UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

TESIS DOCTORAL

Evolución epidemiológica y control de la peste equina africana
Epidemiological evolution and control of African horse sickness

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Departamento de Sanidad Animal
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Evolución Epidemiológica y Control de la Peste Equina Africana

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Epidemiological Evolution and Control of African Horse Sickness

MEMORIA DE TESIS DOCTORAL

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DIRIGIDA POR LOS DOCTORES

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A mis padres y a Pablo.

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GLOSARIO DE ABREVIATURAS

ADN	Ácido desoxirribonucleico
ADNc	Ácido desoxirribonucleico complementario
AHS	African horse sickness
APHIS	Servicio de Inspección Sanitaria de Plantas y Animales
ARN	Ácido ribonucleico
ARNds	Ácido ribonucleico bicatenario
ARNm	Ácido ribonucleico mensajero
CC.AA	Comunidades Autónomas
cELISA	Enzimoinmuno ensayo de competición o bloqueo
DIVA	Diferenciación de animales infectados y vacunados
dpi	Días post-infección
ELISA	Enzimoinmuno ensayo
EHE	Enfermedad hemorrágica epizoótica del ciervo
EIP	Periodo de incubación extrínseca
FAO	Organización de las Naciones Unidas para la Alimentación y la Agricultura
FC	Fijación del complemento
iELISA	Enzimoinmuno ensayo indirecto
IL-1	Interleukina 1
LA	Lengua azul
LFA	Ensayo de flujo lateral
MAGRAMA	Ministerio de Agricultura, Alimentación y Medio Ambiente
NS	Proteínas no estructurales
OIE	Organización Mundial de Sanidad Animal
PEA	Peste equina o peste equina africana
REMO	Registro General de Movimientos de Ganado en España
RT-PCR	Reacción en cadena de la polimerasa con retrotranscriptasa inversa
rRT-PCR	Reacción en cadena de la polimerasa con retrotranscriptasa inversa en tiempo real
SNV	Seroneutralización vírica
TNFα	Factor de necrosis tumoral alfa
UE	Unión Europea
USDA	Departamento de Agricultura de los Estados Unidos de América
VP	Proteínas estructurales
vPEA	Virus de la peste equina
WAHID	Base de datos del sistema mundial de información zoosanitaria

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Fuente de la imagen: Cedidas por Carlos Escribano, Yeguada “La Cartuja”.

RESUMEN

La **peste equina o peste equina africana** (PEA) es una de las enfermedades vectoriales más importantes que afecta a todas las especies de la Familia *Equidae*, siendo transmitida por artrópodos del género *Culicoides* principalmente. Se trata de una enfermedad vírica altamente infecciosa pero no contagiosa, que puede ocasionar diversas formas clínicas en los animales infectados dependiendo de la sintomatología manifestada. La alta patogenicidad y gran poder de difusión de la misma ha ocasionado, que a pesar de no ser zoonótica, sea incluida en la lista única de enfermedades de **declaración obligatoria** de la Organización Mundial de Sanidad Animal (OIE). La situación actual de la enfermedad, endémica en el continente africano y más concretamente en los países del África Sub-Sahariana, supone un peligro constante por la posible difusión a países libres de otros continentes. Este hecho, en combinación con la reciente expansión en Europa de enfermedades vectoriales como la lengua azul, Schmallenberg o West Nile, ha puesto de manifiesto el posible carácter re-emergente de la PEA. España, por sus características geográficas y climáticas, es una de las potenciales vías de entrada de la enfermedad en Europa.

Esta tesis doctoral, titulada "**Evolución Epidemiológica y Control de la Peste Equina"**, tiene como objetivo principal desarrollar y proporcionar nuevas estrategias de prevención y control para la PEA en España. El trabajo desarrollado en la presente tesis, ha generado siete artículos científicos publicados en revistas de impacto internacional. Estos artículos abarcan, desde los estudios inmunológicos desarrollados para alcanzar una mejor comprensión de la respuesta inmune ocurrida tras la infección, hasta el desarrollo de nuevas técnicas de diagnóstico de la misma. El análisis integral de la enfermedad se ve completado con la evaluación epidemiológica y económica de la difusión de la misma en España.

El primer objetivo de esta tesis doctoral consistió en la **evaluación de la respuesta inmune de los équidos frente al virus de la PEA** (vPEA) mediante la evaluación de los patrones de expresión de citoquinas y de la producción de anticuerpos. La cuantificación de la expresión de citoquinas en caballos fue realizada tras el desarrollo y optimización de técnicas de reacción en cadena de la polimerasa con retrotranscriptasa inversa en tiempo real (rRT-PCR) con SYBR Green para ocho citoquinas involucradas en la respuesta inmune innata y adaptativa, junto con un gen de expresión constante (β-Actina). La posibilidad de cuantificación relativa de los niveles de expresión de citoquinas fue combinada con la detección de anticuerpos frente al virus. Estos análisis permitieron llevar a cabo la evaluación de la respuesta inmune en animales

infectados experimentalmente con el vPEA y relacionarlos con las formas clínicas de la enfermedad que presentaban los caballos. En este estudio se observó que los équidos presentaron un patrón de expresión de citoquinas variable, pudiéndose identificar algunos perfiles comunes en función de la forma clínica de la enfermedad. De esta manera, se han descrito nuevos conocimientos de los mecanismos inmunitarios existentes frente a la infección, los cuales ayudarán al desarrollo de nuevas herramientas terapéuticas y preventivas (vacunas), y por ende, a la mejora de los métodos de prevención y control en la lucha contra la enfermedad.

En el segundo objetivo se han desarrollado **dos nuevas técnicas de diagnóstico para la PEA** mediante la utilización de la tecnología Luminex®. Específicamente, se llevó a cabo la mejora de la sensibilidad en la detección de anticuerpos frente al virus en comparación con las técnicas convencionales. Para ello, se desarrolló y evaluó por primera vez un ensayo Luminex frente a la proteina estructural 7 (VP7) analizando un panel de 300 muestras de suero y plasma, cuyos resultados fueron comparados con un enzimoinmuno ensayo (ELISA) y un ensayo de inmunocromatografía o de flujo lateral (FLA) comerciales. La nueva técnica desarrollada permitió la detección de anticuerpos frente a todos serotipos de vPEA, en muestras de animales infectados experimentalmente, analizando la cinética de producción de anticuerpos, y en muestras de campo de animales infectados de manera natural. Posteriormente, este nuevo test fue adaptado para la obtención de un ensayo que permitiera la diferenciación de animales vacunados e infectados (DIVA) mediante la detección de anticuerpos frente a la VP7 y la proteína no estructural (NS3). La técnica desarrollada permite la diferenciación de animales infectados con el serotipo 4, vacunados con vacuna inactivada frente al serotipo 4, y animales sanos en función de la respuesta de anticuerpos. Las ventajas de estos ensayos y la posibilidad de adaptación de la tecnología Luminex® a la detección de otros anticuerpos del virus simultáneamente, lo posicionan como una alternativa al ELISA en los laboratorios de diagnóstico. La mejora en la identificación de los animales infectados permitirá establecer nuevos planes de prevención y control de PEA más eficientes que los actuales.

El tercer objetivo consistió en realizar un **análisis epidemiológico de la PEA en España** que permita mejorar los planes de vigilancia y control de la enfermedad en el país. En un primer estudio, se llevó a cabo la evaluación de las zonas y periodos de mayor riesgo para la PEA mediante la realización de un modelo de decisión multicriterio de epidemiologia espacial. Tras una extensa revisión bibliográfica, se identificaron los principales factores de riesgo de la enfermedad, como son: el periodo de incubación extrínseco, la densidad equina y la distribución de vectores competentes. La superposición espacial de estos factores permitió la localización de

las zonas de mayor riesgo para PEA en el sur-oeste de España, Cantabria y las Islas Baleares, siendo los meses de mayor riesgo julio y agosto. Por otro lado; las zonas que se identifican como escasamente adecuadas se localizan en Galicia, Castilla y León y La Rioja. Los resultados del modelo combinado con las características de la producción equina, determinarán las consecuencias de la difusión de la enfermedad en el sector equino español; de igual manera afianzarán las bases para diseñar estrategias de vigilancia basadas en riesgo.

En un segundo estudio, se evaluó la posible propagación de la enfermedad por movimientos animales. Para ello, se llevó a cabo el análisis de la red de movimientos equinos en Castilla y León, una de las regiones más importantes de producción equina de España. El análisis de redes sociales y de agrupaciones espacio-temporales ha permitido caracterizar los patrones de contacto de la red equina e identificar los individuos, zonas y períodos de tiempo de mayor riesgo para la introducción o difusión de enfermedades en la región. En general, los tipos de explotaciones más importantes de la red fueron aquellas destinadas a la producción y reproducción de equinos, así como los centros de competición de ganado por sus roles en el movimiento de animales. Además fueron identificadas diecisiete agrupaciones espacio-temporales significativas como origen y destino de los movimientos, que formaron cuatro compartimentos interconectados. Los resultados de este estudio pueden ser una valiosa herramienta para diseñar programas de vigilancia basados en el riesgo de enfermedades de los équidos, aumentando la velocidad de detección y control de posibles brotes secundarios en futuras epidemias.

En el cuarto objetivo se **evaluaron las consecuencias económicas de la difusión de la PEA en España**. Concretamente, se desarrolló un meta-modelo matemático que integra el análisis epidemiológico y económico de difusión de la PEA en Andalucía en varios escenarios de temperatura y de ratio de vectores por hospedador. Este modelo fue utilizado para evaluar las estrategias actuales de control de la enfermedad variando la utilización o no de la estrategia de vacunación. Todos los escenarios permitieron la difusión de la enfermedad en Andalucía, sin embargo, el impacto sanitario y económico de la epidemia de PEA sin vacunación fue mayor en comparación con el mismo escenario cuando se aplicaba la estrategia de vacunación. Este trabajo es la primera evaluación económica de la PEA en un país no endémico. Los resultados obtenidos podrán ser de gran utilidad para la toma de decisiones de las autoridades sanitarias proveyendo información tanto del posible impacto de la enfermedad como la estrategia de control a aplicar más adecuada, lo que mejorará la optimización de la relación coste-eficacia de los programas sanitarios.

Finalmente, destacar que los estudios desarrollados en esta **tesis doctoral** proporcionan nueva información acerca de los **mecanismos inmunitarios del hospedador,** los cuales podrían ser usados como marcadores para el desarrollo de nuevas herramientas de control en la lucha contra la enfermedad; **nuevas herramientas de diagnóstico** que permitirán aumentar la sensibilidad y con propiedades DIVA que, junto con los nuevos conocimientos aportados por **estudios epidemiológicos y económicos** realizados en esta tesis doctoral, aportan novedosas metodologías y resultados con una aplicación directa y estratégica en la mejora de los **programas de prevención y control** de la enfermedad en España, orientándolos a una vigilancia basada en riesgo con la mejor relación coste-beneficio.

SUMMARY

African horse sickness (AHS) is one of the most important vector-borne diseases that affects all *Equidae spp*., being transmitted mainly by the *Culicoides* genus. This is a non-contagious but highly infectious viral disease, which may result in different clinical manifestations in infected animals depending of the clinical signs and lesions. Due to the high mortality rates and rapid AHS spread, it is included in the World Organization for Animal Health (OIE) listed diseases. The current situation of the disease, endemic in Africa and particularly, in the countries of Sub-Saharan Africa, has increased the risk of spread to free countries in other continents. This fact, together with the recent spread of vector-borne diseases in Europe such as bluetongue, West Nile and Schmallenberg, supports the concern about AHS being considered as a possible re-emerging disease in Europe. Spain is one of the potential routes of introduction of these vector-borne diseases in Europe by its geographical and climatic characteristics.

This PhD thesis entitled "**Epidemiological Evolution and Control of African Horse Sickness**", has the goal of developing and providing new methodologies and tools for improving the surveillance and control programs against AHS in Spain. The work developed in this thesis consists of seven scientific articles published in journals of international impact. These studies describe immunological studies, diagnostic techniques and epidemiological and economic evaluations of the spread of the disease in Spain.

The first objective consisted in the evaluation of the **horse immune response against AHS virus (AHSV)** by analyzing the cytokine expression pattern and antibody response after AHSV experimental infection. The quantification of the cytokine expression in horses was performed after the development and optimization of real time reverse transcription polymerase chain reactions (rRT-PCRs) with SYBR Green. Eight cytokines involved in innate and adaptive immune responses and a housekeeping gene (β-actin) were detected and amplified with this rRT-PCRs, allowing quantification of relative expression levels. The combination of this technique and the detection of antibodies production against AHSV enabled to carry out the evaluation of the immune response in AHSV experimentally infected horses manifesting different clinical forms. The results showed a different cytokine expression profile in each horse, although some common characteristics were identified within the clinical forms. This study provides new data about the immune response in naïve horses infected with AHSV, and they might be useful to help improving different treatments and vaccines as control and prevention methods.

The second objetive focused on the development of **two new AHS diagnostic techniques** using the Luminex technology®. Specifically, a first study had the aim of improving the sensitivity of immunoassays. To this end, a Luminex assay, using recombinant AHS virus structural protein 7 (VP7), has been developed for serological detection of antibodies against this virus. The new diagnostic assay was compared with commercial enzyme-linked immunosorbent assay (ELISA) and lateral flow assay (LFA) commercial kits by testing a large panel of 300 serum and plasma samples. This new technique allowed the detection of antibodies against VP7 for all serotypes of AHSV, the investigation of the kinetics of antibody responses in experimentally-infected horses and the detection of field samples. Subsequently, this novel immunoassay was adapted to develop a multiplexed Luminex assay for the differentiation of infected and vaccinated animals (DIVA) for AHS serotype 4 using both recombinant VP7 and non-structural protein 3 (NS3). This DIVA assay allows a clear differentiation between infected and vaccinated horses with inactivated vaccine against serotype 4 or uninfected horses. The advantages of these tests and the potential of multiplexing the Luminex asay for the detection of other viral antibodies simultaneously may be an alternative to ELISA in the diagnostic laboratories improving the surveillance and control plans against AHS.

The third objective was to conduct an **AHS epidemiological analysis in Spain** that allowed enhancing the surveillance and control system of the disease in the country. As first study, a GIS-based multicriteria decision model was built up to identify the risk areas and time periods for AHS occurrence in Spain. An extensive literature review allows the identification of main risk factors for the disease, namely the extrinsic incubation period, the equine density and the distribution of competent vectors. The weighted linear combination of these factors revealed that the south-western and north-central areas of Spain and the Balearic Islands are the most suitable areas for AHSV infections, particularly in late summer months. Conversely, Galicia, Castile and Leon and La Rioja can be considered poorly suitable regions. The model results, together with current Spanish equine production features, determine the consequences of the AHS spread in the Spanish horse industry; and should provide the foundations to design risk-based surveillance strategies.

In a second study, the possible spread of the disease by animal movements was evaluated. To do this, we carried out the social network analysis of equine movements in one of the most important regions of Spain's equine production, Castile and Leon. Social network analysis and space-time clusters analysis were used to describe the contact patterns of the equidae network and to identify the most important premises, areas and time periods for potential

disease introduction or spread into the region. In general, production and reproduction farms and centres of livestock competition were the most important type of premises in the studied network, due to their roles in the movement of animals. Furthermore, cluster analyses allowed identifying seventeen significant spatio-temporal clusters of premises at high risk of dispatching or receiving equidae, which formed four interconnected compartments. The results of this study may be useful to design risk-based surveillance and control programmes of equidae diseases and increase the speed of detection and control of potential secondary outbreaks in future epidemics.

The fourth and last objective was **the evaluation of the socio-economic consequences of the AHS epidemic for the horse industry in Spain**. Specifically, a meta-mathematical model has been carefully parameterized to evaluate the epidemiological and economic consequences of AHS spread in Andalusia analyzing different temperature and vector to host ratio, as well as the implementation of vaccination strategy. All scenarios allowed the spread of the disease in Andalusia, however, the health and economic impact of the epidemic without AHS vaccination was higher than the same strategy including the vaccination. This work is the first economic evaluation of the AHS epidemic in a non-endemic country. The model results could be useful for improving decision-making by Spanish national policy makers, as well as to predict the socio-economic consequences of AHS outbreak having into a count the possible scenarios, enhancing the optimization of cost-effectiveness of control programs.

The studies in this thesis provide new information about the host's immune response mechanisms, which could be used as markers for the development of new control tools against the disease. In addition, new diagnostic tools that will increase the sensitivity and DIVA diagnosis are presented in combination with epidemiological and economic studies regarding AHSV. These novel knowledges provide new methodologies and results with a direct application on the development and update of surveillance and control programs of the disease in Spain based on risk-based surveillance with the best cost-benefit relation.

CAPÍTULO I

INTRODUCCIÓN

Fuente de la imagen: Arte en Cantabria (Universidad de Cantabria), Caballo rupestre de las Cuevas de Altamira [dominio público].

La **peste equina o peste equina africana** (PEA) (African horse sickness (AHS), Peste equine) es una de las enfermedades vectoriales re-emergentes que actualmente representa una de las mayores amenazas en Europa. Se trata de una enfermedad vírica altamente infecciosa, no contagiosa, la cual se transmite principalmente por vectores del **género *Culicoides***. Esta enfermedad es considerada como una de las enfermedades más graves que afectan a los **équidos** debido a su alta patogenicidad y gran poder de difusión. Por ello, a pesar de no ser zoonótica, es una enfermedad incluida en la lista única de enfermedades de **declaración obligatoria** de la Organización Mundial de Sanidad Animal (OIE, 2014) y siendo en la Unión Europea (UE) enfermedad de declaración obligatoria (2012/737/UE). La aparición y difusión de esta enfermedad en un país provoca **graves repercusiones socio-económicas**, asociadas tanto a las pérdidas directas por la mortalidad de la enfermedad, como a las pérdidas indirectas relacionadas con la restricción de movimientos de animales e implementación de las medidas para el control y erradicación de la misma.

En este capítulo se describen brevemente los aspectos más relevantes sobre el agente etiológico; las especies susceptibles y los vectores; la inmunopatogenia; las técnicas de diagnóstico y las medidas de prevención y control de la enfermedad; así como la importancia histórica y actual de la PEA en Europa, y más concretamente en España. El dominio de estos conceptos sobre la enfermedad es fundamental para el desarrollo de los **objetivos de esta tesis doctoral**, cuya finalidad será la mejora de la comprensión de la respuesta inmune desarrollada tras la infección, el desarrollo de nuevas técnicas de diagnóstico y la evaluación epidemiológica y económica de la difusión de la enfermedad en España, con el fin de desarrollar nuevas estrategias de prevención y control.

1. PESTE EQUINA

1.1. Etiología

La PEA es una enfermedad vírica cuyo agente etiológico es el **virus de la peste equina** (vPEA) (Figura 1), perteneciente al género *Orbivirus* y la familia *Reoviridae* (Attoui et al., 2012). Este género también engloba otros virus responsables de enfermedades vectoriales animales como la lengua azul (LA) de los rumiantes y la enfermedad hemorrágica epizoótica del ciervo (EHE), presentando todos los orbivirus una organización estructural muy similar, aunque con distintas propiedades epidemiológicas, patológicas y antigénicas, destacando la infección de

distinto rango de hospedadores (Verwoerd et al., 1979; Spence et al., 1984; Attoui et al., 2012; Stanley, 2012). Mediante **seroneutralización vírica (SNV)** se han reconocido **9 serotipos** del virus antigénicamente distintos, únicamente presentando entre algunos de ellos inmunidad cruzada de la siguiente manera: el 1 con el 2; el 3 con el 7; el 5 con el 8; y el 6 con el 9; y en ningún caso con otros orbivirus (McIntosh, 1958; Howell, 1962).

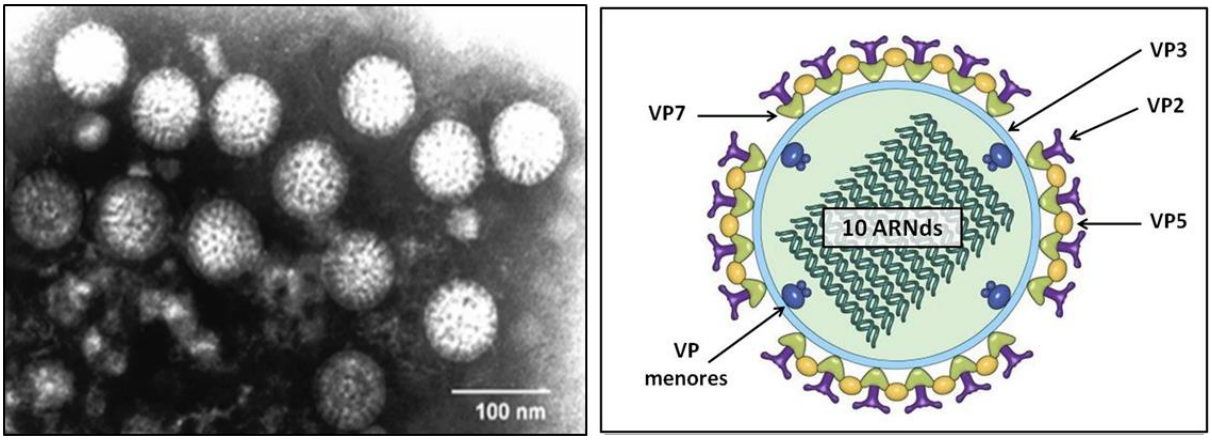

Figura 1: Imagen obtenida mediante microscopía electrónica de virus de la familia *Reoviridae*, al que pertenece la peste equina (izquierda) y diagrama esquemático de la estructura del virus de la peste equina (derecha). Composición propia. Fuente de la imágenes: Centro de Seguridad Alimentaria y Salud Publica, Universidad de Iowa (izquierda), y adaptación de Manole et al., 2012 (derecha).

El vPEA presenta una morfología icosaédrica con un tamaño de alrededor de 70 nm de diámetro. Estructuralmente, el virión es una partícula sin envoltura, cubierta por dos cápsides concéntricas compuestas por 32 capsómeros de las **siete proteínas víricas estructurales** (VP1-7) (Figura 1). La cápside externa está compuesta por dos de las proteínas mayores del virus, VP2 y VP5, principales responsables de la variabilidad antigénica del virus. En la cápside interna se pueden diferenciar dos capas, la constituida por las otras dos proteínas mayores, VP3 y VP7, y la constituida por las proteínas menores VP1, VP4 y VP6. En el interior del virión se localiza el núcleo interno de la partícula vírica, donde se alberga el genoma vírico (Roy et al., 1994) (Figura 1). El genoma vírico se compone de **10 segmentos de ácido ribonucleico bicatenario** (ARNds). Cada segmento de ácido ribonucleico (ARN), excepto los segmentos 9 y 10, codifica una única proteína vírica, incluyendo las proteínas estructurales (VP) y las **cuatro proteínas no estructurales** (NS) (Bremer et al., 1990; Grubman and Lewis, 1992; Belhouchet et al., 2011).

Las distintas proteínas tienen diferentes propiedades y funciones específicas. La **proteína VP2** es la más variable de las proteínas víricas, siendo específica de cada serotipo (Potgieter et al., 2003). Se caracteriza por ser la responsable de la hemoaglutinación viral (Cowley and Gorman,

1987; Kaname et al., 2013) y por ser una de las proteínas responsable de la formación de anticuerpos neutralizantes en el hospedador, ya que en su superficie se localizan varios epítopos (Burrage et al., 1993; Martínez-Torrecuadrada and Casal, 1995; Roy et al., 1996; Stone-Marschat et al., 1996; Bentley et al., 2000; Martínez-Torrecuadrada et al., 2001; Scanlen et al., 2002; Manole et al., 2012; Alberca et al., 2014). Esta respuesta humoral se incrementa en presencia de la **proteína VP5** (Martínez-Torrecuadrada et al., 1996; Guthrie et al., 2009). Al contrario de la VP2, esta proteína se caracteriza por ser altamente conservada en todos los serotipos (du Plessis and Nel, 1997).

La **VP7** es la proteína más conservada del virus, cuya función es la unión del virus a las células de insecto (Bremer et al., 1990; Roy et al., 1991; Mertens et al., 1996). Asimismo, la **VP3** es la proteína responsable de lo organización física del genoma y, consecuentemente, de la integridad del virus. Tanto la VP3 como la VP7 se caracterizan por determinar el serogrupo, ya que son unas proteínas conservadas y similares en todos los serotipos del virus que permiten la identificación del agente infeccioso, sin presentar reacciones cruzadas con las correspondientes proteínas de otros orbivirus (Roy et al., 1991; Williams et al., 1998).

Por último, las **tres proteínas estructurales menores**, VP1, VP4 y VP6, están implicadas en la transcripción, replicación y empaquetamiento de los ARNs virales (Roy et al., 1994).

Las **proteínas no estructurales**, NS1, NS2, NS3, NS3a y la recién descubierta NS4, pueden ser detectadas en el citoplasma y en las membranas de las células infectadas, ya que se sintetizan durante las infecciones virales, resultado de su participación en diferentes funciones de la replicación viral y salida del virus de la célula (van Staden et al., 1991; Grubman and Lewis, 1992; Roy et al., 1994; Van Staden et al., 1998; Belhouchet et al., 2011; Ratinier et al., 2011). Recientemente se ha observado que la **proteína NS3** es la segunda proteína más variable, la cual puede ser dividida en tres grupos mediante su análisis filogenético (Sailleau et al., 1997; Martín et al., 1998; Van Niekerk et al., 2001). Se cree que esta proteína está involucrada en la determinación de la virulencia del agente, por su participación en la liberación de las partículas víricas del interior de la célula (Stoltz et al., 1996; Meiring et al., 2009). Además, esta proteína ha sido identificada en las vacunas inactivadas purificadas frente al serotipo 4 del vPEA, como posible proteína diana para la diferenciación de animales infectados y vacunados (DIVA) (Laviada et al., 1993).

1.2. Epidemiología

La PEA es una enfermedad infecciosa pero no contagiosa, siendo necesaria la presencia de hospedadores susceptibles y vectores competentes para el mantenimiento y difusión de la misma. Los **hospedadores principales** de la enfermedad son todas las especies del género *Equus*, aunque otros animales pueden actuar como hospedadores accidentales del virus. La transmisión del vPEA se realiza principalmente a través de la picadura de artrópodos hematófagos, siendo el **principal vector** del virus los jejenes del género *Culicoides,* aunque otros artrópodos, como mosquitos y garrapatas, pueden estar involucrados (Du Toit, 1944; Ozawa and Nakata, 1965; Mellor et al., 1990; Mellor, 2000; Wilson et al., 2009).

El **ciclo biológico de transmisión** se inicia cuando un jején competente ingiere sangre de un animal infectado con el vPEA durante el periodo de viremia. Una vez el jején ha ingerido el virus, éste alcanza el intestino, donde debe sobrevivir el tiempo suficiente para penetrar e infectar las células de la pared intestinal replicándose en su interior. Desde ahí, se produce una migración, que se extiende por el interior del vector llegando hasta las glándulas salivares, donde el virus se replica por segunda vez activamente, para finalmente ser liberado con la saliva (Mellor, 2000). De esta manera, cuando este vector competente infectado entre en contacto con un hospedador susceptible no infectado le trasmitirá el vPEA (Figura 2).

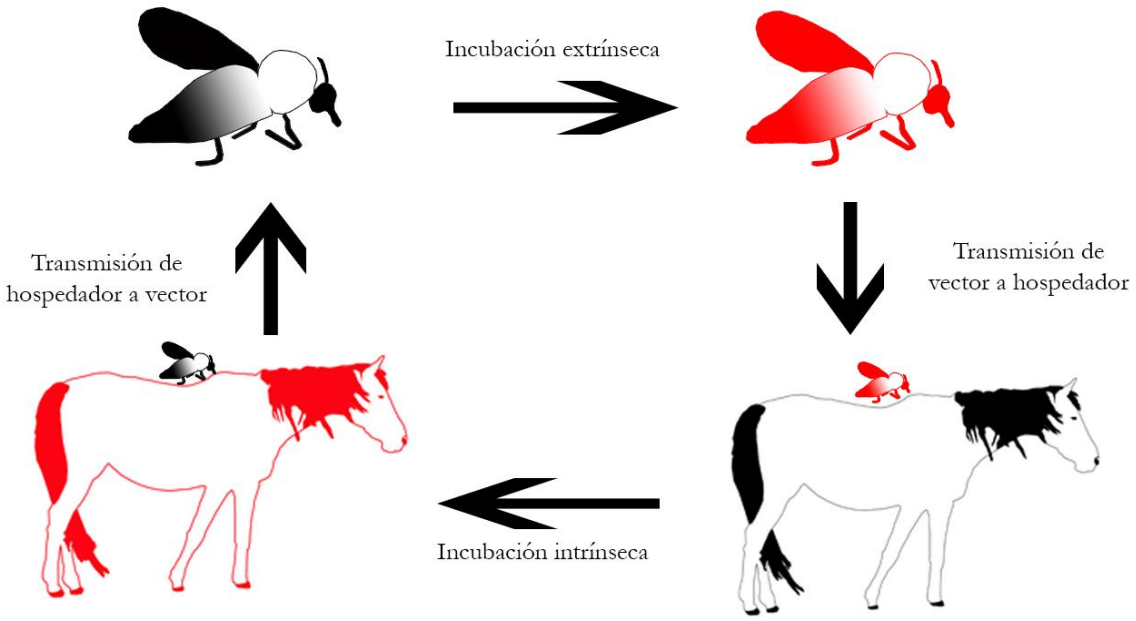

Figura 2: Ciclo de trasmisión del virus de la peste equina. Fuente de la imagen: Elaboración propia.

El **periodo de incubación extrínseca** (EIP) es el tiempo que transcurre entre la ingestión del virus por el jején hasta que el jején adquiere la capacidad de transmitir el vPEA. Este periodo está estrechamente ligado a la **temperatura ambiental** experimentada por el vector, ya que éstos son poiquilotermos (Smith, 1987). La temperatura óptima de replicación del virus está entre los 27ºC y los 30ºC (EFSA, 2007), resultando un EIP de 9 días tras la ingesta del virus por el vector en condiciones de temperatura de 25ºC. De igual modo, el límite de replicación del virus ha sido estimado entre los 10ºC y 15ºC (Mellor, 2000; Wittmann et al., 2002; Carpenter et al., 2011).

Esta asociación de la replicación del virus con la temperatura concreta que, en algunas localizaciones, las condiciones climáticas en determinadas épocas del año no sean las óptimas para la difusión de la enfermedad, apareciendo interrupciones en la transmisión de la misma. Este periodo de tiempo se caracteriza por un silencio epidemiológico de ausencia de brotes de la enfermedad, apareciendo de nuevo cuando las condiciones climáticas son las adecuadas. Este fenómeno se ha denominado **"*overwintering*"** o **hibernación**, y es habitual en todos los orbivirus. Sin embargo, la información disponible sobre las causas que determinan este fenómeno en estas enfermedades es hasta la fecha muy limitada (Wilson et al., 2009; Thompson et al., 2012).

1.2.1. Hospedadores

Los **équidos** son los principales hospedadores de la PEA (Figura 3). Sin embargo, el vPEA también puede infectar a otras especies como **carnívoros, camellos, elefantes** o **rinocerontes**, aunque estas especies no parecen tener un papel relevante en la epidemiología de la enfermedad.

Los **hospedadores naturales** y **principales reservorios** de la enfermedad son las **cebras** (*Equus burchelli*) y los **asnos africanos** (*Equus asinus somalicus*) (Figura 3), los cuales no suelen mostrar signos clínicos de la enfermedad y presentan unas viremias prolongadas, hasta 48 días post-infección (dpi) (Barnard et al., 1994). Por estos motivos, se considera que la distribución endémica de la enfermedad está ligada a la presencia de estos animales, teniendo un rol fundamental en la persistencia de la misma (Barnard, 1998; Lord et al., 1998). Esta presencia necesaria se ha visto confirmada cuando la disminución de las poblaciones salvajes de cebras en el último siglo ha coincidido con una reducción de los focos de la enfermedad. Sin embargo, se ha determinado que aunque estos hospedadores naturales parecen esenciales para el

mantenimiento de la enfermedad, no es necesaria su presencia para que se produzcan brotes esporádicos del virus (Mellor, 1993).

El **resto de équidos** son susceptibles a la enfermedad, presentando formas clínicas más graves con tasas de mortalidad superiores y tiempos de viremia inferiores a los reservorios de la enfermedad (Coetzer and Erasmus, 1994). Los **caballos** son la especie más susceptible al virus, especialmente en zonas no endémicas, donde la mortalidad puede llegar al 100%. En las **mulas**, el índice de mortalidad es aproximadamente del 50%; y en los **asnos europeos** y **asiáticos**, entre un 5 y un 10%. Del mismo modo, las viremias más cortas suelen observarse en caballos, alrededor de 4 u 8 dpi pudiendo alcanzar hasta los 21 dpi en estudios experimentales, mientras que en mulas y asnos pueden durar hasta 28 dpi (Coetzer and Erasmus, 1994; el Hasnaoui et al., 1998; Hamblin et al., 1998; Mellor and Hamblin, 2004). La escasa adaptación del virus a la supervivencia en caballos y mulas dificulta que estos animales puedan convertirse en reservorios a largo plazo de la enfermedad, considerándose **hospedadores accidentales** o **indicadores** (Figura 3).

Figura 3: Principales hospedadores naturales (izquierda - azul) y hospedadores accidentales (derecha - naranja) del virus de la peste equina. Composición propia. Fuente de las imágenes: Cebra (Nigel Dennis Wildlife Photography), asno africano (Hampel Group), caballo y asno europeo (imágenes propias), mula (wikipedia, dominio libre) y onagro (Jean-Christophe Vié).

Otras especies susceptibles a la enfermedad son los **carnívoros domésticos** y **salvajes**, los cuales se infectan por la ingestión de carne contaminada. Estos animales desarrollan una forma clínica severa con una viremia leve, muriendo a los pocos días (Van Rensberg et al., 1981; Alexander et al., 1995). El rol de estas especies en la transmisión de la enfermedad es aún una cuestión sin resolver, aunque se cree que actúan como **hospedador fondo de saco** (Wilson et al., 2009). De igual modo, se han descrito infecciones asintomáticas en **camellos**, en los que se desconoce el nivel y duración de la viremia, pero se ha detectado la presencia de anticuerpos (Awad et al., 1981b). También, se ha identificado la presencia de anticuerpos contra el vPEA en **elefantes** y varias especies de **rumiantes** (ovejas, cabras, bovinos y búfalos), aunque no es posible asegurar si estos anticuerpos han sido producidos debido a la replicación viral o por continuas exposiciones al antígeno (Davies and Otieno, 1977; Awad et al., 1981a; Binepal et al., 1992; Barnard et al., 1995).

1.2.2. Jejenes competentes

Los jejenes del **género *Culicoides*** son el principal vector del vPEA (Du Toit, 1944). Actualmente, han sido descritas alrededor de 1500 especies de *Culicoides*, sin embargo, no todas parecen ser capaces de actuar como vectores eficientes, siendo las hembras adultas de unas 50 especies, las únicas implicadas en la transmisión de enfermedades vectoriales (Figura 4) (Mellor et al., 2000). ***Culicoides imicola*** es la especie más importante en la transmisión de la enfermedad, situada históricamente entre los paralelos 35ºS y 40ºN los cuales abarcan regiones de África, Asia y Europa (Mellor, 1993; Mellor and Hamblin, 2004). Otras especies del genero *Culicoides* pueden o podrían estar implicadas en la transmisión de la enfermedad, como *C.bolitinos* en África, *C.sonorensis* en América del norte o en el complejo de *C.obsoletus* y *C. pulicaris* en Europa (Boorman et al., 1975; Mellor et al., 1975; Mellor et al., 1990; Meiswinkel and Paweska, 2003). Destacar que las dos últimas especies de jejenes han sido los principales vectores de la transmisión de la LA en el norte de Europa llegando a latitudes de 65ºN (Nielsen et al., 2010).

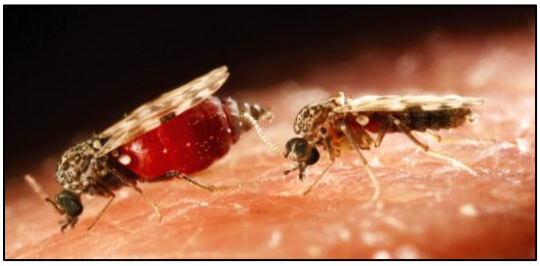

Figura 4: Jejenes hembras del género *Culicoide,* responsable de la transmisión de la peste equina, post y pre-ingesta de sangre. Fuente de la imagen: Instituto de Sanidad Animal de Reino Unido.

Para estos jejenes, al ser insectos poiquilotermos, las **condiciones ambientales** son determinantes para el desarrollo de su ciclo biológico y su distribución geográfica (Mellor et al., 2000), siendo diferentes en función de la especie de *Culicoides* y delimitando de este modo la localización geográfica de las distintas especies. Entre los factores que influyen en la supervivencia y replicación del vector destacan la temperatura y la humedad.

La **temperatura** es fundamental en el ciclo vital del vector, ya que determina funciones básicas tales como la supervivencia de las formas inmaduras así como de las adultas o su reproducción (Mellor et al., 2000). La temperatura óptima para el principal vector de la enfermedad, *C. imicola*, se encuentran entre 12,5ºC y 38ºC (Sellers and Mellor, 1993; Ortega et al., 1999). Aunque otras especies pueden estar más adaptadas al frio, como *C. obsoletus,* requiriendo temperaturas relativamente más bajas para su desarrollo y la supervivencia, 11ºC y 27,5ºC (Dzhafarov, 1964). Otro factor ambiental de gran importancia es la **humedad**, ya que las precipitaciones regulan el tamaño y la persistencia de ambientes adecuados para el vector repercutiendo en la densidad de culicoides adultos (Meiswinkel, 1998). En este aspecto, destaca la asociación ocurrida entre el fenómeno de "El Niño" con el incremento que se produjo en el número de brotes de PEA en África (Baylis et al., 1999). En consecuencia, la PEA se observa principalmente durante estaciones cálidas y lluviosas, que favorecen la propagación de los vectores, y desaparece cuando el frío detiene o reduce de manera significativa la actividad del vector. El estudio de estos factores permitirá conocer la distribución de los vectores y predecir el riesgo de la enfermedad en una región.

1.2.3. Distribución histórica

La PEA es una enfermedad endémica en la zona del **África Subsahariana**, ocupando una ancha banda desde Senegal al oeste, hasta Etiopia y Somalia al este; y en **Yemen** en la Península de Arabia. Sin embargo, en ciertas ocasiones se han notificado brotes fuera de estas áreas en el siglo XX, como en el **Oriente Medio**, **Magreb** y la **Península Ibérica**.

Mellor (1993) realizó la revisión de la historia cronológica de la enfermedad datando las primeras referencias de la enfermedad en 1327 en Yemen, aunque probablemente el virus procediese de África. Sin embargo, no se observaron las primeras manifestaciones clínicas de la enfermedad en este continente hasta 1569, por la introducción de caballos europeos durante su colonización. Desde entonces y hasta el siglo XX, numerosas epidemias más o menos graves han sido notificadas en el continente africano. A lo largo de la última década, la frecuencia y

severidad de los brotes en el continente africano ha ido decreciendo asociado a la disminución de la población de cebras salvajes y a la introducción de vacunas (Mellor and Hamblin, 2004).

Fuera de la zona endémica de la enfermedad, excepto pequeñas incursiones en el norte de África y Península Arábiga, no se detectó la enfermedad hasta 1959 (Rafyi, 1961; Mirchamsy and Hazrati, 1973). Durante ese año, el serotipo 9 del vPEA se expandió por Arabia Saudí, Siria, Líbano, Jordania, Irak, Turquía, Chipre, Irán, Afganistán, Pakistán e India, siendo necesario una campaña de vacunación y sacrificio masivo para conseguir erradicar la enfermedad en 1961 (Howell, 1960; Gohre et al., 1964–1965; Mellor, 1993; Wilson et al., 2009). El mismo serotipo re-emergió en 1965 en el Norte de África, afectando a Argelia, Túnez y Marruecos, y llegando a España en 1966 (OIE, 1966; Díaz-Montilla and Paños Marti, 1967; Laaberki, 1969). La enfermedad fue erradicada rápidamente en España, sin embargo en África persistió 2 años. La última incursión de la enfermedad fuera de la zona endémica en el siglo XX fue en 1987, de nuevo en España, cuando la enfermedad fue introducida accidentalmente. Esta epidemia duró hasta 1990 en la Península Ibérica y se difundió hasta Marruecos, donde permaneció hasta 1991 (Rodríguez et al., 1992; Sánchez-Vizcaíno, 2004).

En el siglo XXI la PEA ha sido detectada en numerosas ocasiones fuera de las regiones endémicas en el continente africano (OIE, 2014b). Desde 2010, los países que han informado de la sospecha o presencia de la PEA a la OIE (año de la última notificación) han sido Botsuana (2010), Ghana (2010), Mauritania (2010), Lesoto (2011), Somalia (2011), Eritrea (2012), Gambia (2013), Namibia (2013), Senegal (2013), Etiopía (2014), Mozambique (2014), Suazilandia (2014) y Sudáfrica (2014); aunque muchos países africanos no notifican el estatus sanitario de esta enfermedad a la OIE (Figura 5).

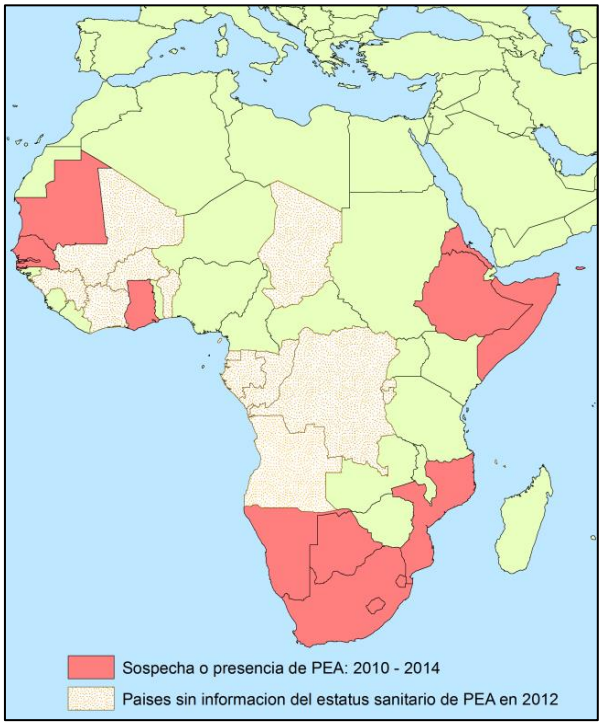

Figura 5: Estatus oficial de la peste equina según datos de la OIE en África. Fuente: OIE-WAHID, elaboración propia.

La situación actual de la enfermedad, con el mantenimiento de la circulación del virus en el continente africano, la adaptación de los vectores a nuevas localizaciones gracias al calentamiento global, y el incremento de los movimientos internacionales de los équidos, hacen que esta enfermedad sea considerada una **enfermedad re-emergente** en Europa (Gould and Higgs, 2009; Gale et al., 2010; Maclachlan and Guthrie, 2010; Members of the Med_Reo_Net, 2010).

1.2.4. Rutas de introducción y difusión de la enfermedad

Dadas las características epidemiológicas de la enfermedad podemos decir que las posibles **vías de entrada y difusión** de la enfermedad son la importación legal e ilegal de animales o vectores infectados; la introducción de vectores infectados en corrientes de aire; la introducción de vectores infectados en vehículos; el bioterrorismo; o los escapes biológicos (Martínez-López et al., 2008).

Las **importaciones legales e ilegales de équidos vivos** son una de las principales rutas de introducción y difusión de enfermedades. Este hecho es debido a que los équidos se caracterizan por ser una de la especies con **mayor cantidad de relaciones internacionales**, favorecidas por el elevado número de eventos hípicos internacionales, la libre comercialización dentro de la UE y la globalización (FEI, 2012; TRACES, 2012). Aunque estos movimientos legales deben realizarse de forma segura tanto entre los países libres de enfermedades como entre aquellos que se vean afectados por ellas, **las relaciones comerciales entrañan ciertos riesgos de introducción de enfermedades**, ya que siempre pueden ocurrir fallos en los controles o en el transporte. Además, la posible existencia de movimientos ilegales de équidos entre países, los cuales son por su propia definición, incontrolables, incrementan el riesgo de introducción de enfermedades. Históricamente esta vía de entrada de enfermedades ha sido el origen de varias de las epidemias de PEA. Por ejemplo, el brote de PEA en España de 1987-1990 tuvo como origen la importación legal de cebras desde Namibia y su posible difusión en el territorio español por movimientos ilegales de équidos (ver Capítulo I, Sección 2). Del mismo modo, los movimientos animales también fueron la ruta de introducción de la enfermedad en el norte de África en 1965 (Mellor and Hamblin, 2004).

Otra de las rutas que históricamente ha tenido una mayor relevancia en la introducción y difusión de la PEA es la **introducción de vectores infectados en corrientes de aire.** Este hecho es debido a que el pequeño tamaño de los jejenes (1–3 mm de longitud) les permite ser desplazados en corrientes de aire a través de largas distancias (Sellers, 1992). De este modo, y

siempre que el vector infectado sobreviva al transporte y entre en contacto con un hospedador, se podrá producir la transmisión de la enfermedad. Sellers et al. (1977) pusieron de manifiesto que el movimiento de vectores en corrientes de aire fue la ruta más probable de introducción de la enfermedad en España en 1966, Chipre en 1960 y Cabo Verde en 1943.

Otras posibles rutas de introducción de la enfermedad podrían ser la **introducción de vectores infectados en vehículos;** el **bioterrorismo** y/o los **escapes biológicos**. Sin embargo hasta este momento, estas rutas no han tenido ninguna relevancia histórica.

1.3. Patogenia

Tras el EIP del virus en el vector llegando hasta las glándulas salivares, el vector es capaz de transmitir nuevamente el virus a los hospedadores vertebrados en posteriores alimentaciones. Durante la picadura del jején, el virus penetra en el hospedador llegando hasta el nódulo linfoide regional, produciéndose la replicación primaria. Seguidamente, se produce la liberación del virus al torrente circulatorio asociándose a los eritrocitos y a los monocitos de la sangre, ocurriendo la viremia, y diseminándose a los órganos diana de replicación secundaria tales como pulmones, corazón, bazo y otros órganos linfoides. Tras la replicación secundaria, se produce otra posterior viremia, la cual es variable en duración y título (Burrage and Laegreid, 1994; Coetzer and Erasmus, 1994; Mellor and Hamblin, 2004; Clift and Penrith, 2010). En la sangre, el virus está asociado a la fracción celular tanto de las células rojas de la sangre como de la capa leucocitaria, no estando presente en el plasma. En los órganos diana, el virus infecta a las células endoteliales microvasculares y a la línea celular monocitos-macrófagos (Laegreid et al., 1992; Brown et al., 1994; Burrage and Laegreid, 1994; Wohlsein et al., 1998; Carrasco et al., 1999; Gómez-Villamandos et al., 1999; Clift and Penrith, 2010).

La difusión y acción patógena del vPEA en el hospedador es capaz de provocar cuatro formas clínicas en los équidos. Se producen desde infecciones subclínicas e incluso asintomáticas, hasta enfermedades de curso agudo que desencadenan la muerte del animal (Burrage and Laegreid, 1994). Cada forma clínica se caracteriza por presentar unos signos clínicos y lesiones específicos, así como por tener asociadas diferentes tasas de mortalidad. Estas diferencias han sido descritas tanto en animales infectados de forma natural como experimentalmente (Maurer and McCully, 1963; Rodríguez et al., 1987; Coetzer and Erasmus, 1994; Skowronek et al., 1995; Long and Guthrie, 2013). Estas características suelen ser útiles para el diagnóstico preliminar y la clasificación de la gravedad de la enfermedad (Rodríguez et al., 1987), aunque no existan lesiones patognomónicas que la definan. Hasta el momento, se

desconoce cuales son los factores involucrados en el desarrollo de las diferentes formas clínicas de la enfermedad, conociéndose que no esta relacionado con el serotipo, la dosis ni la cepa vírica, ya que la misma cepa de un mismo serotipo en una epidemia de PEA o en infecciones experimentales es capaz de producir diferentes formas clínicas de la enfermedad (Rodríguez et al., 1992; Gómez-Villamandos et al., 1999; Sánchez-Matamoros et al., 2014b).

1.3.1. Forma pulmonar o hiperaguda.

Forma clínica más patógena de la enfermedad, la cual es común en los caballos que nunca han entrado en contacto con el virus y no han sido vacunados, así como en potros que no poseen inmunidad pasiva (Coetzer and Erasmus, 1994).

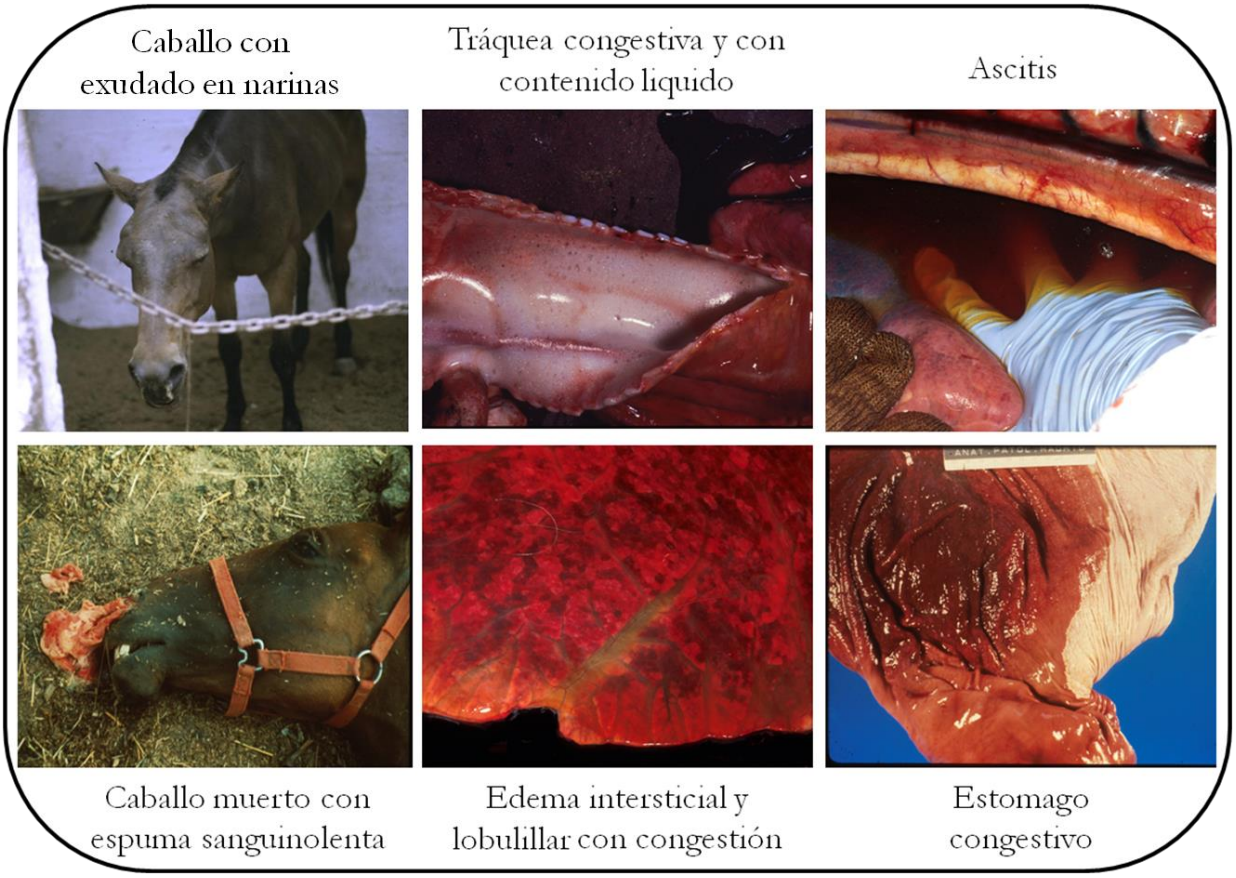

Figura 6: Signos clínicos y lesiones macroscópicas características de la forma pulmonar de la enfermedad. Composición propia. Fuente de las imágenes: APHIS –USDA; y María Castaño y Manuel Rodríguez, UCM.

La forma pulmonar se caracteriza por un período de incubación de 3 a 5 días, tras el cual los animales sufren un pico de fiebre de entre 39 y 41ºC de 1 o 2 días. La muerte se produce de manera repentina en menos de una semana, por lo que en ocasiones no se llega a observar ningún signo clínico. Los animales pueden manifestar disnea severa, tos espasmódica, dificultad

respiratoria grave con ollares dilatados, posición ortopneica y taquipnea. Otros signos clínicos que pueden observarse son depresión, abatimiento, sudoración, mucosas congestivas o cianóticas, pulso rápido y débil y taquicardia. En ocasiones se acompaña con periodos de postración del animal y, en las fases finales de la enfermedad, se puede observar descarga nasal (Rodríguez et al., 1987). La tasa de mortalidad de esta forma clínica es muy elevada, rondando el 95%, por lo que la recuperación de los enfermos no es frecuente. El animal muere debido a la alteración de la función pulmonar, por anoxia o insuficiencia cardíaca congestiva o ambos. En los animales muertos un exudado espumoso puede liberarse por las fosas nasales (Coetzer and Erasmus, 1994; Long and Guthrie, 2013) (Figura 6).

En la necropsia la principal lesión macroscópica que se observa es el edema pulmonar, especialmente visible en los tabiques interlobulillares, acompañado de hidrotórax. Los pulmones se encuentran aumentados de tamaño, pesados, congestivos, con ensanchamiento de los tabiques interlobulillares y petequias subpleurales. El espacio subpleural y los tabiques interlobulillares presentan un infiltrado de exudado gelatinoso ambarino y todo el árbol bronquial (tráquea, bronquios, y bronquiolos) presenta líquido espumoso en su interior debido al edema pulmonar agudo. Además, se pueden observar petequias y equimosis en la mucosa de la tráquea. La congestión y el edema se extienden también al mediastino, pleura y ganglios linfáticos del tórax. Se puede observar hidropericardio y petequias en el pericardio; sin embargo, las lesiones cardiacas no son frecuentes. Se puede producir ascitis en las cavidades torácica y abdominal, así como la mucosa del estómago puede observarse hiperémica y edematosa (Coetzer and Erasmus, 1994; Long and Guthrie, 2013) (Figura 6).

1.3.2. Forma cardiaca o subaguda.

Esta forma clínica de la enfermedad es la más común en caballos y mulas de las zonas donde la enfermedad es endémica, siendo la menos grave de las formas clínicas que aparecen en los caballos sin inmunizar.

La forma cardiaca se caracteriza por un periodo de incubación de 5 o 7 días, que cursa con fiebre entre los 39 ºC y 40ºC; y signos cardiovasculares. Los principales signos clínicos son el edema subcutáneo, el cual se puede extender por los párpados, tejidos faciales, cuello, tórax, pecho y en ocasiones hasta el abdomen, siendo los edemas más característicos los localizados en la fosa supraorbital, conjuntiva palpebral y espacio intermandibular (Figura 7). Además se suele acompañar de taquicardia, petequias y congestión de las mucosas explorables y equimosis en la superficie ventral de la lengua (Coetzer and Erasmus, 1994). La tasa de mortalidad se estima en el

50% de los animales infectados, ocurriendo generalmente a los 4 u 8 días de la reacción febril y puede ser precedido por un cólico y prolapso rectal si la infección intestinal es importante (Rodríguez et al., 1987; Long and Guthrie, 2013).

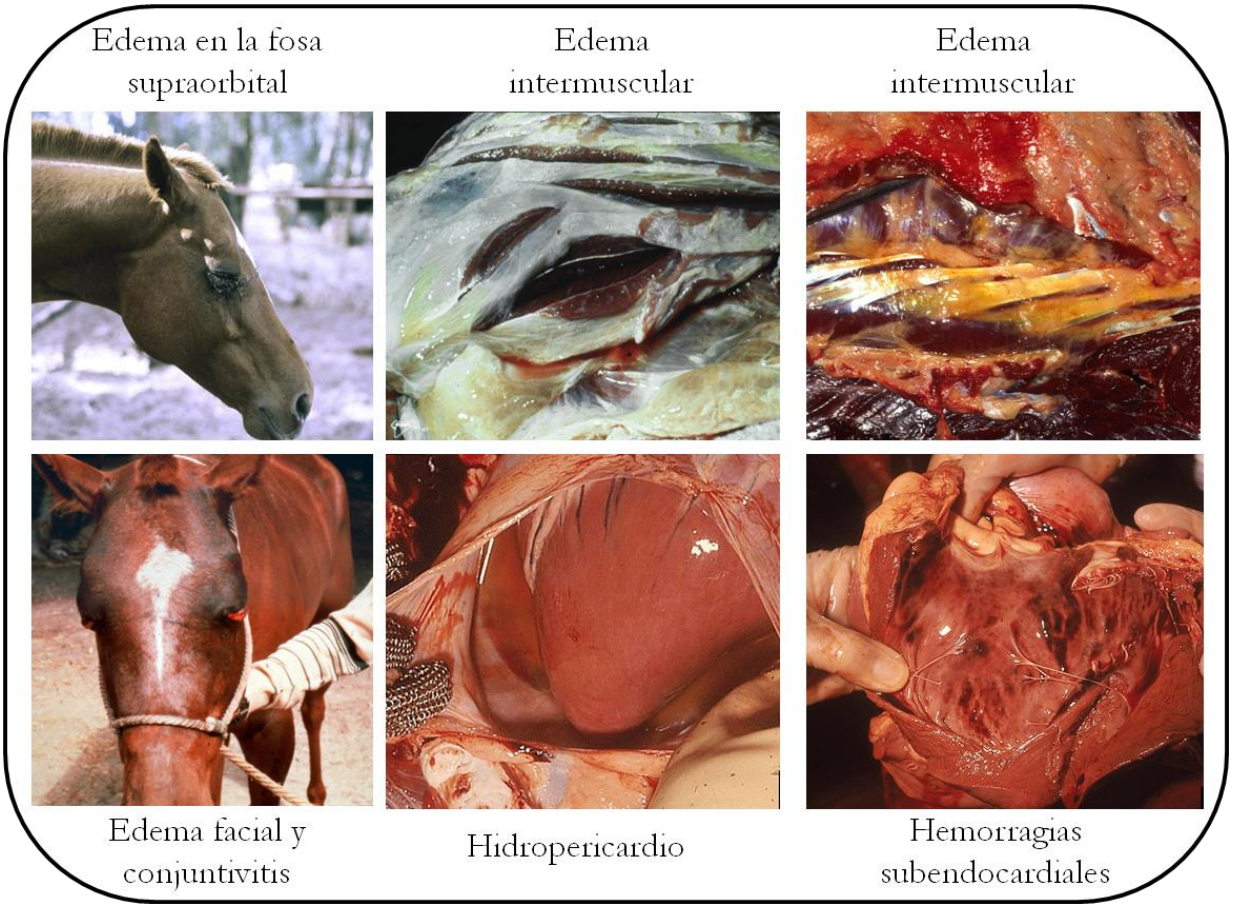

Figura 7: Signos clínicos y lesiones macroscópicas características de la forma cardiaca de la enfermedad. Composición propia. Fuente de las imágenes: APHIS –USDA.

En la necropsia de los animales con la forma cardiaca de la enfermedad, la lesión más importante es la presencia de exudado gelatinoso a nivel subcutáneo e intramuscular en los tejidos y los ganglios linfáticos, especialmente a lo largo de las venas yugulares y en el ligamento de la nuca. Se observa hidropericardio y miocarditis junto con prominentes petequias y equimosis en el epicardio y endocardio. Otras lesiones incluyen la presencia de hemorragias y/o cianosis petequiales en las superficies serosas del ciego y el colon. Al igual que en la forma pulmonar puede aparecer ascitis, pero el edema de los pulmones es leve o ausente (Coetzer and Erasmus, 1994; Long and Guthrie, 2013) (Figura 7).

1.3.3. Forma mixta o aguda.

Es la forma clínica más frecuente de los brotes de la enfermedad, y se caracteriza por una combinación de síntomas respiratorios y cardíacos en los animales afectados. Generalmente, presenta una tasa de mortalidad que excede el 70% de los animales infectados, con un periodo de incubación y curso clínico de duración intermedia. Los animales suelen morir a los 3 o 6 días de la reacción febril (Coetzer and Erasmus, 1994; Long and Guthrie, 2013).

Las lesiones características de esta forma clínica son una combinación de las lesiones de la forma pulmonar y cardiaca, aunque siempre predomina una de las dos (Rodríguez et al., 1987).

1.3.4. Forma febril

Es la forma clínica menos patógena de la enfermedad, la cual aparece en los équidos tras la infección con cepas poco virulentas del virus o en animales parcialmente inmunizados, siendo la única forma clínica que se manifiesta en especies resistentes al virus, cebras y asnos africanos (Coetzer and Erasmus, 1994).

La forma febril se caracteriza por provocar una ligera fiebre y en algunos casos edema en la fosa supraorbital. Aunque otros signos clínicos son raros, algunos animales pueden mostrar depresión, pérdida de apetito, congestión de la conjuntiva o taquicardia. Tras estos leves signos clínicos transitorios, los animales se recuperan completamente no existiendo mortalidad asociada a esta forma clínica de la enfermedad (Coetzer and Erasmus, 1994; Long and Guthrie, 2013).

1.4. Inmunología

Actualmente, los conocimientos de la respuesta inmune del hospedador a la infección con el vPEA son muy escasos. Sin embargo, la principal peculiaridad de todos los animales que sobreviven a la infección es que desarrollan una **sólida inmunidad** contra el vPEA, la cual se caracteriza por ser específica de serotipo (Burrage and Laegreid, 1994). Esta inmunidad ha sido atribuida a la producción de altos niveles de **anticuerpos neutralizantes** en el hospedador, los cuales únicamente son efectivos en cepas del mismo serotipo (Burrage and Laegreid, 1994). Apoyando esta teoría, ha sido demostrada la **transferencia de inmunidad calostral pasiva** de anticuerpos neutralizantes desde yeguas vacunadas a sus potros, así como la transferencia pasiva de anticuerpos monoclonales neutralizantes a ratones neonatales (Blackburn and Swanepoel, 1988; Burrage et al., 1993).

La respuesta serológica de anticuerpos frente a una o dos proteínas virales ha sido estudiada en varias ocasiones, aunque la respuesta de anticuerpos frente a todas las proteínas del virus sólo ha sido descrita en la publicación de Martínez-Torrecuadrada et al. (1997). En este estudio se evaluaron los anticuerpos producidos en animales infectados experimentalmente por inmunobloting, observándose que sólo eran detectables a partir del 9 dpi. Los marcadores tempranos de infección fueron los anticuerpos frente a las proteínas VP6 y NS2; y en menor extensión frente a las proteínas VP3 y NS3. Sin embargo, los anticuerpos frente a la proteína VP2 no fueron detectados mediante esta técnica y frente a la proteína VP7 fueron dependientes de la conformación, detectándose a los 15 dpi, sugiriendo que los anticuerpos frente a esta proteína no eran buenos marcadores para la detección del virus. Sin embargo, posteriormente se han detectado los anticuerpos frente a la proteína VP7 a los 7 dpi (Maree and Paweska, 2005; Sánchez-Matamoros et al., 2014a; Sánchez-Matamoros et al., 2014b).

Múltiples trabajos han determinado que los anticuerpos neutralizantes son fundamentales en la prevención de la infección por virus, sin embargo, otros estudios han demostrado la supervivencia de animales sin el desarrollo de estos anticuerpos (Martínez-Torrecuadrada et al., 1996; Romito et al., 1999; Guthrie et al., 2009). Por ello, deben existir otros mecanismos de defensa en los hospedadores que les capaciten para eliminar las infecciones virales una vez que se hayan establecido en el huésped, como en LA y otras enfermedades equinas (Slater and Hannant, 2000; Abbas et al., 2005; Maclachlan et al., 2014). Sin embargo, actualmente el rol de la **inmunidad celular** frente a la infección con el vPEA no ha sido estudiado. Únicamente podemos mencionar algunos estudios relevantes desarrollados en este campo para la LA (revisados en Maclachlan et al., 2014) y en animales vacunados contra el vPEA (Romito et al., 1999; El Garch et al., 2012; Pretorius et al., 2012).

Finalmente, uno de los mecanismos empleados para el estudio de la respuesta inmune en animales infectados es el estudio del patrón de **respuesta de citoquinas**, como se ha realizado en LA (Drew et al., 2010; Umeshappa et al., 2011; Channappanavar et al., 2012) u otras enfermedades equinas (Quinlivan et al., 2007; Hughes et al., 2011). Sin embargo, la respuesta inmune de animales infectados con el vPEA mediante el estudio de estos parámetros no ha sido analizada aún, existiendo una importante deficiencia en la comprensión de la respuesta inmune de la enfermedad.

El **objetivo 1** de esta tesis ha sido evaluar la respuesta inmune de caballos infectados con el vPEA. Concretamente, se desarrolló una técnica de biología molecular para la evaluación de la expresión de genes de algunas de las citoquinas más importantes involucradas en la respuesta

inmune innata y adaptativa en caballos. Posteriormente, esta técnica fue empleada para evaluar la respuesta inmune en animales infectados experimentalmente con el vPEA.

1.5. Diagnóstico de la enfermedad

Los signos clínicos y las lesiones macroscópicas descritas en los apartados anteriores no son patognomónicos, por ello, el análisis laboratorial es esencial para la confirmación del diagnóstico de la enfermedad, especialmente para la determinación del serotipo del vPEA. Además, es fundamental realizar un diagnóstico diferencial de aquellas enfermedades que presenten similitudes y analogías como son la encefalitis equina, anemia infecciosa equina, carbunco bacteridiano, púrpura hemorrágica, arteritis viral equina y piroplasmosis (OIE, 2013b).

A día de hoy, no hay estándares internacionales para el diagnóstico de la enfermedad. A continuación se describen las técnicas de diagnóstico más utilizadas para detectar la enfermedad, identificando aquellas que la OIE recomienda para el comercio internacional (OIE, 2013b). En el diagnósticos de la enfermedad se pueden diferenciar, por una parte, las técnicas de **diagnóstico directo** que permiten la identificación del agente patógeno o de alguno de sus constituyentes (antígenos o genoma viral), principalmente durante el periodo de viremia; y por otra, las técnicas de **diagnóstico indirecto, o técnicas serológicas,** que se basan en la detección de anticuerpos específicos, tanto de serogrupo como de serotipo. Debido al curso agudo de esta enfermedad, que puede finalizar con la muerte del animal sin que éste llegue a producir una respuesta inmune de anticuerpos detectable, especialmente en zonas no endémicas, las técnicas serológicas no se emplean para el diagnóstico inicial de un brote, aunque son técnicas de elección para controlar la evolución del brote, controlar el estatus sanitario antes de movimientos animales, evaluar la protección humoral de animales vacunados e incluso en función de las características de la vacuna diferenciar animales vacunados e infectados.

Actualmente, la técnica empleada para la detección del virus es la **reacción en cadena de la polimerasa con retrotranscriptasa inversa (RT-PCR)** por su sensibilidad, especificidad y rapidez, siendo una de las *técnicas alternativas aprobadas por la OIE para el comercio internacional.* Se han descrito varias RT-PCR convencionales, basadas en gel de agarosa, para la detección específica de ARN del vPEA de todos los serotipos (Zientara et al., 1993; Mizukoshi et al., 1994; Sakamoto et al., 1994; Stone-Marschat et al., 1994; Zientara et al., 1994, 1995a; Zientara et al., 1995b; Laviada et al., 1997; Bremer and Viljoen, 1998; Zientara et al., 1998; Aradaib et al., 2006; Rodríguez-Sánchez et al., 2008; Aradaib, 2009) o la detección específica de ARN del vPEA que permita el serotipado del virus (Sailleau et al., 2000; Maan et al., 2011). Sin embargo,

recientemente, el diagnóstico de la enfermedad ha sido mejorado mediante el desarrollo de métodos basados en la RT-PCR en tiempo real (rRT-PCR) que mejoran las características de la RT-PCR convencional permitiendo la cuantificación del ARN. Se han descrito rRT-PCR tanto específicas de serogrupo (Agüero et al., 2008; Rodríguez-Sánchez et al., 2008; Férnandez-Pinero et al., 2009; Quan et al., 2010; Monaco et al., 2011; Guthrie et al., 2013) como de serotipo (Koekemoer, 2008; Tena, 2009; Bachanek-Bankowska et al., 2014). Destacar que tanto las RT-PCR convencionales como las rRT-PCR para el serotipado de la enfermedad diseñadas en el fragmento de ARN que codifica la proteína VP2, no están exentas de cierta controversia debido a la variación génica que puede aparecer en este fragmento, lo que dificultaría el reconocimiento mediante esta técnica de todas las variantes genéticas (Koekemoer, 2008).

El **aislamiento viral** es la técnica laboratorial que permite diagnosticar la presencia activa del virus en el hospedador, siendo su principal limitación que se trata de un proceso muy lento y laborioso (House et al., 1990). Para el aislamiento del vPEA se pueden emplear diferentes métodos como son la inoculación en distintas líneas celulares (insecto o mamíferos) o intracerebral en ratón lactante (Howell, 1962; Verwoerd et al., 1979; OIE, 2013b). Tradicionalmente, este método se usa con pruebas como la fluorescencia directa e indirecta para la identificación del virus, siendo el aislamiento viral junto con la tipificación por **SNV** la prueba de referencia o "gold standard" para serotipar el virus (Koekemoer, 2008; OIE, 2013b).

Otras técnicas directas como el **enzimoinmuno ensayo (ELISA) de captura de antígeno** se usan actualmente con menor frecuencia, si bien continúan estando disponibles. Además, nuevas técnicas de diagnóstico han sido publicadas para mejorar el diagnóstico de la enfermedad, como las **sondas de hibridación** que permiten el serotipado del vPEA (Koekemoer et al., 2000; Koekemoer and Dijk, 2004) o la estandarización y validación de ensayos de detección por **inmunoperoxidasa** en tejidos (Clift et al., 2009).

Para el **diagnóstico serológico,** las técnicas aprobadas como *técnicas alternativas por la OIE para el comercio internacional* (Rubio et al., 1998; OIE, 2013b) son el **ELISA de detección de anticuerpos** y la **fijación del Complemento (FC)**, siendo la primera la que se utiliza más frecuentemente. Diferentes variaciones de la técnica han sido adaptadas para la detección de anticuerpos frente al vPEA: **ELISA de competición o bloqueo** (cELISA) (Hamblin et al., 1990; Wade-Evans et al., 1993; House et al., 1996; Kweon et al., 2003) y **ELISA indirecto** (iELISA) (Williams, 1987; House et al., 1990; Wade-Evans et al., 1993; Williams et al., 1993; Laviada et al., 1995; Kweon et al., 2003; Maree and Paweska, 2005). Ambos se caracterizan por presentar unas altas sensibilidad y especificidad, pudiendo analizar un gran número de muestras

simultáneamente, si bien, el cELISA puede emplearse en todas las especies (Figura 8), mientras que el iELISA solamente en équidos. La **FC** es una técnica inmunológica ampliamente usada en el pasado para el diagnóstico de la enfermedad, sin embargo, tras el desarrollo del ELISA ha sido reemplazada, aunque en las zonas endémicas es útil para la titulación de anticuerpos por la detección de IgM (McIntosh, 1956; Blackburn and Swanepoel, 1988) (OIE, 2013b).

Actualmente, otras técnicas como la **serotipificación por SNV** se usan con menor frecuencia, ya que únicamente se utilizan para la confirmación de los resultados del ELISA, permitiendo determinar el serotipo del vPEA responsable de la enfermedad y obtener el título de anticuerpos neutralizantes desarrollado por el animal (Hazrati and Ozawa, 1965; House et al., 1990) (Figura 8). Aunque es la única técnica que permite la detección de anticuerpos neutralizantes del virus, por lo que no presenta falsos positivos, su principal limitación es que requiere de al menos 5 días para obtener los resultados y es necesario enfrentar el suero problema a cada uno de los nueve serotipos de vPEA para poder serotiparlo. Finalmente, en los últimos años se han diseñado nuevas técnicas serológicas como el **ensayo de flujo lateral (LFA) para la detección de anticuerpos** frente al vPEA (Figura 8).

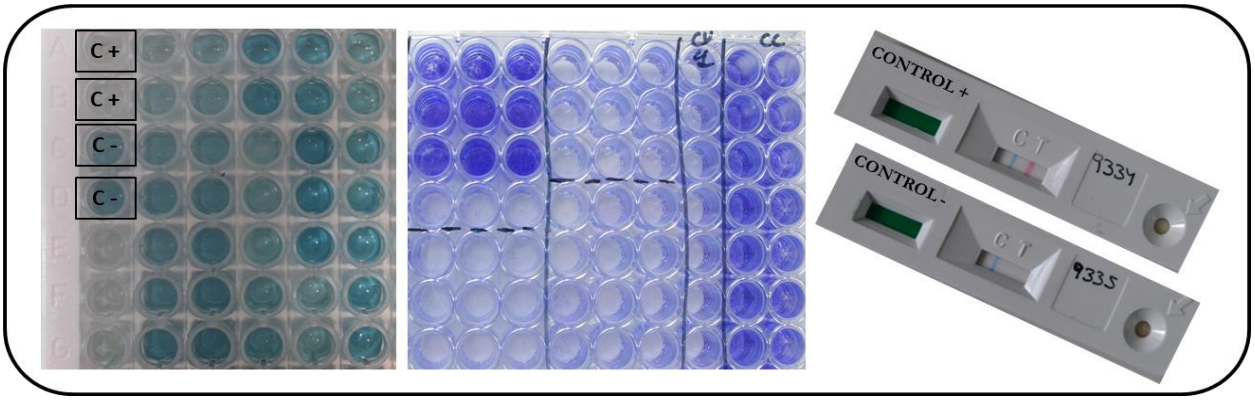

Figura 8: Diagnóstico indirecto del virus de la peste equina: cELISA (derecha), SNV (centro) y LFA para la detección de anticuerpo (izquierda). Composición propia. Fuente de las imágenes: Elaboración propia.

En el **objetivo 2** de esta tesis doctoral se han desarrollado nuevas metodologías para el diagnóstico de la enfermedad que hacen frente a los nuevos retos encaminados a alcanzar una mayor rapidez y sensibilidad de dichas técnicas, permitiendo la detección en campo de los animales infectados, el diagnóstico simultaneo de varios agentes o la diferenciación de animales vacunados de infectados.

1.6. Prevención y control de la enfermedad.

La PEA es una enfermedad incluida en la **lista de enfermedades de declaración obligatoria** tanto para la **OIE** (OIE, 2014a) como para la **UE** (2012/737/UE), por su gran poder de difusión y las graves consecuencias socio-económicas asociadas a la entrada de la enfermedad. Estas repercusiones económicas están principalmente ligadas a las restricciones comerciales y a las altas tasas de morbilidad y mortalidad de la enfermedad, no existiendo un tratamiento específico frente a la misma, únicamente paliando los signos clínicos con tratamientos sintomáticos, basados principalmente en antibióticos, antiinflamatorios y corticoides.

Para reducir estas consecuencias y ser capaces de responder más rápidamente frente a la enfermedad, la OIE, en el **capítulo de peste equina (capítulo 12.1) del Código Sanitario para los Animales Terrestres** (OIE, 2013a), provee textos normativos para garantizar un comercio internacional seguro y medidas sanitarias para la detección temprana, notificación y control de agentes; mientras que la UE ha regulado en la **legislación europea** (Directiva 92/35/CEE) las normas mínimas de control y medidas de lucha que los países miembros deben acatar.

Entre las medidas de control establecidas, es necesario remarcar que la **vacunación** de los animales susceptibles no infectados con su pertinente registro, constituye la medida más eficaz para prevenir la propagación de la enfermedad. En este aspecto, debido a la rápida difusión de la enfermedad por su caracter vectorial, la UE ha establecido la compra y almacenamiento de reservas comunitarias de vacunas contra la peste equina (2009/3/CE). Esta decisión se ha enmarcado dentro de la situación epidemiológica de la enfermedad y la susceptibilidad de los países europeos frente a la enfermedad por presentar vectores competentes de la misma y poblaciones de équidos de gran valor. Teniendo en cuenta estos factores, que actualmente todos los países miembros de la UE son países libres de la enfermedad, y la normativa de la OIE que contempla las directrices para el movimiento de équidos y recuperar la condición de libre, la UE comprará 100.000 dosis de vacunas vivas atenuadas monovalentes liofilizadas, incluidos los diluyentes necesarios, contra la peste equina de cada uno de los serotipos 1, 2, 3, 4, 6, 7 y 8.

1.6.1. Vacunación de las especies sensibles.

La vacunación es una medida de control que permite interrumpir eventualmente el ciclo de transmisión entre el animal infectado y el vector, de modo que disminuye la circulación viral.

De esta manera, se reducen al mínimo las pérdidas asociadas a la enfermedad, y sería posible la erradicación de la enfermedad a largo plazo (Sánchez-Vizcaíno, 2004). Destacar, que al no existir inmunidad cruzada entre todos los serotipos, la vacunación se debe realizar frente al serotipo o serotipos que están circulando en el momento y los animales sólo estarían protegidos frente a esass mismas variantes antigénicas.

Debido a la variedad de serotipos y las diferencias en las estrategias de control de la enfermedad entre países endémicos y países no-endémicos, se han desarrollado diferentes vacunas para combatir la enfermedad: **vacuna viva atenuada** y **vacuna inactivada**. Sin embargo, las nuevas estrategias en el desarrollo de vacunas van orientadas a la producción de vacunas que permitan realizar un diagnóstico DIVA, lo que disminuiría las restricciones al comercio internacional, y que permitan controlar varios serotipos simultáneamente. En este campo se engloban los avances desarrollados en el campo de las **vacunas de nueva generación**.

1.6.1.1. Vacuna viva atenuada.

Las vacunas atenuadas consisten en la obtención de virus con una **menor patogenicidad**, permitiendo desarrollar una respuesta inmune efectiva en el hospedador pero sin desarrollar el cuadro clínico de la enfermedad (Alexander et al., 1936). Estas vacunas fueron desarrolladas en los años 60, manteniendo su producción en Onderstepoort Biological Products (Onderstepoort, Sudáfrica), siendo actualmente la única vacuna disponible en el mercado. Estas vacunas pueden ser monovalentes (un único serotipo) o polivalentes (varios serotipos). Dentro de las polivalentes están disponibles una trivalente (serotipos 1, 3 y 4) y otra tetravalente (serotipos 2, 6, 7 y 8), los serotipos 9 y 5 no está incluidos por presentar inmunidad cruzada en la tetravalente con los serotipos presentes (Mellor and Hamblin, 2004; MacLachlan et al., 2007).

Las **principales ventajas** de estas vacunas atenuadas son su fácil uso, protección frente a múltiples serotipos y bajos costes de producción que permiten su aplicación en países endémicos (von Teichman et al., 2010). Sin embargo, los estudios de producción de anticuerpos en animales vacunados muestran una respuesta inmunológica variable de los mismos incluso tras repetidas revacunaciones, siendo una respuesta no detectable en ocasiones frente alguno de los serotipos (Crafford et al., 2013; Crafford et al., 2014). Estos estudios explican que se hayan detectado viremias en algunos animales vacunados (von Teichman et al., 2010; Weyer et al., 2013). Destacar que a pesar de existir transferencia maternal, el patrón de duración de la inmunidad calostral es variable (Crafford et al., 2013). Otras de las **principales desventajas** de la utilización de este tipo de vacunas, especialmente polivalentes, son la posibilidad de recombinación genética entre los serotipos o con cepas de campo, creando nuevas cepas víricas; o que el vector sea capaz de

replicar la cepa vacunal y difundirla entre la población no vacunada como un nuevo virus (Wenske et al., 1985; Stott et al., 1987; MacLachlan et al., 2007; Venter and Paweska, 2007; Oura et al., 2012). También se ha descrito la existencia de virulencia residual, así como la capacidad de producir viremia y efectos secundarios de la enfermedad, no siendo recomendables en yeguas en gestación por sus efectos teratógenos (Mellor and Hamblin, 2004). Aunque diversos estudios que avalan la seguridad de esta vacuna, hacen especial hincapié en seguir las recomendaciones en su aplicación (von Teichman and Smit, 2008; von Teichman et al., 2010). Finalmente, entre los posibles inconvenientes de la utilización de esta vacuna cabe destacar la imposibilidad de diferenciar animales vacunados e infectados.

A pesar de que estas potenciales limitaciones hacen complicado el establecimiento de una política de control común, actualmente, es la **principal estrategia de control** frente a la enfermedad en zonas endémicas de África habiendo conseguido reducir la prevalencia de la enfermedad, pero **no logrando la erradicación** de la misma, no siendo una vacuna adecuada para este objetivo. Sin embargo, en epidemias fuera de la zona endémica, la vacunación con vacunas atenuadas monovalentes junto con otras medidas de control ha permitido la erradicación de la enfermedad (Howell, 1960; Díaz-Montilla and Paños Marti, 1967; Portas et al., 1999).

1.6.1.2. Vacunas inactivadas.

Los inconvenientes que presentaba la utilización de las vacunas atenuadas en zonas no endémicas, desencadenaron el desarrollo de las vacunas inactivadas frente a la PEA en la **epidemia de 1987-1991** en España y Marruecos. Durante este brote, la casa comercial Merial desarrolló una vacuna inactivada frente al serotipo 4, "Equipest®", la cual demostró ser razonablemente eficaz (Dubourget et al., 1992). Sin embargo, actualmente no está disponible (House et al., 1994; Mellor and Hamblin, 2004). Estas vacunas se **caracterizan** por ser más seguras, disminuyendo las posibilidades de recombinación genética, reversión de la virulencia y sin provocar reacciones adversas. Si bien, son más caras de producir, siendo necesaria la inactivación completa del virus. Además, genera una inmunidad de aparición más lenta, requiriendo al menos dos dosis. No obstante, esta vacuna permitió la erradicación de la enfermedad en el brote de 1987-1991, pudiéndose aplicar en la época de actividad del vector sin riesgos. Posteriormente se descubrió su potencial aplicación como vacuna DIVA, ya que en los animales vacunados no se expresaban anticuerpos frente a la proteína NS3 del virus (Laviada et al., 1993; Laviada et al., 1995).

Recientemente se han publicado los resultados de la evaluación de una vacuna inactivada frente al serotipo 5 y al 9 en caballos, obteniéndose una alta protección de los animales los cuales

desarrollan altos niveles de anticuerpos neutralizantes que impiden la replicación viral (Ronchi et al., 2012; Lelli et al., 2013). Aunque actualmente, no se comercializa ninguna vacuna inactivada.

1.6.1.3. Vacunas de nueva generación.

Actualmente, los esfuerzos en el diseño de vacunas van encaminados al desarrollo de vacunas polivalentes de nueva generación que permitan la diferenciación de animales vacunados e infectados, las cuales sean adaptables a los posibles serotipos responsables de los brotes de la enfermedad. De esta manera, se podrá disponer de una medida eficaz para el control y erradicación de la enfermedad, especialmente útil en países no endémicos.

En los últimos años, se han realizado numerosos estudios sobre el desarrollo de vacunas de nueva generación frente a la PEA, aunque ninguna de ellas está disponible a nivel comercial. Estas vacunas han seguido diferentes estrategias, algunas de las más desarrolladas que han sido evaluadas en équidos son las vacunas recombinantes frente a la PEA en *Baculovirus* (Martínez-Torrecuadrada et al., 1996; Roy et al., 1996; Scanlen et al., 2002), en *Canaripox virus* (Guthrie et al., 2009; El Garch et al., 2012), la cual se encuentra patentada en la actualidad (US 8168200 B2); y en el virus modificado de Ankara (Chiam et al., 2009; Alberca et al., 2014), así como el desarrollo de vacunas de ácido desoxirribonucleico (ADN) (Stone-Marschat et al., 1996; Romito et al., 1999). Estas vacunas emplean como inmunógenos algunas de las proteínas del virus, lo que permite usar el resto de proteínas no presentes en el diseño como diferenciadores entre animales infectados y vacunados, pudiéndose realizar un diagnóstico DIVA de las mismas.

De forma general, las principales características de las vacunas de nueva generación son que son seguras y eficaces, ya que no pueden replicarse en el hospedador eliminando así el riesgo de diseminación viral y la reversión a la virulencia, pudiéndose aplicar en la época de actividad del vector. Además, provocan un inicio rápido de la respuesta inmune altamente específica proporcionando una amplia inmunidad. Finalmente, se pueden utilizar como parte de la estrategia DIVA. Sin embargo los costes de producción y desarrollo son mucho mayores que las vacunas atenuadas y monovalentes.

2. PESTE EQUINA EN ESPAÑA

Las condiciones climáticas y medio ambientales de la Península Ibérica han favorecido la introducción y difusión de la PEA en España. Dos epidemias han azotado el país, en 1966 y 1987-1990, provocando **gravísimas pérdidas para el sector equino**. La diferente situación epidemiológica, capacidad de respuesta y medidas de control establecidas frente a cada una de ellas se describen a continuación.

2.1. Evolución Epidemiológica de la Peste Equina

2.1.1 Primera aparición de la PEA en España: 1966

El primer brote sucedido en España se produjo en la **comarca Campo de Gibraltar** (municipios de Los Barrios y La Línea), provincia de Cádiz. Fue declarado a la OIE el 21 de octubre de 1966 (Díaz-Montilla and Paños Marti, 1967) (Figura 9). La epidemia fue causada por el mismo serotipo circulante en el norte de áfrica, el **serotipo 9**. Los datos oficiales publicados sobre esta epidemia en el sur de España indican una difusión escasa, estimando la muerte de 77 animales por la enfermedad con una mortalidad cercana al 100%. La última muerte asociada a este brote fue el 29 de octubre del mismo año, fecha en la que se consideró **extinguido el foco** (Díaz-Montilla and Paños Marti, 1967). La capacidad de respuesta a este brote en España se encuentra asociada al conocimiento de la situación epidemiológica de la enfermedad promovido por la OIE.

Figura 9: Comarca afectada por los brotes de peste equina y área de control de la enfermedad en España en la epidemia de 1966. Fuente: Elaboración propia con datos del diario ABC 1935. 24 de septiembre de 1967. Edición de Andalucía pág. 82.

Ante la **alarmante situación epidemiológica de la PEA en la Cuenca Mediterránea** desde 1964, la OIE promovió una reunión de urgencia, para informar a los diferentes delegados internacionales de la **situación epidemiológica** y recalcar el uso de las medidas de vigilancia y control en países colindantes (OIE, 1966). **España**, acatando las medidas dispuestas por la OIE, estableció un **efectivo sistema de prevención y vigilancia** permitiendo la rápida detección del primer foco en Campo de Gibraltar (Díaz-Montilla and Paños Marti, 1967). A pesar de la entrada de la enfermedad, las medidas de prevención establecidas fueron las adecuadas, ya que estudios epidemiológicos posteriores indican que la vía de entrada de la enfermedad fue el transporte de jejenes infectados por corrientes de aire provenientes de África (Sellers et al., 1977) y no por movimientos animales.

La detección de la enfermedad en España activó el **programa de control** para la PEA, el cual se regía por la legislación vigente, Ley de Epizootias de 1952 y Reglamento de Epizootias de 1955. La estrategia de control se basó en la vacunación obligatoria en anillo con una vacuna atenuada, el control de movimientos, la desinfección de vehículos y cuadras y el sacrificio de todos los équidos en un radio de 15 km desde el foco primario. Esta estrategia abarcó desde Castellón de la Plana hasta Málaga, Sevilla y Huelva (Rodríguez et al., 2001) (Figura 9). La rápida puesta en marcha del programa de control tras la detección de la enfermedad, permitió la **erradicación de la enfermedad** aunque supuso la muerte o sacrificio de 637 animales y la vacunación masiva de las explotaciones equinas, alrededor de 600.000 animales (Díaz-Montilla and Paños Marti, 1967).

2.1.2. Desde Namibia a España: 1987 - 1991

Tras 21 años sin declararse ningún foco de PEA en la Cuenca Mediterránea, la PEA se consideraba una enfermedad exótica recluida a la zona endémica de la enfermedad. Por ello, en España, los veterinarios carecían de la actualización en el conocimiento de la enfermedad, los laboratorios nacionales no disponían de los recursos necesarios para el diagnóstico laboratorial de la misma, y para el control de la enfermedad únicamente estaban disponibles vacunas atenuadas, con las desventajas que tiene su uso. En esta situación, España se enfrentó a una nueva epidemia de la enfermedad, cuyo control era fundamental especialmente por la futura celebración de los Juegos Olímpicos de Barcelona'92.

2.1.2.1 Nuevo brote de la enfermedad: 1987

El primer foco de la enfermedad se observó el 5 de agosto de 1987 en el safari park "El Rincón" localizado en el **municipio de Aldea del Fresno**, provincia de Madrid (BOCG, 1987).

A pesar de ello, el veterinario de la explotación tras desacatar un proceso de intoxicación y con la colaboración de la Cátedra de Anatomía Patológica de la Facultad de Veterinaria de Madrid (UCM), no informó hasta principios de septiembre de la sospecha de un proceso infecto-contagioso, probablemente PEA, a los Servicios de Sanidad Animal del Ministerio de Agricultura y a los de la Comunidad Autónoma de Madrid. Ante esta situación, el 9 de septiembre, la Subdirección General de Sanidad Animal solicita a los Servicios de Sanidad Animal de Francia los antígenos necesarios para realizar un diagnóstico laboratorial preciso. El 12 de septiembre se produce la **confirmación laboratorial del vPEA** en los animales infectados, notificándose el 14 de septiembre a los servicios sanitarios de la UE y OIE (OIE, 1988; Rodríguez et al., 2001). El **serotipo 4** fue aislado e identificado como responsable de la enfermedad en una serie de explotaciones localizadas en el suroeste de la Comunidad Autónoma de Madrid (municipios de Aldea del Fresno, Villamanta, San Martín de Valdeiglesias y Quijorna) y en la zona limítrofe de la provincia de Toledo (Alrnodóvar, Escalona y Quismondo) y también una pequeña zona de la provincia de Ávila, entre julio y septiembre de 1987 (BOCG, 1987) (Figura 10).

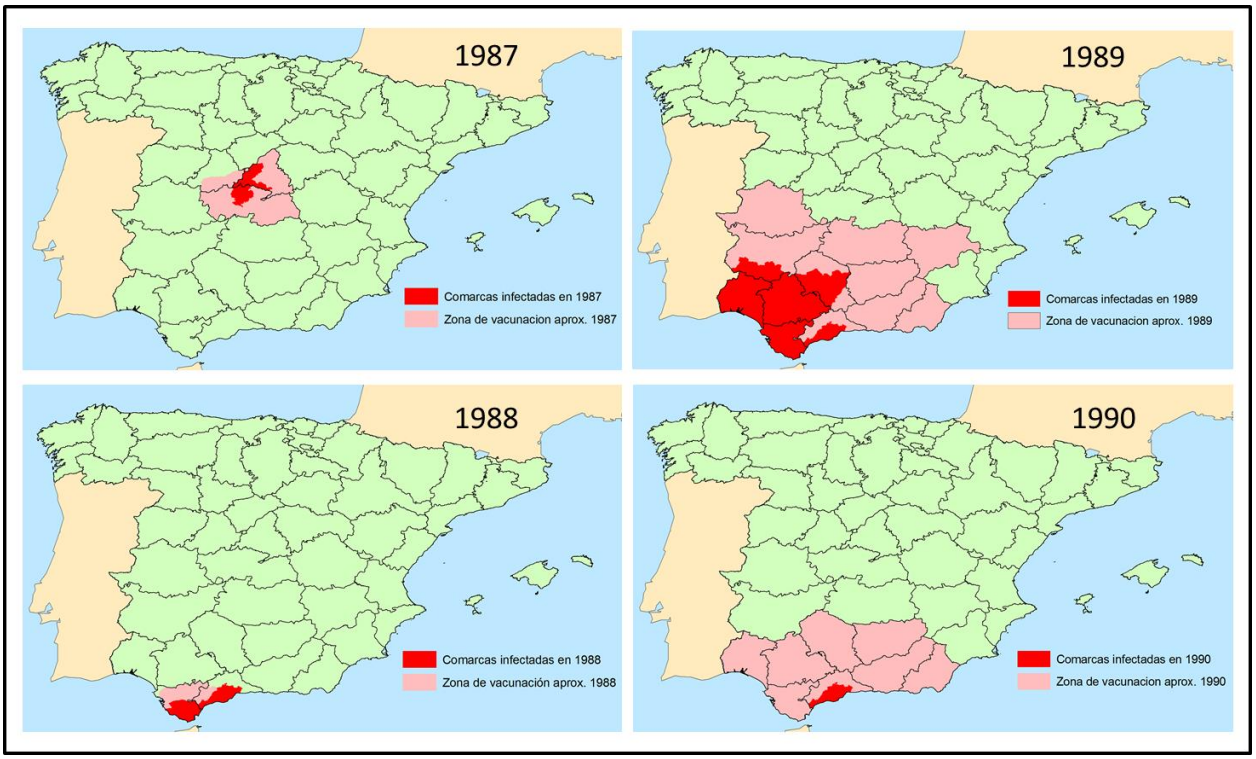

Figura 10: Comarcas afectadas por los brotes de peste equina declarados y zona de seguridad de la enfermedad en España en la epidemia de 1987 (derecha-arriba), 1988 (derecha-bajo), 1989 (izquierda-arriba) y 1990 (izquierda-bajo). Composición propia. Fuente de las imágenes: Elaboración propia con datos proporcionados por la Dra. María Castaño (Dpto. Patología Animal, Facultad de Veterinaria, UCM)

Tras la identificación del vPEA como el agente responsable de las muertes, se realizaron los pertinentes **estudios epidemiológicos** para identificar su origen y se activaron las **medidas de control** de la enfermedad. Para llevar a cabo estas dos tareas se contó con el trascendental apoyo internacional de la OIE y la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO), así como la colaboración del Prof. Erasmus, máximo experto mundial de su tiempo de la PEA (BOCG, 1987).

La **introducción de la PEA** en las inmediaciones de Madrid cuando la distribución de la enfermedad en aquel tiempo estaba limitada a la zona endémica, no habiéndose declarado ningún brote en los países del norte de África, sólo permitía pensar en alguna causa exógena. En esa tesitura, se identificó que el safari en Madrid y el safari de Aitana en Alicante habían importado cebras y asnos africanos provenientes de Namibia por vía marítima, llegando desde Lisboa a Madrid y Alicante (Rodríguez et al., 1992; Sánchez-Vizcaíno, 2004). La ausencia de sintomatología clínica en estos animales favoreció que no fueran identificados en las cuarentenas realizadas, llegando a Madrid y Alicante. Sin embargo, el diagnóstico laboratorial reveló que estos animales eran portadores del virus, siendo sacrificados el 24 de septiembre. Es importante destacar otro evento de confusión en el estudio epidemiológico del origen de la enfermedad, como fue la ausencia de animales enfermos en Alicante. Estudios posteriores asociados al brote de LA en las Islas Baleares han resuelto esta cuestión, ya que no se observa la presencia de *Culicoides* en este área lo que impide la difusión de la enfermedad, remarcando el importante rol de los vectores en la transmisión de los orbivirus (Sánchez-Vizcaíno, 2004).

El **programa de control** consistió en la inmovilización del ganado equino en toda España, la prohibición de concentraciones del mismo (ferias, concursos hípicos, etc.), el secuestro de los équidos en la zona infecta y sospechosa, las desinsectaciones intensas y continuadas, la profilaxis vacunal, el aislamiento de focos y la eliminación de animales enfermos y sospechosos, así como el control de vectores en las zonas afectadas y vigilancia serológica (BOCG, 1987; OIE, 1988). La vacunación, con vacuna polivalente suministrada por el Veterinary Research Institute de Onderstepoort (República Sudafricana), de todos los equinos de la provincia de Madrid y Toledo y de las zonas limítrofes de la provincia de Ávila fue aceptada con controversia entre los ganaderos por las repercusiones que podría acarrearles en el comercio internacional (BOCG, 1987). Además, se creó una mesa de expertos de trabajo permanente para tomar las decisiones pertinentes. Tras la muerte de 146 animales y la vacunación de 38.000 équidos con una vacuna atenuada polivalente, España declaró extinguido el brote de PEA en diciembre de 1987 (OIE, 1988; Rodríguez et al., 1992).

2.1.2.2 De Madrid a Andalucía en 1988

Casi un año después, en octubre de 1988, comienzan a morir caballos en los **municipios de Vejer de la Frontera, Benalup de Sidonia, Casares y San Roque** en las provincias de Cádiz y Málaga, a unos 600 km de los brotes de 1987 (Figura 10). Declarándose oficialmente el 18 de octubre de 1988 un nuevo brote de PEA del serotipo 4 (BOJA, 1988). Diferentes hipótesis intentan explicar la epidemiología de este brote, aunque ninguna de ellas ha sido verificada. Una de las opciones más convincentes es el silencio epizoótico de la enfermedad durante el invierno asociado a un supuesto **movimiento ilegal de animales** infectados de Madrid a Cádiz (ABC, 1989; Rodríguez et al., 1991; Sánchez-Vizcaíno, 2004). Tres focos primarios aparecieron simultáneamente en 1988, que causaron la muerte o sacrificio de alrededor de 150 animales (Sánchez-Vizcaíno, 2004).

La **estrategia de control** que se aplicó fue similar a la adoptada en el brote de 1987, aunque mejorando aspectos de la campaña de vacunación, el control de animales muertos, la monitorización de vectores y la implementación de animales centinelas en áreas afectadas (Rico Mansilla, 1990). La vacunación se aplicó en dos etapas, antes del conocimiento del serotipo responsable de la enfermedad se vacunaron 8.204 y 8.735 caballos en Cádiz y Málaga, respectivamente, con una vacuna atenuada polivalente. Tras el serotipado del virus, se aplicó una vacuna atenuada monovalente a 17.829 caballos en Cádiz (Sánchez-Vizcaíno, 2004). La monitorización de vectores permitió identificar a la especie *C. immicola* como principal vector de la enfermedad en España tras aislar el virus en culicoides y caballos de las mismas zonas, así como la identificación de otras especies capaces de actuar como vectores como *C. obsoletus* y *C. pulicaris* (Mellor et al., 1990). El uso de animales centinelas permitió una mejor monitorización del estatus sanitario de las zonas afectadas. En abril de 1989, la Junta de Andalucía consideró erradicado el brote tras la ausencia de muertes causadas por la enfermedad y la ausencia de virus en la población de vectores tras el 8 de diciembre de 1988 (BOJA, 1989).

2.1.2.3 Recrudescencia en 1989

La enfermedad vuelve a reaparecer en a finales de julio de 1989 con un caballo muerto tras presentar síntomas de PEA en el **municipio de San Roque**, provincia de Cádiz, siendo confirmado el diagnóstico del **serotipo 4 del vPEA** por el laboratorio nacional de Algete. Posteriormente se informa de la situación a todas las Comunidades Autónomas (CC.AA) y se activa el nuevo **programa de control** de la enfermedad modificado según la normativa de 18 de marzo de 1989 (BOE, 18-03-89). Entre las mejoras del nuevo programa de control se incluye la zonificación del territorio, diferenciándose la zona afectada, la zona de seguridad y la zona libre.

En el primer momento del brote de 1989, la zona afectada integró 16 municipios de la provincia de Cádiz y 5 de Málaga mientras que la zona de seguridad comprendía el resto de municipios de Cádiz, 2 de Huelva, 41 de Málaga y 7 de Sevilla. La campaña de vacunación se inició inmediatamente tras la confirmación de la enfermedad, aplicando una vacuna atenuada monovalente inmediatamente en los 10 km alrededor del foco. Además, se prohibió el movimiento equino en Andalucía y se restringió en el resto de España (Rico Mansilla, 1990).

Estas medidas no fueron suficientes para controlar la difusión inicial de la enfermedad, ya que nuevos focos secundarios se detectaron en municipios de Cádiz, Sevilla, Huelva, Córdoba y Badajoz; llegando a difundirse la enfermedad a Portugal y Marruecos (Rodríguez et al., 1992) (Figura 10). Finalmente, para controlar la enfermedad se necesitó **vacunar a toda la Comunidad Autónoma** de Andalucía, **Extremadura y dos provincias de Castilla-La Mancha**, vacunando alrededor de 250.000 équidos (OIE, 1990; Rodríguez et al., 1991) (Figura 10). Además, este brote fue el peor de los brotes ocurridos en España, muriendo alrededor de 1.000 équidos (OIE, 1990). El 98% de animales muertos provenían de Sevilla, Huelva y Córdoba, áreas donde no se habían practicado vacunaciones previas (Sánchez-Vizcaíno, 2004). La epidemia se logró controlar a finales de año, declarándose la **erradicación oficial** en enero de 1990 (Rodríguez et al., 1992).

2.1.2.4 Un brote peculiar: 1990

Tras 9 meses, en septiembre de 1990, un nuevo brote de PEA fue confirmado en los **municipios de Alora, Cártama, Pizarra y Marbella**, provincia de Málaga (BOJA, 1990). Este nuevo brote se limitó a la provincia de Málaga causando 66 muertes hasta noviembre de ese mismo año (OIE, 1991; Rodríguez et al., 1991) (Figura 10). La peculiaridad de estas muertes fue que únicamente se produjeron en animales jóvenes, sin vacunar, recientemente vacunados o con baja protección calostral. Por ello, en esta situación de "semi-control" de la enfermedad se realizó un refuerzo del programa de erradicación de la enfermedad. Este principalmente consistía en la vacunación y marcaje de toda la población equina de Andalucía. Unos 250.000 equinos fueron vacunados hasta julio de 1991, aunque durante 1992 se mantuvo la vacunación de equinos nacidos ese mismo año (MAGRAMA, 1992). También, se incrementó la vigilancia, el uso de animales centinelas y el control de vectores (Sánchez-Vizcaíno, 2004). La enfermedad fue **declarada extinguida** el 13 de diciembre de 1990, 53 días después del último diagnóstico de la enfermedad en un caballo infectado (OIE, 1991).

2.1.2.5 Reflexiones finales de la epizootia 1987-1990

España fue declarada libre de la enfermedad el 1 de diciembre de 1993, transcurrido un año desde la última campaña de vacunación, y sin que se hubiesen detectado en dos años indicios clínicos, serológicos y epidemiológicos de la enfermedad (RD 1347/1992; 93/616/CE). La epidemia que azotó a España de 1987 a 1990 tuvo **dramáticas consecuencias socio-económicas** para la producción equina española, llegando a peligrar las competiciones hípicas de los Juegos Olímpicos de Barcelona'92.

Dos **escenarios epidemiológicos** fueron observados en los brotes de 1987 a 1990, la zona **centro y Andalucía**. En ambos escenarios se establecieron programas similares para el control y erradicación de la enfermedad, sin embargo, en Andalucía la enfermedad re-apareció tres años consecutivos necesitando mayores esfuerzos para conseguir la erradicación de la misma. Varios factores pudieron influenciar la **persistencia del virus** en esta zona de España (Sánchez-Vizcaíno, 2004). Uno de ellos fueron las **adecuadas condiciones climáticas** de la región, que permitirían la supervivencia del vector durante todo el año, a diferencia de la zona centro, donde las temperaturas no son tan óptimas (Rodríguez et al., 1992; Sánchez-Vizcaíno, 2004). Este hecho pudo estar asociado con una falta de reconocimiento de la enfermedad en los meses de invierno, o con el posible rol de los asnos como reservorios de la enfermedad, detectándose únicamente la enfermedad durante los meses de mayor actividad del vector (Rodríguez et al., 1992). Otro factor determinante pudieron ser las dificultades encontradas al llevar a cabo la **campaña de vacunación**, que impidieron una protección vacunal completa de la cabaña ganadera (Rodríguez et al., 1992). Estas dificultades fueron el difícil control de las poblaciones de caballos salvajes y la falta de cooperación de los propietarios equinos por las posibles repercusiones comerciales que les podía ocasionar. Además, la reaparición de la enfermedad en 1989 y 1990 en zonas vacunadas previamente, permitió predecir que estaba existiendo **replicación viral en animales vacunados** probablemente asociado a títulos bajos de anticuerpos neutralizantes. Finalmente, la aplicación de vacunaciones y revacunaciones a toda la cabaña equina de Andalucía y la zona centro, convirtiéndolas en población resistente al virus, permitió la **erradicación de la enfermedad** (Sánchez-Vizcaíno, 2004).

Los caballos, los équidos predominantes en España, son la especie más susceptible al vPEA. Por ello pudo ser que esta epidemia cursará con una **alta tasa de mortalidad** en el país (Rico Mansilla, 1990). Los caballos se caracterizaron por mostrar predominantemente la forma clínica mixta de la enfermedad predominando los daños pulmonares en 1987, 1989 y 1990; y los

signos cardiacos en 1988. Reseñar que también se observó la forma respiratoria y cardiaca en algunos casos (Rodríguez et al., 1987).

La aparición de la PEA en España promovió la **adecuación y actualización de la legislación** referente a la enfermedad, tanto a nivel nacional como internacional (Rodríguez et al., 1991). La legislación española de 1966 referente a PEA, fue modificada para mejorar la prevención, erradicación y control de la misma durante la epizootia de 1987-1990 (BOE, 18/03/1989; BOE 30/12/1989; BOE 04/08/1990). Además, a posteriori ha sido creado el Plan de Alerta contra la PEA en España en el que se establecen las normas de control y medidas de lucha contra la enfermedad (BOE 03/06/1993). En el ámbito internacional, la OIE revisó el capítulo de PEA del Código Sanitario Internacional incluyendo nuevos conceptos como la regionalización y el periodo de actividad del vector, así como los requisitos para considerar una zona libre de la enfermedad y los requisitos para el movimiento de animales (Rodríguez et al., 1991). La UE estableció la regulación pertinente para el movimiento de équidos en países miembros y no miembros de la UE, la delimitación del territorio y sus requisitos en caso de aparecer un brote de PEA (EEC 90/426). El desarrollo de esta legislación parece fundamental cuando el origen del foco se debe a la importación de equinos y cuando una de las teorías más factible en la difusión del virus de Madrid a Andalucía fue el movimiento de caballos.

Las **repercusiones** más importantes de la entrada de la enfermedad en España fueron: miles de animales muertos o sacrificados, muchos de ellos con un gran valor genético y económico; la implementación de restrictivas medidas de control que impedían el libre desplazamiento de animales y las masivas campañas de vacunación que tuvieron que realizarse para lograr controlar y erradicar la enfermedad. Andalucía era y es una región ampliamente ligada a la producción equina, por ello, las medidas de control establecidas influenciaron la vida cotidiana de los andaluces, entre ellos numerosos festejos ligados al caballo como "El Rocío" o la "Feria de Abril".

2.1.3. Situación actual de la PEA en España

Tras la **erradicación de la enfermedad en 1993** ningún brote de PEA ha sido declarado en España. Actualmente, la PEA en España se considera una enfermedad exótica aunque con carácter re-emergente que podría ocasionar fatídicas consecuencias socio-económicas para la producción equina española. En consecuencia, el Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA) ha establecido diferentes **estrategias para prevenir y controlar la enfermedad**.

2.2. Medidas de Prevención y Control frente a la Peste Equina en España

La legislación española reconoce a la PEA como una **enfermedad de declaración obligatoria** (526/2014), y determina las **medidas de prevención y control** que se deben implantar para evitar la entrada y difusión de esta enfermedad en España, las cuales siguen las directrices de la legislación europea y la normativa internacional (OIE) en materia de sanidad animal. Además, en conjunto con estas medidas, el MAGRAMA y las CC.AA mejoran la formación y evalúan a los servicios veterinarios oficiales y privados en materia de respuesta frente a esta enfermedad, para que estén prevenidos. En este marco podemos encontrar diferentes **cursos** y la realización de **simulacros reales y vía internet**.

2.2.1. Medidas de prevención contra la enfermedad: Análisis de riesgo y sistema de vigilancia.

Las medidas de prevención contra la PEA en España están establecidas en base al **análisis de riesgo de entrada** de la misma y la implantación de un **sistema de vigilancia** acorde.

2.2.1.1. Análisis de riesgo de la enfermedad

El análisis de cada una de las posibles vías de entrada de la enfermedad (ver Capítulo I, Sección 1.2.4) en España, nos permite identificar un mayor riesgo en la **introducción de vectores infectados en corrientes de aire** y en la **importación de animales o vectores infectados**. Actualmente, esta información junto con la distribución epidemiológica histórica de la enfermedad, permite identificar que la llegada accidental de vectores infectados desde el norte de África es escasa, presentando un mayor riesgo las importaciones animales (MAGRAMA, 2013).

2.2.1.2. Sistema de vigilancia actual

La implantación del sistema de vigilancia actual se fundamenta en el **análisis de riesgo**. Por ello, las medidas preventivas para evitar la entrada de la PEA en España son los controles sanitarios en los puestos de inspección fronteriza (MAGRAMA, 2013). Al mismo tiempo, se lleva a cabo un plan de vigilancia específica frente a la PEA con dos componentes, vigilancia activa y pasiva; el cual se complementa con un programa de vigilancia entomológica común para las enfermedades vectoriales (MAGRAMA, 2014b).

El **plan de vigilancia pasivo** se basa en la observación clínica de los animales que permita sospechar de la aparición de la enfermedad. Este programa se llevará a cabo a lo largo de

todo el año y en el que deben participar todas las personas responsables de explotaciones equinas del territorio nacional.

El **plan de vigilancia activa** consiste en muestreos serológicos en explotaciones centinelas de équidos que permitan detectar la evidencia de circulación vírica. Estas explotaciones se situarán en la zona de mayor riesgo de entrada, franja litoral de Andalucía y en Canarias, realizándose al principio y final de la época de actividad del vector.

El **programa de vigilancia entomológica** se engloba dentro del marco del Programa Nacional de Vigilancia Entomológica de la LA. Su objetivo es identificar las distribución y los periodos de actividad de los vectores de PEA y LA.

2.2.2. Medidas de lucha contra la enfermedad: Control y erradicación.

Todas las actuaciones de control que se deben implantar en caso de re-introducción de la enfermedad en España se encuentran recogidas en el **Manual Práctico de Operaciones en la Lucha Contra la Peste Equina Africana** (PEA) (MAGRAMA, 2013). Estas medidas se utilizan en conjunto con el **Plan Coordinado Estatal de Alerta Sanitaria Veterinaria** (MAGRAMA, 2004) y la **normativa de Sanidad y Bienestar Animal** (R.D. 804/2011). De forma general, la lucha contra la PEA está basada en los siguientes puntos:

1. Rápida notificación obligatoria de los casos o sospechas.
2. Restricción de movimientos de las zonas afectadas.
3. Establecimiento de áreas de protección y vigilancia a 100 y 150 km. respectivamente.
4. Control de vectores mediante el confinamiento de los animales, especialmente durante las horas de máxima actividad de los vectores, y el uso de desinsectantes y repelentes en las naves, alojamientos e incluso en los propios animales.
5. Investigaciones clínicas, epidemiológicas, serológicas y entomológicas para conocer el posible alcance de la enfermedad.
6. Programas de vacunación sistemática de todos los équidos presentes en las explotaciones incluidas en la zona de protección.

Destacar que entre las medidas de control establecidas no está incluido el sacrificio de todos los animales de especies sensibles de la explotación, pudiendo autorizarse en algunos casos para reducir la carga viral del medio o por motivos de bienestar animal.

Para aplicar la normativa de control y erradicación de la enfermedad conviene definir cuándo se sospecha y se confirma un foco de PEA, ya que las medidas de control se deben

establecer desde el momento de la sospecha de la enfermedad. La legislación establece que se considera una **sospecha de PEA** si: se observa clínica o patología compatible con la enfermedad en los animales; existen indicios epidemiológicos; o resultados en pruebas serológicas. Esta sospecha es **confirmada** si se observa un animal que cumple algunos de los siguientes requisitos: presenta un cuadro clínico atribuible a la presencia de la PEA; o ha dado resultados positivos en las pruebas serológicas; o ha dado positivo a la detección de antígenos o ARN viral a uno o varios serotipos del virus; o cuando se haya podido aislar e identificar el vPEA en un animal. Estos datos deben ir acompañados de estudios epidemiológicos que confirmen la circulación del virus en la explotación de la que procede el animal, descartando la hipótesis de animales vacunados previamente (MAGRAMA, 2013).

En el **objetivo 3** de esta tesis ha sido realizado un análisis epidemiológico de la PEA en España mediante el análisis de los factores epidemiológicos de la enfermedad, así como los movimientos de animales y la difusión de la enfermedad.

3. IMPORTANCIA ECONÓMICA DE LA PESTE EQUINA EN ESPAÑA

La entrada de la PEA en un país acarrea consecuencias dramáticas para su producción equina, asociadas a las restricciones internacionales impuestas, así como por la muerte de los animales y la implementación de las medidas de control y erradicación. Por ello, para evaluar la importancia económica de la enfermedad en España, es fundamental conocer la situación actual del sector equino.

Actualmente el sector equino es de gran relevancia en la producción ganadera española cuyo impacto económico ha sido estimado en más de 5.300 millones de euros en 2012 (Deloitte, 2013). Se trata de un sector singular, pues su demanda no es sólo el consumo de carne sino también actividades de competición; y de recreo y ocio. España se caracteriza por tener el segundo mayor censo equino en Europa tras Rusia (OIE, 2013c), con 629.536 animales los cuales se distribuyen en 184.523 explotaciones a julio de 2014 (MAGRAMA, 2014a). Estas explotaciones se agrupan en las distintas clasificaciones zootécnicas de los équidos (Figura 11), estando censados 219.997 caballos por las asociaciones de pura raza y existiendo 1664 caballos autorizados para participar en competiciones internacionales (Deloitte, 2013).

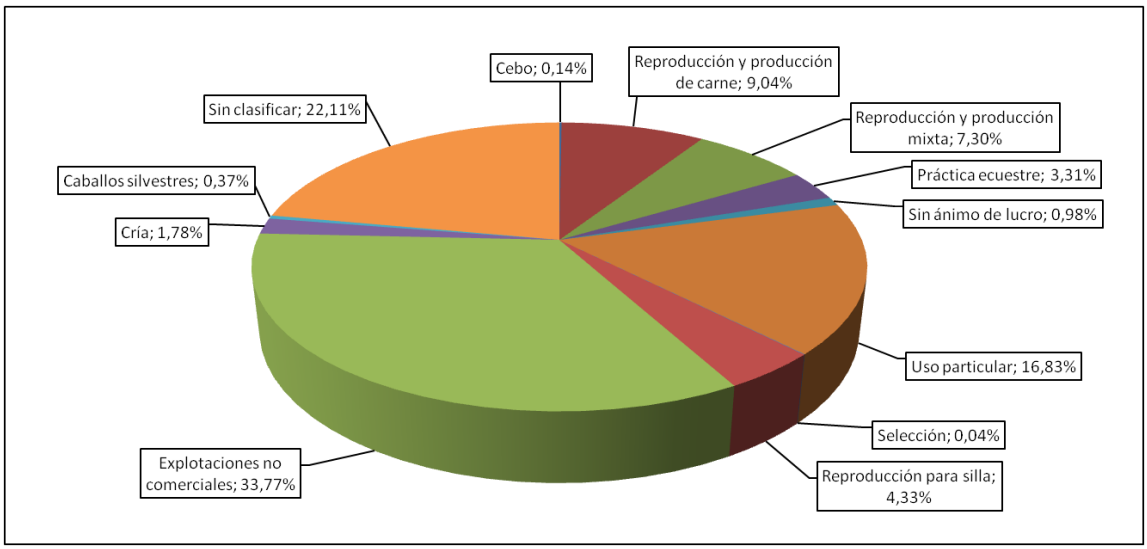

Figura 11: Distribución de las explotaciones de ganado equino según la clasificación zootécnica a junio de 2014. Fuente: Elaboración propia con los datos del MAGRAMA: El sector equino en cifras. Principales indicadores económicos en 2013.

Las principales pérdidas económicas ocasionadas por este patógeno se asocian con la alta tasa de mortalidad que provoca en los caballos no inmunizados, entre el 75 – 90%. Este hecho

no tiene importancia únicamente por el valor económico del caballo, aunque en España existen équidos de razas selectas, sino que además, los caballos se caracterizan por tener un importante valor sentimental, incalculable para muchos de sus propietarios. Por ello, sólo las consecuencias socio-económicas de la mortalidad asociada a la enfermedad en España serían devastadoras.

Estas pérdidas económicas se ven agravadas por el hecho de ser una enfermedad de declaración obligatoria para la OIE y la UE, lo que provoca la restricción de los movimientos nacionales e internacionales de estos animales, así como las concentraciones de los mismos. La peculiaridad de este sector, en el que encontramos una rama del mismo dedicada a las competiciones internacionales, junto con el carácter exportador de la producción equina destinada a la carne, hace que esta medida de control suponga un duro golpe para el sector ecuestre de España. Además, España se caracteriza por realizar numerosas competiciones ecuestres, de las cuales 411 son de ámbito nacional y 67 de ámbito internacional, y festejos populares asociadas a la tradición equina tales como la fiesta de San Joan en Menorca o "El Rocío" en Huelva, por lo que la restricción de los mismos acarrearía la pérdidas de millones de euros y supondría un duro golpe a la actividad deportiva y sociocultural de gran importancia para España (Deloitte, 2013).

Debido a las gravísimas consecuencias que tiene la difusión de la enfermedad, es fundamental implantar un adecuado programa de control y erradicación en el momento que la introducción de la misma es detectada. De este modo, a pesar de que se incrementen los costes, se logrará la erradicación de la enfermedad ya que no erradicar la PEA supondría consecuencias mucho más negativas para España.

Finalmente, recalcar que España nunca ha evaluado los costes asociados a las epidemias de 1966 y 1987-1990, aunque los datos de mortalidad y sacrificio junto con las medidas establecidas para conseguir la erradicación de la enfermedad, permiten suponer los altos costes socio-económicos que causó al país.

En el **objetivo 4** ha sido desarrollado un modelo económico para evaluar los costes que la entrada de la enfermedad ocasionaría en España actualmente.

CAPÍTULO II

JUSTIFICACIÓN Y OBJETIVOS

Fuente de la imagen: Ferran Pestaña, Caballos y garcillas bueyeras 03 [CC BY-SA 2.0]

La PEA es una enfermedad de declaración obligatoria para la OIE, debido a su alta patogenicidad y gran poder de difusión que provoca consecuencias socio-económicas devastadoras en los países que la padecen. La situación actual de la enfermedad, endémica en el continente africano (ver sección 1.2.3) y asociado al incremento de densidad y distribución de vectores competentes en la UE, probablemente como consecuencia de fenómenos ambientales como el calentamiento global (Gould and Higgs, 2009; Gale et al., 2010), hacen que esta enfermedad sea considerada una enfermedad re-emergente en Europa (Maclachlan and Guthrie, 2010). Esta hipótesis ha sido confirmada con la re-emergencia de varias enfermedades vectoriales como la LA, Schmallenberg y West Nile. De entre todos ellos, y por la semejanza con la PEA, destacan los brotes de LA, especialmente tras la epidemia del serotipo 8 en el año 2006, el cual se expandió por el norte de Europa causando graves pérdidas económicas, redefiniendo el rango de expansión de la enfermedad del paralelo 40ºN al paralelo 53ºN, y descubriendo a nuevas especies de jejenes competentes (Toussaint et al., 2007; De Deken et al., 2008; Carpenter et al., 2009).

España desempeña un papel de gran relevancia en la epidemiología de esta enfermedad. Por su localización y climatología, podría ser una de las principales vías de entrada de la enfermedad en Europa. Además, España se caracteriza por tener una importante cabaña equina y una abundante distribución de vectores competentes, los cuales han permitido la difusión de la enfermedad en las dos epidemias previas en nuestro país (1966 y 1987-1990) provocando importantes pérdidas económicas. Por todo ello, el carácter re-emergente de la enfermedad ha hecho saltar todas las alarmas del sector equino español y europeo ante las graves consecuencias que una posible re-introducción de PEA podría ocasionar.

Objetivo general

El objetivo de esta tesis doctoral engloba aspectos inmunológicos, de diagnóstico laboratorial, epidemiológicos y económicos en el estudio de la peste equina. De esta manera esta tesis doctoral pretende abarcar desde la comprensión de la respuesta inmune desarrollada tras la infección, hasta el conocimiento de la importancia económica de la difusión de la enfermedad en España. De esta forma se pretende dotar al sector equino de unas herramientas y conocimientos que permitan un mayor dominio de los distintos fundamentos de la enfermedad que sean de gran utilidad para la prevención y el control de la peste equina.

Objetivos concretos

OBJETIVO 1: Evaluar la **respuesta inmune generada en caballos infectados con el virus de la peste equina**. Para ello, se ha desarrollado un panel de rRT-PCRs para la cuantificación de la expresión de citoquinas en caballos, el cual ha permitido el estudio *in vivo* de la respuesta inmune generada frente al vPEA.

OBJETIVO 2: Desarrollo de **nuevas técnicas de diagnóstico para la peste equina** que permitan afrontar los nuevos retos del diagnóstico de la enfermedad. Concretamente, se han desarrollado dos técnicas serológicas usando la **tecnología Luminex®**, centrando el estudio en el ensayo frente a la VP7 del vPEA, y una posterior adaptación a la evaluación múltiple de anticuerpos frente a las proteínas VP7 y NS3 para un diagnóstico DIVA de la enfermedad cuando se usan vacunas inactivadas.

OBJETIVO 3: Análisis epidemiológico de la peste equina en España mediante la determinación de las áreas de mayor riesgo y el estudio de los movimientos equinos en el territorio.

OBJETIVO 4: Evaluación económica de una epidemia de la peste equina en España. En este estudio se analizarán las consecuencias económicas de un brote de PEA comparándose diferentes estrategias de control.

CAPÍTULO III

RESULTADOS

Fuente de la imagen: Cedidas por Fundación Real Escuela Andaluza Del Arte Ecuestre.

OBJETIVO 1: Evaluación de la respuesta inmune del hospedador frente al virus de la peste equina

El principal mecanismo de defensa del hospedador frente a los patógenos externos es la **respuesta inmune**. El conocimiento de la respuesta inmune del hospedador frente a los agentes infecciosos permite establecer los **marcadores inmunológicos** involucrados en la **respuesta inmune protectora.** En el caso de la PEA, desconocemos muchos aspectos de la respuesta inmune protectora del hospedador frente al virus. Por ello, el **objetivo 1** de esta tesis doctoral consiste en la evaluación de la respuesta inmune generada en caballos infectados experimentalmente con el vPEA, de forma que se puedan identificar marcadores inmunológicos que permitan el desarrollo de nuevos **tratamientos terapéuticos** o **vacunas.** Para ello, se evaluaron los patrones de expresión de varias citoquinas involucradas en la respuesta inmune, así como la producción de anticuerpos en animales infectados experimentalmente y se relacionó con las formas clínicas de la enfermedad en animales.

Con el objetivo de **cuantificar la expression de citoquinas en caballos**, se desarrolló y optimizó un panel de rRT-PCRs con SYBR Green para ocho citoquinas distintas y para un gen de expression constant (B-actina). La seleccion de las citoquinas incluidas se basó en su implicación en la respuesta immune innata y en la adaptativa. Estas rRT-PCR permitieron la detección y amplificación de todas las citoquinas en un programa de termociclador común, con la misma temperatura de alineamiento. Posteriormente, fueron validadas con el empleo de ARN mensajero (ARNm) extraido de linfocitos estimulados con mitógenos y con muestras de sangre de animales infectados. La alta eficiencia y la posibilidad de cuantificación relativa de los niveles de expresión de esta técnica hacen que sea de gran utilidad para evaluar la respuesta inmune de los caballos frente al vPEA y seguramente frente a otros patógenos.

Dados los buenos resultados obtenidos, se decidió combinar esta técnica junto con la detección de anticuerpos, para llevar a cabo la **evaluación de la respuesta inmune** en animales infectados con el vPEA. Con este fin, se realizó una infección experimental en caballos con dos serotipos del virus (serotipo 2 y serotipo 4) en la cual se analizó la **respuesta inmune** en función de la forma clínica de los animales. En la evaluación del perfil de expresión de citoquinas, se encontraron algunos perfiles comunes según la forma clínica de la enfermedad, aunque los resultados entre los animales fueron variables. La mayoría de los animales infectados se caracterizaron por mostrar una sobreexpresión del ARNm de interleukina 1 (IL-1), la cual no

parece jugar un papel protector. Por otra parte, todos los animales que sobrevivieron mostraron la forma febril y cardiaca. Estos animales se caracterizaron por una sobreexpresión continua del factor de necrosis tumoral alfa (TNFα) desde el inicio de la infección, junto con la producción de anticuerpos a los 7 dpi. Sin embargo, los caballos que murieron en el curso de la infección mostraron una falta de expresión de TNFα, aunque se detectó la producción de anticuerpos a los 7 dpi. A pesar de estos perfiles variables de expresión de citoquinas, este estudio ha permitido mejorar la comprensión de los mecanismos de inmunidad contra la infección.

Artículos científicos:

- A. Sánchez-Matamoros, D. Kukielka, A.I. De las Heras, and J.M. Sánchez-Vizcaíno, 2013. **Development and evaluation of a SYBR Green real-time RT-PCR assay for evaluation of cytokine gene expression in horse**. Cytokine 61, 50–53
- A. Sánchez-Matamoros, D. Kukielka, B. Alberca, M. Cabana, E. Viaplana, A. Urniza, J.M. Sánchez-Vizcaíno, 2014. **Evaluation of immune mechanisms in horses exposed to two serotypes of African horse sickness virus and their relation to the clinical form**. Sometido a publicación en Research in Veterinary Science.

Ponencias y congresos:

- A. Sánchez-Matamoros, D. Kukielka, and J.M. Sánchez-Vizcaíno, 2013. **Cytokine and antibody responses in horses showing different clinical forms of African horse sickness virus (AHSV).** *7th VACCINE & ISV CONGRESS*. Annual Global Conference.
- A. Sánchez-Matamoros, A.I. De las Heras, y J.M. Sánchez-Vizcaíno, 2013. **Evaluación de la respuesta inmune en caballos: Cuantificación de citoquinas y citometría de flujo**. *V Congreso Nacional de Investigación para alumnos de Pregrado en Ciencias de la Salud y X Congreso de Ciencias Veterinarias y Biomédicas.*

Development and Evaluation of a SYBR Green Real-Time RT-PCR Assay for Evaluation of Cytokine Gene Expression in Horse

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Abstract

Cytokine secretion is one of the main mechanisms by which the immune system is regulated in response to pathogens. Therefore, the measurement of cytokine expression is fundamental to characterizing the immune response to infections. Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) is widely used to measure cytokine mRNA levels, but assay conditions should be properly evaluated before analyzing important equine infections through relative quantification of gene expression. The aim of this study was to develop and evaluate a set of RT-qPCR assays for a panel of the most common cytokines in horses involved in innate and adaptive immune responses. Eight cytokines (interleukin (IL)-1β, IL-2, IL-4, IL-10, IL-12, TNFα, IFNβ and IFNγ) and a housekeeping gene (β-actin) were detected and amplified with the same annealing temperature in a SYBR Green RT-qPCR assay of samples of mitogen-stimulated peripheral blood mononuclear cells from a healthy horse and whole blood from a horse infected with African horse sickness virus. The method gave good efficiency for all genes tested, allowing quantification of relative expression levels. These SYBR Green RT-qPCR assays may be useful for examining cytokine gene expression in horses in response to exposure to economically important pathogens.

Keywords: Cytokine expression, Horse, Immune system, RT-qPCR SYBR Green, African horse sickness

1. Introduction

The immune system is responsible for defending the host against pathogens through innate and adaptive responses, which are regulated by a network of interactions among cytokines produced in response to infection [1]. Cytokines are a heterogeneous group of low-molecular-weight regulatory proteins that play major roles in regulating the intensity and duration of the immune response. They do so by controlling the activation, proliferation and/or differentiation of various cells types, as well as the secretion of antibodies and mediators [2]. Therefore, characterization and quantification of cytokine production is important for understanding the immune response [1]. In this way, cytokines may be useful as immunological markers to understand disease pathogenesis, help treat diseases or increase vaccine efficacy [3,4].

Various technologies are routinely applied for cytokine detection and quantification; they are based on detection of secreted cytokine proteins or expression of cytokine mRNA. Currently, there are few optimized assays for the measurement of secreted equine cytokine proteins [3] and many more for quantification of cytokine mRNA expression [5]. One of the most widely used methods to quantify cytokine expression is real-time quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR). This technique allows to estimate the secretion of cytokine proteins [6,7] and to establish a cytokine profile. Its main characteristic is the multiple and simultaneous determination of cytokines using small quantities of different types of samples (fluids, organs or cell cultures) with a high specificity, sensitivity and accuracy, however, it require expensive specific material and reagents [7,8]. The most widely used methods for fluorescence monitoring of PCR products are intercalating dyes such as SYBR Green and specific probes like TaqMan. SYBR Green can be as sensitive, robust, and reproducible as specific probes, as long as primer design and set up are properly optimized. In addition, the intercalating dyes are usually less expensive than specific probes [9]. Optimization and validation of SYBR Green RT-qPCRs involves analyzing the RT-qPCR product melting curve, reaction efficiency (E), its linear correlation (RSq), sensitivity (S) and reproducibility (intra- and inter-assay variability). These values are essential for precise and accurate measurement of relative gene expression, and they allow us to decide whether the results of one cytokine RT-qPCR can be compared to others [10,11]. Despite their importance, these validation parameters are not reported in many published studies [3,4]. In particular, there are very few published studies that have carefully validated RT-qPCR assays for cytokine genes important in regulating the equine immune system. This validation is essential to provide new tools for studies of economically significant equine diseases and their treatment.

The aim of this study was to develop and validate a set of SYBR Green RT-qPCR assays for detecting cytokines in horses that would provide insights into the innate and adaptive immune response. These assays were optimized by measuring the effects of mitogens on cytokine expression in peripheral blood mononuclear cells (PBMC) from a healthy horse. Once optimized, assays were validated by analyzing the pattern of cytokine gene expression in the blood of a horse infected with African horse sickness virus (AHSV). The results of this study should provide a basis for future work aimed at understanding the immune response and treating horse infections.

2. Materials and methods

2.1. Isolation and stimulation of horse PBMCs.

Whole blood from a single healthy horse was collected by venopuncture in EDTA vacuum collection tubes. PBMCs were obtained by density separation over Histopaque®-1077 (Sigma) according to the manufacturer's instructions. Cell viability was assessed by the trypan blue method, and PBMCs were resuspended at concentration of 1x105 cells/ml in Dulbecco's Modified Eagle's Medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2mML-glutamine and 2% horse serum. The cells were seeded and cultured in 6-well plates at 37ºC and 5% CO2.

PBMCs were cultured 24 h, then stimulated at 37ºC for 12 h with naive medium (negative control) or with 5 μg/ml of concanavalin A (Con A) and lipopolysaccharide (LPS) (Sigma) (positive controls) [5,6].

2.2. RNA extraction and reverse transcription.

RNA was extracted from PBMC cultures according to the manufacturer's instructions using the RNeasy Mini kit (Qiagen) including the optional step of on-column digestion of DNA (RNase-Free DNase Set, Qiagen) during RNA

isolation. Subsequently, first-strand cDNA was synthesized using an Affinity Script™ QPCR cDNA kit (Agilent technologies) following the kit protocol. Briefly, the total reaction volumes were 20 µL, consisting of 3 µL RNA, 10 µL first strand master mix, 3 µL Oligo (dT) primer, 1 µL Affinity transcript reverse transcriptase, and RNAase-DNAse free water. The reactions were performed with an initial stage of 25 ºC for 5 min, 42 ºC for 5 min, 55 ºC for 15 min and 95 ºC for 5 min and chilled on ice. Following synthesis, the cDNA was stored at -20ºC until use.

2.3. Cytokine primer design.

Primers for interleukin (IL)-1β, IL-2, IL-4 and IL-10 genes were designed based on the following sequences of horse cytokines deposited in GenBank: IL-1β, accession NM_001082526.1; IL-2, NM_001085433.1; IL-4, NM_001082519.1; and IL-10, NM_001082490.1. Primers were designed against completely conserved sequences free of base ambiguities. Primer sets were designed and optimized using Primer 3 (version 0.4.0) based on the default parameters. Four primer pairs were selected using the following criteria: annealing temperature of 60ºC, primer length (18–24 pb) and amplicon size (100–200 pb). Primer pairs predicted that have all requirements were synthesized (Table 1).

Primer sequences against the TNFα [3] and IFNγ [4] genes were taken from previous studies and used without TaqMan probes but instead with SYBR Green. IL-12 and IFNβ primers were obtained from Figueiredo et al. [12] and modified for optimal performance under our conditions (Table 1).

2.4. Real-time PCR.

A qPCR method based on SYBR Green detection was optimized and validated for cytokine gene expression quantification using the primers described in Section 2.3.

To optimize amplification reactions, different primer concentrations were tested. A matrix of primer concentrations in which both primers ranged from 0.2 to 0.8 µM was tested. Reactions (10 µL) were contained 5 lL KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems), the primer pair and 1 µL of cDNA template and RNAase-DNAse free water. PCR reactions were subjected to 95ºC for 5 min; followed by 40 cycles at 95ºC for 3s and 60ºC for 30s; and finally 72ºC for 15s. This was followed by melting curve analysis. All PCRs were performed in an Eco™ Real-Time PCR System (Illumina). The specificity and size of PCR products for each gene were confirmed by gel electrophoresis. PCR products were purified by the QIAquick PCR Purification kit (Qiagen), sequenced by SECUGEN S.A. (Madrid, Spain) and analyzed using the BLAST® tool at the National Center for Biotechnology Information (NCBI).

Reactions were validated under identical qPCR conditions, using optimized primer concentrations and RNA template prepared from PBMC cultures. E, S, RSq and reproducibility were calculated for each gene using Eco Real-Time PCR System Software (version 4.0). In addition, qPCR reactions were carried out in the presence of RNA template but without reverse transcriptase (no RT control) in order to detect possible genomic DNA (gDNA) contamination. All experiments were performed in duplicate and repeated at least three times.

2.5. Validation of housekeeping gene amplification by RT-qPCR.

The housekeeping gene b-actin [13] was amplified under the same conditions as the ones reported in Section 2.4.

2.6. Validation of the RT-qPCR method using blood samples from AHSV-infected horse

One horse negative for antigens and antibodies against AHSV was housed in Biosafety Level 3 facilities (Pfizer Olot S.L.U.,Vall de Bianya, Spain). Horse was intravenously (i.v.) inoculated with strain of AHSV (serotype 4; TCID50/ml = 106). Whole blood was collected by venopuncture in EDTA vacuum collection tubes on day 0 prior to infection and at different times of infection (3 day, 5 day and 7 day). The mRNA from blood samples was extracted, cDNA synthesized and real-time qPCR performed as described above. The use of horses for this study was carried out according to Spanish and European regulations (Directive 2010/63/EU) on animal welfare.

Table 1
Primer sets for SYBR Green RT-qPCR analysis of equine cytokine gene expression with validation values[a].

Target gene	Primer sequence (5′- 3)	First nt pos.[b]	*E* (%)	RSq	*S*	Inter-assay		Intra-assay	
						Mean Ct	CV	Mean Ct	CV from-to
β-actin	F: GGACCTGACGGACTACCTC R: CACGCACGATTTCCCTCTC	549 631	105.2	0.992	10^{2}	25.02	0.14	25.01	0.14–0.17
IL-1β	F: TGTACCTGTCTTGTGGGATGAAA R: TTCTGCTTGAGAGGTGCTGA	606 790	101.6	0.995	10^{1}	21.41	0.19	21.51	0.19–0.26
IL-2	F: TGCATCGCACTAACTCTTGC R: CAATTCTGTGGCCTTCTTGG	56 250	99.5	0.997	10^{1}	30.17	0.52	30.1	0.52–0.62
IL-4	F: CAAAACGCTGAACAACCTCA R: CTGTTGAAGCACCTTTGCAG	105 237	103	0.997	10^{3}	29.85	0.29	30.03	0.290.32
IL-10	F: GTCATCGATTTCTGCCCTGT R: GCTTCGTTCCCTAGGATGC	377 557	101.4	0.994	10^{1}	30.06	0.08	29.98	0.08–0.26
IL-12	F: CCAGACGCTGTGCCTTAGC R: TCTGCCTCTGAGGATCTATCAACA	670 770	94.1	0.996	10^{2}	32.89	0.09	33.08	0.09–0.64
TNFα	F[c]: TTACCGAATGCCTTCCAGTC R: GGGCTACAGGCTTGTCACTT	299 383	99.3	0.996	10^{1}	29.5	0.36	29.51	0.19–0.48
IFNγ	F: TGGACACCATCAAGGAGGAC R: GGACCTTCAGATCATTTACCG	278 385	101.7	0.995	10^{1}	30.91	0.3	31.12	0.03–0.57
IFNβ	F: CCCCGAGGACACAATGAACT R: ACCAATGCAGCATCCTCCTT	156 236	103.2	0.992	10^{2}	35.83	0.03	34.85	0.03–0.07

[a] Abbreviations: F, forward; R, reverse; E, reaction efficiency; RSq, linear correlation; S, limit of sensitivity, Ct, cycle threshold; CV, coefficients of variation.
[b] Numbering according to the Genbank sequence (see Section 2.3).
[c] Three nucleotides were removed from the original forward primer sequence.

3. Results

A set of SYBR Green real-time PCR assays was optimized and validated to quantify the expression of eight cytokine genes in horse. Primer concentrations were optimized to obtain a single specific PCR product, as confirmed by melting curve analysis, gel electrophoresis, and sequencing. Optimized primer concentrations were as follows: IL-2, IL-4, IL-10, IL-12, TNFα, IFNβ and IFNγ, 0.2 μM; IL-1β, and β-actin, 0.8 μM. All primer sets share the same annealing temperature and therefore can be run in parallel in the same thermocycler, making the analyses around 1 h, hence, less time-consuming.

During assay validation, S, RSq and E coefficients were obtained from standard curves of each individual cytokine as well as reproducibility (Table 1). Since it is recommended that quantitative assays include a housekeeping gene as an internal control [9], β-actin expression quantification was adapted and validated to the RTqPCR cytokine method, and E and RSq coefficients similar to those for the cytokine genes were obtained (Table 1). Furthermore, no amplification for cytokine cDNA was observed in the no RT controls, showing that the primer pairs did not amplify potential contaminating gDNA in the samples.

Finally, the cytokine mRNA expression was detectable in unstimulated and stimulated PBMC (Table 2). In addition, the RTqPCR method was validated using blood samples from a horse infected with AHSV obtained at different times of infection and their cytokine mRNA expression levels were determined (Table 2).

Table 2
Measure of the relative gene expression of each cytokine in PBMC stimulated in vitro with 5 μg/ml of Con A and LPS and in horse blood samples stimulated with AHSV 4 at different times.[a] Results are reported as the *n*-fold difference relative to cytokine mRNA expression of calibrator sample, 0 h always that had cytokine expression.

Gene	In vitro		AHSV 4			
	0 h	12 h	0 h	3 day	5 day	7 day
IL-1β	1.00	1.88	1.00	20.28	3.17	4.86
IL-2	1.00	2.89	N/A	N/A	N/A	N/A
IL-4	1.00	1.40	N/A	N/A	N/A	N/A
IL-10	1.00	0.70	N/A	3.87	13.30	1.00[b]
IL-12 p35	1.00	12.83	1.00	4.10	5.11	5.10
TNFα	1.00	9.89	1.00	9.44	11.19	13.30
IFNγ	1.00	1.92	1.00	8.53	3.29	2.48
IFN β	1.00	2.20	1.00	2.12	3.09	0.49

[a] Abbreviations: N/A, not applicable (Ct of sample does not cross the threshold).
[b] Calibrator sample of IL-10 is 7 day because 0 h sample does not applicable.

4. Discussion

This study presents the optimization and validation of eight RT-qPCR based on SYBR Green to quantify the relative expression of cytokine genes in horse. The SYBR Green method in this assay allows rapid quantification (around 1h) of low concentrations of DNA (Table 1). The RT-qPCR described here should allow characterization of the expression of a wide range of cytokines involved in the immune response of horses. The cytokines analyzed here were selected

for their roles in the inflammatory response (IL-1β, IL-12, TNFα and IFNβ) and in the activation of cellular immunity (IL-2 and IFNγ) and humoral immunity (IL-4 and IL-10) [2]. Description of the cytokine gene expression patterns elicited by a given pathogen indicates the type of immune response that has been activated. This information is important for establishing immunological markers of infection, and it can help guide development of effective therapies.

Few studies report assay validation parameters, usually E and RSq, for each primer pair used, even though these values are essential to study the cytokine response, since they determine whether it is possible to compare results for different cytokines [10,11]. The E values obtained for each cytokine gene were similar to one another (Table 1), as well as to the few E values described in the literature [5,12,14]. These slight differences allow the use of relative quantification based on efficiency-corrected Ct models [11] or efficiency-adjusted ΔΔCt [15] calculations in order to avoid inaccurate results due to different E values. Furthermore, the high sensibilities allow detecting the basal level of all cytokines from PBMC culture in vitro. Validation of these SYBR Green RT-qPCR allows to measure fast and simultaneously the relative quantification of eight cytokine genes in horse, which enable to establish the main cytokine expression pattern to different horse pathogens.

To be interpreted precisely, cytokine gene expression levels must be normalized. Typically, this is done by quantifying the expression level of a housekeeping gene in parallel [9]. Several horse housekeeping genes have been validated for use with SYBR Green qPCR assays at annealing temperatures similar to the one used for cytokine primers in the present study [9,13]. Of these, β-actin [13] was selected for adaptation to the optimized RT-qPCR assay. In the optimized assay, all cytokine genes as well as the β-actin gene can be measured in the same thermocycler run, allowing simultaneous normalization of the expression of different cytokine genes. This reduces the time and cost of the analysis, and may improve reproducibility.

To be complete, method validation should be performed using the same kinds of samples as those for which the assay is intended. Studies of the immune response are usually based on whole blood [3] or PBMC [4] samples, since these most closely reflect the systemic immune response. The optimized assay was applied to both types of samples in the present study to validate the methodology and to establish the cytokine expression pattern of the PBMC stimulated with mitogens and the whole blood samples from a horse infected with AHSV. However, the validation of this method in other samples would be also interest.

The aim of treating with Con A and LPS simultaneously is the stimulation of all studied cytokines, since they are potent activators of the immune system. The stimulated PBMC showed a variation of expression at 12h regarding to the basal level (Table 2). The cytokine pattern of PBMC presents mainly an inflammatory response, probably caused by the together stimulation of both mitogens. The adaptive immunity shows a lesser activation at this time, prevailing Th1 response over Th2. In this study, stimulation of PBMCs was done with both mitogens simultaneously. Hence, the results cannot be compared directly with other studies because they stimulated only with Con A or LPS. However, a higher cytokine response, except IL-12, was detected when the PBMC were stimulated with only Con A at 10 μg/ml [6]. This is the first study where PBMCs are stimulated with both mitogens simultaneously in horse. Results show that at 12 h it induces a potent activation of innate immune system and a slight adaptive immunity, allowing the validation of RT-qPCR of eight cytokine genes in horse.

Validation of the method was performed with whole blood samples from a horse stimulated with AHSV, which were obtained at different times of infection. In addition, it allows knowing the tendency of cytokine pattern of AHSV infected animals (Table 2) and it could provide important information regarding disease processes. In this study, all cytokines were detected, except IL-2 and IL-4. Immune response at 3 days was characterized by an increase of IL-1β and TNFα mRNA expression, which may be correlated with initial clinical sign of illness (fever) [16]. This fact allows to determine the important role of the monocytes-macrophages in innate immunity of AHSV since it are one of main target cells for primary virus replication [17]. In addition, IFN type II prevailed over IFN type I, although transcription of both was activated. At 5 days, there was an increase of IL-10 production, which mainly has anti-inflammatory effects, probably associated with decrease of IL-1β, TNFα and IFNγ [18]. Finally, at 7 days it is possible to

observe an initial cellular response, which is predominant during the transcription of Th1 response cytokines. This result is supported by the ones published by Pretorius et al. [19]. The lack of IL-2 and IL-4 mRNA induction of these cytokines may be related with the need to expand the time period for their detection. The cytokine pattern to AHSV-4 showed an evolution from initial inflammatory cascade to initial adaptive cellular immune response. However, further research is required for a more complete explanation of the cytokine pattern of infected horses with AHSV, since individual equine variation can exist [19].

The method described here may serve as the basis for highly efficient and relatively rapid and inexpensive studies aimed at quantifying cytokine gene expression and characterizing the immune response in horses. This SYBR Green RT-qPCR technique works equally well with whole blood and PBMC samples.

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Evaluation of Immune Mechanisms in Horses Exposed to Two Serotypes of African Horse Sickness Virus and their Relation to the Clinical Form.

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Abstract

As knowledge about host defense systems against African horse sickness virus (AHSV) is limited, we aimed to associate the clinical form of the disease with immune mechanisms so that protective immune responses could be identified and presumably used to develop vaccines based on these immune markers. To this end, we evaluated the cytokine expression and the antibody production in AHSV serotype 4 and 2 experimentally infected horses manifesting different clinical forms. The main results show that the mRNA of IL-1 was expressed in the majority of infected animals. Surviving horses showing the fever or the cardiac form showed an overexpression of TNFα together with the presence of antibodies at day 7 post-infection. Dead horses presenting the mixed form showed a lack of TNFα expression while producing antibodies at the same time as surviving horses. These findings shed light on how to better evaluate immune mechanisms against AHSV infection in its different forms.

Keywords: African horse sickness (AHS); immune response; cytokine profile; antibody production; *in vivo* AHSV.

Highlights:

- An experimental infection with AHSV serotype 4 and 2 was performed in different groups of horses.
- Evaluation of cytokine expression and antibody production were associated with clinical forms.
- New data about the immune response in naïve AHS-infected horses is presented here.

1. Introduction

African horse sickness (AHS) is a non-contagious infectious disease that affects equines and is included in the World Organization for Animal Health (OIE) listed diseases. It is caused by the African horse sickness virus (AHSV) belonging to the genus Orbivirus, whereas other viruses such as Bluetongue Virus (BTV) have also been included (House, 1993). This disease causes a high morbidity and mortality rate in naïve horses, with Culicoides spp. considered the most important biological vectors for transmitting the disease (Mellor, 1993). Nowadays, nine different serotypes have been identified by virus neutralization. Besides this, cross-reactivity among some of these serotypes has been described (1 and 2, 3 and 7, 5 and 8, and 6 and 9), but not with other orbivirus (OIE, 2012). AHSV serotype 4 (AHSV 4) and serotype 2 (AHSV 2) have both displayed a marked ability to spread, as AHSV 4 appeared in Spain between 1987 and 1990 (Rodriguez et al., 1992) and AHSV 2 had been notified in different countries outside of the endemic area (Senegal, Nigeria and Ethiopia) in recent years (Wilson et al., 2009). The AHS introduction in non-endemic countries causes dramatic socio-economic impact for equine production by causing disease in animals and world trade restrictions. The presence of Culicoides spp. and the re-emergence of vector-borne diseases in Europe, especially some orbivirus as BTV, has led to increasing concern about the real risk of AHS for European equidae populations (Maclachlan and Guthrie, 2010).

Equids are infected by the bite of a competent vector. The first replication sites of the inoculated virus are regional lymph nodes. Then, the virus is disseminated, causing viremia and reaching secondary replication sites such as lungs, heart, spleen and other lymphoid tissues (Clift and Penrith, 2010; Coetzer and Erasmus, 1994). Within these organs, endothelial cells and the monocyte-macrophage lineage have been described as the main targets cells of AHSV (Clift and Penrith, 2010). The spread of the virus in the host can cause four forms of the disease: pulmonary, mixed, cardiac and fever forms (Burrage and Laegreid, 1994). Each clinical form is characterized by a specific mortality rate, clinical signs and post-mortem lesions (Table 1), as these parameters usually are used for preliminary diagnosis and classification. In this sense, the evolution of clinical forms might be associated with the host's immune response, and an understanding of them is necessary to carry out studies focused on clarifying these links.

Horses recovering from infection have been reported to develop solid lifelong serotype-specific immunity to AHSV (Burrage and Laegreid, 1994), being the neutralizing antibodies the main responsible of immunological protection (Burrage and Laegreid, 1994). However, some studies about the role of neutralizing antibodies in AHSV protection have proven controversial (Guthrie et al., 2009; Martinez-Torrecuadrada et al., 1996; Romito et al., 1999). Therefore, other mechanisms of defense against AHSV may exist within the host, as in BTV (Maclachlan et al., 2013). Recent studies have suggested the role of cell-mediated immunity in the process of protection against AHSV in vaccinated horses (El Garch et al., 2012; Pretorius et al., 2012; Romito et al., 1999) or vaccinated mice (Calvo-Pinilla et al., 2014; de la Poza et al., 2013). However, there are no publications that evaluate how the virus stimulates the equine innate and adaptive immune response in experimental infection, which requires further research.

Cytokines are essential regulators of the immune response to viral infections because they establish an antiviral state by communicating with cells of the immune system (Trinchieri et al., 1993). Therefore, cytokine expression measurement has been used to characterize the immune response in horses against other diseases (Hughes et al., 2011; Quinlivan et al., 2007) or in sheep infected with BTV (Channappanavar et al., 2012; Drew et al., 2010; Umeshappa et al., 2011). The innate response to viruses is characterized by the release of various antiviral and inflammatory cytokines (such as IL-1β, TNFα, IL-10 and IL-12) (Abbas et al., 2011), while the adaptive response could be analyzed by the pattern of cytokines involved in the balance between helper T lymphocytes (such as IL-2, IL-4, IL-10 and IFNγ), IL-12 and TNFα (Street and Mosmann, 1991). In the absence of experimental data on the cytokine profile of horses infected with AHSV, there are significant deficiencies in our understanding of the equine response to this pathogen. Therefore, this study aimed to clarify the horse immune response against AHSV by analyzing the relation of the clinical form of the disease with secreted cytokines and antibody response after AHSV experimental infection. This information will improve our knowledge of the existing host defense mechanisms against AHSV in order to better

understand the disease, allowing the development of new tools to enhance the immune system.

2. Materials and methods

2.1 Animals and virus infection

For the analysis of the immune response against AHSV, horses were experimentally infected with AHSV 4 and AHSV 2. These infections were performed in the experimental facilities of Zoetis Manufacturing & Research Spain, S.L.,Vall de Bianya, Spain. The study was performed following the Spanish and European regulations on animal welfare (RD 53/2013, Directive 2010/63/UE) and was reviewed and approved by the Ethical Committee of Zoetis Manufacturing & Research Spain, S.L. The Animal Care and Use Program of Zoetis Manufacturing & Research Spain, S.L. site is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care.

Eleven healthy adult (cross-breed) horses of mixed gender were used. During the quarantine period, animals were housed outdoors. During this period, routine veterinary health checks were performed. Horses tested negative to antigens (Fernandez-Pinero et al., 2009) and antibodies against AHSV (INgezim AHSV compact plus 14.AHS.K.3; Ingenasa Laboratory, Madrid, Spain).The horses were moved to the experimental facilities one week before inoculation for acclimatization to the new environment.

Table 1. Main clinical signs associated with each form of African Horse sickness allowing the classification of each animal.

Form of the disease	Clinical signs	Post-mortem lesions	Mortality rates
"Fever" Form	- Fever (mild to moderate) - Oedema: supraorbital fossae - Depression - Anorexia - Congestion of mucous membranes	- No gross lesions	No mortality
"Cardiac" or "Dikkop" Form	- Fever - Oedema: supraorbital fossae, palpebral conjunctivae, intermandibular space, head, lips, neck or/and chest - Congested conjunctivae - Petechial haemorrhages in the eyes on the conjunctivae - Petechial haemorrhages on the ventral surface of the tongue - Dyspnoea and cyanosis associated with oedematous swelling - Severe colic	- Yellow gelatinous exudate in the subcutaneous, subfascial and intramuscular tissues and lymph nodes in the head and neck - Severe hydropericardium - Petechial and ecchymotic haemorrhages on epicardial and/or endocardial surfaces of heart - Pale-gray areas in myocardium - Moderate to severe oedema, congestion, petechial haemorrhages and/or cyanosis on the serosal surfaces of the caecum, colon and rectum - Ascites - Swollen and oedematous lymph nodes - Petechial or ecchymotic haemorrhages on the ventral surface of the tongue, which is occasionally swollen and cyanotic - Oedema of the lungs is either slight or absent	Exceed 50%
"Pulmonary" or "Dunkop" Form	- Die without indication of illness - Fever - Marked depression - Respiratory distress - Severe dyspnoea - Coughing spasms - Anomalous position: the head and neck are extended and severe sweating develops - Frothy serofibrinous nasal discharge	- Severe hydrothorax: transparent, pale-yellow and gelatinous fluid. - Interlobular oedema of the lungs - Yellowish gelatinous exudate infiltrated in the sub-pleural and interlobular tissues - Presence of surfactant and froth on the bronchial tree - Froth and yellow serous fluid in the trachea - Petechia and ecchymosis on the mucosa of the trachea - Ascites in abdominal and thoracic cavities - Hyperaemic and oedematous mucosa of the stomach - Swollen and oedematous bronchial and mediastinal lymph nodes - Oedema in the mediastinum, base of the heart and the parietal pleura - Scattered petechiation on the intestinal serosa	Exceed 95%
"Mixed" Form	- Combination of the cardiac and pulmonary forms of disease.	- Lesions common to both the pulmonary and cardiac forms of the disease.	Around 70%

For the experimental infection with AHSV 4, five horses were inoculated with a high dose of a highly virulent AHSV 4 strain (horses 1-5), and one horse was not inoculated and acted as a control (horse 6).

For the experimental infection with AHSV 2, four different horses were inoculated with a high dose of a highly virulent AHSV 2 strain (horse A, horse B, horse C and horse D), and one horse was not inoculated and acted as a control (horse E).

Administration and sampling procedures and clinical examinations of the animals were performed by an experienced veterinary surgeon. Trained animal husbandry technicians were responsible for day-to-day husbandry procedures.

2.2 Form of the disease: Clinical signs and gross lesions

Throughout the study, the animals were monitored to determine the clinical form of the disease. Animals were observed twice a day for clinical signs, appearance and rectal temperature recorded at day of sampling. A rectal temperature above 39.5 °C was considered as a criterion for fever. Humane endpoints were established prior to the start of the study. Euthanasia was practiced, when applicable, according to the guidelines of the American Veterinary Medical Association for euthanasia in the equine species. A post-mortem examination was carried out in order to evaluate the presence of gross lesions characteristic of the ASH. Due to a legal agreement of confidentiality with Zoetis Manufacturing & Research Spain S.L., the specific clinical signs and gross findings observed are not described in this article. However, based on this information, a classification of the disease into one of the four clinical forms (Table 1) was assigned to each animal.

2.3 Blood and serum samples collection

Whole blood was collected by venipuncture in EDTA vacuum collection tubes on day 0 prior to infection. After virus inoculation, blood samples were taken at different time-points. Animals infected with AHSV 4 were sampled on days 0, 3, 5, 7, 10, 12, 14, 17 and 21 post-infection (PI), while animals infected with AHSV 2 were sampled on days 0, 2, 4, 7, 9, 11, 14, 17 and 21 PI. In the same way, serum samples were also collected on day 0 prior to infection as well as on days 7, 14 and 21 PI.

2.4 Virus and antibody detection

The presence of AHSV in the blood of horses was quantified by RT-PCR, as published by Fernández-Pinero et al. (2009). This RT-PCR allows detecting a fragment of the VP7 segment of the viral RNA, which is a highly conserved region in all serotypes. A commercial blocking immunoenzymatic assay (INgezim AHSV compact plus 14.AHS.K.3; Ingenasa Laboratory, Madrid, Spain) and a commercial lateral flow assay (LFA) (INgezim AHSV Crom; Ingenasa Laboratory, Madrid, Spain) were used to detect specific AHSV antibodies. These tests were carried out according to the manufacturer's instructions, allowing the detection of VP7 antibodies in the serum samples.

2.5 Quantification of the cytokine mRNA expression

Seven horse cytokines (IL-1β, TNFα, IL-12, IL-10, IFNγ, IL-2 and IL-4) were selected to evaluate the innate and adaptive immune responses of the infected horses (AHSV 4 and AHSV 2) from day 0 until day 12 PI.

Total RNA was extracted from each whole blood sample of the infected horses, according to the manufacturer's instructions, using the commercial NucleoSpin RNA II® kit (Macherey Nagel®). Subsequently, first-strand cDNA was synthesized using an Affinity Script™ QPCR cDNA kit (Agilent technologies). Synthesized cDNA was stored at −20 °C until use.

Gene expression quantification of cDNA was carried out using the KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems) with the specific primer sequences for seven horse cytokines (IL-1β, TNFα, IL-12, IL-10, IFNγ, IL-2 and IL-4) and β-actin, a technique which was used previously by Sánchez-Matamoros et al. (2013). PCR analysis of each cytokine was performed in triplicate for each sample, while β-actin analysis was carried out in duplicate. The relative target gene expression level was calculated in reference to the β-actin gene, and the calibrator sample for each horse was day 0. Relative quantifications were determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), allowing us to compare the gene expression levels in fold changes. A 1-fold increase in the cycle threshold (Ct) value indicates double the amount of cytokine mRNA in the infected sample as compared to that of a non-infected one. Therefore, cytokines were considered to be up-regulated once the mRNA fold increase difference was more than 2 when compared to the β-actin gene tested.

3. Results

This study analyzes the immune response against AHSV, including serological responses and cytokine profiles in horses experimentally infected with two different serotypes of AHSV (AHSV 4 and AHSV 2) manifesting different forms of the disease.

3.1 Form of the disease

Clinical signs as well as gross lesions indicative of AHS were observed in all horses experimentally infected with AHSV 4 and AHSV 2. A specific form of the disease was assigned to each animal according to clinical signs and gross lesions as described in the literature (Coetzer and Erasmus, 1994) (Table 2) (data not shown due to legal agreement of confidentiality). Uninfected controls remained clinically unaffected throughout the study, and no gross lesions were found during necropsy.

Table 2. Form of the disease, date of death, viraemia and antibody detection of each infected horse by serotypes (AHSV 4 and AHSV 2). The commercial lateral flow assay (LFA) (INgezim AHSV Crom; Ingenasa Laboratory, Madrid, Spain) and commercial blocking immunoenzymatic assay (E) (INgezim AHSV compact plus 14.AHS.K.3; Ingenasa Laboratory, Madrid, Spain) were used to perform the antibody detection.

Animal	Form of the disease	Date of death	Viraemia	Antibody detection
AHSV 4 horse 1	Fever	End of the study	5 – 10 PI (1 peak)	LFA: 7 PI E: 14 PI
AHSV 4 horse 2	Fever	End of the study	7 – 17 PI (2 peak)	LFA: 7 PI E: 14 PI
AHSV 4 horse 3	Cardiac	End of the study	7 – 14 PI (2 peak)	LFA: 7 PI E: 14 PI
AHSV 4 horse 4	Mixed	14 PI	7 – 14 PI (2 peak)	LFA: 7 PI E: 14 PI
AHSV 4 horse 5	Fever	End of the study	7 -14 PI (2 peak)	LFA: 7 PI E: 14 PI
AHSV 2 horse A	Fever	End of the study	9 - 14 PI (1 peak)	LFA: 7 PI E: 14 PI
AHSV 2 horse B	Mixed	9 PI	4 – 7 PI (1 peak)	LFA: 7 PI E: neg
AHSV 2 horse C	Mixed	9 PI	4 – 7 PI (1 peak)	LFA: 7 PI E: neg
AHSV 2 horse D	Mixed	11 PI	7 – 11 PI (1 peak)	LFA: 7 PI E: neg

Abbreviations: PI: post-infection; neg: negative

Horses 1, 2 and 5 suffered the fever form, since they only showed one fever peak. Horse 3 presented gross lesions in the necropsy which were compatible with the cardiac form. Likewise, horse 4 showed characteristic clinical signs and gross findings of the mixed form. The virus caused mortality only in horse 4 (mixed form), which died on day 14 PI, while the remaining horses with the cardiac and fever forms survived the disease (Table 2).

Horse A showed a peak of fever and survived, which was considered the fever form; however, horses B, C and D presented clinical signs and post-mortem lesions which were compatible with the mixed form. The disease resulted in the death of horses B and C on day 9 PI and horse D on day 11 PI (Table 2).

3.2 Viremia and AHS-antibody detection

The presence of the viral genome of AHSV 4 and of AHSV 2 in the blood of each horse was quantified by quantitative RT-PCR. The background Ct for all samples in the RT-PCR test was 40. Viremia was neither detected in the control animals nor present prior to infection. The nine horses infected with AHSV presented individual variations in the results and showed different profiles of viral response (Fig. 1). Viremia levels were slightly higher in the AHSV 2 group than in the AHSV 4 group. In the AHSV 2 group, the animals presented a short viremia period (4 – 5 days) with a peak of viremia, while the animals infected with AHSV 4 showed a viremia period of 8 or 11 days with two viremia peaks, except horse 1.

The serological response was evaluated employing two methods: the ELISA test and a LFA test. The LFA analyses allowed the detection of a specific antibody response against AHSV from day 7 PI until the end of the study in all the sera from the infected animals; however, the detection of antibodies using the ELISA technique was upon day 14 PI. None of the control animals developed antibodies against AHSV.

3.3 Cytokine response

The expression levels of several equine mRNA (IL-1β, TNFα, IL-12, IL-10, IFNγ, IL-2 and IL-4) were quantified in relation to the day 0 PI level for each horse. Analyses were only performed until day 12 PI because most of the animals with the mixed form died at this time, not allowing comparison with the data from the other animals. In addition, the antibodies against AHS were detected on day 7 PI so that the adaptive response in all animals had been activated at that time. The results of all 7 cytokine mRNA samples examined are provided in Table S1.

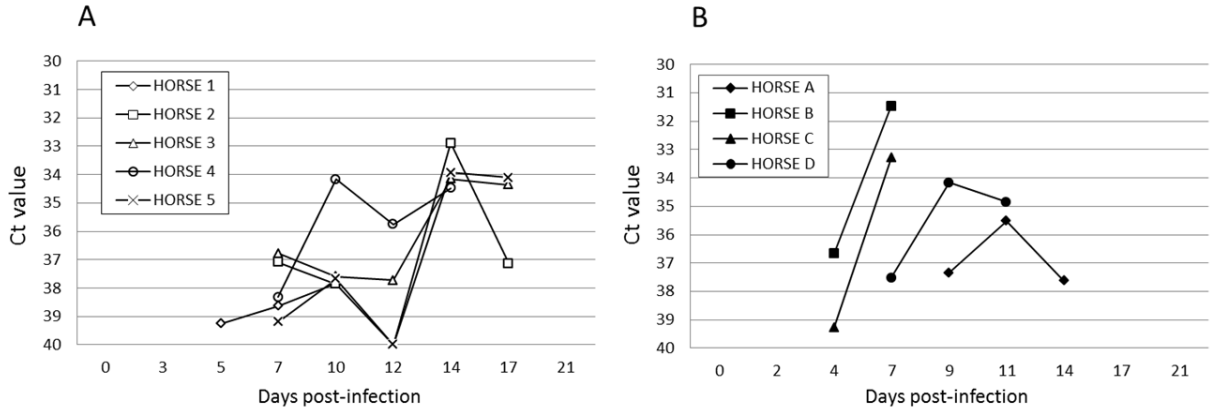


Figure 1: Viremia detection in animals infected with AHSV 4 (A) and AHSV 2 (B). The AHSV viral genome was detected in blood samples by quantitative RT-PCR throughout the experiment. Each line represents the Ct values of each horse. Horses were infected with AHSV 4 or AHSV 2 at 106 TCID50/ml. Each horse is represented by one line with different symbols. Control animals (Horse 6 and Horse E) did not show viremia at any of different infection times, hence, they are not represented.
*AHSV 4 (A): Horse 1 (rhombus), Horse 2 (square), Horse 3 (triangle), Horse 4 (cross) and Horse 5 (star). * On day 14 post-infection (PI), horse 4 died.*
*AHSV 2 (B): Horse A (rhombus), Horse B (square), Horse C (triangle) and Horse D (cross). * On days 7, 9 and 14 PI, horses C, B and D died, respectively.*

The pro-inflammatory cytokines IL-1β, TNFα and IL-12, and anti-inflammatory cytokine IL-10 allow the analysis of the innate response. The expression of IL-1β was induced in most of the horses (except horses 1, A and B) by AHSV infection between days 2 and 4 PI (Fig. 2, Table S1). The overexpression of TNFα was observed only in horses 1, 2, 3, 5 and A, which survived infection (Fig. 2, Table S1), presenting overexpression on days 3 and 4 PI. Only horse 3 showed a substantially enhanced IL-12 expression (Fig. 2, Table S1). Finally, the anti-inflammatory cytokine IL-10 expression was quite variable during the study (Table S1).

The adaptive response was analyzed in parallel. The expressions of IFNγ, IL-2, IL-10 and IL-4 did not present a similar profile in the infected horses (Table S1). TNFα showed a constant overexpression from day 5 to 12 PI (Fig. 2, Table S1) in surviving animals. Horse D showed a slight increase on day 7 PI, but then TNFα expression dropped.

The horses infected with AHSV presented a variable cytokine profile; however, there are common characteristics in these cytokine patterns depending on the form of the disease. Horses with the mixed form showed an immune response mainly characterized by the lack of TNFα production while they presented overexpression of IL-1. The horse showing the cardiac form presented increased IL-1, IL-12 and TNFα expressions. This profile was similar to the surviving horses with the fever form which showed expression of the IL-1 and TNFα.

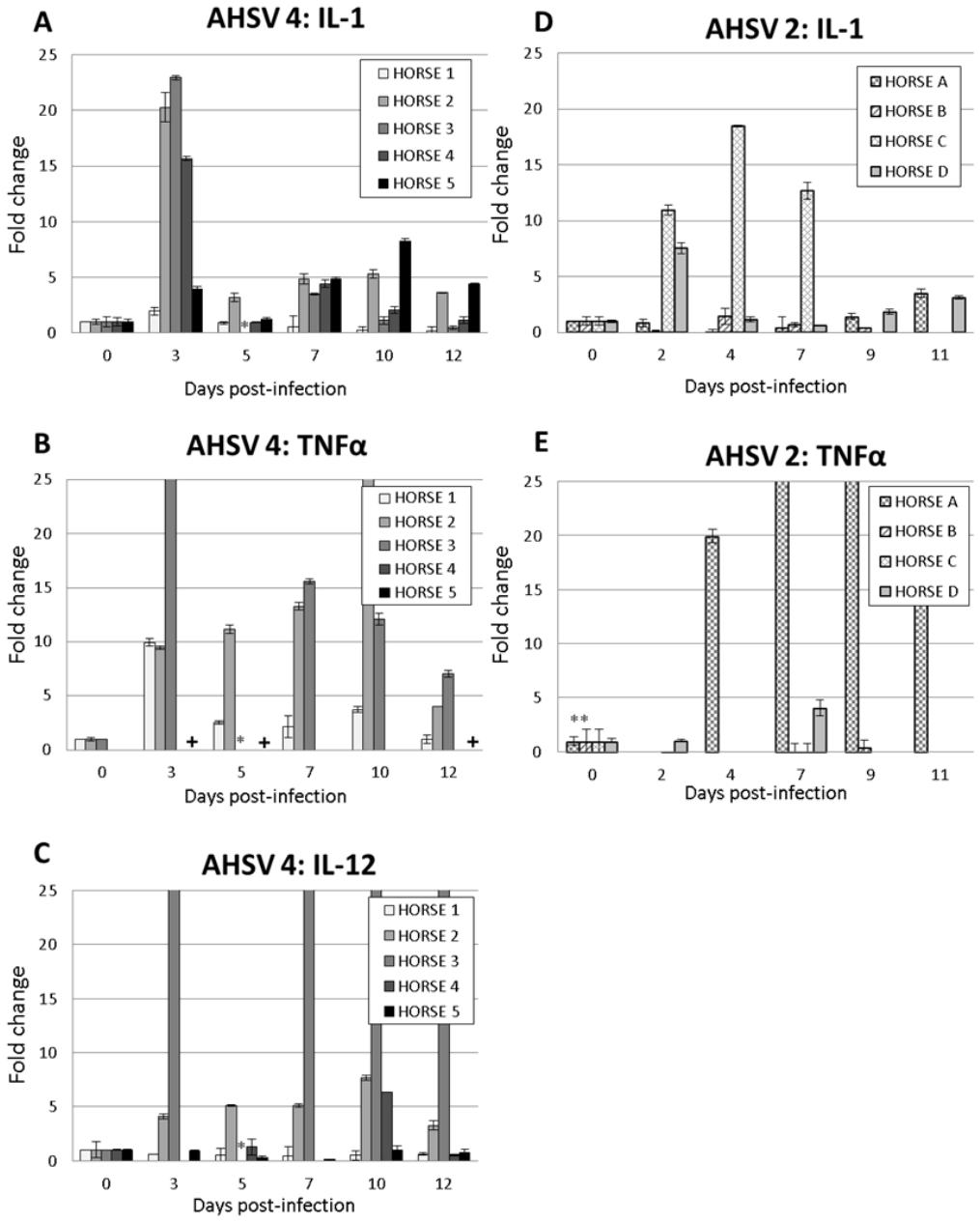
4. Discussion

In this study, the immune response against two serotypes of AHSV has been analyzed by evaluating and correlating the cytokine expression and the antibody response with the manifested disease form in each experimentally infected horse. Pathogenesis of infections has been traditionally related to immunity (Licastro et al., 2005); therefore, the infected horses were classified according to the clinical form of the disease. In consequence, the results obtained in this study demonstrate that the analysis of the cytokine expression and the antibody response associated with the clinical form allow us to improve our knowledge of the immune mechanisms against AHSV infection and its different clinical forms.

Two different AHSV serotypes have been tested to evaluate the immune system in horses. All the horses were infected with the same infectious dose. However, they manifested different clinical forms of the disease (Table 2). Several of these clinical forms have been found in previous AHSV outbreaks caused by a single serotype and during experimental infections with only one serotype, which supports our findings (Gomez-Villamandos et al., 1999; Rodriguez et al., 1992). Therefore, we suggest that the form of the AHSV disease is not linked to serotype or infectious dose and that it could be related to the host's immune response. This is in contrast to results published by Burrage and Laegreid (1994) where they hypothesized that immunity has no influence on the clinical presentation of the

disease. In this work, the mixed, cardiac and fever forms have been studied. The mixed form of AHS was found in four horses in this study, which presented a 100% mortality rate between days 9 and 14 PI. However, the horse with the cardiac form and the four horses showing the fever form survived the disease. The differences in their immune responses could allow clarification of the immune mechanisms of each clinical form, especially in surviving horses. In this study, horses

Figure 2. Representation of the relative expression of most important cytokines discussed in this study: IL-1 of the samples infected with AHSV 4 (A) and AHSV 2 (D), TNFα of the samples infected with AHSV 4 (B) and AHSV 2 (E), and IL-12 of the samples infected with AHSV 4 (C). Samples were analysed at different post-infection times. Boxes represent horse 1, horse 2, horse 3, horse 4 and horse 5 for AHSV 4, and horse A, horse B, horse C and horse D for AHSV 2.



Abbreviations:
+, Positive samples (Ct of the sample crosses the threshold) with no measure of the n-fold difference because the calibrator sample 0 day PI does not show expression levels (Ct of the sample does not cross the threshold).
** Poor quality RNA.*

presented a variable cytokine expression profile similar to those previously described (Pretorius et al., 2012), which are probably associated with genetic differences (Babiuk et al., 2003) or with other cellular-based responses. Therefore, to understand the complete immune mechanisms responsible for the clinical outcome a more detailed study is needed. However, there were some common characteristics in these cytokine profiles that allowed us to characterize part of the immune response in each clinical form.

IL-1 is a pro-inflammatory cytokine secreted by activated monocytes (Boraschi et al., 1996). In this study, the response pattern for IL-1 was similar in the horses infected with both serotypes (Fig. 2, Table S1). All the horses, except horses 1, A and B, presented increased IL-1 between days 2 and 4 (Fig. 2) and previously or simultaneously with viremia detection (Fig. 1). Hence, the primary replication of the virus in the monocyte lineage (Clift and Penrith, 2010) could be associated with the secretion of this cytokine in the horses. However, its sole expression in the AHSV-infected horses seems insufficient to establish an adequate innate immune response in order to allow host survival, as observed in those horses with the mixed form.

TNFα is a potent pro-inflammatory cytokine that performs important antiviral activity and is produced mainly by activated monocytes - macrophages and lymphocytes (Wong and Goeddel, 1986). In this study, horses 1, 2, 3, 5 and A (all the surviving horses) presented an increased TNFα expression on days 3 - 4 PI, and its detection is indicative of continuous overproduction (Harding et al., 1997). Afterwards, different TNFα profiles were observed in each animal (Fig. 2, Table S1). Horse D showed a slight increase on day 7 PI, but then expression dropped. Thus, only surviving horses showed a characteristic maintained increase in TNFα expression in the innate and adaptive responses. The high TNFα expression levels from day 5 to 12 PI (Fig. 2, Table S1) observed in surviving animals (fever and cardiac forms) may be related to an increase in cytotoxic T lymphocytes (LTc CD8+), which was detected by Pretorius et al. (2012) in horses infected with AHSV and Channappanavar et al. (2012) in sheep infected with BTV. Similar TNFα response profiles have been described in other diseases (Channappanavar et al., 2012; Shrestha et al., 2008), suggesting that TNFα plays an important antiviral role in the organism. In our study, increased TNFα levels occurred mainly in the horses with the fever form and were not associated with other clinical signs. Therefore, this cytokine might play an important immune-regulatory role in the host's survival and recovery.

IL-12 acts as a key regulator molecule in the immune system and induces IFNγ production (Sartori et al., 1997). In the present study, the cardiac form of the disease identified in horse 3 showed a cytokine profile leading to higher IL-12 levels than those observed in other clinical forms of the disease (Fig. 2, Table S1). However, the overexpression of this cytokine was not accompanied by the usual increase of IFNγ production. This fact can be associated with high levels of IL-1 and TNFα in this animal because the expression of these cytokines decreases the effects of IL-12 on IFNγ production (Trinchieri, 2003). This immune response could imply that the horses showing the cardiac form of the disease have a better prognosis than those suffering the mixed form (Coetzer and Erasmus, 1994).

IL-10 is a regulatory cytokine that potently inhibits the production of pro-inflammatory cytokines such as IL-1, IL-12 and TNFα. Therefore, IL-10 expression is usually associated with inflammatory response (Moore et al., 2001). However, in some studies, the production of this cytokine has been stimulated by the virus as a mechanism of evasion of the host immune response (Slobedman et al., 2009). In this work, the IL-10 expression profile is quite variable; it slightly increased at the beginning of infection in some of the animals (Table S1). Its expression could well be stimulated by the virus as a viral mechanism of immune evasion.

IFNγ, IL-2, IL-4 and IL-10 are important regulators of the adaptive immune response; more specifically, they influence the balance between helper T lymphocytes LTh 1 CD4+ and LTh 2 CD4+. They are secreted mainly by helper T lymphocytes (LTh CD4+), and the division of these cells into LTh 1 CD4+ (IL-2 and IFNγ) and LTh 2 CD4+ (IL-4 and IL-10) cells is based on their cytokine profiles (Street and Mosmann, 1991). Sánchez-Matamoros et al. (2013) did not detect the IL-2, IL-4 and IL-10 mRNA expressions until day 7 PI in one of the experimentally AHSV 4 infected horses. In this work, detection of IFNγ, IL-2, IL-4 and IL-10 mRNA expression was analyzed up to day 12 PI. However, their mRNAs were not detected. This result is in line with the paper published by Pretorius et al. (2012), who described a reduced

CD4+ T-cell population which could correspond to the non-detection of cytokines secreted by LTh in this work. Nevertheless, antibodies against AHSV were detected in all animals at day 7 PI. The humoral response is related to an efficient response of B lymphocytes that is closely linked to LTh 2 CD4+ (Street and Mosmann, 1991) and which was not detected in this analysis. Nonetheless, other published studies about vaccination against AHS and BTV vaccination and infection have detected the association between enhanced IFNγ expression and LTc CD8+ populations (Channappanavar et al., 2012; El Garch et al., 2012; Umeshappa et al., 2010). By taking this into account, the decrease in LTh CD4+ population could be related to the low levels of cytokines secreted by LTh-1 CD4+ and LTh-2 CD4+ that were not detectable by this method (Sánchez-Matamoros et al., 2013). However, lymphocyte population analyses are necessary to obtain a better knowledge of adaptive responses to AHSV infection.

Summarizing, all the dead horses presenting the mixed form showed an immune response that was mainly characterized by the lack of TNFα production. In addition, all animals showed an activation of the humoral immune response by antibody production at day 7 PI, but it was not enough for the horses to survive. Therefore, in this study, the humoral response, when combined with an absence of TNFα, does not play a sufficient defensive role in the infected animals showing the mixed clinical form. Regarding the cardiac form, the surviving horse presented IL-1, IL-12 and TNFα expressions and a humoral response. This profile was similar to the surviving horses with the fever form, which showed expression of IL-1 and TNFα and a humoral response. The innate immune response appears to be responsible for controlling early AHSV infection until specific adaptive immunity is developed. The studied adaptive immune response seems to reveal the important role of TNFα together with antibodies in obtaining an effective host immune response. However, further analyses to completely characterize the whole immune response in each clinical form of the disease are needed.

5. Conclusions

This is the first study to evaluate innate and adaptive immunity in horses infected with two serotypes of AHSV by molecular and immunological techniques, such as RT-real time PCR and two serological tests (ELISA and LFA test). These results provide new data about the immune response in naïve horses infected with AHSV, and they might be useful to help improve different treatments and vaccines as control and prevention methods. In conclusion, the experimental findings show that there is not a complete association between the clinical form of the disease and the cytokine production pattern. Each horse presented a different cytokine expression profile, although some common characteristics were identified. Consequently, the characterization of the immune response needs further study, such as of flow cytometry of immune cell populations, in order to understand the animals' complete defense mechanisms against disease.

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Table S1. The average relative gene expression of each cytokine in horse blood samples stimulated with AHSV 4 and AHSV 2 at different times [days post-infection (PI)]. The results are reported as the n-fold difference in relation to the cytokine mRNA expression of calibrator sample 0 PI. Horse 1, horse 2, horse 3, horse 4 and horse 5 were infected with AHSV 4; horse A, horse B, horse C and horse D were infected with AHSV 2.

		AHSV 4						AHSV 2			
		1	2	3	4	5		A	B	C	D
IL-1	*0 PI*	1	1	1	1	1	*0 PI*	1*	1*	1	1
	3 PI	1.98	20.28	22.94	15.68	3.94	*2 PI*	0.86	0.17	10.94	7.53
	5 PI	0.90	3.17	*	0.96	1.25	*4 PI*	0.11	1.46	18.45	1.16
	7 PI	0.57	4.86	3.50	4.43	4.86	*7 PI*	0.41	0.69	12.68	0.63
	10 PI	0.26	5.31	1.14	2.07	8.26	*9 PI*	1.43	0.43		1.86
	12 PI	0.15	3.63	0.50	1.19	4.38	*11 PI*	3.49			3.12
TNFα	*0 PI*	1	1	1	N.A	N.A	*0 PI*	1*	1*	1	1
	3 PI	9.93	9.44	52.09	N.A	+ (*36.4*)	*2 PI*	N.A	N.A	0.01	1.03
	5 PI	2.56	11.19	*	N.A	+ (*36.2*)	*4 PI*	19.93	N.A	N.A	N.A
	7 PI	2.14	13.30	15.58	N.A	N.A	*7 PI*	37.52	0.15	0.14	4.06
	10 PI	3.72	31.24	12.08	N.A	N.A	*9 PI*	30.60	0.41		N.A
	12 PI	0.96	4.01	7.06	N.A	+ (*38.2*)	*11 PI*	21.34			N.A
IL-12	*0 PI*	1	1	1	1	1	*0 PI*	N.A	1*	1	N.A
	3 PI	0.58	4.10	155.83	N.A	0.88	*2 PI*	+ (*38.1*)	N.A	0.00	+ (*35.6*)
	5 PI	0.50	5.11	*	1.25	0.24	*4 PI*	+ (*36.2*)	N.A	0.00	N.A
	7 PI	0.42	5.10	79.57	N.A	0.11	*7 PI*	+ (*36.6*)	0.43	0.04	+ (*37.4*)
	10 PI	0.48	7.65	36.14	6.37	0.98	*9 PI*	+ (*37.2*)	1.14		+ (*35.8*)
	12 PI	0.62	3.25	30.43	0.52	0.77	*11 PI*	+ (*35.6*)			N.A
IL-10	*0 PI*	1	N.A	1	N.A	N.A	*0 PI*	N.A	N.A	1	N.A
	3 PI	2.67	+ (*34.2*)	2.1	+ (*34.2*)	+ (*34.4*)	*2 PI*	N.A	+ (*37.1*)	1.75	N.A
	5 PI	0.41	+ (*32.9*)	*	+ (*35.3*)	N.A	*4 PI*	+ (*34.4*)	N.A	2.11	N.A
	7 PI	0.38	+ (*35.8*)	0.48	N.A	N.A	*7 PI*	N.A	N.A	N.A	N.A
	10 PI	0.40	N.A	1.02	N.A	N.A	*9 PI*	+ (*35.0*)	+ (*33.8*)		N.A
	12 PI	0.53	N.A	0.45	N.A	N.A	*11 PI*	+ (*38.0*)			N.A
IFNγ	*0 PI*	1	1	1	N.A	1	*0 PI*	N.A	N.A	N.A	N.A
	3 PI	0.40	8.53	0.51	N.A	N.A	*2 PI*	N.A	+ (*36.3*)	N.A	N.A
	5 PI	0.25	3.29	N.A	+ (*34.5*)	0.34	*4 PI*	N.A	N.A	N.A	N.A
	7 PI	0.47	2.48	0.16	N.A	N.A	*7 PI*	N.A	N.A	N.A	N.A
	10 PI	0.29	N.A	0.40	N.A	0.64	*9 PI*	N.A	N.A		N.A
	12 PI	0.08	1.12	0.05	+ (*34.7*)	1.52	*11 PI*	N.A			N.A
IL-2	*0 PI*	N.A	N.A	N.A	N.A	N.A	*0 PI*	N.A	N.A	N.A	1
	3 PI	N.A	N.A	N.A	N.A	+ (*38.9*)	*2 PI*	N.A	N.A	N.A	N.A
	5 PI	+ (*35.6*)	N.A	N.A	N.A	+ (*37.5*)	*4 PI*	N.A	N.A	N.A	N.A
	7 PI	N.A	N.A	N.A	N.A	N.A	*7 PI*	N.A	N.A	N.A	1.19
	10 PI	N.A	N.A	N.A	N.A	N.A	*9 PI*	N.A	N.A		N.A
	12 PI	N.A	N.A	N.A	N.A	N.A	*11 PI*	N.A			N.A
IL-4	*0 PI*	1	N.A	N.A	N.A	N.A	*0 PI*	N.A	N.A	N.A	N.A
	3 PI	N.A	N.A	+ (*34.4*)	+ (*36.1*)	+ (*30.0*)	*2 PI*	N.A	N.A	N.A	N.A
	5 PI	N.A	N.A	*	+ (*34.3*)	+ (*35.3*)	*4 PI*	N.A	N.A	N.A	N.A
	7 PI	N.A	N.A	+ (*35.8*)	N.A	+ (*36.0*)	*7 PI*	N.A	N.A	N.A	N.A
	10 PI	N.A	N.A	N.A	+ (*34.5*)	N.A	*9 PI*	N.A	N.A		N.A
	12 PI	0.03	N.A	N.A	+ (*35.0*)	N.A	*11 PI*	N.A			N.A

Abbreviations:
*PI, post-infection.; N.A, not applicable (Ct of the sample does not cross the threshold); + (Ct), Positive samples (Ct of the sample crosses the threshold) with no measure of the n-fold difference because the calibrator sample 0 PI does not show expression levels (Ct of the sample does not cross the threshold); * Poor quality RNA.*

OBJETIVO 2: Desarrollo de nuevas técnicas de diagnóstico para la peste equina.

El **diagnóstico laboratorial** de la PEA es una de las herramientas **fundamentales** para confirmar la presencia de la enfermedad, lo que es de especial importancia en zonas no endémicas, así como para el control de los movimientos de animales evitando la difusión de la enfermedad. Por ello, las técnicas laboratoriales usadas para este fin, tanto directas como serológicas, deben ir adaptándose a los **nuevos retos** que se exijan desde los programas de vigilancia y control de esta enfermedad. Actualmente, la mejora del diagnóstico de la enfermedad va encaminada hacia una **mayor rapidez, el incremento de la sensibilidad** y la **diferenciación de animales vacunados de infectados.** Otro aspecto importante es disponer de técnicas que permitan la **detección en condiciones de campo** de los animales infectados y/o el **diagnóstico simultáneo** de varios agentes; para lo cual es necesario recurrir a las **nuevas tecnologías** disponibles en el mercado.

En este contexto se ha enfocado el **segundo objetivo** de la tesis doctoral, el cual consiste en el desarrollo de **nuevas técnicas de diagnóstico para la PEA**. Concretamente, se han desarrollado dos técnicas serológicas usando la novedosa **tecnología Luminex®**, la cual es una técnica *multiplex* que permite el análisis de hasta **100 ligandos distintos simultáneamente en el mismo pocillo**, basándose en la identificación de 100 microesferas diferentes, específicamente acopladas a los ligandos buscados, mediante la utilización de los principios de citometría de flujo, óptica y procesamiento de señales digitales (Angeloni et al., 2013).

El objetivo inicial surgido del empleo de esta tecnología era **alcanzar una mejora en la sensibilidad** de la detección de anticuerpos frente a la proteína VP7 del virus en animales infectados. Para ellos, se decidió usar una proteína VP7 recombinante, lo que permitiría comparar la nueva técnica con otras ya existentes consideradas como convencionales. La capacidad de diagnóstico de este nuevo ensayo fue evaluada y comparada con un cELISA y un LFA comerciales de detección de anticuerpos mediante el análisis de un panel de 300 sueros. La nueva técnica desarrollada permitió la detección de los diferentes serotipos de vPEA presentando sensibilidades superiores al cELISA y similares al LFA. Además, se realizó el análisis de la cinética de la producción de anticuerpos en animales experimentalmente infectados, permitiendo la detección de los mismos a 7 dpi. Así mismo, se validó la posible utilización de la técnica con muestras de campo de animales infectados de manera natural.

Con estos resultados tan prometedores se decidió adaptar este ensayo al diagnóstico múltiple, respondiendo a la necesidad de **diagnósticos DIVA** de la PEA. Para ello partimos de los conocimientos adquiridos en el desarrollo de vacunas inactivadas frente al serotipo 4 para diferenciar animales vacunados e infectados, analizando la presencia de anticuerpos frente a las proteínas VP7 y NS3 del virus. Se evaluaron muestras de animales experimentalmente infectados con este serotipo y animales vacunados con la vacuna inactivada. Los resultados obtenidos permitieron realizar la diferenciación entre anticuerpos producidos frente a ambas proteínas, así como analizar la cinética de la respuesta de los mismos simultáneamente, detectándose de manera más temprana los anticuerpos frente a la VP7 que frente a la NS3.

Estos dos ensayos son los **primeros puestos a punto con la tecnología Luminex®** para el diagnóstico múltiple de PEA, siendo posible su adaptación en el futuro al estudio serológico de la respuesta humoral contra múltiples antígenos del virus en un único test de gran utilidad en el desarrollo de vacunas, serotipado de los animales o diferenciación de otros tipos de vacunas.

Además, con el fin de mejorar el **diagnóstico en campo** de la enfermedad hemos trabajado en la validación de **ensayo de flujo lateral (LFA) de captura de antígeno y anticuerpo**. Los resultados de estas nuevas técnicas laboratoriales no se incluyen en esta tesis doctoral pero han sido presentadas en congresos internacionales.

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Development of a microsphere-based immunoassay for serological detection of African horse sickness virus and comparison with other diagnostic techniques.

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Abstract

African horse sickness (AHS) is a viral disease that causes high morbidity and mortality rates in susceptible *Equidae* and therefore significant economic losses. More rapid, sensitive and specific assays are required by diagnostic laboratories to support effective surveillance programmes. A novel microsphere-based immunoassay (Luminex assay) in which beads are coated with recombinant AHS virus (AHSV) structural protein 7 (VP7) has been developed for serological detection of antibodies against VP7 of any AHSV serotype. The performance of this assay was compared with that of a commercial enzyme-linked immunosorbent assay (ELISA) and commercial lateral flow assay (LFA) on a large panel of serum samples from uninfected horses (n = 92), from a reference library of all AHSV serotypes (n = 9), from horses experimentally infected with AHSV (n = 114), and from West African horses suspected of having AHS (n = 85).

The Luminex assay gave the same results as ELISA for all non-infected samples and positive controls detecting all serotypes. In contrast, the Luminex assay detected a higher rate of anti-VP7 positivity in the West African field samples than did ELISA or LFA. The Luminex assay detected anti-VP7 positivity in experimentally infected horses at 7 days post-infection, compared to 13 days for ELISA. This novel immunoassay provides a platform for developing multiplex assays, in which the presence of antibodies against multiple ASHV antigens can be detected simultaneously. This would be useful for serotyping or for differentiating infected from vaccinated animals.

Keywords: African horse sickness; Antibody detection; Bead-based multiplex immunoassay; Diagnosis; Serology.

Introduction

African horse sickness (AHS) is a highly fatal vector-borne disease of horses. Its severity and rapid spread led to its registration as a World Organization for Animal Health (OIE)-listed disease (OIE, 2012). While AHS is endemic in sub-Saharan Africa, the virus has repeatedly spread beyond this region, causing an important socio-economic impact in affected countries (Portas et al., 1999; Sánchez-Vizcaíno, 2004). Presently the disease is limited to endemic areas. However, the rise of the vector distribution due to climate change (Maclachlan and Guthrie, 2010), the increasing international equidae movements (FEI, 2012), and the potentially devastating consequences of disease introduction into previously disease-free areas have led to increased awareness of the risk of AHS in Europe. In this context, rapid and effective diagnostic techniques are required for early detection. This allows the potentially affected area to organise adequate control measures to avoid AHS spread.

AHS is caused by AHS virus (AHSV), a member of the family *Reoviridae* and genus *Orbivirus* for which nine serotypes have been described (AHSV1-9). The viral genome consists of 10 segments of double-stranded RNA encoding 11 proteins (Grubman and Lewis, 1992; Roy et al., 1994). The outer layer of the viral particle contains seven structural proteins (VP1 to VP7). VP7 is the major outer core protein and group-specific antigen. Therefore, this protein is highly conserved among all AHSV serotypes and is the target protein in several AHS diagnostic assays (Chuma et al., 1992; OIE, 2012).

The OIE officially recognises various serological techniques to detect AHS-related antibodies, including complement fixation, the serum neutralisation test (SNT) and enzyme-linked immunosorbent assays (ELISA) (OIE, 2012). More recently, the lateral flow assay (LFA) has been developed. The Luminex assay is a recent modification of conventional ELISA tests (Elshal and McCoy, 2006). The Luminex assay platform, based on fluorescent beads coated with one or more proteins to interact with target analyte(s), supports the development of multiplex diagnostic assays (Elshal and McCoy, 2006) (http://www.luminexcorp.com/). By allowing simultaneous assay of various targets, Luminex assays may reduce the time, labour, costs and sample volume needed for a more complete analysis over conventional test (Vignali, 2000). Evidence suggests that Luminex assays can be more sensitive than traditional immunoassays (van Gageldonk et al., 2008). As a result, Luminex assays have already been applied to diagnosis of various veterinary diseases (Christopher-Hennings et al., 2013).

The aim of the present study was to develop a sensitive and specific Luminex assay to detect anti-VP7 antibodies in horse serum. The method was validated in horses experimentally infected with two AHSV serotypes and in field samples from West Africa. The performance of this novel assay was compared with that of two commercial serological kits for detection of anti-VP7 antibodies.

Material and methods

Samples

A total of 300 horse serum or plasma samples were evaluated in this study. Of this total, 92 serum samples were from uninfected animals (negative controls), comprising 61 from uninfected horses from AHSV-free areas and 31 from uninfected horses previously used in AHSV challenge studies. A set of nine serum samples were reference sera for each of the nine AHSV serotypes, provided by the Pirbright Institute (UK). A set of 114 serum samples were from specific pathogen-free horses experimentally infected with AHSV2 and AHSV9 as described in section 2.2. Finally, a set of 85 serum and plasma samples were ones that had been sent to ANSES (Maisons-Alfort, France) from the Ivory Coast in 2012 (n = 52 serum samples) and from Cameroon in 2013 (n = 33 plasma samples) because the animals were suspected of having AHS

Experimental infection with ASHV

Experimental infections were performed at Zoetis Manufacturing & Research Spain S.L. (Olot, Spain) in accordance with Spanish and European regulations on animal welfare (RD 53/2013, Directive 2010/63/UE). The studies performed at these facilities were approved by the Ethical and Animal Welfare Committee of Zoetis.

In the first study, nine horses were inoculated intravenously with 2 mL of AHSV9 (10^6 or 10^7 $TCID_{50}$), and one horse was used as a negative control. Serum samples were collected on 0, 3, 5, 7, 10, 13, 17, 20, 24, 27 and 32 days post-infection (dpi). In the second study, six horses were

inoculated intravenously with 2 mL of AHSV2 (10^5, 10^6 or 10^7 $TCID_{50}$), and one horse served as a negative control. In contrast to the animals infected with AHSV9, animals infected with AHSV2 were sampled weekly basis (on 0, 7, 14, 21 and 30 dpi). Successful infection was defined as viraemia based on RT-PCR performed as described (Fernandez-Pinero et al., 2009). A total of 114 samples, collected between 3 and 32 dpi, were tested by Luminex assay.

Labelling of microspheres with AHSV VP7 antigen

Recombinant VP7, produced and purified by INGENASA (Madrid, Spain) as described (Chuma et al., 1992), was used as the antigen in the Luminex assay. Before the purified protein was coupled to the Luminex microspheres, it was dialysed in phosphate-buffered saline (PBS) to enhance coupling efficiency.

The amine-based coupling assay of VP7 to microspheres was performed using the Bio-Plex Amine Coupling Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. A typical coupling reaction at 100-µL scale was conducted as follows: 100 µL of carboxylated, fluorescent magnetic microbeads (1.25×10^6 beads; bead region 37, Bio-Plex Pro Magnetic COOH Beads, Bio-Rad Laboratories) were transferred to a 1.5-mL coupling reaction tube and washed twice with bead-wash buffer. After carefully removing the supernatant, the beads were resuspended in 80 µL of bead activation buffer. To activate the beads, 10 µL of freshly prepared N-hydroxysulfosuccinimide sodium salt (S-NHS; 50 mg/mL, Sigma–Aldrich) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; 50 mg/mL, Sigma–Aldrich) were added to the bead suspension, and the mixture was gently vortexed. The beads were incubated for 20 min with rotation. Activated beads were washed twice with 150 µL of PBS (pH 7.4), resuspended in purified VP7 solution at a final concentration of 1–100 µg/mL VP7, and incubated for 2 h with rotation. The coupled beads were washed once with 500 µL of PBS (pH 7.4) and resuspended in 250 µL of blocking buffer, followed by gentle vortexing. The VP7-coupled microspheres were resuspended in 150 µL of storage buffer, counted, and stored at 2–8 ºC in the dark.

Luminex assay procedure

All serum and plasma samples were first inactivated for 30 min at 56 ºC ± 1 ºC. Coupled beads were diluted to a final concentration of 25 beads/µL in assay buffer consisting of PBS (pH 7.4) containing 10% goat serum (MPBiomedicals) and 0.02% Tween-20. To each well of Bio-Plex Pro flat-bottom plates (Bio-Rad Laboratories), 50 µL of bead suspension (1250 beads) was added to 50 µL of serum sample diluted 1:100. After 30 min incubation with agitation, plates were washed three times with PBS supplemented with 0.05% Tween-20 (PBST). Then 50 µL of biotin-conjugated goat anti-horse IgG (1:2000 dilution; Jackson Immuno Research) was added to each well. Plates were incubated for 30 min on a plate shaker, then washed three times, after which 50 µL of streptavidin R-phycoerythrin conjugate (SAPE; 1 µg/mL, Qiagen) was added to each well. After 15 min incubation and three washes, beads were resuspended in 125 µL of sheath fluid (Luminex Corporation) and analysed using a Bio-Plex 200 system (Bio-Rad Laboratories).

All samples were tested in duplicate in two independent assays and the median fluorescence intensity (MFI) of the reporter signal from at least 50 beads/well was used for data analysis. The results obtained from different assays were normalised by dividing the MFI from the test in question (T) by the MFI of the positive control (PC), then multiplying by 100 to give the T/PC ratio. Dilutions of serum samples and the secondary antibody were optimised by performing, in duplicate, a two-dimensional titration matrix with positive and negative control samples at dilutions ranging from 1:25 to 1:200 and with secondary antibody at dilutions from 1:500 to 1:8000.

Table 1. Comparison of the limits of detection (LOD) by Luminex assay and ELISA in dilution factor of one positive sera controls of each serotype.

	SNT	Luminex assay			ELISA kit		
	Titer of the sample	T/PC ratio of the sample	LOD of the sample	T/PC ratio in the LOD	BP of the sample	LOD of the sample	BP in the LOD
AHSV 2	32	120.8	64	8.4	66	4	53
AHSV 9	128	128.3	128	5.84	68	4	52

SNT: Serum neutralization test; Luminex assay: VP7 Luminex assay; commercial ELISA kit: INgezim AHSV compac plus 14.AHS.K.3; LOD: limit of detection as lowest dilution factor; BP: blocking percentage (commercial ELISA kit).

Each plate contained a high positive internal control, which had an MFI of approximately 8,500 and a T/PC ratio of 100, and a negative internal control, which had an MFI of approximately 35 and a T/PC ratio of 0.41. Each plate also included controls for secondary antibody and SAPE. Samples that did not yield unambiguously negative or positive results were re-tested.

Luminex assay performance characterization: cut-off for positivity, sensitivity, specificity, reproducibility and limit of detection

The cut-off value for positivity was determined using MedCalc 12.5.0.0 (MedCalc Software, Ostrend, Belgium). The sensitivity and specificity of the Luminex assay were calculated using receiver operator characteristic (ROC) analysis and the following equations:

sensitivity = 100 x [true positives / (true positives + false negatives)]

specificity = 100 x [true negatives / (true negatives + false positives)]

Intra-assay (within-plate) and inter-assay variation were calculated. Intra-assay variation was defined as the mean coefficient of variation percentage (CV%) for 20 samples measured in triplicate. Inter-assay variation was assessed by measuring five plates, each containing 10 samples. The limit of detection (LOD), defined as the lowest dilution of a sample that can be detected above the cut-off value, was determined using two-fold serial dilutions of one positive control serum obtained from experimental infection with each serotype. These positive controls were also evaluated by SNT using the procedure already described (OIE, 2012).

Comparison of Luminex assay with commercial assays

The results of the Luminex assay were compared with those from two commercially available immunoassays (Ingenasa Laboratory): the INgezim AHSV compac plus 14.AHS.K.3, a blocking ELISA kit that uses VP7-coated wells to detect anti-VP7 antibodies in samples; and INgezim AHSV Crom 1.4.AHS.K.41, an LFA based on immunochromatography, which is capable of detecting specific anti-VP7 antibodies. The ELISA kit is recommended for use in international trade by both the OIE (OIE, 2012) and European Commission (Council Directive 90/426/EEC) because of its high sensitivity and specificity. Both methods were carried out according to the manufacturer's instructions.

Samples that gave different results in the Luminex and ELISA tests were also analysed using an OIE-recommended AHSV SNT (OIE, 2012). In this study, the SNT was performed with ASHV serotypes 2, 7 and 9, which are the most prevalent serotypes in the area (Bachanek-Bankowska et al., 2014; Diouf et al., 2013).

Agreement between the results of the Luminex assay and commercial ELISA and LFA was assessed using kappa statistics and the criteria of Landis and Koch (1977). McNemar's test for pair-wise comparisons was used to determine whether proportions of positive samples differed significantly between assays. Statistical analyses were performed using MedCalc 12.5.0.0.

Results

Optimisation of VP7 coupling to Luminex microspheres

The coupling efficiency of different amounts of recombinant VP7 to microspheres was assessed, and the highest MFI value was obtained when 5 µg of VP7 was used. Two-dimensional titration matrix tests indicated that the optimal dilution factor was 1:100 for serum and 1:2000 for secondary antibody (Figure 1).

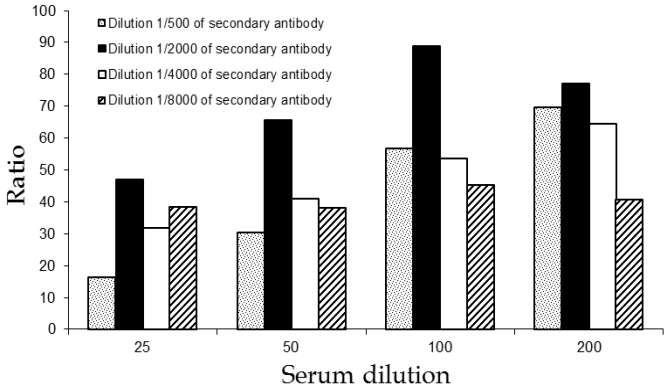

Fig. 1. Two-dimensional titration matrix to determine dilutions of horse serum and of horse secondary antibody that maximise discriminatory power between positive and negative samples in the Luminex assay. The ratios refer to the mean MFI of the positive controls divided by the mean MFI of the negative controls.

Determination of cut-off value for anti-VP7 antibodies positivity in the Luminex assay

A total of 92 negative control samples and 61 positive control samples, comprising 52 positive sera from experimentally infected horses and nine reference sera, which were previously assayed by ELISA, were used to determine the cut-off value of Luminex assay. ROC analysis was used to determine a T/PC ratio of 4.1, equivalent to an MFI of 350, as the cut-off value for positivity (Figure 2). Serum samples with a T/PC ratio above 4.1 were considered positive, while those with a ratio below 4.1 were considered negative.

Sensitivity and specificity of the Luminex assay

The sensitivity of the Luminex assay was measured on 113 positive control samples that tested positive in the ELISA. These samples comprised 9 reference sera, 52 sera from experimentally infected horses and 52 samples from naturally infected horses. All these samples also tested positive in the Luminex assay.

The specificity of the Luminex test was measured on 31 negative control samples from uninfected animals from challenge experiments and 61 negative samples from uninfected horses from AHS-free areas. All 92 samples tested negative in the Luminex assay.

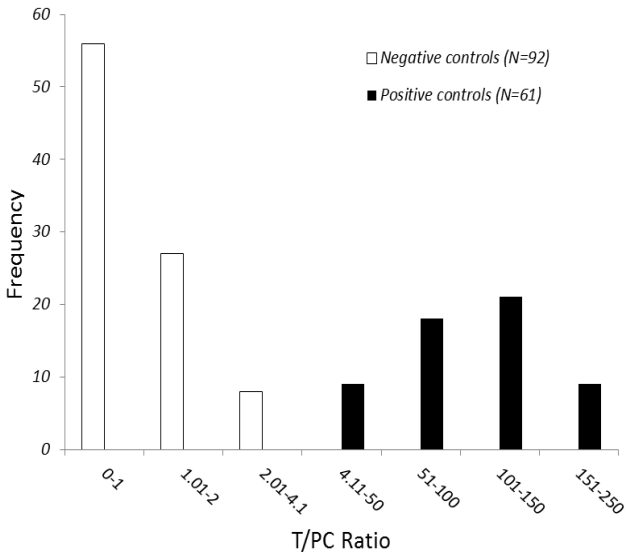

Fig. 2. Negative and positive control samples were assessed against African horse sickness virus structural protein 7 by the Luminex assay to determine Luminex cut-off value (T/PC ratio). The cut-off value was determined to be 4.1.

Reproducibility of the Luminex assay

Intra- and inter-assay variability of the Luminex procedure was measured, and the mean intra-assay CV% was 2.56%. Inter-assay variation was measured by comparing the results for the positive and negative controls in five separate runs, which gave a mean CV% of 8.5%.

Limit of detection of the Luminex assay

Samples positive for AHSV2 or AHSV9 were tested in appropriate SNTs and found to show high neutralising antibody titers of 1:32 and 1:128, respectively (Table 1). These samples were then analysed by both Luminex assay and ELISA to compare the limits of detection. For both serotypes, the Luminex assay showed a lower limit of detection (Table 1).

Antibody response in horses experimentally infected with AHSV

A panel of 145 sera sampled from 15 horses following experimental infection with AHSV2 or AHSV9 was analysed using the Luminex assay, ELISA and LFA. The results of all three methods were compared (Figure 3, Table 2).

In the first experiment, nine horses were inoculated with AHSV9. In 1 of these animals, the Luminex assay detected anti-VP7 antibodies as early as 7 dpi. All horses were identified as being positive at 17 dpi. The LFA yielded comparable results than Luminex assy, although not all animals were positive until 20 dpi. The ELISA did not detect antibodies in any of the animals until 13 dpi, and antibodies were not detected in all animals until 32 dpi. Kappa analysis showed nearly complete agreement between LFA and our Luminex assay on the kinetics of antibody response (kappa = 0.91, 95%CI 0.83 to 0.99). However, the kinetics were significantly different between the ELISA and Luminex assay (kappa = 0.63, 95%CI 0.49 to 0.77; McNemar test = 18.35%, P<0.0001).

In the second experiment, six horses were inoculated with AHSV2. In the Luminex assay, seroconversion was detected in all animals by 14 dpi, and MFI increased up to 30 dpi. The ELISA test gave similar results, whereas LFA detected an antibody response in one animal at 7 dpi and seroconversion in all animals by 14 dpi. All three assays showed nearly complete agreement (kappa = 0.94, 95%CI 0.83 to 1.00).

Field samples from horses of unknown AHSV exposure

Analysis of 85 samples from the Ivory Coast and Cameroon showed the presence of AHSV based on all three assay platforms (Table 2), though the assays gave different prevalence results. The prevalence of antibodies against VP7 among Ivory Coast samples was 57.7% (30/52) for the Luminex assay, 48.1% (25/52) for ELISA and 46.1% (24/52) for LFA. The corresponding values for anti-VP7 antibodies among Cameroon samples were 87.9% (29/33) for the Luminex assay and 81.8% (27/33) for ELISA. LFA did not provide reliable results for 39.4% (13/33) of samples, consistent with the fact that the kit manufacturer does not recommend this method for plasma. Based on the results for both Ivory Coast and Cameroon together, the Luminex assay and ELISA showed nearly complete agreement (kappa = 0.82, 95%CI 0.70 to 0.95).

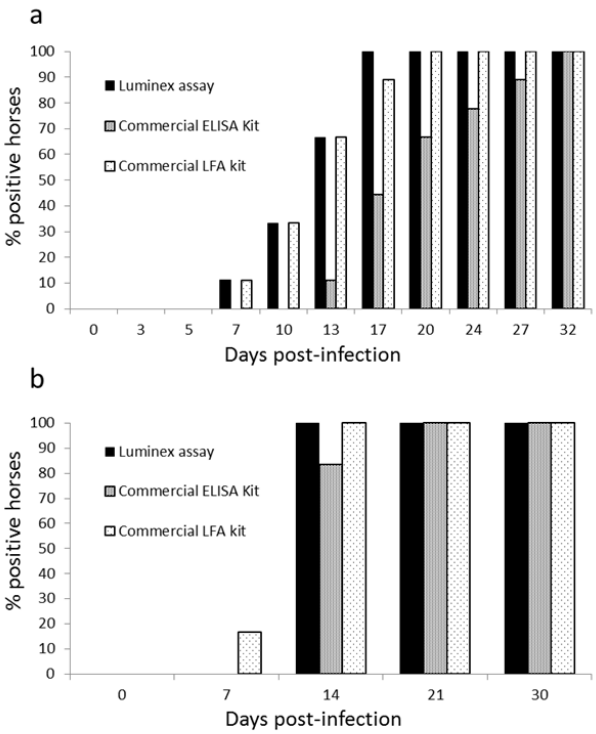

Fig. 3. Proportions of samples testing positive for anti-VP7 antibodies in different VP7-based immunoassays at different times following experimental inoculation with either AHSV9 (a) or AHSV2 (b). Control animals did not show an antibody response at any of the different infection times. The VP7 Luminex assay was compared with a commercial ELISA kit (INgezim AHSV compac plus 14.AHS.K.3) and a commercial LFA kit (INgezim AHSV Crom 1.4.AHS.K.41).

Of the 85 plasma samples, 7% (6/85) gave different results in the Luminex assay and ELISA (Table 2), and these samples were analysed further by SNT. One sample that tested positive in the Luminex assay but negative in ELISA showed low titers of AHSV neutralising antibodies, with results varying from 1:2 to 1:4 for the three AHSV serotypes tested. The remaining five sera tested positive in the Luminex assay but negative in SNT.

Discussion

Global warming and the international livestock trade have increased the risk of unexpected outbreaks of animal diseases, including AHS (Maclachlan and Guthrie, 2010). Therefore, implementation of effective surveillance programmes based on reliable diagnostic methods and epidemiological studies are essential to achieve the most efficient AHS detection. A laboratory diagnosis is indispensable to confirm the disease and to establish appropriate health control measures (OIE, 2012). This study describes the first Luminex assay for rapid, sensitive detection of antibodies against VP7 of all AHSV serotypes. Comparison of this method with an OIE-recommended commercial ELISA kit and a commercial LFA kit on a diverse panel of 300 horse serum and plasma samples suggests that the Luminex method may be more sensitive than the commercial tests and similarly specific as ELISA.

In the last few years, Luminex technology has already proven useful for diagnosis of some animal diseases thanks to its numerous advantages (Christopher-Hennings et al., 2013; Gimenez-Lirola et al., 2012), and here we extend its usefulness to diagnosis of AHSV infection in horses. The novel developed Luminex assay offers several advantages over ELISA. It requires a smaller amount of viral antigen (5 µg VP7 per 1.2 x 10^6 beads, enough for approximately 100 plates) and a smaller amount of serum sample (1:100 dilution). The Luminex assay also shows better sensitivity than ELISA for the same samples, using the same protein. This difference may reflect the fact that the Luminex beads interact with analyte in liquid suspension instead of relying on analyte adsorption on a surface, which may allow faster hybridisation and increase effective binding capacity (Chen et al., 2013; Gimenez-Lirola et al., 2012; Kellar and Iannone, 2002). The Luminex assay showed high reproducibility, specificity and sensitivity with both serum and plasma samples,

allowing reliable calculation of a cut-off value for positivity.

This study assessed the performance of the Luminex assay on experimental samples, allowing us to examine the kinetics of the antibody response and compare it with results obtained using the two commercial kits. The three serological tests showed similar kinetics of anti-VP7 antibody response for serum from AHSV2-infected horses sampled weekly; seroconversion was detected in most infected horses within 14 dpi. However, significant differences between tests were observed in the case of experimental AHSV9 infection, when samples were taken every 3 days. LFA and the Luminex assay detected anti-VP7 antibodies within 7 dpi, while ELISA did not detect antibody response until 13 dpi. Both these results are consistent with previous studies reporting the appearance of specific antibodies at 8 or 12 dpi (Maree and Paweska, 2005; OIE, 2012). The Luminex assay demonstrated good sensitivity over the course of AHSV infection, revealing a rapid increase in antibody levels which could be quantified when a standard anti-VP7 were established. Indeed, the limit of detection was lower with the Luminex assay than with ELISA (Table 1). Given that the Luminex assay gave similar or occasionally better results than ELISA or LFA, and given that it allows more samples to be processed in parallel in smaller volumes, we suggest that it may be superior to conventional assays now on the market. This should be verified in larger studies because of the significant implications for controlling the spread of AHS, since ELISA is currently the recommended test for international trade. The Luminex assay may be a useful alternative to ELISA for mass serological screening in diagnostic and reference laboratories, while the LFA, which is much faster than the Luminex method and performs similarly, may be useful for rapid confirmation of diagnosis in the course of clinical suspicions of having AHS.

In addition to validating our Luminex method using experimental samples, we tested it on field samples that had been sent to the French AHSV reference laboratory ANSES because of clinical suspicion of AHS. The rate of positive field samples with the Luminex assay was 9.6% higher than with ELISA in the case of Ivory Coast samples and 6.1% higher in the case of Cameroon samples. The rate of positive samples with the Luminex assay was 11.6% higher than with LFA in the case of Ivory Coast samples. The different results for the Luminex assay and ELISA may reflect the lower limit of detection (higher sensitivity) of the former method (Table 1), which was confirmed when we used SNT to test the samples that gave different results by Luminex assay and ELISA. The Luminex assay was able to detect one sample with a low titer of neutralising antibodies that the ELISA missed, although neutralizing antibodies were not observed in all samples that gave different results in the two assays.

One possible explanation for why the Luminex platform detects antibodies that the SNT misses is because of differences in the antigenic specificities of the antibodies in the two methods, therefore the SNT could not detect small amounts of anti-VP7 antibodies non-neutralizing (although evidenced by Luminex). The Luminex assay detects antibodies against all VP7 epitopes,

Table 2. Number of positive samples (P), number of negative samples (N) and total number of samples (T) tested for the detection of antibodies against African horse sickness virus structural protein 7 in samples from horses as determined by the Luminex assay, VP7 Luminex assay; commercial ELISA kit, INgezim AHSV compac plus 14.AHS.K.3 and commercial LFA kit, INgezim AHSV Crom 1.4 AHS.K.41.

	Luminex assay			ELISA kit			Kappa value	LFA kit			Kappa value
	P	N	T	P	N	T	(95% CI)	P	N	T	(95% CI)
AHSV 9	55	55	110	35	75	110	0.63 (0.49-0.77)	54	56	110	0.91 (0.83-0.99)
AHSV 2	18	17	35	17	18	35	0.94 (0.83-1.00)	19	16	35	0.94 (0.83-1.00)
Ivory Coast	30	22	52	25	27	52	0.77 (0.60-094)	24	28	52	0.77 (0.60-0.94)
Cameroon	29	4	33	27	6	33	0.76 (0.46-1.00)				
Total	132	98	230	104	126	230	0.74 (0.66-0.83)	97	100	197	0.90 (0.84-0.96)

including group-specific neutralising antibodies, while the AHSV SNT detects only antibodies viral neutralising epitopes, mainly against VP2. Similar discrepancies were observed when an ELISA to detect anti-VP7 antibodies was compared with SNT (Maree and Paweska, 2005b). A second but less probable explanation for the discrepancies among ELISA, LFA, SNT and Luminex assays could be that the Luminex method generates false positives. To identify the likely cause of these discrepancies, future studies should examine a larger number of positive and negative control samples from horses in various AHSV-endemic areas of Africa. These studies should also look more closely at the course of AHSV infection and kinetics of production of anti-VP7 and neutralizing antibodies.

Since our Luminex assay targets the most highly conserved protein of AHSV, it was able to detect anti-VP7 antibodies against all nine serotypes of the virus. This result should be considered preliminary, since only one serum sample of each serotype was tested. If verified, this serotype flexibility, coupled with the multiplex capacity of the Luminex assay, promises to extend the applicability of the method. It would be interesting to develop a Luminex multiplex assay to allow simultaneous detection of antibodies against VP7 and other AHSV proteins. This may allow the differentiation of infected from vaccinated animals (DIVA) (Laviada et al., 1995a), AHSV serotyping (Burrage et al., 1993; Manole et al., 2012a; Martinez-Torrecuadrada et al., 2001) and monitoring of antibody production during vaccine design (Chiam et al., 2009). Another possibility for a Luminex multiplex assay would be to include the detection of antibodies against other diseases that cause similar clinical signs as AHS, thereby generating a syndromic assay, or to include detection of antibodies against other OIE-listed horse pathogens, allowing robust differential diagnosis. Such a multiplex assay would save time, labour, reagents and sample because it would provide information about more than one protein or pathogen in a single reaction.

In conclusion, we have developed a rapid, specific and sensitive Luminex assay to detect anti-VP7 antibodies. This test shows good correlation with commercial ELISA and LFA methods on field samples as well as samples from horses experimentally infected with AHSV2 or AHSV9. The Luminex assay and LFA showed higher sensitivity than ELISA in our hands, suggesting them to be superior for laboratory diagnostics (Luminex) and clinical diagnostics (LFA). If the Luminex assay can be multiplexed, it may allow fast and reliable serotyping, DIVA, vaccination monitoring and differential diagnosis of look-alike diseases.

Conflict of interest statement

The authors declare that they have no conflict of interests.

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Development of a Luminex-based DIVA assay for serological detection of African horse sickness virus in horses

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Vaccine (2014), Enviado

Abstract

African horse sickness (AHS) is considered a fatal re-emergent vector-borne disease of horses. Since detecting AHS in AHSV-free countries may lead to restrictions on international animal movements and thereby cause significant economic damage, detection methods that can differentiate infected and vaccinated animals (DIVA assays) are crucial for reducing the movement restrictions. Here we describe a Luminex-based multiplex assay for DIVA diagnosis of AHS, and we validate it in a duplex format to detect antibodies against structural protein 7 (VP7) and non-structural protein 3 (NS3) in serum samples from horses vaccinated with an inactivated AHS virus (serotype 4) or infected with a live virus of the same serotype. Results of the Luminex-based assay for detecting anti-NS3 antibodies showed good positive correlation with results using an in-house enzyme-linked immunosorbent assay (ELISA). Thus, the Luminex-based technique described here may allow multiplex DIVA antibody detection in a single sample in less than 2 hours.

Keywords: African horse sickness; DIVA diagnosis; antibody detection; NS3; VP7; movement restriction

Introduction

African horse sickness (AHS) is one of the most economically devastating OIE-notifiable viral diseases in the equine industry. This fatal vector-borne disease is caused by nine serotypes of the AHS virus (AHSV1-9), a double-stranded RNA virus of the genus *Orbivirus* that encodes seven structural proteins (VP1-VP7) and at least three non-structural proteins (NS1-NS3) [1-3]. Recent European expansions of such vector-borne diseases as bluetongue or West Nile disease have increased concerns about AHS emergence and spread [4]. As a result, AHS notification carries the threat of international movement restrictions for animals and animal products, which can have devastating economic consequences for equine and associated industries [5].

In the absence of any effective treatment for AHS, vaccination remains one of the most effective interventions to combat the disease [6]. Commercially available live attenuated vaccines have allowed effective control of the disease in endemic areas, but the fact that they can produce adverse vaccine reactions or the possibility of reversion to virulence of vaccine virus or to be transmitted by vectors makes them unsuitable for use in non-endemic regions such as Europe [7]. To avoid these drawbacks, new vaccines have been developed as an alternative to attenuated vaccines [8-10], and this new generation of vaccines enables the development of diagnostic methods that differentiate infected from vaccinated animals (DIVA). One example of these DIVA vaccines is a purified, inactivated AHSV4 vaccine that does not induce antibodies against NS3 [10,11]. The use of such vaccines, combined with the development of reliable DIVA test against AHS, that can simultaneously detect antibodies against different AHSV antigens in a single sample, would allow the rapid assessment of AHSV status in individual horses, e.g. infection, vaccination or absence of infection.

Luminex-based assays are a promising technology allowing the simultaneous detection of multiple antibodies from a small volume of sample [12]. Luminex-based assays have been successfully used for DIVA diagnosis of various veterinary diseases [13,14]. A monoplex Luminex assay based on the most conserved protein of all AHSV serotypes, VP7, has been described for detecting anti-AHSV antibodies in horses [15]. In the present study, we extended this assay to include the simultaneous detection of antibodies against VP7 and NS3, thereby allowing DIVA diagnosis.

Material and methods

2.1. Serum samples

A total of 123 serum samples were evaluated, comprising 53 samples from horses experimentally infected with AHSV4 (ESP P2 BHK 120210), 50 samples from non-infected horses from AHSV-free countries (Spain in 2012 and France), and 20 samples from animals vaccinated with inactivated AHSV4 vaccine. This last group of 20 samples came from the serum bank managed by Prof. J.M. Sánchez-Vizcaino at the VISAVET Health Surveillance Centre. Of these 20 samples, 14 were obtained at 57 days post-vaccination.

Challenge with AHSV4 was performed in BSL-3 facilities (Zoetis Manufacturing & Research, Olot, Spain) in accordance with guidelines of Spain and Europe (RD 53/2013, Directive 2010/63/UE). The 14 horses experimentally infected with AHSV4 were intravenously inoculated with 2 ml of virus corresponding to 10^6, 10^7, or 10^8 $TCID_{50}$. One non-inoculated horse served as a negative control. Blood was drawn on days 0, 7, 14 and 21 post-infection (dpi).

2.2 Production of AHSV antigens

The VP7 protein used in the Luminex-based duplex assay was the same recombinant VP7 (Ingenasa Laboratory, Madrid, Spain) used as an antigen in the previously described Luminex-based monoplex assay [15]. NS3 protein from AHSV4 was produced in *Escherichia coli* BL21 as a fusion protein with a T7 tag [10], and purified using the T7•Tag® Affinity Purification Kit (Novagen) following the manufacturer's instructions. The protein was concentrated by ultrafiltration using an Amicon® Ultra-4 centrifugal filter (Millipore) with a 30-kDa molecular weight cut-off and resuspended in phosphate-buffered saline (PBS).

2.3 Coupling of AHSV antigens to carboxylated paramagnetic beads

Recombinant VP7 protein (5 µg) and recombinant NS3 protein (5 µg) were each covalently conjugated to the free carboxyl groups

on fluorescent carboxylated beads (bead regions 37 and 43, Bio-Plex Pro™ Magnetic COOH Beads, Bio-Rad Laboratories). Coupling was performed using the Bio-Plex® Amine Coupling Kit (Bio-Rad Laboratories) according to the manufacturer's instructions.

2.4 Luminex-based duplex assay

The assay was performed as previously reported [15]. Briefly, 50 µl of each bead suspension containing 1250 beads conjugated to recombinant VP7 or NS3 were added to each well of a Bio-Plex Pro™ flat-bottom plate. Each plate included positive, negative and secondary antibody controls. The positive control was a serum sample containing a median fluorescence intensity (MFI) of approximately 8500 for VP7 and 1200 for NS3; the negative control was a serum sample containing MFI of approximately 35 for VP7 and 100 for NS3.

2.5 Validation of the Luminex-based assay: determination of sensitivity, specificity, repeatability and cut-off value for positivity

Sensitivity and specificity of the Luminex-based duplex assay for detecting anti-NS3 antibodies in horse serum samples were measured using the 50 sera sampled from uninfected horses from AHSV-free areas, the 18 pre-inoculation samples from horses experimentally infected with ASHV4 or the uninfected horse used as a negative control, as well as from 25 samples from experimentally infected animals taken at 14 and 21 dpi. Receiver operating characteristic (ROC) analysis was conducted in order to determine the cut-off value for positivity. This analysis was carried out using MedCalc 12.5.0.0 (MedCalc Software, Ostend, Belgium).

Using the same approach, we determined the sensitivity and specificity of the Luminex-based duplex assay for detecting anti-VP7 antibodies. The cut-off value for positivity was taken from Sánchez-Matamoros et al. [15].

2.6 Comparison of Luminex-based duplex assay with ELISA detection

Anti-NS3 antibodies in horse serum samples were detected using an in-house, indirect enzyme-linked immunosorbent assay (ELISA) as described [10]. The results were compared with those obtained using the Luminex-based duplex assay; inter-assay agreement was assessed using kappa statistics.

Table 1. Performance of the Luminex-based duplex assay for the detection of antibodies against ASHV VP7 or NS3 antigens in horse serum.*

Parameter	Detection of anti-VP7 antibodies	Detection of anti-NS3 antibodies
Sensitivity	100% (25/25)	100% (25/25)
Specificity	100% (68/68)	95.6% (65/68)
Positive likelihood ratio	-	22.7
Negative likelihood ratio	0	0
Cut-off value (T/PC ratio)#	4.1	60
Repeatability		
Intra-assay	5%	5%
Inter-assay	10%	8%

** True positive samples (n = 25) were taken from experimentally infected horses at 14 and 21 days after challenge. True negative samples (n = 68) were taken from uninfected horses.*
 # T/PC ratio: MFI from the test sample (T) divided by MFI from the positive control (PC) and multiplied by 100.

Results and discussion

DIVA assays are essential tools for establishing adequate control measures against AHSV given extensive vaccination and the need to minimize the restriction of international animal movements to decrease one of the most significant economic losses associated with this disease [10]. A new generation of vaccines against AHSV, including vaccines based on inactivated purified viral proteins, have made possible DIVA diagnosis (e.g.[8-10]), and the Luminex platform is optimal for DIVA assays because it can test for multiple antigens in a single sample.

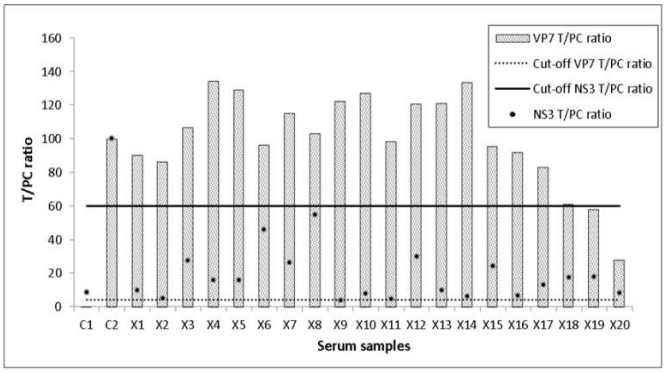

Fig. 1. Ratio of MFI from the test to positive control (T/PC ratio) in individual horse serum samples tested for the presence of antibodies against ASHV VP7 or NS3 using a Luminex-based duplex assay. Samples were taken from animals treated in the following ways: C1, internal negative control; C2, internal positive control; X1-X20, horses vaccinated with an inactivated AHSV4 vaccine. Internal negative control C1 showed low T/PC ratio values of approximately 0.41 for anti-VP7 antibody and 8.3 for anti-NS3 antibody. Internal positive control C2 showed a T/PC ratio of 100 for both types of antibodies. The assay showed an overall T/PC ratio of approximately 4.1 for anti-VP7 antibody and 60 for anti-NS3 antibody.

Here we describe a duplex Luminex-based assay for simultaneous detection of antibodies against ASHV4 VP7 or NS3 proteins. Analysis of serum samples from uninfected, experimentally infected and vaccinated horses suggests that the assay offers high sensitivity, specificity and reproducibility (Table 1). VP7 was selected as a viral antigen because it is the main target protein for group-specific diagnosis [1], while NS3, the second most variable AHSV protein, was selected because it is not expressed in horses vaccinated with inactivated AHSV4 vaccine, allowing DIVA diagnosis [11]. All animals vaccinated with inactivated AHSV4 vaccine in our study were positive for VP7 and negative for NS3 (Fig. 1). These findings suggest that our Luminex-based duplex assay may be appropriate as a DIVA assay for animals vaccinated with inactivated, subunit or recombinant AHSV4 vaccine.

Indirect or competitive ELISA tests have been published using both proteins as antigens [10,16-17], but ELISA assays often require several rounds of testing in order to measure the antibodies response against each protein, larger sample volumes and therefore more labour and time than multiplex assays. Comparison of results for our Luminex-based duplex assay and a conventional ELISA to detect anti-NS3 antibodies indicated good agreement (Kappa 0.953, 95%CI 0.887 to 1.000). In previous work, we showed good agreement between our Luminex-based assay and a commercial ELISA kit for the detection of anti-VP7 antibodies [15].

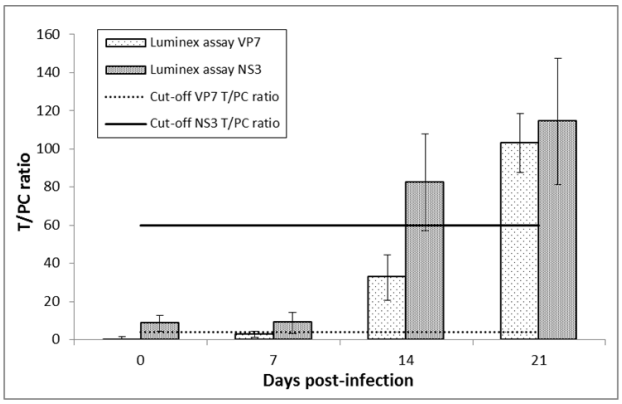

Fig. 2. Mean T/PC ratios in serum samples from horses experimentally infected with AHSV, as determined on different days after infection using a Luminex-based duplex assay to detect antibodies against VP7 or NS3 proteins. The negative control animal was negative for both types of antibodies at all time points after infection. Error bars refer to the standard deviation of the mean. Internal cut-off values for T/PC ratio were 4.1 in the case of anti-VP7 antibody and 60 in the case of anti-NS3 antibody.

The ease and speed of our Luminex-based duplex assay may facilitate monitoring of the kinetics of circulating anti-AHSV antibodies (Fig. 2). Experimentally infected horses in our study first became positive for anti-VP7 antibodies at 7 dpi, whereas they became positive for anti-NS3 antibodies at 14 dpi. By 14 dpi, all experimentally infected animals were positive for antibodies against VP7 and NS3. This delay in NS3 production may reflect the role of the protein at a later stage in the virus lifecycle, during virus replication and exit from the cell [18] or perhaps because NS3 is less immunogenic than VP7. These findings should be taken into account when quarantine tests are performed.

Our Luminex-based duplex assay gave false positive results for 3 of 68 (4.4%) uninfected animals from AHS-free regions (data not shown). In the conventional ELISA to detect anti-NS3 antibodies, 1 of the 68 samples (1.5%) gave a false positive result. These non-specific reactions may reflect the fact that the recombinant NS3 used to coat the beads in our Luminex-based assay or the plates in the ELISA was produced in *E. coli*. Some bacterially overexpressed viral antigens have been shown to give higher false positive rates than native antigens [19]. Whatever the cause of these false positives, the duplex format of our Luminex-based assay facilitated their detection, since all the false positive NS3 samples were negative for anti-VP7 antibodies. In case of NS3 positive and VP7 negative results, the sample would be considered as negative.

Large screening studies should be performed to validate our findings, to establish whether our Luminex-based duplex assay is useful as a DIVA assay for animals vaccinated with inactivated, subunit or recombinant AHSV4 vaccine and to optimize various aspects of the assay procedure, including when animals should be assayed after vaccination. The flexibility of this open diagnostic platform may allow the addition of more viral antigens and the use of other sample types such as oral fluid utilized for diagnosis of swine diseases.

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OBJETIVO 3: Análisis epidemiológico de la peste equina en España.

La distribución de la PEA está asociada a la presencia de los hospedadores de la enfermedad y sus vectores competentes que, simultáneamente, posibilitan la circulación y el mantenimiento la enfermedad. Esta compleja epidemiología, junto con el incremento del riesgo de entrada de la enfermedad en España, ha alarmado a las autoridades, siendo necesario el establecimiento de adecuados planes de vigilancia que permitan la prevención y control de la enfermedad. Actualmente estos programas de vigilancia deben estar basados en el riesgo de la enfermedad (risk-based surveillance), de modo que permitan la optimización de recursos, tanto desde el punto de vista logístico como económico. Por ello, para llevar a cabo esta tarea es fundamental conocer los **aspectos epidemiológicos** de la enfermedad en el país.

El tercer objetivo consiste en realizar un **análisis epidemiológico de la PEA en España** que permita mejorar los conocimientos de las zonas de mayor riesgo para la PEA (**objetivo 3.1**), así como la posible propagación de la enfermedad por movimientos animales (**objetivo 3.2**).

Objetivo 3.1: Zonas y periodos de mayor riesgo para la PEA en España.

Las **zonas y periodos que presentan un mayor riesgo** para la ocurrencia de la enfermedad fueron identificadas mediante la realización de un **modelo de decisión multicriterio** de epidemiología espacial que permitió la superposición de los principales **factores de riesgo de la enfermedad**: el periodo de incubación extrínseco, la densidad equina y la distribución de vectores competentes. El modelo localizó las **zonas más adecuadas** para las infecciones de vPEA en el sur-oeste de España, Cantabria y las Islas Baleares, especialmente en los meses estivales; mientras que las zonas que se identifican como **escasamente adecuadas** se localizan en Galicia, Castilla y León y La Rioja. Los resultados fueron validados con los brotes históricos de la PEA en España, y comparados con el área de distribución del vector culicoides y la LA. Los resultados del modelo, junto con las características de producción equina, deben proporcionar las bases para diseñar **estrategias de vigilancia basadas en riesgo** que permitan la **optimización de la relación coste-eficacia** de los programas sanitarios realizándose la detección temprana y rápido control de la difusión de la enfermedad en España.

Objetivo 3.2: Análisis de la red de movimientos equinos.

Los **movimientos de animales** son una de las vías de introducción y difusión de las enfermedades más frecuente entre países. Los équidos, por su diversidad de usos y naturaleza, son una de las especies animales más complejas en términos de movimientos animales, sin embargo, los registros de estos movimientos por lo general no están disponibles. Este estudio es la primera caracterización de una red completa y fiable de los movimientos de los équidos dentro de Castilla y León, una de las regiones más importantes de producción equina de España. El análisis de redes sociales y análisis de agrupaciones espacio-temporales han permitido analizar los patrones de contacto de la red equina e identificar los individuos, zonas y períodos de tiempo de mayor riesgo para la introducción o difusión de enfermedades en la región.

La **red de movimientos equinos dentro Castilla y León** es estructuralmente compleja. En ella intervienen diferentes tipos de explotaciones que se distribuyen prácticamente por todo el territorio y se realizan movimientos de diferente naturaleza y extensión en comparación con el resto de producciones ganaderas. Las medidas de centralidad permitieron identificar a las explotaciones más importantes de la red, explotaciones de producción y reproducción, y centros de competición de ganado. Además mediante el análisis de agrupaciones, se observaron diecisiete agrupaciones espacio-temporales significativas como origen y destino de los movimientos, que formaron cuatro compartimentos interconectados. Los resultados de este estudio pueden ser útiles para diseñar **programas de vigilancia basadas en el riesgo** de enfermedades de los équidos, aumentando la velocidad de detección y control de posibles brotes secundarios en futuras epidemias. En consecuencia, estos resultados ayudarán a **minimizar el gran impacto económico y sanitario** de los programas de vigilancia de las enfermedades de los équidos. El enfoque analítico utilizado en este estudio se puede extrapolar fácilmente para caracterizar los patrones de movimiento de équidos en otros países y regiones del mundo.

Artículos científicos:

- A. Sánchez-Matamoros, B. Martínez-López, F. Sánchez-Vizcaíno and J.M. Sánchez-Vizcaíno, 2013**. Social Network Analysis of Equidae Movements and Its Application to Risk-Based Surveillance and to Control of Spread of Potential Equidae Diseases**. Transboundary and Emerging Diseases Oct, 60(5), 448-459.

- A. Sánchez-Matamoros, J.M. Sánchez-Vizcaíno, V. Rodríguez-Prieto, E. Iglesias and B. Martínez-López, 2014. **Identification of suitable areas for African horse sickness virus infections in Spanish equine populations.** Transboundary and Emerging Diseases, *in press.*

Ponencias y congresos:

- A. Sánchez-Matamoros, B. Martínez-López, V. Rodríguez-Prieto, E. Iglesias, and J.M Sánchez-Vizcaíno, 2013. **Identifying high risk areas for African horse sickness epidemics in Spain**. *Society for Veterinary Epidemiology and Preventive Medicine. 2013 Annual Conference.*
- A. Sánchez-Matamoros, B. Martínez-López, E. Iglesias, and J.M Sánchez-Vizcaíno, 2012. **Identification of suitable areas for African horse sickness virus infections in Spain**. *13th International Symposium on Veterinary Epidemiology and Economics.*
- A. Sánchez-Matamoros, B. Martínez-López, y J.M Sánchez-Vizcaíno, 2010. **Caracterización de la red de movimientos equinos y su aplicación para la prevención de enfermedades como la peste equina africana en Castilla y León**. *II Congreso Ibérico De Epidemiología Veterinaria.*

Identification of suitable areas for African horse sickness virus infections in Spanish equine populations

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Summary

African horse sickness (AHS) is one of the most important vector-borne viral infectious diseases of equines, transmitted mainly by *Culicoides* spp. The re-emergence of *Culicoides*-borne diseases in Europe, such as the recent bluetongue (BT) or Schmallenberg outbreaks, has raised concern about the potential re-introduction and further spread of AHS virus (AHSV) in Europe. Spain has one of the largest European equine populations. In addition, its geographical, environmental and entomological conditions favour AHSV infections, as shown by the historical outbreaks in the 1990s. The establishment of risk-based surveillance strategies would allow the early detection and rapid control of any potential AHSV outbreak. This study aimed to identify the areas and time periods that are suitable or at high risk for AHS occurrence in Spain using a GIS-based multicriteria decision framework. Specifically risk maps for AHS occurrence were produced by using a weighted linear combination of the main risk factors of disease, namely extrinsic incubation period, equine density and distribution of competent *Culicoides* populations. Model results revealed that the south-western and north-central areas of Spain and the Balearic Islands are the areas at the highest risk for AHSV infections, particularly in late summer months. Conversely, Galicia, Castile and Leon and La Rioja can be considered as low risk regions. This result was validated with historic AHS and BT outbreaks in Spain, and with the *Culicoides* vector distribution area. The model results, together with current Spanish equine production features, should provide the foundations to design risk-based and more cost-effective surveillance strategies for the early detection and rapid control potential of AHS outbreaks in Spain.

Keywords: African horse sickness, vector-borne disease, risk mapping, suitability maps, risk-based surveillance, Spain.

Introduction

African horse sickness (AHS) is one of the most economically devastating notifiable viral diseases of the equine industry (OIE, 2013). The AHS virus (AHSV) is closely related to the bluetongue virus (BTV), belonging to the family *Reoviridae*, genus *Orbivirus*, and transmitted by the same vectors (*Culicoides* spp. midges) but with different host preferences (Mellor, 2000). Sub-Saharan Africa and Yemen are endemic regions, although occasional major outbreaks have appeared in North Africa, Europe and Asia, with dramatic socio-economic consequences for equine production of the affected countries (Mellor and Hamblin, 2004). Currently, the re-emergence of *Culicoides*-borne diseases in Europe, such as BTV or Schmallenberg virus, has led to increasing concern about the potential re-introduction and spread of AHS in European equine populations (Gale et al., 2010). The design and implementation of risk-based surveillance strategies for AHSV should help the early detection and rapid control of the disease, and thus mitigate the health and economic consequences of its spread.

Identification of high-risk areas and time periods for disease occurrence using risk mapping methods is becoming increasingly popular in vector-borne diseases (e.g., Racloz et al., 2008; Rodríguez-Prieto et al., 2012; Sánchez-Vizcaíno et al., 2013). Methods such as the GIS-based multicriteria decision framework (MCDF) allow the integration of maps that contain information about risk factors of the disease to provide a final suitability or a risk map where outbreaks are likely to occur (Malczewski, 2004). This methodology has been previously used to evaluate, for example, the risk of Rift Valley Fever (RFV) epidemics in Africa (Clements et al., 2006), in Spain (Sánchez-Vizcaíno et al., 2013) and in Italy (Tran et al., 2013); West Nile fever (WNF) outbreaks in Castile and Leon (Rodríguez-Prieto et al., 2012); malaria (MD) outbreaks in Iran (Hanafi-Bojd et al., 2012); visceral leishmaniasis (VL) epidemics in the province of East Azerbaijan (Rajabi et al., 2012) among other diseases. As for AHS outbreaks, the main factors related with their occurrence are presence of susceptible hosts and abundance of vectors associated with adequate climatic conditions for virus replication. The virus can infect all *Equidae* species, such as horses, mules, donkeys and zebras, but differences are found in the disease pathogenesis (Mellor and Hamblin, 2004). Regarding vectors, the most important species belong to the *Culicoides* genus, specifically *C. imicola, C. bolitinos* and *C. sonorensis*, although other *Culicoides* species could be involved in transmission, such as *C. pulicaris* and *C. obsoletus* (Mellor et al., 1990; Nielsen et al., 2010). The integration of environmental, entomological and epidemiological risk factors into GIS-based MCDF will help identify the areas and time periods where disease is most likely to occur if AHSV was introduced, and where surveillance should be targeted to provide an early detection system for AHS introduction and spread in Europe.

Spain has the second largest equine census in Europe (OIE, 2012) and adequate climatic conditions for *Culicoides* abundance and virus replication (Calvete et al., 2008). For these reasons, this country is one of the most susceptible European countries where the potential re-introduction and spread of AHSV may have devastating consequences. As an example, the Spanish equine industry obtained profits of €5.3 billion in 2012 and has breeds of high economic and genetic value (Deloite, 2013). Consequently, incursion of AHSV into Spain could have devastating socio-economic effects, as already occurred in the two historic AHS outbreaks in 1966 and 1987-1990 (Rodríguez et al., 2001; Rodríguez et al., 1992; Sánchez-Vizcaíno, 2004). Therefore, this study aimed to identify highly risk areas for AHS epidemics in its equine populations in Spain. For this purpose, a GIS-based MCDF using weighted linear combination (WLC) was used. Knowledge of high-risk areas for AHS infection will provide the foundations to design a risk-based AHS surveillance program in Spain adapted to the Spanish equine production and epidemiological features. The ultimate goal is to improve the early detection of AHS and mitigate the devastating effect that an AHSV outbreak may have on the Spanish equine industry.

Material and methods

The GIS-based MCDF approach was similar to that described by Rodríguez-Prieto et al. (2012) to estimate high risk areas for West Nile in Castile and Leon and by Sánchez-Vizcaíno et al. (2013) to estimate high risk areas for Rift Valley Fever outbreaks. In this study, the GIS-based MCDF framework has been adapted to estimate the risk areas for AHS in Spain considering the AHS epidemiological features and the peculiarities of the Spanish equine population. Firstly, data for the risk factors related to AHS occurrence were identified and collected, and a standardized layer

depicting the spatial distribution of each factor was generated. Secondly, the weights for each factor were determined based on expert opinions. Thirdly, all the layers for each risk factor were combined using WLC to produce an AHS risk map for each month and a global map for the high-risk season (April-October). Finally, the AHS risk map for the high-risk season was evaluated by performing an extensive sensitivity analysis and a validation exercise using historic AHS outbreaks.

1. Identification, data collection and mapping of the risk factors related to AHS suitability

A review of the peer-reviewed scientific literature was conducted to identify the factors related with potential AHSV infections and to determine the relative importance of each factor. Given the similarity of the environmental, entomological and epidemiological features of AHS and BT, both diseases were considered in the literature search. The literature search was conducted in PubMed database by combining keywords such as "African horse sickness" or "Bluetongue" and "risk factors", "*Culicoides*" and "temperature", and so on. As a result of the literature review, three key factors for AHSV infection were identified: temperature (which affects the viral extrinsic incubation period), equine density and *Culicoides* distribution.

Extrinsic incubation period (EIP) is the time required for virus extrinsic replication and dissemination within the vector (Smith, 1987). As midges are poikilothermic, the environmental temperature is an essential factor to understand the virus extrinsic replication in the vector and to predict geographical and temporal limits of transmission (Carpenter et al., 2011). The optimal range for faster midge maturation and a shorter EIP has been found to range from 27ºC to 30ºC (EFSA, 2007). The limits for AHSV replication in *Culicoides* have been analyzed at 15ºC (Mellor, 2000) and the presence of the vector has been limited up to 38ºC (Ortega et al., 1999; Sellers and Mellor, 1993). Consequently, it was assumed that the temperature distribution for virus replication in midges was related with AHS suitability as follows: linearly increasing between 15ºC and 27ºC, maximum value from 27ºC to 30ºC, and linearly decreasing between 30ºC and 38ºC. The monthly temperature data during 2009 obtained from the Spanish National Meteorological Institute (AEMET, 2009) was used for such estimation. Specifically, we used the monthly temperature layer from the AEMET kriging model, which was based on data from 1,803 meteorological stations.

***Equidae* species** (i.e., horses, mules, donkeys, etc.) are susceptible hosts for AHSV allowing the virus multiplication inside them (intrinsic virus replication) (Mellor and Hamblin, 2004). Therefore, areas with high equine density were considered to be at higher risk for AHSV outbreaks. A monotic increasing relationship between equine density and AHS suitability was specifically assumed. The Spanish equine census was provided by the Spanish Government (INE, 2011) according to municipalities (*comarca* in Spanish), for 2009. This census was divided into municipality areas to obtain equine density locally by assuming that values were constant for the whole municipality. This layer was then converted into a raster map of equine density for the whole of Spain.

***Culicoides* distribution** is another factor that influences the spatial and temporal incidence of AHSV transmission. In Spain, *C. imicola* was revealed as being mainly responsible for AHSV transmission during the 1987-1990 outbreaks (Mellor et al., 1990). However, the *C. obsoletus* group could potentially serve as an effective AHSV vector, similar to BTV (Mellor et al., 1990; Nielsen et al., 2010). Given the considerable importance attached to *Orbiviruses* since the 2006 BTV outbreak in Europe, the bioclimatic variables affecting *Culicoides* distribution have been extensively studied (e.g., Calvete et al., 2008; Conte et al., 2007; Ducheyne et al., 2013; EFSA, 2007). In addition, the Spanish government has carried out extensive entomological surveillance framed within the BT eradication program. Both entomological surveillance data and ecoclimatic variables relating to vector abundance were used to predict *Culicoides* distribution in Spain as follows. Since climate has a delayed effect of 1 month on *Culicoides* abundance, data about *Culicoides* traps for a specific month were combined with the climate data for the previous month (Eksteen and Breetzke, 2011).

Culicoides *traps:* raster maps were produced from the data on *C. imicola* and *C. obsoletus* collected between April and October from 2005 to 2008. Data from each trap were used to estimate *Culicoides* distribution by the inverse distance weighted (IDW) method. The IDW was limited to a 50-km radius because this is the distance established in the entomological surveillance program of BT eradication in Spain (MAGRAMA, 2013b).

Temperature affects Culicoides *population:* Temperature is the main climatic factor for the distribution and abundance of *Culicoides* populations. This factor affects many lifecycle parameters of vectors, such as the gonadotrophic cycle or the survival rate. The limits for *Culicoides* survival between the possible vectors in Spain differed slightly. Presence of *C. imicola* has been limited between 12.5ºC and 38ºC (Ortega et al., 1999; Sellers and Mellor, 1993). *C. obsoletus* requires relatively low temperatures for optimal development and survival, and its optimal temperature ranges between 11ºC and 27.5ºC (Dzhafarov, 1964). Consequently, the temperature distribution for *Culicoides* populations is assumed to increase between 11ºC and 27.5ºC, and to decrease between 27.5ºC and 38ºC.

Rainfall: The literature review provided contradictory results about the relation between rainfall and *Culicoides* distribution. *C. imicola* pupae do not tolerate rainfall above 700 mm/period (Nevill, 1971). However, heavy rainfall has been associated with increased adult *Culicoides* abundance (Meiswinkel, 1998), and has also been linked to AHS outbreaks in Africa (Baylis et al., 1999). Thus, the worst-case scenario was assumed with a monotonic increasing relationship between rainfall and abundance of AHS vectors. Monthly average rainfall models in Spain were obtained from the AEMET database (AEMET, 2009). The monthly rainfall layer was obtained from the AEMET kriging model, which was based on the data from 4,189 stations.

Normalized difference vegetation index (NDVI): Satellite-derived NDVI has been previously used as a predictor of both the distribution and abundance of *C. imicola* and *C. obsoletus* (Calvete et al., 2008; Conte et al., 2007; Peters et al., 2011). This parameter, when correlated with soil moisture, rainfall and vegetation biomass (Campbell, 2002), allows the determination of *Culicoides* distribution. This distribution and BT outbreaks are more likely to occur when moist conditions prevail, as indicated by a high NDVI (Calvete et al., 2008; Purse et al., 2005). The monthly mean values of NDVI in Spain are derived from the MODerate-resolution Imaging Spectroradiometer (MODIS, 2004) imagery of the NASA Terra satellite (Scharlemann et al., 2008).

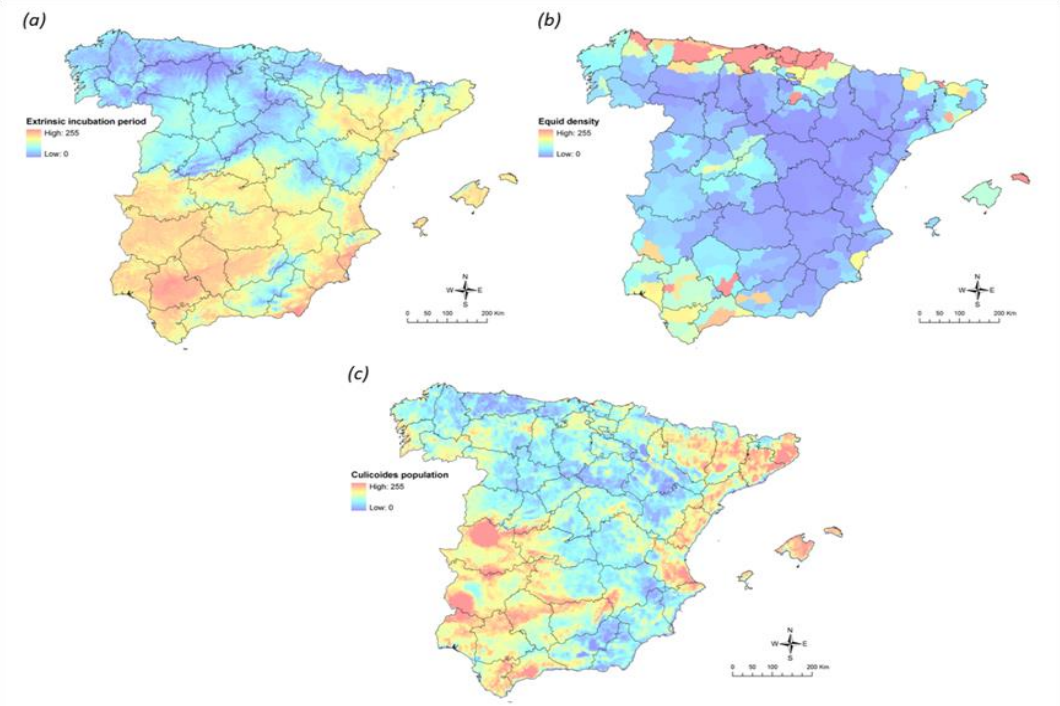

Figure 1: Input risk maps used to obtain the African horse sickness (AHS) risk map: (a) temperature map (which affects the extrinsic incubation period), (b) equine density map, and (c) Culicoides spp. distribution map for the whole risk period. Risk maps are presented on a graduated blue-green-yellow-orange-red scale, ranging from low to high risk. Divisions in maps correspond to provinces in Spain.

Table 1

Results of the sensitivity analysis. The weights of the risk factors used in the study to identify suitable areas for occurrence of outbreaks of African horse sickness in Spain are shown for the reference model and for all the twelve scenarios simulated in the sensitivity analysis. Asterisks identify the weight of each risk factor, which increased or decreased by a total of +/-25% in each scenario, respectively. EIP, temperature affecting the extrinsic incubation period; E, Equidae density; C, *Culicoides* traps; T, temperature affecting Culicoides population; R, rainfall; and NDVI, normalized difference vegetation index.

	Weight (%) of EIP	Weight (%) of E	Individual vector factors			
			Weight (%) of C	Weight (%) of T	Weight (%) of R	Weight (%) of NDVI
Reference model	33	33	8.25	8.25	8.25	8.25
Scenario 1	41.23*	33	8.25	8.25	8.25	8.25
Scenario 2	24.75*	33	8.25	8.25	8.25	8.25
Scenario 3	33	41.25*	8.25	8.25	8.25	8.25
Scenario 4	33	24.75*	8.25	8.25	8.25	8.25
Scenario 5	33	33	10.31*	8.25	8.25	8.25
Scenario 6	33	33	6.18*	8.25	8.25	8.25
Scenario 7	33	33	8.25	10.31*	8.25	8.25
Scenario 8	33	33	8.25	6.18*	8.25	8.25
Scenario 9	33	33	8.25	8.25	10.31*	8.25
Scenario 10	33	33	8.25	8.25	6.18*	8.25
Scenario 11	33	33	8.25	8.25	8.25	10.31*
Scenario 12	33	33	8.25	8.25	8.25	6.18*

Given that the period of activity and abundance of *Culicoides* spp. in Spain have been described to range from April to October, a risk map was produced for all these months as well as for the whole period (Calvete et al., 2008), and AHS risk was also quantified for this period. All the factors were mapped in the same raster with a 1-km^2 cell size format. All the factors were also standardized on a byte scale ranging from 0 to 255 to make them comparable and to improve the combination of all the factors in order to produce the final AHS risk maps (Drobne and Lisec, 2009).

2. Relative importance and combination of factors to produce AHS risk maps.

After producing layers for the risk factors, each layer received a specific importance percentage, or "weight", based on the expert opinions, and were combined using the WLC. The WLC is a method that allows the aggregation of standardized risk maps after considering their relative weight to obtain a final AHS risk map (Malczewski, 2004). By using WLC, AHS risk maps were produced for each month from April to October. Then monthly maps were combined to obtain the final AHS risk map for the whole risk period (April-October).

The AHS cycle requires the presence of susceptible hosts, *Culicoides* spp. midges and adequate environmental conditions at the same time so that all the factors receive a similar weight (0.33) for this framework.

As mentioned above, vector distribution was estimated from the WLC of the annual capture data and temperature, rainfall and NDVI for the previous month. It was assumed that the same weights applied for these factors (i.e., 0.25 for them all).

All the spatial analyses were performed with the ArcGIS 9.3.1 (ESRI©, 2009) software using the "weighted sum" function. Lastly, the final AHS risk map was normalized by the maximum value of all the AHS risk maps, on a scale range from 0 (low risk) to 1 (high risk) in order to make them comparable. The low to high risk areas on the map were represented on a graduated color scale from blue to red, respectively.

3. Sensitivity analysis.

One critical aspect of the WLC method is the uncertainty associated with selection of weights for each risk factor. For this reason, evaluating the impact that changes in model weights may have on the final risk map using a sensitivity analysis is of particular interest.

Specifically, changes in the model outcomes were quantified when modifying each risk factor weight by a total of +/-25% of their initial values (i.e., 12 different scenarios were tested; see Table 1). The non-parametric Spearman's correlation coefficient (Rho) was used to compare the cell values of the maps obtained in the different scenarios. R-language (v. 2.11.1, R Foundation for Statistical Computing, Vienna, Austria 2010) was used to analyze the results. The model was assumed to be robust if there were changes of <10% in the correlation coefficients.

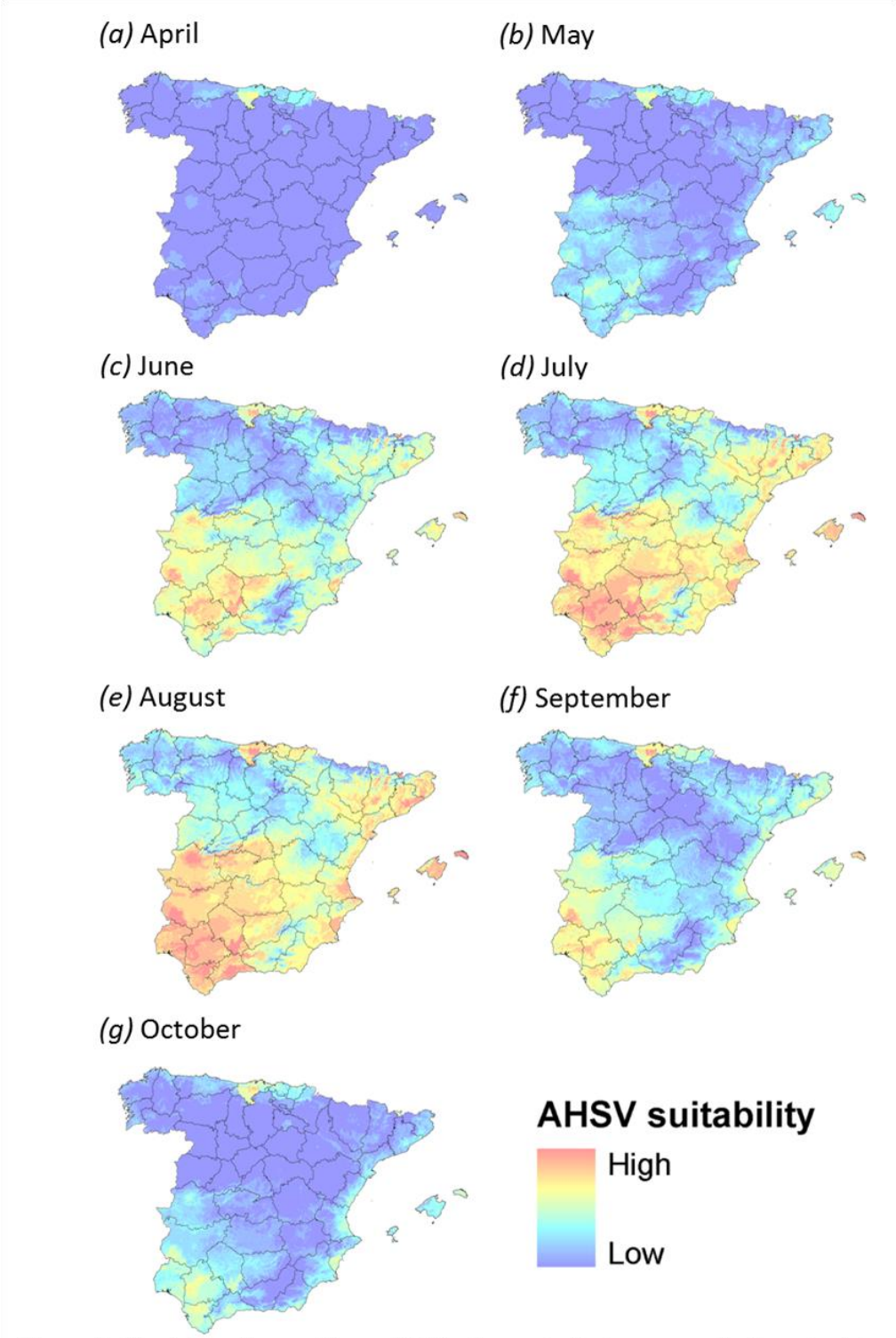

Figure 2: The African horse sickness (AHS) risk map in Spain per month, from April to October. Risk maps are presented on a graduated blue-green-yellow-orange-red scale, with blue as an indicator of AHS low risk and red as an indicator of AHS high risk. Divisions in maps correspond to provinces in Spain.

4. Validation with historical outbreaks

Information about the infected areas during the historic AHS outbreaks in Spain from 1987 to 1990 was used to validate the AHS risk map for the high-risk period (April-October) obtained by the GIS-MCDF approach. The data published on these AHS outbreaks were used to obtain the location of the infected areas at municipality level (Mansilla, 1990; Rodríguez et al., 1992) since the locations of outbreaks were not available. These data were overlaid on the final AHS risk map using the ArcGIS 9.3.1 (ESRI©, 2009) software. The input value of the final AHS risk map for each overlay infected area per year was extracted by the "clip" function. In this way, the median risk value on the final AHS risk map was calculated for each infected area per year.

Results

1. Description of risk factor suitability maps

The AHS risk factors were heterogeneously distributed in Spain (Fig. 1). Temperatures fostering the EIP were higher in southern Spain, specifically in Almeria, Alicante and Seville. Conversely, the highest equine density was located mainly in two areas in northern and southern Spain. Finally, suitability of *Culicoides* spp. was higher in the west-central (Cáceres and Badajoz) and north-eastern areas (Gerona and the Balearic Islands) of Spain.

2. AHS risk maps

The AHS risk map for each month and the final AHS risk map for the whole risk period (April-October) are shown in Fig. 2 and Fig. 3, respectively. In general, the highest risk areas for AHS outbreak occurrence were concentrated in south-western (SW) and north-central (NC) areas of Spain (Fig. 3). SW Spain included the provinces of Badajoz, Huelva, Seville, Cadiz, Cordoba and Malaga, while NC Spain consisted of the Cantabria region. Some other highly risk areas were situated in the Balearic Islands, especially Minorca, and to the south of Alicante (Fig. 3). Furthermore, the lowest risk areas for AHS outbreak occurrence in Spain were concentrated in the regions of Galicia, Castile and Leon, La Rioja and in the province of Teruel (Fig. 3).

July and August were highlighted as the highest risk months for AHS occurrence (Fig. 3). Interestingly, the month during the risk period with the lowest risk was April. During this month, the zone with the highest risk was concentrated in NC Spain, which coincides with the highest equine density areas of Spain. The lowest risk of this area was related with the climatic conditions required by both the virus and vector.

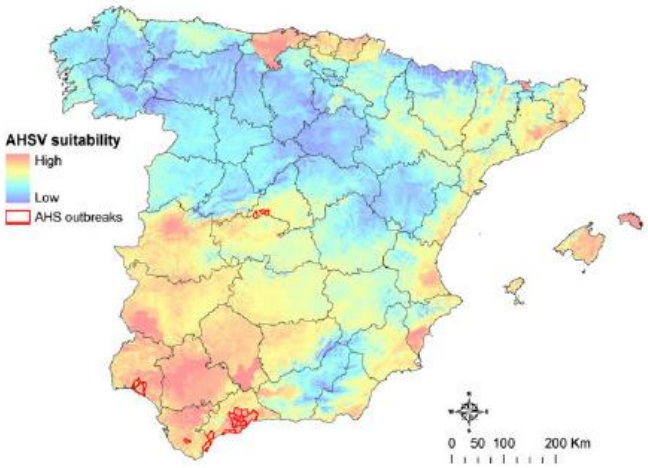

Fig. 3. The African horse sickness (AHS) risk map in Spain considering the whole risk period, from April to October. The risk map is presented on a graduated blue-green-yellow-orange-red scale, ranging from 0 to 1, with 0 as an indicator of AHS low risk and 1 as an indicator of AHS high risk. Divisions in the map correspond to provinces in Spain. Red lines represent the distribution of the infected areas of AHS outbreaks between 1987 and 1990.

3. Sensitivity analysis

The results of the twelve sensitivity analysis scenarios correlated significantly with the outputs on the AHS risk maps obtained for the reference model (Rho not lower than 0.982; $p < 0.01$) (Fig. 4). Consequently, the sensitivity analysis results showed a robust model since AHS risk areas were not sensitive to changes in the relative weights of the AHS risk factors.

4. Validation with historical outbreaks

The historic AHS outbreaks in 1987-1990 were reported in central and southern Spain. Risk mapping detected these areas to be at a medium-high risk of AHS infection (Fig 3), with a median risk value of 0.53, 0.60, 0.61 and 0.68 for the infected areas in 1987, 1988, 1989 and 1990, respectively.

Discussion

In this study, the application of a GIS-based MCDF based on WLC allowed the identification

and mapping of high risk areas and time periods for AHS outbreaks in equine populations in Spain, considered to be a high-risk country for AHS re-emergence in Europe (Martínez-López et al., 2011). AHS occurrence is affected by the complex virus-vector-host-environment interaction. In this context, the GIS-based MCDF allows the simultaneous combination of detailed information on factors related with the extrinsic incubation period of the virus, equine density and *Culicoides* spp. distribution to produce AHS risk maps for each month (Fig. 2) or for the whole high-risk season (April-October) (Fig. 3), which could be used to design risk-based surveillance strategies in Spain. High risk areas for AHS outbreaks were identified in SW Spain (Fig. 3). This area is characterized mainly by optimum temperature for the extrinsic incubation period and high density of domestic equines (Fig. 1). Interestingly, this area overlaps the distribution of the previous AHS and BT outbreaks (Pérez de Diego et al., 2013; Rodríguez et al., 1992), and with the main distribution area of *C. imicola* (Calvete et al., 2008). Eradication of the AHS outbreak in 1987-1990 was achieved after the death or slaughter of 1500 animals (Sánchez-Vizcaíno, 2004). Nowadays, the equine population in SW Spain is estimated at approximately 105000 animals, which represents 33% of the Spanish equine census (INE, 2011). Equine production in this region is characterized by breeding animals for recreational purposes, many with a high genetic value, especially purebred Spanish horses and endangered local donkey breeds (Deloite, 2013; MAGRAMA, 2013a). Therefore, any potential AHS re-introduction and spread in equine populations in SW Spain, which may lead to mortality rates in horses of up to 95%, may have serious economic consequences and could lead to the loss of the genetic diversity of local horses and donkeys.

High risk areas in NC Spain can be explained by the highest equine density (Fig. 1). However, the AHS risk maps need to be interpreted with caution because the mean low temperature estimated in this area could modify the virus extrinsic replication period. Although no historical AHS outbreaks have been reported in this area, the numerous notified BT outbreaks highlight the potential of an orbivirus to spread here. NC risk regions mainly overlapped *C. obsoletus* distribution, which is the main vector responsible for the spread of BTV in northern Europe (Calvete et al., 2008). Equine farms in NC Spain are mostly devoted to horse meat production, being Spain the seventh European exporter of live equines and horse meat (DATACOMEX, 2014; Deloitte, 2013). Therefore, the main consequences of introduction of AHS in this region will be associated with losses in the horse meat industry as potential movement restrictions would have a major economic impact on horsemeat exportation.

There are other regions of interest for AHSV risk in Spain. Firstly, the Balearic Islands, especially Minorca, is a region that has been historically related with BT outbreaks and with the distribution of *C. imicola* and *C. obsoletus* (Calvete et al., 2008; Pérez de Diego et al., 2013). The equine sector is associated with this island's cultural heritage, where the genetic value of the Menorcan horse is stressed. Therefore, the value of these festivals for local economy has been

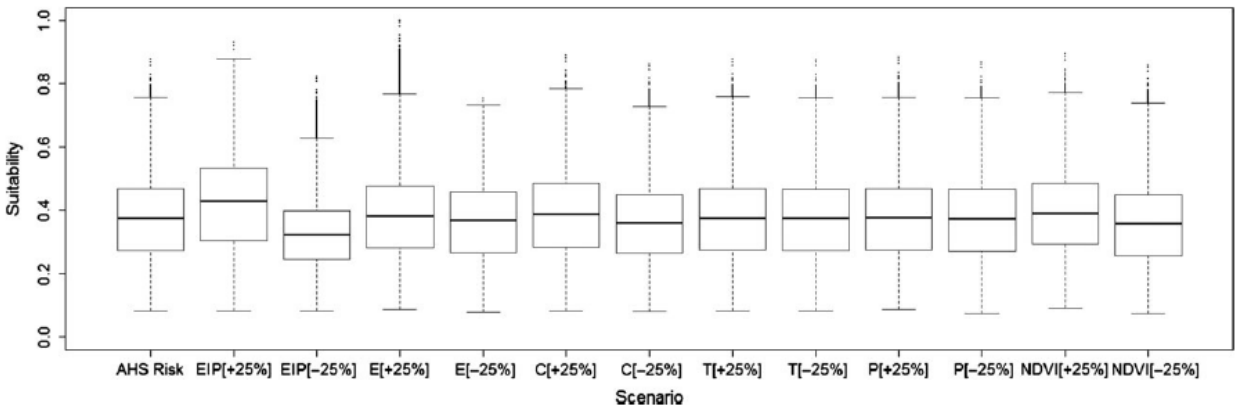

Fig. 4. Boxplot of the estimated African horse sickness (AHS) risk values for raster cells in Spain for the reference scenario (AHSV risk) and for each scenario with changes in the relative weights of the input risk factors. The black line in the boxes indicates the median AHS risk score for raster cells. The extent of the boxes ranges from 25% to 75% of the data, while the extent of the confident intervals ranges from 5% to 95% of the data. EIP, temperature affecting the extrinsic incubation period; E, equine density; C, *Culicoides* spp. traps; T, temperature affecting *Culicoides* spp. population; P, rainfall; NDVI, normalized difference vegetation index; (+25) and (−25) represent the increase and decrease in the relative weights for each scenario, respectively.

estimated to be around 5 million euros (Deloitte, 2013). Secondly, the southern region of Alicante in the Spanish Levant seems to offer good environmental conditions for vector survival and virus replication. However, studies into *Culicoides* spp. populations have described their low abundance in the Levant area (Calvete et al., 2006; 2008) that could be associated with the use of insecticides in this region (Martinez Salvador A., 2004) and the low density of *Culicoides* spp. feeding preferential species such as ruminants and equines (Ninio et al. 2011; Sánchez-Vizcaíno et al., 2013). This fact could explain the lack of AHS spread in Alicante during the 1987 outbreak (Sánchez-Vizcaíno, 2004) and the lack of BT spread in this area (Pérez de Diego et al., 2013). Finally, Galicia, Castile and León, La Rioja and the province of Teruel are the areas at the lowest risk in Spain. These regions could create a natural barrier for AHSV spread between northern and central Spain, which could explain the absence of AHS spread in northern Spain during the AHSV 4 outbreak (1987-1990).

The months at highest risk for AHS occurrence are July and August (Fig. 2). The high temperatures recorded in these months lead to higher virus replication and vector survival. Interestingly, the month during the risk period at lowest risk is April. This low risk is likely associated with inadequate temperature for virus replication and vector survival. During these months, the zones with the highest risk are concentrated in NC Spain. However, the AHS spread in this area should be limited by low temperature for virus replication (Fig. 1).

Risk mapping included most of the main large-scale spatial determinants of AHS ecology (i.e., equine density, temperature affecting the EIP and *Culicoides* spp. distribution), whose data were available for us. However, some limitations should be noted when assessing AHS risk in Spain via this method. Firstly, equine density was assumed constant for the whole municipality, which may lead to underestimate or overestimate the risk within each municipality. Municipality was the smallest administrative division for which data were available. However, the model's accuracy and spatial resolution can be improved by including the geographic locations of equine farms instead of using municipality-based information. In addition, neither the different AHS susceptibilities of infection between *Equidae* species nor their different roles in AHS spread were incorporated into the study. For example, infection of zebras, the natural reservoir of AHSV (Barnard, 1998), was not detected at an early stage, which allowed AHS to spread further, as in 1987. Abundance of vectors can also be improved by including more information about *Culicoides* prediction, which was not available when this work was written, such as use of land cover or density of feeding preferential hosts, e.g., domestic and wild ruminants (Ducheyne et al., 2013; Lo Iacono et al., 2013). Certainly the model developed herein can be easily updated to incorporate these improvements if data become available. Another main limitation of this GIS-based model is the possibility of overestimating risk in regions where there are several factors, but not all of them, with higher values. For example, in this study, the high risk area in NC Spain presents higher values for the factors equine density and *Culicoides* population, but not the EIP factor, given the low mean temperatures for extrinsic virus replication in some months. However, an adequate interpretation of disease epidemiology allows a distinction to be made between these possible false 'risky' areas.

Different authors have described subjectivity associated with selection and weighting risk factors as one of the most important limitations of the GIS-based WLC method (Clements et al., 2006; Rodríguez-Prieto et al., 2012; Sánchez-Vizcaíno et al., 2013). In this study, an extensive literature review was conducted to identify factors associated with AHS occurrence whereas expert opinion allows to define the weights for each factor. For that reason, and due to the potential subjectivity of the weights assigned to each factor, sensitivity analysis was conducted to assess the impact that changes in weights have on model outcomes. The weighting scores of the AHS risk factors did not meaningfully modify the model's results, which suggests that the model is robust (i.e., not sensitive to changes in the weights of risk factors).

Information about historic AHS outbreaks in 1987-1990 in central and southern Spain was used to validate the final AHS risk map. Infected municipalities were located in areas predicted to be medium-high AHS risk (Fig. 3). Unfortunately, this validation does not include all the AHS-infected areas during the 1987-1990 epidemics due to lack of spatial resolution for some outbreak data (i.e., no information at municipality level). For example, the provinces of Seville, Córdoba and Badajoz were affected by AHS in 1989 (Rodríguez et al., 1992). However, reports

of these affected areas do not include outbreak locations at municipality level. Inclusion of those outbreaks could have improved the AHS model validation since most of the areas in these provinces show a high AHS risk.

GIS-based MCDF approach has been recently implemented for support decisions related with vector-borne and other emerging diseases (Hongoh et al. 2011). Several authors have used this methodology to predict the spatial distribution of diseases, their vector and/or hosts (e.g. Sánchez-Vizcaíno et al., 2013; Tran et al., 2013; Rodríguez-Prieto et al., 2012). Results are particularly useful to inform the design of risk-based surveillance and control programs, as well as to identify knowledge gaps. For example, West Nile risk maps obtained by Rodríguez-Prieto et al. (2012) were compared with the sampling locations of the ongoing surveillance program, which allowed the identification of uncovered high risk areas and to better define priority sites for future surveillance activities.

In this study, a GIS-based MCDF was employed to identify risk areas and time periods for potential AHS occurrence in Spain if the virus was introduced. These results, combined with studies that aim to identify the risk of potential AHS introduction into Spain, should provide the foundations to support the design of cost-effective strategies that better prevent and mitigate potential AHS incursions in Spain.

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Social Network Analysis of Equidae Movements and its Application to Risk-Based Surveillance and to Control of Spread of Potential Equidae Diseases

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Summary

Movements of animals and animal products are one of the most important ways of disease introduction and spread between regions and countries. Maybe one of the most complex animal species in terms of diversity of uses, nature and extent of movements are equidae, for which animal movement records are usually not available. The study presented here is the first characterization of a complete and reliable network of equidae movements in Castile and Leon, which is one of the most important equidae production regions of Spain. Social network analysis and space–time cluster analysis were used to describe the contact patterns of the equidae network and to identify the most important premises, areas and time periods for potential disease introduction or spread into the region. The studied network was complex, with very heterogeneous types of premises and diverse nature and extent of the movements compared with other livestock species, which have important implications for prevention and control of equidae diseases. Centrality measures revealed that production and reproduction farms and centres of livestock competition were the most important type of premises in the studied network. Cluster analyses allowed to identify seventeen significant spatio-temporal clusters of premises at high risk of dispatching or receiving equidae, which formed four interconnected compartments. These clusters were mainly located in the north-west region and in the second part of the year. The results of this study may be useful to design risk-based surveillance and control programmes of equidae diseases and increase the speed of detection and control of potential secondary outbreaks in future epidemics. Consequently, these results will help to minimize the great economic and sanitary impact of equidae diseases. The analytical approach used here may be easily extended to characterize the equidae movement patterns in other countries and regions of the world.

Keywords: Social network analysis; spatio-temporal cluster analysis; equidae movements; risk-based surveillance; Spain.

Introduction

In this globalized world, the transmission of infectious animal diseases within and between regions is facilitated by the great distances that animals and animal products travel in short periods of time. For that reason, a proper register and analysis of the animal movement patterns in a region may help to rapidly and effectively identify potential risks of disease introduction and/or spread. In the last years, the register and storage of livestock movement records in databases worldwide is contributing to the development of useful epidemiological analysis and risk assessments aimed at supporting policies for prevention and eventual control of infectious animal disease epidemics (Dubé et al., 2009). However, the movements of pets and animals used for human activities, such as dogs, cats, equidae, are not so well traced or, sometimes, are not even registered. It is of note that these animals may move more frequently, more freely and in a less predictable way than livestock animals, for example, in movements associated with human travels. Therefore, the register and evaluation of the movements of such animals may have critical implications for preventing and controlling the potential spread of infectious diseases affecting these species.

Equidae is one of the animal species with highest social and economical importance in the European Union (EU) not only due to their wide use for human activities associated to work, sport and/or entertainment, but also due to their use for meat production (EU Equus, 2001). However, equidae is likely one of the livestock species with less register and storage of movement records not only in the EU but also worldwide. The lack of equidae movement records is not only one of the most important shortcomings for the development of risk assessments and for the implementation of risk-based surveillance and control policies, but also represents an important challenge to effectively protect equidae populations from diseases.

It is of note that equidae industry may be impacted by up to 21 notifiable diseases of World Organisation for Animal Health (OIE, 2010a); most of them are potential zoonosis which may be a threat for the public health in affected regions; and some of them such as African horse sickness (AHS) may have a considerable impact on the economy and lead to social disruption in affected countries and regions. For those reasons, the characterization of equidae movement patterns would be highly recommended since it would provide valuable information in terms of equidae diseases prevention and preparedness (Herholz et al., 2008).

Spain is the second European country in terms of equidae population, with 669.071 animals censed, behind the United Kingdom (841.400) (OIE, 2010b). A high percentage of animals censed into Spain belong to native-Spanish breeds (MARM, 2003) and breeds at risk of extinction (FAO, 2011) which have a high genetic and economic value for the country. As a consequence, an eventual exotic equidae disease introduction into Spain could have devastating consequences for the country. See for example, the impact that AHS had in 1987 for the Spanish equidae industry (Sánchez-Vizcaíno, 2004). Moreover, Spanish equidae sector has some peculiarities that make this sector more complex for disease surveillance, control and prevention compared with others agricultural industries. Firstly, it is a sector with a high variety of production systems. Secondly, there is an intense and frequent national and international movement of equidae (FEI, 2010; TRACES, 2010) which may increase the risk for disease introduction or spread into the Spanish territory. Spanish authorities, conscious of the vulnerabilities of the Spanish equidae sector regarding disease prevention and control started, as consequence of the AHS epidemic in 1987, to implement a complete register and storage of all equidae movements at the early 1990s. However, to the best of our knowledge, no study aimed at characterizing the equidae movement patterns into an EU member state such as Spain has been published in the peer-reviewed literature.

Social network and cluster analyses methods were used here to characterize equidae movement patterns in the Spanish region of Castile and Leon (CyL), and to identify premises, areas and time periods that may be target for surveillance programs of significant diseases, such as AHS. Characterization of equidae movement patterns allowed the identification of usual equidae movements for each farm into CyL which may be used along with epidemiological surveys to increase the efficacy of detection of possible secondary outbreaks in case of an equidae disease is introduced into the region. CyL is the second largest Spanish region in terms of equidae census (MARM, 2010) and one of the regions that earliest

implemented and accomplished the compulsory registration of all equidae movements.

The objective of the present study was to provide a full description and characterization of a complete and reliable network of equidae movements in the Spanish region of CyL in order to provide a better knowledge of the potential risks associated with equidae movements. This information will be useful for the development of risk assessments and for supporting the design of risk-based surveillance and control programs of equidae diseases, and for increasing the speed of detection of possible secondary outbreaks during the epidemic of a disease that affects the Spanish equidae industry. The analytical approach used here may be easily extended to characterize the equidae movement patterns in other countries and regions of the world.

Material and Methods

Data and study region

The region of Castile and Leon was selected to illustrate the applicability of the methods and results of this study for three reasons. Firstly, because it is the second largest region in Spain in terms of equidae census (MARM, 2010); secondly, because it was one of the first Spanish regions to implement and accomplish the compulsory registration of all equidae movements and, for that reason, the information collected in the CyL database likely represents an accurate estimate of the real number of movements occurring into the region; and finally, because regional authorities made available to us all the epidemiological and demographical data required for the analysis.

The information provided by CyL authorities was the equidae movement records within CyL during 2008, which was the most recent year for which information was available and complete. Specifically, the day of movement, the number of animals moved, the herd size, the geographical location (latitude and longitude) and type of premises of origin and destination were provided. According to the Spanish legislation (R.D 479/2004) the equidae sector is classified in seven types of premises: production and reproduction farms (PR); traders (TO); centres of livestock competition (CCA); entertainment, teaching and researching centres (OEI); slaughterhouses (MAT); permanent bullrings (PT) and pastures with/without common use (PC). This information allowed to generate a contact network with premises represented by nodes and equidae movements among the nodes represented by directed links (i.e. one link is each movement of one or more animals ranging from a premise to another of CyL during 2008) (Fig. 1).

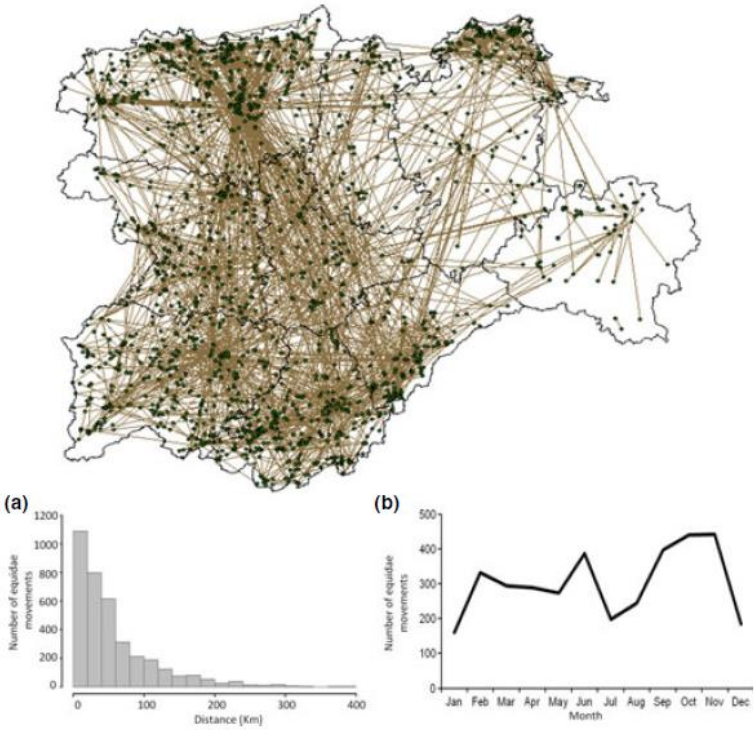

Fig. 1. Geographical representation of the network of equidae movements within the Spanish region of Castile and Leon in 2008. Circles and lines represent, respectively, equidae nodes and at least one equidae link. Histogram (a) shows the frequency of movements by theirs distances and temporal pattern (b) shows the number of movements per month.

Data analysis

Social Network Analysis (SNA) in combination with statistical methods for detection of space-temporal clusters were used, firstly, to characterize the nature and extent of the network of equidae movements within CyL and, secondly, to identify premises, areas and time periods at high risk of receiving or dispatching equidae within CyL during 2008. This approach was an adaptation and practical application to equidae populations of the methods presented in Martínez-López et al. (2009a).

Characterization of the nature and extent of the network of equidae movements within Castile and Leon.

A comprehensive review of general concepts and application of SNA and graph theory in preventive veterinary medicine is available elsewhere (Martínez-López et al., 2009a). But briefly, SNA is an analytical tool which allows exploring, characterizing and quantifying the nature and extent of contacts among a group of nodes. Because animal movements have a specific direction, which is determined by the origin and destination of the movement, the network is referred to as "direct network". One of the most interesting metrics to be computed in SNA to identify important or highly connected nodes within networks are referred to as centrality measures (Koschützki et al., 2005). In animal movement networks, the two centrality measures that have been more extensively used to evaluate the potential risk of disease introduction or spread into a premise are the degree and closeness centrality (Dubé et al., 2009; Martinez-Lopez et al., 2009b). The degree centrality measures the number of incoming (in-degree) or outgoing (out-degree) contacts that a node has. The normalized degree centrality of a node *i* (NDc_i) may be estimated as: $NDc_i = \frac{\mathbf{Dc_i}}{\mathbf{N-1}}$, where Dc_i is the degree centrality of node *i*, and *N* is the total number of nodes in the network (Kleinberg, 1999). Similarly, the closeness centrality is an estimate of how closely connected each node is to all other nodes of the network. Closeness centrality of a node *i* (Cc_i) may be calculated as: $Cc_i = \left[\sum_{j=1}^{N} d(n_i, n_j)\right]^{-1}$, where *d (ni, nj)* is the geodesic length of the shortest path between nodes *i* and *j*, and *N* is the total number of nodes in the network (Beauchamp, 1965; Sabidussi, 1966). Thus, the normalized closeness centrality of a node *i* (NCc_i) may be estimated as: $NCc_i = (N-1) \times Cc_i$. In the direct network here, closeness was computed for outgoing (out-closeness) and incoming (in-closeness) contacts. The relative or normalized degree and closeness centrality measures were computed here to allow comparing the relative importance of nodes that belong to networks of different sizes.

In this study both centrality measures (i.e degree and closeness) were used to identify premises at high risk of receiving and/or sending equidae within the network of equidae movements of CyL, which may be target for surveillance and control strategies of equidae diseases in the region. SNA was performed using R language (v 2.12.0) (available at www.r-project) with the Igraph package (v 0.5.4) (Csardi, 2010). Results were mapped using ArcMap 9.2 (ESRI® 2006). The spatial distribution of the normalized centrality values was represented by using the Kernel density function (Silverman, 1986).

Identification of premises, areas and time periods at high risk of receiving and/or dispatching equidae movements within Castile and Leon in 2008.

Clusters of premises at high risk to receive or send equidae in a particular area and during a specific period of time were identified by space–time permutation scan statistic (Kulldorff et al., 2005). A full description of the formulation and parameters used in the model is available elsewhere (Martínez-López et al. 2009b). Two space–time permutation scan statistic models were run. The first one aimed to identify clusters of premises at high risk of dispatching (OR group) equidae to other premises of CyL and, the second, intended to identify clusters of premises at high risk of receiving (DST group) equidae from other premises of CyL. The reception or dispatch of an animal into/or from a premise was assumed to be a proxy for disease introduction or spread, respectively. The cylindrical windows defined in the model here had a maximum spatial and temporal size of 50% of the population at risk. Time aggregation was seven days to account for the intra-week variation in the probability of the equine movements. In this way, different overlapping cylinders (i.e. possible clusters) may cover the entire study region in defined time. Clusters with a computed P-value < 0.01 were assumed to be significant. Once significant clusters were obtained, spatial and temporal relations among clusters of premises at high risk for disease spread (OR group) and clusters of premises at high risk for disease introduction

(DST group) were quantified, as described by Martínez-López et al. (2009b). Specifically, the extent of the relation between pairs of clusters was computed by estimating the probability that premises belonging to clusters OR send equidae to premises allocated in clusters DST, $P_{OR \rightarrow DST}$ and, by the probability that premises allocated in clusters DST receive equidae from premises belonging to clusters OR, $P_{DST \leftarrow OR}$. Both probabilities were estimated only for the time period when clusters OR and DST overlapped. A group of two or more clusters connected by the movement of equidae was referred to as a compartment.

Cluster analyses were performed using the SaTScan software version 9.1 (Kulldorff, 2003). Results were mapped using ArcMap 9.2 (ESRI® 2006).

Results

Characterization of the nature and extent of the network of equidae movements within Castile and Leon.

The network of equidae movements within CyL during 2008 was compound by 2,118 nodes and 3,645 directed links, with a total of 13,283 animals moved. The network' nodes represented 20.4% of the equidae premises in CyL. The nodes number of PR, TO, CCA, OEI, MAT, PT and PC represent, respectively, the 78.56 %, 0.90%, 2.60%, 14.87%, 0.71%, 0.19%, and 2.17% of total nodes in the network. The mean (standard deviation), 50%, 75% and 95% percentiles of the number of equidae movements per node was 2.39 (4.44), 1, 2 and 7, respectively. Similarly, these measures for the number of equidae moved per shipment were 3.64 (5.88), 2, 4 and 12, respectively. Equidae movements were not distributed homogeneously during the year. Months with more number of movements were November, October, September, June and February (Fig. 1). The distances covered by 25%, 50% and 75% of the equidae movements within CyL were of 15.2, 37.9 and 74.0 Km, respectively (Fig. 1).

Equidae movement patterns were also evaluated for each different type of premises. This evaluation allowed to identify that production and reproduction farms (PR) were the ones with the highest volume of equidae movements. Specifically, PR accounted for the 47.9% and 73% of the total number of outgoing and incoming movements, respectively. The number of outgoing movements was also significant in entertainment, teaching and researching centres (OEI: 10.8% of total number of outgoing movements), centres of livestock competition (CCA: 9.2%) and traders (TO: 5.1%). A significant volume of incoming movements was also allocated in centres of livestock competition (CCA: 26.8% of total number of incoming movements), entertainment, teaching and researching centres (OEI: 10.6%) and slaughterhouses (MAT: 9.7%). All other types of premises were responsible of less than 4% of total number of incoming and outgoing movements. A centre of livestock competition concentrated the highest number of incoming movements (270), whereas a trader concentrated the highest number of outgoing movements (80) in the study period.

The highest number of animals was dispatched by production and reproduction farms (PR: 74.1% of total number of dispatched animals), centres of livestock competition (CCA: 10.9%), entertainment, teaching and researching centres (OEI: 6.6%) and traders (TO: 5.3%). Similarly, the reception of animals was mainly allocated in production and reproduction farms (PR: 42.1%), centres of livestock competition (CCA: 36.9%), slaughterhouses (MAT: 7.6%) and entertainment, teaching and researching centres (OEI: 5.5%).

The mean normalized values for in- and out-degree centrality were of 8.1×10^{-4} (95% PI = 6.5×10^{-4} – 9.7×10^{-4}) and 8.1×10^{-4} (95% PI = 7.3×10^{-4} – 8.9×10^{-4}), respectively. The mean normalized values for in- and out- closeness centrality were of 5.4×10^{-4} (95% PI = 5.4×10^{-4} – 5.5×10^{-4}) and 5.3×10^{-4} (95% PI = 5.2×10^{-4} – 5.3×10^{-4}), respectively. Geographical variation and distribution of the normalized degree and closeness centrality measures is represented on Fig. 2. The distribution of the normalized degree and closeness centrality values was meaningfully different (Kruskal–Wallis test: $P<0.001$) when comparing the seven types of premises of the network of equidae movements in CyL (Fig. 3). Most important premises in terms of potential disease introduction (i.e. high in- degree and in-closeness) were found to be slaughterhouses, centres of livestock competition, traders and permanent bullrings. Conversely, most important premises in terms of potential disease spread (i.e. high out-degree and out-closeness) were found to be traders and CCA.

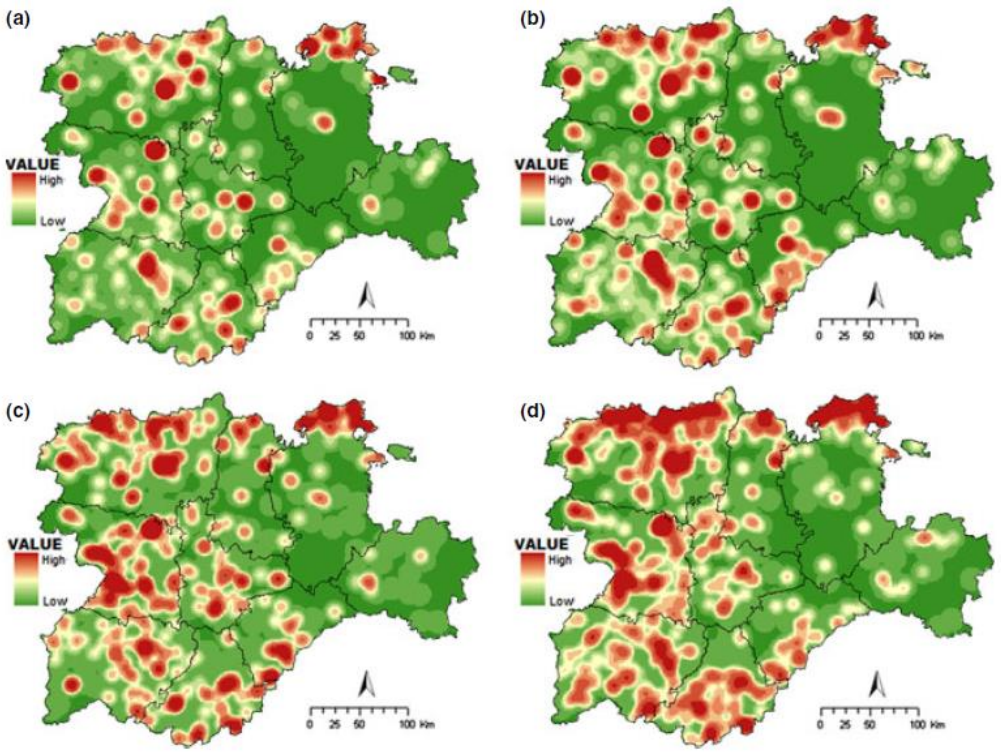

Fig. 2. Geographical representation of the normalized values of input degree (a), output degree (b), input closeness (c) and output closeness (d) of the network of equidae movements within the Spanish region of Castile and Leon in 2008 using the Kernel density. The highest and the lowest value represent 1 and 0, respectively.

Identification of premises, areas and time periods at high risk of receiving and/or dispatching equidae movements within Castile and Leon in 2008.

Six significant (P<0.01) space-temporal clusters of premises at high risk of sending equidae (OR group) were identified (Table 1 and Fig. 4). These clusters were allocated mainly in the North-West part of CyL and in the second part of the year. Clusters with the highest values of observed versus expected number of outgoing movements were cluster 6-OR (observed outgoing movements were 49 times higher than expected outgoing movements) and cluster 4-OR (observed outgoing movements were 40 times higher than expected outgoing movements). Similarly, eleven significant (P<0.01) clusters of premises at high risk of receiving equidae movements (DST group) were identified (Table 1 and Fig. 4). These clusters were mainly allocated in the periphery of CyL and in the second part of the year. Clusters with the highest values of observed versus expected number of incoming movements were cluster 8-DST (observed incoming movements were 57 times higher than expected incoming movements) and cluster 7-DST (observed incoming movements were 37 times higher than expected incoming movements). All clusters of OR and DST group, except clusters 2-OR and 3-OR, had a single predominant premise which concentrated, respectively, most of the outgoing and incoming movements of the clusters. This predominant premise was a centre of livestock competition in all clusters except in cluster 11-DST whose principal premise was a production and reproduction farm.

Space-temporal significant clusters of premises at high risk of sending or receiving equidae movements were grouped into four different compartments refer to as: Zamora South, Zamora North, Burgos and Leon. Zamora South compartment included the cluster of outgoing movements 1-OR and the cluster of incoming movements 1-DST which occurred between 13th and 26th March. Most (87%) of the shipments from cluster 1-OR were shipped to premises located within cluster 1-DST and most (94.5%) of the received equidae movements into cluster 1-DST coming from premises located within cluster 1-OR ($P_{1\text{-}OR \rightarrow 1\text{-}DST} = 0.87$; $P_{1\text{-}DST \leftarrow 1\text{-}OR} = 0.94$). In the compartment, the most important premise was a CCA that received and sent, respectively, 45.3% and 34.9% of movements that constituted the compartment. Similarly, Zamora North compartment was composed by two clusters named 5-OR and 6-DST. Most (77%) of the equidae movements from cluster 5-OR were

dispatched to premises located within cluster 6-DST and most (78%) of received equidae movements into cluster 6-DST coming from premises located within cluster 5-OR ($P_{5\text{-OR}\rightarrow 6\text{ DST}} = 0.77$; $P_{6\text{-DST}\leftarrow 5\text{-OR}} = 0.78$). These movements occurred between 4th and 10th September. A premise of the type CCA received 63.9% and dispatched 33.3% of the equidae movements that constituted the compartment. Burgos compartment was composed by three clusters named 2-OR, 2-DST and 3-DST, which lasted from September to October. Cluster 2-OR dispatched and received equidae movements to and from cluster 2-DST ($P_{2\text{-OR}\rightarrow 2\text{-DST}} = 0.42$; $P_{2\text{-DST}\leftarrow 2\text{-OR}} = 0.91$) between 25th September and 1st October; and dispatched and received equidae movements to and from cluster 3-DST ($P_{2\text{-OR}\rightarrow 3\text{-DST}} = 0.32$; $P_{3\text{-DST}\leftarrow 2\text{-OR}} = 0.76$) between 16th and 22th October. In the compartment, the most important premise was a CCA that received 43.5% of the equidae movements that constituted the compartment. Finally, Leon compartment was composed by two clusters named 3-OR and 4-DST. Cluster 3-OR dispatched and received equidae movements to and from cluster 4-DST ($P_{3\text{-OR}\rightarrow 4\text{-DST}} = 0.25$; $P_{4\text{-DST}\leftarrow 3\text{-OR}} = 0.28$) between 27th and 28th November. A premise of the type CCA received all movements that constituted the compartment. All other clusters did not constitute compartments because they were not related temporal.

Discussion

To the best of the authors' knowledge, this is the first analysis of the network of equidae movements into a country, which combines social network and cluster analysis methods. Specifically, the study here provided a full description and characterization of a complete and reliable network of equidae movements within the Spanish region of Castile and Leon. Results allowed to identify, (i) the most important premises and type of premises in terms of volume of equidae movements and the number of animals dispatched and received; (ii) the most important types of premises in terms of potential disease introduction and disease spread; (iii) the significant space-temporal clusters of premises at high risk of dispatching and receiving equidae movements; and (iv) the relation between clusters (compartments). These results will help to strategically allocate the resources in order to design surveillance program and control future equidae diseases into the region.

There are several routes or paths through which equidae diseases may be introduced and subsequently disseminated into disease-free equidae populations, for example, trade of live equidae and their products (Lubroth, 1988), movement of equidae for human activity (Callinan, 2008), and mechanical transmission associated with movement of people, insects and contaminated objects (Rappole et al., 2000; Sellers et al., 1977). Nevertheless, the report of a large number of outbreaks of equidae diseases that have been associated to equidae movements through history, notably those of equine influenza, equine infectious anaemia and AHS reported, respectively, in Australia in 2007 (Callinan, 2008), in Great Britain in 2006 (OIE, 2011) and Spain in 1987 (Lubroth, 1988; Rodriguez et al., 1992; Sánchez-Vizcaíno, 2004), may lead to consider equidae movement as one of the most important routes for the introduction and spread of equidae diseases within and between regions. Therefore, characterization of equidae movement patterns into a region may help to identify potential risks for disease introduction or spread. In this regard, previous studies were aimed to evaluate the network of contacts between racehorse trainers into the UK (Christley and French, 2003), the potential introduction of vector-borne diseases into equine premises of Spain (Martínez-López et al., 2011), and the movements of equine influenza infected horses into Australia (Firestone et al., 2011). However, those earlier studies did not describe nor characterize a complete network of equidae movements into a region such as the study here.

Social network characterized here was composed by equidae nodes distributed throughout CyL (Fig. 1). In contrast with the mean number of animals moved per shipment in social networks described for species such as swine (48.36 pigs per shipment in Bigras-Poulin et al. (2007) or 58 pigs per movement in Martinez-Lopez et al. (2009b)) or cattle (13.5 bovine per shipment in Robinson and Christley (2007)), the number of equidae moved per shipment into CyL were much lower (3.64). This result may be explained, at least in part, by the personalized care attributed to each of these animals, which usually count on a single box, private veterinarian, and individual horse tack. Months with more trade activity were mainly associated to horse competitions, specifically in one centre of livestock competition located in Leon province, which received 270 movements of 157 premises. The distance covered by 50% of the equidae

Table 1. Clusters of premises at high risk of dispatching (OR group) and receiving (DST group) equidae movements within Castile and Leon during 2008

Cluster name	Radius (km)	Starting date (dd/mm/yy)	Ending date (dd/mm/yy)	*P*-value	Operations	Observed movements (O)	Expected movements (E)	O/E
OR group								
1 OR	34.27	13/03/08	26/03/08	0.001	100	100	10.642	9.39
2 OR	45.47	25/09/08	22/10/08	0.001	109	154	35.099	4.38
3 OR	40.36	23/10/08	10/12/08	0.001	196	189	81.062	2.33
4 OR	0.00	12/06/08	18/06/08	0.001	1	18	0.441	40.76
5 OR	21.39	04/09/08	10/09/08	0.001	46	47	7.395	6.35
6 OR	3.74	07/08/08	31/08/08	0.001	5	12	0.242	49.43
DST group								
1 DST	32.39	13/03/08	26/03/08	0.001	73	92	8.836	10.41
2 DST	15.69	25/09/08	01/10/08	0.001	31	70	4.561	15.34
3 DST	0.00	16/10/08	22/10/08	0.001	1	63	3.050	20.65
4 DST	1.70	27/11/08	03/12/08	0.001	5	93	11.447	8.12
5 DST	0.00	05/06/08	18/06/08	0.001	1	34	1.957	17.36
6 DST	8.79	04/09/08	10/09/08	0.001	22	46	6.289	7.31
7 DST	10.61	14/08/08	20/08/08	0.001	3	19	0.504	37.64
8 DST	0.31	31/07/08	06/08/08	0.001	3	12	0.211	56.82
9 DST	0.00	10/07/08	23/07/08	0.001	1	12	0.403	29.72
10 DST	0.00	06/11/08	12/11/08	0.001	1	10	0.307	32.49
11 DST	62.27	15/05/08	21/05/08	0.001	11	8	0.295	27.05

movements within CyL was similar to the median of distances covered in the movement networks described for cattle and swine within CyL (Martinez-Lopez et al., 2010) and similar also to the distances describe for cattle and swine within France (Rautureau et al. 2011 and 2012). However, that distance was greater than the median of distances covered by the movements of equine influenza infected horses in Australia in 2007 (Firestone et al., 2011) and cattle into Italy in 2007 (Natale et al., 2009). The wide distribution of equidae nodes throughout CyL joined to the fact that these nodes send animals to long distances within the region (Fig. 1) may imply long-distance spread in case of disease introduction. Nowadays, in the context of animal health, the investigation of the sources of infection and potential further spread after an outbreak is based on epidemiological surveys which are filled up by the farmers after the notification of the disease. These surveys have limited ability to detect possible secondary outbreaks since their results are certainly influenced by the stress/apprehension of the farmers after reporting the disease. For that reason, studies as the one presented here, in which animal movement patterns are characterized for a whole territory, during "freedom of disease" periods, may help to identify frequent contacts between farms and areas in particular time periods, and together with the epidemiological surveys, may help to increase the rapidness and sensibility of detecting secondary outbreaks.

Equidae nodes of CyL with more importance in the network of equidae movements were those classified as production and reproduction farms (PR), centres of livestock competition (CCA) and traders (TO). PR were the production type with more number of nodes within the network (78.56% of total nodes) but had low normalized degree and closeness values (Fig. 3) since received and dispatched a low mean number of movements per node (1.0 (95% PI = 1.17 – 0.93) and 1.6 (95% PI = 1.74 – 1.47) movements, respectively). These low values could be associated with the number of animals that hold the PR farms in CyL, since the 25%, 50% and 75% of these farms had only 4, 10 and 28 animals, respectively during 2008. Therefore, as a consequence of such low number of animals in PR in CyL, one may expect that the mean number of incoming and outgoing equidae movements per each of these farms be as low as 1 and 1.6, respectively. However, CCA, although represented 2.6% of total nodes within the network had significant normalized degree and closeness values (Fig. 3), receiving and dispatching the highest mean number of movements per node (22.33 and 65.13 movements, respectively). Similarly, TO, although represented only 0.9% of total nodes within the network had a high normalized degree and closeness values (Fig. 3), receiving and dispatching a high mean number of movements per node (9.74 and 5.21 movements, respectively). As a consequence, one may expect that the infection of CCA or TO would produce larger epidemics (in magnitude and duration) than the infection of PR.

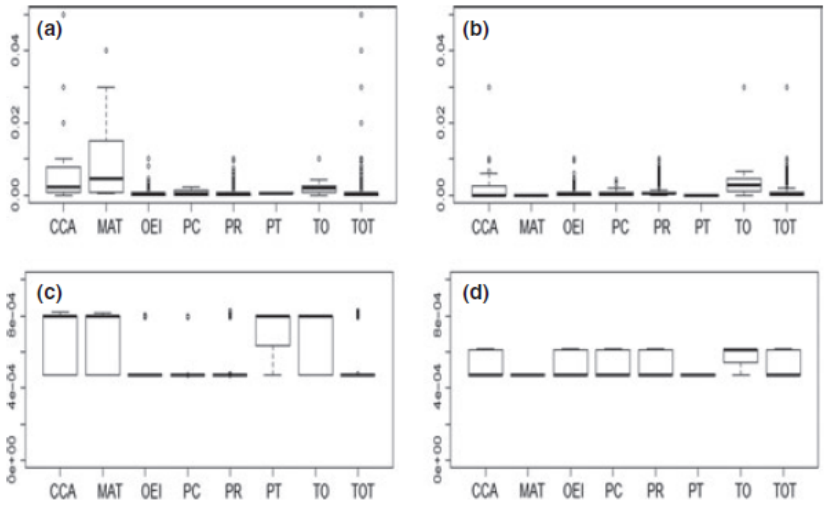

Fig. 3. Distribution of the normalized values of input degree (a), output degree (b), input closeness (c) and output closeness (d) per type of premises and total (TOT) of the network of equidae movements within the Spanish region of Castile and Leon in 2008. Types of premises are centres of livestock competition (CCA); slaughterhouses (MAT); entertainment, teaching and researching centres (OEI); pastures with/without common use (PC); production and reproduction farms (PR); permanent bullrings (PT) and traders (TO).

The mean normalized degree centrality of the network was estimated to be very low. This result could be due to the fact that 90% of nodes of the network received and dispatched less than 3 movements in the year 2008. The mean normalized closeness value of the network was very low indicating that not exist an important number of direct links between all nodes of the network. The normalized degree and closeness values were estimated for each node of the network and mapped using the Kernel density function in order to identify areas of CyL which receive or dispatch the largest number of equidae movements and areas with the nodes most closely connected, respectively (Fig. 2). It is true that the kernel algorithm smoothes and projects the point-data over an area, but certainly this is the intention when we want to represent spatial hot spots in terms of contacts. This tool has been used extensively to spatially represent and evaluate properties of premises located in specific point locations (Savill et al., 2006; Van Steenwinkel et al., 2011). Similarly to those previous works, here we were interesting on spatially visualizing areas important in terms of contacts (i.e. areas concentrating a group of farms with high centrality values). Unfortunately, other representation methods, such as the use of graduated points for individual farms, in areas where farms are very close one to each other, as it is the case here, do not allow to properly visualize the premise values (i.e. big points representing high values are overlapping and covering the small points). For that reason, the use of raster maps produced using the kernel algorithm in GIS was considered appropriate for the purpose of rapidly visualizing areas where "influential" premises are particularly concentrated.

It is important to note here that centrality measures alone should be used with caution since they has been calculated for a static network, without considering the temporal/chronological dimension of the contacts and their use may be limited in time-varying graphs (Natale et al., 2011). Moreover, centrality measures are not useful to identify individual nodes or contacts that, despite not being central, may act as bridges or cut-points (i.e. connecting groups or sub-populations within the network) which may significantly impact the infection dynamics in a particular time period (Keeling et al., 2010). Certainly other centrality measures should be developed to specifically address the epidemiological potential role of a node within an epidemic, similarly to those presented by Natale et al., 2011, but hopefully applicable for more extensive regions and time periods and not so computer-intensive. For that reason, in the study presented here we do not use the centrality measures alone but in combination with space-time cluster analysis methods. The aim was to incorporate time, space as well as directionality and nature of contacts by combining the in-out degree centrality measures with the space-time cluster analysis and type of premise. As a consequence, we are able to capture the behaviour of time-varying graphs and provide time periods, areas as well as directionality of the risk (introduction/spread), which was consider useful to support risk-based surveillance and control programs of equidae diseases.

Six spatio-temporal clusters of premises at high risk of dispatching equidae and eleven clusters of premises at high risk of receiving equidae were identified with described methodology. In all the clusters of the network except one, the premise that dispatched or received the highest percentage of shipments was a CCA. OR group and DST group of clusters were mainly allocated, respectively, in the North-West part and the periphery of CyL, and all clusters except two were concentrated in the second part of the year (Fig. 4). Nine of these clusters identified between July and December seem to be associated with the celebration of several national championships of great importance (CCA). These findings and observations allowed us to identify the premises that in a specific period of the year have higher risk for disease spread or introduction through equidae movement than the expected risk in the same period of time. Consequently, the risk for equidae disease introduction and/or spread into CyL through equidae movements may be reduced by allocating surveillance and control strategies in the identified spatio-temporal clusters and, particularly, in the centres of livestock competition.

Four compartments were identified within the network. Clusters OR and clusters DST that composed the compartments were spatially superimposed. It is of note that in the compartments named Zamora South, Zamora North, and Burgos most of the incoming and outgoing equidae movements occurred between the clusters that composed the compartments. Therefore, one may expect that if an equidae disease affects one premise of those compartments, then the potential spread of the disease through equidae movements would most likely be concentrated within the compartment, or in other words, the potential spread of the disease would be restricted in a reduced area of CyL. It should be noted here that other method to define compartments or modules within a network has been described by Girvan and Newman, 2002. However, this method was not used here mainly because the algorithm, which works properly for undirected networks, has not been properly adapted to analyze directed networks like the one presented here (Leicht and Newman, 2008).

Analysis of the network of equidae movements within CyL was performed with data obtained for the year 2008. Therefore, results presented here will remain valid only if conditions,

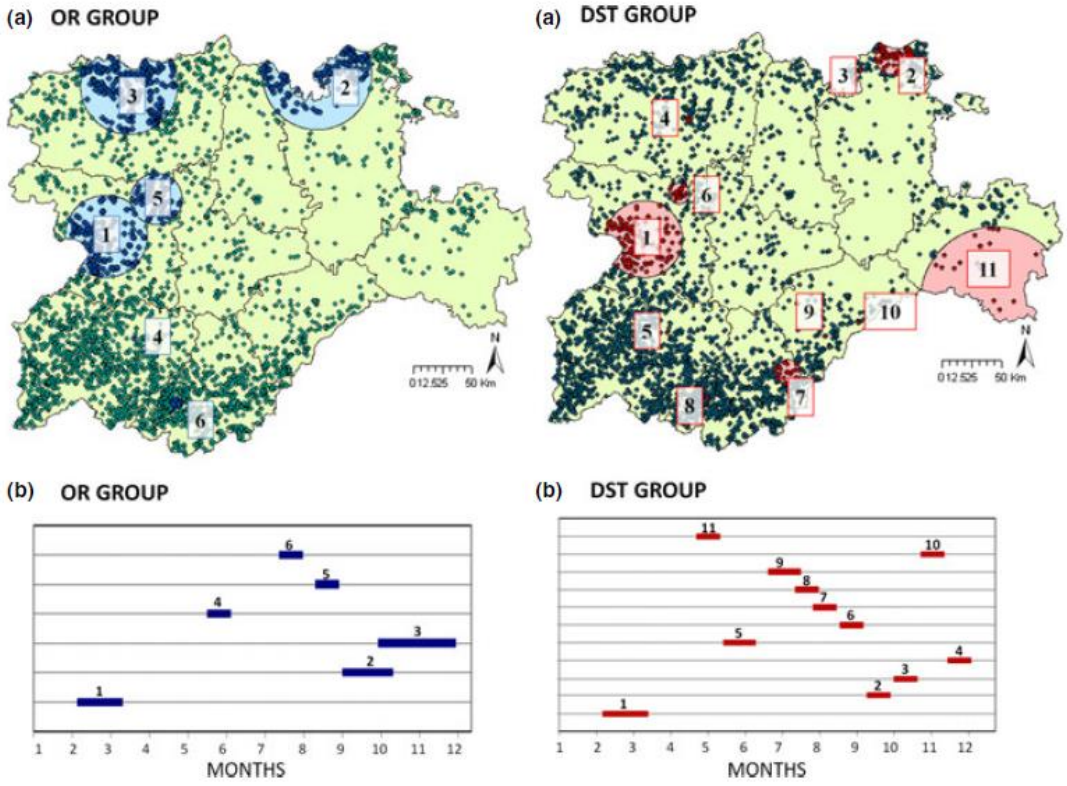

Fig. 4. Geographical (circles) (a) and temporal (horizontal bars) (b) representation of the spatio-temporal clusters of premises at high risk of dispatching (OR group: blue) and receiving (DST group: red) equidae movements within Castile and Leon during 2008. Numbers correspond with clusters' name. Internal boundaries and dark points indicate, respectively, the limit of municipalities and the location of equidae premises.

assumptions and values used to formulate and parameterize the model persist. Because quality of available information is continuously evolving and trade contacts may significantly change from one year to another, analysis presented here should be periodically revised and updated in order to provide the most recent estimates and improve the quality of conclusions, particularly if results are going to support risk-based policies.SNA was used here to characterize the nature and extent of the network of equidae movements only within CyL (i.e. the network of CyL was considered as "closed" network), so centrality measures presented here may change if incoming or outgoing movements from/into the CyL region are included in future studies. Nevertheless, for example a previous study described that the number of equidae movements that CyL receives from other territories is relatively small comparing with the number of contacts within the region (Martínez-López et al, 2011). In fact, if we compared this number of incoming movements from outside to inside CyL with the number of movements occurring within the region, they represent only a 2.82%. Similarly, the premises that receive incoming movements from other regions outside CyL are only a 2.21% of the premises that made movements within CyL in 2008. No available data about the specific number of equidae that CyL dispatches to other regions was available. However, we assume that this number is low since only two regions of CyL, Salamanca and Segovia, registered exports of equidae during 2008 (DATACOMEX, 2012). For that reason, we may conclude, that even if some bias may be present when considering a the CyL network as a closed network, the centrality values presented here for the most of the premises will not be significantly impacted since this region receive and dispatch few movements from/to others sites. However, further studies including the complete network of equidae movements within, from other regions to CyL region and from CyL to other regions will be recommended since it will allow to identify not only the regions that most contribute to the risk of spread of equidae diseases into CyL but also to identify the areas at highest risk for disease introduction into the region or for spread from CyL to other territories through equidae movements.

In conclusion, this is the first comprehensive analysis of the network of equidae movements into a country, which combines social network and cluster analysis method. The studied network was complex involving many different types of premises with diverse nature and extent of contacts. Production and reproduction farms and centres of livestock competition were the premises with more importance in the network of equidae movements of CyL. Premises with potentially high risk for equidae diseases spread and introduction through equidae movements were mainly allocated in, respectively, the North-West part and the periphery of CyL and in the second part of the year. In general, introduction of an equidae disease into CyL may imply long-distance spread (i.e. up to 40 Km.). However, if an equidae disease affects one premise of the compartments named Zamora South, Zamora North, and Burgos, then the potential spread of the disease through equidae movement would likely be concentrated within of the compartment in a reduced area of CyL. Results presented here are of interest because the identification of premises, areas and time periods with potentially high risk for equidae disease introduction and/or spread will be useful for the development of risk assessments and for supporting the design of risk-based surveillance and control programs of equidae diseases, and for increasing the efficacy and speed of detection of secondary outbreaks during the epidemic of a disease that affects the Spanish equidae industry.

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OBJETIVO 4: Evaluación económica de la difusión de la peste equina en España.

Las nuevas directrices de los organismos internacionales junto a la actual crisis financiera que sufre Europa, hacen que la **evaluación económica** sea un pilar fundamental de la **toma de decisiones** para los gobiernos europeos (UE, 2007-2013). El uso combinado de la economía y la epidemiologia, permite el establecimiento de programas de vigilancia y control más **rentables** pero con el objetivo final de **mejorar el bienestar de la sociedad**. La PEA es una enfermedad de gran repercusión socio-económica por las altas tasas de mortalidad en caballos que presenta y los costes asociados al control de la misma, especialmente en países no endémicos, así como las importantes restricciones comerciales que provoca la declaración de una enfermedad de esta importancia. Por ello, para optimizar los recursos y disminuir las consecuencias asociadas a la enfermedad aplicando las **estrategias de control óptimas**, es necesario realizar la evaluación económica de las mismas.

En este contexto se ha enfocado el **cuarto objetivo** de la tesis doctoral, el cual consiste en el desarrollo de un **modelo de difusión de la enfermedad** en nuestro país que junto con su **evaluación económica** permita analizar las consecuencias epidemiológicas y económicas de un brote de PEA comparando diferentes estrategias de control de la enfermedad.

Se desarrolló un meta-modelo matemático que integraba el análisis epidemiológico y económico de difusión de la PEA en Andalucía en varios escenarios de temperatura y ratio de vectores por hospedador. Este modelo fue utilizado para evaluar las estrategias actuales de control de la enfermedad variando la utilización o no de vacunación de emergencia. Los resultados obtenidos permitieron evaluar y comparar las consecuencias sociales y económicas que un brote de PEA podría tener esta región. Todos los escenarios permitieron la difusión de la enfermedad en Andalucía, sin embargo el impacto sanitario y económico de la epidemia de PEA sin vacunación fue mayor en comparación con el mismo escenario cuando se aplicaba la estrategia de vacunación. El impacto económico total varió entre 138 y 584 millones de euros en función del escenario, los mayores costes del impacto económico estuvieron relacionados con las pérdidas de producción y las medidas de control. El coste de la vacunación supuso únicamente entre el 2% y 3% de los costes de las medidas de control cuando era aplicada. Este trabajo es la primera evaluación económica de la PEA en un país no endémico, los resultados obtenidos

podrán ser de gran utilidad para la toma de decisiones de los servicios sanitarios proveyendo tanto el posible impacto de la enfermedad como la estrategia de control a aplicar más adecuada.

Artículos científicos:

- A. Sánchez-Matamoros, M. Martínez-Avilés, E. García-Carrión, B. Martínez-López, E. Iglesias and J.M. Sánchez-Vizcaíno, 2014. **Epidemiological and economic assessment of an epidemic of African horse sickness virus in Andalusia, Spain**. En preparación.

Epidemiological and economic assessment of an epidemic of African horse sickness virus in Andalusia, Spain

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Summary

African horse sickness (AHS) is a lethal vector-borne viral disease of equines included in the notifiable disease list of the World Organization for Animal Health (OIE), and for which Europe is officially disease-free. Given the serious socio-economic effects of AHS in the equine population, a re-emergence should be controlled through efficient decision-making based on epidemic and economic assessments. The aim of this study is to develop an epidemiological and economic meta-model to assess the impact of an AHS epidemic in the equine sector of a non-endemic country under different environmental scenarios and to evaluate whether vaccination would be recommended in this area. Specifically, the meta-model developed has been carefully parameterized to evaluate the epidemiological and economic consequences of the AHS spread, with different climatic and entomological scenarios and comparing different control strategies, in one of the regions where the impact of the disease has been historically higher in Spain, Andalusia.

A spatial model allows the prediction of the number of premises and animals involved in the control measures that follow the detection of an AHS outbreak. These data were included in the transmission and economic meta-model that assesses the epidemiological and economic consequences of two different control strategies following an outbreak confirmation: the application or not of vaccination. The socio-economic consequences of an AHS epidemic varied widely depending on the scenario for both control strategies. The most important epidemic consequence was the large number of dead and slaughtered equines, as well as the high number of immobilized and vaccinated animals. In all scenarios, the highest economic impacts were the cost of production losses and the cost of control measures. The cost of vaccination was 2 to 3% of the cost of the control measures. The control measures with a vaccination strategy allows the reduction of the socio-economic consequences of an AHS epidemic compared with the results of the control measures without vaccination, obtaining a benefit of between €2 mln and €117 mln. Furthermore, the vaccination strategy was efficient in all scenarios because the overall benefit-cost ratios are upper than one. The meta-model developed can be adapted and used under other scenarios to decide under which environmental conditions is vaccination an effective benefit-cost control measure and its use be recommended.

Keywords: African horse sickness, cost-benefit, host-vector model, economic evaluation, vaccination strategy.

Introduction

African horse sickness (AHS) is a serious vector-borne viral disease of equines with mortality rates that can exceed 95% in horses and drastic socio-economic effects. For these reasons, AHS is a notifiable disease for the World Organization for Animal Health (OIE). As a consequence, the presence of AHS infection in a country involves the restriction of all movements of susceptible animals and equine products [1].

Currently, AHS is only present in the African continent, but sporadic outbreaks have occurred in Europe in the past [2,3]. The last epidemic in Europe happened between 1987 and 1990 in Spain and had devastating effects for the Spanish equine industry, with around 1,500 dead or slaughtered equine. The epidemic was successfully controlled and eradicated with the application of control measures including the vaccination of around 500,000 equines (Sanchez-Vizcaino, 2004). All of Europe was granted AHS official freedom by the OIE in 2014 [4].

The AHS virus (AHSV) is closely related to Bluetongue virus (BTV), since both are *Orbivirus* transmitted by the same vector genus (*Culicoides* spp). Over the past decade, the spread of BTV throughout Europe evidenced the presence of new competent *Culicoides* vector species [5,6]. The underestimation of this vector population led to the re-occurrence of BTV during the following vector-season, which resulted in an estimated cost of between 164 and 175 million euros [7]. The similarity between these two vector-borne diseases supports the consideration of AHS as a re-emerging disease in Europe [6].

Today, an AHS outbreak in the European Union (EU) could have worse epidemic and economic consequences than in the past because of a wider distribution of new potential *Culicoides* vectors. The maintenance of the AHS official free status implies the application of surveillance to support such freedom, an early detection system and measures to prevent the introduction of AHS, as well as an effective contingency plan that minimizes the spread of the infection.

The control and prevention of epidemics should be achieved through an adequate decision-making, to avoid a crisis situation like the re-emergence of AHS in a disease-free country. Currently, the European Union legislation on AHS control (Council Directive 92/35/EEC) mandates, upon confirmation of a suspicion on AHS, the establishment of a protection zone (PZ), in an area of a minimum of 100 km of radius around the infected premises, and a surveillance zone (SZ) within an additional 50 km. Measures in the PZ include vector control, movement restriction, potential vaccination, and clinical inspection and sampling. In the SZ, there are additional movement controls and regular veterinary checks; however vaccination is forbidden. Vaccination is one of the most important control measures for the AHS eradication; however its implementation depends on the decision of governments based on non-described epidemiological situation. Therefore, an economic evaluation of the cost of the control measures without vaccination in place against the difference between the cost of the impact of the disease with control measures with vaccination and its cost is a formal analysis to measure the advantages of the control program against its cost.

The economic assessment has been recently promoted for the international organisms for the evaluation of animal disease and its mitigation strategies [8,9]. This analysis should be used to estimate the economic impact of diseases or compare the cost and benefit of management measures for the improvement of the decision-making. Therefore, the economic models have been used for the evaluation of economic impact of different animal diseases such as Bluetongue [7], Foot and Mouth Disease [10] or Avian Influenza [11]; and its mitigation strategies [12-15]. However, any study about economic consequences for AHS spread or the comparison of different control strategies for this disease in vulnerable disease-free country has been performed. Consequently, the economic assessment of AHS could be useful to design AHS control programs in vulnerable disease-free countries based on economic efficiency.

The aim of this study was to perform an epidemiological and economic meta-model to assess an AHS epidemic for the equine sector in one of the AHS risk regions where the impact of the disease would be higher in Spain, Andalusia, by developing different scenarios and comparing different control strategies. This study tries to evaluate whether the vaccination strategy in the PZ would be recommended in this area or if non-vaccination would be a better cost-benefit measure. This study is the first evaluation of the economic consequences of this disease in a vulnerable non-endemic country.

Methodology

2.1. Study region and population

The region of Andalusia was selected to assess the economic consequences of AHS for three reasons: first, it is the largest region in Spain in terms of equine census [16]; second, it is one of the first Spanish regions with higher percentage of equines belong to native Spanish breeds [17], which have a high genetic and economic value for the country; and finally, this region was affected by the two AHS epidemics in Spain, 1966 and 1987-1990 [18].

The premises and equine numbers in Andalusia in 2013 have been obtained from the Spanish Ministry of Agriculture [16] (220,764 equines and 72,192 premises). The model differentiates between purebred horses, of high monetary value, and other equines. We estimated a census of 92,721 purebred horses in Andalusia, 30,598 of which were in breeding premises, based on the estimations by Deloitte [17] and Sánchez Bazán [19].

2.2. General overview and scenarios

We developed a meta-model, integrated by an epidemiological and an economical model, to evaluate the consequences of an AHS epidemic in Andalusia under two control strategies, one with and the other without vaccination (baseline strategy) in the PZ. Both strategies were assessed by modelling an AHS epidemic under two different climatic scenarios of temperature (10-20 ºC and 20-30 ºC), which represent the average temperature range in Andalusian in its AHS risk period (April-December) [20]. Within each climatic scenario, we tested three different vector-host ratios (low, medium and high). Combining vector-host ratios and climatic scenarios, we obtain six possible scenarios that embrace the three seasons that occur under the identified AHS risk period for Andalusia [21]. This way, during spring there could be low to medium value of vector-host ratios and temperatures between 10 and 20ºC, in the summer we would expect medium to high vector-host ratios and temperatures between 20 and 30ºC and above, and in autumn we assume all possible vector-host ratios and temperatures between 10 and 20ºC (Table 1).

For the initial condition (t=0), the number of susceptible animals was assumed to be equal to the total number of equines in Andalusia minus one infectious animal. This model was evaluated daily (time step of 1 day) for a period of 90 days, which represents the duration of each of the seasons (3 months).

Table 1. Scenarios of meta-model in Andalusia based on literature review and database.

	Low ratio	Medium ratio	High ratio
10 - 20 ºC	ES1A	ES2A	ES3A
20 - 30 ºC	ES1B	ES2B	ES3B

2.3. Epidemiological transmission and spread model

A deterministic compartment transmission model (Figure 1) was developed to simulate the disease dynamics in Andalusia. It is a vector-host model similar to the ones developed by Lord et al. [22] and Backer and Nodelijk [23] but with modifications, since we want to estimate the number of animals that would be either immobilized or vaccinated in a spatial context. To estimate the number of animals under each control strategy, we first developed a spatial spread model. Both the transmission and the spread model were developed in MATLAB software (2013) with ode45 function.

2.3.1. Development and inputs of the spatial spread model

To estimate the number of vaccinated animals (VA) in the PZ, and the number of immobilized animals (ZA) in the PZ and SZ following a confirmation, we modelled the spread of AHS from an infectious holding (IF) to a varying number of final infectious holdings (FIF). Firstly, we randomly distributed the 72,192 equine premises in a map of Andalusia assuming a spatial uniform distribution. Next, we estimated the distribution of the 220,764 equines in Andalusia per farm by fitting a gamma distribution (shape = 0.46 and scale = 4.5), where the majority of holdings have a small number of equines (one or two) and very few have a large number.

We run 10,000 Monte Carlo simulations until the following number of FIF was reached (1, 5, 10, 25, 50, 75, 100, 250, 500, 750, 1000). The first step of each simulation starts with one IF, considering that, for every IF, the model would search for other premises within a 20-km radius, which was the distance of 85% BTV8 transmission herds in 2006 [24]. When the specified FIF was reached,

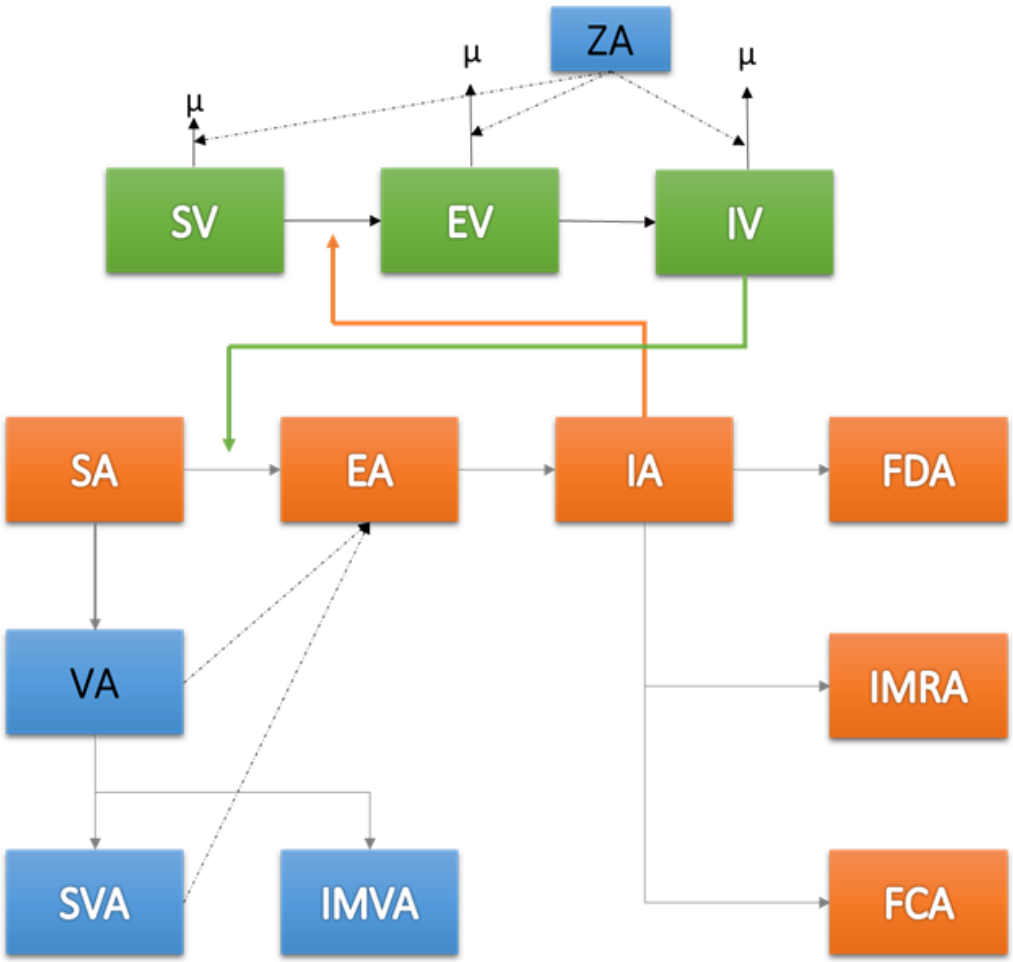
the sum of the total number of premises that were within the different PZ and the different PZ and SZ resulted, respectively, in the number of vaccinated and immobilized premises and the number of VA and ZA. Since we had run 10,000 Monte Carlo simulations, we calculated the median number of VA and ZA for each specified NIF. Finally, we fitted a regression function, with SPSS 2.1 software, to estimate the number of VA and ZA that would feed the transmission model.

2.3.2. Development and inputs of the transmission model

This AHS transmission model includes one vector species (*Culicoides*) and one host species (horse). A vector can only be susceptible (SV), latently infected (EV) or infectious (IV), being this the last stratum before the end of vector-life. The equine host comprises the stages of susceptible (SA), latently infected (EA), infectious (IA), and the final stages of dead animals (FDA), natural recovered animals (IMRA) and slaughtered animals (FCA). In addition, the host and vector could be affected by AHS control measures.

Vaccination takes between 14 and 21 days to produce an effective immunization. In the model, we have differentiated between animals injected with the vaccine but still susceptible (VA), and animals immunized by the vaccine (IMVA) and therefore no longer susceptible. This way, we have considered that for around 30% of the vaccinated animals (expert opinion), the vaccine has not been

Figure 1. AHS transmission model. In orange, the equine host strata comprises the stages of susceptible (SA), exposed (EA), infectious (IA) and the final stages of dead animals (FDA), natural recovered animals (IMRA) and slaughtered animals (FCA). Control measures (in blue) comprise immobilized animals (ZA) and vaccinated animals (VA). If the vaccine was not effective, animals are again susceptible (VA and SVA). The immunized animals through vaccination were identified as IMVA. In green, the vector strata comprise the compartments of susceptible (SV), exposed (EV), infectious (IV) or dead caused by AHS (DV).



effective, and would therefore remain susceptible (SVA). Also, there is an average time lapse between vaccination and effective immunization.

Immobilization of animals (ZA) has been directly related to the spraying of vector in the premises, which will influence the vector mortality in the model (M_v). We assumed that around 70% of the immobilized animals are sprayed. Furthermore, we estimated a time period between 2 and 3 days from immobilization to carry out an efficient insecticide treatment of the premises.

The model can start with either an infectious vector (*IV*) or an infectious equine (*IA*). Transmission of AHS from one *IV* to a susceptible animal (*SA*) will depend on the number of vector bites per animal per day (λ); on the probability that an *IV* bites a *SA* within the population (*SA*/*NA*), and also on the probability that the bite successfully transmits the virus, p_{va}.. This is represented by the force of infection from *IV* to *SA (B),* that can be expressed as $B = p_{va} \cdot \lambda \cdot IV \cdot SA/NA$.

Table 2. Parameters in the epidemiological model for the AHS spread.

Parameter	Description	Units	Value	Source
p_{va}	Probability of transmission from vector-host	[0,1]	Beta distribution: Beta(6,2)	[23]
p_{av}	Probability of transmission from host to vector	[0,1]	Gamma distribution: Γ(1.77,0.0225)	[23]
λ	Reciprocal of the time interval between blood meals	day^{-1}	$(1.71 \cdot 10^{-4}\ T) \cdot (T - 3.7) \cdot (41.9 - T)^{1/2.7}$	[26], *
$1/\varepsilon_a$	Latent period in animals	day	Gamma distribution: Γ(16.9,0.219)	[23]
$1/\varepsilon_v$	Period of extrinsic incubation (EIP)	day	Gamma distribution: Γ(κ, θ)	[29]
p_m	Proportion of dead animals due to disease	[0,1]	Uniform distribution: U(0.43,0.97)	[23]
p_c	Proportion of culled animals	[0,1]	0.20	[B]
p_{ef}	Proportion of effectiveness of the attenuated live vaccine	[0,1]	0.70	[C]
$1/\gamma$	Duration of viraemia dying animals	day	Gamma distribution: Γ(19.36,0.23)	[23]
$1/\delta$	Viraemia recovering animals	day	Gamma distribution: Γ(24.59,0.202)	[23]
p_v	Proportion of vaccinate animal	[0,1]	0.95	[B]
$f(IF)_V$	Estimation of vaccinated animals	animal	$46925.3 * IF^{0.208}$	[A], **
mva	Maximum number of vaccinated animals per day	animal	5000	[B]
$1/\tau$	Time period until immunization of the host with attenuated live vaccine	day	Uniform distribution: U(14,28)	[C]
$f(IF)_Z$	Estimation of immobilized animals	animal	$90724.6 * IF^{0.126}$	[A], **
α	Vector recruitment rate	day^{-1}	For simplicity assumed to be equal to the vector mortality rate	-
μ	Vector mortality rate	day^{-1}	$\mu = 0.015^{0.063\ T}$	[25], *
M_v	Increase in vector mortality due to spraying		3	[15]
$1/x$	Time period until insecticide treatment of the host and premise	day	Normal distribution: N(3,2)	[B]
p_s	Probability of immobilized animal was treated with spraying	[0,1]	0.7	[B]

[A] Estimation in MATLAB software; [B] Expert assessment; [C] Expert assessment and Onderstepoort Biological Products Information; * T = Temperature; **IF = Infected Premises

Similarly, AHS transmission from one *IA* to one susceptible vector (*SV*) will depend on the number of vector bites received per animal per day (λ); on the probability that a *SV* bites an *IA* is the fraction *IA/NA*, and also on the probability that the virus is successfully transmitted to the *SV* through the bite, $p_{av.}$ Ultimately, the force of infection from an *IA* to *SV (b)* is expressed as $b = p_{av} \cdot \lambda \cdot SV \cdot IA/NA$.

The natural host mortality and reproduction rate were not included because the natural lifespan is longer than the AHS epidemic duration. However, we did consider the animals that died because of AHS ($\mathbf{p_m}$), and also those that could be humanely sacrificed because of severe clinical signs ($\mathbf{p_c}$). Furthermore, we assume that the animals that do not die or are euthanized, recover from the disease having lasting immunity (IMRA). We also assume that these animals remain infectious until they reach IMRA.

The biting rate λ, the extrinsic incubation period ε_v, the vector mortality rate μ and the recruitment rate α increase with rising temperature [25-27], hence, temperature dependent functions have been used to evaluate these parameters (Table 2).

All of the AHS epidemic model inputs were determined based on a literature review and expert opinion (Table 1), except for the estimation of the proportion of animals vaccinated (*VA*) and immobilized (*ZA*) which were obtained from the spatial spread model.

The differential equations that express the transmission of AHS between each population state in both the host and the vector strata are provided as supplementary material (Supplementary data, Annex 1).

2.4. Economic model

To estimate the potential economic consequences of an AHS epidemic (C_{TOT}) in Andalusia under different scenarios, we adapted the methodology of cost-benefit analysis to AHS and its mitigation strategies. In this model, the baseline cost of each scenario implemented control strategies without vaccination (baseline strategy) was compared with the costs and benefits of alternative AHS control with vaccination. The total benefit was defined as the disease costs estimated to have been avoided as a result of the vaccination strategy.

The economic model estimated the costs of AHS incursion in the different evaluated control strategies in Andalusia. We summed the costs involved in an epidemic following the detection of a symptomatic horse with AHS compatible signs, which include the treatment and diagnostic costs upon suspicion, the costs associated to control measures upon confirmation (immobilization, vaccination, control vectors and surveillance), and the production losses associated to AHS or to the immobilization control measure. Therefore, C_{TOT} is formulated as:

$$C_{TOT} = C_P + C_D + C_T + C_M, \qquad (2)$$

where C_P represents production losses, C_D diagnostic costs, C_T treatment costs and C_M the direct costs associated to control measures.

The sources of the inputs for the economic model are detailed in Table 3 and Table 4.

2.4.1. Production losses of AHS-suspect infectious animals

The production losses C_P derived from an epidemic depend on the mortality costs C_{mo}, slaughter for welfare reasons (humane slaughter) costs C_{hu} and lower activity costs C_{la}.

Mortality costs are proportional to the number of dead animals and their costs per type of animal:

$$C_{mo} = \sum_{j \in J} DA_j \cdot (AC_j + KC + FC), \qquad (3)$$

where J is the type of animal (purebred horses for reproduction; purebred horses; other equines), DA_j is the number of dead animals of type j, AC_j is the estimated monetary value per animal of type j, KC the cost incurred to have carcass removed per animal and FC the cost of cleaning of each box.

Humane slaughter costs depend on the estimated proportion of animal culled earlier:

$$C_{hu} = \sum_{j \in J} CA_j \cdot (AC_j + KC + FC + CC), \qquad (4)$$

where J is the type of animal, CA_j is the number of culled animals of type j, AC_j is the estimated monetary value per animal of type j, KC the cost incurred to have carcass removed per animal, FC the cost of cleaning the box and CC is the cost of slaughtering for each animal.

Opportunity cost arise form low activity losses per infectious animal which have been estimated per infectious animal based on expert assessment.

$$C_{la} = IA \cdot PC, \quad (5)$$

where IA is the number of infectious animals and PC is the estimated loss for low activity for the whole period following infection. These losses have been valued based in the expert opinion of several Andalusian holding owners, mainly competition and leisure. This value includes the government compensation.

Finally,

$$C_P = C_{mo} + C_{hu} + C_{la}. \quad (6)$$

Table 3. Epidemiological input of the financial calculations of the AHSV spread regarding without and with control measures.

Description	Symbol	AHSV spread	Control measures	Source
Proportion of diseased animal treated	PN	Uniform (70, 80)	Uniform (70, 80)	Expert assessment
Number of samples taken per premise	SN	3	3	Estimation based on the average of size of premise and MAGRAMA [16]
Proportion of samples tested PCR (%)	SP_{PCR}	50	50	[39]
Proportion of samples tested ELISA (%)	SP_{ELISA}	50	50	[39]
Number of doses of vaccine per animal	ND	0	1	Available attenuated vaccine
Number of desinsected treatments per premise	IN_f	4	4	Manufacturer instructions
Number of desinsected treatments per animal	IN_a	2	2	[7]
Number of desinsected vehicle per premise	VN	Uniform (0, 50)	Uniform (0, 50)	Expert assessment for a 90 days period

2.4.2. Diagnostic cost of AHS-suspect infectious animals

The diagnostic cost, C_D, is composed of the sampling cost taken by the official veterinarian (VC_o), the submission cost and the test cost. In this study, a holding with at least one infectious animal is considered an infected holding (IF), that is, we are assuming that every infectious animal exhibits detectable clinical signs. The average of IF was obtained by dividing the number of IA by the average of equines per premise.

One blood sample and one serum sample per animal is then tested by PCR and ELISA respectively.

$$C_D = IF \cdot (VC_o + SC + SN \cdot (SP_{PCR} \cdot SC_{PCR} + SP_{ELISA} \cdot SC_{ELISA})), \quad (7)$$

where IF is the number of infected premises, VC_o is the labour cost of the official veterinarian, SC is the laboratory submission costs, SN is the mean number of samples taken in each premises, SP_{PCR} and SP_{ELISA} are the proportion of samples tested per PCR and ELISA, and SC_{PCR} and SC_{ELISA} the costs per test.

2.4.3. Treatment cost of AHS-suspect infectious animals

Nowadays, palliative care is the only treatment available to relieve the suffering from AHS signs and to prevent secondary infections. The treatment cost includes the cost of the private

veterinarian labour and the cost of veterinary medicines. The veterinary medicines can include pain killers, antibiotic or corticosteroids. The treatment costs are defined as follows:

$$C_T = IF \cdot VC_p + IA \cdot PN \cdot TC, \quad (8)$$

where IF is the number of infected premises, VC_p is the labour cost of the private veterinarian, IA is the number of infectious animals, PN is the proportion of animals treated and TC is the cost of treatment.

Table 4. Economic input for the cost calculations of consequences of the AHS epidemic

Input	Symbol	Value or distribution	Description /Source
Production losses	C_P		
Mortality cost	C_{mo}		
Purebred equine value for reproduction (€/animal)	AC_1	Uniform (3600, 9000)	Expert assessment based on information collected from BOE [32]
Purebred equine value (€/animal)	AC_2	Uniform (600, 4000)	Expert assessment based on information collected from BOE [32]
Equine value (€/animal)	AC_3	Uniform (350, 1100)	Expert assessment based on information collected from BOE [33]
Carcass removed (€/animal)	KC	200	Expert assessment based on information collected from owners
Cleaning (€/premise)	FC	50	Expert assessment based on information collected from owners
Early culling	C_{cu}		
Slaughter cost (€/animal)	CC	Normal (100, 40)	Expert assessment based on information collected from private veterinarian
Lower activity	C_{la}		
Production value (€/animal per day)	PC_1	Uniform (3.5, 17)	Expert assessment based on information collected from owners and BOE [33]
Diagnostic costs	C_D		
Official veterinarian (€/visit)	VC_o	Normal (30, 10)	Expert assessment based on information of official retributions
PCR (€/sample)	SC_{PCR}	22	OIE reference laboratory
ELISA(€/sample)	SC_{ELISA}	2.7	OIE reference laboratory
laboratory submission (€/sample)	SC	20	Private company of transportation
Treatment cost	C_T		
Private veterinarian (€/visit)	VC_p	Normal (50, 25)	Expert assessment based on information collected from private veterinarian
Price veterinary treatment (€/animal)	TC	Uniform (50, 120)	Estimation based on price list of private company and veterinary practitioners.
Control measures	C_M		
Vaccination cost	C_{va}		
Dosage vaccine (incl. material)	DC	10	Estimation based on price list of Department of Veterinary Services of Botswana
Registration	RC	0.05	Estimation based on price list of bluetongue vaccine [12]
Vector mitigation measure	C_{ve}		
One insecticide per box of 35 m³ (€/box)	IC_f	Uniform (0.84, 2.94)	Estimation based on price list of private company
Price of insecticides (€/animal)	IC_a	1.09	Estimation based on price list of bluetongue [7]
Price of insecticides (€/vehicle)	IC_v	Uniform (0.96, 1.92)	Estimation based on price list of private company

2.4.4. Costs of the control measures after confirmation of AHS.

Following confirmation of a suspect case, the total cost of the implemented control program has been estimated based on the cost of four control measures: animal movement restriction costs, vaccination costs, vector mitigation costs and surveillance costs.

The cost associated to the imposed by the animal movement restriction C_{mr} has been evaluated in the protection zone (PZ) and surveillance zone (SZ). The cost included the cost for absence of the activity in this period in the two zones (ZA). To avoid double counting we detract the production losses due to AHS of the infectious animals (section 2.4.1) already considered.

$$C_{mr} = (ZA - IA) \cdot PC, \quad (9)$$

where ZA is the number of animals in restricted movements area, IA is the number of infectious animals, and PC is the estimated losses caused by non-production. Once immobilized, animals remain so for the rest of the simulation period.

The animals in the protection zone are included in the vaccination campaign (VAC). The corresponding costs are defined as follows:

$$C_{va} = VC_p \cdot VF + (VA) \cdot (ND \cdot DC + RC), \quad (10)$$

where VC_p is the labour cost of private veterinarian, VF is the number of vaccinated premises, ND is the number of vaccine doses per animal, DC the cost of one vaccine doses and RC the registration cost per animal.

The vector mitigation measure (VEC) included the treatment of their animals and facilities with insecticides, as well as the vehicles entering and leaving the holding.

$$C_{ve} = (ZF) \cdot \left(IC_f \cdot IN_f + AF \cdot IC_a \cdot IN_a + VN \cdot \mathrm{IC}_v\right), \quad (11)$$

where ZF is the number of premises in the restricted area, IC_f and IC_a the cost of insecticides per holding and animal, IN_f and IN_a the number of insecticides treatments needed in 90 days per holding and animal, AF is the mean number of animals per holding, VN the number of vehicles to be desinsected in 90 days, and IC_v the cost of insecticides per vehicle.

The cost of AHS surveillance (C_{ss}) takes into account similar variables to the diagnosis cost although for all premises in the protection and surveillance zones, except previously diagnosed infectious premises.

$$C_{ss} = (ZF - IF) \cdot (VC_o + SC + SN \cdot (SP_{PCR} \cdot SC_{PCR} + SP_{ELISA} \cdot SC_{ELISA})), \quad (12)$$

where ZF is the number of premises in restricted movements area, IF is the number of infected premises, VC_o is the labour cost of the official veterinarian, SC represents the laboratory submission costs, SN is the mean number of samples taken in each holding, SP_{PCR} and SP_{ELISA} are the proportion of samples tested per PCR and ELISA, and SC_{PCR} and SC_{ELISA} the costs per test.

Finally,

$$C_M = C_{mr} + C_{va} + C_{ve} + C_{ss}. \quad (13)$$

2.5. Outputs of meta-model

The meta-model combines the outputs of the epidemiological and the economic models for each of the two control strategies explored and for each climatic and vector scenario, so that they can be compared.

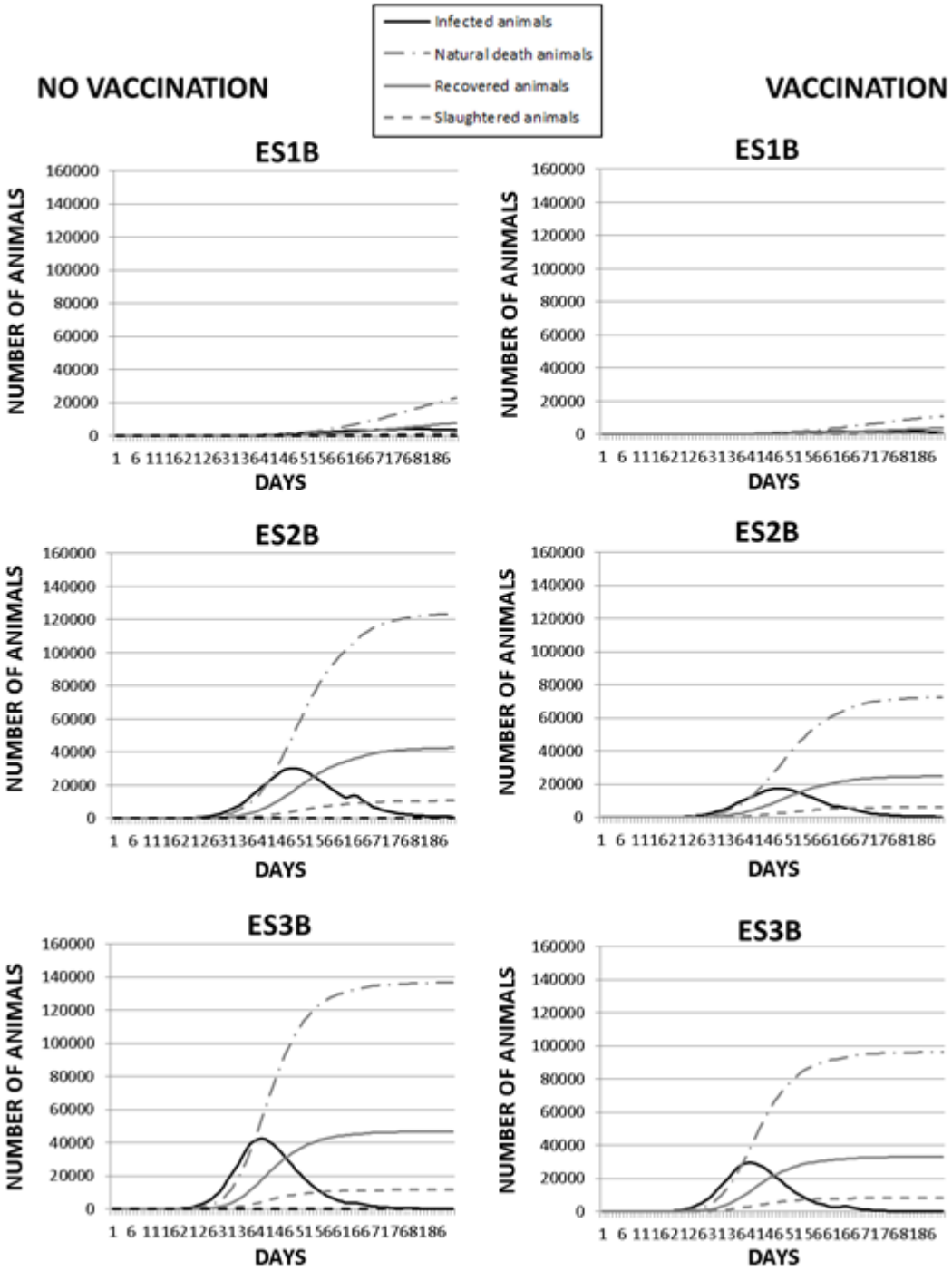
The epidemiological outputs for each scenario are number of infectious animals (IA), dead animals (FDA), naturally recovered animals (IMRA), slaughtered animals (FCA), vaccinated animals (VA) and immobilized animals (ZA), as well as the number of infected premises (IF), vaccinated premises (VF) and immobilized premises (ZF) through the average number of host per premise (AF).

The predicted economic outputs for each scenario are the production losses (C_P), diagnostic costs (C_D), treatment costs (C_T) and the global cost related to the control measures cost (C_M) and estimated for each measure (animal movement restriction costs (C_{mr}), vaccination costs (C_{va}), vector mitigation measure costs (C_{ve}) and surveillance of disease costs (C_{ss})).

2.6. Economic criteria to compare the vaccination strategies.

The comparison between the different implemented control strategies was performed analyzing the economic benefit of vaccination and the benefit-cost ratio. The economic benefit of vaccination is the reduction of the potential economic impact when implementing the vaccination strategy compared with the estimation cost when the vaccination strategy would be not applied. The benefit-cost ratio is the total benefit divided by the total cost of vaccination (C_{va}). When the results exceed one, the strategy is economically efficient, since it shows how benefit is generated per euro invested in the control measure.

Figure 2. Dynamic behavior of the transmission model of scenario B (temperature of 20-30º) under control measure without or with vaccination.



2.7. Sensitivity analysis

The modification of the most influence inputs of the model was performed using a sensitivity analysis. A change of ±10% of each individual input was performed to evaluate its impact on benefit-cost ratio. Sensitivity analysis was conducted for the following parameters: equine value for reproduction, purebred equine value, equine value, production value, price veterinary treatment, vaccine.

Results

3.1. Output of the spatial spread model

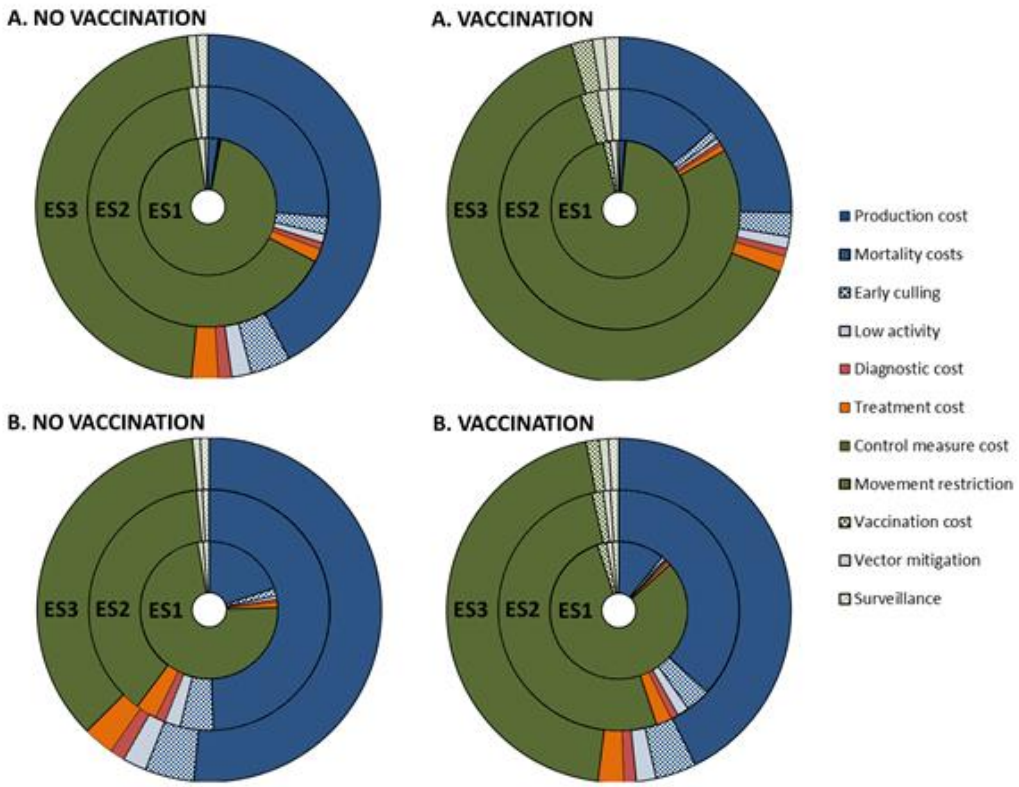
With the real data on census and equine holdings [16], we simulated, through the spatial model, the estimated number of vaccinated and immobilized animals based on a range of potential final infected premises (FIF) from a single infectious holding source in Andalusia. The fitted regression functions of the average number of vaccinated and immobilized animals were, respectively: $Y = 46925.3 * IF^{0.208}$ and $Y = 90724.6 * IF^{0.126}$ (Supplementary data, Annex 2). When the number of FIF exceeded 1000, the model results converged to that the total number of premises in Andalusia was vaccinated and immobilized. For this reason, we stopped the simulations at NIF=1,000.

3.2. Output of the transmission and economic meta-model

The meta-model allows the assessment of the epidemiological and economic consequences of an AHS epidemic under different climatic and entomological scenarios that mimic the conditions that favour AHS spread in the Spanish region of Andalusia. For every day until day 90, the meta-model estimated an epidemiological and an economic output.

The effects of the temperature and vector-host ratios are directly related with the force of infection of AHS epidemic disease, hence for the scenarios with the highest temperature and vector-host ratio values (ES3B in Table 5 and Table 6), there is a higher total number of infectious animals (195,400 without vaccination and 137,600 with vaccination). In this same scenario, the outbreak peak is achieved with 42,600 (without vaccination) and 29,800 (with vaccination) infectious animals.

Figure 3. Percentage of different cost categories of the AHSV epidemic according the different scenarios and the control strategy. A: Temperatures from 10 to 20ºC; B: Temperatures from 20 to 30ºC.



The peak is reached in less time compared with other scenarios, that is, at t = 41 (without vaccination) and 39 days (with vaccination) (Table 5, Table 6, Figure 2). However, for the scenarios with the lowest temperature and vector-host ratio values (ES1A in Table 5 and Table 6), the outbreak peaks with 463 and 161 infectious animals, without or with vaccination respectively, at the end of the simulation period (t=90 days). It is interesting to note here that not all Andalusian equines were infectious in all scenarios, although in ES2B and ES3B without vaccination more than 80% of total population were infectious.

The main epidemiological consequences of this disease in horses are the high mortality rates. For all scenarios, the median percentage of dead and humanely slaughtered equines due to AHSV was of 76% of the infectious equines and between 0.4% and 67.3% of the total population (Table 5, Table 6). The epidemic results of the application of control measures showed a greater variability: the number of immobilized animals was between 50% and 100% of the total population (Table 5), whereas the vaccinated animals varied between 30% and 85% of the total population (Table 6).

The economic impact of an AHS epidemic varied widely based on the climatic and entomologic characteristics considered (Table 5, Table 6). For example, the difference between the worst (ES3B) and best scenario (ES1A) was €443.5 mln and €356.5 mln, without or with vaccination respectively.

In all scenarios, the highest economic impacts are due to the production losses and the control measures (Table 5, Table 6, Figure 3). The production losses are directly related with the number of infectious animals, that is an output of the transmission model. Eighty-eight percent of this loss was caused by the mortality cost in all scenarios. The daily production losses overcome the daily cost of the control measures in the scenarios with medium and high vector-host ratios at 10-20ªC and 20-30ªC under control strategies without vaccination. With vaccination, the daily production losses overcome the daily cost of the control measures only in the scenarios with medium and high vector-host ratios at 20-30ªC.

One of the most important daily economic losses was related with the implementation of the control measure. After the first identification of AHS infectious animal, the cost of the control measures was about €1 mln per day. The main cost related to control strategies was the economic impact associated with movement restriction, being estimated at 93-98% of the control measures' losses (Figure 3).

The diagnostic and treatment costs of AHS-suspects are insignificant (0 – 3%) compared with the others costs (Figure 3).

Table 5. Epidemiological impact (* 1000 equines) and cost (* €1000) of the AHSV epidemic according the different scenarios with a control program without vaccination strategy.

	Temperature 10-20°C			Temperature 20-30°C		
Culicoides	↓	↔	↑	↓	↔	↑
Symbol	**ES1A**	**ES2A**	**ES3A**	**ES1B**	**ES2B**	**ES3B**
Outbreak peak						
IA	0.5	8.5	16.3	4.2	30.2	42.6
Day *(t)*	90	90	71	78	49	41
Final epidemic						
IA	2.3	50.5	118.8	32.9	176.9	195.4
FDA	1.6	35.6	83.5	23	123.7	136.8
FCA	0.1	3.0	7.1	2.0	10.6	11.7
IMRA	0.5	12.0	28.2	7.9	42.5	46.9
VA	0	0	0	0	0	0
ZA	114.9	195.8	204.6	181.1	211.5	213.8
C_P	3,900	88,410	207,281	57,169	308,103	340,389
C_D	123	2,514	5,269	1,545	7,412	8,103
C_T	244	5,257	11,003	3,229	15,588	16,935
C_M	136,358	199,494	210,196	191,564	218,963	218,600
C_{TOT}	140,626	295,675	433,749	253,507	550,065	584,026

3.2.1. Consequences of AHS epidemic without vaccination in Andalusia (baseline strategy).

The simulation of AHS epidemic without an emergency vaccination in the PZ following the presence of one infectious host estimated a number of infectious animals between 2,250 and 195,500 in the best (ES1A) and worst scenarios (ES3B), respectively (Table 5). As shown in the Table 5, the estimated percentage of infectious animals was less than 25% of total population in the ES1A, ES1B and ES2A; the 53.8% of total population in the ES3A; and higher than 80% of total population in the ES2B and ES3B. The average number of infectious animals increases almost 2.4 times from 10-20 ºC to 20-30 ºC.

The number of specific AHS deaths and humanely slaughtered equines varied between 2,255 in the best scenario (ES1A) and 148,500 in the worst scenario (ES3B) (Table 5), being greater than 50,000 animals in all scenarios, except for ES1A and ES1B. The number of immobilized animals in all scenarios was between 181,000 (82.0%) and almost the total equine population (213,850 – 96.9%); except for the scenario ES1A where only 114,900 (52.0%) equines were immobilized.

The economic impact of an AHS epidemic under control strategies without vaccination in Andalusia was estimated in more than €140.5 mln belonging to the scenario with less epidemic consequences (ES1A in Table 5). The estimated cost at temperatures scenario of 10-20ºC were €140.5 mln, €295.5 mln or €433.5 mln in the ES1, ES2 and ES3 respectively; whereas at temperatures of 20-30ºC were €253.5 mln, €550.0 mln or €584.0 mln in the ES1, ES2 and ES3 respectively. The average cost at temperatures of 20-30ºC was €462.5 mln, representing 1.59 times the average of cost at temperatures of 10-20ºC.

Looking at the different cost categories, the cost due to production losses varied widely among scenarios (Figure 3). The production losses of the best scenario (ES1A) cost only €4 mln that represents 3% of the total disease cost; whereas the worst scenario (ES3B) cost €340 mln that is, 58% of the total disease cost (Table 5). This variation is due to the direct relation between production losses and number of infectious animals. The economic impact of the control measures was higher in scenarios with lower vector-host ratios, representing 97% (ES1A), 76% (ES1B) and 67% (ES2A) of the total cost. When the epidemic dynamic affects a larger number of infectious hosts (around 110,000 equines), the percentage of economic cost associated with the production losses was higher than the percentage of economic impact of the control measures, representing the cost of control measures 48% (ES3A), 40% (ES2B) and 37% (ES3B) of the total cost (Figure 3).

Table 6. Epidemiological impact (* 1000 equines) and cost (* €1000) of the AHSV epidemic according the different scenarios with a control program with vaccination strategy.

	Temperature 10-20°C			Temperature 20-30°C		
Culicoides	↓	↔	↑	↓	↔	↑
Symbol	ES1A	ES2A	ES3A	ES1B	ES2B	ES3B
Outbreak peak						
IA	0.2	2.7	6.5	1.8	17.6	29.8
Day *(t)*	90	90	71	78	48	40
Final epidemic						
IA	1.1	21.8	53.0	15.7	103.8	137.6
FDA	0.8	15.3	37.2	11.0	72.7	96.3
FCA	0.1	1.3	3.1	0.9	6.2	8.3
IMRA	0.2	5.2	12.6	3.8	24.9	33.0
VA	66.3	167.3	188.2	139.8	188.7	176.1
ZA	109.8	189.6	202.5	172.1	210.7	213.5
C_P	1,980	38,068	92,427	27,273	181,046	239,559
C_D	61	1,032	2,340	720	4,328	5,718
C_T	116	2,152	4,885	1,504	9,054	11,976
C_M	136,372	202,956	222,676	191,979	238,551	237,915
C_{TOT}	138,529	244,208	322,327	221,477	432,979	495,168

3.2.2. Consequences of AHS epidemic with vaccination in Andalusia.

The number of infectious animals ranged between 1,000 and 138,000 depending on the climatic and entomologic characteristics when the vaccination strategy was implemented in the PZ after the confirmation of an AHS outbreak (Table 6). As shown in the Table 6, the estimated percentage of infectious animals was less than 25% of total population in all scenarios, except ES2B (47.1%) and ES3B (62.3%). The average number of infectious animals increases almost 3.4 times from 10-20 ºC to 20-30 ºC.

The minimum and maximum number of specific AHS deaths and humanely slaughtered equines were 850 (0.4% of total population) and 104,500 (48% of total population), respectively (Table 6). The animals affected by the restricted area were between 49.8% and 96.7% of the total population. The vaccinated animals varied between 63% and 85% of the total population, except in ES1A in which the minimum number of vaccinated equines was 66,000 (30%). The scenarios with a higher number of vaccinated animals were ES3A and ES2B with 188,750 (85.5%) and 188,200 (85.2%).

The estimated economic impact of an AHS epidemic under control strategies with vaccination was €135.5 mln, €244.0 mln or €322.5 mln in ES1, ES2 and ES3 at temperatures of 10-20ºC respectively; whereas at temperatures of 20-30ºC these were €221.5 mln, €433.0 mln or €495.0 mln in ES1, ES2 and ES3 respectively (Table 6). The average AHS economic impact at 10-20ºC was 235.0 mln, increasing almost 1.63 times from 10-20 ºC to 20-30 ºC.

In these simulations, the cost of the control measures has the highest importance in all scenarios, reducing the impact of production losses (Figure 3). The cost of control measures was higher in the scenarios with low and medium values of vector-host ratio (€191.6 mln and €238.5 mln), except for ES1A, where the value was of €136.4 mln.

Looking at the different costs of the control measures (Figure 3), movement restriction resulted in 93-96% of control measure costs, similar results were obtained in simulations without vaccination strategy. The cost of the vaccination strategies varied widely, although it always represented 2-3% of cost of the control measures. The estimated cost of vaccination at temperatures of 10-20ºC was €2.3 mln, €5.7 mln and €6.4 mln in ES1, ES2 and ES3 respectively; whereas at temperatures of 20-30ºC it was €4.8 mln, €6.5 mln and €6.0 mln in ES1, ES2 and ES3 respectively. The vaccination cost per animal was around €34, and the maximum vaccination cost per day was approximately €148.000.

3.3. Comparison of epidemic and financial consequences without and with vaccination.

The comparison of AHS epidemic under the control programme without vaccination (baseline strategy) and under the control programme with vaccination for each scenario allowed predicting the advantages or disadvantages of using a vaccination programme in Andalusia.

Table 7. Difference of epidemiological impact (* 1000 equines) and cost (* €1000) of the AHSV epidemic between scenarios without vaccination (baseline strategy) and with vaccination.

	Temperature 10-20°C			Temperature 20-30°C		
Culicoides	↓	↔	↑	↓	↔	↑
Symbol	**ES1A**	**ES2A**	**ES3A**	**ES1B**	**ES2B**	**ES3B**
IA	1.2	28.7	65.8	17.2	73.1	57.8
FDA	0.8	20.3	46.3	12	51	40.5
FCA	0	1.7	4	1.1	4.4	3.4
IMRA	0.3	6.8	15.6	4.1	17.6	13.9
VA	-66.3	-167.3	-188.2	-139.8	-188.7	-176.1
ZA	5.1	6.2	2.1	9	0.8	0.3
C_P	1,920	50,342	114,854	29,896	127,057	100,830
C_D	62	1,482	2,929	825	3,084	2,385
C_T	128	3105	6118	1725	6534	4959
C_M	-14	-3,462	-12,480	-415	-19,588	-19,315
C_{TOT}	2,096	51,467	111,422	32,031	117,086	88,859

The simulations with the vaccination strategy showed a decrease in the epidemiological consequences of AHS epidemic, with declining number of specific AHS deaths and humanely slaughtered equines between the 56% and 41% of the infectious animals compared with the baseline strategy, except in ES3B where the reduction was of 30% of the infectious animals. However, the number of animals in the restricted zone showed a slighter decline, between 0.1% and 5% (Table 7).

All scenarios with control measures with vaccination decreased the epidemiological outputs, hence, the economic impact of AHS epidemic also decreased compared to the control measures without vaccination, obtaining a benefit towards vaccination strategy between €2 mln and €117 mln (Figure 4) corresponding to the 1.5% and 25.7% of the total cost without vaccination. The highest benefit is gained in scenarios ES2B, ES3A and ES3B, which is about €117,000, €111,500 and €88,900, respectively. The least benefit was obtained in scenario ES1A, €2,100.

The control measures with vaccination resulted in significant lowest economic impact in the production losses (29.6% to 57% of cost the baseline strategy), the diagnostic cost (29.4% to 59% of cost the baseline strategy) and the treatment cost (29.3% to 59% of cost the baseline strategy) of AHS-suspect infectious animals (Figure 3). However, the costs of the control measures after confirmation of AHS in the strategy with vaccination showed similar or higher values than control measures without vaccination.

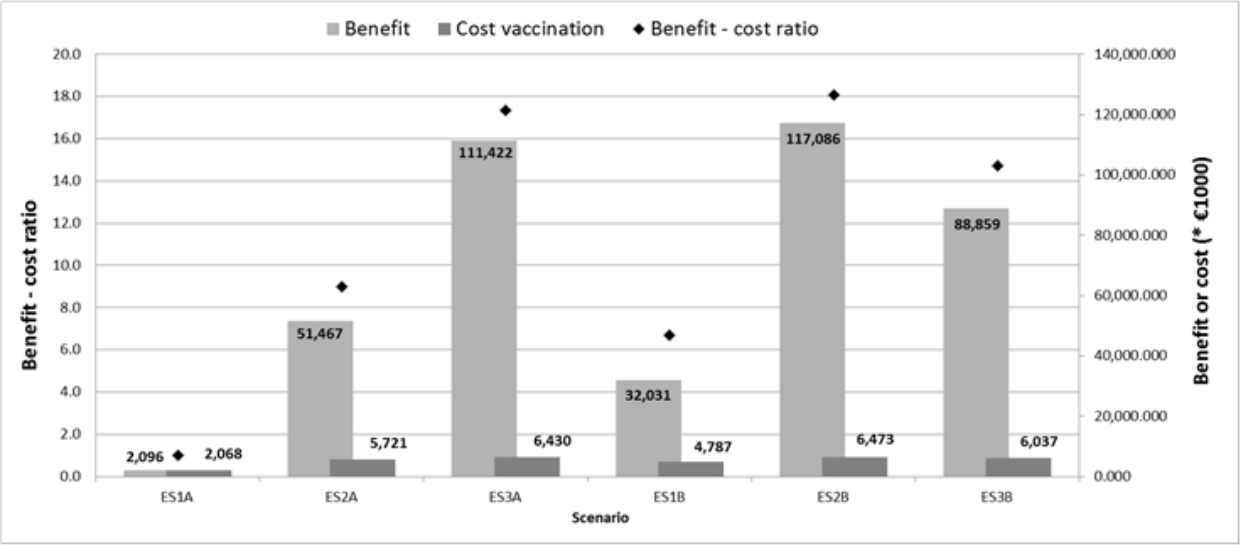
Figure 4 represents the benefits of the vaccination strategy, the vaccination cost and the benefit-cost ratios for each scenario. Results show that the vaccination strategy is an efficient strategy in all scenarios because the benefit-cost ratio is always higher than one (Figure 4). The highest benefit-cost ratio was observed in scenario ES2B (value of 18.1) followed by ES3A (value of 17). The lowest efficient strategy was the scenario ES1A showing a cost-ratio value of 1, being the cost of vaccination similar to the benefit.

Summarizing, the implementation of control measures with vaccination in the protection zone is a recommended strategy in all scenarios, even when it does not yield much profit, as is the case in scenario ES1A, due to the reduction in the epidemiological and economic consequences of AHS outbreak. In this meta-model, the implementation of vaccination in the scenario ES2B (temperature of 20-30ºC and medium vector-host ratio) allowed the highest reduction in the number of infectious and dead animals giving the greatest benefits of the vaccination strategy and benefit-cost ratios. Vaccination under the environmental conditions considered is therefore an efficient tool to control AHS.

3.4. Sensitivity analysis

The sensitivity analysis allows the evaluation the robustness of the results. In this study, the sensitivity analysis indicates change of ± 0.5% in the benefit-cost ratios for all scenarios when the input parameters were varied.

Figure 4. Results of the benefit and cost of vaccination (* €1000) of the different scenarios.



Discussion

The epidemiological and economic meta-model we have developed has allowed to estimate, for the first time, the potential economic impact of AHS in Spain. With the selection of Andalusia as the study vulnerable area, we have dealt with one of the worst-case scenario for AHS spread in Spain [28]. The region complies with two of the most influential parameters for AHS occurrence ([22,23,29]: a high density of exposed susceptible equine population (2.53 equines/km^2, [16,21]), and excellent climatic and environmental conditions for abundant *Culicoides* breeding [20]. Andalusia's warm temperature also favour increased and prolonged risk of AHS transmission, and was the worst affected region in the AHS 1989 epidemic in Spain [18]. The different annual fluctuations of climatological and entomological characteristic of the region has been used to establish the epidemiological scenarios of this model (Table 1) covering the possible circumstances of AHS spread in the region. For Andalusia, AHS has been the worst animal health epidemic in the region, not only because of the number of equines that died, but also because Andalusia has a very strong historical horse tradition. In particular, the region is a highly vulnerable region with famous for breeding Andalusian-pure bred horses, for horse fairs and markets, horse riding in local fairs, or horse riding to help managing the outdoor-bred beef and bull-fighting cattle, among other activities.

The situation of the equine population in Andalusia has evolved since 1989, since, at the time, there were many unidentified equines, which greatly hindered the implementation of effective and quick control measures. Today, with the census of equines and equine holdings, we have been able to build an epidemiological and economic meta-model, that could gain higher accuracy if we could have obtained the exact coordinates and geographic distribution of holdings. Instead, we used a mapping tool to randomly distribute geographically the holdings. However, the estimated spatial distribution might be a proxy for the real average distribution in 100 and 150-km- radii, which was the input used to assess the spread consequences of AHS. This spatial simulated model predicts the number of premises and animals involved in the first emerging control measures of an AHS outbreak in the PZ and SZ (Council Directive 92/35/EEC).

The epidemiological model in the meta-model includes the current knowledge on the virus, the host, the vector and two current control strategies of AHS under the environmental conditions of Andalusia; while the economic model takes into account the monetary value of the Andalusian horse sector. All costs were selected and estimated based on expert assessment of the affected sector (equine premises), related industry (such as veterinarian, laboratory ...), and authorities (MAGRAMA). The selected epidemiological and economic outputs of this study reflect the most important socio-economic consequences of AHS epidemic in a disease-free country, where the quick detection and eradication of the disease is the main objective.

Different studies have identified the vector-host ratio and/or temperature as the most influential factor in the AHS epidemic [22,23,29]. In Andalusia, the temperature and vector-host ratio fluctuate during the year and per regions. Therefore, in this model the different scenarios developed respond to the environmental conditions of the area. The results of this model showed significant differences in the epidemiological and economic output between the scenarios. A better understanding of the socio-economic consequences of the disease allows to be better prepared, knowing the necessary resources and designing effective mitigation measure, for a potential incursion and to improve communication as necessary to the affected sector. Thereby, if the virus was introduced in Andalusia, the government or agricultural insurance companies could use these scenarios to predict the potential economic impact of the epidemic and raising awareness to the equine industry of the importance of control measures against AHS based on technical information. For example, the combination of the climatic and entomological scenarios in Andalusia with the seasons allows the identification of the summer as the most vulnerable season and the worst socio-economic consequences of AHS.

The results of this model corroborate that the AHS introduction and spread in a disease-free region, as Andalusia, has important socio-economic impact mainly associated with the fatal consequences in horses and the establishment of the control measures [2,3]. The number of infectious, specific AHS deaths and humanely slaughtered equines was directly associated with the production losses. However, given the high value of horses (between €350 and €9000), and the high mortality rates of AHS epidemic, the most

important production losses were the mortality costs (Figure 3). Furthermore, the high number of restricted and vaccinated animals was reflected in the cost of the control measures, the other most important economic impact (Figure 3). In the case of vector-borne diseases, the restriction of movement and vaccination is implemented in a radius of 100 and 150 km, respectively. Therefore, these control measures affect many premises, allowing the reduction of the consequences of AHS spread.

Nowadays, Council Directive 92/35/EEC recommends to apply or not vaccination depending on the epidemiological conditions. Vaccination is the most effective measure for the AHS control although it cannot be relied on to fully protect all horses against infection [2]. The current vaccine is not 100% effective, and several doses are required in subsequent years to produce sufficient immunity in the total population [30]. This fact has been included in the meta-model assuming an efficiency of 70%. Despite this potential limitation, the epidemiological and economic results of the meta-model allow to conclude that the control measures with vaccination in PZ is the best strategy based on the benefit-cost ratio for all climatic and entomologic scenarios. The vaccination strategy increases the immunity in the susceptible population, decreasing the probability that a vector infects a susceptible animal and limiting AHS spread. This fact was reflected in the number of specific AHS deaths and humanely slaughtered equines, as well as the associated economic consequences, mainly, the production losses of AHS-suspect animals. Furthermore, this meta-model allows the identification of the one scenario (ES1A) which cost of vaccination was similar to the benefit, being the cost-benefit ratio 1. This fact could be associated with a low number of infectious animals in this scenario without and with vaccination, compared with the other scenarios (Table 5, Table 6). This model could be used to assist policy makers choose the most efficient strategy for AHS control based on the epidemiological and economic principles, as well as to define the epidemiological situations where the implementation of emergency vaccination should be recommended. Furthermore, the results of this model showed that the vaccination strategy should be implemented in all simulated scenarios of this study.

The quality of the input data is fundamental to obtain robust and representative outputs of the meta-model. One of the limitations of this study was the lack of data to estimate the disease epidemic and cost using as alternative the assumption or extrapolation of analogous data from BTV epidemics, taking into account the main differences between *Orbivirus spp*. [23]. One of the assumptions of the epidemiological analysis of the meta-model has been the estimation of the number of infectious equines and holdings without considering the transmission between herds mainly because of the absence of information on geographical location and on movements between herds. For the same reasons, the number of immobilized and vaccinated animals was calculated by assuming a random geographical distribution of premises.

Second, this model is limited to the Andalusian region; but AHS could spread to other areas in a real epidemic increasing the cost of epidemic. In the model, the role of the different equines host, as donkeys, was not incorporated because of their relatively low numbers in Andalusia (5.6% of equines), although these species could play a role in virus spread [19,31]. Moreover, this study has assumed the subjective prices using the estimation provided by individual breeder, veterinarian or other affected sector. The horse value was estimated using the government data [32,33]. Therefore, if the policy makers supply more information about the number and economic details of equines premises with different production goals in Andalusia, we could improve the estimation of AHS economic consequences using the different horse value according to their production goals [17,34]. In addition, the indirect costs associated with AHS, such as consumer reaction, restriction of popular celebrations, loss of international reputation or other affected equine industries, have not been included in the model, due to the absence of available information about them. However, in this model we assumed that the direct cost, mainly associated with the mortality, was the highest impact in an AHS epidemic. Thus, the results of this meta-model may be a conservative estimation of the economic impact of AHS outbreak in Andalusia.

The current vaccination strategy evaluated in the model is with live attenuated vaccines, which does not differentiate between infected and vaccinated animals (DIVA) among other disadvantages such as reversion to virulence or vaccine reactions [35]. However, new vaccines with fewer drawbacks as inactivated or new

generation vaccines have been developed in the last years [36-38], although currently they are not permitted to use. Although, these vaccines are more expensive, they are more effective proved by experimental studies, and could also reduce trade restrictions using the different DIVA test. This meta-model could be adapted to evaluate and compare different vaccines to allow the selection of the best vaccination strategy.

The development of a combined economic and epidemiological meta-model in disease-free countries is an useful tool for policy makers to support decision-making, since this methodology allows the selection of the most efficient cost-benefit control programme using estimated technical and economic information. Nowadays, the emergency AHS control programme does not specify under which epidemiological circumstances is the vaccination recommended. Therefore, the model as developed in this study could help to stablish this situation. For example, all climatic and entomologic scenarios of this study in Andalusia justify the use of the emergency vaccination in PZ to reduce the socio-economic consequences, being especially beneficial when the vector-host ratio, as determined by Culicoides traps, is medium to high. This model could be a starting point to evaluate other surveillance and control strategies against AHS, including the estimation of the economic impact.

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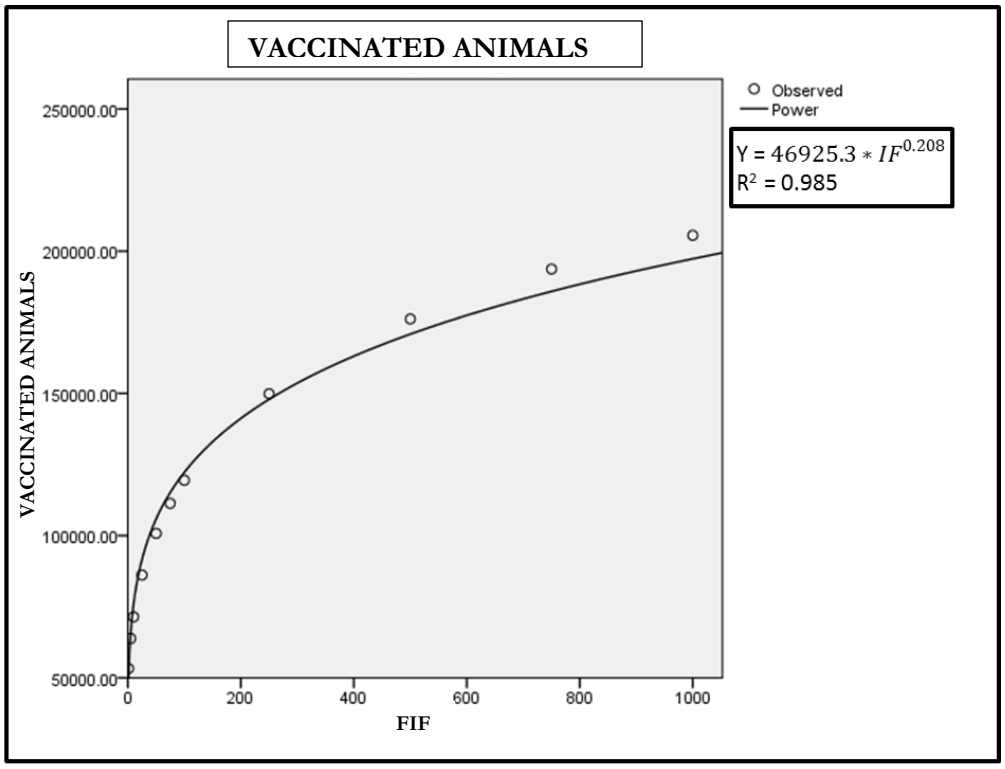
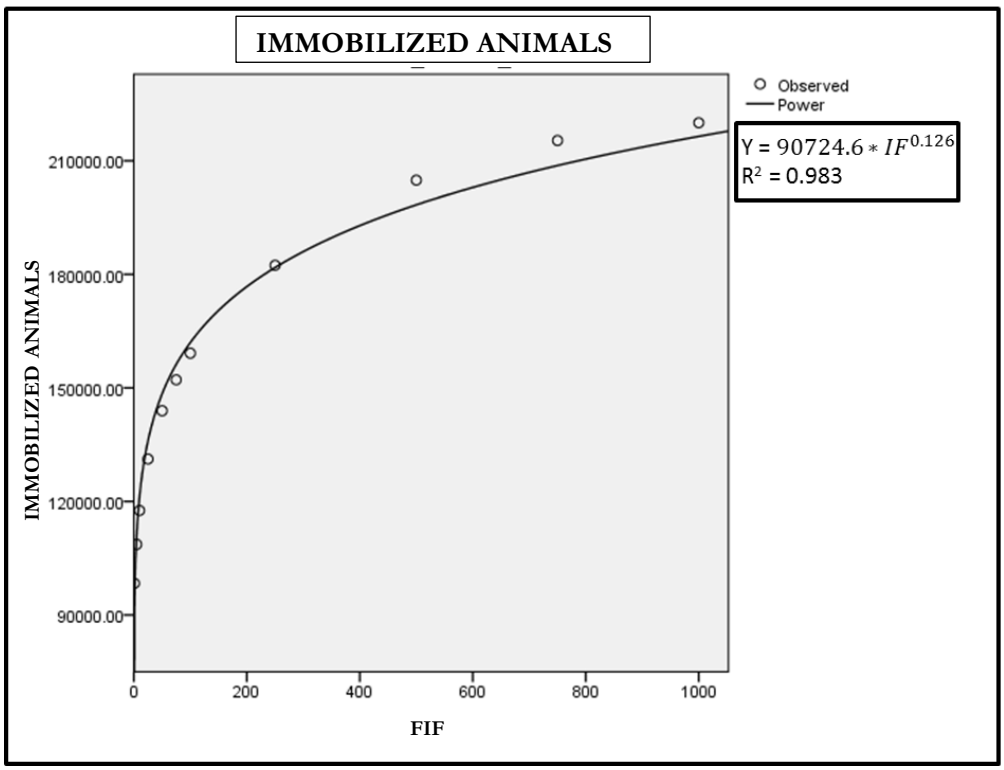
Supplementary data

Annex 1. Differential equations of the epidemiological model.

$$\frac{\mathrm{dSA}}{\mathrm{dt}} = -\mathrm{p_{va}}\,\lambda\,\mathrm{IV}\,\frac{\mathrm{SA}}{\mathrm{NA}} - \mathrm{p_v} f(IF)$$
$$\frac{\mathrm{dEA}}{\mathrm{dt}} = \mathrm{p_{va}}\,\lambda\,\mathrm{IV}\,\frac{(\mathrm{SA}+\mathrm{VA}+\mathrm{SVA})}{\mathrm{NA}} - \varepsilon_a\,\mathrm{EA}$$
$$\frac{\mathrm{dIA}}{\mathrm{dt}} = \varepsilon_a\,\mathrm{EA} - (\mathrm{p_m}\,\gamma + (1-\mathrm{p_m})\,\mathrm{p_c}\,\delta + (1-\mathrm{p_m})(1-\mathrm{p_c})\,\delta)\,\mathrm{IA}$$
$$\frac{\mathrm{dDA}}{\mathrm{dt}} = \mathrm{p_m}\,\varepsilon_a\,\mathrm{EA} - \gamma\,\mathrm{DA}$$
$$\frac{\mathrm{dRA}}{\mathrm{dt}} = (1-\mathrm{p_m})\,(1-\mathrm{p_c})\varepsilon_a\,\mathrm{EA} - \delta\,\mathrm{RA}$$
$$\frac{\mathrm{dCA}}{\mathrm{dt}} = (1-\mathrm{p_m})\,\mathrm{p_c}\,\varepsilon_a\,\mathrm{EA} - \delta\,\mathrm{CA}$$
$$\frac{\mathrm{dFDA}}{\mathrm{dt}} = \gamma\,\mathrm{DA}$$
$$\frac{\mathrm{dIMRA}}{\mathrm{dt}} = \delta\,\mathrm{RA}$$
$$\frac{\mathrm{dFCA}}{\mathrm{dt}} = \delta\,\mathrm{CA}$$
$$\frac{\mathrm{dVA}}{\mathrm{dt}} = \mathrm{p_v}\,f(IF) - \mathrm{p_{va}}\,\lambda\,\mathrm{IV}\,\frac{\mathrm{VA}}{\mathrm{NA}} - \tau\,\mathrm{VA}$$
$$\frac{\mathrm{dSVA}}{\mathrm{dt}} = (1-\mathrm{p_{ef}})\,\tau\,\mathrm{VA}$$
$$\frac{\mathrm{dIMVA}}{\mathrm{dt}} = \mathrm{p_{ef}}\,\tau\mathrm{VA}$$
$$\frac{\mathrm{dNA}}{\mathrm{dt}} = -((1-\mathrm{p_m})\,\mathrm{p_c}\,\delta - \mathrm{p_m}\,\gamma)\,\mathrm{IA}$$
$$\frac{\mathrm{dSV}}{\mathrm{dt}} = \alpha\,\mathrm{NV} - \left(\mathrm{p_{av}}\lambda\,\frac{\mathrm{IA}}{\mathrm{NA}} + \mu + M_V\mu\frac{\mathrm{ZA}}{\mathrm{NA}}\mathrm{p_s}x\right)\mathrm{SV}$$
$$\frac{\mathrm{dEV}}{\mathrm{dt}} = \mathrm{p_{av}}\,\lambda\,\frac{\mathrm{IA}}{\mathrm{NA}}\,\mathrm{SV} - \left(\varepsilon_v + \mu + M_V\mu\frac{\mathrm{ZA}}{\mathrm{NA}}\mathrm{p_s}x\right)\,\mathrm{EV}$$
$$\frac{\mathrm{dIV}}{\mathrm{dt}} = \varepsilon_v\,\mathrm{EV} - \left(\mu + M_V\mu\frac{\mathrm{ZA}}{\mathrm{NA}}\mathrm{p_s}x\right)\mathrm{IV}$$
$$\frac{\mathrm{dNV}}{\mathrm{dt}} = \alpha\mathrm{NV} - \left(\mu + M_V\mu\frac{\mathrm{ZA}}{\mathrm{NA}}\,\mathrm{p_s}x\right)\mathrm{NV} \qquad (1)$$

Annex 2. Estimation of the number of vaccinated (VA) and immobilized animals (ZA) based on a range of potential final infected premises





CAPÍTULO IV

DISCUSIÓN

Fuente de la imagen: Cedidas por Carlos Escribano, Yeguada “La Cartuja”.

La **peste equina** es una de las enfermedades vectoriales **más devastadoras** y con **mayor poder de difusión** de cuantas afectan a los **équidos**, y así lo releja su pertenencia a la lista de **enfermedades de declaración obligatoria de la OIE**. Desde varios años atrás hasta hoy en día, se considera que el riesgo de la enfermedad ha aumentado en Europa como consecuencia de la situación epidemiológica de la enfermedad y la expansión en la distribución de las poblaciones de vectores. España, por sus características geográficas y climáticas, es en concreto una de las potenciales vías de entrada de la enfermedad en Europa. Por este motivo, numerosos esfuerzos se están llevando a cabo para **incrementar los conocimientos** que permitan mejorar las **estrategias de prevención y control** de esta enfermedad, **optimizando la relación coste-beneficio** de los programas sanitarios.

En esta tesis se han abordado diferentes retos en el conocimiento de la PEA, que abarcan desde la evaluación de la **respuesta inmune** generada en caballos infectados con el vPEA y el desarrollo de nuevas **técnicas de diagnóstico**, hasta el **análisis epidemiológico y económico** de la enfermedad en España. El cumplimiento de todos estos objetivos permitirá la mejora de las **herramientas de vigilancia y control de la PEA**.

OBJETIVO 1: Evaluación de la respuesta inmune del hospedador frente al vPEA.

Como se ha mencionado anteriormente, la introducción de la PEA podría acarrear gravísimas consecuencias socio-económicas, principalmente asociadas a su alta tasa de mortalidad, a las estrictas medidas de control basadas en vacunación, y a la restricción de todos los movimientos de équidos en el país afectado. Por estas razones es fundamental el desarrollo de nuevos **tratamientos terapéuticos** que permitan reducir la mortalidad asociada a la enfermedad, así como el desarrollo de nuevas **vacunas** que protejan a los animales reduciendo las limitaciones de las vacunas comercializadas actualmente. El conocimiento de la **respuesta inmune natural de los hospedadores** es fundamental para establecer los mecanismos de lucha frente a la enfermedad más eficientes (Sánchez-Cordón et al., 2009), y como consecuencia, usarlos para el desarrollo de nuevas herramientas terapéuticas y vacunas que protejan a los animales.

Los **patrones de expresión de citoquinas** son unos de los mecanismos disponibles para evaluar la **respuesta inmune**. La producción de las **citoquinas** es peculiar por su carácter temporal, siendo necesaria la activación de los genes de las citoquinas y la síntesis de ARNm para su liberación (Arai et al., 1990; Murtaugh et al., 1996; Karupiah, 1998). Por esta razón, la técnica

seleccionada para la evaluación del patrón de citoquinas fue la detección y cuantificación de la **expresión de genes (ARNm) que codifican citoquinas mediante la rRT-PCR**. Esta técnica permite detectar la expresión de citoquinas, pero no su secreción, por lo que los resultados obtenidos no implican necesariamente la producción de un efecto biológico, ya que la producción de citoquinas se puede regular durante la transcripción o a nivel post- transcripcional (Tizard, 2009). Sin embargo, varios estudios han mostrado una alta correlación entre la expresión de ARNm y la producción de proteínas o su efecto biológico (Cherwinski et al., 1987; Rottman et al., 1995; Giguére and Prescott, 1998). Este hecho junto con el resto de ventajas de la rRT-PCR para la cuantificación de la expresión de genes, como son la alta sensibilidad y especificidad, hacen que esta técnica haya sido la elegida en numerosos estudios sobre la respuesta inmune equina (Quinlivan et al., 2007; Hughes et al., 2011; Pretorius et al., 2012; Berghaus et al., 2014; Betancourt et al., 2014).

En esta tesis se ha **desarrollado y validado una rRT-PCR** usando el agente intercalante **SYBR-Green** para la evaluación de la **respuesta inmune** mediante la **cuantificación simultánea de ocho citoquinas** y un gen de expresión constante. Se seleccionó un abanico de citoquinas de entre las más representativas de la respuesta inmune innata y de la respuesta inmune adaptativa de los caballos, lo que permitiría analizar los principales mecanismos de defensa frente a un agente vírico.

La rRT-PCR incluye un **gen de expresión constante** o **normalizador** (β-Actina) que permite mediante la estandarización de su expresión, puesto que es un gen que no varía, **cuantificar** la expresión de las citoquinas (Zhang et al., 2009). Es gracias a la detección de este normalizador, que a la hora de realizar la cuantificación, en los casos en los que no se produjo una adecuada amplificación del mismo, las **muestras** fueron consideradas **no válidas** puesto que podría estar relacionado con una baja calidad de las muestras, por problemas en la extracción de ácidos nucléicos o de manejo.

Esta técnica rRT-PCR ha sido desarrollada en **dos pasos**, ya que dicho procedimiento permite optimizar el número de PCRs con la misma cantidad inicial de muestra y utilizando la misma concentración inicial de ADN complementario (ADNc) en todas ellas. De este modo, se disminuyen las posibles diferencias de eficiencia de la enzima retrotranscriptasa que puedan darse en la rRT-PCR en un paso (Stahlberg et al., 2004; Wong and Medrano, 2005). La **retrotranscripción** se llevó a cabo con cebadores inespecíficos, oligo (dT), obteniendo copias de todo el ARNm de la muestra, lo que determinó que la proporción de la expresión obtenida fuese equivalente a la encontrada en la sangre. Finalmente, **esta técnica fue evaluada** para su uso

tanto en células mononucleares de sangre periférica *in vitro*, como en sangre completa de caballos *in vivo*. Por las razones anteriormente descritas, esta rRT-PCR fue seleccionada para la evaluación del **patrón de citoquinas en animales experimentalmente infectados con PEA**, y de esta manera analizar la respuesta inmune.

Concretamente en esta tesis doctoral, se ha llevado a cabo el **primer análisis de la respuesta inmune innata y adaptativa** mediante la evaluación del **patrón de citoquinas** y la producción de **anticuerpos** en **caballos infectados experimentalmente** con dos serotipos distintos del **vPEA**. Las distintas formas clínicas de la enfermedad fueron relacionadas con los resultados obtenidos en dichos parámetros, observándose algunas características que muestran una **tendencia común en la evolución de la respuesta inmune para cada una de las formas clínicas**.

Durante el **estudio de la respuesta inmune** de los caballos frente al vPEA, aparecieron varias **limitaciones**. Uno de los problemas más importantes asociado a este virus es la **alta tasa de mortalidad en caballos en los estadíos tempranos** de la enfermedad (Coetzer and Erasmus, 1994), la cual es fundamental para determinar el periodo de estudio. En nuestro caso se vio reducido a los 12 dpi, cuando todos los animales que manifestaron la forma mixta de la enfermedad habían muerto. Otra de las peculiaridades de esta enfermedad es el hecho de que los **animales manifiesten distintas formas clínicas** (Coetzer and Erasmus, 1994), esto hace que el número de animales obtenido de cada una de las distintas formas no siempre sea adecuado para obtener resultados estadísticamente significativos. Debido al impedimento de no poder predecir la forma clínica que cada animal desarrolla, en este estudio de la respuesta inmune en caballos infectados experimentalmente con el vPEA pudimos analizar la respuesta inmune de 4 animales con la forma febril, 4 animales con la forma mixta y 1 animal con la forma cardiaca, no detectando ninguno con la forma pulmonar de la enfermedad. Además, otra de las limitaciones que se puede encontrar en el estudio de la expresión de ARNm de las citoquinas es la dificultad de **conservación de la muestra** (Fleige and Pfaffl, 2006). Sin embargo, como ya hemos indicado con anterioridad, la utilización de la β-Actina como normalizador permitió la identificación y eliminación de aquellas muestras que no se encontraban en un estado adecuado para su análisis.

Los resultados de este trabajo mostraron que los caballos presentaron un **patrón de expresión de citoquinas variable**, al igual que los resultados previamente descritos por Pretorius et al. (2012) en animales vacunados, lo cual ha sido relacionado con variaciones genéticas (Babiuk et al., 2003). No obstante se han podido identificar algunos **perfiles comunes**

en función de la forma clínica de la enfermedad. Además, si estos resultados los relacionamos principalmente a la supervivencia de los animales, en este estudio hemos podido ratificar los resultados publicados por Martínez-Torrecuadrada et al. (1996) ya que solamente la activación de la respuesta inmune humoral no pareció suficiente para la supervivencia de los caballos. Por ello, sería conveniente estudiar el título de anticuerpos neutralizantes necesario para una adecuada protección frente al virus, o el rol de otros posibles mecanismos de defensa en animales que sobreviven sin desarrollar anticuerpos neutralizantes (Martínez-Torrecuadrada et al., 1996; Romito et al., 1999; Guthrie et al., 2009).

Asimismo, se identificó un incremento mantenido de la expresión de **TNFα** durante el desarrollo de las respuestas innata y adaptativa en los animales que sobrevivieron, no detectándose su expresión en animales que no superaron la infeción. El **TNFα** es una poderosa citoquina pro-inflamatoria con propiedades pleiotrópicas que actúa, entre otras cosas, como un agente antiviral del organismo jugando un importante papel en la gravedad y recuperación de muchas infecciones víricas (Herbein and O'Brien, 2000) como por ejemplo West Nile (Shrestha et al., 2008). Sin embargo, su expresión a grandes concentraciones puede ser de especial relevancia en la patogenia de enfermedades como la LA o PEA, en las que se podría asociar a los daños endoteliales y tisulares (revisado en Maclachlan et al. (2014)). No obstante en este estudio, el TNFα parece actuar como un agente protector en la infección, siendo necesario continuar indagando el posible papel de esta citoquina en la patogénesis e inmunidad de PEA, así como su supresión en las formas clínicas más virulentas.

Este estudio ha permitido sentar las **bases para futuras investigaciones de la infección** y siguiendo esta línea, se ha identificado la necesidad de mejorar la comprensión de los mecanismos de defensa del hospedador frente a la enfermedad en las distintas formas clínicas analizadas, para lo cual, actualmente consideramos que además de las herramientas desarrolladas en esta tesis doctoral se deberían analizar otros parámetros inmunitarios como podrían ser la **evolución de poblaciones celulares** o posibles **receptores celulares del virus**.

Esta tesis doctoral recoge el **desarrollo y validación de técnicas de rRT-PCR** para el estudio de las principales citoquinas involucradas en la respuesta inmune en caballos, así como su posterior aplicación para **evaluar los mecanismos esenciales implicados en la respuesta inmune innata y adaptativa en animales infectados con vPEA**. La aplicación de esta técnica ha permitido obtener los primeros resultados del patrón de citoquinas en caballos infectados con vPEA, proporcionando nuevos conocimientos sobre los mecanismos inmunitarios del

hospedador, los cuales ayudaran al desarrollo de nuevas herramientas terapéuticas y vacunas en la lucha contra la enfermedad, y de esta manera los **métodos de prevención y control**.

OBJETIVO 2: Desarrollo de nuevas técnicas de diagnóstico para la peste equina.

El **diagnóstico de la PEA** es un pilar básico para el establecimiento de **programas de vigilancia efectivos**, tanto en países endémicos como libres de la enfermedad. Un adecuado diagnóstico es un requisito fundamental para la rápida identificación de animales infectados permitiendo establecer **adecuados planes de control y evaluando su eficacia**, así como la herramienta más empleada para reconocer los animales infectados anulando **su desplazamiento y evitando la difusión** de la enfermedad. Por ello, la optimización de las técnicas de diagnóstico repercutirá directamente en la mejora de la vigilancia de la enfermedad. En este contexto, el avance en el diagnóstico ha sido orientado hacia una **mayor sensibilidad y rapidez** de las técnicas. Además, la actual situación de re-emergencia de la enfermedad en Europa, ha implementado el desarrollo de nuevas vacunas más seguras y adecuadas para países libres, siendo una de sus peculiaridades la **posibilidad de diferenciar animales infectados de vacunados**, al igual que la vacuna inactivada desarrollada en la epidemia de España de 1987-1990. En esta situación, el desarrollo de diagnósticos DIVA es esencial para diferenciar estos animales y disminuir sus restricciones comerciales (OIE, 2013a). En estas dos líneas se ha llevado a cabo el segundo objetivo de la tesis doctoral, el cual ha consistido en el desarrollo y adaptación de la tecnología Luminex® para mejorar el diagnóstico de PEA.

La **tecnología Luminex** es una técnica revolucionaria que permite la mejora de la rapidez y rentabilidad de los diagnósticos múltiples de enfermedades, manteniendo una precisión óptima. Esta tecnología se basa en la identificación de hasta 100 microesferas diferentes, marcadas internamente con un único espectro fluorescente (Angeloni et al., 2013). El fundamento de esta técnica consiste en la **unión covalente** de estas microesferas a distintos ligandos como proteínas o ADN, entre otros; los cuales son reconocidos por **reacciones biomoleculares específicas** en la superficie de la microesfera en una matriz de suspensión líquida, y cuyos resultados serán revelados mediante un **marcador fluorescente**. La principal ventaja de esta técnica es la posibilidad de realizar múltiples análisis de una muestra simultáneamente (Vignali, 2000). Esta característica hace de esta tecnología la óptima para poder diseñar diagnósticos DIVA. Concretamente, en esta tesis ha sido adaptada para diferenciar así los animales vacunados con la vacuna inactivada del serotipo 4 de los animales infectados, lo que

hemos denominado como **ensayo Luminex DIVA frente a la VP7 y a la NS3**. Además, previamente y teniendo en cuenta otra de las peculiaridades de la técnica, como es la posibilidad de una cinética más rápida y una mayor sensibilidad que los inmuno-ensayos tradicionales(van Gageldonk et al., 2008), esta tecnología ha sido aplicada a la mejora de la sensibilidad y rapidez del diagnóstico de PEA usando la VP7 recombinante, principal responsable del diagnóstico de serogrupo de la enfermedad (Roy et al., 1991), lo que hemos denominado como **ensayo Luminex frente a la VP7**.

Las características del **ensayo Luminex frente a la VP7** lo proponen como una alternativa al ELISA como test de diagnóstico para la detección de todos los serotipos de la enfermedad a nivel laboratorial. El ensayo Luminex mostró un incremento sustancial de sensibilidad frente al cELISA, el cual podría ser fundamental para la vigilancia de la enfermedad, especialmente cuando este tipo de cELISA es recomendado como test para el comercio internacional de la OIE (OIE, 2013b). Este hecho quedó demostrado cuando se analizaron 85 muestras de campo de animales infectados de forma natural y, 6 muestras con resultado positivo en el ensayo Luminex fueron identificadas como negativas en el ensayo cELISA. Tanto la posible falta de sensibilidad del cELISA como la posible falta de especificidad del Luminex podrían tener graves repercusiones para la sanidad ecuestre internacional. Una menor de sensibilidad supone que no se identifiquen animales infectados con bajos títulos de anticuerpos, generalmente sucede en etapas tempranas de la infección, permitiendo su desplazamiento y la posible difusión de la enfermedad; mientras que una menor especificidad supondría importantes restricciones de movimiento a animales sanos que fueran erróneamente identificados como enfermos. Dada la importancia de estos hechos y la imposibilidad de conocer la historia clínica de los animales con resultados no coincidentes, utilizamos la técnica de SNV como test diagnóstico alternativo. De las 6 muestras no coincidentes, sólo una mostró un título bajo de anticuerpos neutralizantes, confirmando la mayor sensibilidad del ensayo Luminex. Por otro lado, en las otras cinco muestras negativas, no se detectaron anticuerpos neutralizantes lo que podría explicarse por una falta de especificidad del ensayo Luminex. Sin embargo, este resultado podría estar asociado con el tipo de anticuerpo detectado en cada ensayo, ya que la seroneutralizacion detecta únicamente anticuerpos neutralizantes, mientras que este ensayo Luminex detecta todos los anticuerpos, neutralizantes o no, frente a la proteína VP7. Este hecho ha sido señalado previamente por Maree and Paweska (2005). La mayor sensibilidad del ensayo Luminex le propone como una futura alternativa al ELISA, aunque actualmente sería conveniente seguir validando su especificidad con muestras de zonas endémicas.

Tras los prometedores resultados obtenidos con el ensayo Luminex frente a la VP7, se diseñó el **ensayo Luminex DIVA frente a la VP7 y la NS3**. En concordancia con estudios previamente realizados con animales infectados y con animales vacunados con la vacuna inactivada del serotipo 4 (Laviada et al., 1993), los animales infectados mostraron una respuesta positiva de anticuerpos frente a ambas proteínas, mientras que los animales vacunados solamente frente a la VP7; permitiendo su utilización como ensayo DIVA para animales vacunados con dicha vacuna. Además, los 68 caballos animales sanos no mostraron respuesta a ninguna de las dos proteínas, a excepción de tres individuos (falsos positivos a la proteína NS3). Estos falsos positivos han sido fácilmente identificados al observarse en animales sin infectar, ya que mostraban resultados incongruentes para la VP7 (negativo) y la NS3 (positivo). Dado que el Luminex para la VP7 presentó muy alta sensibilidad y especificidad, estos animales se deberían considerar negativos. Sin embargo, un falso positivo frente a la NS3 en un animal vacunado, sería considerado como falso infectado, provocando la limitación de movimientos para el mismo. Este hecho podría estar relacionado con el sistema de expresión de la proteína NS3 en el organismo *Escherichia coli*. Aunque en esta tesis la obtención de la proteína NS3 se ha llevado a cabo mediante la purificación de la misma, es plausible pensar en la presencia de trazas de proteínas de *E. coli* en la muestra. Este tipo de organismos, saprofitos en el medio ambiente y en el intestino del hospedador, interactúan de forma natural con el individuo, por ello, las proteínas de *E.coli* pueden reaccionar inespecíficamente con anticuerpos del hospedador, detectándose como falsos positivos del análisis (De Diego et al., 1997). La modificación del sistema de expresión de esta proteína a sistemas de células eucariotas podría ser una alternativa para resolver este problema (Rai and Padh, 2001). No obstante, dado que actualmente no existe ningún test diagnóstico múltiples DIVA para la PEA, podemos asumir que los resultados preliminares de este ensayo permitirán diferenciar animales infectados, vacunados y sanos con una sensibilidad del 100% y una especificidad del 95.6%, lo cual a priori, permitiría reducir las limitaciones de movimientos internacionales en animales vacunados con esta vacuna, ya que todos los animales infectados serían reconocidos, disminuyéndose uno de los principales motivos de pérdidas económicas asociadas a la enfermedad.

Estos estudios han abierto un **nuevo horizonte** de trabajo para la **mejora del diagnóstico de la PEA**. La posibilidad que ofrece la tecnología Luminex de diagnósticos múltiples podría ser utilizada para el desarrollo de nuevos ensayos que junto con los dispuestos en esta tesis doctoral, permitan incrementar el valor del diagnóstico de una muestra. Respecto a nuevos retos en el diagnóstico de PEA podemos incluir el serotipado simultáneo de la enfermedad mediante la detección de anticuerpos frente a la VP2 del virus, el desarrollo de

nuevos diagnósticos DIVA usando otras proteínas marcadoras de vacunas recombinantes, e incluso ampliar el conocimiento de la cinética de producción de anticuerpos frente a las proteínas del virus, entre otras opciones. Asimismo, asumiendo los potenciales diagnósticos múltiples de la técnica, cabría pensar en un único diagnóstico en los laboratorios a partir de una sola muestra frente a todas aquellas enfermedades equinas adaptadas a esta tecnología (Balasuriya et al., 2006; Go et al., 2008; Wagner et al., 2011; McNabb et al., 2014), permitiendo el procesado de un gran número de muestras simultáneamente, reduciendo su manipulación, cantidad y el tiempo necesario para la obtención de resultados. Por otro lado, las ventajas de la técnica Luminex también pueden ser aplicadas a la identificación del agente infeccioso, tanto a nivel antígeno como la detección de su material genético. De esta manera, en nuestro grupo hemos comenzado la adaptación de la tecnología Luminex a la detección y serotipado del vPEA simultáneamente.

Por último, cada día es más necesario desarrollar nuevos test diagnósticos que permitan la **detección en campo de los animales infectados o el diagnóstico simultáneo de varios agentes**, ya que en condiciones de campo el diagnóstico se dificulta. Por ese motivo los recientemente desarrollados LFA indirectos fueron comparados con el ensayo Luminex frente a la VP7. Los resultados de ambos test mostraron una alta correlación en el análisis de las muestras de animales infectados experimentalmente (Valor kapa = 0.96, intervalo de confianza del 95% = 0.91 – 1.0), por lo que hacen que este tipo de test sea idóneo para el diagnóstico clínico de la enfermedad, aunque no tanto a nivel laboratorial por el laborioso manejo de los test individuales en el procesamiento de un gran número de muestras. Sin embargo, sería conveniente seguir evaluando los test por las limitaciones que se observaron al procesar muestras de plasma y sueros de campo. Además, dada la importancia del diagnóstico en campo, seguimos trabajando en esta línea realizando estudios para evaluar el uso de LFA para la detección simultánea de anticuerpos frente a PEA y frente a anemia infecciosa equina; y el uso de LFA para la detección de la proteína VP7 (Sastre et al., 2014).

En la presente tesis se utilizó por primera vez la técnica Luminex para el diagnóstico de la PEA, demostrando la utilidad y ventajas que esta plataforma ofrece para su uso en laboratorios de diagnóstico de enfermedades equinas donde se procesen numerosas muestras, mejorando tanto la eficacia y sensibilidad como el tiempo de procesado respecto a los métodos convencionales como el ELISA, y haciendo frente a los nuevos retos del diagnóstico de la enfermedad como el desarrollo de ensayos DIVA. Todas estas ventajas permitirán establecer planes de prevención y control de PEA más eficaces, ya que se mejorara la identificación de los

animales infectados limitando su movimiento, e identificando los animales vacunados lo que podrá disminuir las restricciones a los mismos.

OBJETIVO 3: Análisis epidemiológico de la peste equina en España.

En los últimos años, la re-emergencia de enfermedades vectoriales como la LA o West Nile, ha puesto de manifiesto la importancia de desarrollar **nuevas estrategias de prevención y control** que permitan la detección temprana y eviten la difusión de enfermedades, optimizando los recursos económicos de los programas sanitarios (Council, 2007). Mediante la **vigilancia basada en riesgo**, la cual consiste en el diseño de planes de vigilancia teniendo en cuenta la información del riesgo de la enfermedad (zonas donde es más probable la entrada o exposición al patógeno) y la época del año de mayor riesgo, se incrementará la eficacia de la detección del agente en cuestión (Stark et al., 2006). Este sistema de vigilancia presenta grandes diferencias con los **sistemas convencionales de prevención y control**, los cuales se basan en la detección pasiva de animales infectados junto con muestreos activos al azar en la población susceptible (Tataryn et al., 2007). La utilización de estas técnicas convencionales ha permitido la detección de numerosas enfermedades, sin embargo, esto se ha producido de forma lenta, habiéndose favorecido la difusión de las enfermedades en los territorios, como ya ocurriese con la catástrofe sanitaria de la fiebre aftosa en Reino Unido en 2001, la cual provocó notables pérdidas económicas que no justifican la relación coste-beneficio de este sistema de prevención convencional (Gibbens et al., 2001; Thompson et al., 2002; Kitching et al., 2006).

Para la mejora del sistema de vigilancia de la PEA en España, en esta tesis doctoral se ha llevado a cabo la identificación de las **zonas y periodos de mayor riesgo para la ocurrencia de la PEA** en el territorio. La adecuada identificación de los factores de riesgo y su superposición en un modelo de decisión multicriterio de epidemiologia espacial, permitió conocer y visualizar los niveles de riesgo de cada zona de España para los meses de mayor riesgo así como para el periodo global. Si aplicamos los resultados obtenidos a las zonas de vigilancia activa serológica que se aplican actualmente en España (MAGRAMA, 2014b), las explotaciones centinelas se deberían localizar en las zonas identificadas de mayor riesgo; además, se deberían incluir las Islas Baleares atendiendo a los antecedentes de brotes de LA y el alto riesgo identificado en este estudio. De esta manera se orientarían los esfuerzos sanitarios en las zonas donde es más probable la introducción y difusión de la enfermedad.

Esta información del riesgo de la enfermedad y los distintos roles productivos del sector ecuestre español, han permitido **evaluar la importancia de la entrada de la enfermedad** en cada una de las zonas, y determinar cuáles son las ramas productivas del sector ecuestre que se verían más afectadas. Asimismo, dado el carácter vectorial de esta enfermedad y las distintas peculiaridades de la producción equina española, la información del mapeo de riesgo es útil para el estudio de determinadas **poblaciones equinas** que se puedan presuponer **de alto riesgo**. Dentro de este grupo destacan las explotaciones de carne en semilibertad de la costa norte de España, y los caballos semisalvajes de las marismas de Doñana. El escaso control sanitario de estas poblaciones, las amplias y/o agrestes extensiones en las que se localizan y el curso clínico de la PEA, son los factores que pueden permitir que la infección de estos animales pase desapercibida, favoreciendo su difusión. Como agravante de esta situación, encontramos que las explotaciones de carne en Cantabria y País Vasco, así como los caballos semisalvajes de las marismas de Doñana, se encuentran situados en zonas de alto riesgo. Por lo que, y teniendo en cuenta esta información, sería fundamental establecer adecuadas medidas de vigilancia situando explotaciones centinelas en las inmediaciones de estas localizaciones, permitiendo mantener las tradiciones productivas, muy especialmente en las marismas de Doñana donde el riesgo de introducción por corrientes de aire se considera muy elevado.

Durante este estudio han sido identificadas algunas **limitaciones**, sin embargo, la adecuada interpretación epidemiológica de los resultados y la flexibilidad del modelo permitirían solventarlas. Además, tanto la robustez como la precisión del modelo han sido evaluadas mediante el **análisis de sensibilidad** y la **validación**. Esta última fue realizada con algunos de los brotes de PEA entre 1987-1990, sin embargo, la validación se podría haber mejorado incluyendo todos los brotes de la epidemia de haber estado disponible la información al respecto.

Uno de los factores de mayor riesgo para la potencial introducción y difusión de enfermedades son los **movimientos animales** (Dubé et al., 2009), los cuales en el contexto de expansión de la globalización y libre comercio incrementan el riesgo de **transmisión de patógenos**. Concretamente en esta tesis doctoral se llevó a cabo el estudio de la **red de movimientos equinos en Castilla y León del año 2008**, la cual proporcionará información de gran utilidad para mejorar los planes de control de las enfermedades equinas en esta Comunidad Autónoma. La caracterización de la red nos permitió conocer el patrón de movimientos, determinar las explotaciones más relevantes y sus roles productivos, así como la posibilidad de difusión de una enfermedad por movimientos en la Comunidad Autónoma. En la red también fueron analizadas las posibles agrupaciones espacio-temporales de los movimientos equinos y sus

interrelaciones (compartimentos), lo que permitió caracterizar las zonas y periodos de mayor riesgo para la potencial difusión de las enfermedades. Estos resultados permitirán adelantarnos en los **programas de vigilancia y control** de las enfermedades que afectan a los equinos, siempre y cuando se combine esta información con el estudio del riesgo de las enfermedades.

Dado que disponemos del mapa de riesgo de PEA para España y que los movimientos de animales infectados pueden ser una de las formas para la difusión de la misma**, siempre que haya vectores competentes**, es fundamental identificar los movimientos y agrupaciones espacio-temporales que podrían tener una mayor importancia en la difusión de la enfermedad. En el caso de Castilla y León, el resultado de esta combinación se aprecia en la Figura 12.

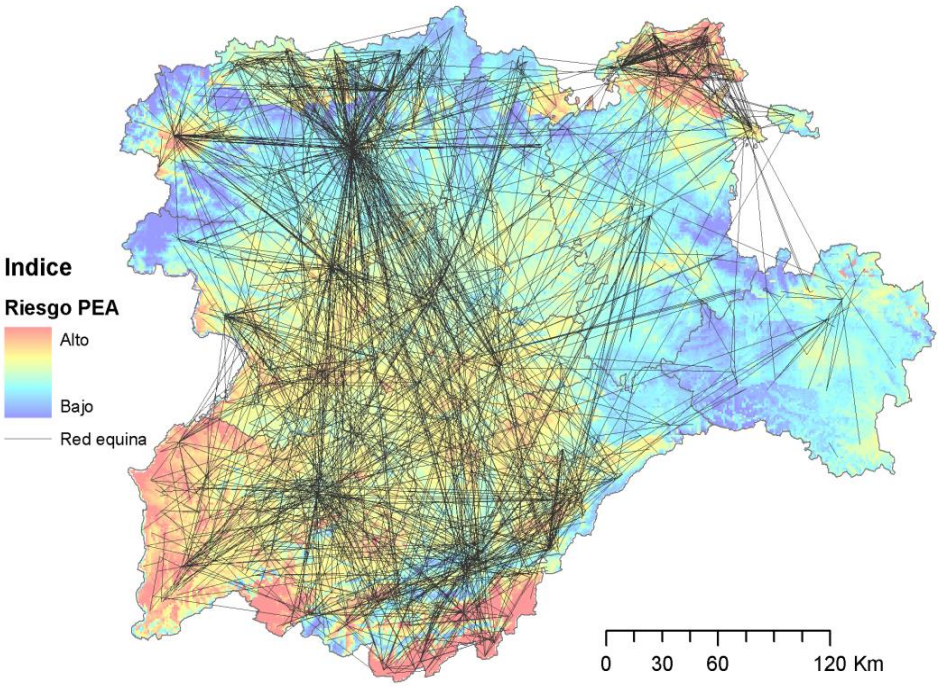


Figura 12: Superposición del mapa de riesgo de peste equina en Castilla y León y la red de movimientos equinos dentro de Castilla y León en 2008. Fuente: Elaboración propia con datos de la Junta de Castilla y León.

Lo que se aprecia en la figura es que numerosos movimientos equinos coinciden con zonas de alto riesgo para la enfermedad, lo que permitiría la rápida difusión de la misma en Castilla y León. Disponer del patrón de movimientos y sus agrupaciones permitirá la **focalización** de los esfuerzos de prevención y control en los **individuos, zonas y/o periodos con mayor riesgo.** Además, en caso de detectarse un brote de la PEA, esta información junto con las encuestas epidemiológicas habituales, permitirá adelantarse y minimizar la difusión de la enfermedad mediante la identificación y control de los movimientos de riesgo hacía una zona

infectada con vectores capacitados. De esta manera, se podrán establecer unas **medidas de control más efectivas** reduciendo los costes económicos asociados al control de enfermedades, así como las repercusiones sociales que puede suponer la entrada de una enfermedad que afecte a la población equina. Por último destacar la importancia de los movimientos que realizan los centros de concentración de animales, y de estos principalmente el que se localiza en el compartimento denominado Burgos, ya que se encuentra en una zona de alto riesgo y sus movimientos se realizan dentro del periodo de riesgo de la enfermedad. En caso de declararse un brote de PEA en la zona previamente a la realización del evento, este se vería anulado, por lo que no supondrían un riesgo para la difusión posterior de la enfermedad; sin embargo, en caso de detectarse con posterioridad sería fundamental tenerlo en cuenta en el estudio de la posible difusión de la misma.

Actualmente, España debe continuar avanzando en la mejora del programa de prevención de la PEA basado en riesgo de la enfermedad. Para ello, dado que se han implementado las redes sociales para el estudio de los movimientos equinos, y se dispone del Registro General de Movimientos de Ganado en España (REMO), sería fundamental poder seguir avanzando en el estudio del patrón de movimientos equinos en el territorio nacional, tanto internamente como desde países externos. Teniendo en cuenta el carácter vectorial de la PEA, otra de las mejoras que se podrían implementar en la vigilancia basada en riesgo, es el estudio de la difusión de la enfermedad por el movimiento natural y/o corrientes de aire de los vectores, dentro de nuestro territorio o como vía de entrada desde países próximos como se ha podido estudiar para la LA (Ducheyne et al., 2011; Sedda et al., 2012; Burgin et al., 2013; Graesboll et al., 2014; Kelso and Milne, 2014). Estos resultados deberían ser combinados con la identificación de las zonas de alto riesgo de entrada de la enfermedad (Defra, 2008; Aznar et al., 2011; Martínez-López et al., 2011; de Vos et al., 2012). La utilización conjunta de estas herramientas podría permitir avanzar en el estudio de modelos del riesgo de entrada y difusión de la enfermedad nuestro país mejorando los sistemas de prevención y control.

En definitiva, los estudios realizados han permitido identificar las zonas de mayor riesgo para la ocurrencia de la enfermedad y estudiar su posible propagación mediante el análisis de redes sociales. Los resultados obtenidos podrán ser utilizados para la mejora de los programas de prevención y control de la PEA en España basándose en el principio de vigilancia basada en riesgo que permitirán la optimización de los recursos sanitarios.

OBJETIVO 4: Evaluación económica de la difusión de la peste equina en España.

La demanda de nuevas estrategias de vigilancia y control de enfermedades animales más eficaces, en combinación con la actual limitación de recursos financieros para este fin, hacen necesaria la **optimización** de los mismos. En este contexto, **nuevas herramientas** como los **análisis económicos** son fundamentales para optimizar **la toma de decisiones**, puesto que permiten la evaluación y comparación de la eficacia de los programas sanitarios desde un punto de vista económico, contribuyendo a la elección de la estrategia más adecuada con el menor coste para la sociedad (Morris, 1999; Dehove et al., 2012). Este motivo unido a las devastadoras epidemias de los últimos años, como fiebre aftosa o influenza aviar (Elci, 2006; Knight-Jones and Rushton, 2013), han favorecido el desarrollo de las **evaluaciones económicas en sanidad animal**, aunque siempre en combinación con los aspectos epidemiológicos de las enfermedades.

Desde principios del siglo XXI, diferentes organismos internacionales como la OIE y la FAO han promovido la implantación de herramientas para la evaluación de los costes y beneficios de los programas sanitarios nacionales (Perry, 1999; Otte et al., 2004). Siguiendo estas recomendaciones, la estrategia de la UE en sanidad animal para 2007-2013 ha sido promover el uso de análisis económicos para un uso eficiente de los recursos (UE, 2007-2013). Diferentes revisiones han sido publicadas para mejorar la comprensión en materia económica en el campo de la sanidad animal enfocada a la toma de decisiones (p. ej. Dehove et al. (2012); Häsler et al. (2013); Howe et al. (2013) y Rich and Perry (2011)). De igual forma, diferentes investigadores han trabajado en la evaluación de los impactos económicos de diferentes enfermedades animales víricas (p. ej. Nieuwenhuis et al. (2012) y Velthuis et al. (2010)), bacterianas (p. ej. van Asseldonk et al. (2013) y Cho et al. (2012)) y parasitarias (p. ej. Bandyopadhyay et al. (2010) y Gharbi et al. (2006)); así como en la optimización de las herramientas de vigilancia y control mediante la comparación de distintas estrategias (p. ej. Häsler et al. (2012), Rutten et al. (2012), Boden et al. (2012) y Wera et al. (2013), entre otros). El trabajo presentado en esta tesis doctoral es el primer estudio del **impacto económico** que la difusión y los mecanismos de control de una enfermedad transfronteriza de caballos, la PEA, puede causar en un país no endémico.

Para realizar este estudio, se seleccionó Andalucía por ser una de las zonas de alto riesgo para la difusión de la enfermedad en España (ver Capítulo III, Objetivo 3.1), y dado que no conocemos la probabilidad de introducción de la PEA en esta Comunidad Autónoma, se establecieron diferentes **escenarios** de la enfermedad en base a las **características climáticas y estudios entomológicos actuales.** Seleccionando estas variables, en caso de una re-introducción de la PEA, se podrán predecir el impacto de la enfermedad y las pérdidas

económicas que más se ajusta a la realidad del momento, ya que como se observa en los resultados del modelo, las consecuencias de las epidemias varían en función de los escenarios. Al mismo tiempo, los resultados de estos escenarios permitirán la adecuada toma de decisiones de las autoridades veterinarias conociendo las estrategias de control más eficaces, así como reunir los recursos necesarios para los programas de prevención y lucha basándose en datos socioeconómicos estimados.

Los modelos epidemiológicos han sido desarrollados para comprender la evolución de las enfermedades, por lo que son cruciales para estudiar las consecuencias de la entrada de la enfermedad en un país libre. Sin embargo para determinar las estrategias de control más efectivas y económicamente rentables, es necesario analizarlos conjuntamente con el contexto económico de la enfermedad (Fenichel et al., 2010). En este estudio ha sido integrada la evaluación epidemiológica con la evaluación económica de la difusión de la enfermedad, la cual ha sido realizada mediante un análisis coste-beneficio, una de las metodologías más utilizada para la evaluación de las enfermedades animales (Rushton, 2009). Los resultados de este análisis revelan la utilidad de evaluar conjuntamente los impactos epidemiológicos y económicos para una **optimización de la relación coste-eficacia** de las estrategias de control.

Una de las principales ventajas de esta metodología es la **flexibilidad del modelo matemático**, ya que es fácilmente modificable permitiendo la inclusión o adaptación de parámetros que permitan mejorar la evaluación de la enfermedad. De esta manera, se puede realizar la evaluación de alternativas a las estrategias de control actuales (p.ej. avalar la utilización de vacunas de nueva generación); adaptarse a las variaciones de los parámetros (p.ej. cambios climatológicos, ratio de vectores o población equina, entre otros); o mejorar el desglose de variables permitiendo evaluar los impactos por tipo de explotación o diferenciando los costes gubernamentales de los que asumiría el propietario del animal.

A la luz de los resultados obtenidos, las medidas de control que incluyen la estrategia de vacunación se plantean como la mejor alternativa en la lucha contra la enfermedad, reduciendo tanto las consecuencias sociales como económicas. Además, ante los numerosos cambios en la legislación de control en los últimos tiempos en España frente a otra enfermedad vectorial como es la LA, deberíamos continuar utilizando este tipo de herramientas para plantear otras situaciones, por ejemplo, la evaluación de las repercusiones de modificar las estrategias de vacunación tras varios años de enfermedad o modificar las zonas de vacunación.

Dado que actualmente no existe ningún modelo económico sobre las consecuencias de la entrada y difusión de la enfermedad en España, este trabajo permitirá mejorar y avalar la **toma de decisiones** haciendo los programas sanitarios de PEA económicamente justificables. Teniendo en cuenta las peculiaridades de la producción equina española, el modelo propuesto e implementado en Andalucía sería el patrón inicial para la evaluación de las consecuencias de la difusión en otras regiones españolas con características de producción diferentes, así como para la evaluación de las estrategias de vigilancia empleadas actualmente.

En definitiva en cuanto al objetivo 4, para llevar a cabo estos estudios es fundamental tener en cuenta la importancia de los equipos multidisciplinares. En este caso ha sido posible el diálogo entre economistas, matemáticos, epidemiólogos y veterinarios clínicos, desde los inicios de la planificación y diseño del estudio, hasta su finalización y publicación. Esta visión multifocal de la enfermedad favorece un acercamiento entre profesionales gracias a una mayor integración de sus diferentes puntos de vista, revelándose nuevos aspectos y matices alejados de los cánones clásicos en los que se han basado los estudios sobre las enfermedades animales hasta el día de hoy. La implantación de estas evaluaciones epidemiológicas y económicas de la sanidad animal, permitirá comprender los **impactos de las enfermedades**, lo que mejorará la **toma de decisiones públicas de los Estados miembros** mediante la **optimización de la relación coste-beneficio** de los programas sanitarios, así como ayudará a establecer directrices para el diseño de los seguros agrarios equinos.

Finalmente, podemos asegurar que actualmente la respuesta de los servicios veterinarios españoles a la enfermedad no sería la misma que la ocurrida entre 1987 y 1990. Las **medidas de prevención y lucha contra la PEA** se encuentran recogidas en manuales actualizados de la enfermedad (MAGRAMA, 2004, 2013), R.D. 804/2011). Las medidas de prevención en España se basan en el análisis de riesgo de introducción, las cuales han sido implementadas en el sistema de vigilancia actual frente a la PEA, equiparándose a otros países europeos que siguen las recomendaciones de la UE en materia de vigilancia basada en riesgo. Por ejemplo, países como Reino Unido, Escocia, Irlanda u Holanda han implantado planes de prevención basados en el riesgo de introducción o difusión de la enfermedad (Defra, 2008; Aznar et al., 2011; Backer and Nodelijk, 2011; de Vos et al., 2012; Iacono et al., 2013). Además, tanto los veterinarios oficiales como privados españoles, actualmente son formados y evaluados mediante diferentes cursos y simulacros, para intentar mejorar la eficiencia de futuras actuaciones frente a esta enfermedad. No obstante, los programas de prevención y control deben continuar actualizándose y

mejorándose integrando nuevos conocimientos y herramientas disponibles, como pueden ser los **descritos en esta tesis,** permitiendo aumentar la sensibilidad del sistema y optimizando la utilización de los recursos disponibles.

Durante todo el desarrollo de esta tesis, el fin último ha sido incrementar los conocimientos de la enfermedad para la mejora de adecuados planes de prevención y control de la misma, para lo cual es inestimable la concienciación de las autoridades sanitarias y personal involucrado en la sanidad animal en la vigilancia basada en riesgo y la optimización de recursos.

Finalmente los trabajos inmunológicos, diagnósticos, epidemiológicos y económicos realizados en esta **tesis doctoral,** han cumplido los objetivos propuestos proporcionando un avance significativo para la lucha contra la enfermedad. Los resultados de la evaluación de la **respuesta inmune de caballos experimentalmente infectados con el vPEA** podrían ser usados para el desarrollo de nuevas herramientas terapéuticas y vacunas; las **nuevas herramientas diagnósticas** desarrolladas podrían permitir aumentar la sensibilidad y eficiencia de la detección de animales infectados, así como la realización de un diagnóstico DIVA para la vacuna inactivada del serotipo 4; además, si son implementados con los nuevos conocimientos aportados por los **análisis epidemiológicos y económicos** de la enfermedad en España (**identificación de las zonas de mayor riesgo del país**, **caracterización de los movimientos equinos** y **simulación del impactos de la epidemia**), permitirán mejorar los **programas de prevención y control** de la enfermedad orientándolos a una vigilancia basada en riesgo con la mejor relación coste-beneficio.

CAPÍTULO V

CONCLUSIONES/CONCLUSIONS

Fuente de la imagen: Señor Hans, Dos Caballos en La Playa [CC BY-NC-ND 2.0].

PRIMERA / FIRST

Se desarrolló un panel de rRT-PCRs con SYBR Green para la cuantificación simultánea de la expresión de ocho citoquinas involucradas en la respuesta inmune de los équidos: IL-1β, IL-2, IL-4, IL-10, IL-12, TNFα, IFNβ e IFNγ; y un gen de expresión constante (β-Actina). Esta nueva herramienta fue validada tanto en células mononucleares de sangre periférica estimuladas *in vitro*, como en sangre completa de un caballo infectado con el vPEA, permitiendo la cuantificación relativa de los niveles de expresión de las citoquinas.

A panel of RTqPCR based on SYBR Green was developed to simultaneously quantify the relative expression of eight cytokine genes in horse: IL-1β, IL-2, IL-4, IL-10, IL-12, TNFα, IFNβ e IFNγ; and a housekeeping gene (β-Actina). This tool was validated with mitogen-stimulated peripheral blood mononuclear cells from a healthy horse and with whole blood from a horse infected with AHSV, allowing quantification of relative cytokine expression levels.

SEGUNDA / SECOND

Se ha analizado por primera vez la respuesta inmune de caballos infectados experimentalmente con dos serotipos del vPEA (serotipo 2 y 4) mediante el análisis del patrón de expresión de citoquinas (IL-1β, TNFα, IL-12, IL-10, IFNγ, IL-2 y IL-4) y la producción de anticuerpos. En este estudio se observó que los caballos presentaron un patrón de expresión de citoquinas variable, pudiéndose identificar algunos perfiles comunes en función de la forma clínica de la enfermedad, así como se detectó la producción de anticuerpos desde el día 7 post-infección hasta el final del estudio mediante ensayos de flujo lateral (LFA).

For first time, the immune response in horses infected with two serotypes of AHSV (serotype 2 and 4) has been analyzed based on the cytokine expression patterns (IL-1β, TNFα, IL-12, IL-10, IFNγ, IL-2 and IL-4) and on antibodies production. The experimental findings show that each horse presented a different cytokine expression profile, although some common characteristics were identified depending on the clinical form of the disease.The antibody production was detected from day 7 post-infection until the end of the study using a lateral flow assay (LFA).

TERCERA / THIRD

Se ha desarrollado por vez primera un ensayo basado en la tecnología Luminex para la detección de anticuerpos frente a la VP7. Este nuevo método diagnóstico aporta rapidez, sensibilidad y especificidad para la detección de anticuerpos frente a la proteína VP7 de todos los serotipos de la PEA. Las ventajas de este ensayo lo posicionan como una posible alternativa al ELISA en los laboratorios de diagnóstico.

This study is the first adaptation of Luminex technology to detect antibodies against AHSV VP7. This novel diagnostic tool is a rapid, specific and sensitive test for the detection of horse VP7 antibodies against all serotypes of AHSV. Therefore, the features of this technique make it to be a good alternative to ELISA for diagnostic laboratories.

CUARTA / FOURTH

Se ha realizado la primera adaptación de la tecnología Luminex al diagnóstico DIVA del serotipo 4 del vPEA. Mediante la detección de anticuerpos frente a las proteínas VP7 y NS3, la técnica desarrollada permite la diferenciación de animales infectados, vacunados con vacuna inactivada y animales sanos. La utilización de este ensayo podría ayudar a reducir las limitaciones de movimientos equinos de los animales vacunados, disminuyendo una de las principales pérdidas económicas asociada a la enfermedad.

For the first time, an adaptation of the Luminex assay to the DIVA diagnosis of AHSV serotype 4 has been described. The detection of antibodies against VP7 and NS3 proteins allow the differentiation of infected, vaccinated with inactivated vaccine and uninfected animals. This DIVA assay could help to reduce the equine movement restrictions of vaccinated animals, decreasing one of the most important economic losses associated with AHS.

QUINTA / FIFTH

Mediante un modelo de decisión multicriterio de epidemiología espacial se ha evaluado por primera vez el riesgo para la ocurrencia de PEA en España. Las zonas de mayor riesgo fueron localizadas en el sur-oeste de España, Cantabria y las Islas Baleares, siendo los meses de mayor riesgo julio y agosto. Por otro lado, las zonas de menor riesgo fueron identificadas en Galicia, Castilla y León y La Rioja. La asociación del riesgo de las zonas con los diferentes tipos de producción determinarán las consecuencias de la difusión de la enfermedad en los sectores de la producción equina española en función de la región.

For first time, the risk areas for AHSV infection have been evaluated in Spain using a GIS-based multricriteria framework. The areas presenting a higher risk were located at the South-Western Spain, Cantabria and the Balearic Islands, particularly in July and August. Conversely, Galicia, Castile and Leon and La Rioja can be considered as low-risk regions. The features of the productions premises, depending on the area, determine the consequences of the AHS spread in the current Spanish equine production.

SEXTA / SIXTH

La red de movimientos equinos dentro de Castilla y León ha sido caracterizada por primera vez identificando los individuos, áreas y periodos de mayor riesgo para la difusión de las enfermedades por movimientos equinos. Estos resultados serán de gran utilidad para el diseño de programas de vigilancia y control basados en el riesgo y para aumentar la eficacia y la rapidez de la detección de focos secundarios durante la epidemia de una enfermedad que afecta al sector equino español.

This study is the first characterization of the network of equine movements in Castile and Leon identifying the premises, areas and time periods with potentially high risk of equine disease introduction and/or spread. These results will be useful for supporting the design of risk-based surveillance and control programmes of equidae diseases and for increasing the efficacy and speed of detection of secondary outbreaks during the epidemic of a disease that affects the Spanish equine sector.

SEPTIMA / SEVENTH

En este trabajo han sido analizadas por primera vez las consecuencias epidemiológicas y económicas de la difusión de la PEA en un país no endémico mediante la evaluación de diferentes escenarios de temperatura y vectores, así como de diferentes estrategias de control de la enfermedad. Todos los escenarios de la enfermedad en Andalucía justificaron el uso de la vacunación de emergencia para reducir las consecuencias socio-económicas de una posible epidemia basándose en los resultados epidemiológicos y en el ratio coste-beneficio de su aplicación. Los responsables de las políticas sanitarias podrían mejorar o modificar el programa de control de la PEA utilizando los resultados de este análisis epidemiológico y económico de la enfermedad, así como predecir las consecuencias socio-económicas de una posible epidemia en función de las características climáticas y entomológicas de la zona afectada.

This work is the first evaluation of the epidemiological and economic consequences of the AHS spread in a non-endemic country through the assessment of different temperature and vector scenarios as well as different strategies for disease control. All scenarios of this study in Andalusia justify the use of the emergency vaccination to reduce the socio-economic consequences of an epidemic based on epidemiological results and benefit-cost ratio. The model results could be used by the Spanish national policy makers to improve or modify the AHS control program, as well as to predict the socio-economic consequences of AHS outbreak according to the climatic and entomological characteristics of the infected area.

OCTAVA / EIGHTH

Todas las metodologías y resultados obtenidos en esta tesis tienen una aplicación directa y estratégica en la mejora del sistema de vigilancia, prevención y control actual de la PEA en España, permitiendo diseñar una vigilancia basada en riesgo que facilitará priorizar y reducir los recursos económicos disponibles para incrementar el ratio coste-beneficio.

All the methodologies and results described in this thesis have a direct strategical application for improving the surveillance, prevention and control of AHS in Spain, allowing to design a risk-based surveillance that will facilitate to prioritize and reduce the economic available resources to increase the benefit-cost ratio.

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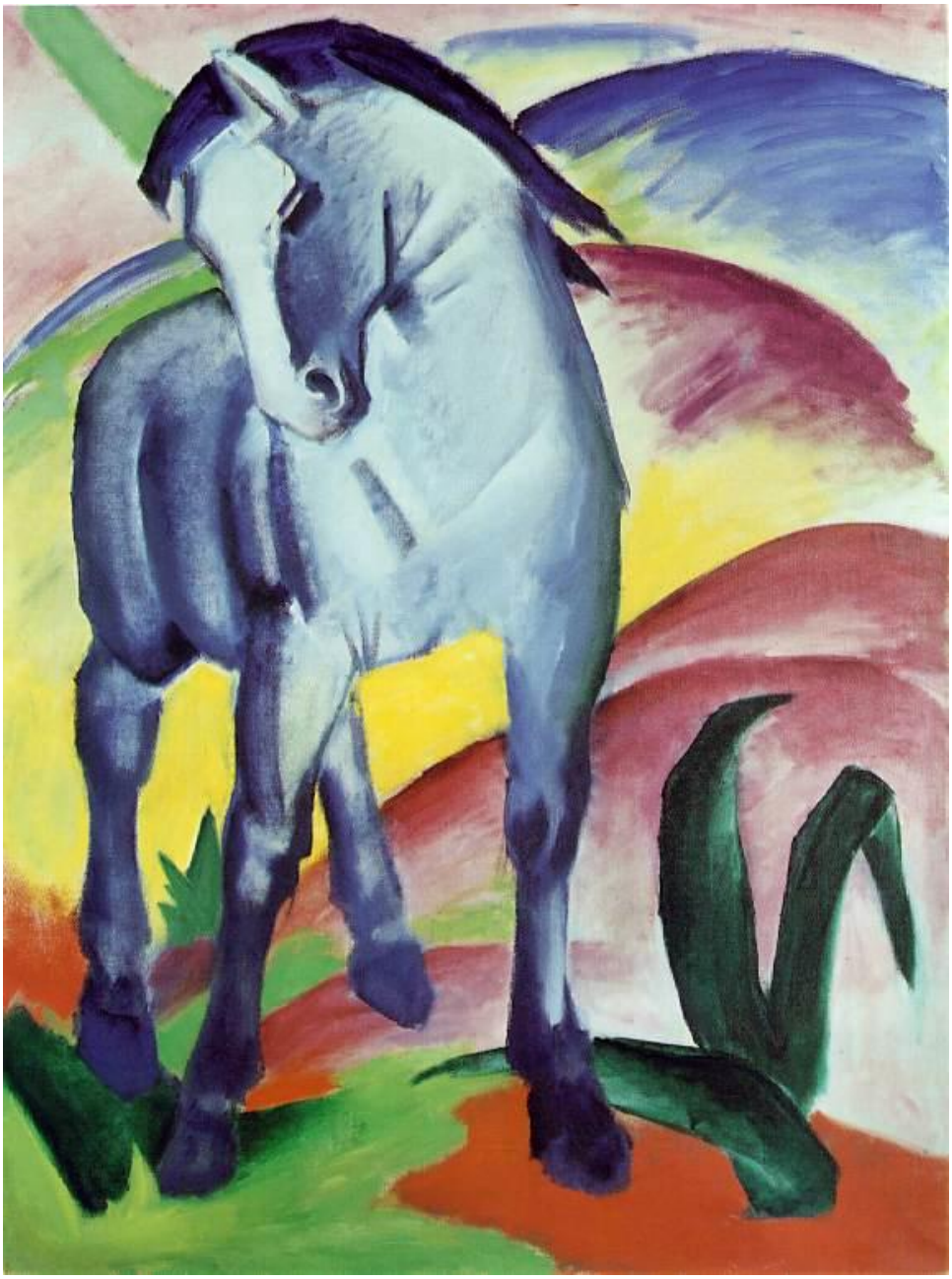
ANEXOS



Fuente de la imagen: Franz Marc, El caballo azul I [dominio público].

OTRAS APORTACIONES EN SANIDAD

1. Publicaciones indexadas

1. Rodríguez-Prieto V., Vicente-Rubiano M., Sánchez Matamoros A., Rubio-Guerri C., Melero M., Martínez-López B., Martínez-Avilés M., Hoinville L,, Vergne T., Comin A., Schauer B., Dórea F., Pfeiffer D.U. and Sánchez-Vizcaíno J.M. 2014. **Systematic review of surveillance systems and methods for early detection of exotic, new and re-emerging diseases in animal populations** Epidemiology and Infection (2014). *In press*
2. Ruiz-Fons F., Sánchez Matamoros A., Gortázar C. and Sánchez-Vizcaíno J.M. 2014. **The role of wildlife in bluetongue virus maintenance in Europe: Lessons learned after the natural infection in Spain.** Virus Research. DOI 10.1016/j.virusres.2013.12.031

2. Publicaciones no indexadas

1. Sánchez-Matamoros A., Rubio-Guerri C., Nieto-Pelegrín E., y Sánchez-Vizcaíno J.M., 2014. **Nuevas herramientas de diagnóstico en enfermedades animales: Metagenómica, Luminex y Proteómica**. Academia de Veterinaria de la Región de Murcia, Anales 2014. *En edición*
2. Sánchez-Vizcaíno J.M., Martínez-Avilés M., Sánchez-Matamoros A.and Rodríguez-Prieto V., 2014. **Emerging vector-borne diseases and any potential to prevent their spread.** CAB REVIEW. *In press.*
3. Sánchez-Vizcaíno J.M., Mur L., Sánchez-Matamoros A. y Martínez-López B., 2014. **Peste porcina africana: nuevos retos y medidas para evitar su propagación.** 82ª Sesión General OIE. Asamblea Mundial. París 25-30 mayo, 2014.

3. Libros

1. Martínez-López B., Martínez Avilés M., Iglesias Martín I., Rodríguez-Prieto V., Sánchez Matamoros A., Fernández Calle L.M., Fernández Salazar M., González González Y., Grau Vila A., Marqués Prendes S., Muñoz Reoyo M.J., de la Torre Reoyo A., Linares F.J, Sánchez-Vizcaíno Buendía F., Perez A., López de Ayala M., Mínguez González O., Sánchez-Vizcaíno J.M. (2013) **Desarrollo de un sistema integral para la mejora de la vigilancia, control y gestión de enfermedades exóticas, emergentes y zoonosicas que afectan al sector ganadero en Castilla y León.** ISBN 978-84-616-3244-2
2. Martínez-López B., Sánchez-Vizcaíno F., Linares F., Rodríguez V., Mur L., Vicente M., Sánchez A., Martínez M. and Sánchez-Vizcaíno J.M. (2012) **Análisis de riesgo epidemiológico predictivo (cualitativo) de entrada en España del resto de enfermedades vectoriales de interés veterinario y su posible incursión en los seguros ganaderos.** ISBN 978-84-690-3507-8.

4. Congresos internacionales y nacionales

1. Vicente-Rubiano M., Rodríguez-Prieto V., Martínez-Avilés M., Sánchez-Matamoros A., Rubio-Guerri C., Melero M., Martínez-López B., Hoinville L,, Vergne T., Comin A., Schauer B., Dórea F., Pfeiffer D.U. and Sánchez-Vizcaíno J.M. **Literature review of surveillance systems and approaches for the early detection of new, exotic and re-emerging diseases.** Comunicación oral en la *Society for Veterinary Epidemiology and Preventive Medicine (SVEPM)*. 2014 Annual Conference, marzo 2014.
2. Sánchez-Matamoros A., Martínez-López B. Tutorizado por Iglesias E. y Sánchez-Vizcaíno, J.M. **Serotipos de peste equina africana de mayor riesgo en España.** Comunicación oral en la *VII Jornadas Complutenses, VI Congreso Nacional de Investigación para alumnos de Pregrado en Ciencias de la Salud y XI Congreso de Ciencias Veterinarias y Biomédicas*, abril 2012.
3. Sánchez A. Tutorizado por Sánchez-Matamoros A. y Sánchez-Vizcaíno J.M. **RT-PCR convencional y a tiempo real con SYBR Green para el diagnóstico de peste equina africana**. Comunicación oral en las *VII Jornadas Complutenses, VI Congreso Nacional de Investigación para alumnos de Pregrado en Ciencias de la Salud y XI Congreso de Ciencias Veterinarias y Biomédicas*, abril 2012.
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5. Sánchez-Matamoros A., Tutorizado por De las Heras A.I. y Sánchez-Vizcaíno J.M. **Evaluación de la respuesta inmune en caballos: Cuantificación de citoquinas y citometría de flujo.** Comunicación oral en la *V Congreso Nacional de Investigación para alumnos de Pregrado en Ciencias de la Salud y X Congreso de Ciencias Veterinarias y Biomédicas*, abril 2011.
6. Sánchez-Matamoros A., Martínez-López B., Merchante E., Gómez A., Trillo J. Tutorizado por Sánchez-Vizcaíno J.M. **Evaluación de la vigilancia sanitaria en seguridad alimentaria.** Comunicación oral en la *V Congreso Nacional de Investigación para alumnos de Pregrado en Ciencias de la Salud y X Congreso de Ciencias Veterinarias y Biomédicas*, abril 2011.
7. Sánchez-Matamoros A., Martínez-López B., Linares F., Vinuesa L. Tutorizado por Sánchez-Vizcaíno J.M. **Análisis probabilístico del riesgo de introducción del virus de la peste equina africana por animales vivos en España**. Comunicación oral en la *IX Congreso De Ciencias Veterinarias y Biomédicas*, abril 2010.

5. Cursos y seminarios impartidos

1. Sánchez-Matamoros A: **Training Course on Diagnosis of Equine Diseases. African horse sickness: Epidemiology, global distribution, clinical disease and diagnosis.** Organizador el Istituto Zooprofilattico Sperimentale of Sicily, 2014.
2. Sánchez-Matamoros A: **African horse sickness: Diagnostic tools.** Organizador SUAT-VISAVET en Madrid, 2013.
3. Sánchez-Matamoros A: **Curso sobre el Análisis económico aplicado a la Sanidad Animal. Análisis económico mediante árboles de decisión. Ejemplos prácticos.** Organizador el grupo de investigación SUAT-VISAVET (UCM) impartido al Ministerio de Agricultura, Alimentación y Medio Ambiente, 2012.

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