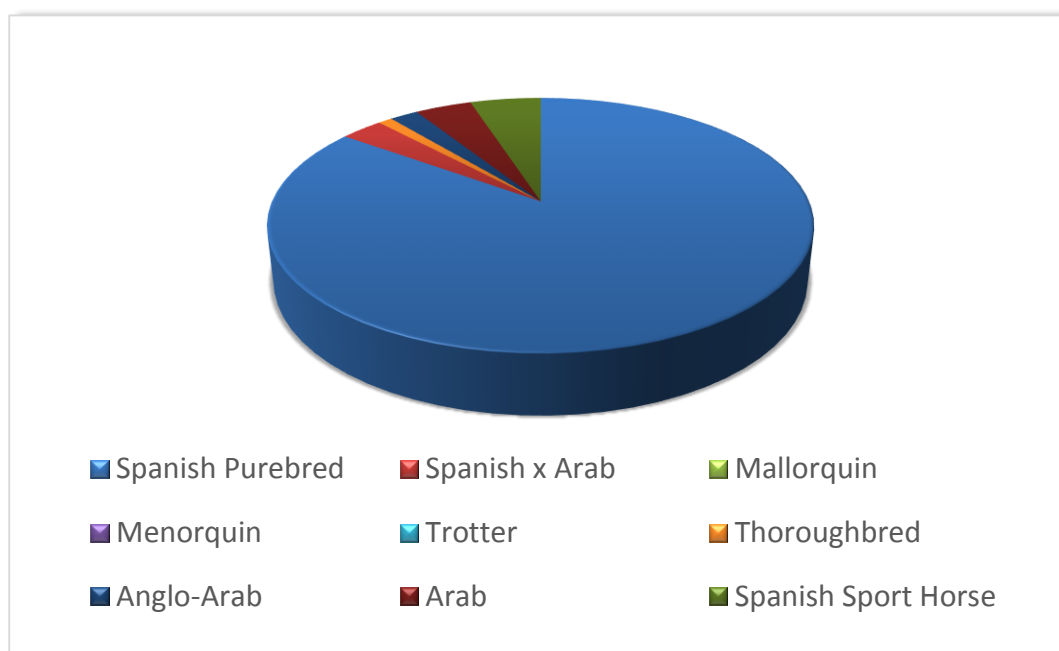
| <b>Autonomous Community</b> | <b>Spanish Purebred<br/>Total Horses</b> | <b>Spanish Purebred<br/>Horses %</b> |
|-----------------------------|--|--------------------------------------|
| ANDALUCÍA                   | 74,012                                   | 42.1                                 |
| ARAGÓN                      | 1,760                                    | 1.0                                  |
| CANTABRIA                   | 1,476                                    | 0.8                                  |
| CASTILLA LA MANCHA          | 13,327                                   | 7.6                                  |
| CASTILLA LEÓN               | 15,358                                   | 8.7                                  |
| CATALUÑA                    | 16,976                                   | 9.6                                  |
| COMUNITAT VALENCIANA        | 12,132                                   | 6.9                                  |
| EXTREMADURA                 | 13,544                                   | 7.7                                  |
| GALICIA                     | 4,386                                    | 2.5                                  |
| ILLES BALEARS               | 3,300                                    | 1.9                                  |
| ISLAS CANARIAS              | 511                                      | 0.3                                  |
| LA RIOJA                    | 442                                      | 0.3                                  |
| MADRID                      | 7,255                                    | 4.1                                  |
| MURCIA                      | 7,561                                    | 4.3                                  |
| NAVARRA                     | 1,124                                    | 0.6                                  |
| PAÍS VASCO                  | 866                                      | 0.5                                  |
| PRINCIPADO DE ASTURIAS      | 1,915                                    | 1.1                                  |
| <b>TOTAL</b>                | <b>175,945</b>                           | <b>100.0</b>                         |

Source: Arca database, data as of 31<sup>st</sup> December 2012 (MAGRAMA, 2012).

According to the census of 2012, the central region of Spain (Castilla La Mancha, Castilla León and Madrid) included 20% of the total SP horses in Spain (MAGRAMA, 2012).

In addition, a study on the impact of the equestrian sector in Spain was carried out by Deloitte for the Royal Spanish Equestrian Federation and it was published in June 2013. This study revealed that, in April 2013, the number of horses in Spain was 723,496. There were 220,006 purebred horses and of these, 85% were SP horses (187,005 horses) (Deloitte, 2013). According to this study and similarly to the census of 2012, the central region of Spain had 23% of the total SP horses in Spain. The distribution of pure breeds in Spain and the distribution of SP horses by autonomous community published by Deloitte in 2013 are shown in Figure 1 and Table 2 respectively.

Figure 1. Distribution of pure breeds in Spain.



Source: Impact of horse industry in Spain. Study published by Deloitte for the National Equestrian Federation (June 2013) (Deloitte, 2013).

Table 2. Spanish Purebred Horse census by autonomous community in Spain (June 2013).

| Autonomous Community   | Spanish Purebred Total Horses | Spanish Purebred Horses % |
|------------------------|-------------------------------|---------------------------|
| ANDALUCÍA              | 78,539                        | 42.0%                     |
| ARAGÓN                 | 1,870                         | 1.0%                      |
| CANTABRIA              | 1,870                         | 1.0%                      |
| CASTILLA LA MANCHA     | 14,960                        | 8.0%                      |
| CASTILLA LEÓN          | 18,700                        | 10.0%                     |
| CATALUÑA               | 18,700                        | 10.0%                     |
| COMUNITAT VALENCIANA   | 9,350                         | 5.0%                      |
| EXTREMADURA            | 16,830                        | 9.0%                      |
| GALICIA                | 3,740                         | 2.0%                      |
| ILLES BALEARS          | 1,870                         | 1.0%                      |
| LA RIOJA               | 6                             | 0.003%                    |
| MADRID                 | 9,350                         | 5.0%                      |
| MURCIA                 | 7,480                         | 4.0%                      |
| NAVARRA                | 1,870                         | 1.0%                      |
| PAÍS VASCO             | 2                             | 0.001%                    |
| PRINCIPADO DE ASTURIAS | 1,870                         | 1.0%                      |
| <b>TOTAL</b>           | <b>187,005</b>                | <b>100.0</b>              |

Source: Impact of horse industry in Spain. Study published by Deloitte for the Royal Spanish Equestrian Federation (June 2013) (Deloitte, 2013).

According to the data published by the Department of Equine Breeding as of 31<sup>st</sup> December 2012, the number of registered SP breeding stud farms in Spain was 17,933 (MAGRAMA, 2012).

In addition to being the most numerous breed in Spain, the SP breed accounts for the highest number of breeder associations. There are more than 25 different breeder associations (there is at least one for every autonomous community). Most of these associations are grouped into two federations: the ANCCE and the FENACE (*Federación de Asociaciones de Ganaderos de Caballo Español*). These associations continue to play an important role in the development of the SP breed.

All these data show the high presence of the SP breed in Spain and the relevance of the SP horse for the Spanish equine industry.

## I.2 CHARACTERISTICS OF A SP BREEDING STUD FARM IN SPAIN.

Equine stud farms can be classified into five types, according to the activities that they are involved in (MAPA, 2003):

- stud farms for the breeding and selection of pure breeds
- equestrian facilities
- breeding farms for the production of horse meat
- farms associated with other type of farming activity
- small private farms

The vast majority of SP horses in Spain are bred in stud farms for the breeding and selection of pure breeds whether public or private, the latter being the most common. Main objectives of private breeding SP stud farms include technical development (management, breeding, replacement and training of horses) and business development (trade relations, services, sales, etc.). These premises tend to have several broodmares, although they do not always have a stallion. The second type of stud farms are the private artificial insemination (AI) and breeding centres. Breeders from other premises can bring their mares to these centres in search of quality genetic material for them. They usually have stallions or equine semen banks. They can house their own stallions and also stallions from other breeders, as well as mares used for the extraction of the semen ("teaser" mares) and also mares owned by other breeders (MAPA, 2003).

The system of exploitation of pure breeds can be semi-intensive or extensive. The majority of the SP horse breeding is conducted with a semi-intensive system. With this system, the horses are subjected to a more exhaustive control, which facilitates higher production. These premises usually have grazing pastures or other outdoor facilities where the mares and their foals stay during the day, covered paddocks where they stay overnight and stables where the stallions are housed (MAPA, 2003).

Due to the nature of the horses and the high investment in each horse, the handling and management in these stud farms needs to be specialized. The main objective in these farms, given that the gestation period of the mare lasts for 11

months, is to obtain one foal per mare and per year. Therefore it is essential that a mare conceives in the first days after foaling, which is positively influenced by a good reproductive management. Breeding in these stud farms is usually carried out by natural covering or AI with fresh or frozen semen. Some stud farms use the embryo transfer technique, allowing mares with a high genetic value to continue with their career in sport. All these characteristics of this very common type of regime imply a high investment in the facilities, as well as higher maintenance costs than other animal productions (MAPA, 2003).

According to the survey conducted by the Department of Equine Breeding in a study published in 2003, the average number of horses stabled in the stud farms for the breeding and selection of pure breeds was 23. The majority of stud farms (74%) reported having less than 30 horses, while only 4% of the farms that responded to this survey had more than 90 horses (MAPA, 2003).

The average SP horse in Spain would therefore be bred in a privately owned stud farm with a semi-intensive system of exploitation, by natural covering or AI with fresh or frozen semen, and with a total of 23 SP horses.

## I.2.1 Importance of management for the prevention and control of equine infectious diseases in a breeding stud farm.

The concept of health is essential for a breeding stud farm. Horses are susceptible to various infectious diseases, some of which are listed as "Diseases of compulsory Declaration" or "Annual communication diseases". The authorities with competences in the field of Animal Health (*Subdirección General de Sanidad Animal* (SGSA) of the *Dirección General de Ganadería* (DGG) at a central level and the *Servicios de Ganadería de las Comunidades Autónomas* (C.C.A.A.) at a regional level) establish measures for the prevention and control of these diseases, and determine a number of sanitary conditions to be imposed in all facilities that host equidae, as well as in the transport of these animals.

The SP horse breeding industry differs from other livestock sectors (e.g. porcine, poultry, cattle, sheep) in that horses on a SP stud farm are managed at an individual, rather than herd level. Thus, each horse is individually treated, and horses enter and leave the premises for many reasons (for example to attend competitions or morphologic shows, or to other stud farms). This increases the risk of introduction and spread of viral and bacterial diseases (Rogers and Cogger, 2010).

The implementation of good management practices for the prevention and control of equine infectious diseases is considered essential to reduce exposure to these diseases in a stud farm (Timoney and McCollum, 1988). In the United Kingdom (UK), guidelines for the management, prevention and control of equine infectious diseases (predominantly but not exclusively venereally transmitted) are published annually (since 1977) by the Horserace Betting Levy Board (HBLB) via the HBLB Codes of Practice (<http://codes.hblb.org.uk/>). These Codes of Practice have also been adopted by Thoroughbred breeders in France, Germany, Italy and Ireland, and they provide the horse breeding industry with valuable information regarding diseases of the greatest relevance. These diseases are contagious equine metritis (CEM), equine viral arteritis (EVA), equine herpesvirus-1 and/or -4 (EHV-1/-4), equine coital exanthema (equine herpesvirus-3), equine infectious anaemia (EIA), dourine (*Trypanosoma equiperdum*) and strangles (*Streptococcus equi equi*) (HBLB, 2013). These codes of practice have not been implemented yet in Spain and until

publication of the Royal Decree 804/2011 for the regulation of sampling, prevention and control of EVA and CEM in June 2011 there was no regulation at all regarding equine infectious diseases for the equine breeding sector (BOE, 2011). In spite of this Royal Decree being a breakthrough for the industry of horse breeding, it only covers two of the most relevant infectious diseases for this sector, and currently there are no regulations or guidelines for the management, prevention and control of the other diseases included in the HBLB Codes of Practice.

The existence of infectious diseases and/or poor management measures in a stud farm may cause reproductive problems in mares (such as irregularities in the oestrus cycle, anoestrus, repeat breeding, pregnancy loss, dystocia, or problems in the neonate) and also in stallions (alterations in any organs of the reproductive system, alterations in semen, in the ejaculate, or lack of libido). At present there are no data on the prevalence of reproductive problems in SP mares and stallions in Spain and the only studies that have been published on this subject were carried out either in Lusitano mares [a breed that has plenty of genetic similarities to the SP horse (Lopes *et al.*, 2005)] or in Standardbred mares. Lusitano mares had a foaling rate (percentage of foalings in the mares within the covered or inseminated mares) of 74.5%, which means that 25.5% of mares had a problem that impeded foaling (Fradinho *et al.*, 2014), whereas Standardbred mares had a foaling rate of 71% and therefore a 29% of mares with a reproductive problem (Katila *et al.*, 2010).

The only parameter that has been evaluated in the SP breed is the length of gestation in a study that used Arab and SP mares in the South of Spain (Valera *et al.*, 2006). The authors of this study noted that the variability in the length of gestation was higher in SP compared to Arab mares. Within the SP mares, the greatest number of normal foalings took place in the range of ages between 5 and 10 years, whereas mares over 14 years had the lowest number of normal foalings (Valera *et al.*, 2006).

In addition to compromising the welfare of the affected horses, any reproductive problem in the stallions or mares of a stud farm represents economic losses for the owner of the stud farm, since each horse involves a major investment of time and money.

## I.3 EQUINE VIRAL DISEASES OF INTEREST IN A BREEDING STUD FARM.

Equine infectious diseases are always a risk for a breeding stud farm. However, viral diseases tend to have a greater significance, due to their rapid transmission and the lack of specific treatment. Viral diseases associated with reproductive problems and/or economic losses in a breeding stud farm include EIA, EVA and EHV-1/-4. These three diseases are included in the World Organisation for Animal Health (OIE, 2014b) listed diseases and upon appearance, their notification to OIE is compulsory (OIE, 2013d).

### I.3.1 Equine Infectious Anaemia (EIA)

#### I.3.1.1 Aetiology

EIA is a disease caused by EIA virus, which belongs to the *Lentivirus* genus of the *Retroviridae* family, *Orthoretrovirinae* subfamily. Other viruses included in the same genus are bovine immunodeficiency virus, caprine arthritis and encephalitis virus, feline immunodeficiency virus, human immunodeficiency viruses type 1 and type 2 and the maedi/visnae virus (OIE, 2013d).

#### I.3.1.2 Epidemiology

The EIA virus is transmitted mechanically by haematophagous biting insects. The most effective in the transmission of the virus are biting insects of the *Tabanidae* family (*Tabanus spp.* and *Hybomitra spp.*) but the common fly (*Stomoxys calcitrans*)

can also act as a vector (Issel *et al.*, 1988). The virus can also be transmitted through transfusions of blood and by the use of contaminated needles, surgical instruments or dental floats (Hall *et al.*, 1988). In horses, the virus persists in leukocytes for life, and viraemia can occur during episodes of pyrexia (OIE, 2013d). EIA virus is also transmitted vertically in the pregnant mare (Kemen and Coggins, 1972; Stein and Mott, 1942). Other potential routes of transmission are via milk and semen, which carry the virus but appear to need to be inoculated subcutaneously for the virus to be transmitted (Stein *et al.*, 1944). These two routes of transmission, although unlikely, have been shown in cases of nursing foals and in a mare with a vaginal laceration during a natural covering (Stein *et al.*, 1944). The possibility of transmission from infectious material through aerosols by direct contact was suggested during an outbreak of the disease in 2006 in Ireland, although it was not confirmed (More *et al.*, 2008a; More *et al.*, 2008b).

The incubation period is 7 to 45 days or more. Some horses remain asymptomatic until they are stressed. The transmission of the virus depends on the number and species of vectors, their feeding habits, the population density of horses, the level of viraemia in the host and the amount of blood transferred (OIE, 2013d). The infection rate varies with the geographical region; infections are common in humid and swampy regions (Sellon, 1993). In premises where the disease has been endemic for many years up to 70% seroprevalence rates were observed (Tashjian, 1984).

### I.3.1.3 Pathogenesis and clinical signs

There are several forms of the disease: the acute form in which clinical signs include pyrexia, depression, haemorrhages, ataxia, very rapid weight loss, haemorrhagic diarrhoea, oedema, jaundice, and petechiae in the mucosae, while the chronic form is characterised by recurrent episodes of pyrexia, depression, anaemia and weight loss interspersed with periods of normality; and the subclinical form in which clinical signs may be absent but can be exacerbated during periods of concurrent disease or stress (Sellon, 1993).

The morbidity rate and the severity of the clinical signs are influenced by the strain and dose of the virus, and also by the health status of the horse. The disease usually goes unnoticed until some horses develop the chronic form of the disease or a routine EIA screening test is carried out (Cheevers and McGuire, 1985). Even though outbreaks of EIA with high rates of morbidity and mortality have been observed, death in naturally infected horses is uncommon; however, the experimental inoculation of a high dose of the virus has led to mortality rates of up to 80% (Cook *et al.*, 2001).

#### I.3.1.4 Diagnosis

EIA is difficult to diagnose based solely on clinical signs shown by affected horses; therefore a combination of various methods is usually required for a definitive diagnosis.

##### *Clinical diagnosis*

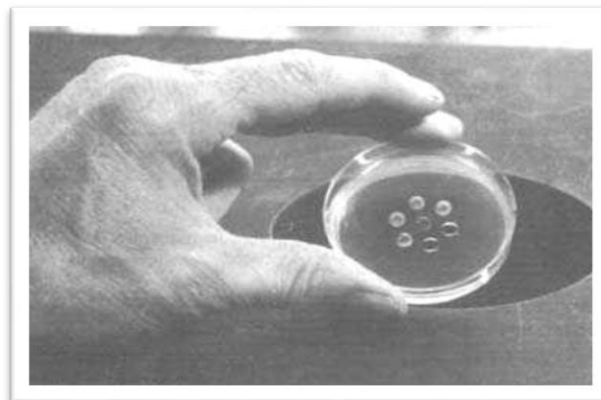
EIA should be among the differentials in horses showing weight loss, oedema and intermittent pyrexia, particularly when new animals have been introduced into the herd (Garner *et al.*, 2003). The differential diagnoses include other diseases that cause pyrexia, oedema and/or anaemia such as EVA, purpura haemorrhagica, leptospirosis, babesiosis, a severe infestation by *strongylidae* or *Fasciola hepatica*, phenothiazine toxicity, autoimmune haemolytic anaemia (Garner *et al.*, 2003).

## *Diagnosis by serology*

Serological techniques are the most frequently used in the diagnosis of EIA. Infected horses are carriers during their lifetime and therefore they remain seropositive. The two most commonly used serological techniques are the agar gel immuno-diffusion test (AGID, also called the Coggins test) and several enzyme-linked immunosorbent assay (ELISA) techniques. There is a third test, called Western blot or immunoelectrotransfer; however, it is not officially used and its use is restricted to cases where there are equivocal results to the ELISA and the Coggins test (Issel and Cook, 1993).

The Coggins test is an internationally recognized standard for the diagnosis of EIA. During its validation, correlation between negative and positive test results with the absence or presence of EIA virus in blood, respectively, was evaluated (Coggins *et al.*, 1972; Pearson *et al.*, 1971). For this purpose blood samples from horses testing positive and negative to the Coggins test were inoculated in susceptible horses in order to validate the fact that horses inoculated with blood positive to the Coggins test (i.e. with antibodies against the EIA virus) were infected while horses that were inoculated with blood negative to the Coggins test (without antibodies to the EIA virus) remained free from infection. Given its proven reliability, the Coggins test is used worldwide as a reference method in order to officially certify absence of infection in horses to be exported or in any movement of horses (OIE, 2013d).

Figure 2. Plate used in the Coggins test.



Source: <http://www.fao.org>

The Coggins test presents the typical limitations of all serological tests. For example, horses infected by EIA virus tend to be seronegative to the Coggins test during the first 2-3 weeks after infection; some cases may even remain seronegative up to 60 days post-infection (Issel and Cook, 1993). Foals that acquire passive immunity via colostrum from EIA seropositive mares may have a positive Coggins test, even though they are not infected by the virus (Issel and Cook, 1993).

When quality reagents are used and the internationally accepted procedures are followed, easily interpretable results are obtained within 24-48 hours (Pearson and Gipson, 1988). It is due to this and other advantages that the Coggins test has been used since its validation to help eradicate EIA in populations of horses around the world (Issel and Cook, 1993).

ELISA techniques can detect antibodies before the Coggins test and they are more sensitive, but can result in false positives (lower specificity) (Singha *et al.*, 2013; Soutullo *et al.*, 2001). For this reason, positive results for EIA obtained by ELISA must be confirmed by the Coggins test, which is the reference technique or "gold standard" according to the OIE (OIE, 2013d). There are different ELISA tests for the diagnosis of EIA: the first ever ELISA developed for the diagnosis of EIA was a competition ELISA (cELISA) that detected antibodies against p26 antigen of EIA virus and it was validated at the end of the 1980s in the United States of America (USA) (Matsushita *et al.*, 1989). The advantage of this test is that it is more objective than the Coggins test when using an ELISA reader, and it is also faster, since the results can be obtained in about two hours (Matsushita *et al.*, 1989). The correlation between the results obtained using the Coggins test and the cELISA test is excellent provided that the standard protocols are followed (Pearson and Gipson, 1988). However, there can be discordant results, mainly due to two causes: the most common reason for a positive cELISA result and a negative Coggins test result is a low level of antibodies against p26 antigen in the sample, which are more readily detectable using the cELISA. The second reason for discordant results is the presence in the sample of antibodies directed to the interspecies determinant of p26 antigen, which are not detected in the Coggins test. This situation could occur in horses after the exposure to other lentiviruses present in nature (Issel and Cook, 1993).

Numerous further ELISA tests have been developed for the diagnosis of EIA. Some of them detect antibodies produced against the nucleic p26 protein antigen, whereas others incorporate p26 protein and gp45 (a viral transmembrane protein) antigen (Pourquier, 2010). The current trend is to use ELISA tests based on synthetic peptides (Soutullo *et al.*, 2001) or a recombinant protein produced by a synthetic gene so that handling a pathogenic strain of EIA virus can be avoided (Reis *et al.*, 2012; Singha *et al.*, 2013). Typical ELISA protocols are used in all these techniques and all of them have very similar sensitivity and specificity (OIE, 2013d).

### *Diagnosis by antigen detection molecular techniques*

In the diagnosis of EIA techniques such as the polymerase chain reaction (PCR) can be used to detect the DNA of EIA virus in the peripheral blood of horses (Nagarajan and Simard, 2001). This technique has proven to be sensitive when detecting natural strains of EIA virus in blood leukocytes from infected horses (Nagarajan and Simard, 2001, 2007). A real time reverse-transcriptase PCR (RT-PCR) has also been described (Cook *et al.*, 2002).

PCR and RT-PCR techniques are useful when trying to diagnose infection by EIA virus in foals born from infected mares, since the foals may have maternal antibodies up to 6-8 months of age. These techniques can also be used as a complement or confirmation for the serologic tests, particularly when there are conflicting results or when the infection by EIA virus is suspected based on clinical signs and serology is negative or equivocal (e.g. in the early stages of the disease when an antibody response has still not been developed). Finally, these techniques are used as screening tests to ensure that the horses used as blood donors or those used in the production of vaccines and antisera are not infected (OIE, 2013d).

## *Diagnosis by whole virus detection techniques*

Viral isolation can be undertaken, although it is not usually necessary for the diagnosis of EIA. EIA virus can be found in the plasma and blood leukocytes during episodes of pyrexia; between these episodes, the virus is only found inside the blood leukocytes. The virus can be isolated from affected horses by inoculating their blood in cultures of white blood cells from uninfected horses. The presence of virus in the cultures can be confirmed by detecting a specific antigen against the EIA virus by ELISA (Shane *et al.*, 1984), by immunofluorescence (Weiland *et al.*, 1982) or using molecular tests. Equine leukocytes are difficult to grow; therefore this technique is not used in the majority of laboratories.

When the infection by the EIA virus in a horse is difficult to confirm, a susceptible horse can be inoculated with blood from the suspected case; a horse that has previously been determined to be negative for antibodies against EIA should be inoculated with blood from the suspected case and the status of its antibodies and clinical condition should be monitored for at least 45 days. Usually, the inoculation of 1-25 ml of blood is enough to prove the infection, but in some cases the use of a greater volume of blood (250 ml) may be required (Coggins and Kemen, 1976).

### **I.3.1.5 Treatment**

There is currently no effective treatment for EIA. Supportive therapy to relieve clinical signs of the disease are recommended until there is a definite confirmation of EIA diagnosis when the slaughter of the horse, which is mandatory in many countries (including Spain), is carried out (OIE, 2013d).

### I.3.1.6 Prevention and control

Many countries have established control measures against EIA. Frequently these measures predominantly consist of applying screening tests in horses due to be imported or transported. Performing a serological test for EIA in horses that come into a premises for the first time is very useful in order to have an EIA-free herd (OIE, 2013d).

Whereas in many countries the slaughter of any EIA-positive horse is mandatory, in other countries such as the United States of America (USA) slaughter of EIA-positive horses is not compulsory, although these should be isolated from other horses and they should be marked with an iron or a tattoo. In these countries, EIA-positive horses can only be transported if they go to their home premises, a slaughterhouse or research facilities (USDA, 2007).

Mares that are asymptomatic EIA carriers normally give birth to foals which are not infected by the virus. The risk of congenital infection increases when the mare presents with clinical signs prior to foaling. Foals born from infected mothers should be isolated from other horses until it is determined that they are free from infection (Garner *et al.*, 2003).

During an outbreak, spraying to control the insects as well as using repellents and mosquito nets on the premises are measures that can help to reduce disease transmission. Horses should be separated into small, isolated groups, and caution should be taken to prevent iatrogenic transmission through the use of contaminated needles. Encapsulated viruses such as EIA virus are easily destroyed with the use of disinfectants. EIA virus does not persist in insects; these are just mechanical vectors (Cheevers and McGuire, 1985).

In EIA-free countries, any outbreaks of this disease can be contained by establishing quarantines, controlling horse movements, investigating the source of infection in the EIA-positive horses and carrying out seroepidemiological surveillance (More *et al.*, 2008a; More *et al.*, 2008b).

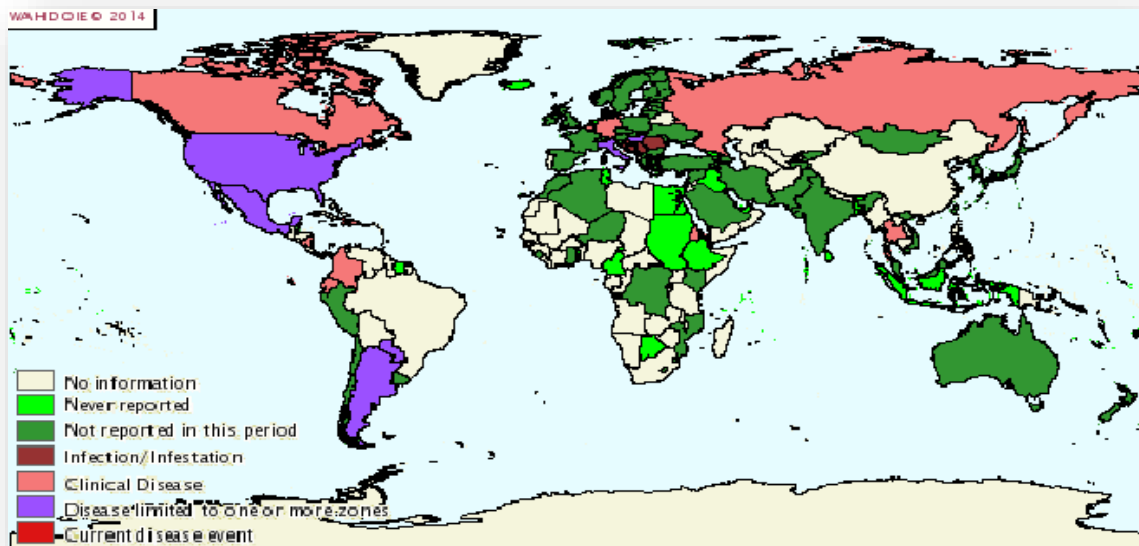
### I.3.1.7 Worldwide distribution and seroprevalence studies

EIA has been reported in North America and South America, Europe, Russia and South Africa at least since 1970 when the technique for the serologic diagnosis was validated (OIE, 2013d).

According to the OIE, since 2005, EIA outbreaks of clinical disease have occurred in countries such as France (2005, 2006, 2007, 2008, 2009, 2010, 2011 and 2012), Germany (2006, 2009, 2010, 2011, 2012 and 2013), Ireland (2006), UK (2006, 2010 and 2012), Uruguay (2007), Greece (2011 and 2012), Belgium (2010, 2012 and 2013), Hungary (2010), Japan (2011) and the Reunion Island (2013) (OIE, 2014b). Even when the situation in Italy and Romania with regards to EIA has not been reported to the OIE, these two countries have had regular EIA outbreaks of clinical as well as subclinical disease since 2006 and the disease is widely spread throughout these countries (Defra).

During the first six months of 2013, the distribution of EIA in the world, according to the OIE, was the following (Figure 3):

Figure 3. Worldwide distribution of EIA in the first six months of 2013 (OIE, 2013a).



This map shows that, during the first six months of 2013, EIA outbreaks of clinical disease occurred in Canada, Colombia, Ecuador, Nicaragua, Belgium, Germany, Russia, Eritrea and Thailand (OIE, 2013a).

In addition to reported outbreaks of EIA clinical disease in many countries of the world, there are numerous studies of EIA seroprevalence in countries of the five continents, since EIA is an OIE listed disease and therefore notifiable and subject to programs of serosurveillance and control in many countries.

In the American continent, EIA seroprevalence studies have been published in countries such as Argentina (Etcheverrigaray *et al.*, 1978), Brazil (Bicout *et al.*, 2006; Borges *et al.*, 2013) and the USA (Issel and Adams, 1979; Loftin *et al.*, 1990). In the African continent there are EIA seroprevalence studies published in Senegal in 1976 (le Jan *et al.*, 1976), Guyana in 1981 (Bamigboye and da Silva, 1981) and Tunisia in 1994 (Boussetta *et al.*, 1994). In Oceania, and more specifically in Australia several studies to detect the presence of EIA carriers were conducted in 1978 in Queensland and Victoria (Harrison, 1978; Thomas and Elder, 1978) and in 1989 in the western part of Australia (Smith *et al.*, 1980). In Asia there is serologic evidence of absence in Turkey and Oman and very limited presence of EIA in India (Albayrak and Ozan, 2010; Body *et al.*, 2011; Malik *et al.*, 2013; Yapklç *et al.*, 2007). Finally, in Europe EIA seroprevalence studies have been published in the Netherlands and Switzerland (de Boer *et al.*, 1979; Kaiser *et al.*, 2009). Table 3 shows a summary of the characteristics and results of the EIA seroprevalence studies published in different countries all around the world.

Table 3. Characteristics and results of the EIA seroprevalence studies published worldwide.

| Study reference                     | Country   | Study period      | Study area    | Number of horses sampled | Serological assay      | Seroprevalence estimate   |
|-------------------------------------|-----------|-------------------|---------------|--------------------------|------------------------|---|
| <i>Etcheverrigaray et al., 1978</i> | Argentina | --                | Buenos Aires  | 870                      | Coggins test           | 21%   |
| <i>Borges et al., 2013</i>          | Brazil    | January-June 2010 | West          | 547                      | Coggins test           | 31.5%   |
| <i>Bicout et al., 2006</i>          | Brazil    | 2002 - 2004       | Southeast     | 8,981                    | Coggins test           | >0 to ≤ 0.5% in 49 cities,<br>>0.5% to ≤1, 5% in 26 cities,<br>>1.5% to ≤5% in 10 cities,<br>>5% to ≤25% in 4 cities. |
| <i>Loftin et al., 1990</i>          | US        | 1984 - 1987       | Southeast     | 891,131                  | Coggins test           | 1% - 2%   |
| <i>Issel and Adams, 1979</i>        | US        | 1975              | Louisiana     | 1,398                    | Coggins test           | 6.7%  |
| <i>Le Jan et al., 1976</i>          | Senegal   | 1971 - 1975       | Whole country | 2,148                    | Coggins test           | 0.5% - 0.9%   |
| <i>Bamigboye and da Silva, 1981</i> | Guyana    | --                | South         | 226                      | Coggins test           | 18.5% – 71.8%   |
| <i>Bousseta et al., 1994</i>        | Tunisia   | --                | North         | 533                      | Coggins test           | 0%  |
| <i>Thomas and Elder, 1978</i>       | Australia | 1973 - 1977       | Queensland    | 1,288                    | Coggins test           | 21.7%   |
| <i>Harrison, 1978</i>               | Australia | 1976              | Victoria      | 1,172                    | Coggins test           | 0.085%  |
| <i>Smith et al., 1980</i>           | Australia | 1975 - 1977       | West          | 630                      | Coggins test           | 0%  |
| <i>Albayrak and Ozan, 2010</i>      | Turkey    | May – August 2009 | Northeast     | 8,769                    | ELISA                  | 0%  |
| <i>Yapklç et al., 2007</i>          | Turkey    | --                | Centre        | 275                      | Coggins test and ELISA | 0%  |
| <i>Body et al., 2011</i>            | Oman      | 2007 - 2009       | Whole country | 593                      | Coggins test           | 0%  |

Table 3 (continuation). Characteristics and results of the EIA seroprevalence studies published worldwide.

| Study reference             | Country     | Study period                   | Study area    | Number of horses sampled   | Serological assay | Seroprevalence estimate   |
|-----------------------------|-------------|--------------------------------|---------------|--|-------------------|---|
| <i>Malik et al., 2013</i>   | India       | 1999-2012                      | Whole country | 67,042   | Coggins test      | 0.003%  |
| <i>De Boer et al., 1979</i> | Netherlands | 1963 - 1966 and<br>1972 - 1979 | Whole country | 3032 resident horses<br>+ 340 horses imported<br>from Eastern Europe | Coggins test      | 0.07% in resident horses<br>4.41% in horses imported<br>from Eastern Europe |
| <i>Kaiser et al., 2009</i>  | Switzerland | 2003 - 2008                    | Whole country | 666  | ELISA             | 0%  |

### I.3.1.8 Equine Infectious Anaemia in Spain

EIA was diagnosed for the first time in 1976, when an outbreak of EIA started in Madrid, with further cases of EIA clinical disease diagnosed in 1978 in two racecourses in Madrid and San Sebastian (Galaz, 1978; OIE, 2014a). Both racecourses were quarantined and nearly 900 Thoroughbred racehorses were considered at risk until 1983, when the last outbreak was declared resolved (Rodríguez, M., personal communication). Spain has been officially free from EIA since then, although strict importation control measures were never implemented. There are no data regarding the seroprevalence of EIA in the Spanish equine population.

### I.3.1.9 Economic importance and association with reproductive pathologies in a stud farm.

Even though the main way of transmission of EIA virus takes place through insect vectors, the virus is also transmitted vertically in pregnant mares (Kemen and Coggins, 1972; Stein and Mott, 1942) and, although much less efficiently, through the semen during the covering of a mare; the presence of EIA virus has been determined in the semen from an infected stallion (Metcalf, 2001) and the transmission of the virus in a mare with a vaginal laceration during the covering by an infected stallion has been confirmed (Stein *et al.*, 1944).

Pregnant mares that show signs of acute EIA are those having the highest risk of infecting the foetus via placenta. Infected foetuses are usually aborted, and when born alive they often die in the first two months (Kemen and Coggins, 1972).

These transmission routes, along with the main through insect vectors, make both the clinical and the economic consequences of EIA in a breeding stud farm more critical than in any other type of equine farm. The consequences of a possible outbreak of EIA in a stud farm include economic losses of great magnitude, mainly

due to mandatory euthanasia of affected horses in many countries, the total loss of economic value in the EIA carriers in countries where EIA-positive horses can be kept alive, the possible abortions caused in infected mares, the restrictions of movement that are established in the affected stud farm, the cost of implementing vector control measures, and other costs related to measures for the diagnosis and control in non-affected horses (OIE, 2013d).

## I.3.2 Equine Viral Arteritis (EVA)

### I.3.2.1 Aetiology

EVA is a contagious disease of equidae, named after the characteristic inflammatory lesions induced by the causal agent in the small arterioles in an acute infection (Bryans *et al.*, 1957a; Bryans *et al.*, 1957b). The disease is caused by a ribonucleic acid (RNA) virus, the equine arteritis virus (EAV), which belongs to the Arterivirus genus, Arteriviridae family, order Nidovirales (Bryans *et al.*, 1957a; Cavanagh, 1997; Snijder and Meulenberg, 1998). Another virus belonging to the same family and of great importance is the virus of the porcine respiratory and reproductive syndrome (PRRS) (Plagemann and Moennig, 1992).

### I.3.2.2 Epidemiology

Exposure to the EAV can occur via respiratory or venereal routes, and can result in clinical or inapparent infections, depending on the viral strain involved, the dose of the virus, the age and physical condition of the infected animal, and several environmental factors (Timoney and McCollum, 1993b). Morbidity rates differ between outbreaks, with rates being higher in large concentrations of horses, for example in racetracks (McCollum and Swerczek, 1978). In clinical infections, the appearance of clinical signs is preceded by an incubation period of 3 to 14 days, although it varies according to the route of infection (shorter in the case of a respiratory infection, longer if the transmission takes place via the venereal route)(Timoney, 2003).

EAV transmission can also occur vertically in utero, resulting in either abortion or the birth of an infected clinically affected foal (Bryans *et al.*, 1957a; Golnik, 1992; Vaala *et al.*, 1992). In these cases, the fluids, placenta and the foetus are important sources of virus (Timoney, 2003).

Finally, less frequently transmission can occur via fomites such as material used for handling the affected horses, the equipment used in AI, etc., contaminated with infective secretions or excretions (Guthrie *et al.*, 2003; Timoney and McCollum, 1993b).

The EAV is not often isolated from affected horses after 28 days post-infection, except from semen in stallions that are carriers (Holyoak *et al.*, 2008). The carrier status has only been identified in the stallion, and in some cases after experimental infection in pre-pubertal colts (Holyoak *et al.*, 1993a; Holyoak *et al.*, 1993b). The carrier status occurs in approximately 10-70% of the infected stallions after the resolution of the clinical signs (Timoney and McCollum, 2000). In the mature stallion the virus can persist for weeks, months, years or even for life in the accessory sex glands, being eliminated with the semen in 85-100% of services, either by natural mating or AI. The EAV can remain infective in frozen semen for years (Timoney, 2000).

### I.3.2.3 Pathogenesis and clinical signs

The most typical clinical signs of EVA are fever, anorexia, depression, leukopenia, the presence of oedema (supraorbital and in the limbs but also in the scrotum, foreskin or mammary glands), conjunctivitis, urticaria, abortion in the mare and fatal interstitial pneumonia in young foals (del Piero *et al.*, 1997; Golnik *et al.*, 1981).

Abortion caused by the EAV in pregnant mares is particularly important due to its economic consequences in a stud farm. Abortion may occur in the late acute phase or the early convalescent phase of the infection, and it may or may not be preceded by clinical signs of EVA in the infected mare (Bryans *et al.*, 1957a; Golnik, 1992; McCollum and Timoney, 1984; Timoney, 1999; Timoney and McCollum, 1993b). Abortions have been reported to occur from the third month until the tenth month of gestation after a natural or experimental infection with EAV (Coignoul and Cheville, 1984; Timoney and McCollum, 1993b; Vaala *et al.*, 1992). Abortion rates

published in EVA outbreaks vary between less than 10% to more than 50-60% (Timoney and McCollum, 1993b). Exposure to the virus in mares during late gestation could lead to vertical transmission and the birth of a foal that is infected with the virus. These foals present with progressive interstitial pneumonia, and they usually die in the first 3-4 days of life (Golnik, 1992; Vaala *et al.*, 1992).

Stallions infected with the EAV are asymptomatic carriers of the virus, although they may show subfertility for a short period that may persist from 16 to 17 weeks after infection (Neu *et al.*, 1992).

#### I.3.2.4 Diagnosis

There are several techniques that can be used for the diagnosis of EVA and each one presents advantages in a particular situation or phase of the disease.

##### *Clinical diagnosis*

In the clinical diagnosis of EVA, several equine infectious and non-infectious respiratory and systemic diseases may be confused due to the similarity of their clinical signs with EVA (Timoney and McCollum, 1993b). Among the most significant diseases to be included in the differential diagnosis of EVA are the infection caused by EHV-1/-4, equine influenza (EI), purpura haemorrhagica, EIA, urticaria and toxicosis by hoary alyssum (*Bertero aincana*). Additional exotic diseases, which may be differential diagnoses for EVA in other countries, include African horse sickness, dourine, and Getah virus infection (Timoney, 2003).

Both abortion and neonatal death caused by EVA are extremely similar to those caused by EHV-1 and less frequently, EHV-4; therefore EHV-1/-4 should always be on top of the differentials when dealing with a possible EVA outbreak of abortions in a stud farm (Timoney, 2003).

## *Diagnosis by serological techniques*

Serological tests are used in different situations in the diagnosis of EVA: firstly, they can be used in the diagnosis of an acute infection, in this case a specific antibody seroconversion in paired sera (acute and convalescent) obtained at an interval of 2 to 4 weeks (Timoney and McCollum, 1993b). Secondly, serology can be used to rule out the possible carrier state in a stallion; it is important to establish if the stallion is positive or negative for antibodies against the EAV (Timoney *et al.*, 1987a). Since the carrier status has never been demonstrated in a seronegative stallion, only stallions with titres of 1:4 or higher to the virus and no history of vaccination against EVA, should be considered potential carriers (Timoney and McCollum, 2000). Finally, the serological techniques are very useful for seroprevalence studies and serosurveillance surveys.

Among the validated serological techniques that are most commonly used for the diagnosis of EVA are the seroneutralization (SN) (Senne *et al.*, 1985), complement fixation (CF) (Fukunaga and McCollum, 1977), indirect immunofluorescence (Crawford and Henson, 1972), AGID (Crawford and Henson, 1972), ELISA tests (Cho *et al.*, 2000; Hedges *et al.*, 1998; Kondo *et al.*, 1998; Nugent *et al.*, 2000) and the microsphere immunoassay (MIA)(Go *et al.*, 2008).

The SN or microneutralization test is currently the reference technique according to the OIE and the most widely used worldwide to diagnose EVA cases, carry out seroprevalence studies and perform screening before any horse movements (OIE, 2013d). Neutralizing antibodies against the EAV persist for many years after natural infection or vaccination with an EVA modified live vaccine (Timoney and McCollum, 1993b). The sensitivity of the SN test for the detection of antibodies against EAV is influenced by many factors, especially the source and the number of culture passes of the strain of virus used in the technique (Edwards *et al.*, 1998).

The CF test has been used in the past to diagnose recent EVA infections, since antibodies that fix the complement have a relatively short average duration equine plasma following infection (Fukunaga and McCollum, 1977). This test has been replaced by the SN test and the different commercially available ELISAs.

Recently a technique of immunoassay based on fluorescent microspheres used to detect antibodies against the major structural proteins of the EAV has been developed (Go *et al.*, 2008).

Among the serological techniques, several ELISA tests with an excellent sensitivity and a similar specificity to the SN test have been developed (Cho *et al.*, 2000; Hedges *et al.*, 1998; Kondo *et al.*, 1998; Nugent *et al.*, 2000). These techniques are based on the use of viral antigens from a purified or recombinant virus. The discovery of the importance of viral GP5 protein in the stimulation of the humoral immune response against the EAV led to the development of several ELISA tests that use a part or the whole of this protein (Cho *et al.*, 2000; Hedges *et al.*, 1998). More recently, a synthetic peptide conjugated with ovalbumin and representing amino acids 81-106 of GP5 protein has been used (Nugent *et al.*, 2000).

Some of these ELISA tests offer similar levels of sensitivity and specificity to the SN technique, and can detect antibodies to the EAV before the SN. Among all the ELISA tests validated for the diagnosis of EVA, two have been described more recently and appear to be very promising (Cho *et al.*, 2000; Nugent *et al.*, 2000). The blocking ELISA validated by Cho *et al.* in 2000 uses monoclonal antibodies against the GP5 protein and has a sensitivity of 99.4% and a specificity of 97.7% with respect to the SN (Cho *et al.*, 2000), while the ELISA validated by Nugent *et al.* in 2000 against a panel of 400 SN positive and 400 SN negative sera uses a synthetic peptide conjugated with ovalbumin and has a sensitivity and specificity of 96.75% and 95.6% respectively (Nugent *et al.*, 2000).

Few of the ELISAs that have been developed have been validated as extensively as the SN test, although some of them have been reported to have comparable sensitivity and specificity to the SN (Cho *et al.*, 2000; Hedges *et al.*, 1998; Nugent *et al.*, 2000). There are also differences in terms of the type of antibodies that detect both techniques; whereas SN detects neutralizing antibodies, thus being a reflection of the immune protection status of the horse, the ELISAs detect both neutralizing and non-neutralizing antibodies (OIE, 2013d).

ELISA tests have the advantage of not being affected by cytotoxicity in serum, and therefore they do not have the delay and difficulties in the interpretation of

results that can pose a significant problem in a SN test (Newton *et al.*, 2004). Due to this and other advantages ELISA tests are increasingly being used around the world for the diagnosis of EVA. Currently there are two commercial ELISA kits for the diagnosis of EVA in Spain, Ingezim Arteritis ELISA® (Ingenasa) (Duthie *et al.*, 2008) and Id Screen® Equine Viral Arteritis Indirect (Id.Vet) (Legrand *et al.*, 2007). Although they both have an excellent sensitivity, in one case (Id Screen® Equine Viral Arteritis Indirect) the specificity is higher and it also presents benefits associated to results being obtained faster (Legrand *et al.*, 2007).

### *Diagnosis by antigen detection molecular techniques*

Diagnosis of EVA by techniques that detect viral antigen or nucleic acid is most useful when an acute infection is suspected. The most suitable samples for diagnosis include nasopharyngeal and conjunctival swabs and also peripheral blood from the affected horse. In case of an EVA suspected abortion, the viral antigen can be detected in placental tissue and fluids, and also in fetal lung, liver and lymphoreticular tissue (del Piero, 2000a; Higgins, 1993). Finally, these techniques are useful in order to confirm the carrier state in a stallion, once it has been established that the stallion is positive to antibodies against EVA by serology; the most appropriate sample for this is semen containing sperm from the ejaculate (Balasuriya *et al.*, 2002; Bryans *et al.*, 1957a; McCollum and Swerczek, 1978).

Several RT-PCR tests, including nested RT-PCR (RT-nPCR) and real-time RT-PCR techniques have been validated. The supernatants of cell cultures used for EVA virus isolation, as well as clinical samples can be used for these techniques (Balasuriya *et al.*, 2002; Chirnside and Spaan, 1990; Ramina *et al.*, 1999; Sekiguchi *et al.*, 1995; St-Laurent *et al.*, 1994; Westcott *et al.*, 2003). RT-PCR techniques have many advantages over viral isolation techniques; however, a well-planned validation has not yet been performed for their use in the diagnosis of abortions and confirmation of carrier stallions (Go *et al.*, 2008; Lu *et al.*, 2008).

EVA antigen can also be detected in tissues and skin biopsies using the immunohistochemistry, a reliable, effective and fast technique (del Piero, 2000b).

Staining with an avidin-biotin complex (ABC) immunoperoxidase has been used for the detection of the viral antigen of the EAV in formalin-fixed tissues, paraffin-embedded tissues and frozen tissues sections (del Piero, 2000a, b; Lopez *et al.*, 1996; MacLachlan *et al.*, 1996).

### *Diagnosis by whole virus isolation techniques*

As with antigen detection by RT-PCR, viral isolation techniques are used when an acute infection is suspected (cases of abortions, neonatal deaths, acute respiratory infections, confirmation of carrier stallions, etc.). The isolation of the EAV is carried out in a limited number of cell lines, of which the RK-13 (rabbit kidney) cell line is one of the most useful (OIE, 2013d). The samples used for viral isolation are similar to those used in RT-PCR techniques (Balasuriya *et al.*, 2002; Bryans *et al.*, 1957a; del Piero, 2000a; Higgins, 1993; McCollum and Swerczek, 1978). Both types of techniques are complementary, have the same sensitivity and are often used together, although the results by RT-PCR techniques are obtained much faster (OIE, 2013d).

### **I.3.2.5 Treatment**

In the absence of a specific anti-viral drug against EVA, the treatment of EVA cases is symptomatic, with an emphasis on controlling the fever and the oedema, especially in the affected stallions (Glaser *et al.*, 1997; Timoney and McCollum, 1993b). Breeding stallions and horses in training should be provided with adequate rest in order to minimize the adverse effects of the disease on performance. In foals with neonatal disease, the prophylactic administration of antibiotics is indicated to control secondary bacterial infections and always considering the risk of lateral spread of the virus to other foals or horses in close proximity to the affected animal. Safe and effective therapeutic measures to eliminate the carrier status in the stallion

have not been developed yet and castration is recommended when a stallion is found to be a carrier of the EAV (Timoney, 2003).

### I.3.2.6 Prevention and control

The methods for the prevention and control of this disease in the equine population include on the one hand an effective diagnosis of EVA in clinical outbreaks of the disease, and on the other hand the identification of carriers (Timoney and McCollum, 1993b; Timoney *et al.*, 1987b).

The carrier stallions should be handled separately in order to ensure that there is no risk of unnoticed spread of the virus, especially to the pregnant mares. Under appropriate management conditions, carriers can continue being used for breeding, although it is recommended that they are only used for covering EVA seropositive mares (Timoney, 2003).

There is currently a significant risk of introducing EVA in the equine breeding population through the use of fresh or frozen infective semen (Balasuriya *et al.*, 1998; Timoney, 2000; Timoney *et al.*, 1987b). Therefore, it is important to determine the presence of the EAV in the semen used for AI, especially when it has been imported from abroad. When working with EVA-positive semen, appropriate precautions when inseminating mares should be followed to avoid the risk of spreading the virus to susceptible horses (Timoney, 2003).

Finally, safe and effective vaccines against EVA have been developed (Doll *et al.*, 1968; McCollum, 1970; McCollum, 1986; Timoney and McCollum, 1993b) and currently there are two commercially available EVA vaccines, one produced with a modified live virus (Arvac<sup>®</sup>, Fort Dodge Animal Health), licensed for use in the US, and one with an inactivated virus and adjuvants (Artervac<sup>®</sup>, Fort Dodge Animal Health), licensed for use in the UK, France, Ireland and Germany, and with a special import permit use in Denmark (Doll *et al.*, 1968; McCollum, 1970; McCollum, 1986; Timoney and McCollum, 1993b). The modified live virus vaccine is safe and

immunogenic in stallions, non-pregnant mares and young horses. The inactivated vaccine, although safe for the use in pregnant mares, is not as immunogenic as the modified live virus vaccine. While vaccination is part of EVA control programmes in some countries, there are no available vaccines in Spain and EVA vaccination is not currently permitted.

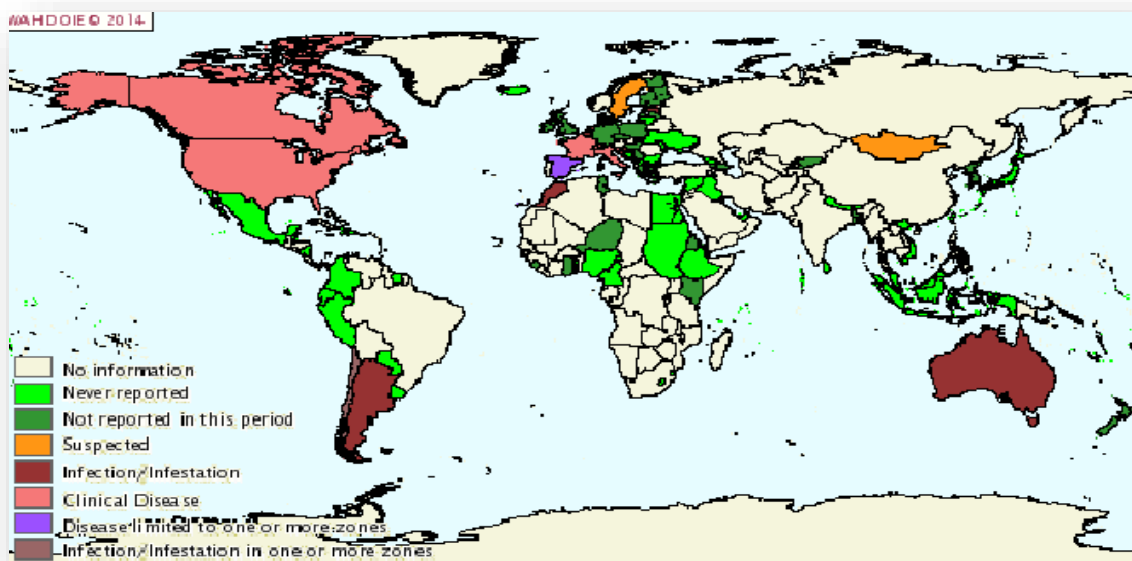
### I.3.2.7 Worldwide distribution and seroprevalence studies

EVA is present in North America, South America, Europe, Africa, Asia and Australia (Huntington *et al.*, 1990a; Timoney and McCollum, 1988, 1991). Recently, as of May 2014, New Zealand was declared to be free from EAV infection (Herrera, 2014).

According to the OIE, since 2005 EVA outbreaks have occurred in the UK (2005, 2010, 2011, 2012), Israel (2008), Croatia (2008), and Argentina (2010) (OIE, 2014b).

During the first six months of 2013, the distribution of EVA in the world, according to the OIE, was the following:

Figure 4 Worldwide distribution of EVA in the first six months of 2013 (OIE, 2013c).



This map shows that, during the first half of 2013, there were outbreaks of clinical disease in Canada, United States of America, France, Slovenia and Switzerland (OIE, 2013c). In the map Spain appears in purple colour, which indicates that there was clinical disease limited to one or more areas. As of April 2013 the International Collating Centre (ICC) published a report of an outbreak of abortions produced by EVA in a breeding stud farm located in the South of Castilla y León autonomous community (ICC, 2013). This report may have been the trigger for the EVA situation of Spain in this map during the first half of 2013.

Further to the confirmation of clinical EVA in outbreaks in many countries, seroprevalence studies have been carried out worldwide. In the American continent there is only one study published in 2001 in the USA (Hullinger *et al.*, 2001). In Oceania two studies of EVA seroprevalence were carried out in New Zealand and Australia (Huntington *et al.*, 1990b; McFadden *et al.*, 2013). In the African continent, there are only two references regarding EVA seroprevalence, a study carried out in the North-East of Tunisia (Ghram *et al.*, 1994) and a study conducted in France where horses residing in several African countries were analysed (Morailon and Morailon, 1978). Finally, Europe is the continent where most of the studies of EVA seroprevalence have been published; there are studies published in Poland (Rola *et al.*, 2011), UK (Newton *et al.*, 1999), Germany (Herbst *et al.*, 1992), the Netherlands (de Boer *et al.*, 1979), Austria (Kölbl *et al.*, 1991) and France (Morailon and Morailon, 1978). Table 4 shows a summary of the characteristics and results of the EVA seroprevalence studies published in different countries all around the world. Most studies were carried out with a representative, random sample of horses; however some of them, such as the one published in the UK in 1999 or the one published in Germany in 1992 were dependent on ad hoc laboratory submissions and a few actively solicited samples (Herbst *et al.*, 1992; Newton *et al.*, 1999).

Table 4. Characteristics and results of the EVA seroprevalence studies published worldwide.

| Study reference                  | Country                   | Study period                | Study area                  | Number of horses sampled   | Serological assay | Seroprevalence estimate  |
|----------------------------------|---------------------------|-----------------------------|-----------------------------|--|-------------------|--|
| Hullinger <i>et al.</i> , 2001   | USA                       | --                          | California                  | 364 horses residing in California and 226 imported   | SN                | 1.9% (resident horses)<br>18.6% (imported horses)  |
| McFadden <i>et al.</i> , 2013    | New Zealand               | 2001 - 2011                 | Whole country               | 7,157  | SN                | 0%   |
| Huntington <i>et al.</i> , 1990b | Australia                 | 1975 - 1989                 | Whole country               | 58 Thoroughbred and 57 Standardbred stallions, 914 Thoroughbred and 481 Standardbred mares | SN                | 73% Standardbred stallions<br>71% Standardbred mares<br>8% Thoroughbred stallions<br>1% Thoroughbred mares |
| Ghram <i>et al.</i> , 1994       | Tunisia                   | --                          | North-East                  | 400  | SN                | 8.75%  |
| Moraillon and Moraillon, 1978    | Several African countries | 1966 - 1974                 | --                          | 468  | SN                | 37.1%  |
| Rola <i>et al.</i> , 2011        | Poland                    | 2006 - 2008                 | --                          | 176 Hucul horses   | SN                | 55.1% (45% of stallions virus-positive in semen)   |
| Newton <i>et al.</i> , 1999      | UK                        | 1995 - 1996                 | Whole country               | 5,478 Standardbred and 12,576 Thoroughbred horses  | SN                | 18.5 – 24% Standardbred horses<br>0-3% Thoroughbred horses   |
| Herbst <i>et al.</i> , 1992      | Germany                   | 1986 - 1987                 | Whole country               | 1905   | SN                | 8.7%   |
| De Boer <i>et al.</i> , 1979     | Netherlands               | 1963 - 1966 and 1972 - 1979 | Whole country               | 283  | SN                | 14%  |
| Kölbl <i>et al.</i> , 1991       | Austria                   | 1988 - 1989                 | Saltzburg and Lower Austria | 944  | SN                | 10.9%  |
| Moraillon and Moraillon, 1978    | France                    | 1966 - 1974                 | Whole country               | 3,324  | SN                | 15.2%  |

### I.3.2.8 Equine Viral Arteritis in Spain

In Spain, serological evidence indicates the presence of EVA since 1978 (Morailon and Morailon, 1978), and in 1992 in Barcelona the first confirmed outbreak of EVA clinical disease was reported (Monreal *et al.*, 1995).

According to the OIE there were serological and/or isolation findings of EVA in Spain in the years 2000, 2001 and 2002. Eleven years later, in April 2013 the ICC published a report regarding an outbreak of abortions produced by the EAV in a breeding stud farm located in the South of Castilla y León autonomous community (ICC, 2013).

The majority of EAV infections in Spain are sporadic cases, which are not usually reported (MAPA, 2003). Despite the surveillance plan published in the APA/212/2003 order, which includes the communication and control of diseases such as EVA and the establishment of an EVA surveillance programme (BOE, 2003), to date there are no studies that have identified or quantified the presence of this disease in Spain and there has been only a preliminary, small study carried out in the autonomous community of Valencia (East of Spain) which determined antibody against EVA by SN in a population of 49 SP horses in 2010-2011. The seroprevalence for EVA was 12.24% in this preliminary study (Gimeno Suarep *et al.*, 2011b). Since then, there have been no further studies to evaluate and quantify the presence of EVA in Spain.

### I.3.2.9 Economic importance and association with reproductive pathologies in a breeding stud farm

EVA can have very significant economic effects for the equine breeding (Timoney and McCollum, 1993a; Timoney and McCollum, 1993b).

There are devastating direct and indirect consequences for the economy of a stud farm when an EVA outbreak occurs. The direct consequences of infection by EAV are related to its association with reproductive pathology in the mares (abortions), death or disease in the newborn foals, and a possible transient decrease in the fertility of the infected stallion. The indirect consequences of an EVA outbreak are related to the decrease in the market value and decreased demand for carrier stallions, the difficulty or impossibility of exporting carrier stallions, EVA positive semen and, in some countries, any EVA seropositive horse; and finally the costs related to implementation of control measures during an outbreak (i.e. the interruption of the training in the stallions, quarantines, restrictions of movement, screening tests in all the horses of the stud farm, etc. (Timoney, 2003).

### I.3.3 Equine Herpesvirus-1 and/or -4 (EHV-1/-4)

#### I.3.3.1 Aetiology

EHV-1 and EHV-4, both members of the *Herpesviridae* family, produce a set of different diseases in horses, including respiratory disease, abortion, neonatal pneumonia, or myeloencephalopathy (Allen and Bryans, 1986; Allen, 1999; Bryans, 1988; Crabb and Studdert, 1995). These diseases represent a challenge to the international equine industry.

Until 1981, EHV-1 and EHV-4 were considered as two subtypes of the same virus, called EHV-1. Even though Sabine *et al.* and Studdert *et al.* showed the differentiation of the two viruses through their viral genomic footprint (Sabine *et al.*, 1981; Studdert *et al.*, 1981), the official recognition of distinction as two different viruses came a decade later, in 1992 (Roizmann *et al.*, 1992).

#### I.3.3.2 Epidemiology

Herpesviruses are enzootic in the majority of the equine population. Both EHV-1 and EHV-4 can be highly contagious and can spread rapidly among animals. In an infected animal, the risk of transmitting the disease during the first 10 days is very high and decreases significantly after 28 days (Allen, 1999). Transmission occurs via the respiratory route, by direct or indirect contact with conjunctival and nasal secretions from infected horses. In stud farms with outbreaks of abortions or affected foals, transmission in mares usually occurs through contact with the placentas and fetal membranes, or the secretions and excretions of infected foals (Patel and Heldens, 2005).

A very important characteristic of these viruses is that they can remain latent. Around the 80% of primary infections by EHV-1 and EHV-4 are followed by a state of latent viral infection in the trigeminal ganglion of the horse; in this latency state

horses are healthy and do not display any clinical signs (Borchers *et al.*, 1999). In situations of immunosuppression or stress, viral reactivation can occur, leading to a release of viral particles through the nasal secretions and the transmission of the virus to other susceptible healthy horses (Allen, 2002). This reactivation is very likely to happen also in pregnant mares, resulting in an abortion and consequently a possible transmission to other individuals through the foetus and foetal membranes (Patel and Heldens, 2005). This mechanism of latency is essential for the survival and transmission of EHV-1 and EHV-4 in the population, since the horses infected with a latent virus are important reservoirs of the disease (Allen, 2002).

### I.3.3.3 Pathogenesis and clinical signs

EHV-1 was first isolated from tissues of aborted foetuses in the USA in 1933 (Dimock *et al.*, 1947); but retrospective studies identified the virus as a cause of abortion in 1921 in Australia (Gilkerson *et al.*, 1998). The infection by EHV-1 can progress through the respiratory mucosae, produce a cell-associated viraemia and be dispersed to other systems, including the uterus resulting in abortion in the last trimester of gestation (Crabb and Studdert, 1995). Foetuses infected with EHV-1 during late pregnancy may be born alive, but they become ill one to two days after birth. These foals present with weakness, lethargy, fever, lymphopenia, hypoxia and severe respiratory problems, with a rapid clinical deterioration and a grave prognosis. In EHV-1 outbreaks, the infection can lead to the presentation of myeloencephalopathy, which can vary from ataxia accompanied by bladder paralysis and retention of faeces to paraplegia or quadriplegia with subsequent death (or more commonly slaughter on humane grounds) (Stierstorfer *et al.*, 2002; van Maanen, 2001).

Infection with EHV-4 has been associated with upper respiratory tract disease; it rarely produces a cell-associated viraemia and in rare occasions it can cause abortion or myeloencephalopathy, although understanding of the role of the

virus in the latter is limited (Allen, 2002; Meyer *et al.*, 1987; Verheyen, 1988). Respiratory disease occurs predominantly in horses between weaning and 2-3 years of age. It is characterized by clinical signs such as fever, lethargy, anorexia, submandibular lymphadenopathy and profuse nasal discharge, which can become mucopurulent as a result of a secondary bacterial infection. The virus can progress to the lungs causing a bronchopneumonia which can be exacerbated by secondary bacterial infections, and with signs of lower respiratory tract disease including cough, abnormal sounds at auscultation and increased inspiratory effort (Allen, 2002; Gilkerson *et al.*, 1994).

#### I.3.3.4 Diagnosis

EHV-1/-4 cause a very contagious disease, with a great potential to explosive outbreaks with high mortality due to abortions or the sequelae of neurological cases. For this reasons, fast and effective methods are needed for the diagnosis of this disease.

##### *Clinical diagnosis*

As described for EVA diagnosis, in the clinical diagnosis of EHV-1/-4, several equine respiratory and systemic diseases may be confused given the similarity of their clinical signs with those produced by EHV-1/-4 (Patel and Heldens, 2005). Among the most significant diseases to be included in the differential diagnosis would be EVA, EI, leptospirosis, diseases caused by other herpesviruses such as equine coital exanthema, infections caused by *Brucella abortus*, *Streptococcus pyogenes equi*, and finally fungal infections such as those produced by fungi of the *Mucor* genus and *Aspergillus* genus (FAO, 2014).

## *Diagnosis by serological techniques*

Serology remains the most popular tool for the diagnosis of EHV-1/-4, even though its utility in some situations, such as abortions, is highly reduced due to the fact that antibody titres to these viruses are frequently already elevated at the time of the abortion (Allen, 1999). It is mostly used in epidemiological investigations of EHV-1/-4 or in the serosurveillance of equine populations (Browning *et al.*, 1988a; Browning *et al.*, 1988b; Gilkerson *et al.*, 1999b; Singh, 1999; Yasunaga *et al.*, 1998).

Diagnosis of EHV-1/-4 by serology relies on the demonstration of a significant increase in the antibody titres (more than four times) in paired samples collected during the acute and convalescent phase of the disease. Serum antibodies against EHV-1 and EHV-4 can be detected by ELISA (Dutta *et al.*, 1983), SN (Thomson *et al.*, 1976), or CF (Thomson *et al.*, 1976).

In 1995 Crabb *et al.* validated a specific ELISA for the diagnosis of EHV-1/-4 that discriminated EHV-1 from EHV-4, and had a sensitivity of 100% and a specificity of 96% (Crabb *et al.*, 1995). More recently, Hartley *et al.* compared different techniques for detecting antibodies against EHV-1/-4 in experimentally and naturally infected foals (Hartley *et al.*, 2005). In this study SN, CF and ELISA were used; whereas the ELISA could clearly identify the horses infected with EHV-1 and those infected with EHV-4, both the SN and CF showed cross-reactivity between both viruses (Hartley *et al.*, 2005).

Even though SN is a very sensitive technique and it is used worldwide, CF and ELISA tests have the advantage of providing results more rapidly than SN and they do not require facilities for cell culture (OIE, 2013d). The lack of standardised reagents or techniques for serological tests in the detection of antibodies against EHV-1/-4 often results in variations in antibody titres in the same sample between different laboratories.

## *Diagnosis by antigen detection molecular techniques*

EHV-1 and EHV-4 nucleic acid or antigen detection techniques are most frequently used when an acute infection is suspected.

Detection of viral antigen by direct immunofluorescence in tissues from aborted fetuses is essential for the diagnosis of abortion caused by EHV-1 (Gunn, 1992). This technique has been compared to viral isolation using cell cultures in more than 100 cases of equine abortion showing that both techniques are similar in terms of diagnostic reliability (OIE, 2013d).

Immunohistochemistry techniques, such as the staining with immunoperoxidase have been validated and are useful in the detection of the EHV-1 antigen in paraffin embedded tissues from aborted fetuses or horses affected by the neurological form of the disease (Schultheiss *et al.*, 1993; Whitwell *et al.*, 1992). Immunoenzymatic staining is particularly useful for the simultaneous evaluation of the morphologic lesions and the identification of the infectious agent when EHV-1 is suspected. This technique can also be used to diagnose EHV-1 or EHV-4 in a monolayer of infected cells (van Maanen *et al.*, 2000).

Several PCRs with primers specific for EHV-1 and EHV-4 have been developed for the detection of virus in clinical samples, paraffin embedded tissues, or cell cultures inoculated with the virus (Borchers and Slater, 1993; Lawrence *et al.*, 1994; O'Keefe *et al.*, 1994; Tekelioglu, 2005; Varrasso *et al.*, 2001; Wagner *et al.*, 1992; Wang, 2007); There is a multiplex PCR that can simultaneously detect and discriminate EHV-1 and EHV-4 (Ataseven *et al.*, 2009). There is a high correlation between PCR techniques and viral isolation in the diagnosis of EHV-1 and EHV-4 (Varrasso *et al.*, 2001) The diagnosis of EHV-1/-4 by PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the sample, thus it has been included in the range of routine diagnostic tests for EHV-1 and EHV-4 (OIE, 2013d). The most appropriate samples for the diagnosis of an acute infection by PCR are samples of aborted fetuses, nasopharyngeal swabs, or peripheral blood from affected horses. The diagnosis by PCR may also be done in samples from the spinal cord, cerebrospinal fluid (CSF) and brain of horses with neurological signs (OIE, 2013d).

## *Diagnosis by whole virus detection techniques*

Viral detection techniques are used in the same scenarios where antigen detection by PCR techniques are used (i.e. when an acute infection - neonatal deaths, abortions, neurological form of the disease, acute respiratory infections – is suspected). For the isolation of EHV-1 and EHV-4 from nasopharyngeal swabs, primary equine foetal kidney cells or cells derived from equine dermal or pulmonary tissue fibroblasts are most used, although EHV-1 can also be isolated by using a range of non-equine cell lines (Barrandeguy, 1999; Taouji *et al.*, 2002). Blood leukocyte cultures can be used to isolate EHV-1 in the early stages of myeloencephalopathy (OIE, 2013d). The basis for the identification of any viruses isolated from clinical samples is the immunoreactivity with specific antisera. Immunofluorescence techniques are used for the detection of viral antigen in infected cell cultures by using specific monoclonal antibodies. This technique is effective and very specific; in laboratories where this technique cannot be carried out, isolates can be identified using the SN test or a PCR (OIE, 2013d).

### **I.3.3.5 Treatment**

Currently there is no specific treatment for EHV-1/-4. In some cases (e.g. in neonatal infections by EHV-1, early stages of an outbreak of abortions or myeloencephalopathy), antiviral drugs can be administered (Murray *et al.*, 1998). In most of the cases, treatment is provided in order to alleviate clinical signs, maintain hydration and minimize complications such as secondary bacterial infections (Allen, 2002). In certain cases of EHV-1 myeloencephalopathy, horses may require catheterization of the bladder and antibiotic treatment. The most severe cases of myeloencephalopathy with recumbency require intensive support measures, as well as lifting the horse with a crane in order to prevent decubitus ulcers; in these cases, several days without an improvement are often followed by euthanasia of the horse (Allen, 2002).

### I.3.3.6 Prevention and control

The elimination of EHV-1/-4 from an equine population is virtually impossible due to the state of latency of these viruses. The most effective method to control EHV-1/-4 relies on prevention through vaccination and the implementation of preventive management practices on the premises (OIE, 2013d).

Vaccination with inactivated or live-attenuated vaccines against EHV-1/-4 is an important part of any programme of prevention and control of infectious diseases in a stud farm. Foals usually have maternal antibodies against EHV-1/-4 up to 5-6 months of age; therefore primary vaccination should be administered just before weaning. In premises with a large number of horses, vaccination of the entire herd can help reduce the spread of infection due to the benefits of herd immunity. Pregnant mares should be vaccinated according to vaccine manufacturers' instructions, thus reducing the risk of abortion storms caused by EHV-1/-4. No vaccine to date has shown protection against myeloencephalopathy caused by EHV-1, and vaccinating does not prevent from the state of latency (Allen, 2002).

Preventive management strategies include segregating horses in small groups in the stud farm, isolating these groups and reducing stress (HBLB, 2013). The highest risk of infection by EHV-1/-4 lies in the introduction of new horses to established groups, especially new horses coming from competitions, auctions, etc. Any newcomers should be isolated for at least 21 days before they are introduced in the herd (HBLB, 2013).

When an outbreak of EHV-1/-4 occurs, the measures already mentioned are essential to prevent the spread of the infection and the economic losses. Infected horses, as well as those in contact with affected horses should be isolated. The movement of horses to and from the stud farm should be restricted until 21 days after the last case of rhinopneumonitis. Finally, after the outbreak has been resolved, all facilities within the premises should be cleaned and disinfected (HBLB, 2013).

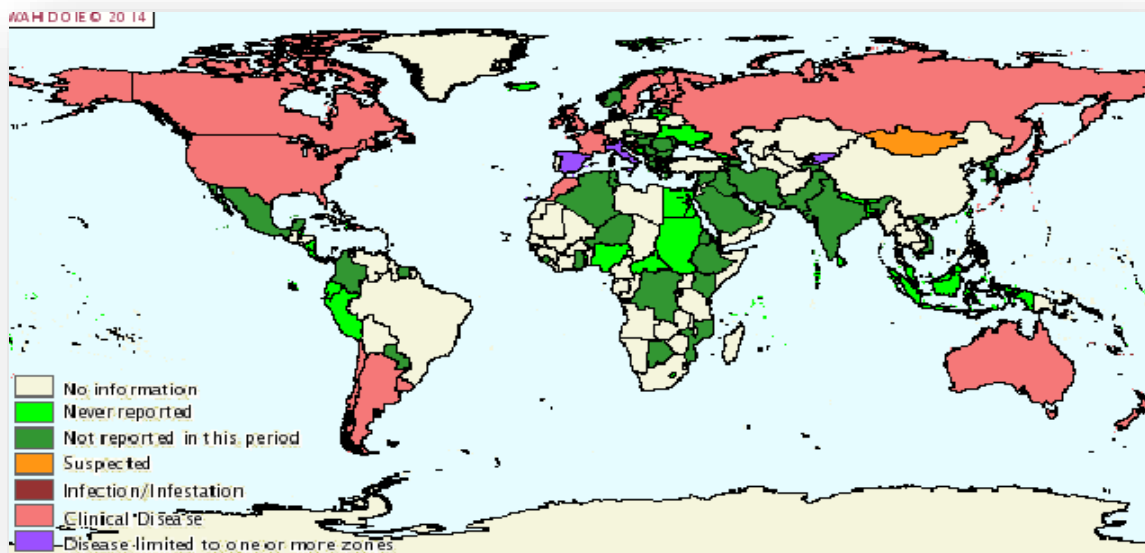
### I.3.3.7 Worldwide distribution and serological studies

EHV-1 and EHV-4 are distributed worldwide. EHV-4 is enzootic and widespread in many equine populations in the world (Allen and Bryans, 1986; Gilkerson *et al.*, 1999a; Matsumura *et al.*, 1992); whereas EHV-1 infection has been described in the USA (Allen, 2002; Brown *et al.*, 2007), Australia (Gilkerson *et al.*, 1999a), Japan (Matsumura *et al.*, 1992), Argentina (Galosi *et al.*, 1998), Brazil (Mori *et al.*), Egypt (Amer, 2011) and Nigeria (Ayedefa, 1992) among other countries.

According to the OIE, since 2005, EHV-1/-4 outbreaks have occurred in Israel (2005, 2008, 2011, 2012), Croatia (2009), South Africa (2009), United Arab Emirates (2010) and Chile (2013) (OIE, 2014b).

During the first six months of 2013, the distribution of EHV-1/-4 in the world, according to the OIE, was the following:

Figure 5. Worldwide distribution of EHV-1/-4 in the first six months of 2013 (OIE, 2013b).



This map shows that, during the first half of 2013, there were outbreaks of clinical disease in Canada, USA, Chile, Argentina, Uruguay, Morocco, UK, France, Switzerland, Finland, Russia, Japan, Australia and New Zealand (OIE, 2013b). During this period, Spain appears in purple colour in the map, indicating that there was clinical disease limited to one or more areas.

In addition to the confirmed worldwide presence of outbreaks of EHV-1/-4, seroprevalence studies have been carried out in several countries of the world such as Colombia (Ruiz-Saenz *et al.*, 2007, 2008), Peru (Rios, 2002), the Netherlands (de Boer *et al.*, 1979), Turkey (Ataseven, 2010; Gür, 2008), Lithuania (Liutkevicien, 2006), and in Tanzania and Namibia in zebras (Borchers and Frolich, 1997; Borchers *et al.*, 2005). Table 5 shows a summary of the characteristics and results of the EHV-1/-4 seroprevalence studies published in different countries all around the world.

Table 5. Characteristics and results of the EHV-1/-4 seroprevalence studies published worldwide.

| Study reference                | Country     | Study period                   | Study area   | Number of horses sampled              | Serological assay                    | Seroprevalence estimate  |
|--------------------------------|-------------|--------------------------------|--|---------------------------------------|--------------------------------------|--|
| <i>Ruiz-Saenz et al., 2007</i> | Colombia    | --                             | Region of Meta                                       | 68                                    | Indirect ELISA                       | 0% (EHV-1)<br>94.1% (EHV-4)  |
| <i>Ruiz-Saenz et al., 2008</i> | Colombia    | --                             | Region of Antioquia and Meta                         | 96 horses in Antioquia and 43 in Meta | Indirect ELISA                       | 18.8% (EHV-1 in Antioquia region)<br>33% (EHV-1 in Meta region)<br>98.7% (EHV-4 in Antioquia region)<br>96.6% (EHV-4 in Meta region) |
| <i>Rios, 2002</i>              | Peru        | 2000 - 2001                    | Surco, Chorrillos, Cieneguilla, Lurín and Pachacamac | 448                                   | SN test                              | 39.6% – 48.8% (EHV-1/-4)   |
| <i>Gür and Yapici, 2008</i>    | Turkey      | --                             | West and Central                                     | 188                                   | Indirect ELISA                       | 3.7% (EHV-1)<br>56.9% (EHV-4)<br>60 % (EHV-1/-4)   |
| <i>Ataseven et al., 2010</i>   | Turkey      | --                             | East   | 405                                   | Indirect ELISA                       | 9.9% (EHV-1)<br>64.7% (EHV-4)  |
| <i>De Boer et al., 1979</i>    | Netherlands | 1963 – 1966 and<br>1972 - 1979 | Whole country  | 283                                   | SN test                              | 76 % (EHV-1/-4)  |
| <i>Liutkevicien, 2006</i>      | Lithuania   | --                             | Seven herds  | 114                                   | Indirect Fluorescent Antibody and SN | 86.8% (EHV-1/-4)   |

### I.3.3.8 EHV-1/-4 in Spain

According to the OIE, the first report of EHV-1/-4 in Spain dates back to 1989, and since then there have been sporadic outbreaks reported in 2008 (two outbreaks with ten cases in total) and 2011 (a single case reported). In addition, according to the OIE, Spain reported the presence of EHV-1/-4 without specifying the number of outbreaks or affected horses in 2007, 2009 and 2010 (OIE, 2014a; OIE, 2014b).

The only epidemiological study to evaluate the presence of EHV-1/-4 in Spain dates back to 1992 and was published by Martín Otero as part of his PhD Thesis (Martin Otero, 1992). In this study 202 samples from horses residing in different military horse breeding centres in Palencia, San Sebastian, Cadiz, Alcalá de Henares and Zaragoza were collected and analysed using an indirect ELISA and SN. The percentage of seropositives for EHV (EHV-1 and/or EHV-4) by ELISA was 6.9% while this percentage was 4.9% by SN. The indirect ELISA resulted in 2% of false positives (Martin Otero, 1992). Since 1992 there has only been a further, small preliminary study carried out in the autonomous community of Valencia (East of Spain) which determined antibody against EHV-1/-4 by SN in a population of 49 SP horses in 2010-2011. The seroprevalence for EHV-1/-4 was 100% in this preliminary study (Gimeno Suarez *et al.*, 2011a). Since then, there have been no further studies to evaluate and quantify the presence of EHV-1/-4 in Spain.

### I.3.3.9 Economic importance and association with reproductive pathologies in a breeding stud farm

The consequences of an outbreak of EHV-1/-4 in the economy of a stud farm are mainly related to its association with reproductive pathology in the mares (abortions), and death or disease in the neonatal foals. Outbreaks of rhinopneumonitis tend to be on a larger scale than other disease outbreaks, such as those of EVA; therefore the economic losses due to abortions and neonatal deaths are often devastating. In addition, the presence of carriers with a latent virus means that outbreaks of EHV-1/-4 are difficult to prevent and control, since any situation of stress or immune suppression in a carrier can trigger an outbreak of abortions (HBLB, 2013).

Even though the respiratory disease caused by EHV-1/-4 can easily be controlled and does not produce sequelae, respiratory disease can spread rapidly amongst young horses and can result in more severe situations, such as an outbreak of abortions in mares or cases of myeloencephalopathy (Allen, 2002).

Finally, other economic consequences of an EHV-1/-4 outbreak would be the costs related to control measures, the interruption of the stallions training, quarantines, movement restrictions, screening tests carried out in all horses, disinfection of the premises, etc. (HBLB, 2013).





## *II. RATIONALE, AIMS AND OBJECTIVES*



## *II. RATIONALE, AIMS AND OBJECTIVES*

### **II.1 RATIONALE**

The equine industry continues to increase in importance in Spain, with a direct and indirect economic impact of more than 5,300 million euro, representing the 0.51% of the Gross Domestic Product (GDP) (Deloitte, 2013).

Equine breeding accounts for an economic impact of nearly 543 million euro, with most of this impact being attributed to the breeding of SP horses, since this breed represents 85% of the pure breed horses in Spain and nearly 100% of exportations to Europe and America standing for more than 58 million euro in 2012 (Deloitte, 2013).

Even though Andalusia is the region with the largest number of registered SP horses in Spain, this region is quite static in terms of horse movements and the majority of the SP horses reside in private premises with less than 4 horses (MAGRAMA, 2012). The central region of Spain (Madrid, Castilla La Mancha and Castilla y León), although with a lower number of SP horses than Andalusia, accounts for the 20.4% of SP horses and represents a high risk for the introduction of equine infectious diseases, being a very frequent stop for horses travelling from/to Europe and the region where the largest number of competitions are held (Deloitte, 2013; MAGRAMA, 2012).

Equine viral diseases such as EIA, EVA and EHV-1/-4 are important causes of breeding problems and economic losses in a stud farm. Most importantly, these diseases compromise welfare in the affected horses, causing death in some cases. The lack of a specific treatment for them and the speed at which they are spread within an equine population strengthen the need for their prevention and control in a breeding stud farm.

In order to plan and apply measures of prevention and control against these diseases, it is essential to know the current scenario regarding the presence of these three diseases and risk factors associated with exposure to these viruses in SP stud farms. There are no recent studies published regarding the seroprevalence of EIA in

Spain and the only studies to evaluate the seroprevalence of EVA and EHV-1/-4 in this country are either historical or carried out in a very small sample (Gimeno Suarez *et al.*, 2011a; Martin Otero, 1992). However, the situation in nearby European countries is of concern, with EIA outbreaks of clinical disease in Belgium and Germany in 2013 (OIE, 2014b) and a seroprevalence estimate of 0.44% for EIA in the Netherlands (de Boer *et al.*, 1979), EVA outbreaks of clinical disease in France, Switzerland and Spain in 2013 (OIE, 2014b) and studies showing EVA seroprevalences ranging from 8.7% in Germany (Herbst *et al.*, 1992) to 55.1% in Hucul horses in Poland (Rola *et al.*, 2011), and EHV-1/-4 outbreaks of clinical disease in the UK, France, Switzerland, Finland and Spain in 2013 (OIE, 2014b) and studies showing EHV-1/-4 seroprevalence results of 76% in the Netherlands (de Boer *et al.*, 1979) or 86.8% in Lithuania (Liutkevicien, 2006).

## II.2 AIMS

The main aim of this study was to evaluate the current situation regarding the presence of EIA, EVA and EHV-1/-4 in the SP breeding population in the central area of Spain. These results are relevant to veterinary surgeons, and those involved in the care of SP horses used in breeding, and they are key in order to establish the need for routine serosurveillance measures in SP breeding stud farms.

Additionally, this study aimed to determine the most important characteristics and measures of prevention and control of equine infectious diseases to take into account when trying to lower the risk of introduction of equine viral diseases into SP stud farms.

Finally, the study aimed to investigate whether the SP breeding stallions and mares residing in this area were healthy in terms of being free from reproductive problems which involve a lower efficiency and higher costs to the breeding.

The information provided by this study will inform the SP breeding industry about the need for raising the awareness of the problems that can pose equine

infectious diseases in the people related to SP breeding stud farms (stud farm owners, veterinary surgeons, stud farm workers) through the implementation of Codes of Practice for the most important infectious diseases affecting the breeding stock in Spain such as the ones already implemented in other nearby European countries (e.g. UK, France, Italy or Germany), which would help to implement targeted management strategies and measures of prevention and control of equine infectious diseases in the Spanish SP stud farms in order to aid disease prevention and, consequently, the associated welfare and financial costs.

## II.3 OBJECTIVES

The main objectives of the study were to:

1. Determine the seroprevalence of EIA, EVA and EHV-1/-4 in the SP breeding population in the central area of Spain.
2. Describe the characteristics and management practices with regards to the prevention and control of equine infectious diseases implemented in the SP breeding stud farms in the central area of Spain.
3. Describe the occurrence and nature of the breeding problems in SP mares and stallions in the central area of Spain.

The secondary objectives of the study were to:

- Identify any potential risk factors associated with the exposure to the infectious diseases of interest (EIA, EVA and EHV-1/-4) within the characteristics and management practices regarding the prevention and control of equine infectious diseases in SP stud farms in central Spain.

- Evaluate associations between the exposure to the infectious diseases of interest (EIA, EVA and EHV-1/-4) and the occurrence of breeding problems in SP mares and stallions in the central area of Spain.



### *III. MATERIALS AND METHODS*



### III. MATERIALS AND METHODS

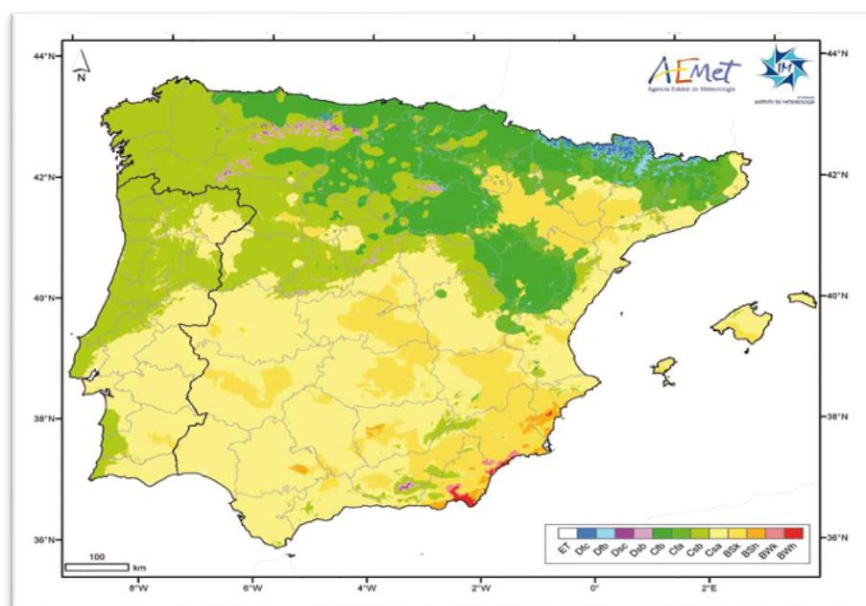
#### III.1 MATERIALS

##### III.1.1 Study site

For the purpose of this study, the central region of Spain, comprising the provinces of Madrid, Segovia, Ávila, Cuenca, Guadalajara and Toledo, was chosen. This area has a total of 67,724 km<sup>2</sup>, representing 13.4% of the total surface of Spain. The total number of inhabitants in this central area as of January 2012 was 8,022,327, representing 17% of the total Spanish population. Of the provinces in this central area, Madrid accounts for the largest number of inhabitants (6,498,560) and also the highest density of population (809.5 inhabitants/km<sup>2</sup>) (INE, 2012).

The central area of Spain has a variety of climate types, which are representative of the whole country. The map with the different climates present in Spain, according to the Köppen-Geiger Climate Classification, is shown in Figure 6.

Figure 6. Distribution of the different climate types in Spain.



Source: AEMET. Iberian Climate Atlas (1971-2000) (AEMET, 2011).

The provinces of Segovia, Ávila and the North-west (NW) of Madrid have similar climate types, according to the Köppen-Geiger Climate Classification, including a temperate with a dry season and hot summer climate (CFA), a temperate with a dry season and temperate summer (CFB), a temperate with dry or hot summer (CSA), a temperate with dry or temperate summer (CSB), a cold with temperate and dry summer (DSB), a cold with dry and fresh summer (DSC) and a cold steppe climate (BSK). The provinces of Cuenca, Guadalajara, Toledo and most of the surface of Madrid have CSA, BSK, CFA and CFB climate types (AEMET, 2011).

Regarding the census of SP horses in the central area of Spain, 21,309 SP horses were registered as of 31st December 2012 in this region, accounting for the 12.1% of the SP horses registered in Spain (MAGRAMA, 2012). Based on census data indicating 175,945 registered SP horses and 17,933 registered SP breeding stud farms in Spain, and an average of 9.8 SP horses per stud farm, the estimated number of SP breeding stud farms in the area of study would be 2,174 (MAGRAMA, 2012).

The area studied has a similar average density of SP horse population to that of Spain (0.39 SP horses/km<sup>2</sup> in the central area of Spain vs. 0.35 SP horses/km<sup>2</sup> in Spain). However, within the area of study there are differences in the density of SP horse population between provinces; with the lowest SP horse densities observed in Guadalajara and Cuenca (0.03 and 0.04 SP horses/km<sup>2</sup> respectively) and the highest SP horse density observed in Madrid (0.90 SP horses/km<sup>2</sup>). SP stud farms densities in the area of study have a similar distribution; the central region of Spain has a comparable average density of SP stud farms to that of Spain (0.039 SP stud farms/km<sup>2</sup> in the central area vs 0.035 SP stud farms/km<sup>2</sup> in Spain), and the same differences apply to the SP stud farm densities between provinces, with the lowest SP stud farm densities in Guadalajara and Cuenca (0.003 and 0.004 SP stud farms/km<sup>2</sup> respectively) and the highest SP stud farm density in Madrid (0.092 SP stud farms/km<sup>2</sup>) (INE, 2012; MAGRAMA, 2012).

The data on the number of registered SP horses, calculated number of SP breeding stud farms, area surface and SP horse and stud farm density of population in each of the provinces in the central region of Spain is shown in Table 6.

Table 6. Distribution of SP horses and stud farms in the central region of Spain.

| Region               | Area Surface (km <sup>2</sup> ) | SP Stud Farms | SP Horses      | SP horses density | SP stud farms density |
|----------------------|---------------------------------|---------------|----------------|-------------------|-----------------------|
| ÁVILA                | 8,049.92                        | 322           | 3,164          | 0.39              | 0.040                 |
| SEGOVIA              | 6,922.75                        | 400           | 3,916          | 0.56              | 0.058                 |
| CUENCA               | 17,141.13                       | 64            | 623            | 0.04              | 0.004                 |
| GUADALAJARA          | 12,212.09                       | 38            | 374            | 0.03              | 0.003                 |
| TOLEDO               | 15,369.69                       | 610           | 5,977          | 0.39              | 0.040                 |
| MADRID               | 8,027.70                        | 740           | 7,255          | 0.90              | 0.092                 |
| TOTAL CENTRAL REGION | 67,723.28                       | 2,174         | 21,309         | 0.39              | 0.039                 |
| <b>TOTAL SPAIN</b>   | <b>505,968.36</b>               | <b>17,933</b> | <b>175,945</b> | <b>0.35</b>       | <b>0.035</b>          |

Source: Arca database, data as of 31<sup>st</sup> December 2012; Instituto Nacional de Estadística, 2012 (INE, 2012; MAGRAMA, 2012).

Finally, the central area of Spain is also the area where the largest number of competitions are held, with nearly 31% of the 1616 competitions held in Spain per year, followed by Andalusia, with 15.6% of the total competitions held in Spain (Deloitte, 2013; MAGRAMA, 2012).

### III.1.2 Population of study

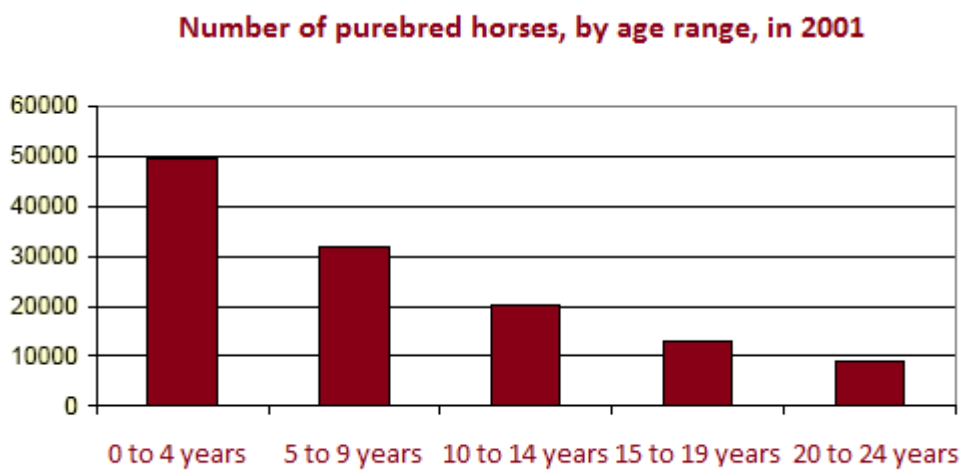
The SP breeding industry differs from the other livestock sectors (such as porcine, poultry, bovine or ovine) as management and healthcare measures are often applied at individual animal level rather than to the herd as a whole. Thus, each horse is individually treated, and horses may enter and leave the premises for many reasons (competitions, morphologic shows, other stud farms). The target population of this study was the population of breeding SP horses in the central area of Spain. Considering the census of SP breeding horses registered as of 31<sup>st</sup> December 2012 in the central area of Spain, the reference population for this study were 21,309 horses (MAGRAMA, 2012).

Out of these 21,309 horses in the central region, 52.7% (11,230) were mares and 47.3% (10,079) were stallions. Within the SP registered mares, 56.3% (6,322)

were registered as active breeders, whereas only the 39.8% (4,011) of all the registered SP stallions were active breeders (MAGRAMA, 2012).

Regarding the distribution by age of the SP horse population in the central area of Spain, there are no data published in the census of 2012; however, the distribution by age within the horses of pure breed (including SP horses but also Arab, Thoroughbred, French trotter and other pure breeds) in Spain, in 2001, is shown in Figure 7.

Figure 7. Distribution of horses of pure breed, by age, in 2001.



Source: Study and characterisation of equine industry in Spain (MAPA, 2003).

### III.1.2.1 Sample size

Assuming a seroprevalence of 50% for any of the three diseases studied (EIA, EVA and EHV-1/-4), a population of 21,309 SP horses in the central area of Spain, with a 95% confidence and 5% precision in the determination of seroprevalence for these diseases, the minimum sample size was determined to be 378 horses. The sample size calculation for the estimation of a proportion (seroprevalence) was carried out with the Win Epi software by using the formula for finite populations (De Blas, 2006):

$$n = \frac{Z_v^2 * p * q * N}{e^2 (N-1) + Z_v^2 p * q}$$

The population of SP registered horses in the central region of Spain was stratified by stud farms. In order to capture the heterogeneity of the SP breeding stud farms, eligible premises were identified based on the number of horses and the population density in the stud farm, climate of the area (following the Köppen-Geiger Climate Classification) and distribution in the central region of Spain. From these premises, a convenience sample of stud farms were included following the methods proposed by Dohoo *et al.* (Dohoo *et al.*, 2003), the inclusion criteria were the presence of any active SP breeders in the stud farm and the willingness of stud farm owner/veterinary surgeon to collaborate in the study, whereas the exclusion criteria were the impossibility to draw blood samples from any SP horses in the stud farm due to the animals presenting a risk for the person in charge of sampling, and the refusal of the veterinary surgeon/stud farm owner to provide the necessary data from the animals sampled or the stud farm.

In every stud farm, 100% of the breeding SP stallions were sampled and at least 25% of the breeding SP mares were conveniently sampled by means of choosing the mares which were easiest to handle. The inclusion criteria for the breeding SP stallions and mares were to actively have bred in the past twelve months or to have been in contact with other SP breeding stallions/mares, and to be considered healthy by means of a clinical examination performed by the stud farm

veterinary surgeon, whereas the exclusion criteria were the impossibility to draw a blood sample due to the animal presenting a risk for the person in charge of sampling, and to be resident in the stud farm for a shorter time than 3 weeks.

Where possible a convenience sample of at least 25% of the SP colts and fillies was also taken following the same method as mares; the inclusion criteria for the SP colts and fillies were to be older than 8 months of age and to be considered healthy by means of a clinical examination performed by the stud farm veterinary surgeon, whereas the exclusion criteria were the impossibility to draw a blood sample due to the animal presenting a risk for the person in charge of sampling, and to be resident in the stud farm for a shorter time than 3 weeks.

In the case of EHV-1/-4, only the samples from horses with a history of non-vaccination against EHV-1/-4 were analysed and used in the determination of EHV-1/-4 seroprevalence.

The inclusion of SP stud farms in the study was carried out consecutively until the target horse sample size was reached. Between September 2011 and November 2013, serum samples were obtained from 555 SP horses residing in 35 different stud farms in the central region of Spain.

### III.1.2.2 Data collection from the horses included in the study.

Data were collected for each horse meeting study inclusion criteria, including sex, age, breeding status, presence/absence of reproductive problems and vaccination history for EHV-1/-4.

In breeding stallions and mares with a record of reproductive problems within 12 months prior to being sampled for the study, a sex-specific questionnaire of reproductive problems was completed. Both questionnaires (reproductive problems in the mare and reproductive problems in the stallion) were designed following the most typical causes for alteration in the male and female sexual function (Smith, 2002).

Both questionnaires consisted of a combination of 5 open and multiple-choice closed ended questions. The questions covered the identification of the mare/stallion in the study, the age of the mare/stallion, the number of years as an active breeder, a multiple-choice question that included all the most typical causes for alteration in the female/male sexual function (as a checkbox list) (Smith, 2002) and two open questions in order to specify the cause and/or treatment for the reproductive problem. Questionnaires were pretested amongst a group of veterinary surgeons, who were not enrolled on the study, and were revised in accordance with their comments. They were then administered in a face-to-face format, and the veterinary surgeon's responses were recorded on a printed copy of the questionnaire. If requested, the survey was sent to the veterinary surgeon prior to collection of data. Data were entered into MS Excel 2007 (Microsoft Corporation, Redmond WA, USA).

The questionnaire of reproductive problems in the stallion is shown in Appendix 1, and the questionnaire of reproductive problems in the mare is presented in Appendix 2.

### III.1.2.3 Data collection from the stud farms included in the study.

Once stud farms had met study inclusion criteria, veterinary surgeons completed a questionnaire regarding general management and health, with the help of the stud farm owner where required.

The questionnaire of general management and health in SP breeding stud farms in central Spain (Appendix 3) was designed to cover the characteristics and management measures regarding the prevention and control of equine infectious diseases in a stud farm. It consisted of 8 sections and a combination of open, closed and multiple-choice questions with space for additional comments. The questionnaire sections aimed to gather information such as contact data and stud farm location, general farm operation size and features, number of horses in the stud

farm, groups established in these horses, movement of horses in the stud farm, health management of the horses, vaccination and worming regimes, disinfection measures, measures against insects and rodents, and finally the breeding performance of the breeding stallions and mares of the stud farm.

As for the other questionnaires, it was pretested amongst a group of veterinary surgeons, and was revised in accordance with their comments. The questionnaire was then administered in a face-to-face format, and the veterinary surgeon's responses with the help of the stud farm owner when necessary were recorded on a printed copy of the questionnaire. If requested, the survey was sent to the veterinary surgeon or the stud farm owner prior to collection of data. Data were entered into MS Excel 2007 (Microsoft Corporation, Redmond WA, USA).

## III.2 METHODS

### III.2.1 Sampling procedures

Each of the SP horses included in the study were sampled by means of jugular venepuncture using needles and tubes with the Vacutainer® system. Ten millilitres (10 ml) of blood were collected in tubes without an anticoagulant and the tubes were identified with the study identification number, consisting of a letter for the stud farm and a number for each of the horses sampled.

The blood samples were transported to the laboratory, where they were allowed to clot, and after the clot was formed the samples were centrifuged at 3000 rpm (revolutions per minute) for 10 minutes using a KUBOTA Compact Tabletop 2010 centrifuge (Kubota Corporation). Serum was separated using 1.5 ml Pasteur pipettes and aliquots of approximately 600 µl serum were placed in 1.5 ml Eppendorf tubes. Samples were then frozen at -40°C until their analysis.

## III.2.2 Serological analysis of the samples

The samples collected in the study were analysed for antibodies against EIA and EVA. Only the samples from unvaccinated horses were analysed for antibodies against EHV-1/-4, since vaccine-induced antibodies cannot be differentiated from viral infection-induced antibodies (Allen, 1999).

### III.2.2.1 Equine Infectious Anaemia serology

#### *EIA serological analysis by ELISA*

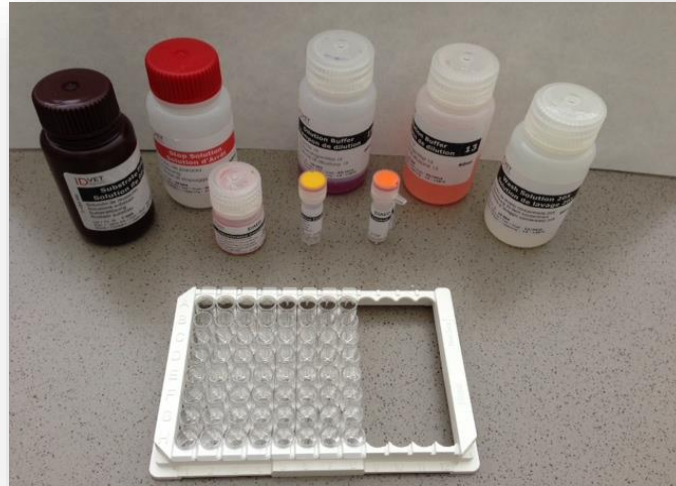
The serological technique used for the analysis of antibodies against EIA in this study was a double antigen ELISA commercial kit (ID Screen Equine Infectious Anaemia Double Antigen ELISA®, ID.vet Innovative Diagnostics) (Pourquier, 2010).

This diagnostic kit is designed to detect antibodies directed against the Equine Infectious Anaemia Virus (EIAV) in horse sera. It uses two fragment antibodies (Fab) on Immunoglobulin G (IgG) and ten Fab on Immunoglobulin M (IgM) which either bind the immunoglobulins to the microplate or bind a peroxidase antigen used as conjugate. The kit is composed of microplates coated with p26 (Gene GAG) recombinant antigen, positive and negative controls, dilution buffer for the samples, dilution buffer for the conjugate, p26 antigen-peroxydase conjugate, wash concentrate, substrate solution and stop solution (H<sub>2</sub>SO<sub>4</sub> 0.5M). Figure 19 shows the different components of the EIA ELISA kit.

The materials required for this technique that were not provided in the commercial kit were a Finnpiptette® digital 12 channel 50-300 µl micropipette (Sigma Aldrich®, Spain), 10 µl micropipette (PIPETMAN Classic P10®, Gilson Inc.), 100 µl micropipette (PIPETMAN Classic P100®, Gilson Inc.), disposable tips, disposable 300 µl 96-well microplates, distilled water, a 96-well microplate reader

(BioTek ELx 800 Absorbance microplate reader, BioTek®) and a printer connected to the microplate reader.

Figure 8. Components of the ID Screen Equine Infectious Anaemia Double Antigen ELISA® used.



The microplate wells are coated with p26 (gene GAG) recombinant antigen. If there are antibodies against the EIAV in the tested sample, antibodies against p26 form an antibody-antigen complex. When a p26 antigen-peroxidase conjugate is added to the well, it fixes to the free Fab of the bound serum anti-p26 antibodies. After adding a substrate solution which binds to the peroxidase producing colour, the resulting coloration depends on the quantity of specific antibodies and can be measured by using a microplate reader.

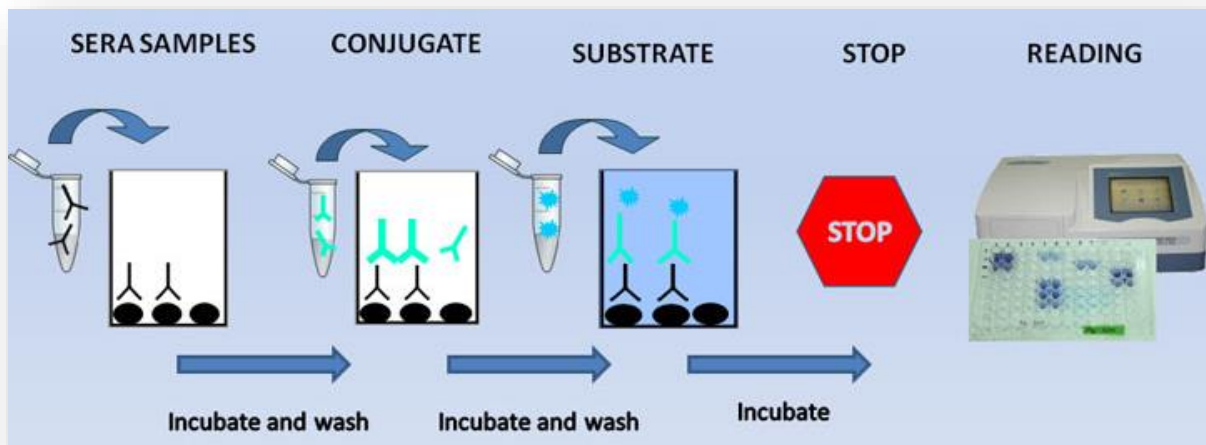
As for the testing procedure, and after all the reagents were allowed to come to room temperature, the first step was to prepare the serum samples for the analysis; samples were defrosted at 37°C in a WS27 SHEL LAB Shaking Water Bath (Shelly Scientific®) and, in order to avoid differences in incubation times between specimens, a 96-well disposable microplate was prepared with 50 µl of the dilution buffer and 50 µl of the sample, positive or negative control. All the specimens, as well as the positive and negative controls, were duplicated. One hundred microliters (100 µl) of the sample/control dilutions were then transferred into the ELISA microplate with p26 coated wells included in the commercial kit using a

multichannel pipette, and the microplate was incubated for 30 minutes at room temperature ( $21^{\circ}\text{C} \pm 5^{\circ}\text{C}$ ).

After the first incubation, the wells were emptied and manually washed 3 times with approximately 300  $\mu\text{l}$  of the wash solution (previously diluted with distilled water). The conjugate was diluted with a dilution buffer, and 100  $\mu\text{l}$  conjugate were added to each well. The microplate was then incubated a second time for 30 minutes at room temperature.

After the second incubation, the wells were emptied and manually washed 3 times with approximately 300  $\mu\text{l}$  of the wash solution (previously diluted with distilled water); 100  $\mu\text{l}$  of the substrate solution were then added to each well, and the microplate was incubated a third time for 15 minutes at room temperature and in the dark. After the last incubation 100  $\mu\text{l}$  of the stop solution were added to each well in order to stop the reaction. The microplate was then read at 450 nm with the microplate reader and the Optical Density (OD) results were printed. Figure 9 shows a scheme of the testing procedure of an indirect ELISA such as the one used in this study.

Figure 9. Scheme of the testing procedure of an indirect ELISA.



Source: <http://www.sanidadanimal.info>

The test was validated if the mean OD value of the positive control was greater than 0.350, and if the ratio of the mean values of the positive and negative controls was greater than 3. For each sample, results were expressed as S/P (sample-to-positive control) percentage, which was calculated with the following formula:

$$S/P \% = \frac{OD_{\text{sample}} - OD_{\text{NC}}}{OD_{\text{PC}} - OD_{\text{NC}}} \times 100$$

OD<sub>NC</sub>: mean OD of the negative control

OD<sub>PC</sub>: mean OD of the positive control

OD<sub>sample</sub>: mean OD of the sample

Samples with an S/P % ≤ 50% were considered **negative**, samples with an S/P % > 50% but < 60% were considered **equivocal** and samples with an S/P & ≥ 60% were considered **positive**.

According to the manufacturers, the ELISA kit used has a published sensitivity and specificity of 100% (Pourquier, 2010). However, while ELISA tests are more rapid and easier to interpret than the Coggins test, there are more chances of getting false positive results, since they have a lower specificity than the Coggins test (Cullinane *et al.*, 2007; Piza *et al.*, 2007). In a study of EIA seroprevalence in the Sultanate of Oman the ID Screen Equine Infectious Anaemia Double Antigen ELISA® used in this study was reported to have a sensitivity of 100% and a specificity of 99.3% (Body *et al.*, 2011). Since the Coggins test is the OIE standard confirmatory test for EIA, any positive or equivocal samples by ELISA would be tested by Coggins test.

### III.2.2.2 Equine Viral Arteritis serology

#### *EVA serological analysis by ELISA*

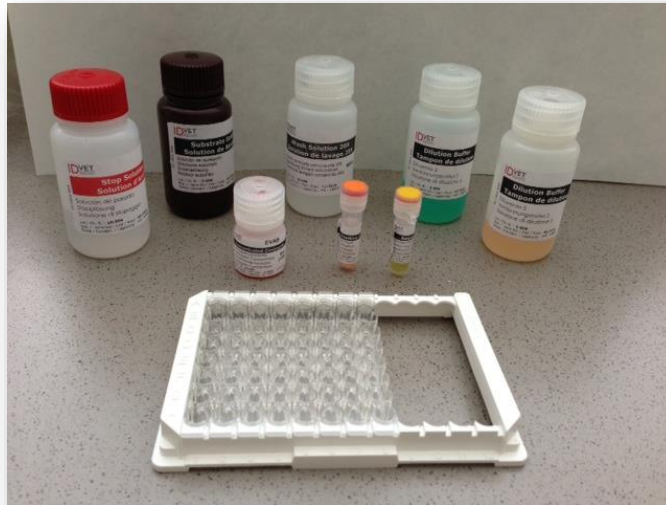
The serological technique used for the analysis of antibodies against EVA in this study was an indirect confirmation ELISA commercial kit (ID Screen® Equine Viral Arteritis Indirect, ID.vet Innovative Diagnostics) (Legrand *et al.*, 2009; Legrand *et al.*, 2007).

This diagnostic kit is designed to detect antibodies directed against EAV in horse sera. It has a bi-well format and uses specific EAV peptides which bind the immunoglobulins to the wells in the even columns of the microplate (the wells in the odd columns of the microplate are not coated). When there are antibodies against the EAV in the sample, the antibodies bind to the specific EAV peptides and these complexes bind to an anti-equine IgG-peroxydase conjugate which will produce colour when the substrate solution is added to the well. The resulting coloration depends on the quantity of specific antibodies and can be measured by using a microplate reader.

The kit is composed of microplates, positive and negative controls, dilution buffer for the samples, dilution buffer for the conjugate, anti-equine IgG-peroxydase conjugate, wash concentrate, substrate solution and stop solution (H<sub>2</sub>SO<sub>4</sub> 0.5M). Figure 20 shows the different components of the EVA ELISA kit.

The materials required for this technique that were not provided in the commercial kit were a Finnpiptette® digital 12 channel 50-300 µl micropipette (Sigma Aldrich®, Spain), 10 µl micropipette (PIPETMAN Classic P10®, Gilson Inc.), 100 µl micropipette (PIPETMAN Classic P100®, Gilson Inc.), disposable tips, disposable 300 µl 96-well microplates, distilled water, a 96-well microplate reader (BioTek ELx 800 Absorbance microplate reader, BioTek®) and a printer connected to the microplate reader.

Figure 10. Components of the ID Screen® Equine Viral Arteritis Indirect ELISA used.



As for the testing procedure already shown in Figure 9, and after all the reagents were allowed to come to room temperature, the first step was to prepare the serum samples for the analysis; samples were defrosted at 37°C in a WS27 SHEL LAB Shaking Water Bath (Shelly Scientific®) and, in order to avoid differences in incubation times between specimens, a 96-well disposable microplate was prepared with 180 µl of the dilution buffer and 200 µl of the sample, positive or negative control. All the specimens, as well as the positive and negative controls, were duplicated. Two hundred microliters (200 µl) of the sample/control dilutions were then transferred into the ELISA microplate (100 µl into the even column wells coated with the EAV specific peptides and 100 µl into the odd column wells, not coated) using a multichannel pipette, and the microplate was incubated for 1 hour 30 minutes at room temperature (21°C ± 5°C).

After the first incubation, the wells were emptied and manually washed 3 times with approximately 300 µl of the wash solution (previously diluted with distilled water). The conjugate was diluted with a dilution buffer, and 100 µl conjugate were added to each well. The microplate was then incubated a second time for 30 minutes at room temperature.

After the second incubation, the wells were emptied and manually washed 3 times with approximately 300 µl of the wash solution (previously diluted with

distilled water); 100 µl of the substrate solution were then added to each well, and the microplate was incubated a third time for 15 minutes at room temperature and in the dark. After the last incubation 100 µl of the stop solution were added to each well in order to stop the reaction. The microplate was then read at 450 nm with the microplate reader and the OD results were printed.

For every sample, the mean corrected OD was calculated with the formula:

$$OD_{\text{corrected sample}} = OD_{\text{even column}} - OD_{\text{odd column}}$$

The test was validated if the mean corrected OD value of the positive control was greater than 0.350, and if the ratio of the mean values of the positive and negative controls was greater than 3. For each sample, results were expressed as an S/P percentage, which was calculated with the following formula:

$$S/P \% = \frac{OD_{\text{corrected sample}}}{OD_{\text{corrected PC}}} \times 100$$

OD<sub>corrected sample</sub>: mean corrected OD of the sample

OD<sub>corrected PC</sub>: mean corrected OD of the positive control

Samples with an S/P % ≤ 50% were considered **negative**, samples with an S/P % > 50% but < 60% were considered **equivocal** and samples with an S/P & ≥ 60% were considered **positive**. When the OD value of the control, uncoated well (OD<sub>odd column</sub>) was higher than the OD value of the coated well (OD<sub>even column</sub>), the result was considered **not interpretable**.

According to the manufacturers, the ELISA kit used has a sensitivity of 100% and a specificity of 100%. However, while ELISA tests are more rapid and easier to interpret than the SN test, there are more chances of getting false positive results, since they have a lower specificity than the SN test (Legrand *et al.*, 2009; Legrand *et al.*, 2007). In a validation study carried out by the Beaufort Cottage Laboratories at Rossdales, Newmarket, Suffolk, the ID Screen® Equine Viral Arteritis Indirect ELISA

used in this study was reported to have a sensitivity of 100% and a specificity of 96.75% (Beaufort Cottage Laboratories, 2008). Given this specificity, samples with a positive, equivocal or not interpretable result were retested by the SN test.

### *EVA serological analysis by SN test*

The virus SN test measures the capacity of antibody present in serum samples to neutralise virus infectivity. In this study, samples with a positive, equivocal or not interpretable result by ELISA were sent to the Diagnostic Laboratory Services, Animal Health Trust, Newmarket, Suffolk, United Kingdom, in order to be retested by SN.

The test procedure in use was that developed by the National Veterinary Service Laboratories of the United States Department of Agriculture, with some modifications (Senne *et al.*, 1985). The neutralisation reaction takes place in micro plates in which pre-diluted sera are incubated with a Standard Virus Dose in the presence of exogenous complement. The samples are then added to preformed monolayers of RK13 indicator cells grown in 96 well cell culture micro plates, these plates are incubated for 2 days and the wells examined for cytopathic effect (CPE) caused by infectious virus. The presence of neutralising antibody is indicated by the absence of CPE in wells containing test sera, relative to the Virus Control wells.

The test was carried out in RK-13 cells using the approved CVL-Bucyrus (Weybridge) strain of EAV as reference virus (Edwards *et al.*, 1998). Serum samples were inactivated for 30 minutes in a water bath at 56°C. Serial twofold dilutions of the inactivated test sera in cell culture medium were made in a 96-well, flat-bottomed, cell-culture microtitre plate starting at a ½ serum dilution and using four rows of wells for each serum to be tested. Serum controls, together with low-, mid and high-titred positive control sera were included in each test. A dilution of stock virus made up to contain from 100 to 300 TCID<sub>50</sub> (50% tissue culture infective dose) per 25 µl was prepared using as diluent, 10% cell culture medium containing antibiotics and fresh guinea-pig complement at a final concentration of 10%. Twenty-five microliters (25 µl) of the appropriate dilution of stock virus was added

to every well containing 25 µl of each serum dilution, except the cell control wells on each plate. The plates were covered and incubated for 1 hour at 37°C in a humid atmosphere of 5% CO<sub>2</sub> in air. The contents of each well were then transferred to an identical set of plates containing a volume of 100 µl of a monolayer of cells from 1 day-old cultures of RK-13 cells. The plates were covered with plate sealers and lids, incubated at 37°C in a humid atmosphere of 5% CO<sub>2</sub> in air and read microscopically for non-viral CPE after 18 - 24 hours and again for viral CPE after 48–72 hours' incubation.

If the control virus titration was  $< 10^{1.6}$  TCID<sub>50</sub>/well, then the plates were incubated again, until reaching this target. If the virus titration was  $>10^{2.4}$  TCID<sub>50</sub>/well, the test was considered invalid. Those wells with CPE covering  $> 50\%$  of the total monolayer were scored positive, whereas those wells with CPE covering  $\leq 50\%$  of the monolayer were scored negative; these wells were antibody positive. The Karber formula [ $\text{Log}_{10} \text{VN50} = L - d(S - 0.5)$ ], where L = negative log of the lowest dilution used, d = log difference between dilution steps and S = sum of the proportion of negative wells (those showing  $\leq 50\%$  CPE, i.e. neutralised)] was used to calculate the  $\text{log}_{10}$  VN50 endpoint titres for the EAV SN titrations. Samples with a  $\text{log}_{10}$  titration of  $\geq 0.6$  were considered **positive**, whereas samples with a  $\text{log}_{10}$  titration of  $< 0.6$  were considered **negative**.

When using this technique, not infrequently, sera are encountered that give rise to toxic changes in the lower dilutions tested. In such cases it may not be possible to establish whether the sample is negative or a low-titred positive. This problem may be overcome by carrying out repeated freeze-thaw cycles of the serum samples (Geraghty *et al.*, 2005), or by retesting the toxic sample using microtitre plates with confluent monolayers of RK-13 cells that had been seeded the previous day. Also, the toxicity in serum samples can be reduced or eliminated if the sample is adsorbed with a packed suspension of RK-13 cells prior to testing or by substituting rabbit in place of guinea-pig complement in the virus diluent (Newton *et al.*, 2004). The sensitivity of the SN test for detection of antibodies to EAV can be significantly influenced by several factors, especially the source and passage history of the strain of virus used (Edwards *et al.*, 1998).

### III.2.2.3 EHV-1/-4 serology

#### *EHV-1/-4 analysis by ELISA*

The serological technique used for the analysis of antibodies against EHV-1/-4 in this study was an indirect ELISA commercial kit (Ingezim Rinoneumonitis 14.HVE.K1®, Ingenasa).

This assay allows the detection of antibody against EHV-1/-4 by using a 96-well plate coated with a soluble extract of EHV-1 proteins. When an equine serum sample contains antibodies against EHV-1/-4, the antibodies bind the antigen adsorbed on the plate. After washing to eliminate all non-fixed material from the serum sample, the presence of immunoglobulins can be detected by using a specific anti-equine IgG-peroxydase conjugate which will produce colour when the substrate solution is added to the well (Nakane and Kawaoi, 1974; Sanz *et al.*, 1985). The resulting coloration depends on the quantity of specific antibodies against EHV-1, EHV-4 or both (there is cross-reactivity since both viruses have epitopes in common) and can be measured by using a microplate reader.

The kit is composed of microplates, positive and negative controls, dilution buffer for the samples, dilution buffer for the conjugate, anti-equine IgG-peroxydase conjugate, wash concentrate, substrate solution, dilution buffer for the substrate solution, stop solution (H<sub>2</sub>SO<sub>4</sub>) and microplate lids. Figure 21 shows the different components of the EHV ELISA kit.

The materials required for this technique that were not provided in the commercial kit were a Finnpipette® digital 12 channel 50-300 µl micropipette (Sigma Aldrich®, Spain), 10 µl micropipette (PIPETMAN Classic P10®, Gilson Inc.), 100 µl micropipette (PIPETMAN Classic P100®, Gilson Inc.), 1000 µl micropipette (PIPETMAN Classic P1000®, Gilson Inc.), disposable tips, disposable 1 ml tubes, distilled water, a 96-well microplate reader (BioTek ELx 800 Absorbance microplate reader, BioTek®) and a printer connected to the microplate reader.

Figure 11. Components of the Ingezim Rinoneumonitis 14.HVE.K1® Indirect ELISA used.



As for the testing procedure (shown in Figure 9), and after all the reagents were allowed to come to room temperature, the first step was to prepare the serum samples for the analysis; samples were defrosted at 37°C in a WS27 SHEL LAB Shaking Water Bath (Shelly Scientific®) and, in order to avoid differences in incubation times between specimens, 1 ml disposable tubes were prepared with 495 µl of the dilution buffer and 5 µl of the sample. The positive and negative controls were not diluted. All the specimens, as well as the positive and negative controls, were duplicated. One hundred microliters (100 µl) of the sample dilutions/controls were then transferred into the ELISA microplate using a multichannel pipette, and the microplate was sealed and incubated for 1 hour at 37°C in a Memmert 854 incubator (Memmert GmbH & Co).

After the first incubation, the wells were emptied and manually washed 4 times with approximately 300 µl of the wash solution (previously diluted with distilled water). The conjugate was diluted with a dilution buffer, and 100 µl conjugate were added to each well. The microplate was then sealed and incubated a second time for 30 minutes at room temperature (25°C).

After the second incubation, the wells were emptied and manually washed 5 times with approximately 300 µl of the wash solution (previously diluted with distilled water); 100 µl of the substrate solution previously diluted with a dilution

buffer were then added to each well, and the microplate was sealed and incubated a third time for 15 minutes at room temperature and in the dark. After the last incubation 100 µl of the stop solution were added to each well in order to stop the reaction. The microplate was then read at 405 nm with the microplate reader and the OD results were printed.

The test was validated if the mean OD value of the positive control was greater than 0.8, and if the mean OD value of the negative control was lower than 0.3. The positive and negative cut-off values for the test were calculated using the following formulas:

- Positive cut-off = mean OD of negative control + 0.3
- Negative cut-off = mean OD of negative control + 0.2

Samples with a mean OD value higher than the Positive cut-off were considered **positive**, whereas samples with a mean OD value lower than the Negative cut-off were considered **negative**, and samples with a mean OD value between the positive and negative cut-off were considered **equivocal**.

According to the manufacturers, the ELISA kit used has a sensitivity of 100% and a specificity of 67%. Given this specificity, samples with a positive or equivocal result were retested by the SN test.

### *EHV-1/-4 serological analysis by SN test*

In this study, samples with a positive or equivocal result by ELISA were sent to the Diagnostic Laboratory Services, Animal Health Trust, Newmarket, Suffolk, United Kingdom, in order to be retested by SN.

This serological test was performed in flat-bottom 96-well microtitre plates using a constant dose of virus and doubling dilutions of equine test sera. Ten percent (10%) medium was used throughout as a diluent and virus stocks of known titre were diluted just before use to contain 100 TCID<sub>50</sub>. Monolayers of E-Derm equine

embryonic cells were monodispersed with EDTA/trypsin and resuspended at a concentration of  $5 \times 10^5$ /ml for their use.

As for the testing procedure, serum samples to be tested were inactivated for 30 minutes in a water bath at 56°C. Twenty-five microliters (25  $\mu$ l) of 10% medium were added to all wells of the microtitre assay plates, and 25  $\mu$ l of each test serum were pipetted into the first wells of triplicate rows of the plate. Doubling dilutions of each serum were made across the plates by sequential mixing and transfer of 25  $\mu$ l to each subsequent well. Four sera were assayed in each plate per antigen.

After that, 25  $\mu$ l of the appropriately diluted EHV-1 or EHV-4 virus stock were added to each well (100 TCID<sub>50</sub>/well). The final serum dilutions, after addition of virus, ran from 1/4 to 1/256. A separate control plate included titration of a positive horse serum of known titre, cell control (no virus), and a virus titration to calculate the actual amount of virus used in the test. The plates were incubated for 1 hour at 37°C in 5% CO<sub>2</sub> atmosphere, and after the incubation 25  $\mu$ l of guinea-pig complement diluted 1:4 in 10% medium was added to each well. The plates were then incubated for a further hour at 37°C in 5% CO<sub>2</sub> atmosphere. 150  $\mu$ l of the prepared of Equine Embryonic cell suspension ( $2 \times 10^5$  cells/ml) were added to each well. Finally the plates were incubated for 4–7 days at 37°C in an atmosphere of 5% CO<sub>2</sub> in air.

The plates were microscopically examined for CPE and the results were recorded on a worksheet. The actual amount of virus added to each well should be between  $10^{1.6}$  and  $10^{2.4}$  TCID<sub>50</sub> for the test to be validated. Wells were scored as positive for neutralisation of virus if 50% of the cell monolayer remained intact. The highest dilution of serum resulting in complete neutralisation of virus (no CPE) in both duplicate wells was the end-point titre for that serum. As for EHV-1, samples with a log<sub>10</sub> titration of  $\geq 2.3$  were considered **positive**, whereas samples with a log<sub>10</sub> titration of  $< 2.3$  were considered **negative**. For EHV-4, samples with a log<sub>10</sub> titration of  $\geq 2.0$  were considered **positive**, whereas samples with a log<sub>10</sub> titration of  $< 2.0$  were considered **negative**.

### III.2.3 Data analysis and statistics

The apparent seroprevalence (AS) for the three diseases of study was estimated by the percentage of seropositive results obtained by each technique (ELISA and SN test) within the total number of horses sampled (555 SP horses sampled for EIA, EVA and 334 non-vaccinated horses for EHV-1/-4).

The determination of true seroprevalence (TS) for ELISA tests used was carried out by using the following formula, which took into account the AS, the sensitivity (SE) and specificity (SP) of the test.:

$$TS = \frac{(AS + SP - 1)}{(SE + SP - 1)}$$

The “gold standard”, reference SN tests carried out for EVA and EHV-1/-4, were assumed to have a perfect sensitivity and specificity and therefore the apparent seroprevalence was assumed to be equal to the true seroprevalence. For every disease analysed, 95% confidence intervals (CI) were calculated for each seroprevalence percentage given a sample size and a reference population size (De Blas, 2006). The results from EVA and EHV-1/-4 SN tests were used for further analysis of data with regards to the identification of risk factors for exposure to these equine diseases.

As for the data from individual horses and the characteristics, management measures and measures of control of equine infectious diseases of each stud farm, the initial examination, data coding and descriptive analyses were conducted using Excel (Microsoft). Descriptive statistics were produced for quantitative variables [mean, standard deviation (SD), median, maximum and minimum, interquartile range (IR)] and qualitative variables (absolute and relative frequencies). All further analyses were conducted using SPSS® software (IBM®).

As for individual variables (i.e. sex, age, etc.) and stud farm variables (variables regarding the characteristics, and management practices in the stud farm), an univariable analysis was made by using a Chi-squared ( $\chi^2$ ) or Fisher’s

exact contingency table analysis as well as an ordinary logistic regression in order to obtain odds ratios (OR) with 95% CI. SPSS for Windows (SPSS Inc. version 18) was used for the data analysis.

A multivariable analysis was performed on all individual and stud farm variables by creating a decision tree through the Chi-square automatic interaction detector (CHAID) (Kass, 1980), as it offered the capacity to combine categorical and continuous variables. This procedure is a non-parametric analysis based on statistically recursive partitioning algorithms that classify independent variables into predictor values of a dependent outcome. EVA VN status or EHV-1/-4 VN status in the SP horses sampled in the study was set as the dependent outcome, while 31 independent outcomes were tested. Exhaustive CHAID analysis results were presented in decision trees, which provide a hierarchical visual depiction of predictor variables interactions. The independent variables that were significantly associated with the dependent outcome were classified in nodes. The variable at the highest level of the tree was determined to have the closest statistical association with the dependent outcome, and was represented as the first node. Subsequent associations were classified in consecutive nodes. Groups were split under the following criteria: tree depth was limited to three levels and the minimum number of cases per node for parent nodes was ten. Statistical significance was established at the 95% confidence level. SPSS for Windows (SPSS Inc. version 18) was used for the data analysis. Standard post-fit diagnostic tests were conducted to evaluate the decision tree model including Hosmer-Lemeshow goodness-of-fit test and a receiver operating characteristic (ROC) curve was generated with calculation of area under the curve (AUC).

Finally, the prevalence of reproductive problems in the SP breeding horses (>2 years old) within the preceding 12 months was calculated and descriptive statistics were produced. The nature of the reproductive problems was reported and these data were used in order to identify any associations with the presence of EVA and/or EHV-1/-4 antibodies using Chi-squared ( $\chi^2$ ) or Fisher's exact contingency table analysis and also ordinary logistic regression analysis to obtain OR with 95% CI.



## *IV. RESULTS*

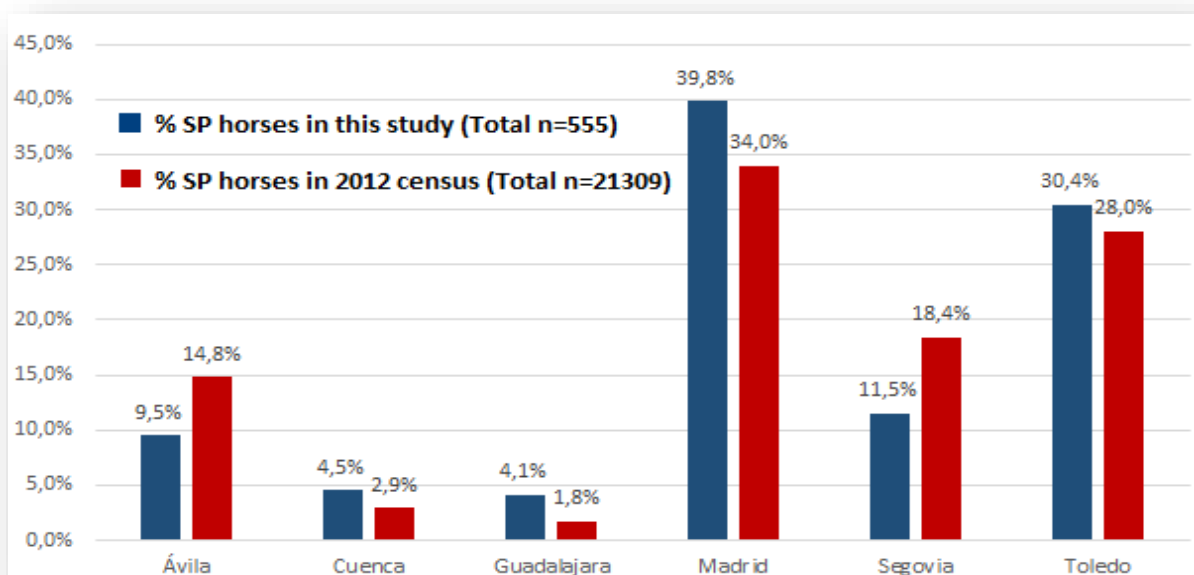


## IV. RESULTS

### IV.1 CHARACTERISTICS OF THE SP HORSE POPULATION SAMPLED.

Between September 2011 and November 2013, serum samples were obtained from 555 SP horses residing in 35 different stud farms in the central region of Spain. The percentages of the horses sampled in each province of this region matched that of 2012 census, as shown in Figure 12.

Figure 12. Distribution by province of 555 SP horses sampled in Central Spain in comparison with the distribution of the general SP breeding population in central Spain published in the census of 2012 (n=21,309).

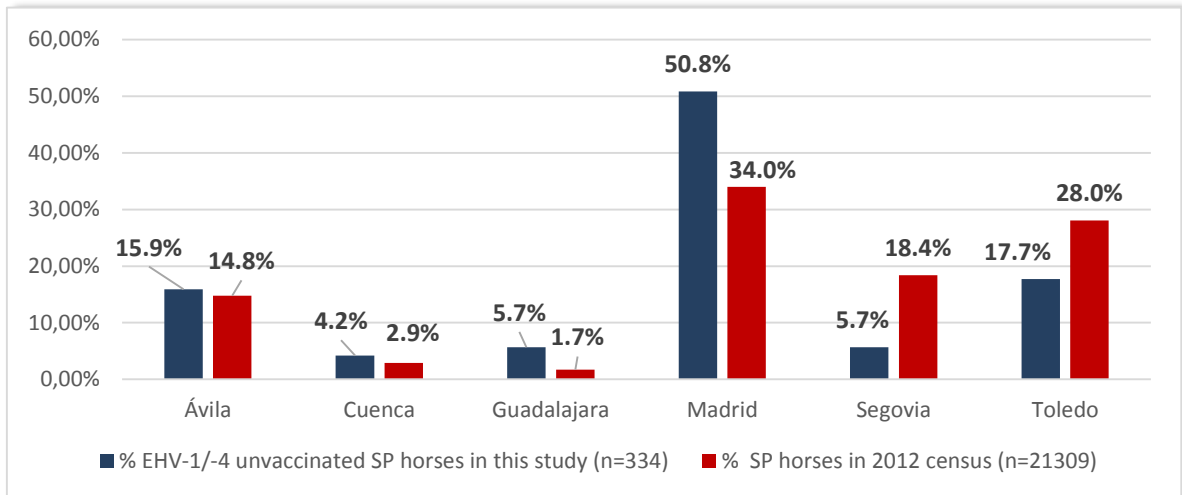


Source: Arca database, data as of 31st December 2012; Instituto Nacional de Estadística, 2012 (INE, 2012; MAGRAMA, 2012).

The subset of horses unvaccinated against EHV-1/-4 used as sample population for EHV-1/-4 seroprevalence (n=334 horses) had a similar distribution by province in central Spain than the whole sample population (n=555 horses). However, there were no data of the EHV-1/-4 unvaccinated horse population in

central Spain and therefore Figure 13 shows the distribution by province of this subset of horses (n=334) in comparison with the general SP breeding horse population in central Spain.

Figure 13. Distribution by province of 334 EHV-1/-4 unvaccinated SP horses sampled in Central Spain in comparison with the distribution of the general SP breeding population in central Spain published in the census of 2012 (n=21,309).

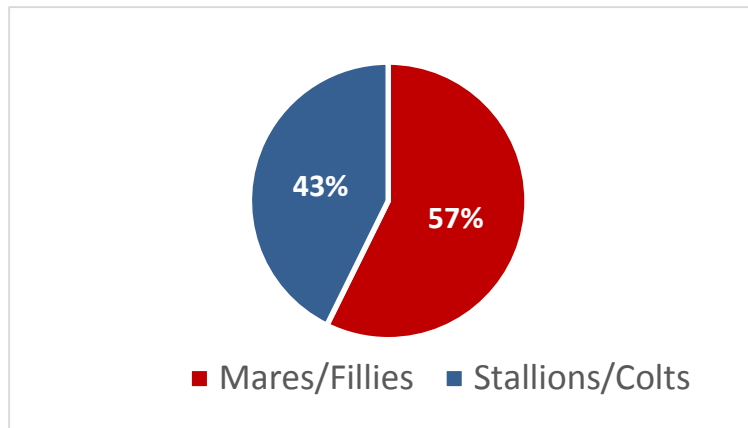


Source: Arca database, data as of 31st December 2012; Instituto Nacional de Estadística, 2012 (INE, 2012; MAGRAMA, 2012).

Figures 14 to 19 show the stratification of the SP horses included in the study (n=555) and the subset used for EHV-1/-4 seroprevalence (n=334) by sex, age, and breeding status.

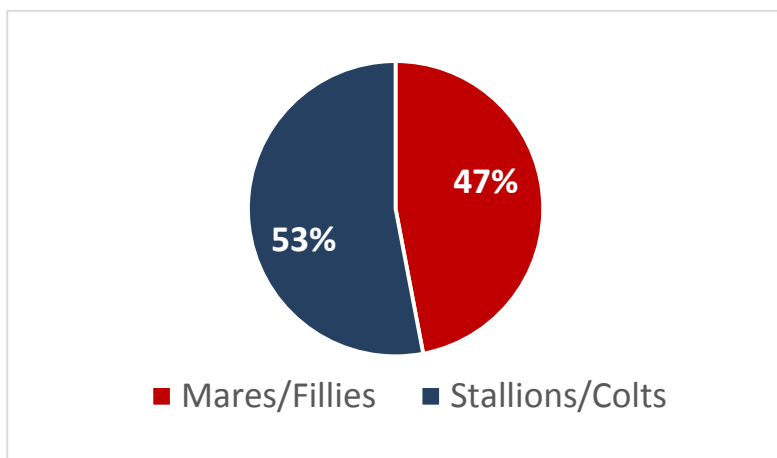
Regarding the distribution of the sample by sex, mares represented a slightly higher proportion of the sample (Figure 14). No geldings were sampled in this study.

Figure 14. Distribution by sex of 555 SP horses sampled in Central Spain.



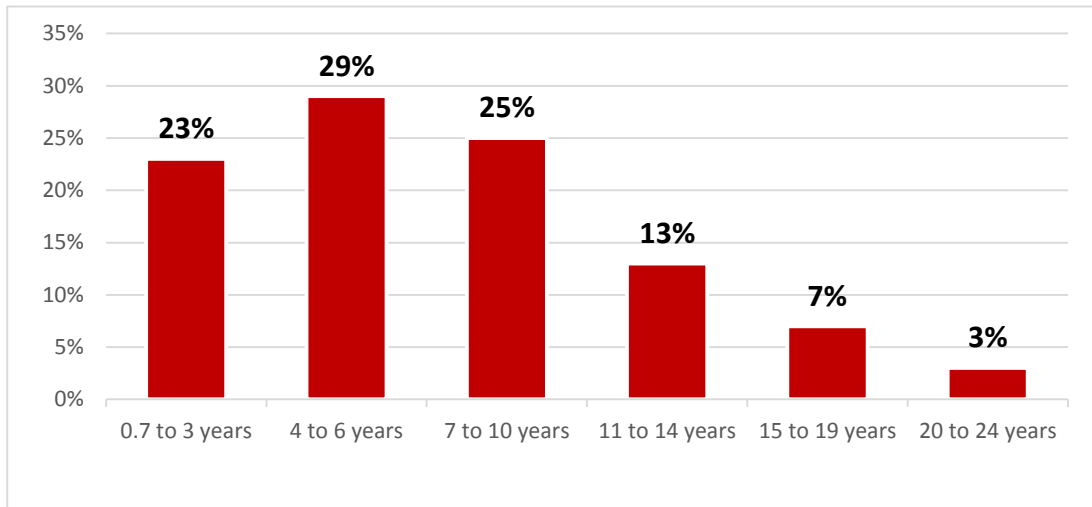
The distribution by sex of the subset of EHV-1/-4 unvaccinated horses used for EHV-1/-4 seroprevalence showed slightly changed percentages, with stallions being more numerous in this sample (Figure 15).

Figure 15. Distribution by sex of 334 EHV-1/-4 unvaccinated SP horses sampled in Central Spain



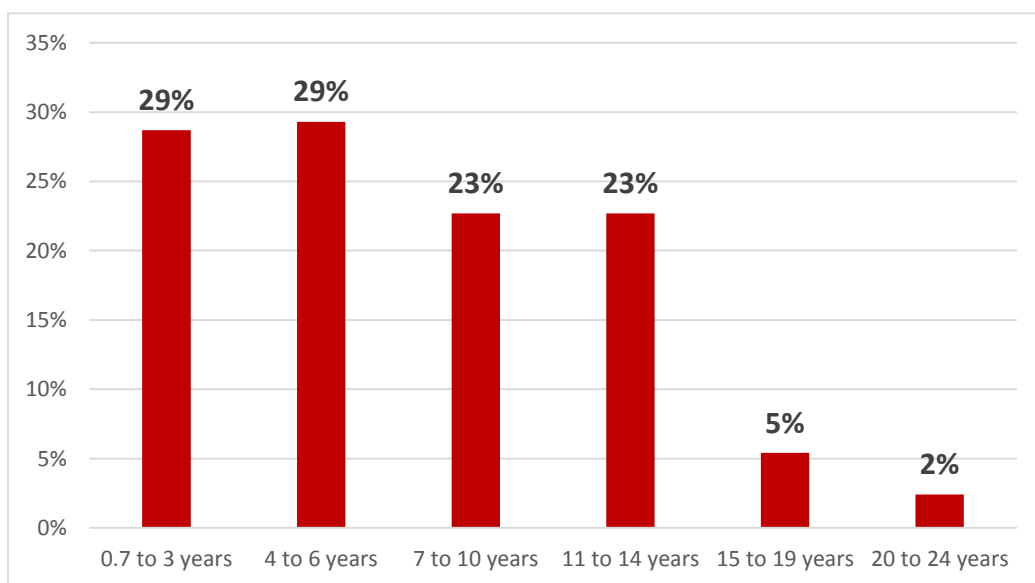
The ages of the horses in the sample (n=555) ranged from 0.7 to 24 years, with a mean age of 7.3 years  $\pm$  SD 5.0 years, a median age of 6 years and interquartile range (IR) of 4 – 10 years. Figure 16 shows the distribution by age group of the SP horses included in the study.

Figure 16. Distribution by age group of the SP horses sampled in Central Spain (n=555).



The ages of the horses in the subset of unvaccinated horses used for EHV-1/-4 seroprevalence (n=334) ranged from 0.7 to 24 years, with a mean age of 6.7 years  $\pm$  SD 4.7 years, a median age of 5.5 years and interquartile range (IR) of 3 – 9 years. Figure 17 shows the distribution by age group of the subset of EHV-1/-4 unvaccinated horses included in the study.

Figure 17. Distribution by age group of the EHV-1/-4 unvaccinated SP horses sampled in Central Spain (n=334).



Regarding the stratification of the sample (n=555) by breeding activity, Figure 18 shows that the majority (63%) of the horses were active breeders. Within the active breeders, nearly three quarters (74%) were mares and 26% were stallions. However, within the inactive breeders (horses aged  $\geq 3$  years old that had been inactive for the past 12 months) and the pre-pubertal horses (horses  $< 3$  years old), the proportions of females and males were the opposite. The stratification of the subset of EHV-1/-4 unvaccinated horses (n=334) by breeding activity showed the same distribution as the general sample population (Figure 19).

Figure 18. Distribution by breeding activity of the 555 SP horses sampled in Central Spain.

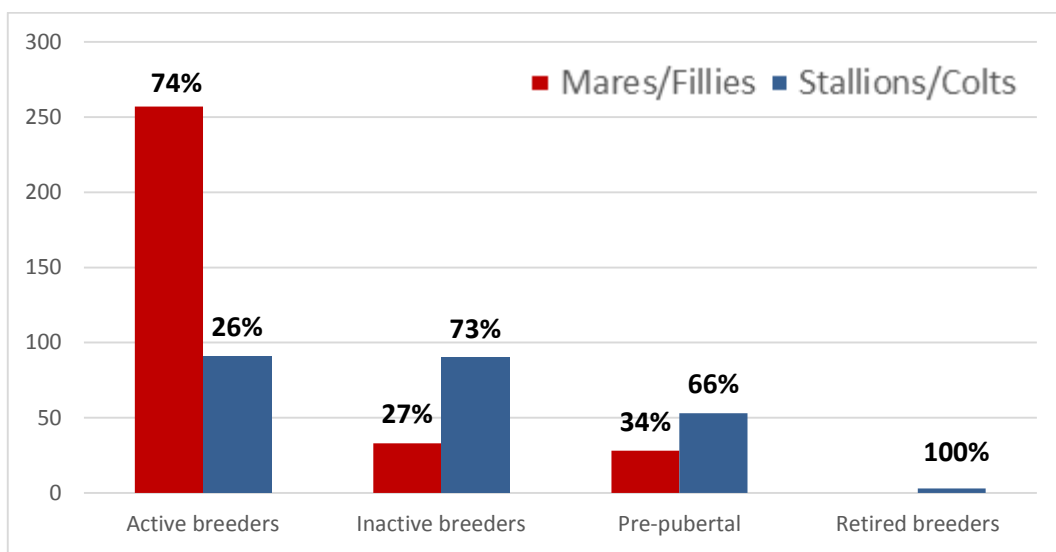
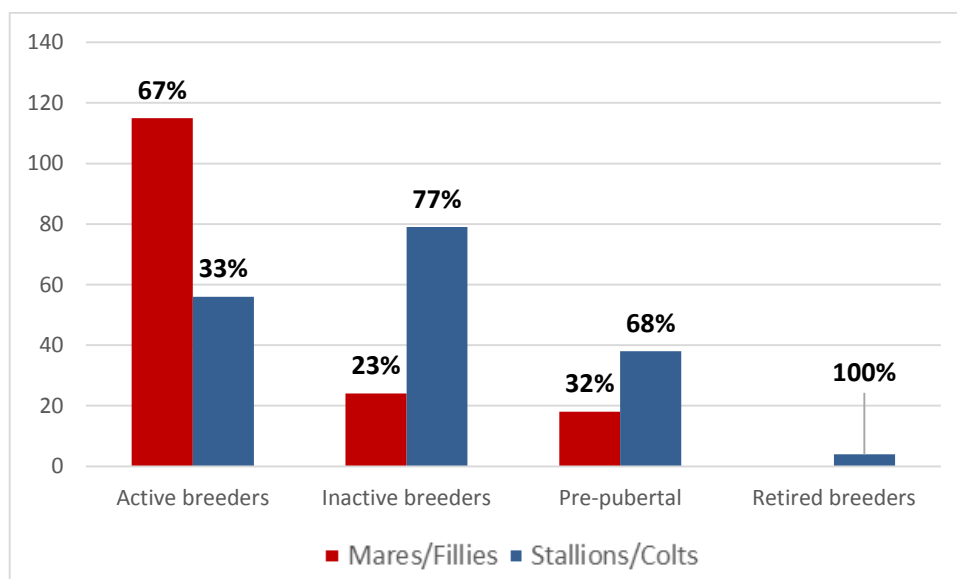


Figure 19. Distribution by breeding activity of the 334 EHV-1/-4 unvaccinated SP horses sampled in Central Spain.



Of the horses currently used for breeding in the sample of SP breeding horses of central Spain, 23% (n=80/348) were reported to have had at least one reproductive problem in the past 12 months. Out of these, 92% were mares and 8% were stallions (Figure 20). Within the active breeding mares, 29% (n=74/257) had a history of reproductive problems, whereas within the active breeding stallions, only 7% (n=6/91) were reported to have reproductive problems. A similar picture was observed in horses used for breeding in the subset of EHV-1/-4 unvaccinated SP horses (n=171), which is shown in Figure 21.

Figure 20. History of reproductive problems within the active SP breeders sampled in Central Spain (n=348, 80 SP breeders with and 268 SP breeders without reproductive problems in the past 12 months).

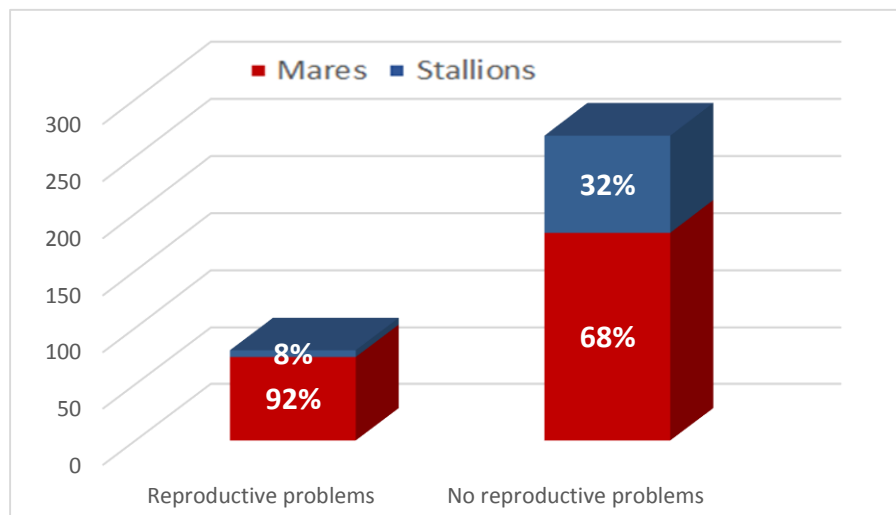


Figure 21 History of reproductive problems within the active EHV-1/-4 unvaccinated SP breeders sampled in Central Spain (n=171, 30 SP breeders with and 141 SP breeders without reproductive problems in the past 12 months).

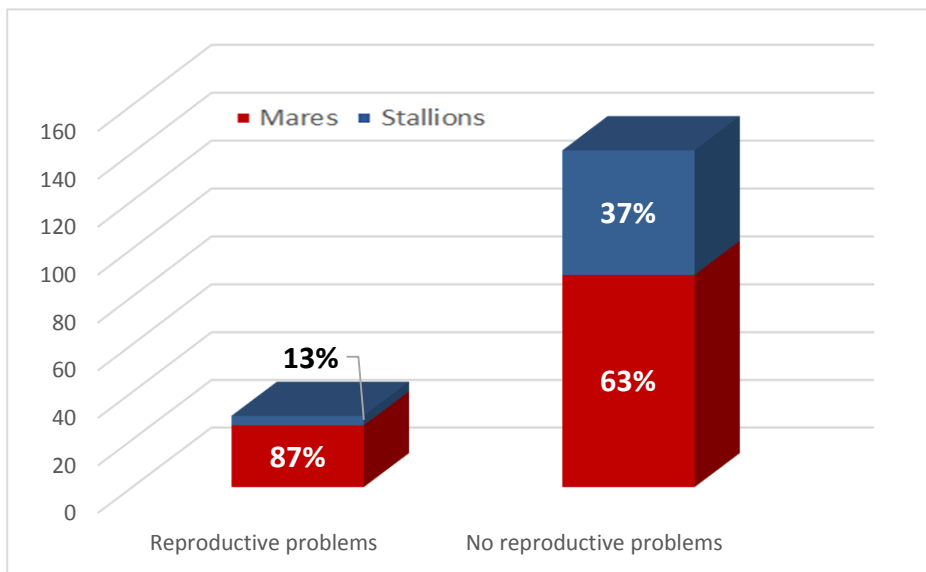
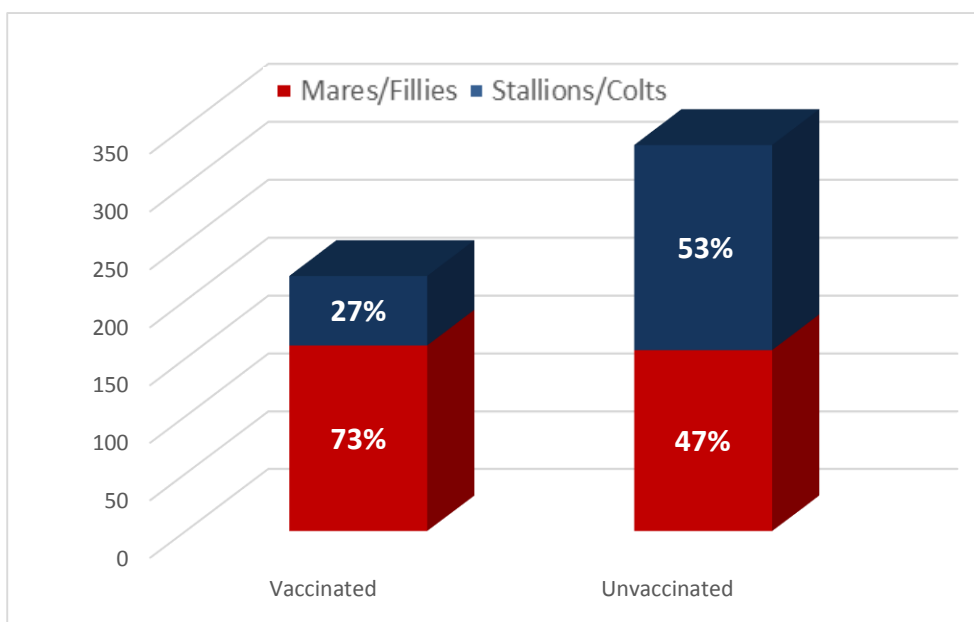


Figure 22 shows the proportion of horses vaccinated and unvaccinated against EHV-1/-4 in the sample (n=555). The majority of horses (60.2%) included in the sample were unvaccinated against EHV-1/-4. Within the vaccinated horses, the number of vaccinated mares was significantly greater than the number of vaccinated stallions ( $p < 0.0001$ ).

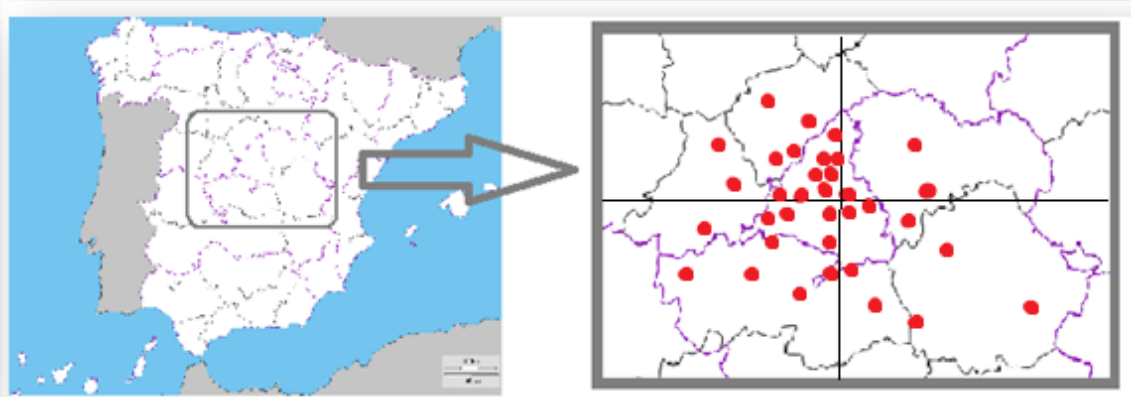
Figure 22. Distribution of horses vaccinated and unvaccinated against EHV-1/-4 in the sample (n=555, 334 unvaccinated and 221 vaccinated SP horses).



## IV.2. DISTRIBUTION OF THE SP BREEDING STUD FARMS SAMPLED.

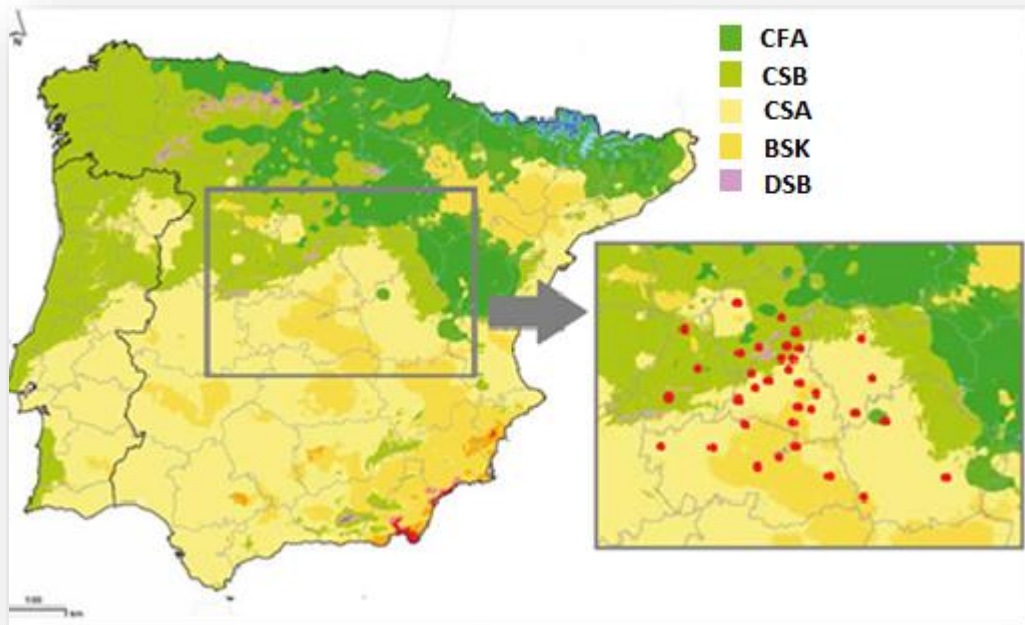
Regarding the distribution of the 35 SP stud farms included in this study, 16 stud farms were located in the province of Madrid, whereas 3 were located in Ávila, 4 were located in Segovia, 6 in Toledo, 3 in Cuenca and 3 in Guadalajara. Figure 23 shows the study area and the distribution of the 35 stud farms sampled in the central area of Spain.

Figure 23. Distribution of the SP stud farms sampled in the central area of Spain.



As shown in Figure 24, the 35 stud farms included in this study had locations with the most representative climates of Spain: 48% of the stud farms with a temperate with dry or hot summer climate (CSA), this climate representing the 40% of the Spanish territory, 23% of the stud farms with a temperate with dry or temperate summer climate (CSB), 23% of the stud farms with a cold steppe climate (BSK), 3% of the stud farms with a cold with temperate and dry summer (DSB) and 3% of the stud farms with a temperate with a dry season and hot summer climate (CFA) (Rubel and Kotttek, 2010).

Figure 24. Köppen-Geiger climatic map with the distribution and different climates of the SP stud farms sampled in this study.



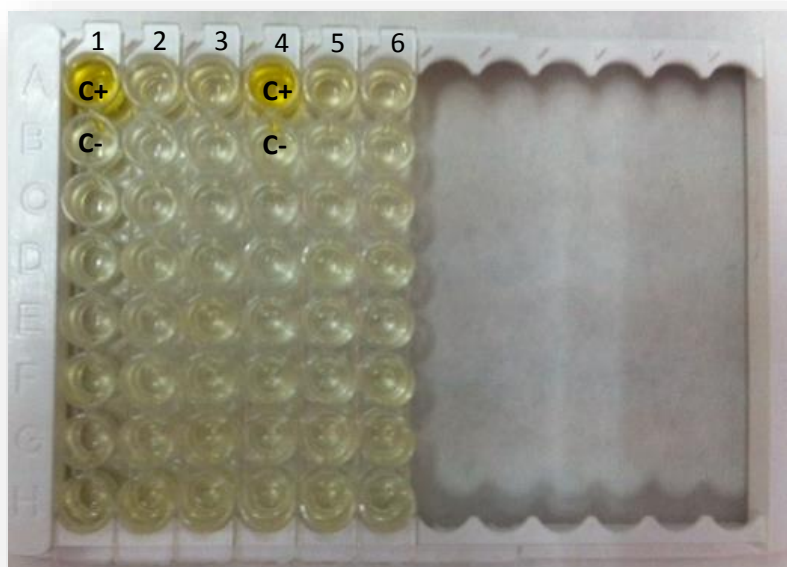
Within the 35 SP stud farms sampled in this study, 5 had an EHV-1/-4 vaccination regime in all the horses and therefore they were excluded from any further analysis related to EHV-1/-4; these stud farms were located in Cuenca (n=1), Madrid (n=1), Toledo (n=2) and Segovia (n=1) and had locations with CSA climate (n=3), BSK climate (n=1) and CSB climate (n=1).

## IV.3 SEROPREVALENCE RESULTS

### IV.3.1 Seroprevalence of Equine Infectious Anaemia

After testing the 555 serum samples for EIA antibodies by ELISA, all samples were identified as negative. Figure 25 shows an EIA ELISA test where wells A1 and A4 were positive controls, wells B1 and B4 were negative controls, and the rest of the wells were samples with a negative result.

Figure 25. Example of an EIA ELISA test carried out in this study.



Based on a census-derived population size of 21,309 SP breeding horses in the central area of Spain (MAGRAMA, 2012) and a sample size of 555 SP horses serologically screened in this study using an ELISA test with a published sensitivity of 100%, there was evidence with 95% confidence that the true prevalence of EIA among SP breeding horses in central Spain was between 0% and 0.53% (De Blas, 2006).

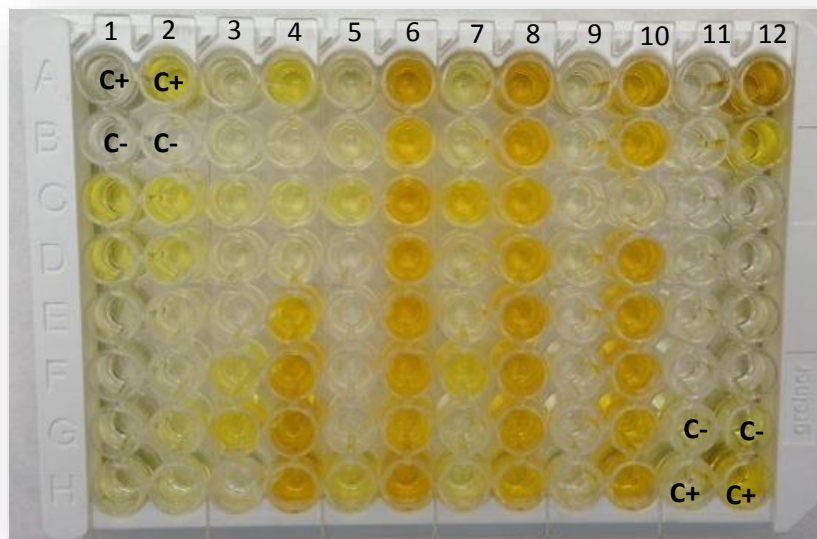
Regarding the maximum possible stud farm seroprevalence, based on a census-derived number of 2,174 SP stud farms registered as of 31st December 2012 in the central area of Spain (provinces of Segovia, Ávila, Madrid, Guadalajara, Cuenca

and Toledo) (MAGRAMA, 2012) and a sample size of 35 SP stud farms serologically screened in this study using an ELISA test with a published sensitivity of 100%, there was evidence with 95% confidence that the true prevalence of EIA among SP stud farms in central Spain was between 0% and 8.14%.

### IV.3.2 Seroprevalence of Equine Viral Arteritis

Using an ELISA with a published sensitivity of 100% and a specificity of 96.75% (Beaufort Cottage Laboratories, 2008), 106 out of 555 serum samples were positive for antibodies against EAV, whereas 8 samples gave equivocal results and 14 were not interpretable (NI). Figure 26 shows an EVA ELISA test in a bi-well format where wells A1-A2 and H11-H12 were positive controls, wells B1-B2 and G11-G12 were negative controls, wells C7-C8 were a sample with a NI result, wells G3-G4 were a sample with a equivocal result, wells E1-E2 for were an example of a serum without antibody against EAV and wells A5-A6 were an example of a serum with antibody against EAV.

Figure 26. Example of an EVA ELISA test carried out in this study.



The true EVA seroprevalence (TS) for the ELISA test used, with a sensitivity of 100% and a specificity of 96.75%, for an apparent seroprevalence of 19.1% and 95% confidence, was 16.4% (12.8% - 20%).

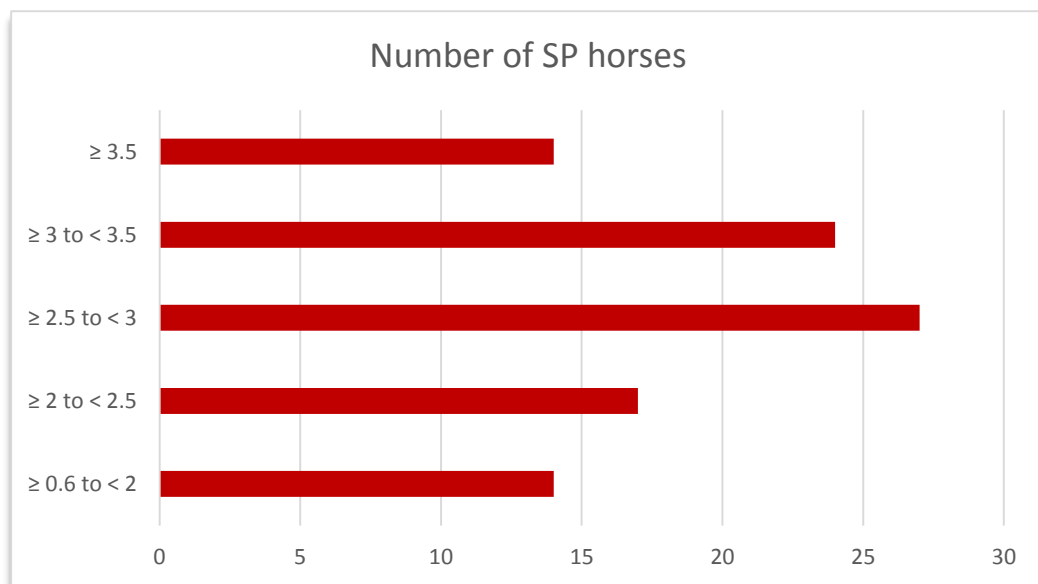
When the positive, equivocal and NI samples were retested using the SN test, 92 out of 106 positive samples by ELISA were positive by SN, 2 out of 8 equivocal samples by ELISA were positive by SN, and 2 out of 14 NI samples by ELISA were positive by SN. A total of 96 samples were therefore positive for EVA by SN (i.e. had

a log<sub>10</sub> titre of ≥ 0.6). The results of both the EVA ELISA and EVA SN for each of the horses sampled in the study are shown in Appendix 4.

The true seroprevalence for EVA using the SN test (gold standard test) in the population of this study, with 95% confidence was 17.3% (14.2% - 20.4%).

Within the 96 EVA seropositive SP horses in the study, 15% had EVA SN log<sub>10</sub> titres ≥ 0.6 and < 2, whereas 18% had EVA SN log<sub>10</sub> titres ≥ 2 and < 2.5, 28% had log<sub>10</sub> titres ≥ 2.5 and < 3, 25% had log<sub>10</sub> titres ≥ 3 and < 3.5, and finally 15% had log<sub>10</sub> titres of ≥ 3.5. Figure 27 shows the number of seropositive SP horses distributed by EVA log<sub>10</sub> titre.

Figure 27. Number of seropositive SP horses distributed by EVA log<sub>10</sub> titre (n=96).

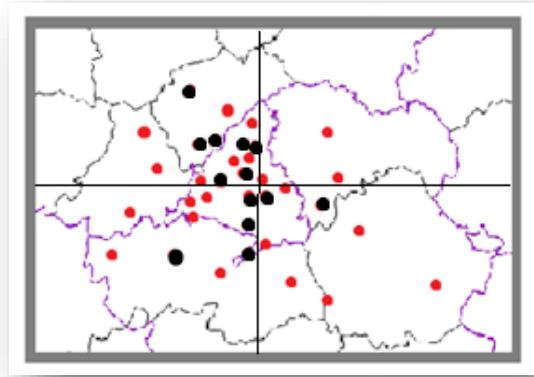


Regarding the stud farms sampled in this study, 13 out of 35 (37.1%) had horses which were seropositive to EAV by SN. The stud farm seroprevalence for EVA in the population of this study, with 95% confidence was between 21.1% and 53.1%. Out of the 13 EVA seropositive stud farms, 5 (38.5%) showed a seroprevalence of < 30%, whereas 6 (46%) showed a seroprevalence between 30% and 60% and 2 (15.5%) showed a seroprevalence of > 60%.

The distribution of the EVA seropositive SP stud farms (black dots) within the central area of Spain is shown in Figure 28. Fifty percent (50%) of the SP stud farms sampled in the NW of this central area were seropositive for EVA, whereas

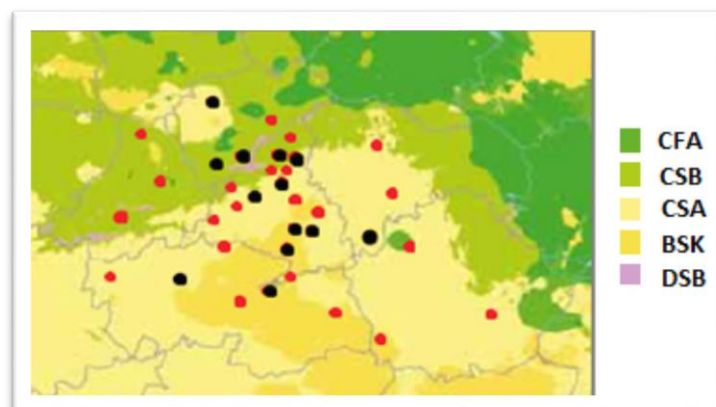
40% were seropositive in the South-West (SW), 25% were seropositive in the South-East (SE) and none were seropositive in the North-East (NE).

Figure 28. Distribution of EVA seropositive stud farms in the area of study.



The distribution of the EVA seropositive SP stud farms (black dots) within the different climate areas in the central area of Spain is shown in Figure 29. Forty-one percent (41%) of the SP stud farms in a location with CSA climate (temperate with dry or hot summer climate) were seropositive, whereas 37.5% of the stud farms in a location with BSK climate (cold steppe climate) were seropositive, 25% of the stud farms in a location with CSB climate (temperate with dry or temperate summer climate) were seropositive, and 100% (1 out of 1 stud farm) in a location with DSB or CFA climate (cold with temperate and dry summer or temperate with a dry season and hot summer respectively) were seropositive.

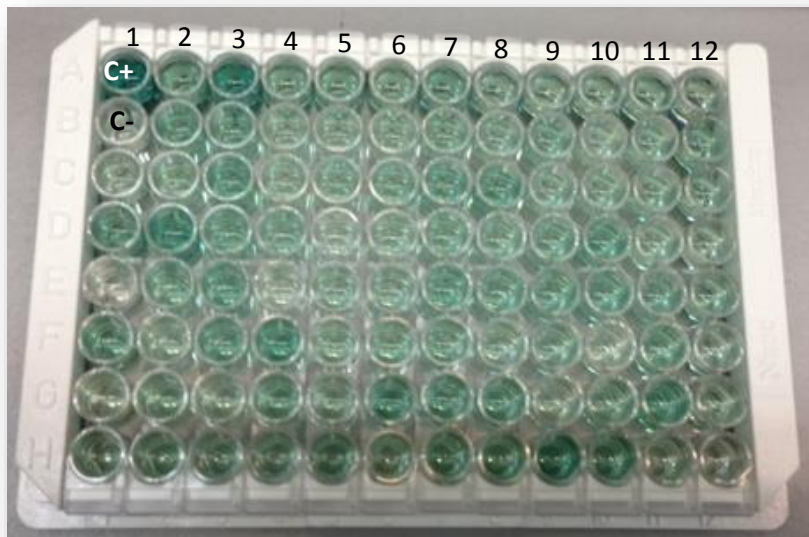
Figure 29. Distribution of EVA seropositive stud farms within the different climate areas in the area of study (n=35).



### IV.3.3 Seroprevalence of EHV-1/-4

Using an ELISA with a sensitivity of 100% and a specificity of 67% according to the manufacturers, 179 out of 334 serum samples were positive for antibodies against EHV-1/-4, whereas 72 samples gave equivocal results. Figure 30 shows an EHV-1/-4 ELISA test where the well A1 is a positive control, well B1 is a negative control, wells A3 or D2 are examples of sera with a positive result, wells C1 or D1 are examples of sera with a negative result and wells A2 or F1 are examples of sera with an equivocal result.

Figure 30. Example of an EHV-1/-4 ELISA test carried out in this study.

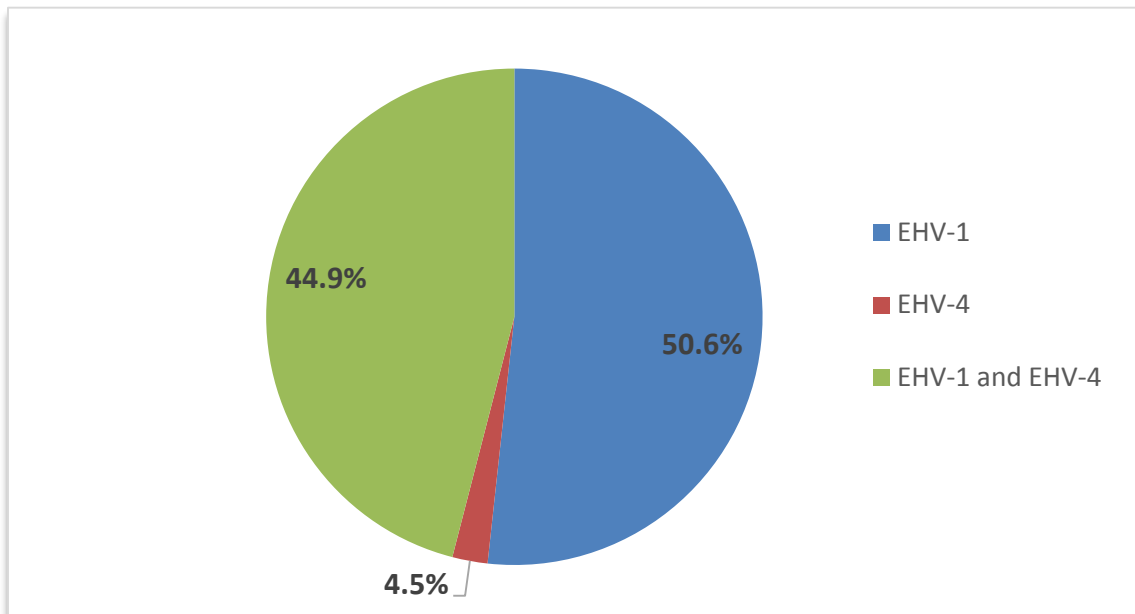


The true EHV-1/-4 seroprevalence (TS) for the ELISA test used, with a sensitivity of 100% and a specificity of 67%, for an apparent seroprevalence of 53.6% and 95% confidence, was 30.7% (22.7% – 38.8%).

When the positive and equivocal samples were retested using the SN test, 141 out of 179 positive samples by ELISA were positive for either EHV-1, EHV-4 or both by SN, and 37 out of 72 equivocal samples by ELISA were positive for either EHV-1, EHV-4 or both by SN. A total of 178 samples were therefore positive for either EHV-1, EHV-4 or both by SN (i.e. had a  $\log_{10}$  titre of  $\geq 2.3$  for EHV-1 and/or  $\geq 2.0$  for EHV-4). Out of these 178 positive samples, 90 (50.6%) were positive for

EHV-1 only, 80 (44.9%) were positive for both EHV-1 and EHV-4 and only 8 (4.5%) were positive for EHV-4 only. Figure 31 shows the percentages of horses that were seropositive to EHV-1, EHV-4 or both EHV-1 and EHV-4 in the VN test.

Figure 31. Percentage of SP horses seropositive to EHV-1, EHV-4 or both in the VN test (n=178, 90 SP horses seropositive to EHV-1 only, 80 SP horses seropositive to both EHV-1 and EHV-4 and 8 SP horses seropositive to EHV-4 only)



The true seroprevalence for EHV-1/-4 using the SN test (gold standard test) in the population of this study, with 95% confidence was 53.3% (47.9% - 58.6%). Since the SN test differentiates between EHV-1 and EHV-4 (although there can be a certain grade of cross-reactions between both viruses), individual seroprevalence for EHV-1 and EHV-4 could be calculated; EHV-1 seroprevalence by SN was 50.9% (45.5% - 56.3% with 95% confidence), whereas EHV-4 seroprevalence by SN was 26.3% (21.6% - 31.1% with 95% confidence). The results of both the EHV-1/-4 ELISA and EHV-1 and EHV-4 SN for each of the horses sampled in the study are shown in Appendix 4.

Within the 178 EHV-1/-4 positive horses, 23 had a positive titre to EAV, therefore the seroprevalence of EVA and EHV-1/-4 in this sample was 6.9% (4.2% - 9.6% with 95% confidence).

Regarding the stud farms sampled in this study where there were any horses non-vaccinated against EHV-1/-4, 27 out of 30 (90%) had horses which were seropositive to EHV-1/-4 by SN within the horses unvaccinated against EHV-1/-4. The stud farm seroprevalence for EHV-1/-4 in the population of this study, with 95% confidence was between 79.3% and 100%. Out of the 27 EHV-1/-4 seropositive stud farms, 4 (15%) showed a seroprevalence of < 30%, whereas 9 (33%) showed a seroprevalence between  $\geq 30\%$  and <60%, 13 (48%) showed a seroprevalence between  $\geq 60\%$  and <90% and only 1 (4%) had a seroprevalence of > 90%.

The three EHV-1/-4 seronegative SP stud farms sampled in this study were located in the NW of the central area of Spain and had different climate types; one of them was located in an area with CFA (temperate with dry season and hot summer) climate, another was located in a location with CSA (temperate with dry or hot summer) climate and the third was located in an area with DSB (cold with temperate and dry summer) climate.

## IV.4 GENERAL MANAGEMENT AND HEALTH IN SP BREEDING STUD FARMS IN CENTRAL SPAIN.

### IV.4.1 Descriptive analysis

Regarding the location of the 35 participating stud farms in the central area of Spain shown in Table 7, 45% of the 35 participating stud farms were located in the province of Madrid. Looking at the area of study as a whole (Table 8), the stud farms were equally distributed in the North and South of the central area of Spain.

Table 7. Distribution by provinces of the 35 SP stud farms sampled in the central area of Spain.

| Province     | SP Stud Farms No | SP Stud Farms % |
|--------------|------------------|-----------------|
| Madrid       | 16               | 45%             |
| Ávila        | 3                | 9%              |
| Segovia      | 4                | 11%             |
| Toledo       | 6                | 17%             |
| Cuenca       | 3                | 9%              |
| Guadalajara  | 3                | 9%              |
| <i>Total</i> | <b>35</b>        | <b>100%</b>     |

Table 8. Distribution by area of the 35 SP stud farms sampled in the central area of Spain.

| Area         | SP Stud Farms No | SP Stud Farms % |
|--------------|------------------|-----------------|
| North        | 17               | 49%             |
| South        | 18               | 51%             |
| <i>Total</i> | <b>35</b>        | <b>100%</b>     |
| North-West   | 14               | 40%             |
| North-East   | 3                | 9%              |
| South-West   | 10               | 29%             |
| South-East   | 8                | 22%             |
| <i>Total</i> | <b>35</b>        | <b>100%</b>     |

Regarding the climates in the stud farms locations, 17 stud farms (48%) had a temperate with dry or hot summer climate (CSA), 8 stud farms (23%) had a temperate with dry or temperate summer climate (CSB), 8 (23%) had a cold steppe climate (BSK), 1 stud farm (3%) had a cold with temperate and dry summer climate (DSB) and 1 stud farm (3%) had a temperate with a dry season and hot summer climate (CFA).

The SP stud farms included in this study had a total area surface ranging from 0.004 to 13 km<sup>2</sup> (mean 0.94 km<sup>2</sup> ± SD 2.35 km<sup>2</sup>; median 0.1 km<sup>2</sup>, IR 0.03 – 0.3). The number and percentage of stud farms by total area surface is shown in Table 9.

Table 9. Distribution by total area surface of the 35 SP stud farms sampled in central Spain.

| Area surface               | SP Stud Farms No | SP Stud Farms % |
|----------------------------|------------------|-----------------|
| < 0.1 km <sup>2</sup>      | 16               | 46%             |
| ≥0.1 to <1 km <sup>2</sup> | 11               | 31%             |
| ≥1 to <4 km <sup>2</sup>   | 7                | 20%             |
| > 4 km <sup>2</sup>        | 1                | 3%              |
| <b>Total</b>               | <b>35</b>        | <b>100%</b>     |

Regarding the area used for breeding, the 35 SP stud farms of this study had a surface ranging from 0.002 to 6.5 km<sup>2</sup> (mean 0.53 km<sup>2</sup> ± SD 1.27 km<sup>2</sup>; median 0.07 km<sup>2</sup>, IR 0.02 – 0.2). The number and percentage of stud farms by area surface used for breeding is shown in Table 10.

Table 10. Distribution by breeding surface of the 35 SP stud farms sampled in central Spain.

| Breeding surface           | SP Stud Farms No | SP Stud Farms % |
|----------------------------|------------------|-----------------|
| < 0.1 km <sup>2</sup>      | 21               | 60%             |
| ≥0.1 to <1 km <sup>2</sup> | 8                | 23%             |
| ≥1 to <4 km <sup>2</sup>   | 5                | 14%             |
| > 4 km <sup>2</sup>        | 1                | 3%              |
| <b>Total</b>               | <b>35</b>        | <b>100%</b>     |

The population density on the stud farms ranged from 7 to 11000 horses/km<sup>2</sup> (mean 1063 horses/km<sup>2</sup> ± SD 2077 horses/km<sup>2</sup>; median 400 horses/km<sup>2</sup>, IR 125 – 950 horses/km<sup>2</sup>). As displayed in Table 11, 60% of the stud farms had ≥100 to <1000 horses/km<sup>2</sup>.

Table 11. Distribution by population density of the 35 SP stud farms sampled in central Spain.

| Population density                    | SP Stud Farms No | SP Stud Farms % |
|---------------------------------------|------------------|-----------------|
| < 100 horses/km <sup>2</sup>          | 6                | 17%             |
| ≥100 to <500 horses/km <sup>2</sup>   | 13               | 37%             |
| ≥500 to <1000 horses/km <sup>2</sup>  | 8                | 23%             |
| ≥1000 to <3000 horses/km <sup>2</sup> | 6                | 17%             |
| ≥3000 horses/km <sup>2</sup>          | 2                | 6%              |
| <b>Total</b>                          | <b>35</b>        | <b>100%</b>     |

Of the 35 SP stud farms, the oldest farm started its activity in 1965 and the newest started in 2013. Stud farms starting their activity before 1990 represented a quarter of the total stud farms (9 farms, 26%), same as those starting between 1990 and 1999 (9 farms, 26%) and those starting between 2000 and 2009 (9 farms, 26%), whereas those starting between 2010 and 2013 were represented by a 22% (8 farms).

Within the stud farms sampled, 60% (21 stud farms) were linked to a breeders association. Of these, 18 (86%) were associated to ANCCE; other breeders associations were ASOCAMAN (*Asociación de Castilla La Mancha de Pura Raza Española (P.R.E.)*), CESCALLE (*Asociación de Criadores de Caballos P.R.E. de Castilla y Leon*) and UCCE (*Unión de Criadores de Caballos P.R.E.*).

In addition to SP breeding, 46% of stud farms sampled (16 stud farms) involved in other equine industry activities and the most frequently reported activities were dressage (n=9/16), liveries (n=6/16), and equine hospitals (n=2/16). Other additional activities less frequently reported included bullfighting, farriers teaching, carriage driving, show jumping or hacking.

All SP stud farms had animals other than horses in the premises. The most common were dogs (86%), and cats (40%). Other animals in the premises were chickens (17%), other birds (20%), goats (14%), cattle (11%), sheep (9%), donkeys (6%), pigs (3%), rabbits and deer (both 3%).

The SP stud farms included in this study had a number of horses ranging 6 to 220 (mean  $48.1 \pm SD 47.3$  horses; median 32 horses, IR 16–61). The majority of them (86%,  $n=30/35$ ) had  $\leq 70$  horses of all breeds and also  $\leq 70$  SP horses. The distributions of SP stud farms by number of horses of all breeds and number of SP horses are shown in Table 12 and Table 13, respectively.

Table 12. Distribution of the 35 SP stud farms sampled in central Spain by number of horses of all breeds.

| Number of horses of all breeds | SP Stud Farms No | SP Stud Farms % |
|--------------------------------|------------------|-----------------|
| $\leq 15$ horses               | 8                | 23%             |
| $>15$ to $\leq 30$ horses      | 9                | 26%             |
| $>30$ to $\leq 70$ horses      | 13               | 37%             |
| $>70$ horses                   | 5                | 14%             |
| <b>Total</b>                   | <b>35</b>        | <b>100%</b>     |

Table 13. Distribution of the 35 SP stud farms sampled in central Spain by number of SP horses.

| Number of SP horses       | SP Stud Farms No | SP Stud Farms % |
|---------------------------|------------------|-----------------|
| $\leq 15$ horses          | 10               | 29%             |
| $>15$ to $\leq 30$ horses | 8                | 23%             |
| $>30$ to $\leq 70$ horses | 12               | 34%             |
| $>70$ horses              | 5                | 14%             |
| <b>Total</b>              | <b>35</b>        | <b>100%</b>     |

The SP stud farms included in this study had a number of SP breeding mares ranging from 0 to 65 (mean  $16.3 \pm SD 15.8$  mares; median 12 mares, IR 4–22). The distribution of SP stud farms by number of SP breeding mares is shown in Table 14.

Most of the SP stud farms (77%) had no SP mares retired from breeding, 12% had only one retired mare and 11% had two retired mares.

Table 14. Distribution of the 35 SP stud farms sampled in central Spain by number of SP breeding mares.

| Number of breeding mares | SP Stud Farms No | SP Stud Farms % |
|--------------------------|------------------|-----------------|
| ≤10 mares                | 17               | 49%             |
| >10 to ≤30 mares         | 12               | 34%             |
| >30 mares                | 6                | 17%             |
| <b>Total</b>             | <b>35</b>        | <b>100%</b>     |

The 35 SP stud farms had a number of SP breeding stallions ranging from 0 to 15 (mean  $3.0 \pm$  SD 3.4 stallions; median 2 stallions, IR 1–3). The distribution of SP stud farms by number of SP breeding stallions is shown in Table 15. Most of the SP stud farms (71%) had no SP stallions retired from breeding, 17% had only one retired stallion and 12% had 2-6 retired stallions.

Table 15. Distribution of the 35 SP stud farms sampled in central Spain by number of SP breeding stallions.

| Number of breeding stallions | SP Stud Farms No | SP Stud Farms % |
|------------------------------|------------------|-----------------|
| 0 to ≤2 stallions            | 21               | 60%             |
| >2 to ≤5 stallions           | 8                | 23%             |
| >5 stallions                 | 6                | 17%             |
| <b>Total</b>                 | <b>35</b>        | <b>100%</b>     |

The number of geldings and/or stallions older than 4 years old but without a history of covering mares ranged from 0 to 80 horses (mean  $6.8 \pm$  SD 15.6 horses; median 1 horse, IR 0–4). Eighty percent (80%) of the stud farms (28 farms) had ≤ 5 and 20% had > 5 geldings and/or stallions older than 4 years old. Regarding teasers, 94% of the stud farms had no teasers, and only 6% of farms had 1 teaser in the premises.

Regarding the presence of young horses in the SP stud farms sampled, the number of suckling foals ranged from 0 to 21 (mean  $4.4 \pm \text{SD } 5.7$  foals; median 3 foals, IR 0–6). The number of yearlings ranged from 0 to 22 (mean  $5.6 \pm \text{SD } 6.7$  yearlings; median 2 yearlings, IR 1-10). Finally, the number of 2 and 3 year-old horses ranged from 0 to 40 (mean  $8.1 \pm \text{SD } 10.3$  horses; median 5 yearlings, IR 2-7).

A total of 17 stud farms (49%) reported that they routinely grouped all breeding mares together with the young horses (fillies and colts < 2 years old), while a further 20% of stud farms (n=7) reported that they grouped all breeding mares together, and segregated the youngsters. Other herd management practices reported were grouping mares with suckling foals in one group, pregnant and barren mares in other group, and youngsters in other group (11% of the stud farms), grouping mares with suckling foals in one group, pregnant mares in other group, and barren mares together with youngsters in other group (6%), and finally having four groups: mares with suckling foals, pregnant mares, barren mares, and youngsters (14%). The presence of smaller groups within these categories was recorded only in 9 stud farms (26%).

The majority of stud farms (74%) housed breeding stallions and colts > 2 years old in stables, while 23% of stud farms reported that breeding stallions were stabled and colts > 2 years old were kept together in a large paddock. The third way of housing these horses, although less frequent (3% of stud farms), was to have each horse in an individual paddock.

Regarding the movements to fairs and/or competitions in the SP stud farms of the study, most of the stud farms (19 out of 35, 54%) had not attended fairs and/or competitions with their horses in the past 12 months. Of those farms that did report movement of horses off the premises, 10 stud farms (29%) went to <5 fairs and/or competitions, whereas 6 (17%) went to  $\geq 5$  fairs and/or competitions in the past 12 months.

Within the SP stud farms, nearly three quarters had not taken their stallions or mares to other studs for breeding purposes in the past 12 months (74% and 77% respectively). The number of new horses acquired by the owners of the stud farms in the past 12 months ranged from 0 to 15 (mean  $3.1 \pm \text{SD } 4.9$  horses; median 1

horse, IR 0–3). The number of horses sold ranged from 0 to 40 (mean  $5.1 \pm$  SD 7.4 horses; median 4 horses, IR 0–6). The distribution of SP stud farms by number of horses acquired and sold is presented in Table 16 and Table 17, respectively.

Table 16. Distribution of the 35 SP stud farms sampled in central Spain by number of horses acquired.

| Number of horses acquired | SP Stud Farms No | SP Stud Farms % |
|---------------------------|------------------|-----------------|
| 0 horses                  | 16               | 46%             |
| >0 to ≤5 horses           | 12               | 34%             |
| >5 horses                 | 7                | 20%             |
| <b>Total</b>              | <b>35</b>        | <b>100%</b>     |

Table 17. Distribution of the 35 SP stud farms sampled in central Spain by number of horses sold.

| Number of horses sold | SP Stud Farms No | SP Stud Farms % |
|-----------------------|------------------|-----------------|
| 0 horses              | 10               | 29%             |
| >0 to ≤5 horses       | 14               | 40%             |
| >5 horses             | 11               | 31%             |
| <b>Total</b>          | <b>35</b>        | <b>100%</b>     |

Regarding movement of stallions and mares onto the stud farm from other stud farms for breeding purposes, 80% of the stud farms reported no movements of stallions onto the premises for breeding purposes and within the remaining 20% there were 2 stud farms which were breeding and AI centres and which had 100 stallions coming onto the premises for semen extraction in the past 12 months. Regarding visitor mares, 20 out of 35 stud farms (57%) had no visitor mares coming into the premises for breeding purposes in the past 12 months and within the remaining 43% there were 4 stud farms which had 30 to 100 mares coming into the

premises for being covered/inseminated with any of the stallions owned by the stud farm.

Finally, the visits from people not belonging to the stud farm into the premises per month ranged from 1 to 1500 (mean  $114.5 \pm$  SD 272.8 visits from people; median 15 visits from people, IR 5–80). The distribution of SP stud farms by number of visits from people coming into the premises per month is presented in Table 18.

Table 18. Distribution of the 35 SP stud farms sampled in central Spain by number of people coming into the premises per month.

| Number of visits from people per month | SP Stud Farms No | SP Stud Farms % |
|--|------------------|-----------------|
| $\leq 10$ people                       | 14               | 40%             |
| >10 to $\leq 50$ people                | 10               | 29%             |
| >50 people                             | 11               | 31%             |
| <i>Total</i>                           | <b>35</b>        | <b>100%</b>     |

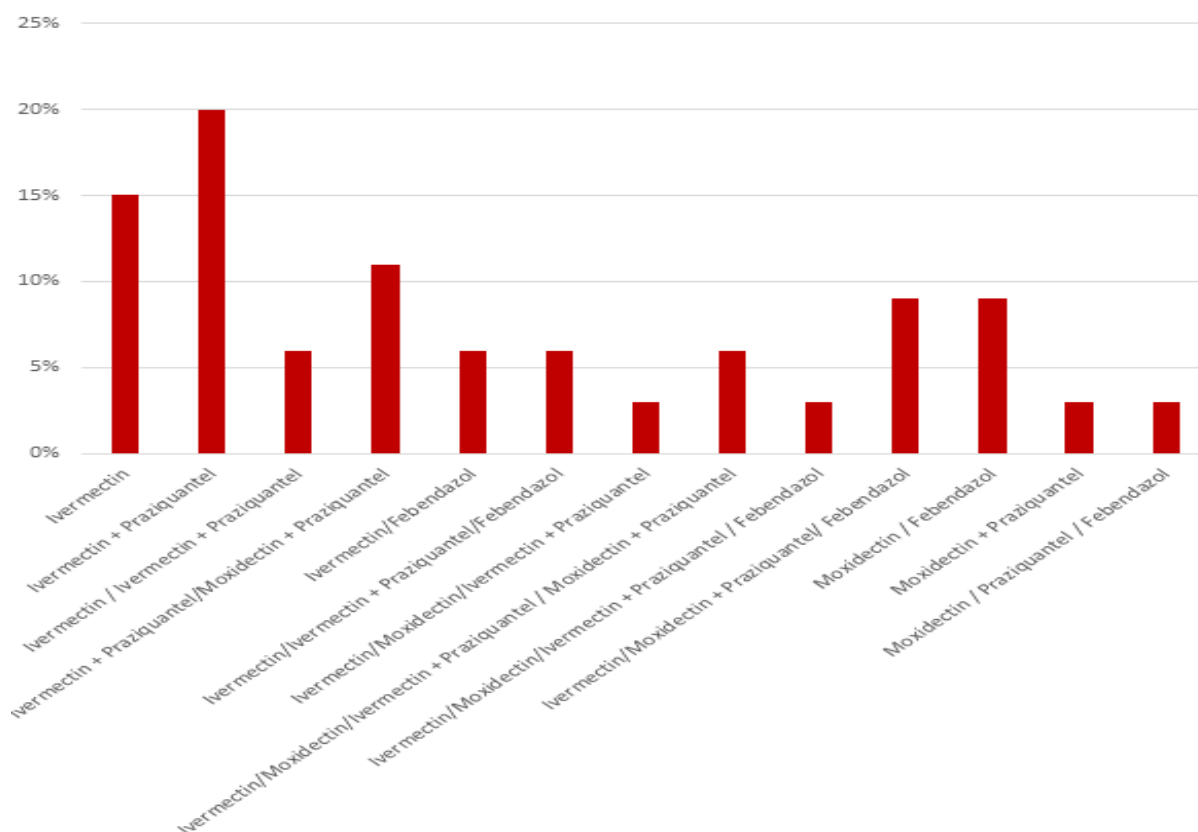
Regarding the implementation of vaccination regimes for several equine diseases, the distributions of SP stud farms by presence of vaccination regimes following the manufacturer instructions for Tetanus, Equine Influenza, EHV-1/-4 and West Nile Virus (WNV) are shown in Table 19.

Table 19. Distribution of the 35 SP stud farms sampled in central Spain by presence of a vaccination regime for Tetanus, Equine Influenza, EHV-1/-4 and WNV.

| Vaccination regime                  | SP Stud Farms No | SP Stud Farms % |
|-------------------------------------|------------------|-----------------|
| <i>Tetanus</i>                      |                  |                 |
| Yes, in all horses                  | 25               | 71%             |
| No                                  | 10               | 29%             |
| <b>Total</b>                        | <b>35</b>        | <b>100%</b>     |
| <i>Equine Influenza</i>             |                  |                 |
| Yes, in all horses                  | 25               | 71%             |
| No                                  | 10               | 29%             |
| <b>Total</b>                        | <b>35</b>        | <b>100%</b>     |
| <i>EHV-1/-4</i>                     |                  |                 |
| Yes, in all horses                  | 5                | 14%             |
| No                                  | 16               | 46%             |
| Active breeders/Pregnant mares only | 14               | 40%             |
| <b>Total</b>                        | <b>35</b>        | <b>100%</b>     |
| <i>West Nile Virus</i>              |                  |                 |
| Yes, in all horses                  | 1                | 3%              |
| No                                  | 34               | 97%             |
| <b>Total</b>                        | <b>35</b>        | <b>100%</b>     |

Within the 35 SP stud farms, a worming regime was implemented in all horses in 32 stud farms (91%), whereas the regime was implemented in active breeders in 2 farms (6%) and no worming regime was implemented in one stud farm (3%). Within the stud farms which had a worming regime (n=34), most of them wormed their horses twice a year (76%), whereas the rest wormed their horses 3 or 4 times a year (24%). The anthelmintic products used in the 34 SP stud farms with a worming regime is shown in Figure 32.

Figure 32. Percentages of the different wormers used in the 35 SP stud farms sampled in central Spain



Regarding the implementation of disinfection regimes, and preventive measures against insects and rodents in the premises, distributions of SP stud farms by presence of these regimes are shown in Table 20, Table 22 and Table 23 respectively, whereas the products or measures used in the disinfection of the SP stud farms of this study are presented in Table 21.

Table 20. Distribution of the 35 SP stud farms sampled in central Spain by presence of a disinfection regime.

| Presence of disinfection regime | SP Stud Farms No | SP Stud Farms % |
|---------------------------------|------------------|-----------------|
| Yes                             | 29               | 83%             |
| No                              | 6                | 17%             |
| <b>Total</b>                    | <b>35</b>        | <b>100%</b>     |

Table 21. Distribution of the 35 SP stud farms sampled in central Spain by use of different products and regimes for disinfection.

| Products and regimes used for disinfection | SP Stud Farms No | SP Stud Farms % |
|--|------------------|-----------------|
| <i>Zotal</i> daily                         | 11               | 38%             |
| <i>Zotal</i> once a week                   | 4                | 14%             |
| <i>Zotal</i> twice a month                 | 3                | 11%             |
| <i>Zotal</i> once a month                  | 4                | 14%             |
| <i>Zotal</i> twice a year                  | 1                | 3%              |
| <i>Despadac</i> once a week                | 3                | 11%             |
| <i>Zotal</i> + <i>Despadac</i> once a week | 1                | 3%              |
| <i>Zotal</i> + Limestone once a month      | 1                | 3%              |
| Bleach once a week                         | 1                | 3%              |
| <b>Total</b>                               | <b>29</b>        | <b>100%</b>     |

*Zotal*: 4-cloro-3-metilphenol 0,8% - 4-cloro-2-bencilphenol 0,4% - 2-fenilphenol 1,5%. *Despadac*: quarternary amonium and aldehydes

The areas in the SP stud farms most commonly disinfected were the stables (disinfected in 100% of stud farms), corridors (disinfected in 24% of stud farms), sand paddocks (disinfected in 14% of stud farms) and the areas where the feed was kept (disinfected in 10% of the stud farms).

Table 22. Distribution of the 35 SP stud farms sampled in central Spain by presence of measures against insects.

| Presence of measures against insects | SP Stud Farms No | SP Stud Farms % |
|--------------------------------------|------------------|-----------------|
| Yes                                  | 29               | 83%             |
| No                                   | 6                | 17%             |
| <b>Total</b>                         | <b>35</b>        | <b>100%</b>     |

Within the 29 stud farms which had measures against insects, *Agita* (Tiametoxam 10%; 9-tricosene 0,05%) was used in the surfaces of 38% of stud farms, whereas cypermethrin was applied topically for horses on 48% of stud farms, deltamethrin was applied topically for the horses on 7% of stud farms, *Zotal*

(4-cloro-3-metilphenol 0,8% - 4-cloro-2-bencilphenol 0,4% - 2-fenilphenol 1,5%) was used in 7% of stud farms and other measures such as ultraviolet lamps, nets, fly-capture sticks, or garlic in feed were implemented in 14% of stud farms.

Table 23. Distribution of the 35 SP stud farms sampled in central Spain by presence of measures against rodents.

| Presence of measures against rodents | SP Stud Farms No | SP Stud Farms % |
|--------------------------------------|------------------|-----------------|
| Yes                                  | 28               | 80%             |
| No                                   | 7                | 20%             |
| <b>Total</b>                         | <b>35</b>        | <b>100%</b>     |

Within the 28 stud farms that had measures implemented against rodents, cats were used in 39% of the stud farms, dogs were used in 21% of farms, poison boxes were used in 29% of stud farms, traps were used in 21% of stud farms and other measures such as electronic devices or hiring a company to treat rodents in the farm were used in 7% of the stud farms.

Regarding the use of natural cover (NC) and artificial insemination (AI) in the stallions of the SP stud farms of this study (n= 29 stud farms with presence of breeding stallions), the distribution of stud farms by the use of NC and AI is shown in Table 24.

Table 24. Distribution of the 35 SP stud farms sampled in central Spain by % use of Natural cover (NC) and Artificial Insemination (AI) in their stallions.

| % use of NC / % use of AI in the stallions | SP Stud Farms No | SP Stud Farms % |
|--|------------------|-----------------|
| 0% NC / 100% AI                            | 6                | 21%             |
| 10 - 50% NC / 50 - 90% AI                  | 4                | 14%             |
| 60 - 90% NC / 10 - 40% AI                  | 5                | 17%             |
| 100% NC / 0% AI                            | 14               | 48%             |
| <b>Total</b>                               | <b>29</b>        | <b>100%</b>     |

Out of the 29 stud farms with presence of breeding stallions, only 5 (18%) reported having stallions with reproductive problems. The percentage of stallions with reproductive problems in the 29 stud farms ranged from 0% to 100% (mean 7.2%  $\pm$  SD 21.9%, median 0, IR 0 – 0).

Regarding the use of NC and AI in the mares of the SP stud farms of this study (n= 34 stud farms with presence of breeding mares), the distribution of stud farms by the use of NC and AI is shown in Table 25.

Table 25. Distribution of the 35 SP stud farms sampled in central Spain by % use of Natural cover (NC) and Artificial Insemination (AI) in their mares.

| <b>% use of NC / % use of AI in the mares</b> | <b>SP Stud Farms No</b> | <b>SP Stud Farms %</b> |
|---|-------------------------|------------------------|
| <b>0% NC / 100% AI</b>                        | 9                       | 26%                    |
| <b>10 – 50% NC / 50 - 90% AI</b>              | 9                       | 26%                    |
| <b>60 – 90% NC / 10 – 40% AI</b>              | 7                       | 22%                    |
| <b>100% NC / 0% AI</b>                        | 9                       | 26%                    |
| <b>Total</b>                                  | <b>34</b>               | <b>100%</b>            |

The percentage of mares with reproductive problems in the 34 stud farms of this study ranged 0 to 100 (mean 24.8%  $\pm$  SD 26.4%, median 15, IR 4.5 – 35.2). The distribution of stud farms by % mares with reproductive problems is shown in Table 26.

Table 26. Distribution of the 35 SP stud farms sampled in central Spain by % mares with reproductive problems.

| <b>% mares with reproductive problems</b> | <b>SP Stud Farms No</b> | <b>SP Stud Farms %</b> |
|---|-------------------------|------------------------|
| <b>0%</b>                                 | 4                       | 12%                    |
| <b>&gt;0 to <math>\leq</math>10%</b>      | 10                      | 29%                    |
| <b>&gt;10 to <math>\leq</math>20%</b>     | 7                       | 21%                    |
| <b>&gt;20 to <math>\leq</math>50%</b>     | 9                       | 26%                    |
| <b>&gt;50%</b>                            | 4                       | 12%                    |
| <b>Total</b>                              | <b>34</b>               | <b>100%</b>            |

Within the stud farms which had mares with reproductive problems, repeat breeding was reported in 21% of the stud farms, early abortions were reported in 29% of the stud farms, late abortions in 32% of the stud farms, stillbirths in 9% of the stud farms, the presence of neonatal deaths or weak foals at birth was reported in 32% of the stud farms, and barren mares (mares which were covered/inseminated several times and did not manage to conceive) were reported in 65% of the stud farms.

## IV.5 ASSOCIATION OF EVA STATUS WITH INDIVIDUAL AND STUD FARM RISK FACTORS.

In order to find associations between the different individual and stud farm risk factors and the seropositivity to EVA, a Chi squared and a univariable ordinary logistic regression analysis was carried out in the SP horses sampled in this study (n=555). The results for the Chi squared analysis, as well as the ordinary logistic regression between the different individual and stud farm variables and EVA status in the 555 SP horses of this study are shown in Table 28 and Table 29, respectively.

Individual risk factors associated a priori with EVA seropositivity included sex, age, breeding activity and reproductive problems and EHV-1/-4 vaccination (Table 27), whereas stud farm factors associated a priori with EVA seropositivity included the stud farm location and climate, the total area and the area used for breeding in the stud farm, the stud farm horse density of population, the stud farm association to a Breeders' association, the presence of dogs and cats in the stud farm, the number of SP breeding stallions in the stud farm, the outings to fairs/competitions and to other stud farms with breeding purposes in the past 12 months, the average number of visits from people coming into the stud farm monthly in the past 12 months, the presence of an Equine Influenza + Tetanus vaccination regime in the stud farm, the presence of a EHV-1/-4 vaccination regime in the stud farm, the presence of measures against insects implemented in the stud farm and the percentage of reproductive problems in the mares of the stud farm (Table 28).

Table 27. Chi squared analysis and univariable logistic regression of risk factors related to individual characteristics for EVA seropositivity in 555 SP horses in the central region of Spain.

| Variable   | Category                          | Frequency %<br>EVA positives | Frequency %<br>EVA negatives | Odds ratio | 95% CI       | $\chi^2$ p value |
|--|-----------------------------------|------------------------------|------------------------------|------------|--------------|------------------|
| <b>Sex (n=555)</b>   | Stallion                          | 26 (11%)                     | 211 (89%)                    | Reference  |              | 0.001            |
|  | Mare                              | 70 (22%)                     | 248 (78%)                    | 2.29       | 1.41 - 3.72  |                  |
| <b>Age (n=555)</b>   | < 3 years                         | 9 (10.8%)                    | 74 (89.2%)                   | 1.02       | 0.43 - 2.39  | 0.002            |
|  | 3 - 6 years                       | 17 (10.7%)                   | 142 (89.3%)                  | Reference  |              |                  |
|  | 7 - 9 years                       | 24 (17.6%)                   | 112 (82.4%)                  | 1.79       | 0.92 - 3.49  |                  |
|  | 10 - 14 years                     | 33 (27.5%)                   | 87 (72.5%)                   | 3.17       | 1.67 - 6.03  |                  |
|  | ≥ 15 years                        | 13 (22.8%)                   | 44 (77.2%)                   | 2.47       | 1.11 - 5.45  |                  |
| <b>Age (n=555)</b>   |                                   |                              |                              | 1.07       | 1.03 - 1.11  | 0.002            |
| <b>Breeding activity and reproductive problems (n=555)</b> | Non breeding                      | 18 (8.7%)                    | 189 (91.3%)                  | Reference  |              | <0.001           |
|  | Breeding-No reproductive problems | 44 (16.4%)                   | 224 (83.6%)                  | 2.06       | 1.15 - 3.69  |                  |
|  | Breeding-Reproductive problems    | 34 (42.5%)                   | 46 (57.5%)                   | 7.76       | 4.03 - 14.95 |                  |
| <b>EHV-1/-4 vaccination (n=555)</b>                        | No                                | 38 (11.3%)                   | 298 (88.7%)                  | Reference  |              | <0.001           |
|  | Yes                               | 58 (26.5%)                   | 161 (73.5%)                  | 2.82       | 1.80 - 4.44  |                  |

Table 28. Univariable logistic regression of risk factors for EVA seropositivity related to stud farm characteristics, general management and measures of prevention and control of infectious diseases in 555 SP horses in the central region of Spain.

| Variable   | Category   | Frequency %<br>EVA positives | Frequency %<br>EVA negatives | Odds<br>ratio | 95% CI      | p value |
|--|--|------------------------------|------------------------------|---------------|-------------|---------|
| Stud farm location in central area of Spain (n=555)    | North  | 61 (25.3%)                   | 180 (74.7%)                  | 2.70          | 1.71 - 4.26 | <0.001  |
|  | South  | 35 (11.1%)                   | 279 (88.9%)                  | Reference     |             |         |
| Stud farm climate (n=555)                              | temperate with dry or hot summer (CSA + CFA)             | 37 (14.0%)                   | 228 (86.0%)                  | Reference     |             | <0.001  |
|  | temperate/cold with temperate and dry summer (CSB + DSB) | 42 (32.6%)                   | 87 (67.4%)                   | 2.97          | 1.79 - 4.93 |         |
|  | cold steppe (BSK)  | 17 (10.6%)                   | 144 (89.4%)                  | 0.73          | 0.39 - 1.34 |         |
| Total area of the stud farm (n=555)                    | < 1 km <sup>2</sup>                                      | 94 (25.2%)                   | 279 (74.8%)                  | Reference     |             | <0.001  |
|  | ≥ 1 km <sup>2</sup>                                      | 2 (1.1%)                     | 180 (98.9%)                  | 0.03          | 0.01 – 0.13 |         |
| Area used for breeding in the stud farm (n=555)        | < 1 km <sup>2</sup>                                      | 31 (30.4%)                   | 71 (69.6%)                   | Reference     |             | <0.001  |
|  | ≥ 1 km <sup>2</sup>                                      | 65 (14.4%)                   | 388 (85.6%)                  | 0.38          | 0.23 – 0.63 |         |
| Stud farm density of population (n=555)                | ≤ 500 horses/km <sup>2</sup>                             | 24 (7.5%)                    | 296 (92.5%)                  | Reference     |             | <0.001  |
|  | > 500 horses/km <sup>2</sup>                             | 72 (30.6%)                   | 163 (69.4%)                  | 5.45          | 3.30 – 8.98 |         |
| Stud farm onset year (n=555)                           | ≤ 2000   | 53 (15.8%)                   | 283 (84.2%)                  | Reference     |             | 0.240   |
|  | > 2000   | 43 (19.6%)                   | 176 (80.4%)                  | 1.30          | 0.84 – 2.03 |         |
| Stud farm association to Breeders' association (n=555) | no   | 15 (9.2%)                    | 148 (90.8%)                  | Reference     |             | 0.001   |
|  | yes  | 81 (20.7%)                   | 311 (79.3%)                  | 2.57          | 1.43 - 4.61 |         |

Table 28 (cont). Univariable logistic regression of risk factors for EVA seropositivity related to stud farm characteristics, general management and measures of prevention and control of infectious diseases in 555 SP horses in the central region of Spain.

| Variable   | Category                  | Frequency %<br>EVA positives | Frequency %<br>EVA negatives | Odds<br>ratio | 95% CI      | p value |
|--|---------------------------|------------------------------|------------------------------|---------------|-------------|---------|
| Other equine activities in the stud farm (n=555)                                 | no                        | 60 (19.2%)                   | 253 (80.8%)                  | Reference     |             | 0.185   |
|  | yes                       | 36 (14.9%)                   | 206 (85.1%)                  | 0.74          | 0.47 - 1.16 |         |
| Dogs in stud farm (n=555)  | no                        | 44 (52.4%)                   | 40 (47.6%)                   | Reference     |             | < 0.001 |
|  | yes                       | 52 (11.0%)                   | 419 (89.0%)                  | 0.11          | 0.07 – 0.19 |         |
| Cats in stud farm (n=555)  | no                        | 84 (28.3%)                   | 213 (71.7%)                  | Reference     |             | < 0.001 |
|  | yes                       | 12 (4.7%)                    | 246 (95.3%)                  | 0.12          | 0.06 – 0.23 |         |
| Number of SP breeding mares in stud farm (n=555)                                 | ≤ 10 SP Breeding mares    | 23 (13.1%)                   | 153 (86.9%)                  | Reference     |             | 0.073   |
|  | > 10 SP Breeding mares    | 73 (19.3%)                   | 306 (80.7%)                  | 1.59          | 0.96 - 2.63 |         |
| Number of SP breeding stallions in stud farm (n=555)                             | ≤ 2 SP Breeding stallions | 29 (10.7%)                   | 241 (89.3%)                  | Reference     |             | < 0.001 |
|  | > 2 SP Breeding stallions | 67 (23.5%)                   | 218 (76.5%)                  | 2.55          | 1.59 – 4.10 |         |
| Breeding mares separated from young horses (n=555)                               | no                        | 50 (20.6%)                   | 193 (79.4%)                  | 1.50          | 0.94 - 2.33 | 0.071   |
|  | yes                       | 46 (14.7%)                   | 266 (85.3%)                  | Reference     |             |         |
| Groups within breeding mares and young horses (n=555)                            | no                        | 67 (19.3%)                   | 280 (80.7%)                  | 1.48          | 0.92 - 2.37 | 0.106   |
|  | yes                       | 29 (13.9%)                   | 179 (86.1%)                  | Reference     |             |         |
| Outings to fairs/competitions in the past 12 months (n=555)                      | no                        | 32 (11.1%)                   | 255 (88.9%)                  | Reference     |             | < 0.001 |
|  | yes                       | 64 (23.9%)                   | 204 (76.1%)                  | 2.50          | 1.57 - 3.97 |         |
| Outings to other stud farms with breeding purposes in the past 12 months (n=555) | no                        | 77 (22.0%)                   | 273 (78.0%)                  | Reference     |             | < 0.001 |
|  | yes                       | 19 (9.3%)                    | 186 (90.7%)                  | 0.36          | 0.21 - 0.62 |         |
| Acquired horses in the past 12 months (n=555)                                    | no                        | 9 (10.3%)                    | 78 (89.7%)                   | Reference     |             | 0.062   |
|  | yes                       | 87 (18.6%)                   | 381 (81.4%)                  | 1.98          | 0.95 - 4.10 |         |

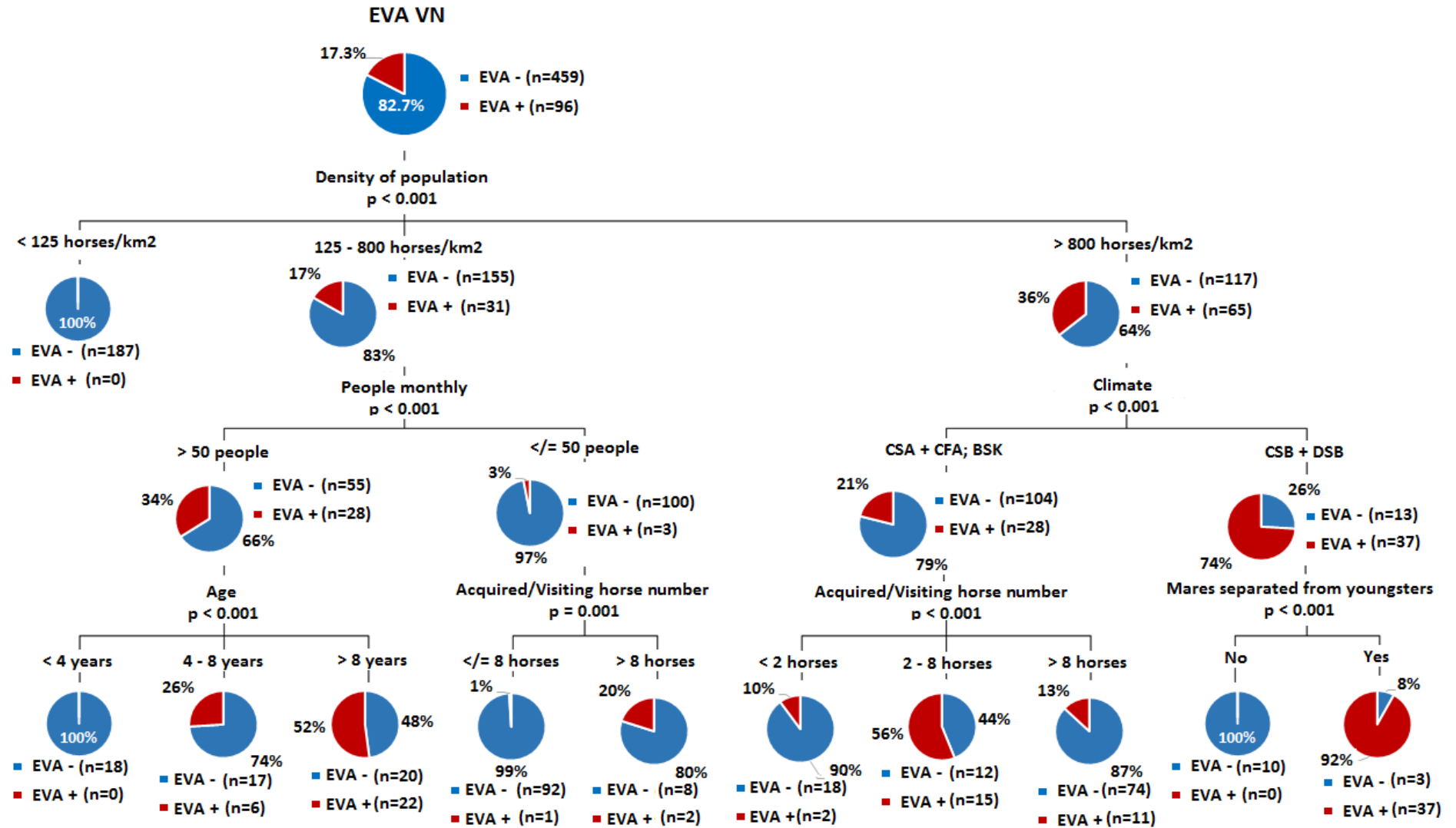
Table 28 (cont). Univariable logistic regression of risk factors for EVA seropositivity related to stud farm characteristics, general management and measures of prevention and control of infectious diseases in 555 SP horses in the central region of Spain.

| Variable   | Category    | Frequency %<br>EVA positives | Frequency %<br>EVA negatives | Odds<br>ratio | 95% CI      | p value |
|--|-------------|------------------------------|------------------------------|---------------|-------------|---------|
| Average number of visits from people coming into the stud farm monthly in the past 12 months (n=555) | ≤ 50 people | 52 (13.8%)                   | 326 (86.2%)                  | Reference     |             | 0.001   |
|  | > 50 people | 44 (24.9%)                   | 133 (75.1%)                  | 2.07          | 1.32 - 3.25 |         |
| Equine Influenza + Tetanus vaccination regime in the stud farm (n=555)                               | no          | 43 (25.6%)                   | 125 (74.4%)                  | 2.17          | 1.38 - 3.41 | 0.001   |
|  | yes         | 53 (13.7%)                   | 334 (86.3%)                  | Reference     |             |         |
| Equine Rhinopneumonitis vaccination regime in the stud farm (n=555)                                  | no          | 16 (7.8%)                    | 188 (92.2%)                  | 0.29          | 0.16 - 0.51 | < 0.001 |
|  | yes         | 80 (22.8%)                   | 271 (77.2%)                  | Reference     |             |         |
| Disinfection regime in the stud farm (n=555)   | no          | 14 (15.7%)                   | 75 (84.3%)                   | 0.87          | 0.47 - 1.62 | 0.670   |
|  | yes         | 82 (17.6%)                   | 384 (82.4%)                  | Reference     |             |         |
| Measures against insects implemented in the stud farm (n=555)  | no          | 46 (43.0%)                   | 61 (57.0%)                   | 6.00          | 3.70 - 9.73 | < 0.001 |
|  | yes         | 50 (11.2%)                   | 398 (88.8%)                  | Reference     |             |         |
| Measures against rodents implemented in the stud farm (n=555)  | no          | 21 (18.9%)                   | 90 (81.1%)                   | 1.15          | 0.67 - 1.96 | 0.614   |
|  | yes         | 75 (16.9%)                   | 369 (83.1%)                  | Reference     |             |         |
| Breeding by natural covering in the stallions of the stud farm (n=501)                               | no          | 19 (17.6%)                   | 89 (82.4%)                   | 0.95          | 0.54 - 1.66 | 0.862   |
|  | yes         | 72 (18.3%)                   | 321 (81.7%)                  | Reference     |             |         |
| Breeding by natural covering in the mares of the stud farm (n=548)                                   | no          | 19 (13.2%)                   | 125 (86.8%)                  | 0.66          | 0.38 - 1.13 | 0.126   |
|  | yes         | 76 (18.8%)                   | 328 (81.2%)                  | Reference     |             |         |
| Percentage of reproductive problems in the mares of the stud farm (n=548)                            | <50%        | 58 (12.8%)                   | 397 (87.2%)                  | Reference     |             | < 0.001 |
|  | ≥50%        | 37 (39.8%)                   | 56 (60.2%)                   | 4.52          | 2.75 - 7.44 |         |

Following the Chi squared and the univariable logistic regression analysis of the association between the EVA status in the horses sampled and individual and stud farm risk factors, and given the complex and clustered nature of the data which impeded a multivariable analysis by ordinary logistic regression at this stage, a non-parametric, multivariable analysis by the construction of a decision tree using the CHAID algorithm was carried out.

The decision tree, which included both individual and stud farm variables (variables related to the characteristics, general management and measures of prevention and control of infectious diseases in the SP stud farms) is shown in Figure 33.

Figure 33. Decision tree for variables associated with seropositivity to EAV in the study population of SP breeding horses sampled in central Spain (n=555).



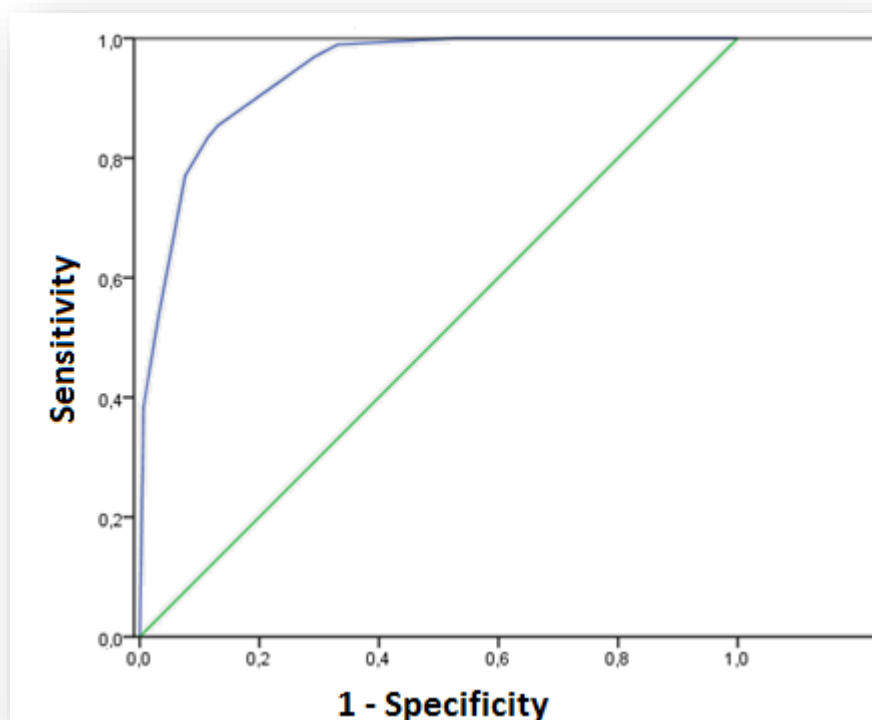
The tree had 17 nodes and 10 terminal nodes, therefore there would be 10 different probabilities for EVA infection in the horses sampled in this study. The first branch would be the variable “Density of population in the stud farm”; horses in stud farms with  $\leq 125$  horses/km<sup>2</sup> were all negative for EVA VN in our sample and would be expected to be negative in the SP breeding horse population in central Spain. Horses living in stud farms with  $>125$  and  $<800$  horses/km<sup>2</sup> would have a probability of 0.167 for being seropositive to EVA. Within these, the next branch would be the variable “average number of visits from people coming monthly into the stud farm in the past 12 months”. Horses in stud farms with  $>50$  visits from people coming monthly into the premises would have a probability of being EVA seropositive of 0.337, and within these and in a final branch, horses  $\leq 4$  years old would all be negative for EVA VN, whereas horses between 4 and 8 years old would have a probability of being EVA positives of 0.26 and horses  $>8$  years old would have a probability of 0.524 of being EVA positives. Horses in stud farms with  $\leq 50$  visits from people coming monthly into the premises would have a probability of being EVA seropositive of 0.03, and within these and in a final branch, horses in stud farms where more than 8 horses were acquired or visited the premises had a probability of being EVA seropositive of 0.20 whereas horses in stud farms where  $\leq 8$  horses were acquired or visited the premises had a very low probability of being EVA seropositive (0.01).

Horses living in premises with more than 800 horses/km<sup>2</sup> would have a probability of being infected by EAV of 0.36, and the next branch would divide this group in two by the climate of the area where the stud farm was located. Horses living in an area with a temperate with dry or hot summer (CFA or CSA) and those living in an area with a cold steppe climate (BSK) had a smaller probability of being infected by EAV (0.21) than horses living in an area with a temperate/cold with dry or temperate summer climate (CSB or DSB) (0.74). The group of horses in stud farms with a CFA, CSA or BSK climate, was divided in three final nodes by number of horses that were acquired or visited the premises; within these the highest probability of being EVA seropositive was in the group of horses living in premises where between 2 and 8 horses were acquired or visited the premises (0.56). Finally, the group of horses in stud farms with a CSB or DSB climate was divided in two final nodes by the presence of a physical separation between breeding mares and youngsters in the

stud farm. Interestingly, the highest probability of being EVA seropositive was that in the group of horses living in premises where broodmares and youngsters were segregated (0.92).

Regarding the predictive power of the tree model, the area under the ROC curve was 0.94 (95% CI 0.92 – 0.96), indicating that the model had good overall predictive power. The ROC curve is shown in Figure 34.

Figure 34. ROC curve for the decision tree model for variables associated with seropositivity to EAV in the study population of 555 SP breeding horses in central Spain (AUC = 0.94).



## IV.6 ASSOCIATION OF EHV-1/-4 STATUS WITH INDIVIDUAL AND STUD FARM RISK FACTORS.

In order to find associations between the different individual and stud farm risk factors and the seropositivity to EHV-1/-4, a Chi squared and a univariable ordinary logistic regression analysis was carried out in the unvaccinated SP horses sampled in this study (n=334). The results for the Chi squared analysis, as well as the ordinary logistic regression between the different individual and stud farm variables and EHV-1/-4 status in the 334 unvaccinated SP horses of this study are shown in Table 29 and Table 30, respectively.

Individual risk factors associated *a priori* with EHV-1/-4 seropositivity included the age and the breeding activity and reproductive problems (Table 30), whereas stud farm factors associated *a priori* with EHV-1/-4 seropositivity included the stud farm location, the total area of the stud farm, the segregation in small groups within the mares and the young horses in the stud farm, the average number of visits from people coming into the stud farm monthly in the past 12 months, and the presence of a EHV-1/-4 vaccination regime in the stud farm (Table 30).

Table 29. Chi squared analysis and univariable logistic regression of risk factors related to individual characteristics for EHV-1/-4 seropositivity in 334 unvaccinated SP horses in the central region of Spain.

| Variable   | Category                          | Frequency (%)<br>EHV-1/-4 positives | Frequency (%)<br>EHV-1/-4 negatives | Odds ratio | 95% CI       | p value |
|--|-----------------------------------|-------------------------------------|-------------------------------------|------------|--------------|---------|
| <b>Sex (n=334)</b>   | Stallion                          | 86 (48.6%)                          | 91 (51.4%)                          | Reference  |              | 0.067   |
|  | Mare                              | 92 (58.6%)                          | 65 (41.4%)                          | 1.50       | 0.97 - 2.31  |         |
| <b>Age (n=334)</b>   | < 3 years                         | 15 (26.3%)                          | 42 (73.7%)                          | 0.47       | 0.23 - 0.96  | < 0.001 |
|  | 3 - 6 years                       | 51 (46.4%)                          | 59 (53.6%)                          | Reference  |              |         |
|  | 7 - 9 years                       | 35 (47.9%)                          | 38 (52.1%)                          | 1.06       | 0.59 - 1.93  |         |
|  | 10 - 14 years                     | 54 (79.4%)                          | 14 (20.6%)                          | 4.46       | 2.22 - 8.96  |         |
|  | ≥ 15 years                        | 23 (88.5%)                          | 3 (11.5%)                           | 8.87       | 2.51 - 31.27 |         |
| <b>Age (n=334)</b>   |                                   |                                     |                                     | 1.20       | 1.13 - 1.28  | <0.001  |
| <b>Breeding activity and reproductive problems (n=334)</b> | Non breeding                      | 68 (41.7%)                          | 95 (58.3%)                          | Reference  |              | <0.001  |
|  | Breeding-No reproductive problems | 89 (63.1%)                          | 52 (36.9%)                          | 2.39       | 1.50 - 3.80  |         |
|  | Breeding-Reproductive problems    | 21 (70.0%)                          | 9 (30.0%)                           | 3.26       | 1.41 - 7.56  |         |

Table 30. Univariable logistic regression of risk factors for EHV-1/-4 seropositivity related to stud farm characteristics, general management and measures of prevention and control of infectious diseases in 334 SP horses in the central region of Spain.

| Variable  | Category   | Frequency (%)<br>EHV-1/-4 positives | Frequency (%)<br>EHV-1/-4 negatives | Odds<br>ratio | 95% CI      | p value |
|---|--|-------------------------------------|-------------------------------------|---------------|-------------|---------|
| <b>Stud farm location in central area of Spain (n=334)</b>    | North  | 78 (44.8%)                          | 96 (55.2%)                          | reference     |             | 0.001   |
|   | South  | 100 (62.5%)                         | 60 (37.5%)                          | 2.05          | 1.32 - 3.18 |         |
| <b>Stud farm climate (n=334)</b>                              | temperate with dry or hot summer (CSA + CFA)             | 80 (47.9%)                          | 87 (52.1%)                          | reference     |             | 0.109   |
|   | temperate/cold with temperate and dry summer (CSB + DSB) | 54 (61.4%)                          | 34 (38.6%)                          | 1.73          | 1.02 - 2.92 |         |
|   | cold steppe (BSK)  | 44 (55.7%)                          | 35 (44.3%)                          | 1.34          | 0.80 - 2.34 |         |
| <b>Total area of the stud farm (n=334)</b>                    | < 1 km <sup>2</sup>                                      | 150 (56.6%)                         | 115 (43.4%)                         | reference     |             | 0.017   |
|   | ≥ 1 km <sup>2</sup>                                      | 28 (40.6%)                          | 41 (59.4%)                          | 0.52          | 0.30 - 0.90 |         |
| <b>Area used for breeding in the stud farm (n=334)</b>        | < 1 km <sup>2</sup>                                      | 155 (55.0%)                         | 127 (45.0%)                         | reference     |             | 0.154   |
|   | ≥ 1 km <sup>2</sup>                                      | 23 (44.2%)                          | 29 (55.8%)                          | 0.65          | 0.36 - 1.18 |         |
| <b>Stud farm density of population (n=334)</b>                | ≤ 500 horses/km <sup>2</sup>                             | 88 (54.7%)                          | 73 (45.3%)                          | reference     |             | 0.630   |
|   | > 500 horses/km <sup>2</sup>                             | 90 (52.0%)                          | 83 (48.0%)                          | 0.90          | 0.58 - 1.38 |         |
| <b>Stud farm onset year (n=334)</b>                           | ≤ 2000   | 103 (55.1%)                         | 84 (44.9%)                          | reference     |             | 0.460   |
|   | >2000  | 75 (51.0%)                          | 72 (49.0%)                          | 0.85          | 0.55 - 1.31 |         |
| <b>Stud farm association to Breeders' association (n=334)</b> | no   | 73 (51.0%)                          | 70 (49.0%)                          | reference     |             | 0.477   |
|   | yes  | 105 (55.0%)                         | 86 (45.0%)                          | 1.17          | 0.76 - 1.81 |         |

Table 30 (cont). Univariable logistic regression of risk factors for EHV-1/-4 seropositivity related to stud farm characteristics, general management and measures of prevention and control of infectious diseases in 334 SP horses in the central region of Spain.

| Variable  | Category                  | Frequency (%)<br>EHV-1/-4 positives | Frequency (%)<br>EHV-1/-4 negatives | Odds<br>ratio | 95% CI      | p value |
|---|---------------------------|-------------------------------------|-------------------------------------|---------------|-------------|---------|
| <b>Other equine activities in the stud farm (n=334)</b>                                 | no                        | 103 (50.2%)                         | 102 (49.8%)                         | reference     |             | 0.159   |
|   | yes                       | 75 (58.1%)                          | 54 (41.9%)                          | 1.37          | 0.88 - 2.14 |         |
| <b>Dogs in stud farm (n=334)</b>  | no                        | 24 (64.9%)                          | 13 (35.1%)                          | reference     |             | 0.135   |
|   | yes                       | 154 (51.8%)                         | 143 (48.2%)                         | 0.58          | 0.29 - 1.19 |         |
| <b>Cats in stud farm (n=334)</b>  | no                        | 104 (52.8%)                         | 93 (47.2%)                          | reference     |             | 0.826   |
|   | yes                       | 74 (54.1%)                          | 63 (46.0%)                          | 1.05          | 0.68 - 1.63 |         |
| <b>Number of SP breeding mares in stud farm (n=334)</b>                                 | ≤ 10 SP breeding mares    | 64 (53.3%)                          | 56 (46.7%)                          | reference     |             | 0.991   |
|   | > 10 SP breeding mares    | 114 (53.3%)                         | 100 (46.7%)                         | 1.00          | 0.64 - 1.56 |         |
| <b>Number of SP breeding stallions in stud farm (n=334)</b>                             | ≤ 2 SP breeding stallions | 110 (51.9%)                         | 102 (48.1%)                         | reference     |             | 0.497   |
|   | > 2 SP breeding stallions | 68 (55.7%)                          | 54 (44.3%)                          | 1.17          | 0.75 - 1.83 |         |
| <b>Breeding mares separated from young horses (n=334)</b>                               | no                        | 93 (55.0%)                          | 76 (45.0%)                          | 1.15          | 0.75 - 1.77 | 0.520   |
|   | yes                       | 85 (51.5%)                          | 80 (48.5%)                          | reference     |             |         |
| <b>Groups within breeding mares and young horses (n=334)</b>                            | no                        | 141 (56.8%)                         | 107 (43.1%)                         | 1.74          | 1.06 - 2.86 | 0.027   |
|   | yes                       | 37 (43.0%)                          | 49 (57.0%)                          | reference     |             |         |
| <b>Outings to fairs/competitions in the past 12 months (n=334)</b>                      | no                        | 103 (55.7%)                         | 82 (44.3%)                          | reference     |             | 0.331   |
|   | yes                       | 75 (50.3%)                          | 74 (49.7%)                          | 0.81          | 0.52 - 1.24 |         |
| <b>Outings to other stud farms with breeding purposes in the past 12 months (n=334)</b> | no                        | 114 (51.6%)                         | 107 (48.4%)                         | reference     |             | 0.381   |
|   | yes                       | 64 (56.6%)                          | 49 (43.4%)                          | 1.22          | 0.78 - 1.93 |         |
| <b>Acquired horses in the past 12 months (n=334)</b>                                    | no                        | 32 (47.8%)                          | 35 (52.2%)                          | reference     |             | 0.310   |
|   | yes                       | 146 (54.7%)                         | 121 (45.3%)                         | 1.32          | 0.77 - 2.26 |         |

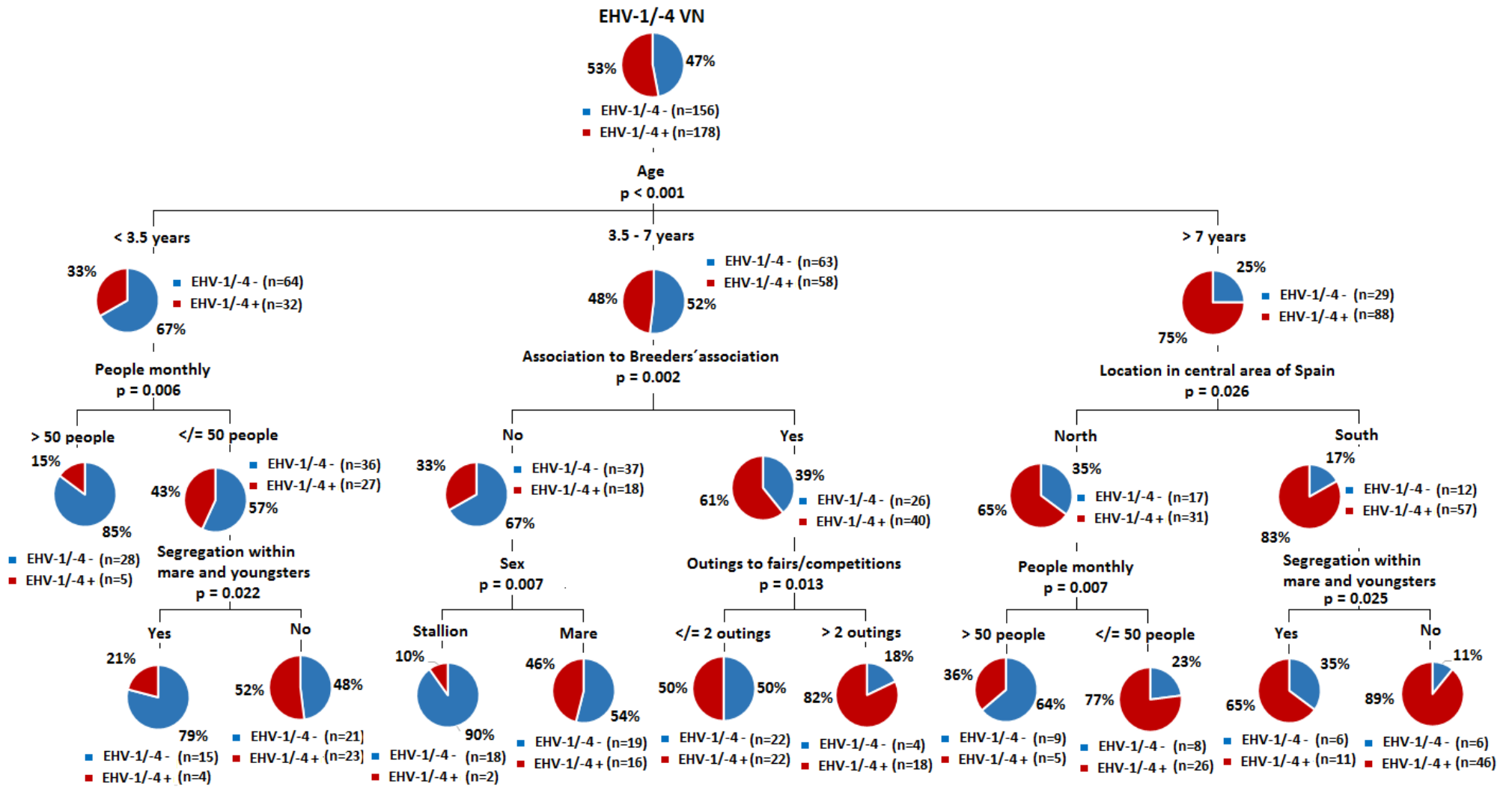
Table 30 (cont). Univariable logistic regression of risk factors for EHV-1/-4 seropositivity related to stud farm characteristics, general management and measures of prevention and control of infectious diseases in 334 SP horses in the central region of Spain.

| Variable   | Category    | Frequency (%)<br>EHV-1/-4 positives | Frequency (%)<br>EHV-1/-4 negatives | Odds<br>ratio | 95% CI      | p value |
|--|-------------|-------------------------------------|-------------------------------------|---------------|-------------|---------|
| Average number of visits from people coming into the stud farm monthly in the past 12 months (n=334) | ≤ 50 people | 132 (61.1%)                         | 84 (38.9%)                          | reference     |             | <0.001  |
|  | > 50 people | 46 (39.0%)                          | 72 (61.0%)                          | 0.41          | 0.26 - 0.64 |         |
| Equine Influenza + Tetanus vaccination regime in the stud farm (n=334)                               | no          | 74 (51.8%)                          | 69 (48.2%)                          | 0.90          | 0.58 - 1.38 | 0.624   |
|  | yes         | 104 (54.4%)                         | 87 (45.5%)                          | reference     |             |         |
| Equine Rhinopneumonitis vaccination regime in the stud farm (n=334)                                  | no          | 120 (58.8%)                         | 84 (41.2%)                          | 1.77          | 1.14 - 2.76 | 0.011   |
|  | yes         | 58 (44.6%)                          | 72 (55.4%)                          | reference     |             |         |
| Disinfection regime in the stud farm (n=334)   | no          | 44 (55.0%)                          | 36 (45.0%)                          | 1.09          | 0.66 - 1.81 | 0.726   |
|  | yes         | 134 (52.8%)                         | 120 (47.2%)                         | reference     |             |         |
| Measures against insects implemented in the stud farm (n=334)  | no          | 46 (57.5%)                          | 34 (42.5%)                          | 1.25          | 0.75 - 2.08 | 0.387   |
|  | yes         | 132 (52.0%)                         | 122 (48.0%)                         | reference     |             |         |
| Measures against rodents implemented in the stud farm (n=334)  | no          | 54 (57.4%)                          | 40 (42.6%)                          | 1.26          | 0.78 - 2.04 | 0.341   |
|  | yes         | 124 (51.7%)                         | 116 (48.3%)                         | reference     |             |         |
| Breeding by natural covering in the stallions of the stud farm (n=265)                               | no          | 25 (50.0%)                          | 25 (50.0%)                          | 0.81          | 0.43 - 1.49 | 0.494   |
|  | yes         | 119 (55.3%)                         | 96 (44.7%)                          | reference     |             |         |
| Breeding by natural covering in the mares of the stud farm (n=327)                                   | no          | 51 (53.7%)                          | 44 (46.3%)                          | 1.03          | 0.64 - 1.66 | 0.913   |
|  | yes         | 123 (53.0%)                         | 109 (47.0%)                         | reference     |             |         |
| Percentage of reproductive problems in the mares of the stud farm (n=327)                            | <50%        | 148 (54.0%)                         | 126 (46.0%)                         | reference     |             | 0.508   |
|  | ≥50%        | 26 (49.1%)                          | 27 (50.9%)                          | 0.82          | 0.45 - 1.48 |         |

Following the Chi squared and the univariable logistic regression analysis of the association between the EHV-1/-4 status in the unvaccinated SP horses sampled and individual and stud farm risk factors, and given the complex and clustered nature of the data which impeded a multivariable analysis by ordinary logistic regression at this stage, a non-parametric, multivariable analysis by the construction of a decision tree using the CHAID algorithm was carried out.

The decision tree, which included both individual and stud farm variables (variables related to the characteristics, general management and measures of prevention and control of infectious diseases in the SP stud farms) is shown in Figure 35.

Figure 35. Decision tree for variables associated with seropositivity to EHV-1/-4 in the 334 unvaccinated SP breeding horses sampled in central Spain.



The tree had 19 nodes and 10 terminal nodes, therefore there would be 10 different probabilities for EHV-1/-4 infection in the horses sampled in this study. The first branch would be the variable “age”; horses  $\leq 3.5$  years of age would have the lowest probability of being seropositive to EHV-1/-4 (0.33), followed by horses between 3.5 and 7 years of age (0.48) and horses  $> 7$  years (0.75).

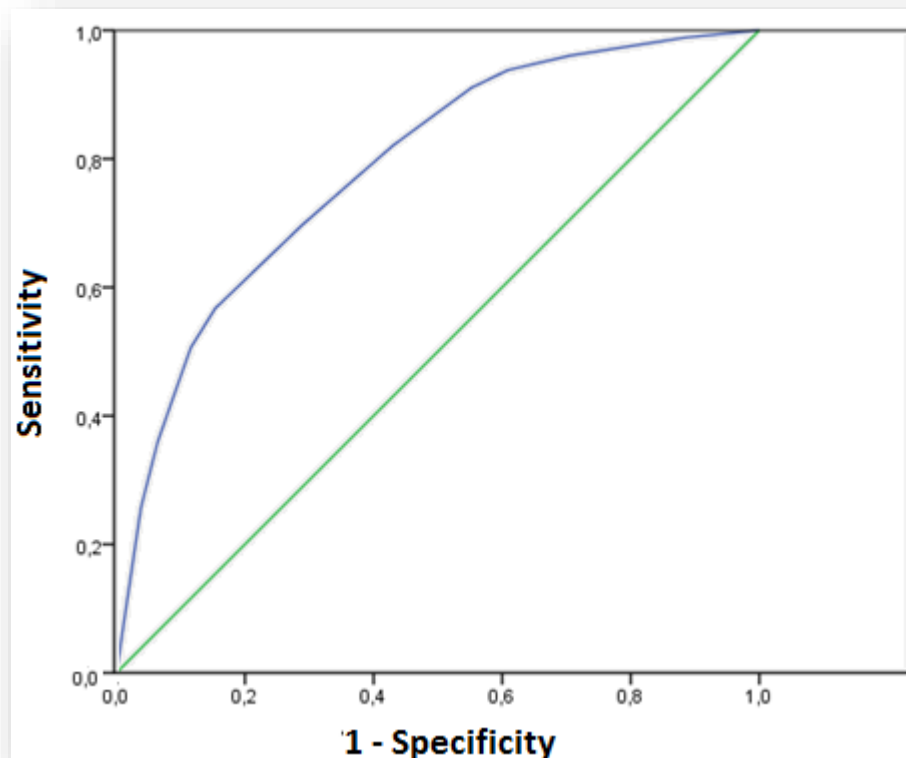
Within the group of horses  $\leq 3.5$  years of age, those living in stud farms with  $> 50$  visits from people coming into the premises monthly had a probability of being EHV-1/-4 seropositive of 0.15, whereas those living in premises where  $\leq 50$  people came in monthly had a probability of 0.43. Within these and in a final node, horses living in stud farms where mares and young horses were segregated into small groups had a lower probability of being EHV-1/-4 positive than those living in stud farms where this segregation did not occur (0.21 vs 0.52 respectively).

Within the group of horses between 3.5 and 7 years of age, there was a higher probability of EHV-1/-4 infection in those living in stud farms that were associated to a breeders association (0.81) and within these, there was a higher probability in horses that lived in premises where there had been more than 2 outings to fairs or competitions (0.82 vs 0.50 respectively). Within the horses living in stud farms that were not associated to a breeders association (probability of EHV-1/-4 infection of 0.32), mares were at a higher risk of being EHV-1/-4 infected (0.46 vs 0.10 in stallions).

Regarding horses with more than 7 years of age, those living in stud farms in the South of the central region of Spain were at a higher risk of being EHV-1/-4 positive (0.83 vs 0.65 in the North). Within the group of horses living in the North, and in a final branch, those living in premises where there were more than 50 visits from people into the stud farm monthly had a probability of being EHV-1/-4 positive of 0.36, whereas those living in premises where there were  $\leq 50$  visits from people coming in monthly had a higher probability (0.78). Finally, within the group of horses living in the South of the central region of Spain and in a final branch, those horses living in premises where mares and young horses were segregated into small groups had a lower probability than those living in premises where this segregation did not occur (0.65 vs 0.88 respectively).

Regarding the predictive power of the tree model, the area under the ROC curve was 0.79 (95% CI 0.74 – 0.84), indicating that the model had acceptable overall predictive power. The ROC curve is shown in Figure 36.

Figure 36. ROC curve for the decision tree model for variables associated with seropositivity to EHV-1/-4 in the study population of SP breeding horses in central Spain (AUC = 0.79).



## IV.7 PRESENCE OF REPRODUCTIVE PROBLEMS IN THE SP MARES AND STALLIONS OF THE STUDY.

### IV.7.1 Descriptive analysis

Within the 257 active breeding mares sampled, 74 mares [29% (23%-34%)] were reported to have had at least one reproductive problem in the past 12 months. The mean age of these mares was  $10.3 \pm 4.5$  years (range 4 - 21), median age was 9 years (IR 7 - 14). The mean age of these mares was significantly different ( $p= 0.02$ ) from the mean age of the breeding mares without reproductive problems ( $8.9 \pm 4.5$  years). The mean number of years breeding was 3.4 years  $\pm$  SD, (range 1 - 17), median was 5 years, IR 3 - 9 years. The number and percentage of the SP mares sampled in this study showing different reproductive problems is shown in Table 31. Several mares (10) showed more than one reproductive problem.

Table 31. Number and percentage of the SP mares sampled in central Spain showing different reproductive problems (n=74).

| Reproductive problem  | SP breeding mares No | SP breeding mares % |
|-----------------------|----------------------|---------------------|
| Repeat breeding       | 21                   | 28%                 |
| Cyclic irregularities | 7                    | 9%                  |
| Early abortion        | 14                   | 19%                 |
| Late abortion         | 20                   | 27%                 |
| Stillbirth            | 4                    | 5%                  |
| Problems with neonate | 16                   | 22%                 |
| Retained membranes    | 2                    | 3%                  |

Within the SP mares of this study, gestation loss was the most frequent problem, reported in 38 mares [51% (40.0% - 62.7%)]. Out of these mares, 14 had early abortions (abortions occurring before day 150), whereas 20 had late abortions (abortions occurring from day 150 to day 300) and 4 had stillbirths (abortions

occurring from day 300 onwards). Repeat breeding was the second most frequent reproductive problem, with 21 of the 74 mares [28.4% (18.1%-38.6%)] suffering from this problem. Out of the 74 mares, 16 [21.6% (12.2%-31.0%)] had problems with the neonate, with 4 giving birth to immature foals, 3 having sick or weak foals, and 9 mares suffering from neonatal death. Cyclic irregularities were reported in 7 of the 74 mares [9.5% (2.8% - 16.1%)]; within these, repeat breeding had occurred in 4 mares and early abortions had occurred in 2 mares. Finally, 2.7% (0%-6.4%) of mares (n=2/74) were reported to have had retained membranes after foaling.

The suspected cause of the reproductive problem/s was specified in 21 mares (28.4%). Old age, chronic joint pain, endometriosis, haemorrhagic follicles and endotoxaemia due to a severe mastitis was considered to be the cause for cyclic irregularities with/without repeat breeding in 5 mares. Treatments with NSAID (Non-Steroidal Anti-inflammatory Drugs) for chronic joint pain, prostaglandin for haemorrhagic follicles and systemic oxytocin for mastitis were implemented in 3 of these 5 mares. The suspected cause for repeat breeding was reported in only 10 out of 21 mares; chronic joint pain, haemorrhagic follicles, endometriosis, myometrial fibrosis, endometritis, subclinical infection of the uterus, bad vulval conformation, lack of uterine defences, uterine infection with *E. coli* and *Staphylococcus* were the specified causes. Treatments with NSAID for chronic joint pain, Caslick and uterine post AI lavages with antibiotics and oxytocin in the mares with bad vulval conformation, endometritis and subclinical infection of the uterus, oxytocin and antibiotics (gentamicin intra-uterine, enrofloxacin orally) for uterine infections were implemented in 6 of these 10 mares. The suspected cause for gestation loss was specified in 4 out of 38 mares; causes were negative energy balance due to lactation, uterine infection with *Staphylococcus*, endotoxaemia due to a severe mastitis and bad vulval conformation. Only the uterine infection and the severe mastitis were treated with antibiotics and oxytocin respectively. Finally, out of the mares with problems in the neonate, the cause of the problem was specified only in 5 of them; causes were pneumonia due to meconium aspiration, pneumonia, placentitis (2 cases), perinatal asphyxia and immaturity in the foal. Neonatal pneumonia was treated with cephalosporin and NSAID in one case and oxytetracyclin, cephalosporin and NSAID in the other case, whereas placentitis in

two mares was treated with Altrenogest and TMS (trimethoprim and sulfadiazine) in both cases.

Within the 91 active breeding stallions sampled, only 6 stallions [7% (1.5%-11.7%)] had shown reproductive problems in the past 12 months. The mean age of these stallions was 11.8 years  $\pm$ SD (range 9 - 20), median age was 12 years (IR 9 - 15). The mean number of years breeding was 7.4 years,  $\pm$ SD (range 5 - 15), and median was 8.5 years, (IR 5 - 10 years).

Of the SP stallions with reproductive problems, 3 stallions [50% (10%-90%)] had lack of libido, while the other 3 stallions [50% (10%-90%)] had problems in the semen. Suspected causes for lack of libido were specified in 2 cases, one of them had a lack of experience covering mares which was managed with freedom teasing, and the other had a subclinical infection by amoebas which had not been treated. Causes for the problems in semen were specified in two cases: an intermittent haemospermia in one case (which was minimised by diluting the semen) and periods of low fertility and seropositivity to EVA in the other case.

## IV.7.2 Analysis of the association of EVA status and reproductive problems

In order to find associations between seropositivity to EVA and different reproductive problems, a Chi squared analysis was carried out in the SP mares with reproductive problems. The association between seropositivity to EVA and the different reproductive problems in the SP stallions could not be done, since the number of stallions presenting with reproductive problems was too low (6 stallions). The results for the Chi squared analysis for the association between EVA status and the different reproductive problems occurring in the 74 SP mares of this study are shown in Table 32.

When comparing EVA status in the SP breeding mares which showed reproductive problems in our study (n=74) to that in the active breeding mares with no reproductive problems (n=183), EVA seropositivity was associated with all the reproductive problems except for early abortion in the 74 SP mares, indicating that EVA seropositivity was linked to the presence of cyclic irregularities/repeat breeding, late abortion and problems with the neonate occurring in the past 12 months in the SP mares of the study.

Table 32. Chi squared analysis of the association between EVA status and specific reproductive problems in the SP mares with reproductive problems compared to the active breeding SP mares with no reproductive problems (n=183) in the central region of Spain.

| Reproductive problems                                | Category                 | Frequency % EVA positives | Frequency % EVA negatives | $\chi^2$ p value |
|--|--------------------------|---------------------------|---------------------------|------------------|
| <b>Cyclic irregularities/repeat breeding (n=207)</b> | No reproductive problems | 33 (18.0%)                | 150 (82.0%)               | 0.006            |
|  | yes                      | 11 (45.8%)                | 13 (54.2%)                |                  |
| <b>Early abortion (n=197)</b>                        | No reproductive problems | 33 (18.0%)                | 150 (82.0%)               | 0.304            |
|  | yes                      | 4 (28.6%)                 | 10 (71.4%)                |                  |
| <b>Late abortion (n=207)</b>                         | No reproductive problems | 33 (18.0%)                | 150 (82.0%)               | 0.006            |
|  | yes                      | 11 (45.8%)                | 13 (54.2%)                |                  |
| <b>Problem with neonate (n=199)</b>                  | No reproductive problems | 33 (18.0%)                | 150 (82.0%)               | 0.006            |
|  | yes                      | 8 (50%)                   | 8 (50%)                   |                  |



## *V. DISCUSSION*



## *V. DISCUSSION*

### V.1 REPRESENTATION OF THE SP BREEDING HORSE POPULATION IN CENTRAL SPAIN BY THE HORSES SAMPLED IN THIS STUDY

The characteristics of the equine population (n = 555 horses) sampled on the 35 SP stud farms in this study were considered highly representative of the wider SP breeding horse population in central Spain. Regarding the percentages of the horses sampled in each province, the situation matched that published in the census of 2012, with the highest percentages of horses sampled in the provinces with a larger SP breeding population (Madrid, followed by Toledo and Segovia) (MAGRAMA, 2012).

The distribution by sex of the sample was similar to that described in the census of 2012 for the SP breeding population in the central area of Spain (57% of mares in this study vs. 53% in the census and 43% of stallions in this study vs. 47% in the census) (MAGRAMA, 2012). Within the mares of our sample, a high percentage (74%) were active breeders whereas only the 26% of stallions were active breeders; this differed slightly from the data in the census, as 56% of mares and 40% of the stallions were active breeders (MAGRAMA, 2012). The differences in the percentages of the stallions may have been due to many stud farm owners registering their stallions but then not breeding with them, whereas the differences in the mares could be caused by a high representation of the breeding mares with respect to non-breeding and pre-pubertal mares in the sample.

Regarding the age of the horses, both the population of SP horses sampled in this study and the population of horses of pure breed (including SP horses but also Arab, Thoroughbred, French trotter and other pure breeds) collected in the census of 2001 had a very similar distribution (MAPA, 2003), except that in our sample horses younger than 4 years could have been slightly misrepresented due to difficulties in their handling that made sampling impossible in some stud farms (these animals could not be caught or restrained in order to draw a blood sample from them).

## V.2 SEROPREVALENCE

### V.2.1 Seroprevalence of Equine Infectious Anaemia

Spain has never been surveyed in order to determine EIA seroprevalence. The central region of Spain that was sampled in this study is considered to represent a high risk area for the potential introduction of equine infectious diseases, as horses travelling from/to Europe frequently stop in this area and it is the region where the largest number of competitions are held (Deloitte, 2013). Additionally, the most recent Spanish EIA outbreaks occurred predominantly in the province of Madrid, consequently this region is likely to reflect the highest risk of EIAV persistence since 1983, although no clinical cases of EIA have been detected and reported through passive surveillance (samples from horses tested prior to exportation to other countries) in Spain since 1983 (OIE, 2014a; OIE, 2014b).

After testing the 555 serum samples for EIA antibodies by ELISA, the absence of any EIA seropositive animals detected in the sample population using a highly sensitive ELISA provided 95% confidence that the true prevalence of EIA in the SP breeding population in central Spain is  $\leq 0.53\%$ . Given the excellent representation of the SP breeding horse population in central Spain in the sample population of this study in terms of location, sex, age and breeding activity and also the variety of SP breeding stud farms sampled in terms of size, density of population and climate, EIA results in this study can be considered highly representative of the SP breeding population in central Spain and could well be representative of the SP breeding population throughout Spain.

Even though the double antigen ELISA technique used to detect antibodies against EIAV was not the reference or “gold standard” technique according to the OIE (which is the Coggins test), ELISA techniques can detect antibodies before the Coggins test and they are more sensitive (Reis *et al.*, 2012; Soutullo *et al.*, 2001). The reason why the Coggins test is the gold standard technique for EIA diagnosis is that it is more specific (Cullinane *et al.*, 2007; Piza *et al.*, 2007); that is, ELISA techniques can result in false positives and therefore any positive results to EIA by ELISA must

be confirmed by using the Coggins test (OIE, 2013d). In this study there were no positives to EIA by ELISA and therefore there was no need to retest any samples by Coggins given the excellent sensitivity of the ELISA used (100%) (Body *et al.*, 2011).

Given the recent situation in Europe regarding the presence of EIAV in countries such as Belgium, Germany or Russia during the first six months of 2013 (OIE, 2013a), the results of this study are good news for the SP breeding industry. Despite the absence of EIA testing prior to importation of horses into Spain from other EU member states and the fact that this area would have a high risk for the introduction of equine infectious diseases due to holding the largest number of national and international competitions in Spain (Deloitte, 2013; MAGRAMA, 2012), the SP breeding population in central Spain has remained free of EIA infection since the last outbreak in 1983 (Galaz, 1978). The situation in Spain can then be compared to the situation in Switzerland, where the absence of EIA was confirmed in a seroprevalence study published in 2009 (Kaiser *et al.*, 2009).

The breeding of SP horses is crucial for the Spanish horse industry, with this breed representing 85% of pure bred horses in Spain and nearly 100% of horse exports to Europe and America. As this export trade had a monetary value of more than 58 million Euros in 2012 (Deloitte, 2013), the evidence for freedom from EIA provided by this study in SP horses in stud farms in central Spain may offer increased confidence when exporting SP horses to other countries. However, judicious biosecurity that includes both pre-export as well as post-arrival EIA testing should be implemented to prevent the movement of subclinically EIA infected horses within Europe.

## V.2.2 Seroprevalence of Equine Viral Arteritis

There is serological evidence that demonstrates the presence of EAV in Spain since 1978 (Moraillon and Moraillon, 1978), and the first outbreak of EVA clinical disease was confirmed in Barcelona in 1992 (Monreal *et al.*, 1995). However, up to present the only information regarding the serological presence of this disease in Spain was published in a preliminary, small study carried out in the autonomous community of Valencia (east of Spain) in 2010-2011 and which determined antibody against EVA by SN in the 12.24% of a population of 49 SP horses (Gimeno Suarep *et al.*, 2011b).

The seroprevalence of EVA in the population of this study was 17.3% (14.2% - 20.4%) and this figure is consistent with the data provided by Gimeno Suarep *et al.* in Valencia, therefore providing evidence of the actual presence of EVA in Spanish SP stud farms where it had always been considered a sporadic disease (MAPA, 2003). Within the 96 EVA seropositive SP horses in the study, the majority (53%) had high EVA SN titres (log<sub>10</sub> titres  $\geq 2.5$  and  $< 3$ ) and 15% had very high EVA SN titres (log<sub>10</sub> titres of  $\geq 3.5$ ); most of these results were from active breeders and these represented the 63% of the sample therefore explaining the high percentage of samples with a high titre. Many of these horses with a high EVA SN titre were mares having continuous contact with the same EVA seropositive stallions, and suggesting the presence of EVA carriers in our study (Rola *et al.*, 2011).

Even though this study was not designed to determine the SP stud farm seroprevalence, 37.1% (21.1% - 53.1%) of the stud farms in this study had horses which were seropositive to EVA VN test. Within these, the highest percentage of stud farms (46%) had a seroprevalence of EVA between 30% and 60%, again suggesting the presence of a high viral activity in the stud farm in the form of outbreaks which are not always attributed to EVA and which could be caused by carrier stallions or introduced in the stud farm by horses that go to competitions or to other stud farms (Holyoak *et al.*, 2008).

The results of this study confirm the presence of EAV in the SP horse population in central Spain, similar to that in other European countries where EVA

seroprevalence studies have been carried out, such as France (15.2% of horses of various breeds) (Morailion and Morailion, 1978), Netherlands (14% of horses of various breeds) (de Boer *et al.*, 1979), Austria (10.9% of horses of various breeds) (Kölbl *et al.*, 1991) and the UK (18.5% – 24% of Standardbred horses)(Newton *et al.*, 1999).

While the SN test is the reference or “gold standard” technique for the diagnosis of EVA according to the OIE (OIE, 2013d), the indirect confirmation ELISA used in this study (ID Screen® Equine Viral Arteritis Indirect, ID.vet Innovative Diagnostics) (Legrand *et al.*, 2009; Legrand *et al.*, 2007) performed well as an initial screening test. Although this study was not designed to assess the sensitivity and specificity of this ELISA test, the specificity of the ELISA when used to test the samples in this study (87%) was lower than that published in their validation study (96.75%) (Beaufort Cottage Laboratories, 2008). Therefore the ELISA used in this study would be valid as a screening test although SN would be needed in order to confirm positive and equivocal results (OIE, 2013d).

Given the fact that EVA outbreaks of clinical disease resemble those caused by EHV-1/-4 (del Piero, 2000b), EVA may be misdiagnosed and there is a low awareness of the disease in the Spanish equine breeding industry (MAPA, 2003). Despite the surveillance plan published in the APA/212/2003 order, which included the communication and control of diseases such as EVA and the establishment of an EVA surveillance programme (BOE, 2003), and the occasional OIE reports showing serological and/or isolation findings of EVA in Spain (OIE, 2013c), the perceptions drawn from the present study are that many stud farm owners ignore the existence and implications of this disease. Furthermore, the outbreak of abortions caused by EAV reported in April 2013 in a breeding stud farm located in the South of Castilla y León (ICC, 2013) was brought to light due to the veterinary surgeon of the affected stud farm being enrolled in this study. A better understanding and awareness of EVA disease in the equine breeding industry in Spain would probably be key in order to apply measures of prevention and control for this disease; the work carried out in other European countries with the publishing of a Codes of Practice which covers the most important equine infectious diseases for the equine breeding supports this fact (HBLB, 2013).

Even though the identification of carrier stallions was not included in the aims of this study, as published elsewhere the presence of carriers in stud farms where there was a high seroprevalence of EVA suggests the same phenomenon within the breeding stallions of these stud farms (Hullinger *et al.*, 1998; Rola *et al.*, 2011). Although annual semen testing for EAV in active breeding stallions was a measure specified in the Royal Decree published in 2011 (BOE, 2011), this is actually not being done in the majority of the small, private stud farms where the stallions do natural coverings; however this is not the case when the semen is due to be exported as the semen needs to be certified free of EIA, CEM and EVA (BOE, 2011). The identification of carrier stallions is key in order to prevent and control EVA transmission within the population of a SP stud farm, but also to prevent the transmission between SP stud farms as there is a high number of SP breeding stallions and mares that move to other stud farms with breeding purposes or go to fairs and competitions where there is a high density of other SP breeding horses (Deloitte, 2013).

Although vaccination against EVA has been successfully implemented in several European countries (e.g. UK and France), there are no available vaccines in Spain and EVA vaccination is not currently permitted. Regarding the situation in the UK, vaccination is carried out mostly in the Thoroughbred horse population, which has a lower EVA seroprevalence than that reported in this Study for SP horses in central Spain (<3% vs. 17.3%, respectively) (Newton *et al.*, 1999); Thoroughbred horses are routinely screened for antibodies against EAV every year and they are tested in order to confirm they are seronegative to EVA before being vaccinated. As drawn from the Codes of Practice for Equine Infectious Diseases adopted in the UK and several European countries, the first step in order to implement a vaccination scheme for EVA in Spain would be to increase the awareness of the disease within the Spanish equine breeding industry and to encourage annual serological testing of the breeding stallions and mares (HBLB, 2013).

The excellent representation of the SP breeding horse population in central Spain in the sample population of this study and also the variety of SP breeding stud farms sampled make possible to extrapolate EVA results in this study to the whole SP breeding population in central Spain and also possibly throughout Spain.

However, several authors have reported differences in the seroprevalence of EVA within breeds: EVA seroprevalence was significantly higher in Standardbred horses compared to Thoroughbreds in the UK (18.5%-24% vs. <3% respectively) (Newton *et al.*, 1999) and the same happened in Australia where the differences were even greater (72% in Standardbred horses vs. 4.5% in Thoroughbreds) (Huntington *et al.*, 1990b); it has also been suggested that certain breeds could be more susceptible to EAV infection (e.g. Hucul horses in Poland which showed a seroprevalence of 55.1% with 45% of stallions being positive to EAV in semen (Rola *et al.*, 2011)). Although breed-specific differences might reflect inherent genetic differences that confer resistance to infection, they are more likely reflective of different cultural and management factors and different perceptions and attitudes towards infectious diseases and their control of the horse owners within the different horse populations and breeds (Holyoak *et al.*, 2008) Therefore these results may not be directly applicable to other equine breeds in Spain.

### V.2.3 Seroprevalence of EHV-1/-4

The first report of EHV-1/-4 in Spain dates back from 1989 (OIE, 2013b), and since then there have been sporadic outbreaks reported. There are only two references about the serological presence of EHV-1/-4 in Spain; both studies reported very different seroprevalence estimates although they were carried out with a time lapse of 18 years (Gimeno Suarez *et al.*, 2011a; Martin Otero, 1992) and sampled a different horse population. While Martin Otero reported a seroprevalence of EHV-1/-4 of 4.9% by SN, Gimeno Suarez *et al.* reported a seroprevalence of EHV-1/-4 of 100% using the same technique (Gimeno Suarez *et al.*, 2011a; Martin Otero, 1992). The results of the present study lie in between these, as the seroprevalence for EHV-1/-4 in the SP breeding horse population in central Spain was deemed to be 53.3% (47.9% - 58.6%). The population sampled in this study would be more similar to the one sampled by Gimeno Suarez *et al.* in Valencia than the one sampled by Martin Otero in 1992; the seroprevalence published by Martin Otero could be low due to this study sampling a population of horses from the Military stud farms that were very static (did not move from their premises) and very controlled by the military. On the other hand, the seroprevalence reported by Gimeno Suarez *et al.* could be high due to this study being small and preliminary; it included only 49 SP horses from 5 stud farms in Valencia and their seroprevalence result would probably change if the study was extended to other stud farms.

The overall EHV-1/-4 seroprevalence in SP horses in central Spain is similar to that published using the same technique in other countries, such as the Netherlands (76%) or Peru (39.6% - 48.8%) (de Boer *et al.*, 1979; Rios, 2002). However, the specific seroprevalences for EHV-1 and EHV-4 in the population sampled in this study are different from those published elsewhere. While the seroprevalence of EHV-1 [50.9% (45.5% - 56.3% with 95% confidence)] was higher than the seroprevalence of EHV-4 [26.3% (21.6% - 31.1% with 95% confidence)] in this study, EHV-4 was more prevalent than EHV-1 in other countries such as Colombia and Turkey (Ataseven, 2010; Gür, 2008; Ruiz-Saenz *et al.*, 2007, 2008). Interestingly, all four studies carried out in these countries used the same technique, (Svanovir® EHV1/ EHV4-Ab ELISA, Svanova Biotech AB, Sweden); this ELISA seemed to always detect EHV-4 and much less frequently EHV-1 and it failed to detect

up against known positive and negative control sera when used at the Animal Health Trust diagnostic laboratories (Newton, R., personal communication). However, our results should be considered with caution since the SN test shows a certain amount of cross-reactivity between the two subtypes of the virus (OIE, 2013d; Wagner *et al.*, 1992).

The results of this study show that EHV-1/-4 was highly extended in the central area of Spain. Out of the 30 SP stud farms sampled for EHV-1/-4, 90% (79.3% – 100%) had at least one EHV-1/-4 seropositive horse and within these, 48% showed a seroprevalence between 60% and 90% in the horses sampled. Due to the long-term nature of neutralising antibodies tested in the horses of this cross-sectional study, the circulation of EHV-1/-4 in SP stud farms (due to horses having the virus latent and it reactivating from time to time, or due to horses moving in and out of the premises and having contact with other potentially infected horses) (Allen, 2002) could not be confirmed, although the presence in our study of EHV-1/-4 seropositive horses in horses with different ages and from different groups within the same stud farm would suggest this idea. In order to confirm the circulation of EHV-1/-4 in the SP stud farms of our study different techniques should be used (such as CF test which determines short-term antibodies) and/or several samples should be taken from the horses in a stud farm in order to evaluate any changes in the antibody titres (Gilkerson *et al.*, 1999b; Patel and Heldens, 2005).

While the SN test is a reference or “gold standard” technique for the diagnosis of EHV-1/-4 according to the OIE (OIE, 2013d), the indirect confirmation ELISA used in this study (Ingezim Rinoneumonitis 14.HVE.K1®, Ingenasa) performed well as an initial screening test. Although this study was not designed to assess the sensitivity and specificity of this ELISA test, the specificity of the ELISA when used to test the samples in this study (78.8%) was higher than that published in their validation study (67%). The ELISA used in this study would be valid as a screening test although SN would be needed in order to confirm positive and equivocal results (OIE, 2013d).

Even though the awareness of EHV-1/-4 by the veterinary surgeons is higher than that of EVA mainly due to the neurological form of the disease, many stud farm owners from the present study ignored the implications of this disease. The

surveillance plan published in the APA/212/2003 order did not include EHV-1/-4 (BOE, 2003) even when the outbreaks of EHV-1/-4 tend to be on a larger scale than other disease outbreaks, such as EVA, and the economic losses due to abortions and neonatal deaths in a stud farm are often devastating (Allen, 2002).

The high seroprevalence of EHV-1/-4 within some of the SP stud farms sampled suggests either the presence of carriers with a latent virus that reactivates and triggers outbreaks from time to time or the periodic introduction of EHV-1/-4 into the SP stud farms through horses that move in and out the premises (HBLB, 2013). The presence of carriers of latent EHV-1/-4 in a breeding stud farm means that outbreaks of EHV-1/-4 are difficult to prevent and control, since any situation of stress or immune suppression in a carrier can trigger an outbreak of abortions (HBLB, 2013).

In order to apply measures of prevention and control for this disease, increased awareness of EHV-1/-4 in the equine breeding industry in Spain would be essential and published Codes of Practice including this disease would be beneficial to both veterinary surgeons and stud farm owners (HBLB, 2013). Given the high seroprevalence of EHV-1/-4 in the SP horses sampled in central Spain and the impossibility of diagnosing horses with a latent virus by serology, there would be two important measures which should be put in place in SP stud farms in Spain: the first one would be a vaccination regime against EHV-1/-4; 54% of the SP stud farms sampled in this study had it already and it has been shown that vaccination contributes to herd immunity and, together with good management practices, lowers the probabilities of abortion storms in a stud farm (Allen, 2002; Mayr, 1987). The second measure would be to educate and increase the awareness regarding management and control measures against an outbreak of EHV-1/-4 in a breeding stud farm in order to minimize the consequences (HBLB, 2013).

The subset of EHV-1/-4 unvaccinated horses sampled in this study and used for the analysis of EHV-1/-4 (n=334) could not be compared with a reference population of EHV-1/-4 unvaccinated horses due to data from this reference population not being available. However, since there was an excellent representation of the SP breeding horse population in central Spain in the sample population of this study and the percentage of EHV-1/-4 unvaccinated horses in the

sample of this study is likely to be similar to that in the wider population in central Spain, EHV-1/-4 results in this study may well be extrapolated to the whole SP breeding population in central Spain and also possibly throughout Spain. However, given the differences in the management of a breeding stud farm and the management of livery yards or other types of premises within the equine industry in Spain (MAPA, 2003), these results could probably not be extended to the whole equine population in this country.

### V.3 CHARACTERISTICS, GENERAL MANAGEMENT AND HEALTH IN SP BREEDING STUD FARMS IN CENTRAL SPAIN.

Information is scarce as to how SP horses are kept and managed in Spain and, data regarding demographics and management have not previously been available exclusively for this breed in Spain. There are only two studies carried out and focused on this subject: the first study dates back to 2003 and collected data from breeding stud farms (including those breeding SP horses but also other breeds) (MAPA, 2003), and the second was published recently and provided information regarding all types of equine premises and all breeds (including stud farms but also livery yards, racetracks, private yards, etc.) (Deloitte, 2013). The results of our study provide a detailed description of the demographic characteristics, general management and health measures implemented in SP stud farms in the central area of Spain.

The SP stud farms in this study were chosen using non-random methods. While random sampling from all SP stud farms in the central area of Spain may have resulted in a more representative sample, this was considered to be impractical and would have posed logistical difficulties. However, the variety of SP breeding stud farms sampled in this study in terms of size, density of population and climate were considered to be representative of the common practices on SP breeding farms in central Spain at the time of the survey.

Regarding the questionnaire of general management and health conducted in the SP stud farms, it is possible that where stud farm owners assisted in questionnaire completion, they may have provided answers that they believed were “correct” (social acceptability bias), but this was considered unlikely as a number of them actually indicated that they had limited or no protocols in place to prevent the spread of disease. It should also be noted that none of the SP stud farms had had a very recent disease outbreak so the questionnaires were conducted in a relatively disease-free environment, which may have influenced their responses since some studies published following the 2007 EI outbreak in Australia have shown that disease outbreak experiences can influence owner perceptions about disease

control (e.g. owners that had no long-term impacts resulting from the 2007 EI outbreak were more likely to have lower biosecurity compliance (Schemann *et al.*, 2011) whereas managers of horse premises that experienced EI infection in their horses in 2007 reported increased levels of perceived vulnerability to a future outbreak (Schemann *et al.*, 2013). Memory decay in responders may have introduced error, as they were asked to provide details regarding characteristics of the stud farm within the previous 12 months; however, most veterinary surgeons and stud farm owners kept data in a stud farm diary which was checked in case of doubts.

The 35 SP breeding stud farms sampled in this study were considered representative of the whole population of SP stud farms in the central area of Spain in terms of location, climate, area size and number of horses. Regarding location, the greatest proportion of stud farms sampled were located in Madrid, Toledo and Segovia. Stud farms in these locations accounted for the 73% of the total, compared to 80.5% of SP stud farms in these locations registered in the census of 2012 (MAGRAMA, 2012). The distribution by area followed this demographic pattern and most of the SP stud farms were located in the NW and SW of the central area (69% in total). The SP stud farms sampled covered locations with all the climate types in central Spain, and 48% of the stud farms had CSA climate, which is the most prevalent in Spain being present in 40% of the country (Rubel and Kottek, 2010). The sample of SP stud farms in this study had slightly larger total surface area than the ones included in the census of 2001 (mean 0.53 km<sup>2</sup> vs. 0.35 km<sup>2</sup> respectively); although the median would have been a better figure to make the comparison, this data was not available in the census (MAPA, 2003). The census of 2001 also reported a lower number of horses per stud farm (30 horses vs. 48.1 horses in the SP stud farms of this study). The differences in these figures could have been due to the present study sampling a small number of SP stud farms compared to the census of 2001, but they could also be due to the increased confidence of stud farm owners in the period of economic growth in Spain from 2001-2007 which could have made that the inversion in land and horses was higher than in the previous years (MAPA, 2003).

Within the 35 SP stud farms sampled, 60% were associated to a Breeder's association, most of them associated to ANCCE. This association has 541 members up to date, which represents nearly the 25% of the SP stud farms in Spain and focuses on the improvement of the SP breed and its promotion through the organisation of competitions (ANCCE, 2014). A higher level of associations within the SP stud farms or the creation of an industry institution such as the HBLB in the UK would involve an easier way to educate stud farm owners with regards to the communication, prevention and control of equine infectious diseases (HBLB, 2013).

Nearly half of the SP stud farms sampled (46%, n= 16/35) were involved in equine activities other than the breeding of SP horses, most commonly dressage or livery; these activities increase the risk of introduction of equine infectious diseases since they involve more movements in and out of the premises and more contact with horses from other premises (Ather and Greene, 2005).

Regarding the horses living in the SP stud farms of this study, a typical SP stud farm in our sample would have 16 breeding mares, no mares retired from breeding, 3 breeding stallions, 7 geldings and/or stallions older than 4 years old but without a history of covering mares, no teasers, 4 suckling foals, 6 yearlings and 8 2-3 year-old horses. This distribution reflects a lower use of the SP breeding stallions compared to other breeds using natural covering such as the Thoroughbred (8 mares bred by a stallion in the SP stud farms of this study vs. 20 mares bred by a stallion in the Thoroughbred breed in America)(The Jockey Club, 2013). It also reflects the difficulties that SP stud farm owners have to sell their products as there were a considerable number of horses that could not be sold at an early age and were trained in order to be sold as riding horses; only an average of 5 horses had been sold in the past 12 months.

Nearly half of the SP stud farms sampled (49%, n= 17/35) did not have any segregation between breeding mares and young horses and only the 26% of stud farm owners (n=9/35) segregated their mares and young horses in small groups. This demonstrates that groups in the SP stud farms were selected in order to ease management and decrease the costs whereas stud farm owners should be encouraged to consider reducing the consequences of an infectious disease outbreak when devising their management strategies (HBLB, 2013).

Regarding the movements of horses in the SP stud farms of this study, 54% of the SP stud farms did not attend any fairs/competitions in the past 12 months, but the elevated costs of attending a competition (e.g. an average of 582 euro per competition in Madrid (Deloitte, 2013)) could have had an influence on this. Stud farm owners were reticent to move their SP stallions and mares to other stud farms for breeding purposes (only 26% moved their stallions and 23% moved their mares to other SP stud farms); however the percentage of stud farm owners accepting visiting mares into their premises was higher (43%, n=15/35). There are no data regarding movement of horses in SP stud farms, and the only study to evaluate the percentage of horses regularly travelling off their premises was published in the UK and reported that only the 13% of horses never left their premises (Mellor *et al.*, 2001). The harsh economic situation in Spain could have decreased the number of movements to fairs, competitions or other stud farms with breeding purposes due to the associated costs.

According to the results of our survey, the number of visits from people coming into the premises varied within the SP stud farms of our study; the high average of 114 visits from people coming into the premises monthly was influenced by the existence of some stud farms that had other activities such as liveries and stud farms with a large number of horses which were trained and sold. Therefore the median of 15 visits from people may represent realistic figure when representing the set of SP stud farms in this study.

Regarding the implementation of vaccination regimes in the SP stud farms of this study, 71% did vaccinate all horses against EI and the same percentage vaccinated against Tetanus. Even though there are no data regarding the use of EI and Tetanus vaccination in SP horses, these data have been published in the UK general horse population (78.8% - 90.5% vaccinated against EI and 81.9% - 95.3% vaccinated against Tetanus) (Boden *et al.*, 2013; Hotchkiss *et al.*, 2007; Ireland *et al.*, 2013), and also in the population of geriatric horses of the UK (66.3% vaccinated against EI and 82.8% vaccinated against Tetanus) (Ireland *et al.*, 2011); the reasons for the differences between these figures could be due to differences between both populations, the desire of some stud farm owners to decrease costs the belief that if horses don't move out of the premises there is no risk of EI infection and the lower

perception of EI risk in Spain where the stud farm owners do not hear of any EI outbreaks reported compared to UK horse owners who have a higher awareness of the disease due to EI outbreaks being sporadically reported. This could have also been the reason of the low percentage of SP stud farms that vaccinated against WNV (3%, n=1/35), since even when there were outbreaks of WNV reported in the South of Spain in 2011, this information somehow does not reach to the stud farm owners (OIE, 2014b).

The use of EHV-1/-4 vaccination in SP breeding horses has not been reviewed in the literature and the studies that have evaluated the use of this vaccine in horses showed large differences in the figures (90.6% of the horse owners in the UK vaccinated their horses against EHV-1/-4 in a study (Boden *et al.*, 2013) compared to 4.1% - 20.6% in other studies using the same population (Hotchkiss *et al.*, 2007; Ireland *et al.*, 2013). These figures are different to the 40% vaccinated SP horses in our study and the 54% SP stud farms with an EHV-1/-4 vaccination regime, but the population used in our study is completely different than that in the UK (horses in riding clubs and livery yards), and the figures provided by Boden *et al.* seem to high compared to the rest of the studies carried out in the UK (Boden *et al.*, 2013; Hotchkiss *et al.*, 2007; Ireland *et al.*, 2013), suggesting the presence of a bias in the responders of their survey.

Within the SP stud farms sampled, the implementation of a worming regime was widespread, and only 1/35 SP stud farms did not have a worming regime. Most veterinary surgeons wormed 2 times a year (76%), a regime which differs greatly to the horse population in other countries, such as the UK, where most of the horses are wormed more than 2 times per year (Hotchkiss *et al.*, 2007; Ireland *et al.*, 2013; Lloyd *et al.*, 2000; Mellor *et al.*, 2001). Veterinary surgeons used a variety of products, although ivermectin was present in most worming regimes, same as in the UK horse population (Hotchkiss *et al.*, 2007; Ireland *et al.*, 2013). This is of some concern considering the recent reports of ivermectin resistance in horses (Kaplan and Nielsen, 2012; Nielsen *et al.*, 2013) and the seroprevalence of cestodes of 18-24% reported in 2005 in slaughtered horses in central Spain (Meana *et al.*, 2005).

A high percentage of the SP stud farms included in this study had disinfection measures (83%, n=29/35), measures against insects (83%, n=29/35) and rodents

(80%, n=28/35). The high percentages of stud farms having these measures implemented in the premises could have been due to the respondents giving the answers that they believed were correct; and while this could be true with the disinfection, the economic implications and disadvantages of having insects and rodents in the premises could have made that stud farm owners actually applied these measures.

Regarding the breeding in the SP farms of this study, 48% bred their stallions by natural covering while only 26% bred their mares exclusively by natural covering. The differences in the breeding protocols between sexes are probably due to the introduction of AI techniques that are often used to breed the SP mares with stallions of other SP stud farms. There are no data published on the use of natural covering and AI in SP breeding horses, therefore these figures could not be contrasted.

Of the SP breeding stud farms sampled, only 18% reported having at least a stallion with reproductive problems, while 88% reported having at least one mare with reproductive problems. The differences in number of breeding mares and breeding stallions in the SP stud farms may have influenced this since the mean number of breeding mares was 8 times higher than the mean number of breeding stallions in our study. Within the stud farms that had mares with reproductive problems, the mean percentage of these mares in the stud farms was very similar to the percentage of mares with reproductive problems in the sample (24.8% vs. 29% respectively); therefore the percentage of mares with reproductive problems sampled would be representative of the SP stud farms sampled in central Spain. The most worrying data pertained to late abortions, since 29% of all SP stud farms sampled reported at least one late abortion in the past 12 months. This would involve a high economic loss for the owner of the stud farm, since the breeding of each horse involves a major investment of time and money (Schulman *et al.*, 2013).

## V.4 ASSOCIATION OF EIA, EVA AND EHV-1/-4 STATUS WITH INDIVIDUAL OR STUD FARM RISK FACTORS

### V.4.1 Association of Equine Infectious Anaemia status with individual or stud farm risk factors

Given the lack of seropositive results for EIA in the population sampled in this study, it was not possible to analyse the data in order to find any significant statistical associations between EIA positive status and individual horse level risk factors or with factors related to management practices regarding the prevention and control of equine infectious diseases implemented in the SP breeding stud farms.

It should be noted that the 83% of the SP breeding stud farms sampled in this study had routine measures against insects implemented in the premises. While most of the stud farms where these measures were implemented used chemicals, interestingly 14% used a more natural approach, by installing ultraviolet lamps, nets, fly-capture sticks, or providing garlic in the horses feed. The presence of any measures, whether chemicals or natural approaches, against insects is important considering that the major route for EIA transmission are biting insects. Even though these measures were probably not implemented due to the fear of an EIA outbreak in the stud farms but most likely to make the horses more comfortable and ease the management in the stud farm, these measures should be implemented in every stud farm, since they can help prevent EIA infection and transmission (Issel and Foil, 1984).

Given the situation in nearby European countries with regards to EIA, other measures for the prevention of EIA to be implemented in order to keep Spanish SP stud farms free from EIA infection would be routine testing of the horses prior to importation into Spain and after their arrival; these measures have already been implemented in other countries (such as the UK) when importing horses from countries where EIA cases have been confirmed (such as Romania) and would be of extreme importance in Spain (HBLB, 2013).

## V.4.2 Association of Equine Viral Arteritis status with individual or stud farm risk factors

The results of the analyses presented in this study did highlight several established as well as some apparently previously unrecognised risk factors for the individual seropositivity to EVA in the SP horses sampled.

Among the individual risk factors identified in the univariable analysis (sex, age, breeding activity and reproductive problems, and EHV-1/-4 vaccination), the only factor that was included in the multivariable analysis (decision tree) was the age ( $p < 0.001$ ). According to the decision tree analysis, horses older than 8 years would have the highest probability of being EVA seropositive (52%) in the 83 horses living in premises where more than 50 people came in monthly to the premises and where there was a density of population of 125 – 800 horses/km<sup>2</sup>. Previous studies have shown that the age was a risk factor for seropositivity to EVA (Gimeno Suarez *et al.*, 2011b; Rola *et al.*, 2011); given that horses only need one encounter with EAV to be seropositive for life (Timoney, 2003), the increased risk of EVA seropositivity with age in the population of our study would be due to an increased risk of encountering at least one exposure to EAV within the history of the horse up to the time of the cross sectional sampling of this study.

Some seroprevalence studies showed that there was a significantly higher proportion of seropositive mares compared to stallions (Gimeno Suarez *et al.*, 2011a; Hullinger *et al.*, 1998; Rola *et al.*, 2011). Even when sex was associated to EVA seropositivity in the univariable analysis (mares were 2.3 times more likely to be EVA positive than stallions), sex was not pointed out as a potential risk factor by the decision tree analysis due to other factors deemed stronger and, in order to confirm the association shown by the univariable analysis, a multivariable, mixed logistic regression analysis (including the stud farm factor as a random effect) should be carried out.

According to the decision tree analysis, within the SP horses sampled in this study (n=555), the stud farm factor highlighted as potentially most influential on the EVA seropositivity of the horses was the density of horse population in the stud farm ( $p < 0.001$ ), followed by the average number of visits from people coming monthly into the stud farm ( $p < 0.001$ ) and the climate of the location where the stud farm was situated ( $p < 0.001$ ). Other stud farm risk factors that were associated to but had less influence on the EVA seropositivity were the number of acquired and/or visiting horses ( $p = 0.001$ ), and the segregation of breeding mares from youngsters in the stud farm ( $p < 0.001$ ).

Within the 555 SP horses sampled, 100% of those living in premises with  $\leq 125$  horses/km<sup>2</sup> were negative to the EVA VN, whereas 16.7% of horses living in premises with  $> 125$  and  $\leq 800$  horses/km<sup>2</sup> were positive and this percentage was even higher (36%) in those horses living in premises with  $> 800$  horses/km<sup>2</sup>. Even though a multivariable, mixed logistic regression analysis (including the stud farm factor as a random effect) should be carried out in order to confirm this risk factor in the population of SP horses of this study, this variable had also been found to be significant in the univariable analysis; horses living in stud farms with more than 500 horses/km<sup>2</sup> were 5.45 times more likely to be EVA seropositive. There are no data in the literature that associates horse density of population to a higher probability of being EVA seropositive, but McCollum and Swerczek reported higher morbidity rates in EVA outbreaks occurring in large concentrations of horses such as racetracks (McCollum and Swerczek, 1978), and some studies focused in other equine diseases did show that a high density of population in a stud farm does increase the risk of transmission of infectious diseases (Backer and Nodelijk, 2011; Glass and Barnes, 2013). Therefore in the SP stud farms with a high density of horse population, measures of prevention and control of EVA related to the detection and isolation of possible carriers or acutely infected horses would be strongly advised (HBLB, 2013; Holyoak *et al.*, 2008). Semen shedding stallions should be identified in these stud farms and only previously EVA seropositive mares should be bred with them in order to reduce the burden of infection within the population; in time (as the shedding stallions retire) SP stud farms should look to eradicate EAV from the SP breeding herd and implement an annual testing routine to make the possibility of vaccinating all seronegative stallions and new stallions worthwhile.

Among the risk factors highlighted by the decision tree and within the group of SP horses that lived in premises with  $>125$  and  $\leq 800$  horses/km<sup>2</sup>, the average number of visits from people coming monthly into the stud farm in the past 12 months was significantly associated to EVA status. Thirty-four percent (34%) of the horses living in stud farms where there were more than 50 visits from people coming monthly into the premises were seropositive to EVA vs. only the 3% in stud farms where there were  $\leq 50$  visits from people. Again this factor had also been found to be significant in the univariable analysis comprising the 555 SP horses of the study; horses living in stud farms where there were more than 50 visits from people coming monthly into the premises were 2 times more likely to be positive to EVA. This could be due to a possible EVA iatrogenic transmission by fomites used by vets or people from the equine industry that visited the stud farms which has previously been reported elsewhere (Timoney and McCollum, 1993b), or it could be due to the fact that most of the SP stud farms with more human visitors did have regular horse movements into the yard such as acquired horses, or mares/stallions visiting the premises with breeding purposes; the number of acquired horses was also a variable associated to EVA status in two of the groups of SP horses in which the decision tree divided the sample population. Therefore biosecurity measures should be extreme in the SP stud farms where there are regular human or horse movements (Ather and Greene, 2005).

Regarding the climate of the locations where the SP stud farms of this study were situated, EVA was more prevalent in locations with a temperate with dry or temperate summer (CSB) and cold with temperate and dry summer (DSB) climate, present in some areas of Segovia, Ávila and the NW of Madrid. Even though the climate has not yet been considered a risk factor for the transmission of EVA, it has been associated to the transmission of other diseases such as EIA due to the association of warm and humid climates to the presence of the vector (Bicout *et al.*, 2006; Borges *et al.*, 2013). The locations with both of these climates are the ones where the temperature during the summer is the lowest of all, and this could have an influence on the virus survival outside the horse (e.g. in fomites), since the survival of EAV has been found to be temperature dependant (it may survive only 20-30 minutes at 56°C, from 2 to 3 days at 37°C and up to 75 days at 4°C (Holyoak *et al.*, 2008; Timoney, 2003). There would also be differences in the management of

the horses which would depend on the climate; during the colder months of the year, stud farms in a location with a colder climate tend to keep horses in larger groups and indoor, whereas stud farms in a location with a milder climate tend to leave horses outside in paddocks where the density of population is lower.

Interestingly, within the population of horses living in locations with a temperate/cold with dry or temperate summer climate (CSB or DSB) of our sample (n=43), 100% of the horses living in stud farms where breeding mares and youngsters were not segregated were negative for EVA VN whereas those living in premises that did segregate these two groups had a probability of being EVA seropositive of 92%. The reasons for this association could be that the SP stud farms where mares were separated from youngsters had a large number of breeding mares, which could increase the chances of EVA respiratory transmission in the event of an outbreak (del Piero, 2000b; Monreal *et al.*, 1995).

To summarise, within the SP horses sampled in this study, those residing in stud farms where there was a high horse density of population would benefit from a solid implementation of measures of prevention and control of EVA related to the detection and isolation of possible carriers or acutely infected horses (HBLB, 2013; Holyoak *et al.*, 2008), SP horses from stud farms with a large number of visits from people or a large number of horses moving in and off the premises would benefit from the implementation of biosecurity measures and routine EVA testing prior to the introduction of new horses to the stud farm (Ather and Greene, 2005; HBLB, 2013), and finally stud farm owners in locations with a cold/temperate with dry and temperate summer should be especially careful during the colder months and a thorough disinfection scheme should be in place in order to get rid of viruses (such as EVA) persisting in the environment and causing further infections in the event of an outbreak (Holyoak *et al.*, 2008; Mumford *et al.*, 1987).

### V.4.3 Analysis of the association of EHV-1/-4 status with individual or stud farm risk factors in the SP horses sampled.

Among the individual risk factors identified in the univariable analysis (age, and breeding activity and reproductive problems), the only of these factors included in the multivariable analysis (decision tree) was the age ( $p < 0.001$ ) in the 334 horses of the study.

The association between age and EHV-1/-4 seropositivity has been studied in the past and a gradual increase in the risk of exposure to EHV-1/-4 was observed in several studies (Allen, 2002; Liutkevicien *et al.*, 2006; Ruiz-Saenz *et al.*, 2008). According to the univariable analysis carried out in the 334 unvaccinated SP horses of this study, the increase in the risk of exposure to EHV-1/-4 was of 1.2 times for every year of age. Even though a multivariable, mixed logistic regression analysis (including the stud farm factor as a random effect) should be carried out in order to confirm this association, age was found to be the variable with more influence on EHV-1/-4 seropositivity of the EHV-1/-4 unvaccinated horses sampled ( $n=334$ ), according to the results of the decision tree analysis ( $p < 0.001$ ).

Even though sex was not considered a risk factor in the whole population of unvaccinated SP horses of this study ( $n=334$ ) according to the univariable analysis ( $p=0.067$ ), it was in a small group of horses ( $n=55$ ) according to the decision tree ( $p=0.007$ ). In this group, mares were more likely to be seropositive than stallions (46% vs. 10%). This has been seen by other authors (Liutkevicien *et al.*, 2006), although it would have to be interpreted with caution in this study due to this risk factor not being significantly associated in the wider population of SP horses sampled and it should be confirmed by a multivariable, mixed logistic regression analysis.

Other risk factors identified in this analysis were the average number of visits from people coming monthly into the stud farm ( $p=0.006$ ), whether the stud farm was associated to a Breeder's association ( $p=0.002$ ), the location of the SP stud farm in the central area of Spain ( $p=0.026$ ), the segregation of mares from youngsters in

the stud farm ( $p=0.02$ ) and the outings to fairs and/or competitions in the previous 12 months ( $p=0.013$ ).

Within the group of SP horses younger than 3.5 years sampled ( $n=96$ ), those living in premises where more than 50 visits from people coming in monthly had a lower probability of being EHV-1/-4 positive and this association had also been found in the univariable analysis carried out in the whole population of unvaccinated SP horses of the study ( $n=334$ ). The stud farms with more human visits would also have had more movements of horses into the premises ( $p<0.001$ ) and therefore the owners and/or veterinary surgeons could have been more aware of the risks of introduction of EHV-1/-4 by the horses coming into the premises, implementing an EHV-1/-4 vaccination regime ( $p<0.001$ ). Therefore and in the SP stud farms with a large number of visits from people or a large number of horses moving in and off the premises, the implementation of biosecurity measures and the routine EHV-1/-4 vaccination of the horses is key in order to lower the risk of introducing EHV-1/-4 into the stud farm (Ather and Greene, 2005 ; Glass and Barnes, 2013).

Interestingly and even though this factor was not considered significant in the univariable analysis carried out in the 334 unvaccinated SP horses, according to the decision tree the association of the SP stud farm to a Breeders' association would be a risk factor for EHV-1/-4 seropositivity in the group of horses between 3.5 and 7 years ( $n=121$ ); those horses living in SP stud farms that were associated to a Breeders' association had a higher probability of being EHV-1/-4 seropositive (61% vs. 33%) ( $p=0.002$ ). The reason for this could be that the different SP Breeders' associations in Spain promote the SP breed by organising fairs, competitions, sales, etc. (ANCCE, 2014). Therefore the real risk factor, which was also highlighted by the decision tree in a group of 66 SP horses and has been associated with a higher risk of EHV-1/-4 infection in the literature, would be the number of horse movements in the premises (Lunn *et al.*, 2009; Mumford *et al.*, 1987). Again, these SP stud farms with a high number of horses moving in and off the premises would benefit from the implementation of biosecurity measures and a routine EHV-1/-4 vaccination of the horses in the stud farm (Ather and Greene, 2005 ; Glass and Barnes, 2013).

Within the group of horses older than 7 years of age (n= 117), which was the group with the highest percentage of EHV-1/-4 seropositives, location was pinpointed as a risk factor. Horses living in SP stud farms located in the South of the central region were 1.3 times more likely to seropositive to EHV-1/-4 than those living in the North (83% vs. 65% respectively); the same relationship had been found in the univariable analysis comprising the 334 SP horses sampled (OR= 2.05). Whereas some studies have found differences in the seroprevalence of EHV-1/-4 between areas of the same country (Ataseven, 2010; Ataseven *et al.*, 2009), other studies revealed no differences between areas (Ruiz-Saenz *et al.*, 2007, 2008). In our study, most of the SP stud farms located in the South of the central region had other equine activities in the premises (dressage, liveries, etc.) (p<0.001) and the horses in these stud farms went out to more fairs and competitions than the horses in SP stud farm in the North of the central region (p<0.001). These factors have previously been related to the presence of EHV-1/-4 and other viral infections in horse premises and could have had an influence in this study (Lunn *et al.*, 2009; Mumford *et al.*, 1987). Therefore the number of human and horse movements in and off the premises would have an influence in SP stud farms, where the implementation of biosecurity measures (e.g. the isolation and quarantine of new horses) and a routine EHV-1/-4 vaccination of the horses would be beneficial (Ather and Greene, 2005 ; Glass and Barnes, 2013).

Even when not considered significant in the univariable analysis comprising the whole population of unvaccinated horses of this study (n=334), according to the decision tree analysis and in two groups of horses (n=69 and n=63 respectively), separating the breeding mares from the young horses in the stud farm was protective against EHV-1/-4 infection; in both groups horses living in stud farms where mares were not separated from youngsters had twice the probability of being EHV-1/-4 seropositive than those living in stud farms where this segregation existed. It has been published that the mare and foal population is a reservoir of EHV-1, from which new cases of infection propagate through the foal population both before and after weaning; the long standing management practices of separating pregnant mares from other groups of horses have demonstrated to reduce the incidence of EHV-1 abortion (Gilkerson *et al.*, 1999a) and this measure would be of much benefit in the SP stud farms in central Spain.

To summarise, within the SP horses sampled in this study, the implementation of biosecurity measures and a routine EHV-1/-4 vaccination regime in the horses of the stud farm would be key in order to reduce the risk of the introduction of EHV-1/-4 into the stud farm (Ather and Greene, 2005 ; Glass and Barnes, 2013), and this would be especially important in those horses residing in stud farms with a large number of visits from people, a large number of horses moving in and off the premises, stud farms with other equine activities in the premises and those where the horses go out to fairs and/or competitions (Lunn *et al.*, 2009; Mumford *et al.*, 1987). All SP stud farms would also benefit from the long standing management practices of separating pregnant mares from other groups of horses, which have demonstrated to reduce the incidence of EHV-1 abortion (Gilkerson *et al.*, 1999a).

## V.5 OCCURRENCE OF REPRODUCTIVE PROBLEMS IN THE SP MARES AND STALLIONS OF THE STUDY.

Of the SP breeding mares sampled in the study, 29% (23%-34%) were reported to have had at least one reproductive problem in the past 12 months. There are no data published regarding the prevalence of reproductive problems in SP mares or stallions; the only studies that have been published on this subject were carried out either in Lusitano mares (a breed that has plenty of genetic similarities to the SP horse (Lopes *et al.*, 2005)) or in Standardbred mares. Lusitano mares had a foaling rate (percentage of foalings in the mares within the covered or inseminated mares) of 74.5%, which means that, similarly to our study, 25.5% of mares had a problem that impeded foaling (Fradinho *et al.*, 2014), whereas Standardbred mares had a foaling rate of 71% and therefore a 29% of mares with a reproductive problem, same as the mares in our study (Katila *et al.*, 2010). In our study, the mean age of the SP breeding mares with reproductive problems was significantly higher than that in the SP breeding mares without reproductive problems ( $p=0.02$ ); the association between age and the occurrence of reproductive problems has already been shown in a study where foaling rates decreased with age (Katila *et al.*, 2010) and a study where mare age had a significant impact on efficiency of becoming pregnant, maintaining pregnancy and producing a live foal (Bosh *et al.*, 2009).

In the SP mares with reproductive problems of this study ( $n=74$ ), gestation loss was the most frequent problem [ $n=38/74$ , 51% (40% - 62.7%)], and the suspected cause for gestation loss was identified only in 4 out of these 38 mares [10.5% (0%-18%)], which is quite low compared to other study where the cause was found in 58.7% of the cases (Marenzoni *et al.*, 2013). Within the mares with gestation loss of our study, most had late abortions [ $n=20/38$  mares, 53% (37%-68%)], which is a cause for concern due to the high economic costs associated to a late abortion (Allen, 2002, 1999; de Mestre, 2013; Schulman *et al.*, 2013). In our study and within the few cases that were investigated by the veterinary surgeon of the stud farm, no viral infections were reported to be the cause for gestation loss or neonatal disease. This is in contrast to other study where EHV was found to be the causing agent for 40% of fetal loss (Marenzoni *et al.*, 2013), showing that EHV-1/-4

was probably not even considered as a cause of abortion in many of the mares of this study. Therefore an effort should be made to increase the awareness of veterinary surgeons and stud farm owners about the investigation of the cause of abortions and the need to analyse the foetus and/or placenta and also to make them consider viral diseases (such as EHV-1/-4 and EVA) as one of the main causes for abortion and neonatal disease (HBLB, 2013; Marenzoni *et al.*, 2013; Timoney and McCollum, 1993b).

Of the SP breeding stallions sampled in the study, only 7% (1.5%-11.7%) were reported to have reproductive problems during the past 12 months, and out of these horses, 50% showed a lack of libido. There are no data published regarding the prevalence of reproductive problems in SP stallions or stallions of other breeds, however, in a study that evaluated the behaviour of SP stallions during mating, 13.8% showed no erection and 17.2% showed no ejaculation (Hernández *et al.*, 2000).

## V.5.1 Association of reproductive problems with EVA status

Even though EVA infection has only been associated to abortions in mares, death or disease in the newborn foals and a possible transient decrease in the fertility in the infected stallion and that no signs of a decrease in problems or fertility have been demonstrated in the infected mare further to the elimination of the virus (Huntington *et al.*, 1990a; Timoney, 2003; Timoney and McCollum, 1993b), when the association between EVA seropositivity and the presence of specific reproductive problems in the past 12 months was analysed in the SP mares sampled showing reproductive problems (n=74) compared to the active breeding mares with no reproductive problems (n=183), significant associations were found for some problems such as cyclic irregularities/repeat breeding (p=0.006), late abortions (p=0.006) and problems with the neonate (p=0.006).

Interestingly, even though EVA has previously been isolated from abortions that occurred from 3 to 10 months (Timoney and McCollum, 1993b), EVA seropositivity was not associated with the occurrence of early abortions in the SP mares of this study.

Since EVA antibodies remain in serum for a long time (even for life) in infected horses (Timoney, 2003; Timoney and McCollum, 1993b), it would be difficult to ascertain whether EVA infections happened in the past 12 months, explaining the presence of reproductive problems in these mares, or if EVA infections happened long prior to the past 12 months, leaving these mares with reproductive sequelae which could have predisposed to cyclic irregularities, late abortions or problems with their neonate. Another reason could be the fact that active breeding mares with reproductive problems would have had an increased number of exposures to several stallions and therefore to possible carriers of the EAV.





## *VI. FUTURE WORK*



## *VI. FUTURE WORK*

In addition to providing valuable information, relevant to both veterinary surgeons and SP stud farm owners, regarding the current situation of EIA, EVA and EHV-1/-4 in SP stud farms in central Spain, this work has also described the characteristics and management measures with regards to the prevention and control of equine infectious diseases in this population of stud farms. Within these characteristics and management measures, this study has identified several potential risk factors associated with EVA and EHV-1/-4 seropositivity in the SP horses in central Spain. The potential risk factors highlighted by this study are areas that would require targeted research and further academic study.

Given the highly complex and clustered nature of the data gathered during this study, additional analysis of the variables presented within this thesis by means of a multivariable mixed logistic regression will take into account a possibly associated stud farm factor and will provide more reliable results regarding risk factors associated with EVA and EHV-1/-4 seropositivity. The model obtained in future work would aid predicting which horses are more likely to be seropositive to EVA and/or EHV-1/-4 and it will be validated in horses different to those sampled in this study but from the same reference population. A predictive model would be of invaluable help, for example when trying to implement a routine testing scheme for EVA and introduce EVA vaccination in Spain.





## *VII. CONCLUSIONS*



## VII. CONCLUSIONS

1. The absence of EIA seropositive animals in our study provided evidence of **freedom from EIA infection** in the SP breeding horse population in central Spain, despite the recent EIA outbreaks in nearby European countries and the frequent national and international horse movements in this area.
2. The seroprevalence of EVA in the horse population [**17.3% (14.2% - 20.4%)**] and in the SP stud farm population [**37.1% (21.1% - 53.1%)**] of this study was highly relevant; therefore EVA should not be considered a sporadic disease in the SP stud farms in central Spain.
3. Within the unvaccinated population of this study, EHV-1/-4 seroprevalence in the horses [**53.3% (47.9% - 58.6%)**] and in the SP stud farms [**90% (79.3% - 100%)**] demonstrated that this disease is widely spread in the SP breeding horse population in central Spain.
4. Nearly half of the SP stud farms sampled in this study (46%) reported having equine activities other than breeding in the premises, the same percentage (46%) reported outings to fairs and competitions with their horse and 54% acquired new horses in the past 12 months, demonstrating that **horse movements** are frequent in the SP stud farms in central Spain.
5. Most of the SP stud farms sampled had **basic measures of prevention and control of equine infectious diseases**, such as a worming regime (97%), a disinfection regime (83%), measures against insects (83%) and rodents (80%). However, nearly half of the SP stud farms showed an **absence of specific measures**, such as an EHV-1/-4 vaccination regime (46%) and segregating breeding mares from young horses (49%).
6. In our study, **29% (23%-34%)** of active breeding SP mares in central Spain showed reproductive problems in the past 12 months, of which gestation loss was the most frequent problem, and only **7% (2-12%)** of active breeding SP stallions showed reproductive problems related to a lack of libido or abnormalities in the semen.

7. In order to maintain the SP breeding horse population in central **Spain free from EIA infection, measures against insects** should be implemented in every stud farm and judicious biosecurity that includes **routine EIA testing** of the horses prior to importation into Spain and after their arrival should be established to prevent the introduction of subclinically EIA infected horses from Europe.
8. Within the SP horses of this study, those residing in stud farms where there was a **high horse density of population**, or had a **large number of visits from people** or **frequent movements of horses** were more likely to be **EVA seropositive**; these stud farms should establish good biosecurity measures in the premises as well as a prompt detection and isolation of possible carriers or acutely infected horses and routine EVA testing prior to the introduction of new horses.
9. **Older** SP horses, and those residing in stud farms with a **large number of visits from people, frequent movements of horses** and **other equine activities in the premises** were more likely to be **EHV-1/-4 seropositive**; the implementation of biosecurity measures, routine EHV-1/-4 vaccination and specific measures such as separating pregnant mares from other horses would be key in order to reduce the risk of the introduction of EHV-1/-4 into these stud farms.
10. In our study, **EVA seropositivity** was associated to the occurrence of **cyclic irregularities/repeat breeding** ( $p=0.006$ ), **late abortions** ( $p=0.006$ ) and **problems with the neonate** ( $p=0.006$ ), suggesting that EVA should be considered when investigating the cause for reproductive problems in the SP mares in central Spain.
11. In our opinion, the data obtained in the present study should encourage the Administration, the different Breeders' associations and veterinary surgeons to pursue the **education and awareness** of stud farm owners regarding the presence, prevention and control of equine infectious diseases in a breeding stud farm.



## *VIII. APENDIXES*



# VIII. APPENDIXES

## APPENDIX 1. Questionnaire of reproductive problems in the SP stallion.



### REPRODUCTIVE PROBLEMS IN THE SPANISH PUREBRED STALLION



1. Name of the stud farm: \_\_\_\_\_

2. Stallion's identification: \_\_\_\_\_

3. Stallion's age: 

|  |  |
|--|--|
|  |  |
|--|--|

  
Y Y

4. Time that the stallion has spent breeding: 

|  |  |
|--|--|
|  |  |
|--|--|

|  |  |
|--|--|
|  |  |
|--|--|

  
Y Y M M

5. Specify which reproductive problem/s has the stallion suffered:

- Abnormalities of the penis
- Abnormalities of the prepuce
- Abnormalities of the testicles, spermatic cord and/or scrotum
- Lack of libido
- Abnormalities of the semen
- Problems when ejaculating

Specify the cause, if known:

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Specify the treatment followed:

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N/A (the stallion did not follow any treatment)



## APPENDIX 2. Questionnaire of reproductive problems in the SP mare.



### REPRODUCTIVE PROBLEMS IN THE SPANISH PUREBRED MARE



1. Name of the stud farm: \_\_\_\_\_

2. Mare's identification: \_\_\_\_\_

3. Mare's age: 

|   |   |
|---|---|
|   |   |
| A | A |

4. Time that the mare has spent breeding: 

|   |   |
|---|---|
|   |   |
| A | A |

|   |   |
|---|---|
|   |   |
| M | M |

5. Specify which reproductive problem/s has the mare suffered:

- Cyclic irregularity
- Anestrus
- Repeat breeding
- Pregnancy loss
  - Early abortion (up to gestation day 150)
  - Late abortion (from gestation day 150 to gestation day 300)
  - Stillbirth (from gestation day 300 onwards)
- Prolonged gestation (more than 370 days of gestation)
- Dystocia
- Retained fetal membranes
- Alterations in lactation
- Problems in the neonate
  - Immature foal
  - Sick/weak foal
  - Neonatal death

Specify the cause, if known:

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Specify the treatment followed:

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N/A (the mare did not follow any treatment)

APPENDIX 3. Questionnaire of general management and health in SP breeding stud farms in central Spain.



**GENERAL MANAGEMENT AND HEALTH IN  
SPANISH PUREBRED STUD FARMS IN  
CENTRAL SPAIN**



**Section 1: CONTACT DETAILS**

1. Title: Miss/Mr/Mrs/Dr/Other: \_\_\_\_\_  
 First Name: \_\_\_\_\_ Surname: \_\_\_\_\_
2. Stud farm name and address:  
 Address: \_\_\_\_\_  
 \_\_\_\_\_  
 Town: \_\_\_\_\_ Province: \_\_\_\_\_  
 Postcode: 

|  |  |  |  |  |
|--|--|--|--|--|
|  |  |  |  |  |
|--|--|--|--|--|
3. Telephone number: \_\_\_\_\_
4. Mobile number: \_\_\_\_\_
5. E-mail address: \_\_\_\_\_

**Contact Preference**

Mail

Email

Phone

**Section 2: GENERAL INFORMATION REGARDING THE STUD FARM**

6. Which is the total size of the stud farm?  
 (Area in  $hm^2$ ): \_\_\_\_\_
7. Which is the size of the area dedicated to equine breeding in the stud farm?  
 (Area in  $hm^2$ ): \_\_\_\_\_
8. Year when you first started running the stud farm: 

|  |  |  |  |
|--|--|--|--|
|  |  |  |  |
|--|--|--|--|

  
Y Y Y Y

9. Is the stud farm a member of any Breeders Association?

Yes

No

If so, which one/s?

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10. Is there any activity (horse-related) associated to the stud farm, other than the breeding of SPH?

Yes

No

If so, which one/s?

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11. Do you own other animals in the stud farm, other than horses?

Yes

No

If so, which animals and how many?

Dogs: \_\_\_\_\_

Cattle: \_\_\_\_\_

Donkeys: \_\_\_\_\_

Goats: \_\_\_\_\_

Cats: \_\_\_\_\_

Sheep: \_\_\_\_\_

Mules: \_\_\_\_\_

Pigs: \_\_\_\_\_

Other: \_\_\_\_\_

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**Section 3: THE SPANISH PUREBRED STUD FARM**

12. Which is the total number of horses of all breeds in the stud farm:

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13. Which is the total number of Spanish Purebred Horses (SPH) in the stud farm:

---

14. Disclose how many of your SPH are:

Mares used for breeding at present:

Geldings 4 years old or older:

Mares retired from breeding at present:

Teasers used for breeding:

Stallions used for breeding at present:

Nursing foals:

Stallions retired for breeding at present:

Yearlings:

Youngsters (two and three years old):



**Section 4: MOVEMENTS WITHIN THE STUD FARM.**

18. Approximately, in the past 12 months, how many outings to sale fairs did you do with the stallions in the stud farm (SPH and other breeds)?

\_\_\_\_\_

19. Approximately, in the past 12 months, how many outings to competitions (dressage, morphological, etc.) did you do with the horses in the stud farm (SPH and other breeds)?

\_\_\_\_\_

20. Approximately, in the past 12 months, how many outings to other studs did you do with the stallions in the stud farm (SP stallions and other breeds)?

\_\_\_\_\_

21. Approximately, in the past 12 months, how many outings to other studs did you do with your breeding mares (SP and other breeds)?

\_\_\_\_\_

22. Approximately, in the past 12 months, how many newcomers (SPH and other breeds) from other studs did you have in the stud farm?

\_\_\_\_\_

23. Approximately, in the past 12 months, how many of your own horses (SPH and other breeds) were sold and left the stud farm?

\_\_\_\_\_

24. Approximately, in the past 12 months, how many visits from stallions from other studs (SPH and other breeds) did you have in the stud farm?

\_\_\_\_\_

25. Approximately, in the past 12 months, how many visits from mares from other studs (SPH and other breeds) did you have in the stud farm?

\_\_\_\_\_

26. In average, in the past 12 months, how many visits from outsiders (visitants, buyers, etc.) did you have per month in the stud farm?

\_\_\_\_\_

27. If you own other animals in the stud farm, were there any movements of these in the past 12 months?

N/A

No, there were no movements in the past 12 months

Yes, there were movements in the past 12 months

Specify:

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**Section 5: GENERAL HEALTH MANAGEMENT IN THE STUD FARM.**

28. In the stud farm, are horses vaccinated against Tetanus as per manufacturer's recommendations?

- No
- Yes, only the breeding stock (SPH and other breeds)
- Yes, all the horses in the stud farm
- Other

Specify: \_\_\_\_\_

29. In the stud farm, are horses vaccinated against Equine Influenza as per manufacturer's recommendations?

- No
- Yes, only the breeding stock (SPH and other breeds)
- Yes, all the horses in the stud farm
- Other

Specify: \_\_\_\_\_

30. In the stud farm, are horses vaccinated against Equine Herpesvirus-1/-4 as per manufacturer's recommendations?

- No
- Yes, only the breeding stock (SPH and other breeds)
- Yes, all the horses in the stud farm
- Yes, only pregnant mares (SPH and other breeds)
- Other

Specify: \_\_\_\_\_

31. In the stud farm, are horses vaccinated against West Nile Virus as per manufacturer's recommendations?

No

Yes, only the breeding stock (SPH and other breeds)

Yes, all the horses in the stud farm

Other

Specify: \_\_\_\_\_

32. In the stud farm and regarding the horses, do you have a routine worming scheme (horses wormed minimum 2 times per year)?

No

Yes, only in the breeding stock (SPH and other breeds)

Yes, in all the horses in the stud farm

Other

Specify: \_\_\_\_\_

Please specify how many times per year are horses wormed and which product/s were used in the past 12 months:

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33. In the stud farm, who coordinates and manages the breeding?

Owner

Vet

Both the owner and vet

Other

Specify: \_\_\_\_\_

**Section 6: DISINFECTION AND DISINFECTATION IN THE STUD FARM.**

34. In the stud farm, is there a disinfection scheme in place?

No

Yes

Specify which parts of the stud farm are disinfected, how often and which products are used:

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35. In the stud farm, is there a specific scheme in order to eliminate insects in place?

No

Yes

Specify which parts of the stud farm are treated against insects, how often and which products/measures are used:

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36. In the stud farm, is there a specific scheme in order to eliminate rodents in place?

No

Yes

Specify which parts of the stud farm are treated against rodents, how often and which products/measures are used:

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**Section 7: REPRODUCTIVE PERFORMANCE OF THE S.P. STALLIONS IN THE STUD FARM.**  
Note this section relates ONLY to Spanish Purebred Stallions in the stud farm

Not applicable – There are no S.P. stallions in the stud farm. Go to section 8.

37. Approximately, in the past 12 months, which percentage of your **SP stallions**’ breeding was done by natural covering and which percentage was done by Artificial insemination?

% by natural covering

% by artificial insemination

38. Approximately, in the past 12 months, which percentage of your **SP stallions** presented any reproductive problems (e.g. abnormalities of the penis, prepuce, testicles, spermatic cord, scrotum, lack of libido, abnormalities of the semen, problems when ejaculating...)?

% with reproductive problems

Specify the nature of the problems in the SP stallions:

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**Section 8: REPRODUCTIVE PERFORMANCE OF THE S.P. MARES IN THE STUD FARM.**  
Note this section relates ONLY to Spanish Purebred Mares in the stud farm

Not applicable – There are no S.P. mares in the stud farm.

39. Approximately, in the past 12 months, which percentage of the breeding with the **S.P. mares** in the stud farm was done by natural covering and which percentage was done by Artificial insemination?

% by natural covering

% by artificial insemination

40. Approximately, in the past 12 months, which percentage of the **S.P. mares** in the stud farm presented any reproductive problems (e.g. cyclic irregularity, anestrus, repeat breeding, pregnancy loss, prolonged gestation, dystocia, retained fetal membranes, alterations in lactation, problems in the neonate...)?

% with reproductive problems

Specify the nature of the problems in the mares:

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41. Approximately, in the past 12 months, how many early abortions (up to gestation day 150) did you have in the **S.P. mares** of the stud farm?

|                                       |
|---------------------------------------|
| Early abortions in the past 12 months |
|---------------------------------------|

42. Approximately, in the past 12 months, how many late abortions (from gestation day 150 to gestation day 300) and/or stillbirths (from gestation day 300 onwards) did you have in the **S.P. mares** of the stud farm?

|                                      |
|--------------------------------------|
| Late abortions in the past 12 months |
|--------------------------------------|

|                                   |
|-----------------------------------|
| Stillbirths in the past 12 months |
|-----------------------------------|

43. Approximately, in the past 12 months, how many neonatal deaths and/or weak foals did you have in the **S.P. mares** of the stud farm?

|   |
|---|
| Neonatal deaths and/or weak foals in the past 12 months |
|---|

44. Approximately, in the past 12 months, how many barren **S.P. mares** did you have left in the stud farm?

|                                    |
|------------------------------------|
| Barren mares in the past 12 months |
|------------------------------------|

45. Approximately, in the past 12 months, which percentage of the **S.P. mares** in the stud farm conceived in the first cycle, second cycle, third cycle, fourth cycle or further cycles?

|                      |
|----------------------|
| % In the first cycle |
|----------------------|

|                       |
|-----------------------|
| % In the second cycle |
|-----------------------|

|                      |
|----------------------|
| % In the third cycle |
|----------------------|

|   |
|---|
| % In the fourth cycle or further cycles |
|---|

**Date of completion of this questionnaire:**

|                      |                      |   |                      |                      |   |                      |                      |                      |                      |
|----------------------|----------------------|---|----------------------|----------------------|---|----------------------|----------------------|----------------------|----------------------|
| <input type="text"/> | <input type="text"/> | / | <input type="text"/> | <input type="text"/> | / | <input type="text"/> | <input type="text"/> | <input type="text"/> | <input type="text"/> |
| D                    | D                    |   | M                    | M                    |   | Y                    | Y                    | Y                    | Y                    |



APPENDIX 4. Individual characteristics and EVA ELISA, EVA SN, EHV-1/-4 ELISA, EHV-1 and EHV-4 SN results of the SP horses included in the study.

| No | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 1  | A1  | Stallion | 13  | yes            | no                    | no                   | 28/09/2011    | Negative  |        | Negative  |          |          |
| 2  | A2  | Stallion | 6   | yes            | no                    | no                   | 28/09/2011    | Negative  |        | Negative  |          |          |
| 3  | A3  | Stallion | 4   | yes            | no                    | no                   | 28/09/2011    | Negative  |        | Positive  | 2.3      | 1.5      |
| 4  | A4  | Mare     | 21  | yes            | yes                   | yes                  | 28/09/2011    | Negative  |        |           |          |          |
| 5  | A5  | Mare     | 6   | yes            | no                    | yes                  | 28/09/2011    | Negative  |        |           |          |          |
| 6  | A6  | Mare     | 9   | yes            | no                    | yes                  | 28/09/2011    | Negative  |        |           |          |          |
| 7  | A7  | Mare     | 10  | no             | n/a                   | no                   | 28/09/2011    | Negative  |        | Positive  | 2.6      | 2        |
| 8  | B1  | Stallion | 5   | no             | n/a                   | no                   | 06/10/2011    | Positive  | 3.2    | Positive  | 1.5      | 1.2      |
| 9  | B2  | Stallion | 7   | no             | n/a                   | no                   | 06/10/2011    | Positive  | 3.5    | Positive  | 1.8      | 1.7      |
| 10 | B3  | Stallion | 12  | no             | n/a                   | no                   | 06/10/2011    | Negative  |        | Positive  | 1.4      | 1.7      |
| 11 | B4  | Stallion | 9   | no             | n/a                   | no                   | 06/10/2011    | Positive  | 3.1    | Positive  | 2.4      | 1.4      |
| 12 | B5  | Stallion | 6   | no             | n/a                   | no                   | 06/10/2011    | Positive  | 2.4    | Negative  |          |          |
| 13 | B6  | Stallion | 4   | no             | n/a                   | no                   | 06/10/2011    | Negative  |        | Negative  |          |          |
| 14 | B7  | Mare     | 2,5 | no             | n/a                   | no                   | 06/10/2011    | Negative  |        | Negative  |          |          |
| 15 | B8  | Mare     | 14  | yes            | yes                   | no                   | 06/10/2011    | Negative  |        | Positive  | >2.6     | 1.8      |
| 16 | B9  | Stallion | 4   | no             | n/a                   | no                   | 06/10/2011    | Negative  |        | Positive  | 1.2      | 1.4      |
| 17 | B10 | Stallion | 4   | no             | n/a                   | no                   | 06/10/2011    | Negative  |        | Equivocal | 2        | 1.5      |
| 18 | B11 | Stallion | 8   | no             | n/a                   | no                   | 06/10/2011    | Positive  | 3.5    | Positive  | 1.7      | 0.9      |
| 19 | C1  | Stallion | 9   | yes            | yes                   | no                   | 07/10/2011    | Negative  |        | Positive  | >2.6     | 2        |
| 20 | C2  | Stallion | 15  | yes            | yes                   | no                   | 07/10/2011    | Positive  | 1.7    | Positive  | >2.6     | 2        |
| 21 | C3  | Stallion | 24  | no             | n/a                   | no                   | 07/10/2011    | Positive  | < 0.6  | Positive  | 2.1      | 1.5      |
| 22 | C4  | Stallion | 6   | no             | n/a                   | no                   | 07/10/2011    | N.I.      | < 0.6  | Positive  | 2.6      | 1.4      |

| No | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 23 | C5  | Stallion | 13  | yes            | no                    | no                   | 07/10/2011    | Negative  |        | Positive  | 2.6      | 2.3      |
| 24 | C6  | Stallion | 15  | no             | n/a                   | no                   | 07/10/2011    | Negative  |        | Positive  | 2.4      | 1.5      |
| 25 | C7  | Mare     | 14  | yes            | yes                   | yes                  | 07/10/2011    | Positive  | 2.3    |           |          |          |
| 26 | C8  | Mare     | 14  | yes            | yes                   | yes                  | 07/10/2011    | Positive  | 2.3    |           |          |          |
| 27 | C9  | Mare     | 17  | yes            | no                    | yes                  | 07/10/2011    | Negative  |        |           |          |          |
| 28 | C10 | Mare     | 13  | yes            | yes                   | yes                  | 07/10/2011    | Positive  | 1.7    |           |          |          |
| 29 | C11 | Mare     | 8   | yes            | yes                   | yes                  | 07/10/2011    | Equivocal | 2.3    |           |          |          |
| 30 | C12 | Mare     | 19  | yes            | yes                   | yes                  | 07/10/2011    | Positive  | 3      |           |          |          |
| 31 | C13 | Mare     | 18  | yes            | yes                   | yes                  | 07/10/2011    | Positive  | 3.4    |           |          |          |
| 32 | C14 | Mare     | 13  | yes            | no                    | yes                  | 07/10/2011    | Negative  |        |           |          |          |
| 33 | C15 | Mare     | 5   | yes            | no                    | yes                  | 07/10/2011    | Negative  |        |           |          |          |
| 34 | C16 | Mare     | 4   | yes            | no                    | yes                  | 07/10/2011    | Negative  |        |           |          |          |
| 35 | C17 | Mare     | 4   | yes            | no                    | yes                  | 07/10/2011    | Negative  |        |           |          |          |
| 36 | C18 | Mare     | 4   | yes            | no                    | yes                  | 07/10/2011    | Negative  |        |           |          |          |
| 37 | C19 | Mare     | 6   | yes            | yes                   | yes                  | 07/10/2011    | Negative  |        |           |          |          |
| 38 | D1  | Mare     | 9   | yes            | no                    | no                   | 20/06/2012    | Negative  |        | Negative  |          |          |
| 39 | D2  | Mare     | 7   | yes            | no                    | no                   | 20/06/2012    | Negative  |        | Positive  | 2.3      | 2        |
| 40 | D3  | Mare     | 4   | yes            | no                    | no                   | 20/06/2012    | Negative  |        | Positive  | >2.6     | 1.8      |
| 41 | D4  | Mare     | 18  | yes            | no                    | no                   | 20/06/2012    | Negative  |        | Positive  | >2.6     | 1.8      |
| 42 | D5  | Stallion | 11  | no             | n/a                   | no                   | 20/06/2012    | Negative  |        | Positive  | 2.4      | 2        |
| 43 | D6  | Stallion | 8   | no             | n/a                   | no                   | 20/06/2012    | Negative  |        | Positive  | 1.8      | 1.5      |
| 44 | D7  | Stallion | 6   | no             | n/a                   | no                   | 20/06/2012    | Negative  |        | Negative  |          |          |
| 45 | D8  | Stallion | 10  | no             | n/a                   | no                   | 20/06/2012    | Positive  | 2.9    | Positive  | 2.3      | 2        |
| 46 | D9  | Stallion | 10  | no             | n/a                   | no                   | 20/06/2012    | Negative  |        | Positive  | 2.4      | 1.7      |

| No | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 47 | D10 | Stallion | 7   | no             | n/a                   | no                   | 20/06/2012    | Negative  |        | Negative  |          |          |
| 48 | D11 | Mare     | 6   | yes            | no                    | no                   | 20/06/2012    | Negative  |        | Positive  | 2.6      | 1.4      |
| 49 | D12 | Mare     | 5   | yes            | yes                   | no                   | 20/06/2012    | Negative  |        | Positive  | 2.3      | 2.3      |
| 50 | D13 | Mare     | 8   | yes            | no                    | no                   | 20/06/2012    | Negative  |        | Negative  |          |          |
| 51 | D14 | Mare     | 12  | yes            | no                    | no                   | 20/06/2012    | Negative  |        | Positive  | 2        | 1.5      |
| 52 | D15 | Mare     | 9   | yes            | no                    | no                   | 20/06/2012    | Negative  |        | Positive  | 2.6      | 2.1      |
| 53 | D16 | Mare     | 6   | yes            | yes                   | no                   | 20/06/2012    | Negative  |        | Positive  | 2        | 1.7      |
| 54 | D17 | Mare     | 3   | yes            | no                    | no                   | 20/06/2012    | Negative  |        | Positive  | 2        | 1.7      |
| 55 | D18 | Stallion | 16  | yes            | no                    | no                   | 20/06/2012    | Negative  |        | Positive  | 2.6      | 2.3      |
| 56 | D19 | Mare     | 8   | yes            | yes                   | no                   | 20/06/2012    | Negative  |        | Positive  | 2.1      | 1.5      |
| 57 | E1  | Mare     | 6   | yes            | no                    | yes                  | 10/10/2011    | Positive  | 2.5    |           |          |          |
| 58 | E2  | Stallion | 7   | no             | n/a                   | no                   | 10/10/2011    | Negative  |        | Equivocal | 1.1      | 0.5      |
| 59 | E3  | Stallion | 8   | no             | n/a                   | no                   | 10/10/2011    | Negative  |        | Positive  | 1.7      | 0.9      |
| 60 | E4  | Stallion | 6   | no             | n/a                   | no                   | 10/10/2011    | Negative  |        | Positive  | 2.6      | 2.1      |
| 61 | E5  | Stallion | 6   | yes            | no                    | no                   | 10/10/2011    | Positive  | 3.4    | Positive  | 1.4      | 0.8      |
| 62 | E6  | Stallion | 5   | no             | n/a                   | no                   | 10/10/2011    | Positive  | 2.5    | Positive  | 2.3      | 1.2      |
| 63 | E7  | Stallion | 4   | no             | n/a                   | no                   | 10/10/2011    | Negative  |        | Positive  | 2.4      | 2        |
| 64 | E8  | Stallion | 4   | no             | n/a                   | no                   | 10/10/2011    | Negative  |        | Positive  | 1.5      | 0.8      |
| 65 | E9  | Mare     | 17  | yes            | yes                   | no                   | 10/10/2011    | Positive  | 2.9    | Positive  | >2.6     | >2.6     |
| 66 | E10 | Mare     | 7   | yes            | yes                   | yes                  | 10/10/2011    | Positive  | 3.5    |           |          |          |
| 67 | E11 | Mare     | 6   | yes            | yes                   | yes                  | 10/10/2011    | Positive  | 3.2    |           |          |          |
| 68 | E12 | Mare     | 8   | yes            | no                    | yes                  | 10/10/2011    | Positive  | 2.6    |           |          |          |
| 69 | E13 | Mare     | 9   | yes            | no                    | yes                  | 10/10/2011    | Positive  | 2.7    |           |          |          |

| No | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 70 | E14 | Mare     | 12  | yes            | yes                   | yes                  | 10/10/2011    | Positive  | 3.6    |           |          |          |
| 71 | E15 | Mare     | 19  | yes            | no                    | yes                  | 10/10/2011    | Positive  | 3.2    |           |          |          |
| 72 | E16 | Stallion | 2   | no             | n/a                   | no                   | 10/05/2012    | Negative  |        | Equivocal | 0.8      | 0.9      |
| 73 | E17 | Mare     | 1   | no             | n/a                   | no                   | 10/05/2012    | Equivocal | 1.4    | Equivocal | 2.4      | 2        |
| 74 | E18 | Mare     | 1   | no             | n/a                   | no                   | 10/05/2012    | Positive  | 2.6    | Positive  | 2.1      | 1.8      |
| 75 | E19 | Stallion | 2   | no             | n/a                   | no                   | 10/05/2012    | Negative  |        | Positive  | 2        | 1.7      |
| 76 | E20 | Stallion | 1   | no             | n/a                   | no                   | 10/05/2012    | Positive  | 3      | Positive  | >2.6     | 2.7      |
| 77 | E21 | Stallion | 1   | no             | n/a                   | no                   | 10/05/2012    | Positive  | 3.2    | Equivocal | 1.1      | 1.8      |
| 78 | E22 | Stallion | 1   | no             | n/a                   | no                   | 10/05/2012    | Positive  | 2.6    | Negative  |          |          |
| 79 | E23 | Stallion | 2   | no             | n/a                   | no                   | 10/05/2012    | Negative  |        | Equivocal | 1.4      | 1.4      |
| 80 | E24 | Stallion | 2   | no             | n/a                   | no                   | 10/05/2012    | Negative  |        | Negative  |          |          |
| 81 | E25 | Stallion | 2   | no             | n/a                   | no                   | 10/05/2012    | Negative  |        | Positive  | 2        | 1.7      |
| 82 | E26 | Stallion | 1   | no             | n/a                   | no                   | 10/05/2012    | Negative  |        | Negative  |          |          |
| 83 | E27 | Stallion | 2   | no             | n/a                   | no                   | 18/03/2012    | Negative  |        | Negative  |          |          |
| 84 | F1  | Stallion | 2,5 | no             | n/a                   | no                   | 11/10/2011    | Negative  |        | Negative  |          |          |
| 85 | F2  | Stallion | 10  | yes            | no                    | no                   | 11/10/2011    | Negative  |        | Negative  |          |          |
| 86 | F3  | Mare     | 9   | yes            | yes                   | no                   | 11/10/2011    | Negative  |        | Negative  |          |          |
| 87 | F4  | Mare     | 5   | yes            | n/a                   | no                   | 11/10/2011    | Negative  |        | Negative  |          |          |
| 88 | F5  | Mare     | 6   | yes            | n/a                   | no                   | 11/10/2011    | Positive  | < 0.6  | Equivocal | 1.4      | 1.4      |
| 89 | F6  | Mare     | 8   | yes            | no                    | no                   | 11/10/2011    | Negative  |        | Equivocal | 1.7      | 1.2      |
| 90 | F7  | Mare     | 8   | yes            | n/a                   | no                   | 11/10/2011    | Negative  |        | Positive  | >2.6     | 1.5      |
| 91 | F8  | Mare     | 4   | yes            | no                    | no                   | 11/10/2011    | Negative  |        | Negative  |          |          |
| 92 | F9  | Stallion | 2,5 | no             | n/a                   | no                   | 11/10/2011    | N.I.      | < 0.6  | Negative  |          |          |
| 93 | F10 | Mare     | 8   | yes            | n/a                   | no                   | 11/10/2011    | Negative  |        | Negative  |          |          |

| No  | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 94  | F11 | Mare     | 5   | yes            | n/a                   | no                   | 11/10/2011    | Negative  |        | Equivocal | 1.7      | 1.1      |
| 95  | G1  | Mare     | 5   | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Positive  | 2        | 2        |
| 96  | G2  | Mare     | 5   | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Positive  | 2        | 0.5      |
| 97  | G3  | Mare     | 5   | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Positive  | 1.7      | 0.8      |
| 98  | G4  | Mare     | 4   | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Positive  | 2.4      | 1.4      |
| 99  | G5  | Mare     | 6   | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Positive  | 2.6      | 2.3      |
| 100 | G6  | Mare     | 8   | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Equivocal | 1.1      | 0.8      |
| 101 | G7  | Mare     | 8   | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Positive  | 2.4      | 1.7      |
| 102 | G8  | Mare     | 15  | yes            | yes                   | no                   | 21/12/2011    | Negative  |        | Positive  | 2.6      | 1.8      |
| 103 | G9  | Mare     | 11  | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Positive  | >2.6     | 2        |
| 104 | G10 | Mare     | 9   | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Positive  | 2.6      | 2.3      |
| 105 | G11 | Mare     | 4   | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Positive  | 2.6      | 2        |
| 106 | G12 | Mare     | 7   | yes            | no                    | no                   | 21/12/2011    | Equivocal | < 0.6  | Positive  | 2.1      | 1.4      |
| 107 | G13 | Mare     | 11  | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Positive  | 2.6      | 1.7      |
| 108 | G14 | Stallion | 11  | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Positive  | >2.6     | 1.7      |
| 109 | G15 | Stallion | 15  | yes            | no                    | no                   | 21/12/2011    | Negative  |        | Positive  | >2.6     | 1.7      |
| 110 | H1  | Mare     | 8   | yes            | no                    | no                   | 26/12/2011    | Equivocal | < 0.6  | Positive  | 2.6      | >2.6     |
| 111 | H2  | Mare     | 3   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Equivocal | 1.7      | >2.6     |
| 112 | H3  | Mare     | 7   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | >2.6     | >2.6     |
| 113 | H4  | Mare     | 3   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | 2        | >2.6     |
| 114 | H5  | Mare     | 7   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | 2.1      | 1.7      |
| 115 | H6  | Mare     | 4   | yes            | yes                   | no                   | 26/12/2011    | Negative  |        | Positive  | >2.6     | 2        |
| 116 | H7  | Mare     | 4   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | 2.3      | 2.1      |
| 117 | H8  | Mare     | 3   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Negative  |          |          |

| No  | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 118 | H9  | Mare     | 4   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Equivocal | 1.4      | 0.9      |
| 119 | H10 | Mare     | 3   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Equivocal | 1.5      | 1.5      |
| 120 | H11 | Mare     | 3   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | 1.7      | 1.4      |
| 121 | H12 | Mare     | 3   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Equivocal | 1.5      | 1.2      |
| 122 | H13 | Stallion | 6   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | 1.7      | 1.4      |
| 123 | H14 | Mare     | 4   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | 1.5      | 1.7      |
| 124 | H15 | Mare     | 3   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | 2.6      | 1.8      |
| 125 | H16 | Mare     | 3   | yes            | no                    | no                   | 26/12/2011    | N.I.      | < 0.6  | Positive  | 1.4      | 1.7      |
| 126 | H17 | Mare     | 2   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | 2.3      | 1.5      |
| 127 | H18 | Mare     | 11  | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | >2.6     | 1.8      |
| 128 | H19 | Mare     | 6   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Negative  |          |          |
| 129 | H20 | Mare     | 4   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | 1.7      | 1.5      |
| 130 | H21 | Mare     | 4   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | 1.7      | 2        |
| 131 | H22 | Mare     | 6   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Equivocal | 1.4      | 1.4      |
| 132 | H23 | Mare     | 4   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | 2.6      | 1.1      |
| 133 | I1  | Mare     | 12  | yes            | no                    | yes                  | 26/12/2011    | Negative  |        |           |          |          |
| 134 | I2  | Mare     | 16  | yes            | no                    | yes                  | 26/12/2011    | Negative  |        |           |          |          |
| 135 | I3  | Mare     | 4   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Negative  |          |          |
| 136 | I4  | Mare     | 10  | yes            | no                    | yes                  | 26/12/2011    | Negative  |        |           |          |          |
| 137 | I5  | Mare     | 4   | yes            | yes                   | no                   | 26/12/2011    | Negative  |        | Positive  | 1.8      | 1.4      |
| 138 | I6  | Mare     | 4   | yes            | yes                   | no                   | 26/12/2011    | Negative  |        | Negative  |          |          |
| 139 | I7  | Mare     | 6   | yes            | no                    | yes                  | 26/12/2011    | Negative  |        |           |          |          |
| 140 | I8  | Mare     | 5   | yes            | yes                   | yes                  | 26/12/2011    | Negative  |        |           |          |          |
| 141 | I9  | Stallion | 8   | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Negative  |          |          |

| No  | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 142 | I10 | Stallion | 17  | yes            | no                    | no                   | 26/12/2011    | Negative  |        | Positive  | >2.6     | 1.2      |
| 143 | I11 | Stallion | 10  | yes            | no                    | no                   | 12/04/2012    | Negative  |        | Positive  | 2        | 1.4      |
| 144 | I12 | Mare     | 3   | no             | n/a                   | no                   | 12/04/2012    | Negative  |        | Equivocal | 2.3      | 1.4      |
| 145 | I13 | Stallion | 9   | no             | n/a                   | no                   | 12/04/2012    | Negative  |        | Positive  | 1.8      | 1.1      |
| 146 | I14 | Stallion | 3   | no             | n/a                   | no                   | 12/04/2012    | Negative  |        | Negative  |          |          |
| 147 | I15 | Stallion | 3   | no             | n/a                   | no                   | 12/04/2012    | Negative  |        | Negative  |          |          |
| 148 | I16 | Mare     | 4   | no             | n/a                   | no                   | 12/04/2012    | Negative  |        | Negative  |          |          |
| 149 | I17 | Stallion | 3   | no             | n/a                   | no                   | 12/04/2012    | Negative  |        | Equivocal | 1.7      | 1.7      |
| 150 | I18 | Stallion | 4   | no             | n/a                   | no                   | 12/04/2012    | Negative  |        | Equivocal | 1.1      | 0.8      |
| 151 | I19 | Stallion | 3   | no             | n/a                   | no                   | 12/04/2012    | Negative  |        | Equivocal | 2        | 1.8      |
| 152 | I20 | Stallion | 3   | no             | n/a                   | no                   | 12/04/2012    | Negative  |        | Negative  |          |          |
| 153 | I21 | Stallion | 4   | no             | n/a                   | no                   | 12/04/2012    | Negative  |        | Equivocal | 0.8      | 1.5      |
| 154 | I22 | Stallion | 4   | no             | n/a                   | no                   | 12/04/2012    | Negative  |        | Negative  |          |          |
| 155 | J1  | Mare     | 9   | yes            | no                    | yes                  | 17/01/2012    | Negative  |        |           |          |          |
| 156 | J2  | Mare     | 6   | yes            | no                    | yes                  | 17/01/2012    | Negative  |        |           |          |          |
| 157 | J3  | Mare     | 8   | yes            | no                    | yes                  | 17/01/2012    | Negative  |        |           |          |          |
| 158 | J4  | Stallion | 8   | yes            | no                    | no                   | 17/01/2012    | Negative  |        | Positive  | >2.6     | 2        |
| 159 | K1  | Stallion | 3   | no             | n/a                   | no                   | 17/10/2011    | Negative  |        | Negative  |          |          |
| 160 | K2  | Stallion | 2,5 | no             | n/a                   | no                   | 17/10/2011    | Negative  |        | Negative  |          |          |
| 161 | K3  | Mare     | 6   | yes            | no                    | yes                  | 17/10/2011    | Negative  |        |           |          |          |
| 162 | K4  | Mare     | 6   | yes            | no                    | yes                  | 17/10/2011    | Negative  |        |           |          |          |
| 163 | K5  | Mare     | 2,5 | no             | n/a                   | no                   | 17/10/2011    | Negative  |        | Negative  |          |          |
| 164 | K6  | Mare     | 6   | yes            | no                    | yes                  | 17/10/2011    | Negative  |        |           |          |          |
| 165 | K7  | Mare     | 6   | yes            | yes                   | yes                  | 17/10/2011    | Negative  |        |           |          |          |

| No  | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 166 | L1  | Stallion | 8   | yes            | no                    | yes                  | 12/04/2012    | Negative  |        |           |          |          |
| 167 | L2  | Stallion | 10  | yes            | no                    | yes                  | 12/04/2012    | Negative  |        |           |          |          |
| 168 | L3  | Stallion | 7   | yes            | no                    | yes                  | 12/04/2012    | Negative  |        |           |          |          |
| 169 | L4  | Stallion | 11  | yes            | no                    | no                   | 12/04/2012    | Negative  |        | Positive  | 2.6      | 1.4      |
| 170 | L5  | Stallion | 4   | yes            | no                    | yes                  | 12/04/2012    | Negative  |        |           |          |          |
| 171 | L6  | Stallion | 18  | yes            | no                    | yes                  | 12/04/2012    | Positive  | 1.5    |           |          |          |
| 172 | L7  | Stallion | 16  | yes            | no                    | yes                  | 12/04/2012    | N.I.      | < 0.6  |           |          |          |
| 173 | L8  | Stallion | 7   | yes            | no                    | yes                  | 12/04/2012    | Negative  |        |           |          |          |
| 174 | L9  | Stallion | 6   | yes            | no                    | yes                  | 12/04/2012    | N.I.      | < 0.6  |           |          |          |
| 175 | L10 | Stallion | 14  | yes            | no                    | yes                  | 12/04/2012    | Positive  | 2.6    |           |          |          |
| 176 | L11 | Mare     | 21  | yes            | yes                   | yes                  | 12/04/2012    | Positive  | 2.8    |           |          |          |
| 177 | L12 | Mare     | 13  | yes            | yes                   | yes                  | 12/04/2012    | Positive  | 1.8    |           |          |          |
| 178 | L13 | Mare     | 7   | yes            | no                    | yes                  | 12/04/2012    | Positive  | 3      |           |          |          |
| 179 | L14 | Mare     | 11  | yes            | yes                   | yes                  | 12/04/2012    | Positive  | 2.6    |           |          |          |
| 180 | M1  | Mare     | 9   | yes            | yes                   | no                   | 12/07/2012    | Positive  | 1.7    | Positive  | 2.3      | 1.8      |
| 181 | M2  | Stallion | 11  | yes            | yes                   | no                   | 12/07/2012    | Positive  | 2.8    | Positive  | >2.6     | 1.4      |
| 182 | M3  | Mare     | 8   | yes            | no                    | no                   | 12/07/2012    | Positive  | 3.6    | Positive  | >2.6     | 1.2      |
| 183 | M4  | Mare     | 9   | yes            | yes                   | no                   | 12/07/2012    | Negative  |        | Positive  | 2.3      | 1.1      |
| 184 | M6  | Mare     | 10  | yes            | no                    | no                   | 12/07/2012    | Negative  |        | Equivocal | 2.1      | 1.1      |
| 185 | M7  | Mare     | 12  | yes            | no                    | no                   | 12/07/2012    | Positive  | 3.2    | Positive  | 2.4      | 1.4      |
| 186 | M8  | Mare     | 12  | yes            | yes                   | no                   | 12/07/2012    | Positive  | 1.4    | Positive  | >2.6     | 1.8      |
| 187 | M9  | Mare     | 7   | yes            | no                    | no                   | 12/07/2012    | Negative  |        | Negative  |          |          |
| 188 | M10 | Stallion | 13  | yes            | no                    | no                   | 12/07/2012    | Positive  | < 0.6  | Positive  | 2.6      | 1.1      |
| 189 | M11 | Stallion | 3   | no             | n/a                   | no                   | 12/07/2012    | Negative  |        | Equivocal | 2.6      | 0.9      |

| No  | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 190 | M12 | Mare     | 11  | yes            | no                    | no                   | 12/07/2012    | Positive  | 2.6    | Positive  | 2.3      | 1.7      |
| 191 | M13 | Stallion | 8   | yes            | no                    | no                   | 12/07/2012    | Negative  |        | Equivocal | 2.6      | 1.7      |
| 192 | M14 | Mare     | 10  | yes            | yes                   | no                   | 12/07/2012    | Positive  | 3.6    | Positive  | 1.4      | 0.8      |
| 193 | M15 | Stallion | 9   | yes            | no                    | no                   | 12/07/2012    | Positive  | 3.4    | Positive  | 1.5      | 1.1      |
| 194 | M16 | Stallion | 12  | yes            | no                    | no                   | 12/07/2012    | Negative  |        | Positive  | >2.6     | 1.7      |
| 195 | M17 | Mare     | 13  | yes            | yes                   | no                   | 12/07/2012    | Positive  | 3.5    | Positive  | >2.6     | 1.7      |
| 196 | N1  | Mare     | 2   | no             | n/a                   | no                   | 29/10/2012    | Negative  |        | Equivocal | 1.8      | 1.7      |
| 197 | N2  | Mare     | 14  | yes            | yes                   | no                   | 29/10/2012    | Negative  |        | Positive  | >2.6     | 1.7      |
| 198 | N3  | Mare     | 12  | no             | n/a                   | no                   | 29/10/2012    | Negative  |        | Positive  | >2.6     | 1.5      |
| 199 | N4  | Mare     | 7   | yes            | no                    | no                   | 29/10/2012    | Negative  |        | Positive  | >2.6     | 1.4      |
| 200 | N5  | Mare     | 7   | no             | n/a                   | no                   | 29/10/2012    | Negative  |        | Positive  | 2.6      | 1.5      |
| 201 | N6  | Mare     | 2   | no             | n/a                   | no                   | 29/10/2012    | Negative  |        | Negative  |          |          |
| 202 | N7  | Mare     | 4   | no             | n/a                   | no                   | 29/10/2012    | Negative  |        | Negative  |          |          |
| 203 | N8  | Stallion | 16  | no             | n/a                   | no                   | 20/06/2012    | Negative  |        | Positive  | 2.6      | 2.3      |
| 204 | O1  | Mare     | 12  | yes            | no                    | no                   | 06/11/2012    | Negative  |        | Positive  | >2.6     | 1.7      |
| 205 | O2  | Mare     | 8   | yes            | no                    | no                   | 06/11/2012    | Negative  |        | Negative  |          |          |
| 206 | O3  | Stallion | 5   | yes            | no                    | no                   | 06/11/2012    | Negative  |        | Negative  |          |          |
| 207 | O4  | Stallion | 1   | no             | n/a                   | no                   | 06/11/2012    | Negative  |        | Negative  |          |          |
| 208 | O5  | Stallion | 2   | no             | n/a                   | no                   | 06/11/2012    | Negative  |        | Positive  | 1.4      | 1.4      |
| 209 | O6  | Mare     | 3   | yes            | no                    | no                   | 06/11/2012    | Negative  |        | Equivocal | 1.8      | 1.4      |
| 210 | O7  | Mare     | 6   | yes            | no                    | no                   | 06/11/2012    | Negative  |        | Negative  |          |          |
| 211 | O8  | Stallion | 8   | no             | n/a                   | no                   | 06/11/2012    | Positive  | < 0.6  | Negative  |          |          |
| 212 | O9  | Mare     | 9   | yes            | no                    | no                   | 06/11/2012    | Negative  |        | Positive  | 2.6      | 1.2      |
| 213 | O10 | Mare     | 7   | yes            | yes                   | no                   | 06/11/2012    | Negative  |        | Equivocal | 1.5      | 1.7      |

| No  | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 214 | O11 | Mare     | 14  | yes            | yes                   | no                   | 20/04/2013    | Positive  | < 0.6  | Positive  | >2.6     | 2.3      |
| 215 | O12 | Stallion | 6   | yes            | no                    | no                   | 20/04/2013    | Negative  |        | Equivocal | >2.6     | 2.3      |
| 216 | P1  | Stallion | 4   | yes            | no                    | no                   | 13/12/2012    | Negative  |        | Negative  |          |          |
| 217 | P2  | Stallion | 4   | no             | n/a                   | no                   | 13/12/2012    | Negative  |        | Negative  |          |          |
| 218 | P3  | Stallion | 8   | no             | n/a                   | no                   | 13/12/2012    | Positive  | < 0.6  | Equivocal | 1.5      | 0.9      |
| 219 | P4  | Stallion | 6   | no             | n/a                   | no                   | 13/12/2012    | Negative  |        | Negative  |          |          |
| 220 | P5  | Stallion | 5   | no             | n/a                   | no                   | 13/12/2012    | Negative  |        | Equivocal | 2        | 1.7      |
| 221 | P6  | Stallion | 1,5 | no             | n/a                   | no                   | 13/12/2012    | Negative  |        | Negative  |          |          |
| 222 | P7  | Mare     | 2   | yes            | no                    | yes                  | 13/12/2012    | Negative  |        |           |          |          |
| 223 | P8  | Mare     | 6   | yes            | yes                   | yes                  | 13/12/2012    | Positive  | 2.9    |           |          |          |
| 224 | P9  | Mare     | 13  | yes            | no                    | yes                  | 13/12/2012    | Negative  |        |           |          |          |
| 225 | P10 | Mare     | 6   | yes            | yes                   | yes                  | 13/12/2012    | Negative  |        |           |          |          |
| 226 | P11 | Mare     | 2   | no             | n/a                   | no                   | 13/12/2012    | Negative  |        | Negative  |          |          |
| 227 | P12 | Mare     | 2   | no             | n/a                   | no                   | 13/12/2012    | Negative  |        | Equivocal | 2        | < 0.5    |
| 228 | Q1  | Mare     | 17  | yes            | yes                   | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 229 | Q2  | Mare     | 10  | yes            | yes                   | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 230 | Q3  | Mare     | 7   | yes            | yes                   | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 231 | Q4  | Mare     | 13  | yes            | yes                   | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 232 | Q5  | Mare     | 18  | yes            | yes                   | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 233 | Q6  | Mare     | 20  | yes            | yes                   | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 234 | Q7  | Mare     | 7   | yes            | yes                   | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 235 | Q8  | Mare     | 22  | yes            | no                    | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 236 | Q9  | Mare     | 4   | yes            | no                    | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 237 | Q10 | Mare     | 20  | yes            | no                    | yes                  | 21/02/2013    | Negative  |        |           |          |          |

| <b>No</b> | <b>ID</b> | <b>SEX</b> | <b>AGE</b> | <b>ACTIVE BREEDER</b> | <b>REPRODUCTIVE PROBLEMS</b> | <b>EHV-1/-4 VACCINATION</b> | <b>SAMPLING DATE</b> | <b>EVA ELISA</b> | <b>EVA SN</b> | <b>EHV ELISA</b> | <b>EHV-1 SN</b> | <b>EHV-4 SN</b> |
|-----------|-----------|------------|------------|-----------------------|------------------------------|-----------------------------|----------------------|------------------|---------------|------------------|-----------------|-----------------|
| 238       | Q11       | Mare       | 4          | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 239       | Q12       | Mare       | 4          | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 240       | Q13       | Mare       | 18         | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 241       | Q14       | Mare       | 18         | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 242       | Q15       | Mare       | 13         | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 243       | Q16       | Mare       | 6          | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 244       | Q17       | Mare       | 17         | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 245       | Q18       | Mare       | 15         | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 246       | Q19       | Stallion   | 20         | yes                   | yes                          | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 247       | Q20       | Stallion   | 10         | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 248       | Q21       | Stallion   | 7          | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 249       | Q22       | Stallion   | 4          | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 250       | Q23       | Stallion   | 9          | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 251       | Q24       | Stallion   | 7          | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 252       | Q25       | Stallion   | 5          | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 253       | Q26       | Stallion   | 22         | yes                   | no                           | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 254       | Q27       | Stallion   | 21         | yes                   | no                           | yes                         | 21/02/2013           | N.I.             | < 0.6         |                  |                 |                 |
| 255       | Q28       | Stallion   | 7          | yes                   | no                           | yes                         | 21/02/2013           | Equivocal        | < 0.6         |                  |                 |                 |
| 256       | Q29       | Mare       | 1          | no                    | n/a                          | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 257       | Q30       | Mare       | 1,1        | no                    | n/a                          | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 258       | Q31       | Stallion   | 1,1        | no                    | n/a                          | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 259       | Q32       | Stallion   | 1,2        | no                    | n/a                          | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 260       | Q33       | Stallion   | 1          | no                    | n/a                          | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |
| 261       | Q34       | Stallion   | 1,25       | no                    | n/a                          | yes                         | 21/02/2013           | Negative         |               |                  |                 |                 |

| No  | ID  | SEX      | AGE  | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|-----|----------|------|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 262 | Q35 | Stallion | 1,25 | no             | n/a                   | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 263 | Q36 | Stallion | 0,9  | no             | n/a                   | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 264 | Q37 | Stallion | 1,25 | no             | n/a                   | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 265 | Q38 | Stallion | 1,1  | no             | n/a                   | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 266 | Q39 | Stallion | 1    | no             | n/a                   | yes                  | 21/02/2013    | Negative  |        |           |          |          |
| 267 | Q40 | Mare     | 10   | yes            | no                    | yes                  | 20/03/2013    | Negative  |        |           |          |          |
| 268 | Q41 | Mare     | 7    | yes            | no                    | yes                  | 20/03/2013    | Positive  | < 0.6  |           |          |          |
| 269 | Q42 | Mare     | 7    | yes            | no                    | yes                  | 20/03/2013    | Negative  |        |           |          |          |
| 270 | R1  | Mare     | 6    | yes            | yes                   | yes                  | 16/03/2013    | Positive  | 2.3    |           |          |          |
| 271 | R2  | Mare     | 8    | yes            | yes                   | yes                  | 16/03/2013    | Positive  | 1.4    |           |          |          |
| 272 | R3  | Mare     | 6    | yes            | no                    | yes                  | 16/03/2013    | Positive  | 2.6    |           |          |          |
| 273 | R4  | Mare     | 7    | yes            | yes                   | yes                  | 16/03/2013    | Positive  | 1.7    |           |          |          |
| 274 | R5  | Mare     | 5    | yes            | yes                   | yes                  | 16/03/2013    | Positive  | 2.5    |           |          |          |
| 275 | R6  | Mare     | 7    | yes            | yes                   | yes                  | 16/03/2013    | Positive  | 2.4    |           |          |          |
| 276 | R7  | Mare     | 7    | yes            | no                    | yes                  | 16/03/2013    | Positive  | 2.5    |           |          |          |
| 277 | R8  | Mare     | 6    | yes            | yes                   | yes                  | 16/03/2013    | Positive  | 2.6    |           |          |          |
| 278 | R9  | Mare     | 3    | yes            | no                    | yes                  | 16/03/2013    | Positive  | 2.3    |           |          |          |
| 279 | R10 | Stallion | 1,3  | no             | n/a                   | no                   | 16/03/2013    | Negative  |        | Negative  |          |          |
| 280 | R11 | Mare     | 0,7  | no             | n/a                   | no                   | 16/03/2013    | N.I.      | < 0.6  | Negative  |          |          |
| 281 | S1  | Mare     | 1,3  | no             | n/a                   | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 282 | S2  | Mare     | 1,3  | no             | n/a                   | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 283 | S3  | Mare     | 2,2  | no             | n/a                   | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 284 | S4  | Mare     | 1,6  | no             | n/a                   | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 285 | S5  | Mare     | 2,2  | no             | n/a                   | yes                  | 20/04/2013    | Negative  |        |           |          |          |

| <b>No</b> | <b>ID</b> | <b>SEX</b> | <b>AGE</b> | <b>ACTIVE BREEDER</b> | <b>REPRODUCTIVE PROBLEMS</b> | <b>EHV-1/-4 VACCINATION</b> | <b>SAMPLING DATE</b> | <b>EVA ELISA</b> | <b>EVA SN</b> | <b>EHV ELISA</b> | <b>EHV-1 SN</b> | <b>EHV-4 SN</b> |
|-----------|-----------|------------|------------|-----------------------|------------------------------|-----------------------------|----------------------|------------------|---------------|------------------|-----------------|-----------------|
| 286       | S6        | Mare       | 2,9        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 287       | S7        | Mare       | 2,2        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 288       | S8        | Mare       | 2,2        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 289       | S9        | Mare       | 3,2        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 290       | S10       | Mare       | 3,1        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 291       | S11       | Mare       | 3,2        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 292       | S12       | Mare       | 3          | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 293       | S13       | Stallion   | 1,2        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 294       | S14       | Stallion   | 1,2        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 295       | S15       | Stallion   | 1,2        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 296       | S16       | Stallion   | 1,2        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 297       | S17       | Stallion   | 1,4        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 298       | S18       | Stallion   | 3,2        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 299       | S19       | Stallion   | 3          | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 300       | S20       | Stallion   | 2,2        | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 301       | S21       | Mare       | 8          | yes                   | no                           | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 302       | S22       | Mare       | 4          | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 303       | S23       | Mare       | 4          | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 304       | S24       | Mare       | 4          | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 305       | S25       | Mare       | 4          | no                    | n/a                          | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 306       | S26       | Mare       | 9          | yes                   | no                           | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 307       | S27       | Mare       | 5          | yes                   | no                           | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 308       | S28       | Mare       | 16         | yes                   | no                           | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |
| 309       | S29       | Mare       | 11         | yes                   | no                           | yes                         | 20/04/2013           | Negative         |               |                  |                 |                 |

| No  | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 310 | S30 | Mare     | 8   | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 311 | S31 | Mare     | 10  | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 312 | S32 | Mare     | 11  | yes            | yes                   | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 313 | S33 | Mare     | 14  | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 314 | S34 | Mare     | 13  | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 315 | S35 | Mare     | 9   | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 316 | S36 | Mare     | 8   | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 317 | S37 | Mare     | 9   | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 318 | S38 | Mare     | 13  | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 319 | S39 | Mare     | 11  | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 320 | S40 | Mare     | 10  | yes            | yes                   | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 321 | S41 | Stallion | 4   | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 322 | S42 | Stallion | 13  | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 323 | S43 | Stallion | 5   | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 324 | S44 | Stallion | 4   | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 325 | S45 | Stallion | 4   | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 326 | S46 | Stallion | 13  | yes            | yes                   | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 327 | S47 | Stallion | 5   | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 328 | S48 | Stallion | 5   | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 329 | S49 | Stallion | 5   | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 330 | S50 | Stallion | 7   | yes            | no                    | yes                  | 20/04/2013    | Negative  |        |           |          |          |
| 331 | S51 | Stallion | 0,7 | no             | n/a                   | no                   | 16/11/2013    | Negative  |        | Negative  |          |          |
| 332 | S52 | Stallion | 0,7 | no             | n/a                   | no                   | 16/11/2013    | Negative  |        | Equivocal | 2.3      | 2.3      |
| 333 | S53 | Stallion | 0,7 | no             | n/a                   | no                   | 16/11/2013    | Positive  | < 0.6  | Negative  |          |          |

| No  | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 334 | S54 | Stallion | 0,7 | no             | n/a                   | no                   | 16/11/2013    | Negative  |        | Negative  |          |          |
| 335 | S55 | Mare     | 0,7 | no             | n/a                   | no                   | 16/11/2013    | Negative  |        | Negative  |          |          |
| 336 | S56 | Mare     | 0,7 | no             | n/a                   | no                   | 16/11/2013    | Negative  |        | Negative  |          |          |
| 337 | S57 | Mare     | 0,7 | no             | n/a                   | no                   | 16/11/2013    | Negative  |        | Negative  |          |          |
| 338 | S58 | Stallion | 0,7 | no             | n/a                   | no                   | 16/11/2013    | Positive  | < 0.6  | Equivocal | 2.4      | 2.2      |
| 339 | T1  | Mare     | 8   | yes            | yes                   | no                   | 20/04/2013    | Negative  |        | Equivocal | 2.5      | 1.6      |
| 340 | T2  | Mare     | 12  | yes            | no                    | no                   | 20/04/2013    | Negative  |        | Positive  | >2.6     | 2.5      |
| 341 | T3  | Mare     | 7   | yes            | yes                   | no                   | 20/04/2013    | Negative  |        | Negative  |          |          |
| 342 | T4  | Mare     | 18  | yes            | yes                   | no                   | 20/04/2013    | Negative  |        | Negative  |          |          |
| 343 | T5  | Mare     | 9   | yes            | no                    | no                   | 20/04/2013    | Negative  |        | Positive  | >2.6     | >2.6     |
| 344 | T6  | Mare     | 8   | yes            | no                    | no                   | 20/04/2013    | Negative  |        | Positive  | >2.6     | >2.6     |
| 345 | T7  | Stallion | 12  | no             | n/a                   | no                   | 20/04/2013    | Negative  |        | Positive  | >2.6     | 2        |
| 346 | T8  | Mare     | 2   | no             | n/a                   | no                   | 20/04/2013    | Negative  |        | Negative  |          |          |
| 347 | T9  | Stallion | 23  | yes            | no                    | no                   | 20/04/2013    | Negative  |        | Positive  | >2.6     | 2.2      |
| 348 | T10 | Stallion | 8   | yes            | no                    | no                   | 20/04/2013    | Negative  |        | Equivocal | 2.3      | 2.1      |
| 349 | U1  | Stallion | 16  | yes            | no                    | yes                  | 13/07/2013    | Negative  |        |           |          |          |
| 350 | U2  | Stallion | 5   | yes            | no                    | yes                  | 13/07/2013    | Negative  |        |           |          |          |
| 351 | U3  | Stallion | 8   | yes            | no                    | yes                  | 13/07/2013    | Negative  |        |           |          |          |
| 352 | U4  | Stallion | 7   | no             | n/a                   | yes                  | 13/07/2013    | Negative  |        |           |          |          |
| 353 | U5  | Stallion | 4   | no             | n/a                   | yes                  | 13/07/2013    | Negative  |        |           |          |          |
| 354 | U6  | Stallion | 4   | no             | n/a                   | yes                  | 13/07/2013    | Negative  |        |           |          |          |
| 355 | U7  | Stallion | 8   | no             | n/a                   | yes                  | 13/07/2013    | Negative  |        |           |          |          |
| 356 | U8  | Stallion | 12  | no             | n/a                   | yes                  | 13/07/2013    | Negative  |        |           |          |          |
| 357 | U9  | Stallion | 8   | no             | n/a                   | yes                  | 13/07/2013    | Negative  |        |           |          |          |

| No  | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 358 | U10 | Stallion | 14  | no             | n/a                   | yes                  | 13/07/2013    | Negative  |        |           |          |          |
| 359 | V1  | Mare     | 14  | yes            | no                    | yes                  | 18/08/2013    | Positive  | 2.3    |           |          |          |
| 360 | V2  | Mare     | 4   | yes            | no                    | yes                  | 18/08/2013    | Positive  | 3.6    |           |          |          |
| 361 | V3  | Mare     | 4   | yes            | no                    | yes                  | 18/08/2013    | Positive  | 3.9    |           |          |          |
| 362 | V4  | Mare     | 10  | yes            | yes                   | yes                  | 18/08/2013    | Positive  | 3.2    |           |          |          |
| 363 | V5  | Mare     | 9   | yes            | yes                   | yes                  | 18/08/2013    | Positive  | 3.6    |           |          |          |
| 364 | V6  | Mare     | 15  | yes            | yes                   | yes                  | 18/08/2013    | Positive  | 1.5    |           |          |          |
| 365 | V7  | Mare     | 10  | yes            | no                    | yes                  | 18/08/2013    | Positive  | 3.4    |           |          |          |
| 366 | V8  | Mare     | 5   | yes            | no                    | yes                  | 18/08/2013    | Positive  | 3.2    |           |          |          |
| 367 | V9  | Mare     | 8   | yes            | no                    | yes                  | 18/08/2013    | Positive  | 3.5    |           |          |          |
| 368 | V10 | Mare     | 8   | yes            | yes                   | yes                  | 18/08/2013    | Positive  | 2.9    |           |          |          |
| 369 | V11 | Mare     | 5   | yes            | no                    | yes                  | 18/08/2013    | Positive  | 2.5    |           |          |          |
| 370 | V12 | Mare     | 8   | yes            | no                    | yes                  | 18/08/2013    | Positive  | 3.2    |           |          |          |
| 371 | V13 | Mare     | 4   | yes            | no                    | yes                  | 18/08/2013    | Positive  | 3      |           |          |          |
| 372 | V14 | Mare     | 5   | yes            | yes                   | yes                  | 18/08/2013    | Positive  | 3.5    |           |          |          |
| 373 | V15 | Mare     | 4   | yes            | no                    | yes                  | 18/08/2013    | N.I.      | 2.6    |           |          |          |
| 374 | V16 | Mare     | 2   | no             | n/a                   | no                   | 18/08/2013    | Positive  | 3.9    | Equivocal | >2.6     | 2.1      |
| 375 | V17 | Mare     | 5   | yes            | yes                   | yes                  | 18/08/2013    | Positive  | 3.2    |           |          |          |
| 376 | V18 | Stallion | 2   | no             | n/a                   | no                   | 18/08/2013    | Positive  | 2.8    | Negative  |          |          |
| 377 | V19 | Stallion | 3   | no             | n/a                   | no                   | 18/08/2013    | Positive  | 3.4    | Positive  | >2.6     | 2.4      |
| 378 | V20 | Stallion | 3   | yes            | no                    | no                   | 18/08/2013    | Positive  | 2      | Negative  |          |          |
| 379 | V21 | Stallion | 10  | yes            | no                    | no                   | 18/08/2013    | Positive  | 2.6    | Positive  | >2.6     | >2.6     |
| 380 | V22 | Stallion | 5   | yes            | no                    | no                   | 18/08/2013    | Positive  | 3.4    | Positive  | >2.6     | 2.2      |
| 381 | V23 | Stallion | 5   | yes            | no                    | no                   | 18/08/2013    | Negative  |        | Positive  | >2.6     | 2.5      |

| No  | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 382 | V24 | Stallion | 9   | yes            | no                    | no                   | 18/08/2013    | Positive  | 3.3    | Positive  | >2.6     | >2.6     |
| 383 | V25 | Mare     | 8   | yes            | no                    | yes                  | 18/08/2013    | Positive  | 3.2    |           |          |          |
| 384 | V26 | Mare     | 9   | yes            | no                    | yes                  | 18/08/2013    | Positive  | 3.4    |           |          |          |
| 385 | V27 | Mare     | 3   | no             | n/a                   | no                   | 18/08/2013    | Positive  | 2.4    | Negative  |          |          |
| 386 | V28 | Stallion | 1   | no             | n/a                   | no                   | 18/08/2013    | Positive  | 2.8    | Negative  |          |          |
| 387 | V29 | Mare     | 1   | no             | n/a                   | no                   | 18/08/2013    | Positive  | 3.5    | Negative  |          |          |
| 388 | W1  | Stallion | 12  | yes            | no                    | no                   | 28/08/2013    | Positive  | 1.5    | Positive  | >2.6     | 2.4      |
| 389 | W2  | Stallion | 6   | yes            | no                    | no                   | 28/08/2013    | Negative  |        | Negative  |          |          |
| 390 | W3  | Stallion | 4   | yes            | no                    | no                   | 28/08/2013    | Negative  |        | Negative  |          |          |
| 391 | W4  | Stallion | 1   | no             | n/a                   | no                   | 28/08/2013    | Negative  |        | Positive  | 2.5      | 2.3      |
| 392 | W5  | Stallion | 1   | no             | n/a                   | no                   | 28/08/2013    | Negative  |        | Equivocal | >2.6     | 2.5      |
| 393 | W6  | Stallion | 1   | no             | n/a                   | no                   | 28/08/2013    | Negative  |        | Negative  |          |          |
| 394 | W7  | Mare     | 3   | no             | n/a                   | no                   | 28/08/2013    | Positive  | < 0.6  | Positive  | 2.4      | 2.3      |
| 395 | X1  | Stallion | 10  | yes            | no                    | yes                  | 29/08/2013    | Negative  |        |           |          |          |
| 396 | X2  | Mare     | 11  | yes            | no                    | yes                  | 29/08/2013    | Negative  |        |           |          |          |
| 397 | X3  | Mare     | 3   | no             | n/a                   | yes                  | 29/08/2013    | Negative  |        |           |          |          |
| 398 | X4  | Stallion | 4   | no             | n/a                   | yes                  | 29/08/2013    | Negative  |        |           |          |          |
| 399 | Y1  | Mare     | 7   | yes            | no                    | yes                  | 30/08/2013    | Negative  |        |           |          |          |
| 400 | Y2  | Mare     | 6   | yes            | no                    | yes                  | 30/08/2013    | Negative  |        |           |          |          |
| 401 | Y3  | Mare     | 3   | no             | n/a                   | no                   | 30/08/2013    | Negative  |        | Equivocal | 2.5      | 1.8      |
| 402 | Y4  | Mare     | 4   | no             | n/a                   | no                   | 30/08/2013    | Negative  |        | Negative  |          |          |
| 403 | Y5  | Mare     | 5   | yes            | no                    | yes                  | 30/08/2013    | Negative  |        |           |          |          |
| 404 | Y6  | Mare     | 16  | yes            | no                    | yes                  | 30/08/2013    | Positive  | 2      |           |          |          |
| 405 | Y7  | Mare     | 14  | yes            | no                    | yes                  | 30/08/2013    | Positive  | 2.5    |           |          |          |

| No  | ID  | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|-----|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 406 | Y8  | Mare     | 6   | yes            | no                    | yes                  | 30/08/2013    | Negative  |        |           |          |          |
| 407 | Y9  | Mare     | 6   | yes            | no                    | yes                  | 30/08/2013    | Negative  |        |           |          |          |
| 408 | Y10 | Mare     | 6   | yes            | yes                   | yes                  | 30/08/2013    | Negative  |        |           |          |          |
| 409 | Y11 | Stallion | 8   | yes            | no                    | no                   | 30/08/2013    | Negative  |        | Positive  | 2.5      | >2.6     |
| 410 | Y12 | Stallion | 4   | no             | n/a                   | no                   | 30/08/2013    | Negative  |        | Equivocal | >2.6     | 2.4      |
| 411 | Y13 | Stallion | 5   | no             | n/a                   | no                   | 30/08/2013    | Positive  | < 0.6  | Equivocal | 2.5      | 2.2      |
| 412 | Y14 | Stallion | 6   | yes            | no                    | no                   | 30/08/2013    | Negative  |        | Negative  |          |          |
| 413 | Z1  | Stallion | 7   | no             | n/a                   | no                   | 31/08/2013    | Negative  |        | Positive  | >2.6     | 2.3      |
| 414 | Z2  | Stallion | 5   | no             | n/a                   | no                   | 31/08/2013    | Negative  |        | Negative  |          |          |
| 415 | Z3  | Stallion | 9   | no             | n/a                   | no                   | 31/08/2013    | Negative  |        | Equivocal | >2.6     | 2.3      |
| 416 | Z4  | Stallion | 12  | yes            | no                    | no                   | 31/08/2013    | Negative  |        | Positive  | >2.6     | 2.4      |
| 417 | Z5  | Stallion | 3   | no             | n/a                   | no                   | 31/08/2013    | Negative  |        | Negative  |          |          |
| 418 | Z6  | Stallion | 2   | no             | n/a                   | no                   | 31/08/2013    | Negative  |        | Equivocal | >2.6     | 2.3      |
| 419 | Z7  | Stallion | 3   | no             | n/a                   | no                   | 31/08/2013    | Negative  |        | Equivocal | 2.5      | 2.5      |
| 420 | Z8  | Stallion | 5   | no             | n/a                   | no                   | 31/08/2013    | Negative  |        | Equivocal | >2.6     | >2.6     |
| 421 | Z9  | Mare     | 11  | yes            | yes                   | yes                  | 31/08/2013    | Negative  |        |           |          |          |
| 422 | Z10 | Mare     | 4   | yes            | no                    | yes                  | 31/08/2013    | Negative  |        |           |          |          |
| 423 | Z11 | Mare     | 5   | yes            | no                    | yes                  | 31/08/2013    | Negative  |        |           |          |          |
| 424 | Z12 | Mare     | 6   | yes            | no                    | yes                  | 31/08/2013    | Negative  |        |           |          |          |
| 425 | Z13 | Mare     | 16  | yes            | no                    | yes                  | 31/08/2013    | Negative  |        |           |          |          |
| 426 | Z14 | Mare     | 12  | yes            | no                    | yes                  | 31/08/2013    | Negative  |        |           |          |          |
| 427 | Z15 | Mare     | 5   | yes            | no                    | yes                  | 31/08/2013    | Negative  |        |           |          |          |
| 428 | AA1 | Mare     | 5   | no             | n/a                   | no                   | 31/08/2013    | Negative  |        | Equivocal | >2.6     | 2.5      |
| 429 | AA2 | Mare     | 13  | yes            | yes                   | no                   | 31/08/2013    | Negative  |        | Positive  | >2.6     | 2.4      |

| No  | ID   | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|------|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 430 | AA3  | Mare     | 10  | no             | n/a                   | no                   | 31/08/2013    | Negative  |        | Equivocal | >2.6     | 2        |
| 431 | AA4  | Mare     | 4   | no             | n/a                   | no                   | 31/08/2013    | Negative  |        | Equivocal | >2.6     | >2.6     |
| 432 | AA5  | Stallion | 4   | no             | n/a                   | no                   | 31/08/2013    | Negative  |        | Negative  |          |          |
| 433 | AA6  | Stallion | 2   | no             | n/a                   | no                   | 31/08/2013    | Negative  |        | Negative  |          |          |
| 434 | AB1  | Stallion | 3   | yes            | no                    | no                   | 03/09/2013    | Negative  |        | Positive  | >2.6     | 2.3      |
| 435 | AB2  | Stallion | 3   | no             | n/a                   | no                   | 03/09/2013    | Negative  |        | Equivocal | 2.5      | 2.2      |
| 436 | AB3  | Stallion | 4   | no             | n/a                   | no                   | 03/09/2013    | Positive  | < 0.6  | Positive  | >2.6     | 2.4      |
| 437 | AB4  | Stallion | 3   | no             | n/a                   | no                   | 03/09/2013    | Negative  |        | Equivocal | 2.5      | 2.4      |
| 438 | AB5  | Stallion | 4   | no             | n/a                   | no                   | 03/09/2013    | Negative  |        | Positive  | >2.6     | >2.6     |
| 439 | AB6  | Stallion | 16  | no             | n/a                   | no                   | 03/09/2013    | Positive  | < 0.6  | Positive  | 2.5      | 1.9      |
| 440 | AB7  | Stallion | 5   | no             | n/a                   | no                   | 03/09/2013    | Negative  |        | Positive  | >2.6     | >2.6     |
| 441 | AB8  | Stallion | 22  | no             | n/a                   | no                   | 03/09/2013    | N.I.      | < 0.6  | Positive  | >2.6     | 2.3      |
| 442 | AB9  | Mare     | 7   | yes            | no                    | no                   | 03/09/2013    | Negative  |        | Negative  |          |          |
| 443 | AB10 | Mare     | 7   | yes            | no                    | no                   | 03/09/2013    | Negative  |        | Negative  |          |          |
| 444 | AB11 | Mare     | 20  | yes            | no                    | no                   | 03/09/2013    | Negative  |        | Positive  | >2.6     | 1.9      |
| 445 | AB12 | Mare     | 4   | yes            | no                    | no                   | 03/09/2013    | Negative  |        | Equivocal | >2.6     | 2.3      |
| 446 | AB13 | Mare     | 14  | yes            | no                    | no                   | 03/09/2013    | Negative  |        | Equivocal | 2.5      | 2.1      |
| 447 | AB14 | Mare     | 6   | yes            | no                    | no                   | 03/09/2013    | Negative  |        | Positive  | >2.6     | 2.1      |
| 448 | AB15 | Mare     | 5   | yes            | no                    | no                   | 03/09/2013    | Negative  |        | Positive  | >2.6     | 2.5      |
| 449 | AB16 | Mare     | 7   | yes            | no                    | no                   | 03/09/2013    | Positive  | < 0.6  | Positive  | 2.5      | 1.7      |
| 450 | AB17 | Mare     | 7   | yes            | no                    | no                   | 03/09/2013    | Negative  |        | Positive  | 2.5      | 2.4      |
| 451 | AC1  | Mare     | 7   | yes            | yes                   | yes                  | 04/09/2013    | Positive  | 2      |           |          |          |
| 452 | AC2  | Mare     | 8   | yes            | yes                   | yes                  | 04/09/2013    | Negative  |        |           |          |          |
| 453 | AC3  | Mare     | 9   | yes            | no                    | no                   | 04/09/2013    | Negative  |        | Positive  | 2.3      | 1.9      |

| No  | ID   | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|------|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 454 | AC4  | Mare     | 8   | yes            | yes                   | yes                  | 04/09/2013    | Negative  |        |           |          |          |
| 455 | AC5  | Mare     | 8   | yes            | yes                   | yes                  | 04/09/2013    | Negative  |        |           |          |          |
| 456 | AC6  | Mare     | 8   | yes            | yes                   | yes                  | 04/09/2013    | Negative  |        |           |          |          |
| 457 | AC7  | Mare     | 4   | yes            | no                    | no                   | 04/09/2013    | Positive  | 2.6    | Equivocal | 2.5      | 2.2      |
| 458 | AC8  | Mare     | 8   | yes            | no                    | yes                  | 04/09/2013    | Negative  |        |           |          |          |
| 459 | AC9  | Stallion | 9   | yes            | yes                   | no                   | 04/09/2013    | Negative  |        | Positive  | >2.6     | 2        |
| 460 | AC10 | Mare     | 6   | yes            | yes                   | no                   | 30/10/2013    | Negative  |        | Positive  | 2.4      | 2.2      |
| 461 | AD1  | Mare     | 4   | yes            | no                    | no                   | 04/09/2013    | Negative  |        | Negative  |          |          |
| 462 | AD2  | Mare     | 15  | yes            | no                    | no                   | 04/09/2013    | Negative  |        | Positive  | >2.6     | 1.3      |
| 463 | AE1  | Stallion | 2,5 | no             | n/a                   | no                   | 15/09/2013    | Negative  |        | Negative  |          |          |
| 464 | AE2  | Stallion | 2,5 | no             | n/a                   | no                   | 15/09/2013    | Negative  |        | Equivocal | 2.1      | 1.2      |
| 465 | AE3  | Stallion | 3,5 | no             | n/a                   | no                   | 15/09/2013    | Positive  | < 0.6  | Positive  | >2.6     | >2.6     |
| 466 | AE4  | Stallion | 15  | yes            | no                    | no                   | 15/09/2013    | Negative  |        | Positive  | >2.6     | 1.8      |
| 467 | AE5  | Stallion | 3,5 | no             | n/a                   | no                   | 15/09/2013    | Negative  |        | Equivocal | >2.6     | 1.8      |
| 468 | AE6  | Stallion | 12  | yes            | no                    | no                   | 15/09/2013    | Negative  |        | Positive  | >2.6     | 1.6      |
| 469 | AE7  | Stallion | 7   | yes            | no                    | no                   | 15/09/2013    | Negative  |        | Positive  | 2.5      | 1.3      |
| 470 | AE8  | Mare     | 4   | no             | n/a                   | no                   | 15/09/2013    | Negative  |        | Equivocal | 2.3      | 1.1      |
| 471 | AE9  | Mare     | 3   | no             | n/a                   | no                   | 15/09/2013    | Negative  |        | Positive  | >2.6     | 1.7      |
| 472 | AE10 | Mare     | 2   | no             | n/a                   | no                   | 15/09/2013    | Negative  |        | Positive  | 2.5      | 1.4      |
| 473 | AE11 | Mare     | 12  | yes            | yes                   | no                   | 15/09/2013    | Negative  |        | Positive  | >2.6     | 2.2      |
| 474 | AE12 | Mare     | 7   | no             | n/a                   | no                   | 15/09/2013    | Equivocal | < 0.6  | Positive  | >2.6     | 2.4      |
| 475 | AE13 | Mare     | 11  | yes            | no                    | no                   | 15/09/2013    | Negative  |        | Positive  | >2.6     | 1.6      |
| 476 | AE14 | Mare     | 7   | no             | n/a                   | no                   | 15/09/2013    | Negative  |        | Positive  | >2.6     | 1.6      |
| 477 | AE15 | Mare     | 15  | yes            | yes                   | no                   | 15/09/2013    | Negative  |        | Positive  | 2.4      | 1.5      |

| No  | ID   | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|------|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 478 | AE16 | Mare     | 2   | no             | n/a                   | no                   | 15/09/2013    | Negative  |        | Positive  | 2.4      | 1.9      |
| 479 | AF1  | Stallion | 1   | no             | n/a                   | no                   | 18/09/2013    | Negative  |        | Negative  |          |          |
| 480 | AF2  | Stallion | 3   | no             | n/a                   | no                   | 18/09/2013    | Negative  |        | Equivocal | 2.2      | 1.4      |
| 481 | AF3  | Stallion | 4   | no             | n/a                   | no                   | 18/09/2013    | Negative  |        | Positive  | 2.4      | 1.4      |
| 482 | AF4  | Stallion | 12  | no             | n/a                   | no                   | 18/09/2013    | Negative  |        | Negative  |          |          |
| 483 | AF5  | Stallion | 5   | no             | n/a                   | no                   | 18/09/2013    | Negative  |        | Equivocal | 2.2      | 1.4      |
| 484 | AF6  | Mare     | 4   | yes            | no                    | yes                  | 18/09/2013    | Negative  |        |           |          |          |
| 485 | AF7  | Mare     | 7   | yes            | no                    | yes                  | 18/09/2013    | N.I.      | < 0.6  |           |          |          |
| 486 | AF8  | Mare     | 14  | yes            | yes                   | yes                  | 18/09/2013    | Negative  |        |           |          |          |
| 487 | AF9  | Mare     | 8   | yes            | no                    | yes                  | 18/09/2013    | N.I.      | < 0.6  |           |          |          |
| 488 | AF10 | Stallion | 24  | no             | n/a                   | no                   | 18/09/2013    | Negative  |        | Positive  | 2.4      | 1.6      |
| 489 | AF11 | Stallion | 4   | no             | n/a                   | no                   | 18/09/2013    | Negative  |        | Negative  |          |          |
| 490 | AF12 | Stallion | 20  | no             | n/a                   | no                   | 18/09/2013    | N.I.      | < 0.6  | Positive  | >2.6     | >2.6     |
| 491 | AF13 | Stallion | 18  | no             | n/a                   | no                   | 18/09/2013    | Negative  |        | Positive  | 2.5      | 1.5      |
| 492 | AF14 | Stallion | 14  | yes            | no                    | no                   | 18/09/2013    | Negative  |        | Equivocal | 2.1      | 1.1      |
| 493 | AG1  | Stallion | 11  | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Positive  | 2.2      | 1        |
| 494 | AG2  | Stallion | 6   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Negative  |          |          |
| 495 | AG3  | Stallion | 4   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Positive  | 2.1      | 0.8      |
| 496 | AG4  | Stallion | 4   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Negative  |          |          |
| 497 | AG5  | Stallion | 3   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Positive  | 2.2      | 1.4      |
| 498 | AG6  | Stallion | 3   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Equivocal | >2.6     | 1.5      |
| 499 | AG7  | Stallion | 2   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Equivocal | 2        | 1.3      |
| 500 | AG8  | Stallion | 2   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Positive  | >2.6     | 1.4      |
| 501 | AG9  | Stallion | 1   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Equivocal | 2.5      | 1.4      |

| No  | ID   | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|------|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 502 | AG10 | Stallion | 1   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Equivocal | >2.6     | 1.8      |
| 503 | AG11 | Mare     | 7   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Positive  | 2.5      | 1.7      |
| 504 | AG12 | Mare     | 6   | yes            | no                    | no                   | 19/09/2013    | Negative  |        | Equivocal | 2.4      | 1.4      |
| 505 | AG13 | Mare     | 6   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Equivocal | 2.4      | 1.7      |
| 506 | AG14 | Mare     | 5   | yes            | no                    | no                   | 19/09/2013    | Negative  |        | Positive  | >2.6     | 1.7      |
| 507 | AG15 | Mare     | 9   | yes            | no                    | no                   | 19/09/2013    | Negative  |        | Positive  | >2.6     | 1.6      |
| 508 | AG16 | Mare     | 5   | yes            | no                    | no                   | 19/09/2013    | Negative  |        | Equivocal | 1.8      | 1.3      |
| 509 | AG17 | Mare     | 6   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Positive  | 2.3      | 1.6      |
| 510 | AG18 | Mare     | 6   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Positive  | 2        | 1.6      |
| 511 | AG19 | Mare     | 14  | no             | n/a                   | no                   | 19/09/2013    | N.I.      | < 0.6  | Positive  | >2.6     | 2.5      |
| 512 | AG20 | Mare     | 9   | no             | n/a                   | no                   | 19/09/2013    | Negative  |        | Positive  | >2.6     | 1.5      |
| 513 | AH1  | Mare     | 6   | yes            | no                    | no                   | 05/11/2013    | Negative  |        | Positive  | 2.5      | 1.8      |
| 514 | AH2  | Mare     | 16  | yes            | no                    | no                   | 05/11/2013    | Negative  |        | Positive  | 2.6      | 1        |
| 515 | AH3  | Mare     | 10  | yes            | no                    | no                   | 05/11/2013    | Negative  |        | Positive  | 2.3      | 1.5      |
| 516 | AH4  | Stallion | 20  | yes            | no                    | no                   | 05/11/2013    | Negative  |        | Positive  | 2        | 1.5      |
| 517 | AH5  | Stallion | 3   | no             | n/a                   | no                   | 05/11/2013    | Negative  |        | Equivocal | 2        | 1.3      |
| 518 | AH6  | Mare     | 3   | no             | n/a                   | no                   | 05/11/2013    | Negative  |        | Equivocal | 2.1      | 1.4      |
| 519 | AH7  | Stallion | 20  | yes            | no                    | no                   | 05/11/2013    | Negative  |        | Positive  | >2.6     | 1.1      |
| 520 | AH8  | Mare     | 11  | yes            | yes                   | no                   | 05/11/2013    | Negative  |        | Positive  | 2.4      | 1.7      |
| 521 | AH9  | Mare     | 8   | yes            | no                    | no                   | 05/11/2013    | Negative  |        | Positive  | 2.5      | 1.7      |
| 522 | AH10 | Mare     | 7   | yes            | no                    | no                   | 05/11/2013    | Negative  |        | Equivocal | 1.5      | 1.2      |
| 523 | AH11 | Mare     | 17  | yes            | no                    | no                   | 05/11/2013    | Negative  |        | Positive  | 2.5      | 1.2      |
| 524 | AI1  | Mare     | 11  | yes            | no                    | yes                  | 06/11/2013    | Positive  | 2.8    |           |          |          |
| 525 | AI2  | Stallion | 8   | yes            | no                    | no                   | 06/11/2013    | Negative  |        | Positive  | >2.6     | 1.3      |

| No  | ID   | SEX      | AGE | ACTIVE BREEDER | REPRODUCTIVE PROBLEMS | EHV-1/-4 VACCINATION | SAMPLING DATE | EVA ELISA | EVA SN | EHV ELISA | EHV-1 SN | EHV-4 SN |
|-----|------|----------|-----|----------------|-----------------------|----------------------|---------------|-----------|--------|-----------|----------|----------|
| 526 | AI3  | Mare     | 16  | yes            | no                    | yes                  | 06/11/2013    | Positive  | 2.1    |           |          |          |
| 527 | AI4  | Mare     | 17  | yes            | yes                   | yes                  | 06/11/2013    | Positive  | 2      |           |          |          |
| 528 | AI5  | Stallion | 2   | no             | n/a                   | no                   | 06/11/2013    | Negative  |        | Equivocal | 1.6      | 0.9      |
| 529 | AI6  | Stallion | 12  | yes            | no                    | no                   | 06/11/2013    | Negative  |        | Positive  | >2.6     | 1.8      |
| 530 | AI7  | Stallion | 3   | no             | n/a                   | no                   | 06/11/2013    | Negative  |        | Equivocal | 2.2      | 1.3      |
| 531 | AI8  | Mare     | 20  | yes            | no                    | yes                  | 06/11/2013    | Negative  |        |           |          |          |
| 532 | AI9  | Mare     | 13  | yes            | no                    | yes                  | 06/11/2013    | Positive  | 2.3    |           |          |          |
| 533 | AI10 | Stallion | 5   | no             | n/a                   | no                   | 06/11/2013    | Negative  |        | Positive  | 1.8      | 2.5      |
| 534 | AI11 | Stallion | 3   | no             | n/a                   | no                   | 06/11/2013    | Negative  |        | Positive  | 1.6      | 2.5      |
| 535 | AI12 | Mare     | 17  | yes            | no                    | yes                  | 06/11/2013    | Positive  | 1.8    |           |          |          |
| 536 | AI13 | Stallion | 19  | yes            | no                    | no                   | 06/11/2013    | Negative  |        | Positive  | 1        | 2.5      |
| 537 | AI14 | Mare     | 2   | no             | n/a                   | no                   | 06/11/2013    | Negative  |        | Negative  |          |          |
| 538 | AI15 | Stallion | 2   | no             | n/a                   | no                   | 06/11/2013    | Negative  |        | Positive  | 1.6      | >2.6     |
| 539 | AI16 | Mare     | 4   | yes            | no                    | no                   | 06/11/2013    | Negative  |        | Equivocal | 2.3      | 1.2      |
| 540 | AI17 | Mare     | 18  | yes            | no                    | yes                  | 06/11/2013    | Positive  | 1.9    |           |          |          |
| 541 | AI18 | Mare     | 11  | yes            | no                    | yes                  | 06/11/2013    | N.I.      | 1.7    |           |          |          |
| 542 | AI19 | Stallion | 3   | no             | n/a                   | no                   | 06/11/2013    | Negative  |        | Negative  |          |          |
| 543 | AI20 | Stallion | 4   | no             | n/a                   | no                   | 06/11/2013    | Negative  |        | Positive  | 1.9      | 1.4      |
| 544 | AI21 | Stallion | 9   | yes            | no                    | no                   | 06/11/2013    | Positive  | 3      | Positive  | >2.6     | 1.9      |
| 545 | AI22 | Stallion | 10  | yes            | no                    | no                   | 06/11/2013    | Negative  |        | Positive  | >2.6     | 1.8      |
| 546 | AI23 | Stallion | 5   | no             | n/a                   | no                   | 06/11/2013    | Positive  | < 0.6  | Negative  |          |          |
| 547 | AI24 | Stallion | 7   | yes            | no                    | no                   | 06/11/2013    | Negative  |        | Positive  | 2.5      | 1.3      |
| 548 | AI25 | Stallion | 11  | yes            | no                    | no                   | 06/11/2013    | Negative  |        | Positive  | >2.6     | 1.9      |
| 549 | AI26 | Stallion | 5   | yes            | no                    | no                   | 06/11/2013    | Negative  |        | Positive  | >2.6     | 2.1      |

| <b>No</b> | <b>ID</b> | <b>SEX</b> | <b>AGE</b> | <b>ACTIVE BREEDER</b> | <b>REPRODUCTIVE PROBLEMS</b> | <b>EHV-1/-4 VACCINATION</b> | <b>SAMPLING DATE</b> | <b>EVA ELISA</b> | <b>EVA SN</b> | <b>EHV ELISA</b> | <b>EHV-1 SN</b> | <b>EHV-4 SN</b> |
|-----------|-----------|------------|------------|-----------------------|------------------------------|-----------------------------|----------------------|------------------|---------------|------------------|-----------------|-----------------|
| 550       | A127      | Stallion   | 11         | yes                   | no                           | no                          | 06/11/2013           | <i>Positive</i>  | <i>2</i>      | <i>Equivocal</i> | <i>2.4</i>      | <i>2.2</i>      |
| 551       | A128      | Stallion   | 5          | yes                   | no                           | no                          | 06/11/2013           | <i>Negative</i>  |               | <i>Positive</i>  | <i>2.5</i>      | <i>1.1</i>      |
| 552       | A129      | Stallion   | 6          | no                    | n/a                          | no                          | 06/11/2013           | <i>Negative</i>  |               | <i>Equivocal</i> | <i>2.3</i>      | <i>1.6</i>      |
| 553       | A130      | Stallion   | 5          | yes                   | no                           | no                          | 06/11/2013           | <i>Negative</i>  |               | <i>Positive</i>  | <i>&gt;2.6</i>  | <i>2.2</i>      |
| 554       | A131      | Mare       | 13         | yes                   | no                           | yes                         | 06/11/2013           | <i>Positive</i>  | <i>2.3</i>    |                  |                 |                 |
| 555       | A132      | Stallion   | 4          | yes                   | no                           | no                          | 06/11/2013           | <i>Negative</i>  |               | <i>Positive</i>  | <i>&gt;2.6</i>  | <i>1.6</i>      |



## *IX. BIBLIOGRAPHY*



## *IX. BIBLIOGRAPHY*

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