ESTUDIO DE NUEVOS FACTORES DE RIESGO PARA LA TRANSMISIÓN DEL VIRUS DE LA HEPATITIS E

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STUDY OF NEW RISK FACTORS FOR THE TRANSMISSION OF THE HEPATITIS E VIRUS

Programa de doctorado:

BIOMEDICINA

Autor de la tesis:

PEDRO LÓPEZ LÓPEZ

Directores de tesis:

Antonio Rivero Román / Antonio Rivero Juárez

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TITULO: *ESTUDIO DE NUEVOS FACTORES DE RIESGO PARA LA TRANSMISIÓN DEL VIRUS DE LA HEPATITIS E*

AUTOR: *Pedro López López*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

https://www.uco.es/ucopress/index.php/es/
ucopress@uco.es

TÍTULO DE LA TESIS: Estudio de nuevos factores de riesgo para la transmisión del virus de la hepatitis E.

DOCTORANDO/A: Pedro López López

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

D. ANTONIO RIVERO ROMÁN, Doctor en Medicina y Cirugía por la Universidad de Málaga, Profesor titular de Medicina de la Universidad de Córdoba, jefe de sección de la Unidad de Gestión Clínica de enfermedades infecciosas del Hospital Universitario Reina Sofía, e Investigador Principal del grupo de investigación en Virología Clínica y Zoonosis del Instituto Maimonides de Investigación Biomédica de Córdoba (IMIBIC).

D. ANTONIO RIVERO JUÁREZ, Doctor con mención internacional y premio extraordinario por la Universidad de Córdoba, e investigador emergente del grupo de investigación en Virología Clínica y Zoonosis del Instituto Maimonides de Investigación Biomédica de Córdoba (IMIBIC).

El presente proyecto de tesis doctoral está constituido por cinco trabajos realizados en su totalidad en el grupo de investigación en Virología Clínica y Zoonosis del IMIBIC, sobre la identificación de nuevos factores de riesgo para la infección de una enfermedad zoonótica emergente como es el virus de la Hepatitis E. Los resultados derivados de estos trabajos han sido comunicados en congresos Nacionales e Internacionales de Enfermedades Infecciosas y publicados en revistas internacionales indexadas en el *Journal Citation Report*. Estas publicaciones acumulan un factor de impacto de 26,18 puntos (factor de impacto medio de 5,23 puntos). Cabe destacar, que dos de los trabajos realizados han permitido optimizar el diagnóstico serológico y molecular de la infección por el virus de la hepatitis E y, por ello, han tenido un gran impacto por su aplicación en la práctica clínica y su citación en documentos de consenso y guías de práctica clínica. Todos estos trabajos han sido realizados por el

doctorado, con la supervisión de sus directores, y el apoyo técnico y científico de nuestro grupo de investigación. Durante el periodo doctoral, el doctorando ha demostrado un gran interés y compromiso con nuestro grupo de investigación, convirtiéndose en parte fundamental del mismo. Además, dado que varios trabajos del presente proyecto de tesis doctoral se han realizado en colaboración con otros centros, el doctorando ha adquirido las capacidades suficientes para realizar la coordinación y ejecución de proyectos multicéntricos. Por último, el doctorando ha realizado una estancia formativa de tres meses en la Universidad de Porto (Portugal), que ha dado como resultado dos publicaciones científicas en revista situada en primer decil. Por todo ello, que el presente proyecto de Tesis Doctoral reúne los méritos suficientes para optar al Grado de Doctor en Biomedicina por la Universidad de Córdoba.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 27 de enero de 2022

Firma de los directores

Fdo.: Antonio Rivero Román

Fdo.: Antonio Rivero Juárez

INDICIOS DE CALIDAD DE LAS PUBLICACIONIES PRESENTADAS EN EL PROYECTO DE TESIS DOCOTORAL

Objetivo	Título	Revista	Categoría	Posición en la categoría	Factor de Impacto (JCR 2020)
Desarrollar y validar clínicamente un método molecular con capacidad de detección de todos los genotipos y subtipos del género *Orthohepevirus* A	Development and clinical validation of a pangenotypic PCR-based assay for the detection and quantification of hepatitis E virus (*Orthopevirus* A genus).	Journal of Clinical Microbiology	Microbiology	22/137 (Q1/T1)	5,948
Optimizar el algoritmo diagnóstico de la infección aguda por el VHE incluyendo la determinación serológica y molecular	Limited Value of Single Sampling for IgM Antibody Determination as a Diagnostic Approach for Acute Hepatitis E Virus Infection.	Microbiology Spectrum	Microbiology	18/137 (Q1/T1)	7,171
Evaluar si los pacientes infectados por el VIH constituyen un grupo de riesgo para la infección por el VHE	Human Immunodeficiency Virus Infected Patients are Not at Higher Risk for Hepatitis E Virus Infection: A Systematic Review and Meta-Analysis.	Microorganisms	Microbiology	52/136 (Q2/T1)	4,128
Evaluar la influencia del gen PROGINS sobre la susceptibilidad y el curso clínico de la infección por el VHE	Mutations in the Progesterone Receptor (PROGINS) May Reduce the Symptoms of Acute Hepatitis E and Protect Against Infection.	Frontiers in Microbiology	Microbiology	28/136 (Q1/T1)	5,64
Evaluar los factores de riesgo asociados a la infección activa por el VHE en cerdos criados en diferentes sistemas de producción	Risk factors associated with hepatitis E virus in pigs from different production systems.	Veterinary Microbiology	Science Edition - VETERINARY SCIENCES	14/146 (Q1/D1/T1)	3,293

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Me gustaría agradecer a todas las personas que durante todos estos años me han apoyado, tanto profesional como personalmente, y que sin ellas no hubiera podido llegar hasta donde he llegado.

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Y por último, a mi familia, por el apoyo que me han dado desde siempre, y sobre todo a mi madre, que siempre ha dado todo lo mejor por mí.

A todos vosotros, gracias.

“Collaboration across disciplines is also crucial for the implementation of prevention and control measures that benefit public health. A One Health approach is necessary to understand and mitigate HEV transmission via food, animals, substances of human origin and the environment”

Mulder AC y col. Eurosurveillance 24 de Marzo de 2019

Índice general

I. Abreviaturas

.col: colaboradores

Ag: Antígeno

ALT: Alanina aminotransferasa

APOE: Apoliproteína E

ARN (RNA): Acido Ribonucleico (Ribonucleic acid)

EASL: Asociación Europea para el Estudio del Hígado (European Association for the Study of the Liver)

ECDC: Centro Europeo para el Control de Enfermedades (European Center for Disease Control)

ELISA: Ensayo de inmunoabsorción ligado a enzimas

H: Región Hipervariable

Hel: Helicasa

HSH: Hombres que tienen sexo con hombres

I2: Heterogeneidad

IC (CI): Intervalo de Confianza (Confidence interval)

IF (JCR): Factor de impacto (Journal Citation Reports)

IgG: Inmunoglobulina G (Inmunoglobuline G)

IgM: Inmunoglobulina M (Inmunoglobuline M)

Kb: Kilobases

kDa: Kilodaltons

Met: Metiltransferasa

ml: mililitro (mililiter)

nt: nucleótidos

OMS (WHO): Organización Mundial de la Salud (World Health Organization)

ORF: Marco de lectura abierta (Open Reading Frame)

PcP: Cisteína Proteasa similar a Papaína (Papain-like cysteine protease)

PCR: Reacción en cadena de la polimerasa (Polymerase Chain Reaction)

PCV2: Circovirus porcino 2 (Porcine circovirus type 2)

PGHS: Proteoglicano de Heparán Sulfato

PhD: Doctorado

PRISMA: Elementos de Informes Preferidos para Revisiones Sistemáticas y Metaanálisis (Preferred Reporting Items for Systematic Reviews and Meta-Analyses)

PRRSV: Virus del síndrome respiratorio y reproductivo porcino (Porcine reproductive and respiratory syndrome virus)

RdRp: Polimerasa de ARN dependiente de ARN (RNA-dependent RNA polymerase)

RE: Retículo endoplasmático

RP (PR): Receptor de la Progesterona (Progesterone Receptor)

RT-qPCR/RT-PCR: Retrotanscriptasa PCR cuantitativa (Quantitative Reverse Transcription PCR)

Th: Linfocito T ayudante (Linfocito T helper)

UI (IU): Unidades Internacionales (International Units)

VHE (HEV): Virus de la Hepatitis E (Hepatitis E Virus)

VIH (HIV): Virus de la Inmunodeficiencia Human (Immunodeficiency Virus Human)

VLP: Partícula viral (Viral-like particle)

vs.: versus

II. Resumen/Summary

Resumen

El objetivo principal del presente proyecto de tesis doctoral es la identificación de factores de riesgo para la infección por el virus de la hepatitis E (VHE). En este sentido, ya que la infección por el VHE en nuestro medio es zoonótica, es necesario el realizar una aproximación One Health para la evaluación de factores de riesgo de la infección; esto es evaluar los factores tanto en población humana como en población animal. Para llevar a cabo esta evaluación es fundamental disponer de una buena herramienta diagnóstica con el objeto de identificar a los sujetos infectados, así como la optimización del algoritmo diagnóstico que permita identificar a la totalidad de pacientes infectados. El primer paso del proyecto de tesis doctoral ha sido el desarrollo y validación de un método molecular para el diagnóstico de la infección por el VHE con una mayor sensibilidad y especificidad que los métodos moleculares disponibles actualmente. En segundo lugar, se ha realizado la optimización del algoritmo diagnóstico de la infección con el objeto de minimizar el número de pacientes falsos negativos. Una vez optimizado el diagnóstico de la infección por el VHE, se han evaluado los factores de riesgo tanto en población humana como en población animal. Para ello se han establecido cinco objetivos, cuya consecución ha dado lugar a 5 publicaciones científicas. Estos se resumen en los siguientes puntos:

Objetivo 1: Desarrollar y validar clínicamente un método molecular con capacidad de detección de todos los genotipos y subtipos de la especie *Orthohepevirus* A. Debido a la considerable variabilidad que el VHE ha mostrado en la actualidad, no existe un método de cribado por PCR que haya sido diseñado para detectar el amplio espectro de genotipos que presenta esta especie viral. La implementación de un método preciso para la detección de todos los genotipos del VHE actualmente descritos, así como la detección de subtipos no identificados, es necesaria para la salud pública tanto humana como animal, constituyendo la base de un modelo de una única Salud (One Health). Por todo ello, el objetivo de nuestro estudio ha sido el diseñar un método basado en PCR pangenotípica para la detección y cuantificación de ARN del VHE para todo el espectro de genotipos descritos.

Los análisis para determinar las condiciones óptimas y el rendimiento de este método se llevaron a cabo mediante el uso del virus estándar de la Organización Mundial de la Salud (OMS) (6219/10) así como el panel VHE de la OMS (8578/13). Del mismo modo, se realizaron comparaciones de rendimiento de nuestro nuevo método con otros dos métodos comerciales para detectar el ARN del VHE en concentraciones inferiores a 1.000 UI/ml del panel viral de la OMS. Nuestro método demostró mayor sensibilidad que los dos kits comerciales con los que fue comparado (sensibilidad de 100% en comparación con 71,4% [ampliCube 2.0.] y 80.9% [RealStar 2.0.]). Posteriormente realizamos la validación clínica de nuestro método frente a los kits comerciales mediante el análisis de 54 muestras de suero de pacientes con hepatitis aguda comparando la sensibilidad y la especificidad. El método de PCR presentado en este estudio fue capaz de detectar todo el espectro de genotipos descritos pertenecientes a la especie *Orthohepevirus* A, mostrando un mejor rendimiento que los dos kits comerciales. Por tanto, este procedimiento puede significar una mejora significativa en el diagnóstico molecular de la infección por VHE.

Objetivo 2: Optimizar el algoritmo diagnóstico de la infección aguda por el VHE incluyendo la determinación serológica y molecular. Para llevar a cabo nuestro objetivo, realizamos un estudio prospectivo en el que se incluyeron pacientes con sospecha de infección por VHE, definidos como individuos con hepatitis aguda que mostraban resultados negativos para los marcadores serológicos y moleculares de otros virus hepatotropos. Todos los pacientes fueron evaluados para la infección por el VHE, incluyendo análisis de anticuerpos IgM y ARN viral. La infección por el VHE se definió como la positividad de cualquiera de estos marcadores. Se incluyeron 182 pacientes, de los cuales 68 (37,4%) fueron diagnosticados de infección por VHE. De estos, 29 (42,6%) fueron positivos tanto para IgM como para ARN del VHE, 25 (36,8%) fueron positivos sólo para anticuerpos IgM, y 14 (20,6%) fueron positivos sólo para ARN del VHE. Considerando sólo los individuos que fueron positivos a los anticuerpos IgM, se pudieron identificar 54 de los 68 casos (79,4%), mostrando un porcentaje de individuos falsos negativos del 20,6%. En conclusión, el

algoritmo de diagnóstico de la infección por VHE en pacientes con hepatitis aguda debería incluir la determinación tanto de anticuerpos IgM como del ARN viral en suero, ya que la toma de muestras única para la determinación de anticuerpos IgM condujo a una proporción importante de pacientes falsos negativos.

Objetivo 3: Evaluar si los pacientes infectados por el VIH constituyen un grupo de riesgo de infección por el VHE. No está establecido si las personas infectadas con el virus de la inmunodeficiencia humana (VIH) son más susceptibles a la infección por VHE que las personas no infectadas por el VIH. Por esta razón, realizamos una revisión sistemática y metaanálisis que incluían estudios que evaluaban la prevalencia del VHE en esta población, comparándola con individuos no infectados. El análisis incluyó estudios que comparaban pacientes que vivían con el VIH y personas VIH negativas, emparejados por edad, género y área geográfica. Encontramos que las personas infectadas por el VIH no muestran un mayor riesgo para la infección por el VHE, obteniendo una Odds Ratio (OR) de 0,87 (IC del 95%: 0,74-1,03) para efectos fijos, y 0,88 (IC del 95%: 0,70-1,11) para efectos aleatorios (I^2 = 47%). De este trabajo concluimos que, en base a los resultados obtenidos, no existen argumentos que recomienden que pacientes infectados por el VIH deban tomar medidas preventivas adicionales a las recomendadas para la población general.

Objetivo 4: Evaluar la influencia del gen PROGINS sobre la susceptibilidad y el curso clínico de la infección por VHE. Diseñamos un estudio prospectivo que incluyó tres grupos de pacientes categorizados según el patrón serológico y molecular del VHE: (i) nunca infectados (anticuerpos IgG e IgM así como ARN viral negativos), (ii) infección pasada (anticuerpos IgG positivos, y negativo para IgM y ARN viral), e (iii) infección aguda (anticuerpos IgM positivos y/o ARN viral detectable). Determinamos el genotipo del PR clasificando a los pacientes como homocigotos wild-type, heterocigotos y homocigotos PROGINS. En los pacientes con infección reciente, se recogieron los síntomas clínicos y alteraciones analíticas. En este estudio se incluyeron 311 pacientes infectados por el VIH. De éstos, 198 eran homocigotos para el genotipo wild-type (63,7%), 91 eran heterocigotos

(29,3%) y 22 eran homocigotos PROGINS (7,1%). En nuestro estudio encontramos que el genotipo homocigótico PROGINS en mujeres se asoció con una menor seroprevalencia del VHE (100% vs. 0%; p < 0,001). Además, ninguno de los pacientes con una infección reciente por VHE con genotipo homocigoto para el gen PROGINS presentó síntomas (100% vs. 0%; p < 0,001). De este trabajo concluimos que la mutación PROGINS juega un papel protector frente a la infección por VHE y se asocia con la infección subclínica en pacientes infectados por el VIH, especialmente en mujeres.

Objetivo 5: Evaluar los factores de riesgo asociados a la infección activa por el VHE en cerdos criados en diferentes sistemas de producción. En este estudio se evaluaron los factores de riesgo asociados con la infección por el VHE en el principal reservorio animal, los cerdos. La identificación de los factores de riesgo asociados con la infección por VHE en esta especie podría ayudar a determinar estrategias de contingencia para minimizar el riesgo de transmisión a los humanos. Se diseñó un estudio prospectivo en el que se incluyeron 1.040 cerdos procedentes de 26 explotaciones de la provincia de Córdoba. La variable desenlace del estudio fue carga viral detectable del VHE en sangre periférica. Se calculó la prevalencia de la infección por VHE en la población global del estudio, así como por diferentes características basales relacionadas con el animal, y variables higiénico sanitarias relacionadas con la explotación de origen. Los factores asociados con la infección por VHE se analizaron mediante análisis multivariante. Ciento setenta y dos cerdos presentaban infección por VHE, resultando una prevalencia del 16,5% (IC del 95%: 14,4% - 18,9%). Los factores asociados con una mayor prevalencia de la infección por VHE fueron: agricultura extensiva [23,9%; OR = 2.239 (1.036–4.837)], ausencia de vado sanitario [33,8%; OR = 3,597 (1,649–7,850)], no usar cuarentena en la reposición de los animales [20,8%; OR = 2.723 (1.450–5.112)], y contacto con otras especies domésticas simpátricas [24,5%; OR = 3,893 (1,453-10,431)]. Nuestro estudio identifica por primera vez el impacto negativo que la ausencia de ciertas medidas higiénico-sanitarias en las explotaciones porcinas en la tasa

de infección por el VHE, por lo que su corrección podría reducir el riesgo de infección por VHE en los cerdos, y, por lo tanto, minimizar el riesgo de transmisión zoonótica.

Summary

The main objective of this doctoral thesis project was to identify risk factors for hepatitis E virus (HEV) infection. Since HEV infection in our environment is zoonotic, the risk factors for infection should be assessed with a One Health approach, that is, by assessing risk factors in both the human and animal populations. To carry out the evaluation, a good diagnostic tool is essential to detect infected subjects, as is an optimized diagnostic algorithm to detect all infected patients. The first step of this doctoral thesis project was to develop and validate a molecular method for diagnosis of HEV infection with higher sensitivity and specificity than the molecular methods currently available. In the second step, the diagnostic algorithm for infection was optimized to minimize the number of false negative patients. The optimized diagnosis of HEV infection was then used to evaluate the risk factors for infection in human as well as animal populations. Five objectives were established, completion of which resulted in 5 scientific publications. These are summarized below:

Objective 1: To develop and clinically validate a molecular method capable of detecting all genotypes and subtypes of *Orthohepevirus* A species. Due to the considerable subtype variability that HEV currently shows, no PCR screening method has been designed to detect the broad spectrum of genotypes exhibited by this viral species. Nevertheless, implementation of an accurate method for the detection of all currently described HEV genotypes, as well as detection of unidentified subtypes, is necessary for both human and animal public health and constitutes the basis of a One Health approach to HEV infection. The objective of our study therefore was to design a PCR-based pangenotypic assay for the detection and quantification of HEV RNA for the entire spectrum of genotypes described. Testing to determine the optimal conditions and performance of this method was performed using the World Health Organization (WHO) standard strain (6219/10) as well as the WHO reference panel of HEV genotypes (8578/13). Comparisons of the performance of our new method against two other commercial methods were also

carried out, based on detection of HEV RNA at concentrations below 1,000 IU/ml of the WHO viral panel. Our method demonstrated higher sensitivity than the two commercial kits with which it was compared (100% sensitivity as against 71.4% [ampliCube 2.0.] and 80.9% [RealStar 2.0.]). Our method was then clinically validated against the commercial kits by testing 54 serum samples from patients with acute hepatitis and comparing sensitivity and specificity values. The PCR method presented in this study was able to detect the entire spectrum of described genotypes of *Orthohepevirus* A species and performed better than the two commercial kits. This procedure could therefore represent a significant improvement in the molecular diagnosis of HEV infection.

Objective 2: To optimize the diagnostic algorithm for acute HEV infection by including serological and molecular determinations. For this objective, we conducted a prospective study including patients with suspected HEV infection, defined as individuals with acute hepatitis who tested negative for the serological and molecular markers of other hepatotropic viruses. All patients were assessed for HEV infection using IgM antibody and viral RNA tests. HEV infection was defined as positivity for either of these markers. One hundred and eighty-two patients were included, 68 (37.4%) of whom were diagnosed with HEV infection. Of these, 29 (42.6%) were positive for both IgM and HEV RNA, 25 (36.8%) were positive only for IgM antibodies, and 14 (20.6%) only for HEV RNA. Focusing on those patients who were positive only for IgM antibodies, 54 out of 68 cases (79.4%) were identified, and the percentage of false negative patients was 20.6%. In conclusion, in patients with acute hepatitis, the diagnostic algorithm should include the determination of both IgM antibodies and viral RNA in serum, since single sampling for IgM antibody determination led to a substantial proportion of false negative patients.

Objective 3: To evaluate whether HIV-infected patients constitute a risk factor for HEV infection. It has not been established whether patients infected with the human immunodeficiency virus (HIV) are more susceptible to HEV infection than those who are not HIV-infected. Accordingly, we conducted a systematic review and meta-analysis including

studies assessing HEV prevalence in the HIV-infected population compared with uninfected individuals. The analysis included studies comparing patients living with HIV and HIV-negative individuals, matched by age, gender and geographical area. We found that patients infected with HIV do not show a greater risk for HEV infection, obtaining an odds ratio (OR) of 0.87 (95% CI: 0.74-1.03) in the fixed effects meta-analysis, and 0.88 (95% CI: 0.70-1.11) in random effects (I2= 47%). Based on the study findings, we concluded that there are no arguments to recommend that HIV-infected patients should adopt extra preventive measures relative to those recommended for the general population.

Objective 4: To evaluate the influence of the PROGINS gene on susceptibility and clinical course of HEV infection. We designed a prospective study that included three groups of patients categorized according to serological and molecular pattern of HEV: (i) never infected (negative for IgG and IgM antibodies and negative for HEV RNA); (ii) past infection (positive for IgG antibodies and negative for IgM and viral RNA), and (iii) acute infection (positive IgM antibodies and/or detectable viral RNA). PR genotype was determined, and patients were classified as homozygous wild type, heterozygous, or homozygous PROGINS. The clinical symptoms and analytical alterations in patients with recent infection were collected. This study included 311 HIV-infected patients: 198 had the homozygous wild-type genotype (63.7%), 91 were heterozygous (29.3%), and 22 were homozygous PROGINS (7.1%). Our study found that in women the homozygous PROGINS gene was associated with a lower seroprevalence of HEV. Furthermore, none of the patients with recent HEV infection who were homozygous for PROGINS showed symptoms (100% vs. 0%; $p < 0.001$). We concluded from this study that the PROGINS mutation has a protective role against HEV infection and is associated with subclinical infection in HEV-infected patients, especially women.

Objective 5: To assess the risk factors associated with active HEV infection in pigs reared in different production systems. This study evaluated the risk factors associated with HEV infection in the main animal reservoir, pigs. Identification of risk

factors associated with HEV infection in this species could help to establish contingency strategies to minimize the risk of transmission to humans. A prospective study was designed that included 1,040 pigs from 26 farms in the province of Cordoba. The outcome variable was detectable HEV viral load in peripheral blood. The prevalence of HEV was calculated for the overall study population, and for different baseline characteristics of the animals and hygiene and sanitation variables linked to the farm of origin. Factors associated with HEV infection were analyzed by multivariate analysis. One hundred and seventy-two pigs were infected with HEV, giving a prevalence of 16.5% (95% CI: 14.4% -18.9%). Factors associated with higher prevalence of HEV infection were extensive farming [23.9%; OR = 2.239 (1.036-4. 837)], absence of a disinfection ford [33.8%; OR = 3.597 (1.649-7.850)], not applying quarantine for replacement stock [20.8%; OR = 2.723 (1.450-5.112)] and contact with other sympatric domestic species [24.5%; OR = 3.893 (1.453-10.431)]. Our study identifies for the first time the negative impact that the absence of certain hygiene / sanitation measures on pig farms has on the rate of HEV infection. Correcting these could reduce the risk of HEV infection in pigs and consequently minimize the risk of zoonotic transmission.

III. Introducción

1. Taxonomía del género *Orthohepevirus*

El Virus de la Hepatitis E (VHE) es un virus ARN monocatenario de sentido positivo (clase IV según clasificación de Baltimore). Su genoma tiene una longitud de 7,2 kb, presentando una caperuza en su extremo 5' y una cola poli (A) en el extremo 3' (Tam y col., 1991). Pertenece a la familia *Hepeviridae*, la cual se divide en dos géneros, *Orthohepevirus* y *Piscihepevirus* (*Purdy y col., 2017*). El género *Orthohepevirus* está formado por cuatro especies, A, B, C y D (**Figura 1**), que han demostrado tener capacidad infectiva a diferentes especies de mamíferos y aves (Smith y col., 2015). El virus de la Hepatitis E es el único representante de la especie *Orthohepevirus* A, agente causal de la hepatitis E (Kamar y col., 2017). La especie *Orthohepevirus* B se ha detectado en aves, la especie *Orthohepevirus* C en roedores y mustélidos, mientras que el *Orthohepevirus* D se ha identificado en quirópteros (Kamar y col., 2017; Purdy y col., 2017). Pese a que estas especies no estaban consideradas como zoonóticas, recientemente se han descrito varios casos de infección por *Orthohepevirus* C en seres humanos en Hong-Kong (Sridhar y col., 2018; Andonov y col., 2019; Sridhar y col., 2021). Sin embargo, se desconoce su verdadera dimensión e impacto a nivel mundial.

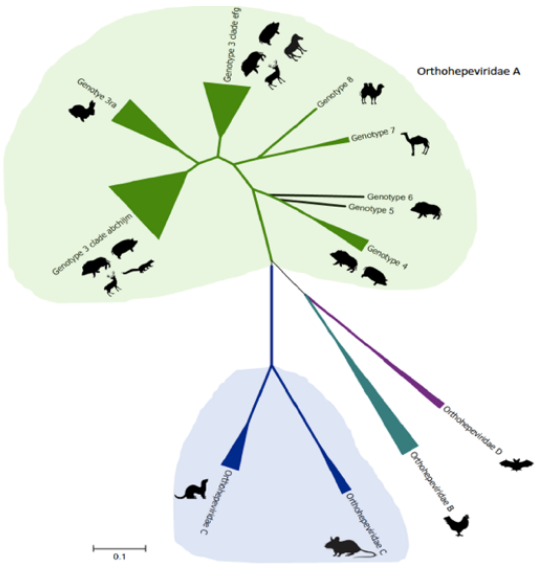

Figura 1. Árbol filogenético de las especies pertenecientes al género *Orthohepevirus* (Figura realizada por Pedro López López y Antonio Rivero Juárez).

2. Epidemiología del VHE

Se estima que cada año se producen unos 20 millones de casos de infección por el VHE, de los cuales 3,3 millones presentan una hepatitis aguda. La OMS estima que en 2015 se produjeron unas 44.000 muertes asociadas al VHE, que representan un 3,3% de la mortalidad por las hepatitis víricas a nivel Mundial (World Health Organization, 2017).

Existen ocho genotipos diferentes del VHE (del 1 al 8), los cuales poseen características particulares respecto a la distribución geográfica (**Figura 2**) y hospedadores (Wang y col., 2021). Las infecciones pueden tener un carácter epidémico o endémico, dependiendo del genotipo involucrado.

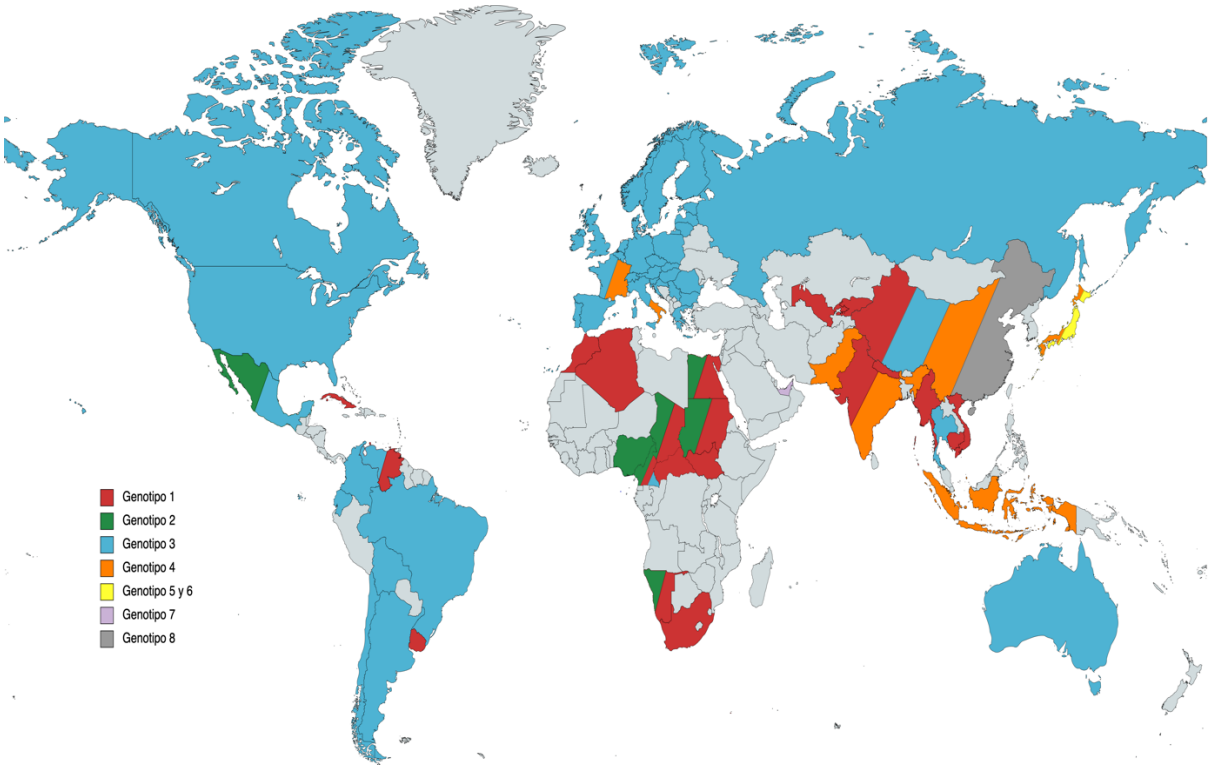

Figura 2. Distribución geográfica donde se han identificado los distintos genotipos del VHE. (Figura realizada por Pedro López López).

2.1. Genotipos 1 y 2

Se ha descrito la presencia del genotipo 1 del VHE en Asia y África subsahariana, mientras que el genotipo 2 se ha descrito en África y México, ambos produciendo grandes brotes epidémicos (Nelson y col., 2019) (**Figura 2**). Los brotes causados por estos genotipos están relacionados con el consumo de agua contaminada (Nelson y col., 2019). Con frecuencia se han notificado grandes brotes en países del sudeste asiático asociados a épocas de lluvias y monzones (Nelson y col., 2019). El mayor brote documentado por genotipo 1 del VHE se produjo en la India entre los años 1986 y 1988, registrándose más de 120.000 casos y 765 muertes (Wang y col., 1989). Del mismo modo, se han registrado numerosos brotes en países africanos, asociados al consumo de agua procedente de depósitos de agua contaminados y en campos de refugiados (Kim y col., 2014). Por otro lado, se han notificado grandes epidemias durante la estación seca, asociadas al consumo de agua contaminada debido a tuberías rotas, ríos o pozos poco profundos sin tratar, así como fallos de potabilización del agua en las propias plantas de tratamiento y suministro (Hakim y col., 2017; Junaid y col., 2014). Por su parte, los primeros brotes epidémicos producidos por el genotipo 2 se identificaron a finales de 1986 en Huitzililla y Telixtac (México) (Velázquez y col., 1990). Desde entonces, se han registrados numerosos brotes en países africanos, donde las secuencias víricas aisladas presentaban una alta homología con las secuencias identificadas en México (Buisson y col., 2000; Escriba y col., 2008). Pese a que la principal vía de transmisión es el consumo de aguda contaminada, se ha descrito la transmisión persona a persona. De esta forma, se registró un gran brote epidémico en Uganda no asociado al consumo de agua, sino al contacto estrecho con personas infectadas (Teshale y col., 2010). Además, se ha demostrado una alta eficiencia en la transmisión vertical, estimándose una tasa de transmisión superior al 70% (Khuroo y col., 2009), presentando una alta mortalidad en mujeres embarazadas, debido al desarrollo de hepatitis fulminante, y neonatos. Se estima que se producen al año alrededor de 3.000 abortos asociados a estos genotipos del VHE en países en desarrollo (Khuroo y col., 2009)(**Figura 3**).

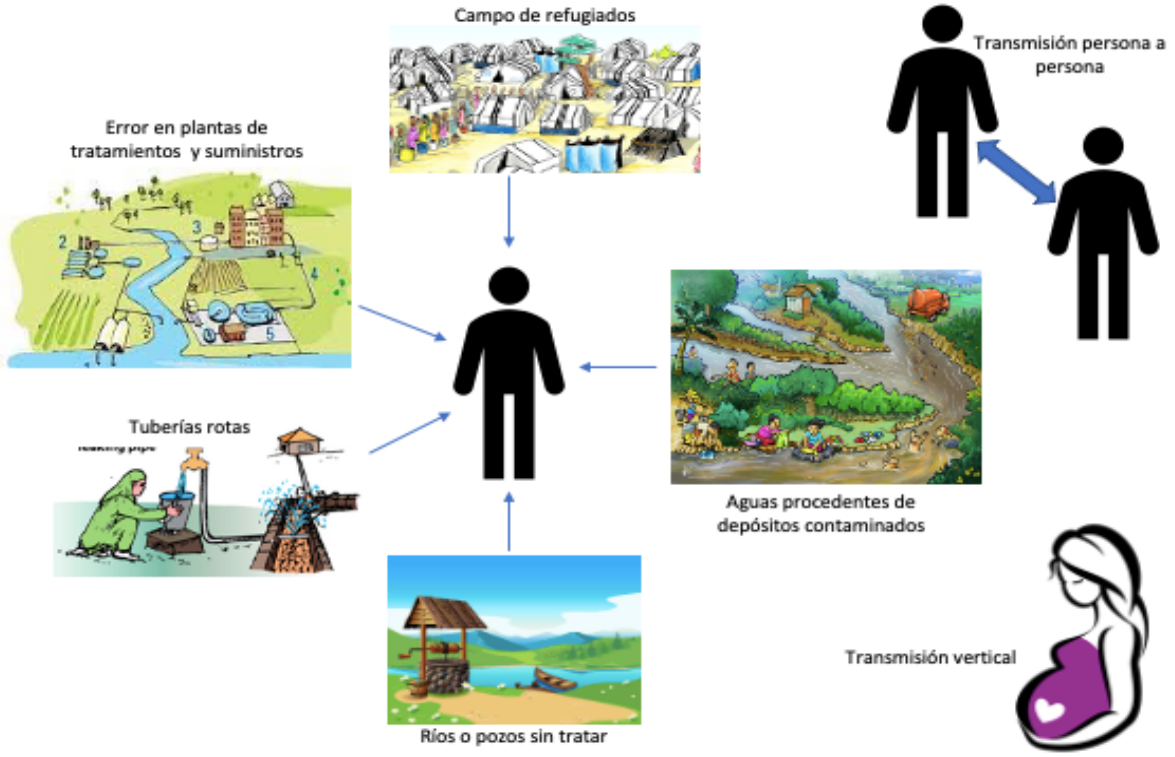

Figura 3. Vías de transmisión de los genotipos 1 y 2 del VHE (Figura adaptada por Pedro López López de Khuroo y col. 2016).

Se han estudiado diferentes factores de riesgo, así como prácticas que podrían estar asociadas con el aumento de las infecciones por estos genotipos. En primer lugar, se han sugerido como factores de riesgo variables relacionadas con el individuo, como la edad y el sexo. En este sentido, Labrique y col. (2009) llevó a cabo un estudio prospectivo con el objetivo de determinar los factores de riesgo del VHE durante varias estaciones y condiciones climáticas. En este estudio se incluyeron 1.134 sujetos de zonas rurales de Bangladesh. La seroprevalencia basal del VHE fue del 22,5%, y tras un seguimiento de 12 meses se detectaron 49 seroconversiones, lo que supuso una tasa de incidencia del 60,3 por 1.000 personas-año, observándose una menor tasa de incidencia en niños con edades por debajo de 10 años (28,9 por 1000 personas-año) (Labrique y col., 2009, 2010). Pese a que se desconoce la razón exacta de esta menor tasa de infección, se ha sugerido que en esta población puede estar relacionada con el nivel de exposición. En este sentido, Kmush y col. (2016) en otro estudio realizado en Bangladesh, encontraron que la exposición a dosis

infectivas del VHE en niños es menor debido a un menor consumo de agua contaminada. En contra de esto, se ha descrito que, en otro entorno, como Egipto, la población pediátrica presenta una mayor seroprevalencia contra el VHE comparado con esta misma población en el sur de Asia (Kamel y col., 1995; Darwish y col., 1996; Fix y col., 2000). Se desconocen las razones de estas diferencias entre las características clínicas y epidemiológicas de las infecciones agudas por VHE en las poblaciones africanas y asiáticas. En Egipto no se registran lluvias monzónicas con inundaciones, sin embargo, la exposición al agua altamente contaminada con VHE puede ser común en los primeros años de vida (Darwish y col., 1996; Fix y col., 2000). Por otro lado, pese a que se ha sugerido que el género puede ser un factor de riesgo para la infección, los resultados son contradictorios. Así, Corwin y col. (1997) encontraron una mayor prevalencia de VHE en mujeres que hombres en Indonesia (55% versus 47%, $p < 0.05$). Sin embargo, Junaid y col. (2014) detectaron una mayor prevalencia del VHE en hombres que mujeres en Nigeria. Sin embargo, otros estudios no han encontrado diferencias entre hombres y mujeres (Labrique y col., 2009, 2010; Takahashi y col., 2004). Esta disparidad podría estar más relacionada con el desarrollo de prácticas o interacciones con el medio más que con el género per se (Junaid y col., 2014).

Los factores y prácticas de riesgo más asociados con una mayor prevalencia del VHE están relacionados con la localización geográfica, así como con diferentes prácticas higiénicas. En este sentido, Junaid *y col*. (2014) detectaron que las zonas rurales presentan mayor número de residentes con anticuerpos IgG que en zonas urbanas (57,1% vs. 36,1%; $p < 0,001$), así como que las regiones del sur de Nigeria presentaban mayor prevalencia de IgG que las regiones del norte (63% vs. 40%; $p < 0,001$). Este gradiente de prevalencia puede ser explicado por la mayor concentración de zonas rurales en el sur del país (Junaid y col., 2014). Estas diferencias entre zonas residenciales se han observado en otros países como Indonesia, China o Egipto (Wibawa y col., 2004; Stoszek y col., 2006; Dong y col., 2007). Adicionalmente, en Nigeria se ha descrito que las personas de hogares donde se han dado más de 2 casos de ictérica, el riesgo de infección del VHE aumenta debido a la transmisión

persona a persona (Teshale y col., 2010). En este sentido, Teshale y col. (2010) describieron un brote de VHE en Madi Opeia (Nigeria) asociado a la asistencia de un funeral de un individuo con ictericia. Posteriormente, se llevó a cabo un estudio de casos y controles, donde se identificó que un deficiente saneamiento, y el uso compartido de recipientes con agua, ya sea de almacenaje (OR= 2,8; 95% IC = 1,16–6,94) o para lavarse las manos (OR= 1,9; 95% IC = 1,07–3,38), se asoció con la transmisión del VHE (Howard y col., 2010). Por otro lado, ciertas profesiones están asociadas con un aumento de la seroprevalencia del VHE, como son las relacionadas con la agricultura, donde el uso de aguas contaminadas para el riego supone un riesgo de infección para los trabajadores (Junaid y col., 2014). Además, las malas condiciones sanitarias ambientales en las fincas junto con el nivel socioeconómico suponen potenciales factores de riesgo. Defecar en campo abierto es una práctica poco higiénica que con el paso del tiempo aumenta la contaminación del terreno y, por consecuencia, el riesgo de contraer infecciones, entre ellas el VHE (Junaid y col., 2014). En este sentido, no usar el inodoro, ni llevar a cabo medidas higiénicas individuales básicas como el lavado de manos y el uso de jabón tras defecar, son prácticas de riesgo para la infección por el VHE (Eker y col., 2008; Junaid y col., 2014; Teshale y col., 2010). Por otro lado, consumir vegetales o frutas sin un adecuado lavado supone otro importante factor de riesgo (Junaid y col., 2014). En este estudio, Junaid y col. (2014) encuentra un mayor porcentaje de personas con anticuerpos IgG frente al VHE que no lavaban las frutas y verduras adecuadamente antes de consumirlas, mientras que en aquellas que si lo hacían disminuía considerablemente la seroprevalencia (del 60,3% al 30,3%; $p < 0,001$). En este sentido, en otro estudio llevado a acabo en Edirne (Turquía) se detectó que las personas que consumían frutas y verduras sin un adecuado lavado antes de consumirlas presentaban una mayor tasa de prevalencia ($p = 0,015$) (Eker y col., 2009).

2.2. Genotipos 3 y 4

El principal reservorio de los genotipos 3 y 4 son los suidos, tanto cerdo doméstico (*Sus scrofa domesticus*) como jabalí (*Sus scrofa*) (Salines y col., 2017; Meng y col., 2009).

Estudios llevados a cabo en cerdos domésticos en Europa han observado que la seroprevalencia media global se sitúa en el 35% (Lange y col., 2017; Thiry y col., 2014), sin embargo, esta puede variar dependiendo de la región de estudio (**Tabla 1**). Por su parte, la prevalencia de infección activa global, definida como presencia de ARN viral en sangre periférica o heces, se sitúa en el 2,5% (**Tabla 2**). Respecto al jabalí, al igual que en cerdo doméstico, las prevalencias de anticuerpos (**Tabla 3**) y presencia del ARN viral pueden variar según el país de estudio, situándose la tasa de infección activa en estos animales entre el 2% y el 68% (Rivero-Juarez y col., 2018) (**Tabla 4**).

Tabla 1. Seroprevalencias de anticuerpos del VHE en cerdo doméstico (*Sus scrofa domesticus*) de países europeos.

Referencia	País	N	Prevalencia Anticuerpos
Takova y col., 2020	Bulgaria	433	60%
Tsachev y col., 2019	Bulgaria	360	60,3%
Tsachev y col., 2020	Bulgaria	171	82,5%
Crossan y col., 2015	Escocia	108	61,4%
Kukielka y col., 2016	España	48	43,8%
García y col., 2021	España	45	73,3%
Feurer y col., 2018	Francia	1.034	60%
Jori y col., 2016	Francia	208	87,9%
Hickey y col., 2016	Irlanda	198	8%
Mughini-Gras y col., 2017	Italia	2.700	41,1%
Caruso y col., 2017	Italia	879	50%
Costanzo y col., 2015	Italia	216	80%
Pavia y col., 2021	Italia	692	56,8 %
Lange y col., 2017	Noruega	153	90%
Grierson y col., 2013	Reino Unido	629	92,8%
Kureljušić y col., 2020	Serbia	150	55,3%
Kelbert y col., 2021	Suecia	376	71,8%

Leyenda: colaboradores (.col); número de individuos (N).

Tabla 2. Prevalencias de ARN del VHE en cerdo doméstico (*Sus scrofa domesticus*) de países europeos.

Referencia	País	Muestra	N	Prevalencia ARN
Jäckel y col., 2016	Alemania	Hígado	115	6,9%
Jemeršić y col., 2019	Croacia	Sangre	1.419	15,2%
		Heces		
Prpić y col., 2015	Croacia	Sangre	848	12,3%
		Bazo		
		Hígado		
Crossan y col., 2015	Escocia	Suero	162	44,4%
Jackova y col., 2021	Eslovaquia	Heces	388	13,7%
Raspor y col., 2017	Eslovenia	Heces	811	5,4%
		Hígado	811	4,9%
		Bilis	811	5,3%
García y col., 2021	España	Hígado	270	9,6%
		Heces		
		Riñón		
		Corazón		
		Suero		
		Diafragma		
Kukielka y col., 2016	España	Suero	48	0%
Rivero-Juarez y col., 2020	España	Suero	328	16,8%
		Heces	328	9,1%
Kantala y col., 2015	Finlandia	Heces	67	22,4%
Jori y col., 2016	Francia	Suero	24	8,3%
Feurer y col., 2018	Francia	Hígado	1.034	2,8%
		Musculo	1.034	0%
Capai y col., 2019	Francia	Heces	919	9,2%
Colson y col., 2015	Francia	Heces	50	68%
Fenaux y col., 2018	Francia	Heces	22	0%
Costanzo y col., 2015	Italia	Heces	216	7,4%
Pavia y col., 2021	Italia	Heces	67	10,6 %
Caruso y col., 2017	Italia	Heces	246	16,3% Destete
			258	6% Cerdas
Chelli y col., 2021	Italia	Hígado	585	2,1%
		Heces	569	1,9%
Ianiro y col., 2021	Italia	Heces	281	23,8%
La Rosa y col., 2017	Italia	Heces	24	75%
Monini y col., 2015	Italia	Heces	242	18,6%
Spancerniene y col., 2018	Lituania	Suero	470	32,9% ORF1

				22,6% ORF2
Grierson y col., 2013	Reino Unido	Heces	629	15%
		Plasma	629	3%
Milojević y col., 2019	Serbia	Hígado	330	15,5%
		Heces	15	53,3%
Wang y col., 2019	Suecia	Heces	363	22%

Leyenda: colaboradores (.col); número de individuos (N); ácido desoxirribonucleico (ARN); Open Reading Frame (ORF).

Tabla 3. Seroprevalencias de anticuerpos del VHE en jabalíes (*Sus scrofa*) de países europeos.

Referencia	País	N	Prevalencia Anticuerpos
Weigand y col., 2018	Alemania	104	11,5%
Anheyer-Behmenburg y col., 2017	Alemania	48	27,1% Estación 1
		132	51,5% Estación 2
Thiry y col., 2017	Bélgica	383	34%
Žele y col., 2016	Eslovenia	288	30,2%
Caballero-Gómez y col., 2019	España	99	57,6%
Kukielka y col., 2016	España	108	57,4%
Jori y col., 2016	Francia	346	29%
Caruso y col., 2015	Italia	594	4,9 %
Martinelli y col., 2015	Italia	2.211	10,2%
Dorn-In y col., 2017	Polonia	163	17,2%
Kozyra y col., 2020	Polonia	470	49,4%
Strakova y col., 2018	República Checa	366	8,5%

Leyenda: colaboradores (.col); número de individuos (N).

Tabla 4. Prevalencias de ARN del VHE en jabalíes (*Sus scrofa*) de países europeos.

Referencia	País	Muestra	N	Prevalencia ARN
Weigand y col., 2018	Alemania	Suero	104	3,8%
Anheyer-Behmenburg y col., 2017	Alemania	Suero	95	6,3% Estación 1
			137	24,1% Estación 2
Thiry y col., 2017	Bélgica	Suero	69	5,8%
		Hígado	61	6,5%
Jemeršić y col., 2019	Croacia	Sangre/Heces	720	15,2%
		Sangre/Tejido	720	11,5%

Prpić y col., 2015	Croacia	Sangre	536	24,5%
		Bazo	536	24,5%
		Hígado	536	24,5%
Žele y col., 2016	Eslovenia	Suero	288	0,4%
Caballero-Gómez y col., 2019	España	Suero	99	1%
Kukielka y col., 2016	España	Suero	158	10,1%
Rivero-Juarez y col., 2020	España	Suero	328	16,8%
		Heces	328	9,1%
Wang y col., 2019	España	Suero	190	16%
		Heces	207	12%
		Global	264	20%
Rivero-Juarez y col., 2018	España	Suero	142	23%
Jori y col., 2016	Francia	Suero	352	2,3%
Fenaux y col., 2018	Francia	Hígado	20	10%
		Heces	19	5,3%
Lhomme y col., 2015	Francia	Hígado	106	5,8%
Caruso y col., 2015	Italia	Suero	594	0%
		Hígado	320	3,7%
Martinelli y col., 2015	Italia	Hígado	40	0%
		Suero	37	0%
Bonardi y col., 2020	Italia	Hígado	73	31,5%
Montagnaro y col., 2015	Italia	Hígado	164	33,5%
De Sabato y col., 2018	Italia	Hígado	92	52,2%
Aprea y col., 2018	Italia	Hígado	291	14%
Serracca y col., 2015	Italia	Hígado	372	1,90%
Di Pasquale y col., 2019	Italia	Hígado	332	16,30%
Di Profio y col., 2016	Italia	Hígado	196	1,50%
Spancerniene y col., 2018	Lituania	Suero	505	25,9% ORF1
		Hígado	505	17,0% ORF2
Dorn-In y col., 2017	Polonia	Suero	216	36,8%
		Heces	216	36,8%
Kozyra y col., 2020	Polonia	Hígado	470	12,1%
		Heces	433	6,2%
Mesquita y col., 2014	Portugal	Hígado	80	25%
		Heces	40	10%
Strakova y col., 2018	República Checa	Suero	31	9,70%
Porea y col., 2018	Rumania	Hígado	45	15,50%
		Bazo	5	40%
		Global	50	18%
Wang y col., 2019	Suiza	Suero	134	9%
		Heces	134	7%
		Global	134	15%

Leyenda: colaboradores (.col); número de individuos (N); ácido desoxirribonucleico (ARN);

Open Reading Frame (ORF).

Aunque los principales reservorios animales son los suidos, se han identificado un amplio número de especies de mamíferos que pueden actuar tanto como reservorios como hospedadores (**Figura 4**). Se ha detectado la presencia del VHE en cérvidos, como el ciervo (*Cervus elaphus*) o el corzo (*Capreolus capreolus*). En ciervos, la prevalencia es variable en función del país de origen. En este sentido, en Alemania la prevalencia de infección activa se sitúa entre el 0 y el 3,45%, dependiendo de la estación de caza analizada (Anheyer-Behmenburg y col., 2017), mientras que en Italia se ha establecido la prevalencia en el 11% (Di Bartolo y col., 2017). También se ha observado la presencia del VHE en otras especies de artiodáctilos como cabras (*Capra hircus aegagrus*) y ovejas (*Ovis aries orientalis)* (Peralta y col., 2009), observándose una prevalencia de infección menor a la observada en cérvidos (Kukielka y col., 2016; Di Bartolo y col., 2017). Otro reservorio del VHE son los lagomorfos, como el conejo (*Oryctologus cuniculus*) y la liebre (*Lepus europaeus*), en los que se ha identificado la presencia del virus en un amplio número de países (Hammerschmidt y col., 2017; Izopet y col., 2012; Cossaboom y col., 2011). Por otro lado, otros potenciales hospedadores del VHE son los équidos, como el caballo (*Equus caballus ferus*), el burro (*Equus asinus*) y las mulas (*Equus asinus* × *Equus caballus*) (García-Bocanegra y col., 2019; Saad y col., 2007), detectándose una prevalencia global de infección activa del 0,8%. Otros estudios han evaluado la presencia del anticuerpos anti-VHE en animales de compañía, como perros (*Canis familiaris*) (Veronesi y col., 2021; Liang y col., 2014), y gatos (*Felis silvestris domesticus*) (Liang y col., 2014). Sin embargo, no hay estudios en los que se haya conseguido detectar infección activa en estas especies (Liang y col., 2014). Otras especies silvestres identificadas como hospedadores del VHE, pero con un papel más residual en la epidemiología del virus y por su capacidad de transmisión al ser humano son el delfín (*Tursiops truncatus*), los primates (Caballero-Gómez y col., 2019), y otras especies del orden Carnivora (Montalvo-Villalba y col., 2017; Nidaira y col., 2012; Li y col., 2016).

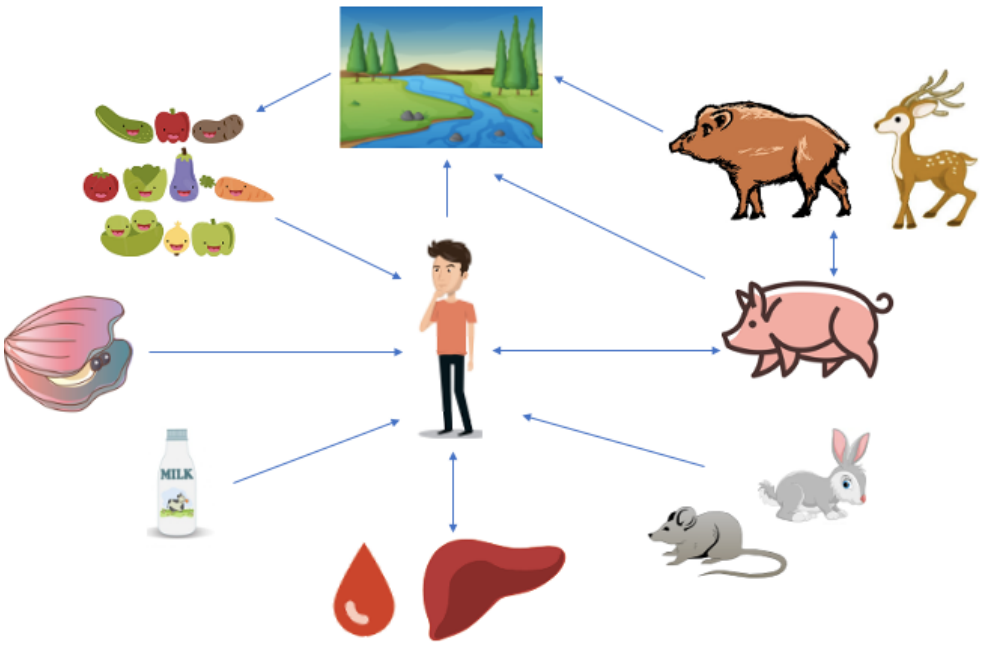

Figura 4. Principales vías de transmisión de los genotipos 3 y 4 del VHE (Figura realizada por Pedro López López y Antonio Rivero Juárez).

Mientras que el genotipo 3 tiene una distribución global (Dalton y col., 2018), el genotipo 4 se encuentra principalmente en países del sureste asiático (Lu y col., 2006) (**Figura 2**). Sin embargo, se ha detectado en los últimos años la presencia del genotipo 4 en suidos de países europeos (Hakze-van der Honing y col., 2011; Colson y col., 2012a; Garbuglia y col., 2013). El primer aislamiento del genotipo 4 del VHE en Europa fue en Países Bajos y Bélgica, como parte del proceso de vigilancia biosanitaria de explotaciones porcinas (Hakze-van der Honing y col., 2011). Posteriormente, se detectó este genotipo en un estudio realizado en Italia entre los años 2012 y 2013 (Monne y col., 2015). Del mismo modo, se han identificado casos en humanos por este genotipo en Europa. En este sentido, en un estudio caso-control se detectó el primer caso de infección autóctona por genotipo 4 del VHE en Alemania (Wichmann y col., 2008). De igual manera, en 2009 se detectó el primer caso autóctono de hepatitis E por genotipo 4 en Francia (Tessé y col., 2012), y posteriormente se detectaron 5 casos más, dos en ese mismo año, observándose que las cepas detectadas presentaban alta homología con secuencias de cerdos de Bélgica (Colson y col., 2012a), y tres casos más entre 2011 y 2012 (Colson y col., 2012b). También en Italia

se han identificado 5 casos de infección por genotipo 4 (Garbuglia y col., 2013). Por todo ello, el genotipo 4 puede ser considerado como emergente en Europa.

Dado su amplia circulación en diferentes especies animales destinadas al consumo humano, la principal vía de transmisión de estos genotipos es el consumo de productos crudos o poco cocinados derivados de animales infectados (Kamar y col., 2014) (**Figura 4**). En Asia se han descrito brotes a consecuencia del consumo de carne de jabalí asada (Li y col., 2005), y carne de ciervo cruda (Tei y col., 2003). Por su parte, en Europa se han descritos numerosos brotes asociados al consumo de carne de cerdo y jabalí procesada de diversas formas, ya sea, asada (Guillois y col., 2016), cocinada (Rivero-Juarez y col., 2017), o productos curados (Renou y col., 2014; Colson y col., 2012a; Garbuglia y col., 2015). Además, en Reino Unido se ha demostrado la transmisión por consumo de salchichas (Said y col., 2014). En este sentido, otros alimentos procesados de venta directa al público han sido identificados como posibles vías de transmisión del VHE, como, por ejemplo, el bacon, el paté, u otros productos de origen porcino (Harrison y col., 2020; Smith y col., 2021). Por otro lado, se ha detectado el VHE en otros productos destinados al consumo humano, diferentes a los derivados cárnicos, como la leche (Huang y col., 2016; Long y col., 2017), sugiriendo que podría ser una vía de transmisión de la infección (Rivero-Juarez y col., 2016) (**Figura 4**). En este sentido, un estudio experimental in vivo demostró la capacidad de transmisión del virus a través de leche cruda o pasteurizada a baja temperatura (Huang y col., 2016). Por lo tanto, dada la alta prevalencia en población animal, y la eficiencia de la transmisión por consumo de productos derivados, el VHE es un importante problema de Salud Pública.

Otras vías de transmisión del VHE no relacionadas con el consumo de productos de origen animal, son las transfusiones de hemoderivados y el trasplante de órgano sólido (Gallian y col., 2019; Lhome y col., 2017) (**Figura 4**). En este sentido, se ha detectado el ARN del VHE en donantes de sangre en gran cantidad de países de Europa, estableciéndose la prevalencia en 1 donación por cada 2.500 (Baylis y col., 2012; Hogema y col., 2012;

Harritshøj y col., 2016; Rivero-Juarez y col., 2019; Mansuy y col., 2016; Gallian y col., 2014; O'Riordan y col., 2016; Hewitt y col., 2014). De igual forma, se ha demostrado la transmisión de la infección por trasplante de órgano sólido, principalmente hígado (Lhome y col., 2017; Protzer y col., 2014; Schlosser y col., 2012; Halleux y col., 2012). Por ello, los receptores de hemoderivados y de trasplante de órgano sólido constituyen una población de riesgo.

Por último, otras vías de transmisión de estos genotipos podrían ser el consumo de moluscos, dado que se ha identificado ARN viral en bivalvos de diferentes países europeos (Mesquita y col., 2016a; Rivadulla y col., 2019; O'Hara y col., 2018) (**Figura 4**). En este sentido, en un crucero se asoció un brote de hepatitis aguda por genotipo 3 al consumo de marisco (Said y col., 2009). Por otro lado, se ha identificado la presencia del virus en la superficie de frutas y verduras (Brassard y col., 2012; Kokkinos y col., 2012; Maunula y col., 2013; Doceul y col., 2016), por lo que podría ser otra vía de transmisión. Sin embargo, la eficiencia de transmisión por el consumo de estos alimentos no ha sido evaluada.

2.2.1. Factores de riesgo asociados a la infección en humanos

Los principales factores de riesgo asociados a la infección por los genotipos 3 y 4 del VHE son el consumo de carne cruda o poco cocinada. En este sentido, se ha observado un mayor riesgo de infección para el VHE por el consumo de carne de cerdo (OR = 3; IC 95% 1,4–6,5) y jabalí (OR = 3,7; IC 95% 1,1–12,4), así como el consumo de hígado de cerdo (OR = 5,3; IC 95% 1,8–15,7) en un estudio caso-control realizado en Alemania (Faber y col., 2018). De igual manera, el consumo de productos derivados del hígado (paté y figatelli) (OR = 2,1; IC 95% 1,5–3) como productos cárnicos cocidos (salchichas) (OR = 1,9; IC 95% 1,3–2,7) se han identificado como factores de riesgo (Faber y col., 2018). En cuanto a alimentos que no tienen un origen animal, el consumo de vegetales crudos también se ha identificado como factor de riesgo (OR = 1,9; IC 95% 1,3–2,9) (Faber y col., 2018). En este sentido, un estudio llevado a cabo en Italia detectó el VHE en 6 de 911 (0,6%) preparados de ensalada (Terio y col., 2017). De igual forma, otro estudio ha detectado la presencia del VHE en 1

de 38 (2,6%) muestras de frambuesas congeladas recogidas en bolsa estéril (Maunula y col., 2013).

Se han identificado varios factores ocupacionales y relacionados con el lugar de residencia como factores de riesgo de la infección por el VHE. En este sentido, las personas con profesiones en las que existe un contacto estrecho con animales, como veterinarios o manipuladores de cerdos, presentan una seroprevalencia del VHE mayor que otras profesiones (Kamar y col., 2014; Galiana y col., 2008; Huang y col., 2019; Teixeira y col., 2017). En este sentido, se han reportado dos casos de veterinarios que se infectaron por contacto estrecho con cerdos en Francia, uno por contacto frecuente con un cerdo mascota (Renou y col., 2007), y otro durante una cirugía (Colson y col., 2007). En esta misma línea, también se ha evidenciado una mayor seroprevalencia en cazadores y trabajadores forestales en diferentes países europeos (Doceul y col., 2016). Esto puede estar relacionado con que vivir en el medio rural se haya identificado también como factor de riesgo para la infección por el VHE (Rivero-Juarez y col., 2017). Por último, se ha descrito el área geográfica como factor de riesgo en Francia, identificando las regiones situadas en el Sur del país como zonas de alto riesgo (Mansuy y col., 2011). Sin embargo, se desconocen las razones por las que se produce este gradiente Norte-Sur.

Por otro lado, variables demográficas como la edad y el género se han identificado como factores de riesgo, como se ha comentado para los genotipos 1 y 2. En primer lugar, se ha observado que la seroprevalencia del VHE aumenta con la edad (Carpentier y col., 2012; Krumbholz y col., 2012; Harrison y col., 2013; Verhoef y col., 2012; Meader y col., 2010). En este sentido, en el 2º Estudio Nacional de Seroprevalencia en España (Ministerio de Sanidad, 2020) se observa una tendencia ascendente en la prevalencia de anticuerpos IgG frente al VHE a medida que aumenta la edad, de tal manera que en menores de 20 años la seroprevalencia es inferior al 5%, mientras que en población mayor de 70 años es del 38,6% (Ministerio de Sanidad, 2020). En segundo lugar, también se ha identificado que los hombres presentan una mayor seroprevalencia que las mujeres (Zeng y col., 2017; Boon y

col., 2018; Gérardin y col., 2019). En este sentido, en el 2º Estudio Nacional de Seroprevalencia en España también se observa una mayor seroprevalencia en hombres que en mujeres, sobre todo a partir del grupo de edad de 30-39 años (Ministerio de Sanidad, 2020).

También se han identificado algunas comorbilidades y procesos hospitalarios como factores de riesgo para la infección por el VHE. Sin embargo, su consideración es controvertida. En primer lugar, en referencia a procesos hospitalarios, se ha especulado que recibir un alto número de transfusiones iguala el riesgo de transmisión del VHE por esta vía al riesgo por consumo de carne de cerdo cruda o poco cocinada (Tedder y col., 2017). En el mismo sentido, se ha identificado la hemodiálisis como una práctica de riesgo de infección por el VHE, al observarse mayor prevalencia en esta población que en población general (Haffar y col., 2017). Sin embargo, estos estudios no han sido replicados. En segundo lugar, comorbilidades como la diabetes o la cirrosis hepática se han identificado como factores de riesgo (Kumar y col., 2007; Chen y col., 2015; Stepanova y col., 2017). Por otro lado, existen estudios que identifican la infección VIH como un factor asociado a una mayor seroprevalencia del VHE (Rapicetta y col., 2013; Riveiro-Barciela y col., 2014; Shrestha y col., 2017). Este hecho puede estar relacionado con la alta proporción de hombres que tienen sexo con hombres (HSH) en esta población, sugerido como práctica de riesgo de infección del VHE (Payne y col., 2013; Lanini y col., 2015; Greco y col., 2018). Sin embargo, otros estudios no han evidenciado que la infección por el VIH (Payne y col., 2013; Junaid y col., 2014; Taha y col., 2015; Madden y col., 2016) ni el ser HSH (Lin y col., 2019) sean factores de riesgo para el VHE. Debido a que la inmunosupresión derivada de la infección por el VIH es un factor de riesgo para presentar formas más graves de la enfermedad, como hepatitis crónica (Dalton y col., 2009), tal y como se comentará en el apartado 5.1. (Patrón serológico y molecular de la infección por el VHE) del presente proyecto de tesis doctoral, es de gran interés el evaluar si realmente esta población

constituye una población de riesgo, debido a que se podrían implementar estrategias preventivas especiales en ella.

Por último, se ha evaluado como algunos factores genéticos pueden modular el riesgo de infección por el VHE (Arruvito y col., 2008; Devadas y col., 2018; Zhang y col., 2015). En este sentido, se ha evaluado la asociación entre la susceptibilidad a la infección por VHE y el gen de la Apoliproteína E (APOE) (Zhang y col., 2015). En este estudio, se observan que las variantes alélicas APOE e3 y APOE e4 protegen frente a la infección por el VHE (Zhang y col., 2015). En esta misma línea, se han estudiado las mutaciones en el gen receptor de la progesterona (PROGINS), que modulan la respuesta Th1 y Th2 haciendo a la gente portadora más susceptibles a sufrir algunos tipos de cáncer (Yuan y col., 2013; Gallegos-Arreola y col., 2015). En este contexto, el receptor de la progesterona ha sido estudiado en relación con la susceptibilidad al VHE (Debes y col., 2018), donde se observa que aquellos portadores de la mutación PROGINS tienen mayor riesgo de infección por el VHE (Debes y col., 2018). Debido a la escasez de estudios en este sentido, es de gran interés la validación de estos marcadores en otras poblaciones de pacientes, así como la identificación de otros posibles genes asociados con la susceptibilidad de la infección.

2.2.2. Factores de riesgo asociados a la infección en animales

Dado que los suidos son el principal reservorio del VHE, y el consumo de productos derivados supone la principal vía de transmisión al ser humano, el estudio de factores de riesgo en los suidos es una prioridad. Al igual que en humanos, en los cerdos se han identificado como factores de riesgo demográficos de infección para el VHE la edad y el área geográfica (Burri y col., 2014). En primer lugar, se ha observado que a medida que aumenta la edad en los cerdos lo hace también la tasa de seroprevalencia (Jori y col., 2016; Kureljušić y col., 2020). Sin embargo, se ha detectado que la prevalencia de anticuerpos anti-VHE en lechones lactantes puede ser muy alta (Kureljušić y col., 2020). La causa de esta elevada seroprevalencia en cerdos jóvenes puede ser explicada debido a la transmisión

de la inmunidad materna a través de la leche (Kureljušić y col., 2020). En este sentido, se han observado menos casos de infección por VHE en lechones que recibieron leche de madres seropositivas (Kureljušić y col., 2020), por lo que los lechones que son destetados prematuramente y no adquieren anticuerpos anti-VHE de la madre tienen mayor riesgo de infección por el VHE. En segundo lugar, la evaluación de la prevalencia del VHE de diferentes países de la Unión Europea pone de manifiesto que el área geográfica es un factor de riesgo a tener en cuenta, encontrando tasas de prevalencias de anticuerpos IgG anti-VHE en cerdos domésticos de entre el 20%-54,5% en Serbia, el 29,2%-50% en Bulgaria, el 38,94%-50% en Rumanía, o del 31,1%-91,7% en Croacia (Mrzljak y col., 2019). Este es un aspecto importante debido a que la mayor parte de los animales de reposición de las explotaciones de países del Oeste Europeo, como España, proceden de estos países.

Por otro lado, la especie de suido y la raza son otro factor de riesgo de infección del VHE. En este sentido, un estudio llevado a cabo en Francia ha observado diferencias significativas entre las prevalencias de cerdo doméstico (88%), jabalí híbrido (43,5%), y jabalí puro (26%) (Jori y col., 2016). Se desconoce la verdadera causa de estas diferencias, sin embargo, podrían estar asociadas con otros factores de riesgo como la producción o el contacto con otros animales. Se ha observado que la alta densidad de población porcina en las explotaciones y el estrecho contacto entre los cerdos contribuyen al aumento de la seroprevalencia. Sin embargo, esto es controvertido, ya que otros estudios encuentran lo opuesto. En este sentido, un estudio llevado acabo en Belgrado observó que los cerdos criados en semi-extensivo presentan mayor seroprevalencia que los criados en explotaciones intensivas (Kureljušić y col., 2020). Estas diferencias de seroprevalencia parecen estar relacionadas con las deficientes medidas sanitarias, así como por el contacto estrecho con el medio, con fuentes de agua externas, o el contacto con animales de vida libre. Estas medidas pueden ser modificables e intervenidas, y, por lo tanto, modular la prevalencia del VHE en las diferentes explotaciones y, como consecuencia, disminuir el riesgo de infección al ser humano. Sin embargo, no hay estudios que evalúen la presencia

o ausencia de medidas biosanitarias como factores de riesgo del VHE en explotaciones porcinas. Este aspecto es de gran importancia debido a que podría tener un gran impacto en el control de la infección a nivel de la Sanidad Animal.

2.3. Otros genotipos (5-8)

En cuanto al resto de genotipos del VHE identificados, los genotipos 5 y 6 del VHE solo se han detectado en jabalíes presentes en Japón (**Figura 2**), sin evidenciarse casos de infecciones por estos genotipos en seres humanos (Nimgaonkar y col., 2017). El genotipo 5 ha sido demostrado ser infeccioso en modelos in vitro de células humanas de hepatocarcinoma, así como en un modelo animal de primates (*Macaca fascicularis*), demostrando la posibilidad de la transmisión zoonótica del virus (Li y col., 2018).

El genotipo 7 se ha identificado en los Emiratos Árabes Unidos (**Figura 2**). Lee y col. (2016) comunicaron su detección por primera vez en un paciente que consumía regularmente leche y carne de dromedario. En este caso, el análisis filogenético demostró una alta homología entre las secuencias identificados en las muestras obtenidas del paciente con las identificadas en la carne y leche del dromedario (Lee y col., 2016). Su circulación ha sido demostrada en otros países donde existe una alta densidad de estos animales. En este sentido, un estudio llevado a acabo en Etiopía detectó una seroprevalencia del 22,4% en esta especie (Li y col., 2017), mientras que otro estudio muestra una tasa de infección activa en esta población animal del 1,5% (Rashe y col., 2016). Por otro lado, recientemente, se ha identificado un nuevo genotipo, clasificado como 8, en camellos bactrianos de China (Woo y col., 2017) (**Figura 2**). A diferencia del genotipo 7, no se han detectado casos en humanos, sin embargo, si ha sido demostrada su potencial zoonótico mediante modelo in vivo (Wang y col., 2019).

La evaluación de estos genotipos en diferentes poblaciones humanas y animales es limitada, principalmente por la falta de sensibilidad para estos genotipos de las técnicas moleculares actuales. Por ello, su epidemiología y circulación en otras poblaciones

diferentes a suidos silvestres de Japón (genotipos 5 y 6) o a los camélidos (genotipos 7 y 8) es desconocida, por lo que su importancia en Salud Pública está por determinar. Para ello, es fundamental el desarrollo de herramientas diagnósticas moleculares que demuestren una alta sensibilidad para estos genotipos, así como para otros que puedan emergen en los próximos años.

3. Aspectos diagnósticos de la infección por el VHE

3.1. Patrón serológico y molecular de la infección por el VHE

El diagnóstico del VHE se lleva a cabo mediante la detección de diferentes marcadores serológicos y moleculares. Por un lado, las técnicas serológicas se usan para detectar la presencia de anticuerpos específicos como IgG e IgM. Por otro lado, las técnicas moleculares detectan el ARN viral (Zhao y col., 2016).

Durante la primera fase de la infección aguda (**Figura 5**), se produce un aumento de la carga viral, pudiéndose detectar tanto en suero, como en orina y heces (Rivero-Juárez y col., 2020). Pocos días después, se produce una respuesta inmunológica caracterizada por la producción de anticuerpos IgM, pudiendo ser detectados a partir del primer mes tras infección (Rivero-Juárez y col., 2020). Desde el segundo mes tras infección aproximadamente, se producen los anticuerpos IgG, que se mantienen detectables durante un largo periodo de tiempo (hasta más de 10 años) (Rivero-Juárez y col., 2020). En la fase de resolución de la infección aguda, se produce un descenso de la carga viral hasta hacerse indetectable en suero y en heces alrededor del mes 3 (Rivero-Juárez y col., 2020). Esta fase de resolución se caracteriza también por la seroreversión de los anticuerpos IgM, los cuales desaparecen entre los meses 6 y 12 tras inicio de la infección (Rivero-Juárez y col., 2020).

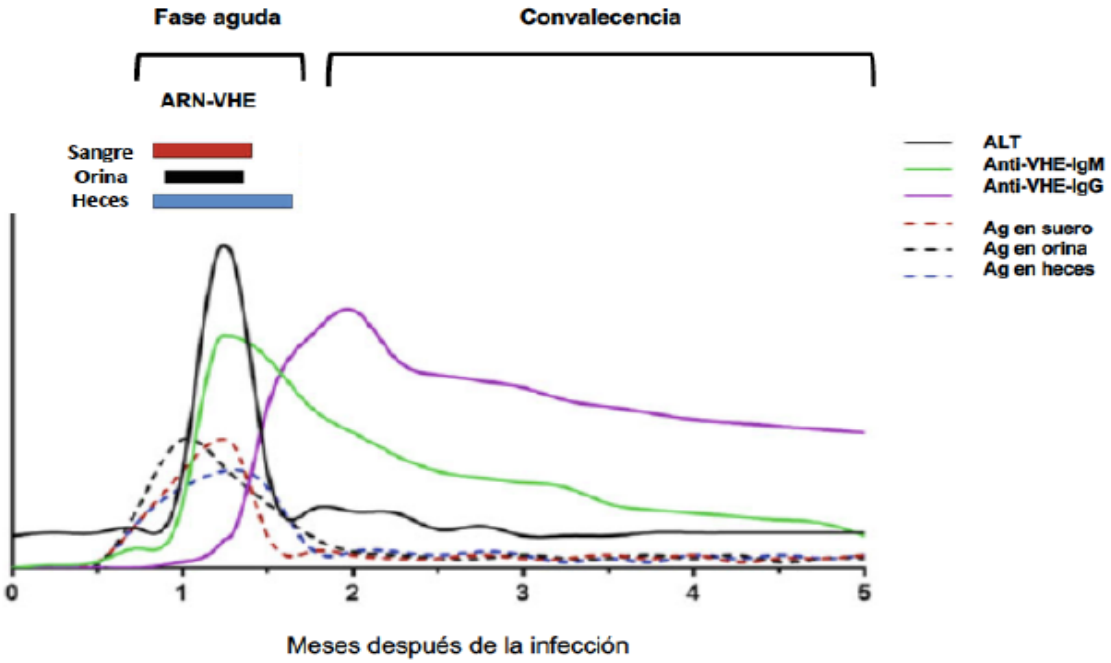

Figura 5. Dinámica de anticuerpos IgG/IgM y ARN de la infección agua del VHE (Rivero-Juárez y col., 2020).

Por otro lado, en ciertas situaciones clínicas, este patrón serológico y molecular puede ser diferente. En pacientes inmunodeprimidos, la carga viral puede permanecer detectable en sangre periférica por más de 3 meses (Rivero-Juárez y col., 2020; Fujiwara y col., 2014). Esta situación se define como infección crónica, permaneciendo la carga viral detectable hasta recuperación de la inmunosupresión o implementación de tratamiento (Fujiwara y col., 2014). Además, durante la infección crónica, los pacientes pueden experimentar un retraso en la seroconversión a anticuerpos IgG e IgM, o incluso no producirse nunca esta seroconversión.

3.2. Organización molecular del VHE y ciclo biológico

El genoma del VHE está estructurado en diferentes marcos de lectura abierta (ORF, siglas en inglés) donde se localizan los genes de los que se sintetizan las diferentes proteínas que conformarán los nuevos viriones durante su ciclo de vida. El conocimiento del genoma viral junto con el ciclo biológico hace posible plantear diversos métodos para la detección

y diagnóstico de la infección por el VHE.

3.2.1. Organización genómica del VHE

El genoma del VHE está formado por tres marcos de lectura abierta que codifican las diferentes proteínas virales (ORF1, ORF2 y ORF3) (Wang y col., 2021) (**Figura 6**). Recientemente se ha identificado en el genoma del genotipo 1 una cuarta ORF (ORF4), incluida en la región ORF1 (Nair y col., 2016; Yadav y col., 2021).

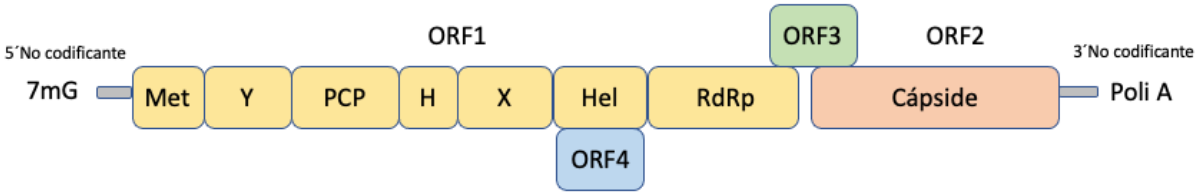

Figura 6. Genoma del VHE. Marcos de lectura abiertos (ORF1,2,3,4) y regiones genéticas de las proteínas virales (Figura realizada por Pedro López López).

La ORF1 codifica una poliproteína de aproximadamente 186 kDa que contiene las proteínas virales no estructurales (Koonin y col., 1992). Las proteínas codificadas en esta región incluyen el dominio de metiltransferasa (Met), dominio Y (Y), cisteína proteasa similar a papaína (PcP), región hipervariable (H), dominio X (X), dominio de la helicasa (Hel) y dominio de la polimerasa de ARN dependiente de ARN (RdRp) (Koonin y col., 1992; Kamar y col., 2014; Kenney y col., 2019) (**Figura 6**). Estas proteínas, codificadas por esta región, son las encargadas de la síntesis de ARN viral, así como su transcripción y replicación (Koonin y col., 1992; Kenney y col., 2019).

La ORF2 codifica una proteína de 72 kDa encargada de formar la cápside vírica. Esta proteína muestra en su estructura un dominio N-terminal, una zona correspondiente a la partícula viral (VLP), y un dominio C-terminal (Wang y col., 2021). El análisis de la estructura cristalina de la VLP muestra tres dominios bien definidos: dominio S (capa), M (medio) y P (sobresaliente) (Yamashita y col., 2009; Xing y col., 2010; Tang y col., 2011).

El dominio S es el bloque de construcción de la cápside (Yamashita y col., 2009). El dominio M está estrechamente asociado con el dominio S y se encuentra en la superficie alrededor de un eje triple icosaédrico, y, además, están vinculado al dominio P con una estructura en forma de bisagra rica en prolina (Yamashita y col., 2009). Por último, el dominio P es responsable de la unión de viriones a células susceptibles, y contiene epítopos neutralizadores de virus, así como la estructura para la homo-oligomerización de la proteína de la cápside (Yamashita y col., 2009).

La ORF3 codifica una proteína multifuncional de pequeño tamaño (vp13), con un peso molecular de 13 kDa (Nan y col., 2016). Su traducción comienza en la ORF2, superponiéndose por 331 nt (Graff y col., 2006; Yamada y col., 2009). Esta proteína viral tiene función reguladora (Nan y col., 2016). Está asociada con los viriones cuasi-envueltos, disociándose de las partículas virales desnudas después de la eliminación de la envoltura (Feng y col., 2014; Yin y col., 2016a). Esta proteína interviene en la liberación del virus tras su formación. Además, la vp13 es necesaria para la infectividad in vivo, sin embargo, no para la replicación in vitro (Graff y col., 2006).

La ORF4 solo se encuentra en el genotipo 1, situada dentro de la ORF1, entre los nucleótidos 2835–3311. Esta región sintetiza una proteína que interviene en la estimulación de la RdRp. Esta proteína solo se produce bajo estrés del retículo endoplásmico (RE), y su expresión es independiente de la caperuza del virus (Nair y col., 2016).

3.2.2. Ciclo biológico del VHE

El virus se une a las moléculas de proteoglicano de heparán sulfato (PGHS) de la membrana celular, e interactúa con un receptor de superficie del hepatocito para internalizarse mediante un proceso dependiente de clatrina (Yin y col., 2016b; Kamar y col., 2017). Una vez dentro del hepatocito, se elimina este recubrimiento de clatrina. El ARN viral es liberado en el citosol de las células, y se traduce en proteínas no estructurales (Kamar y col., 2017). La estructura de la caperuza en el extremo 5' del genoma del VHE

recluta a las subunidades ribosómica 40S de la célula para iniciar la traducción de la ORF1 (Lin y col., 2021). Una vez producida, la RdRp iniciará la transcripción del ARN genómico viral uniéndose a su extremo 3' para producir un ARN intermedio de sentido negativo (Lin y col., 2021). Este ARN de sentido negativo sirve como molde para la síntesis de transcripciones de sentido positivo de longitud completa y un ARN genómico de 2,2 kb (Kamar y col., 2017). El ARN de sentido positivo se traduce en proteínas de ORF2, que corresponden a las proteínas de la cápside, así como a proteínas de la ORF3, que posteriormente servirán para la encapsidación y ensamblaje viral para la producción de nuevos viriones (Kamar y col., 2017). Las proteínas de la cápside pasan a través del RE y el ARN genómico del virus se empaqueta para formar nuevos viriones (Kamar y col., 2017). La liberación del VHE se lleva a cabo mediante la vía exosomal y la proteína ORF3 (Kamar y col., 2017). La mayoría de las partículas víricas infecciosas en forma de viriones envueltos, se liberan desde el lado apical de los hepatocitos hacia los canalículos biliares, donde perderán su envuelta por digestión biliar debido a la acción detergente de las sales biliares (Kamar y col., 2017; Lin y col., 2021). Finalmente, estos llegarán al tracto gastrointestinal, donde se excretarán en las heces, indicativo de transmisión por vía entérica, y perpetuación del ciclo infeccioso (Kamar y col., 2017) (**Figura 7**).

Por otro lado, una porción de viriones se liberará a través del lado basolateral de los hepatocitos, los cuales conservarán su envuelta (Lin y col., 2021) (**Figura 7**). Esta forma viral envuelta del VHE se encuentra presente en la sangre. La envuelta de los viriones es originada de la membrana celular del huésped, la cual es resistente a los anticuerpos neutralizantes (Lin y col., 2021). Estos viriones entran a la célula mediante los mismos mecanismos que los viriones no envueltos, sin embargo, no necesitan la molécula de PGHS para adherirse a las células diana (Yin y col., 2016). Los viriones envueltos pueden encontrarse en la carne cruda o poco cocinada, los cuales, tras ser ingeridos, pierden su envuelta en el tracto gastrointestinal, siguiendo el ciclo natural de la infección, y eliminándose finalmente por heces como viriones no envueltos (Lin y col., 2021).

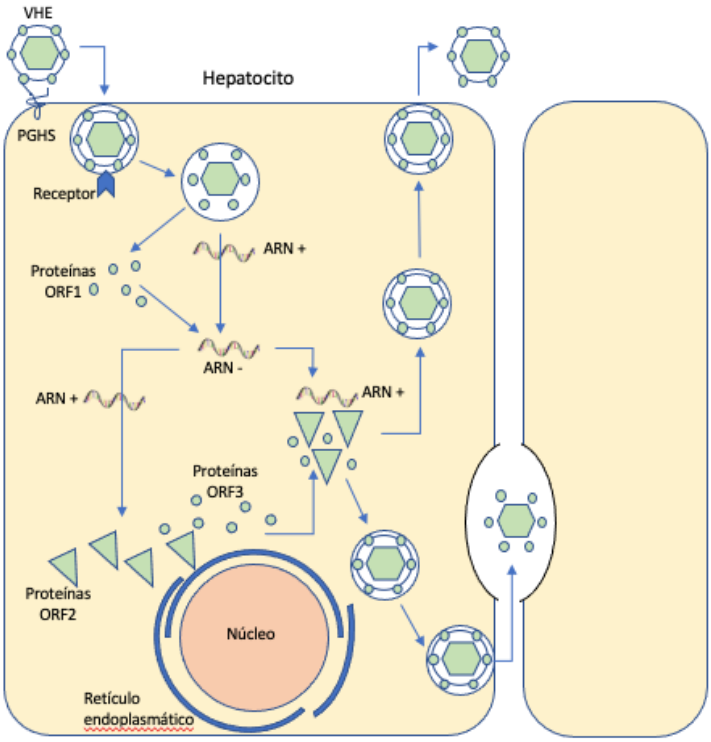

Figura 7. Ciclo biológico del VHE en el hepatocito (Figura realizada por Pedro López López, basada en Kamar y col., 2017).

3.3. Necesidades en el diagnóstico de la infección por el VHE

Las guías de práctica clínica recomiendan basar el diagnóstico de la infección aguda por el VHE en la determinación de anticuerpos IgM mediante ensayo de inmunoabsorción ligado a enzimas (ELISA) (Rivero-Juárez y col., 2020). Esta recomendación se apoya en que es un método de bajo coste y fácil implementación. Por otro lado, la determinación de ARN solo se recomienda para el cribado de pacientes con anticuerpos IgM o en aquellos pacientes inmunodeprimidos para el cribado de la infección crónica (Rivero-Juárez y col., 2020). Esto se debe a que los métodos moleculares requieren un procedimiento más complejo, con la necesidad de equipamiento y personal especializado, y, por lo tanto, conlleva un aumento del coste. Por todo ello, el cribado de la infección por el VHE pivota en la determinación de anticuerpos IgM.

Sin embargo, las pruebas serológicas adolecen de ciertas limitaciones que hacen que este algoritmo diagnóstico pueda no ser el adecuado. En primer lugar, muestran una baja sensibilidad para el diagnóstico de la infección durante las primeras semanas de la infección aguda (Avellon y col., 2015). En cambio, las técnicas moleculares tienen una alta especificidad y mayor sensibilidad durante los primeros días tras inicio de los síntomas (Rivero-Juárez y col., 2020). Esto es crucial para la detección de infecciones en donantes de sangre y de órganos. En segundo lugar, en pacientes inmunodeprimidos su empleo no tiene valor diagnóstico debido a que en esta población puede haber ausencia de anticuerpos (Fujiwara y col., 2014; Kamar y col., 2014). Por ello, el empleo de técnicas moleculares es fundamental en el diagnóstico de la infección aguda en pacientes inmunodeprimidos. Por último, aunque se estima que los anticuerpos IgM permanecen hasta los 6-12 meses tras inicio de la infección, se ha descrito que en el 50% de los pacientes éstos pueden permanecer detectables hasta 3 años, y en el 17% permanecer por más de 3 años (Riveiro-Barciela y col., 2020). Esto supone una importante limitación para el diagnóstico debido a la baja especificidad demostrada en este contexto. Por ello, pese a que la determinación de anticuerpos IgM sigue siendo el método diagnóstico de elección en la mayoría de los países, este algoritmo necesita ser evaluado y optimizado.

La ausencia de recomendación para el uso de técnicas moleculares al cribado de la infección aguda por el VHE puede deberse a las limitaciones que presentan los métodos diagnósticos actuales. Como se ha especificado en el apartado 4. (Epidemiología del VHE), el VHE presenta una gran variedad de genotipos y subtipos. Esto hace que las técnicas moleculares requieran de un diseño pangenotípico para poder detectarlas y así minimizar el número de falsos negativos. En este sentido, los ensayos moleculares disponibles actualmente adolecen de esta limitación. Los más usados y sus limitaciones son:

a. RealStar HEV RT-PCR assay (AltonaDiagnostic; Hamburgo, Alemania). Este método de diagnóstico especifica en su ficha técnica que posee un límite de detección de 200 UI/mL. Sin embargo, este límite de detección ha sido sólo testado con la cadena estándar

de la OMS correspondiente al genotipo 3a (PEI code 6329/10). Además, se ha demostrado que la sensibilidad de la técnica disminuye significativamente hasta en un 33% cuando se analizan infecciones por VHE con cargas virales bajas producidas por genotipo 3f (el de mayor prevalencia en nuestro medio) (Abravanel y col., 2013). Por último, no se especifica su límite de detección de otros genotipos virales en su ficha técnica (RealStar HEV RT-PCR assay, Insert package).

b. AmpliCube HEV RT-PCR kit (Mikrogen; Neuried, Alemania). Este método de diagnóstico especifica en su ficha técnica que tiene un límite de detección de 36,13 UI/mL. Sin embargo, este límite sólo ha sido testado con la cadena estándar de la OMS correspondiente al genotipo 3a (PEI code 6329/10). Además, no se especifica su capacidad de detección de otros genotipos virales en su ficha técnica (AmpliCube HEV RT-PCR assay, Insert package).

Por todo ello, es necesario el desarrollo y validación clínica de un ensayo molecular con alta sensibilidad y capacidad de detección de todos los genotipos y subtipos descritos en la actualidad. Además, la incorporación de este método como parte del cribado de la infección por el VHE, permitiría identificar como positivos individuos mal clasificados como negativos mediante técnicas serológicas, y, por lo tanto, aumentar el número de casos diagnosticados. Esta optimización del diagnóstico es fundamental para el estudio de factores de riesgo de la infección por el VHE, ya que clasificaría correctamente a los pacientes y animales infectados, y permitiría realizar análisis más precisos.

IV. Referencias

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V. Hipótesis y Objetivos

Hipótesis

Las limitaciones de las técnicas empleadas en el diagnóstico de la infección por el VHE, así como la falta de optimización del algoritmo diagnóstico, impiden la correcta evaluación de los factores de riesgo asociados con la infección por el VHE tanto en población humana como animal.

Objetivos

El **objetivo principal** es la identificación de nuevos factores de riesgo asociados a la infección por el VHE en diferentes poblaciones humanas y animales.

Objetivos específicos:

1. Desarrollar y validar clínicamente un método molecular con capacidad de detección de todos los genotipos y subtipos del género *Orthohepevirus* A.

Artículo: "Development and clinical validation of a pangenotypic PCR- based assay for the detection and quantification of hepatitis E virus (*Orthopevirus* A genus)". Journal of Clinical Microbiology (2021) (*Autores con igual contribución).

2. Optimizar el algoritmo diagnóstico de la infección aguda por el VHE incluyendo la determinación serológica y molecular.

Artículo: "Limited Value of Single Sampling for IgM Antibody Determination as a Diagnostic Approach for Acute Hepatitis E Virus Infection". Microbiology Spectrum (2021).

3. Evaluar si los pacientes infectados por el VIH constituyen un grupo de riesgo para la infección por el VHE.

Artículo: "Human Immunodeficiency Virus Infected Patients are Not at Higher Risk for Hepatitis E Virus Infection: A Systematic Review and Meta-Analysis". Microorganisms (2019).

4. Evaluar la influencia del gen PROGINS sobre la susceptibilidad y el curso clínico de la infección por el VHE.

Artículo: "Mutations in the Progesterone Receptor (PROGINS) May Reduce the Symptoms of Acute Hepatitis E and Protect Against Infection". Frontiers in Microbiology (2019).

5. Evaluar los factores de riesgo asociados a la infección activa por el VHE en cerdos criados en diferentes sistemas de producción.

Artículo: "Risk factors associated with hepatitis E virus in pigs from different production systems". Veterinary Microbiology (2018).

VI. Publicación 1

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Development and Clinical Validation of a Pangenotypic PCR-Based Assay for the Detection and Quantification of Hepatitis E Virus (*Orthohepevirus* A Genus)

Mario Frías,[a] Pedro López-López,[a] Ismael Zafra,[a] Javier Caballero-Gómez,[a,b] Isabel Machuca,[a] Ángela Camacho,[a] María A. Risalde,[c] Antonio Rivero-Juárez,[a] Antonio Rivero[a]

[a]Virología Clínica y Zoonosis, Hospital Universitario Reina Sofía de Córdoba, Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Universidad de Córdoba, Córdoba, Spain

[b]Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de Córdoba, Córdoba, Spain

[c]Departamento de Anatomía y Anatomía Patológica Comparadas, Facultad de Veterinaria, Universidad de Córdoba, Córdoba, Spain

Mario Frías and Pedro López-López contributed equally to this article. Author order was determined on the basis of seniority.

Development and clinical validation of a pangenotypic PCR-based assay for the detection and quantification of hepatitis E virus (*Orthopevirus* A genus)

1. Introduction

Hepatitis E virus (HEV) is an RNA virus that belongs to the genus *Orthohepevirus* A. The species included in this genus are a major cause of acute hepatitis worldwide (World Health Organization, 2017). According to epidemiological criteria, the major four genotypes (i.e., genotypes 1-4) can be grouped into two main groups: genotypes 1 and 2, for which humans appear to be the only hosts, that produce large outbreaks in developing countries (primarily Africa and Asia) (World Health Organization, 2017), and genotypes 3 and 4, which can infect humans and a large variety of animals and are usually reported as sporadic cases (Suin *et al.*, 2019). To these four primary genotypes, which have already been widely studied, other genotypes (i.e., genotypes 3ra, 5 to 8) with potential zoonotic capacity must be added (Takahashi *et al.*, 2011; Lee *et al.*, 2016; Woo *et al.*, 2016).

HEV has shown considerable variability among subtypes (Abravanel *et al.*, 2012; Tian *et al.*, 2015; Caballero-Gomez *et al.*, 2019). However, despite the emerging nature of some HEV genotypes and subtypes, at present, there is no PCR screening assay that has been designed to detect the broad spectrum of the species in the *Orthohepevirus* A genus. Thus, the implementation of an accurate quantitative assay for the detection of all currently described HEV genotypes, as well as the potential detection of unidentified subtypes, is necessary in both human and animal public health, constituting the basis for a One-Health model for addressing HEV. In this sense, the diagnosis of HEV infection requires precise and sensitive tools. Serological tests may lack sensitivity or yield biased epidemiological data regarding seroprevalence (Drobeniuc *et al.*, 2010; Kmush *et al.*, 2015). Meanwhile, the molecular diagnosis of HEV infection by PCR exhibits high sensitivity and specificity to and has been crucial from both the epidemiological (blood and organ donor screening) and clinical (management of acute and chronic infection)

perspectives.

For these reasons, our study consists of the development of the concept of this assay, the evaluation of the performance of the pangenotypic RT-PCR assay, and the clinical validation of the detection and quantification of HEV RNA from the *Orthohepevirus* A genus.

2. Methods and Results

2.1. In silico development of primers and probe

The identification of sequences to be used as pangenotypic primers was performed using an *in silico* procedure. The software used was MEGA (Version 10.0) and the MAFFT online service (Katoh *et al.*, 2019). These primers and probes were obtained by aligning all whole-genome sequences of 134 strains from the *Orthohepevirus* A genus (genotypes 1 to 8) with infective capacity in humans and/or animals that were available in GenBank as of December 2018 (**Table S1**). Due to the high homology between sequences, the ORF3 region was selected as a target for the development of primers and probes. The design of these primers was based on the sequence described in the study carried out by Jothikumar *et al.* (2006). Thus, a 70-bp sequence was selected for the development of primers and probes (nucleotides 5,304 to 5,373 in the 3a strain [AB630970]). The primers and probe selected were as follows: forward primer 5´-RGTRGTTTCTGGGGTGAC-3´, reverse primer 5´-AKGGRTTGGTTGGRTGA-3´, and probe 5´-FAM-TGAYTCYCARCCCTTCGC-TAMRA-3´. Subsequently, the specificity for HEV of the region amplified by these sequences was verified using BLAST® (Basic Local Alignment Search Tool) without obtaining any homologous sequence or a sequence with a similarity level greater than 70%.

2.2. RNA extraction and Real-time RT-PCR

All measurements for this study were performed by unique one-step real-time RT-PCR with the Qiagen One-Step RT-PCR Kit (Qiagen, Hilden, Germany) and using a CFX

Connect instrument (Bio-Rad, CA, USA). For the RT-PCR assay employed in this study, the 1st World Health Organization (WHO) International Standard for HEV RNA Nucleic Acid Amplification Techniques (NAT)-Based Assays (PEI code 6219/10) and 1st WHO International Reference Panel for HEV Genotypes for NAT-Based Assays (PEI code 8578/13) supplied by the Paul-Ehrlich-Institut were used. The WHO Standard HEV strain and all genotypes from the WHO HEV panel were reconstituted with 500 µL of DEPC-treated water (Thermo Fisher Scientifics, Waltham, MA, USA). For the WHO strains, RNA was extracted from reconstituted virus solution (200 µL) using the commercial QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) by an automated procedure (QIAcube, Qiagen, Hilden, Germany). The primers and probe, supplied by IDT (Integrated DNA Technologies, Iowa, USA) and purified by the desalting technique, were reconstituted at a concentration of 20 µM. RT-PCR analyses were performed in final volumes of 50 µL containing 2 µL of dNTPs, 2 µL of enzyme mix, 10 µL of 5x buffer, 2.5 µL of each primer (20 µM), 1.12 µL of probe (20 µM) and a variable amount of RNase-free water depending on the amount of RNA extracted used. For the quantification of all the RT-PCRs carried out in this study, a standard curve of 16 points was designed, which consisted of 1:2 dilutions of the WHO HEV standard strain

2.3. Determination of optimal thermocycling conditions

To establish an optimum annealing temperature, a temperature gradient of 8 points was performed between 50°C and 56°C for 45 cycles of amplification and using the WHO HEV standard strain (**Figure S1**). The optimal annealing temperature obtained in this experiment was 51°C. The definitive thermocycling conditions are shown in Table S2 with the indications provided by the manufacturer of the One-Step Kit.

2.4. Detection limit setup

The WHO HEV standard strain was employed to establish the detection limit for 5 µL, 10 µL, 15 µL, 20 µL and 25 µL of RNA extracted. To this end, 16 replicates were

analyzed in decreasing 1:2 dilutions for each amount of RNA extracted. The starting dilution was 1/512, which corresponded to a concentration of 976.5 IU/mL. The detection limit was determined by probit analysis (at the 95% confidence level). Table S3 presents the number of samples tested, as well as the number of positive samples in each of the dilutions. Table 1 presents the detection limit for different concentrations, as determined using probit analysis. For the subsequent development experiments, we chose 25µL of RNA extracted, which exhibit a detection limit of 21.86 IU/mL (17.38-34.30 IU/mL).

Table 1. Limit of detection according to volume of RNA extracted.

RNA extracted volume	Limit of detection (95% CI)
5 µL	100.2 (77.1-161.5)
10 µL	74.1 (60.8-101.2)
15 µL	69.2 (56.7-94)
20 µL	36.39 (29.3-52.2)
25 µL	21.86 (17.38-34.30)

Legend: CI, Confidence interval

2.5. Performance analysis of the WHO HEV panel and emerging genotypes

To analyze the performance of the pangenotypic primers against different genotypes, the WHO HEV panel was used. This sample panel consists of eleven different members, including genotypes 1a (2 strains), 1e, 2a, 3b, 3c, 3e, 3f, 4c, and 4g, as well as a human isolate related to rabbit HEV (3ra). For each of these genotypes, five replicates were performed. Table 2 shows that the assay was able to detect all genotypes present in the WHO HEV panel and was able to measure the viral load of each of the genotypes. To verify the capacity of the pangenotypic assay to detect emerging viral genotypes (genotypes 5, 6, 7 and 8) that are not included in the HEV genotype panel, an in silico-designed a single strand DNA fragment belonging to the ORF3 region for each genotype and supplied by IDT was tested. For genotype 5 a fragment of 339 bp (AB573435), for genotype 6 a fragment of 330 bp (AB602441), for genotype 7 a fragment of 342 bp

(KJ496143) and for genotype 8 a fragment of 342 bp (KX387867) were designed. The fragments were reconstituted at the concentration recommended by the manufacturer (10 ng/µL). As a control, a fragment of strain 1a (8567/13) from the HEV genotype panel was used. In screening these genotypes, the pangenotypic assay was able to detect genotypes 5, 6, 7 and 8 using these synthetic strains.

Table 2. Sensitivity analysis of panHEVnotypic.

Viral load (UI/mL)	Template volume. Number of replicates detected				
	5 µL	10 µL	15 µL	20 µL	25 µL
61	9/16	13/16	14/16	16/16	16/16
30.5	4/16	5/16	5/16	13/16	16/16
15.25	3/16	4/16	4/16	8/16	11/16
7.62	0/16	0/16	0/16	3/16	5/16
3.81	0/16	0/16	0/16	0/16	1/16

2.6. Performance comparison between pangenotypic and commercial kits: HEV panel

The performance of the pangenotypic assay was compared with those of the commercial kits ampliCube 2.0 HEV (Mikrogen Diagnostik, Neuried, Germany), subsequently referred to as kit no. 1, and RealStar HEV RT-PCR Kit 2.0 (Altona Diagnostic, Hamburg, Germany), subsequently known as kit no. 2. The first comparison consisted of the detection at concentrations below 1,000 IU/mL of genotypes 1e, 3c, 3f and 3ra, which are included in the HEV genotype panel. The mean viral load obtained in the WHO report (Baylis *et al.*, 2019) was employed as a reference for the dilutions. For each of the dilutions, 5 replicates were made following the protocol described above for the pangenotypic assay and following the manufacturer's instructions for the two commercial kits. In the comparison with the two commercial kits, the pangenotypic assay showed

better performance in the 4 genotypes tested at low concentrations. The percentage and number of positive replicas detected are shown in Table 3. Except for genotype 3c, the performance of both commercial kits did not exceed 25% of positive replicates at low concentrations.

Table 3. Limit of detection according to volume template.

Template volume	Limit of detection (95% IC)
5 µL	100.2 (77.1-161.5)
10 µL	74.1 (60.8-101.2)
15 µL	69.2 (56.7-94)
20 µL	36.39 (29.3-52.2)
25 µL	21.86 (17.38-34.30)

2.7. Performance comparison between pangenotypic and commercial kits: Testing in a real setting

This comparison consisted of testing clinical specimens of patients with acute hepatitis in care at the Reina Sofía Hospital in Córdoba between 2017 and 2019. Acute hepatitis was defined as acute illness with i) onset of such symptoms as nausea, anorexia, fever or malaise and ii) elevated serum aminotransferase levels. Sera were tested by enzyme immunoassay for anti-hepatitis A IgM, anti-hepatitis B core and hepatitis B surface antibody, total hepatitis C antibodies, total hepatitis D antibodies, anti-Epstein Barr IgM and anti-cytomegalovirus IgM. In addition, sera were tested by means of PCR for the detection of hepatitis B virus DNA, hepatitis C virus RNA, cytomegalovirus virus DNA and Epstein-Barr virus DNA. All samples were screened for HEV infection by enzyme-immunoassay for anti-hepatitis E IgM (Wantai, Beijing Wantai Biological Pharmacy Enterprise © Ltd, Beijing, China) and HEV RNA in triplicate using the pangenotypic assay, kit no. 1 and kit no. 2. RNA extraction from human serum samples was performed following the same procedure described previously in RNA extraction and Real-time RT-

PCR section. Samples with detectable HEV for any assay were genotyped by targeting the ORF2 region using primers HEV_5920S (5´-CAAGGHTGGCGYTCKGTTGAGAC-3´) and HEV_6425A (5´-CAAGGHTGGCGYTCKGTTGAGAC-3´) in the first round and HEV_5930S (5´-GYTCKGTTGAGACCWCBGGBGT-3´) and HEV_6334A (5´-TTMACWGTRGCTCGCCATTGGC-3´) in the second round. The second amplification product of 467 bp was sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). SnapGene software (Version 3.1; GSL Biotech, snapgene.com) was used for sequence analysis. The consensus sequence was obtained using SeqMan Software SeqMan NGen® Version 12.0 (DNASTAR. Madison, WI). Subtype assignment and phylogenetic analyses were performed using the HEVnet genotyping tool (https://www.rivm.nl/mpf/typingtool/hev/) (Mulder *et al.*, 2019) and confirmed by BLAST.

Fifty-four patients were included in the validation procedure (**Figure 1**). The etiology of acute hepatitis is shown in Table S4. Of the 54 patients, 33 (61.1%) were determined to be HEV RNA-negative by the pangenotypic assay. Neither of these patients were determined to be positive by any of the commercial kits. Of these 33 patients, 7 were diagnosed with HEV infection by anti-HEV IgM antibody. These 7 patients were determined to be negative for HEV RNA by both commercial kits.

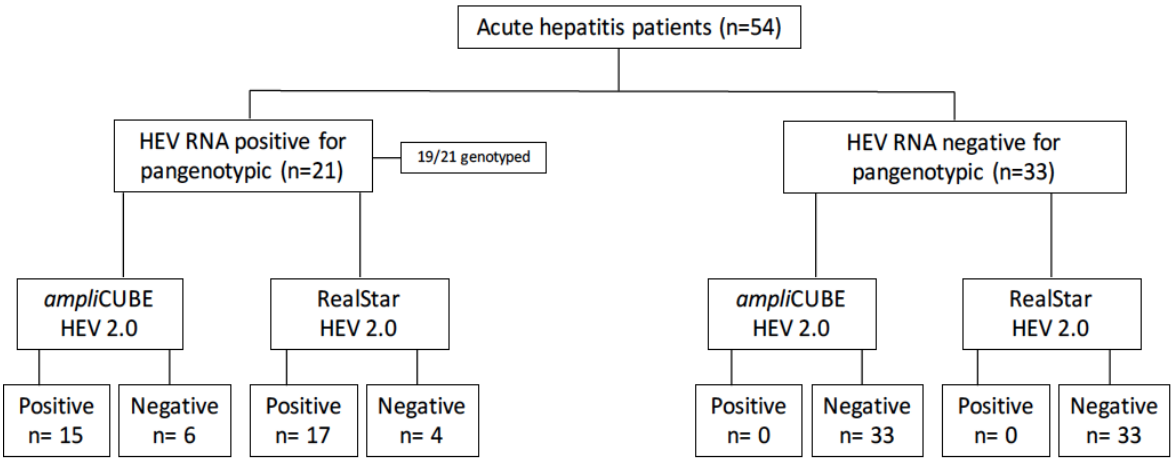

Figure 1. Flow chart of validation procedure.

Twenty-one patient samples (38.9%) were determined to be HEV RNA-positive using the pangenotypic assay, of which 19 could be sequenced. All strains were consistent with genotype 3f. Another RT-PCR determination was repeated in duplicate for the 2 samples that could not be sequenced, and positivity was confirmed. Of these 21 patients, 15 were determined to be HEV RNA-positive by kit no. 1, and 17 were determined to be HEV RNA-positive by kit no. 2 (**Table 4**). Four of the 6 patients who were negative for kit no. 1 were also not detected with kit no. 2.

Table 4. Performance of the three RT-PCR techniques against genotypes 1e, 3c, 3f and 3ra.

	PanHEVnotypic	ampliCube HEV 2.0 quant	RealStar HEV RT-PCR Kit 2.0
	Number of replicates detected (Cq mean ± SD)		
Genotype 1e[a]			
1/20 (88.9 IU/mL)	4/5 (37.6 ± 0.3)	1/5 (37.2)	1/5 (42.3)
1/80 (22.2 IU/mL)	4/5 (37.7 ± 1.3)	0/5 (NA)	2/5 (39.1 ± 0.4)
1/160 (11.1 IU/mL)	1/5 (38)	0/5 (NA)	0/5 (NA)
1/400 (4.4 IU/mL)	2/5 (39.7 ± 4.2)	0/5 (NA)	0/5 (NA)
Total positive/tested (%)	11/20 (55)	1/20 (5)	3/20 (15)
Genotype 3c[a]			
1/5 (625 IU/mL)	5/5 (37.3 ± 0.5)	5/5 (37.9 ± 0.5)	5/5 (37.9 ± 0.4)
1/20 (125 IU/mL)	5/5 (37.7 ± 3.5)	4/5 (38.8 ± 0.4)	5/5 (39.8 ± 2.8)
1/40 (62 IU/mL)	5/5 (39.2 ± 1.1)	3/5 (39.9 ± 3.1)	4/5 (37.8 ± 0.8)
1/100 (25 IU/mL)	5/5 (40.6 ± 1.1)	2/5 (38.7 ± 0.8)	4/5 (39.1 ± 0.6)
Total positive/tested (%)	20/20 (100)	14/20 (70)	18/20 (90)
Genotype 3f[a]			
1/60 (115 IU/mL)	4/5 (37.9 ± 3.4)	1/5 (37.5 ± 3.2)	0/5 (NA)
1/240 (28 IU/mL)	3/5 (39.4 ± 0.9)	3/5 (37.9 ± 0.2)	0/5 (NA)

1/480 (14.41 IU/mL)	0/5 (NA)	0/5 (NA)	0/5 (NA)
1/1,200 (5.76 IU/mL)	2/5 (40.7 ± 1.8)	0/5 (NA)	1/5 (40.3 ± 1.4)
Total positive/tested (%)	9/20 (45)	4/20 (20)	1/20 (5)
Genotype 3ra[a]			
1/1,250 (76 IU/mL)	5/5 (38.3 ± 4.1)	2/5 (37.2 ± 0.1)	5/5 (39.5 ± 2.8)
1/5,000 (19 IU/mL)	3/5 (39.3 ± 0.8)	1/5 (39)	0/5 (NA)
1/10,000 (9.5 IU/mL)	1/5 (38.7)	0/5 (NA)	0/5 (NA)
1/25,000 (3.81 IU/mL)	1/5 (43.1)	0/5 (NA)	0/5 (NA)
Total positive/tested (%)	10/20 (50)	3/20 (15)	5/20 (25)

a. All dilutions are based on the viral load estimated from the analyses performed by the WHO.
SD, standard deviation; NA, not available.

3. Discussion

HEV is an emerging disease with high phylogenetic diversity, especially genotype 3, which has a global distribution and a large number of recently described subtypes (Abravanel *et al.*,2012; Lhomme *et al.*, 2013; Nicot *et al.*; 2018). On the other hand, new species are gradually emerging that may serve as reservoirs of new genotypes and that can be potentially zoonotic (Li *et al.*, 2018; Lee *et al.*, 2016). Therefore, we designed a pangenotypic detection assay that could minimize the number of false negatives and thereby substantially increase the number of isolated cases of the virus. This assay could clinically facilitate the management of patients infected by HEV, both in acute and chronic infections. On the other hand, this assay could increase the number of cases and therefore may provide new strains not previously described.

To the best of our knowledge, there are no studies comparing the performances of HEV diagnostic assays in patients with acute hepatitis. In this study, our assay and two other commercial assays were compared by two analyses. In the first analysis, it was observed that the pangenotypic assay exhibited better performance than commercial kits at very low concentrations of four different genotypes. It should be noted that there was

a greater difference in performance in genotypes 1e, 3f and 3ra. Some studies have shown performance problems in detecting genotype 3f at low concentrations using both in-house and commercial assays. In this vein, Baylis *et al.*, using an in-house RT-PCR procedure, showed that from a concentration of 12,000 IU/mL, the efficiency for the detection of HEV 3f was considerably reduced (Baylis *et al.*, 2011). Similarly, a study conducted by Abravanel *et al.* (2013) observed that the RealStar HEV RT-PCR Kit 1.0 had a poor performance below a concentration of 100 IU/mL (Abravanel *et al.*, 2013). With the assay proposed in this manuscript, genotype 3f was detected with a viral load greater than the mean viral load estimated by the WHO (Baylis *et al.*, 2019). On the other hand, to the best of our knowledge, there are no other studies that have conducted a performance analysis of the assays for genotypes 1e and 3ra at low virus concentrations. In the second analysis, which consisted of a clinical validation procedure, despite the relatively small sampling performed, the two commercial assays were unable to detect between four (kit no. 2) and six samples (kit no. 1), four of which belong to the 3f genotype. Furthermore, this lower performance observed with this genotype was independent of the viral load of the sample (5 of 6 samples had a viral load greater than 10,000 IU/mL). All together show that both commercial kits exhibit a lower performance for genotype 3f, because the fail to detect this genotype at concentration below 100 IU/mL and the lack of detection of two and four (pending of the assay) clinical species infected by this genotype. This result is notable, since genotype 3f is one of the most prevalent genotypes in Europe and shows a worldwide distribution (Abravanel *et al.*, 2012). Therefore, the use of our assay could minimize the number of false negatives.

HEV is the main etiological agent of acute viral hepatitis (World Healthy Organization, 2017). Suboptimal performance in the detection of HEV can lead to an erroneous diagnosis of acute HEV infection in patients with hepatitis of unknown etiology or a non-negligible percentage of false negatives in the detection of the virus in blood donations in countries where the screening is implemented (Webb *et al.*, 2019; Murrison

et al., 2017). From an epidemiological point of view, these false negative results could lead to underestimation of the percentage of infections. In this sense, there is evidence of discrepancies between positive HEV-Ag and negative HEV RNA; therefore, the possibility of biased HEV RNA determinations by false negatives cannot be ruled out. This bias has been observed in studies of human cohorts (El-Sayed Zaki *et al.*, 2006; Gupta *et al.*, 2013; Wen *et al.*, 2015; Majumdar *et al.*, 2013; Geng *et al.*, 2016; Zhao *et al.*, 2015). Some of these studies used broad spectrum primers included in the ORF1 or ORF2 region. In this work, we designed an assay focused on the ORF3 region, since it has been identified as the ideal area for the development of molecular diagnostic assays due to its low profile of mutations between viral genotypes/subtypes (Abravanel *et al.*, 2012; Muñoz-Chimero *et al.*, 2016).

This study has several limitations. First, genotypes not included in the panel provided by the WHO (i.e., genotypes 5, 6, 7 and 8) were tested with synthetic strains, which means the performance of this assay has not been tested on real samples for these genotypes. Second, the study has only been evaluated with the QIAGEN One-step PCR Kit; therefore, the performance demonstrated in this study may not be maintained if other PCR kits are used. Third, the assay has only been designed to detect species of *Orthohepevirus* A, which means that other strains of other species with observed zoonotic potential that was demonstrated after the design of this study, such as those belonging to the *Orthohepevirus* C (Siddharth *et al.*, 2018; Sridhar *et al.*, 2020), have not been considered. Finally, this assay would need to be validated in other real-world settings with a wider distribution of genotypes and subtypes.

4. Conclusions

Our assay was able to detect all described genotypes of the *Orthohepevirus* A genus and showed a stronger performance than the two commercial kits. Our assay may

represent a significant improvement in the molecular diagnosis of infection with important clinical and epidemiological implications.

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6. Suplementary materials

Table S1. Whole-genome sequences of *Orthohepevirus* A used in silico for the design of primers and probe.

Genotype	Accession Number	Host	Location
1a	M73218	Human	India, Pakistan, UK, Myanmar
1a	MH504163	Human	UK
1b	L08816	Human	China, Pakistan
1c	X98292	Human	India
1d	AY230202	Human	Morocco
1e	AY204877	Human	Chad
1f	JF443721	Human	India
1g	LC225387	Human	India, Mongolia, Pakistan, Japan, UK, France
1	FJ457024	Human	India
1	MH918640	Human	Nigeria
2a	KX578717	Human	Mexico
2b	MH809516	Human	Nigeria
3a	KJ507955	Pig	Canada
3a	AB074918	Human	Japan
3a	AB074920	Human	Japan
3a	AB089824	Human	Japan
3a	AB481228	Pig	Japan
3a	AB591734	Mongoose	Japan
3a	AF060668	Human	USA
3a	AF060669	Human	USA
3a	AF082843	Human, Pig, Mongoose	USA, Japan, South Korea, UK, Germany, Canada, Singapore, China, Mexico, Thailand

3a	FJ426403	Pig	Korea
3a	FJ426404	Pig	Korea
3a	HW532736	Human	USA
3a	JN564006	Human	USA
3a	JN837481	Human	USA
3a	KF303502	Pig	Germany
3b	AB073912	Pig	Japan
3b	AB091394	Human	Japan
3b	AB189070	Wild boar	Japan
3b	AB189071	Deer	Japan
3b	AB189072	Human	Japan
3b	AB222182	Wild boar	Japan
3b	AB222183	Wild boar	Japan
3b	AB222184	Wild boar	Japan
3b	AB236320	Mongoose	Japan
3b	AB246676	Human	Japan
3b	AB291955	Human	Japan
3b	AB291962	Human	Japan
3b	AB291963	Human	Japan
3b	AB301710	Human	Japan
3b	AB369691	Human	Japan
3b	AB481229	Pig	Japan
3b	AB630971	Human	Japan
3b	AP003430	Human, Pig, wild boar, deer, mongoose	Japan, China, Canada
3b	FJ527832	Pig	China
3b	KJ507956	Pig	Canada
3c	FJ705359	Human, Wild boar	Germany, France, Netherlands, Sweden, UK, Thailand, Canada
3c	KC618402	Human	Germany
3c	KJ701409	Human	France
3e	AB248521	Human, pig, wild boar, macaque	Japan, France, Germany, Hungary, Italy, UK
3e	AB248522	Pig	Japan
3e	AB291958	Human	Japan
3e	AB780453	Wild boar	Japan

3e	FJ998015	Wild boar	Germany
3e	HM055578	Pig	Hungary
3e	JQ013795	Rabbit	France
3e	JQ026407	Primate	Japan
3e	JQ953665	Pig	France
3e	KF922359	Human	France
3f	EU360977	Pig	Sweden
3f	KT581444	Pig	Sweden
3f	KT581445	Pig	Sweden
3f	KT581446	Pig	Sweden
3f	KT581447	Pig	Sweden
3f	AB291961	Human	Japan
3f	AB369687	Human, pig, wild boar	France, Germany, UK, Spain, Denmark, Sweden, Thailand, Singapore, Japan
3f	AB850879	Human	Japan
3f	EU495148	Human	France
3f	EU723512	Pig	Spain
3f	EU723513	Pig	Spain
3f	EU723514	Pig	Spain
3f	EU723516	Pig	Spain
3f	FJ653660	Human	Thailand
3f	FJ956757	Human	Germany
3f	JN906976	Pig	France
3f	JQ953666	Pig	France
3f	KC166971	Human	France
3g	AF455784	Pig	Kyrgyzstan
3h	KY780957	Pig	Switzerland
3h	MF346773	Pig	Switzerland
3h	JQ013794	Human, pig	France, Switzerland, Mongolia
3i	FJ998008	Human, wild boar	Sweden, Germany
3j	AY115488	Pig	Canada
3k	AB290312	Pig	Mongolia
3k	AB369689	Human, pig	Japan
3l	JQ953664	Human, pig	France, Italy
3m	AB290313	Pig	Mongolia

3m	KU176130	Human	France
3m	KU513561	Human	Spain, France
3ra	AB740221	Rabbit	Mongolia
3ra	AB740222	Rabbit	Mongolia
3ra	FJ906895	Human, rabbit, hare	France, Germany, China, South Korea, USA
3ra	FJ906896	Rabbit	China
3ra	GU937805	Rabbit	China
3ra	JQ013791	Rabbit	France
3ra	JQ013792	Rabbit	France
3ra	JQ013793	Rabbit	France
3ra	JQ768461	Rabbit	China
3ra	JX121233	Rabbit	China
3ra	JX565469	Rabbit	USA
3ra	KX227751	Rabbit	China
3ra	KY436898	Rabbit	Germany
3ra	KY496200	Rabbit	Korea
3ra	MF480297	Rabbit	Germany
3ra	MF480297	Rabbit	Germany
3ra	MG211750	Rabbit	France
3ra	MG211751	Rabbit	France
3	AB290313	Pig	Mongolia
3	MF959765	Wild boar	Italy
3	LC260517	Pig	Japan
3*	MK390971	Pig	Italy
3	MF959764	Wild boar	Italy
3	KP294371	Wild boar	Germany
4a	AB197673	Human, pig	China, Mongolia, Taiwan, South Korea, Japan
4b	DQ279091	Human, pig, Rhesus monkey	Taiwan, China, Cambodia, Japan
4c	AB074915	Human, pig	Japan
4d	AJ272108	Human, pig	China
4e	AY723745	Pig	India
4f	AB220974	Human	Japan
4g	AB108537	Human	Japan, China

4h	GU119961	Human, pig, cow, goat, yak	China
4i	AB369690	Human, pig, wild boar	China, Japan
4	AB369688	Human	Japan
4*	MK410048	Pig	China
5a	AB573435	Wild boar	Japan
6a	AB602441	Wild boar	Japan
6	AB856243	Wild boar	Japan
7a	KJ496143	Dromedary camel	UAE
7	KJ496144	Dromedary camel	UAE
7	KT818608	Human	Saudi Arabia
8a	KX387865	Bactrian Camel	China
8	KX387867	Camel	China
8*	MH410174	Bactrian Camel	China

* These strands were not included in the original alignment but were subsequently aligned in order to check the affinity of the assay

Table S2. Thermal cycling conditions.

Step	Temperature	Time	Cycles
Reverse transcription	50°C	30 minutes	1
Initial denaturation	95°C	15 minutes	1
Denaturation	95°C	10 seconds	45
Annealing	51°C	30 seconds	
Extension	60°C	20 seconds	

Table S3. Performance analysis of the pangenotypic assay with WHO HEV Standard.

Viral load (IU/mL)	Template volume. Number of replicates detected				
	5 µL	10 µL	15 µL	20 µL	25 µL
61	9/16	13/16	14/16	16/16	16/16
30.5	4/16	5/16	5/16	13/16	16/16
15.25	3/16	4/16	4/16	8/16	11/16
7.62	0/16	0/16	0/16	3/16	5/16
3.81	0/16	0/16	0/16	0/16	1/16

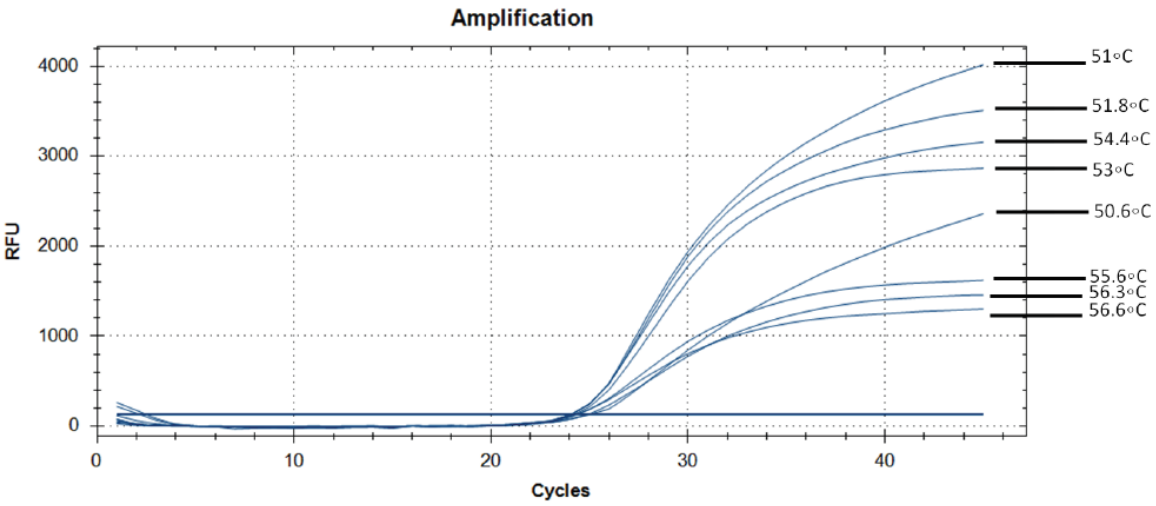
Table S4. Etiology of patients included in the validation.

Diagnosis	N=54 (n, %)
HAV	4 (7.4)
HBV	2 (3.7)
HCV	1 (1.8)
HDV	1 (1.8)
HEV*	28 (51.9)
CMV	5 (9.3)
EBV	1 (1.8)
Unknown etiology	12 (22.2)

*7 cases were IgM+ and HEV RNA negative

HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; HEV, hepatitis E virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus.

Figure S1. Temperature gradient using the WHO HEV standard strain.



VII. Publicación 2

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RESEARCH ARTICLE
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Limited Value of Single Sampling for IgM Antibody Determination as a Diagnostic Approach for Acute Hepatitis E Virus Infection

Antonio Rivero-Juarez [a], Pedro Lopez-Lopez[a], Juan Antonio Pineda[b], Juan Carlos Alados[c], Ana Fuentes-López[d,e], Encarnación Ramirez-Arellano[f,g], Carolina Freyre[h], Ana Belén Perez[i], Mario Frias[a], and Antonio Rivero[a]

[a]Unit of Infectious Diseases, Hospital Universitario Reina Sofia, Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Universidad de Córdoba (UCO), Cordoba, Spain
[b]Unit of Infectious Diseases and Microbiology, Hospital Universitario de Valmegrid.412800.f, Seville, Spain
[c]Clinical Microbiology Unit, Hospital Universitario de Jerez, Cádiz, Spain
[d]Clinical Microbiology Unit, Hospital Universitario Clínico San Cecilio, Granada, Spain
[e]Instituto de Investigacion Biosanitaria Ibs.Granada, Granada, Spain
[f]Infectious Diseases, Microbiology and Preventive Medicine Unit, Virgen Macarena University Hospital, University of Seville/Biomedicine Institute of Seville, Seville, Spain
[g]Department of Medicine, University of Seville/Biomedicine Institute of Seville, Seville, Spain
[h]Clinical Microbiology Unit, University Hospital of Puerto Real, Cádiz, Spain
[i]Clinical Microbiology Unit, Hospital Universitario Reina Sofía, Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Córdoba, Spain

Limited value of a single sampling of IgM antibody determination as a diagnostic approach for acute hepatitis E virus infection

1. Introduction

Hepatitis E virus is recognized as a major cause of acute hepatitis in Europe and worldwide (EASL, 2018; ECDC, 2019). During the acute phase, different serological markers can be applied for diagnosis. Viral RNA can be detected between 2 and 6 weeks before the onset of symptoms and is undetectable in serum approximately 3 weeks later (Rivero-Juárez *et al.*, 2020). Conversely, the immune response follows a transient increase in IgM antibodies, which are detected during the acute phase of the disease and may last up to 12 months (Rivero-Juárez *et al.*, 2020). The most commonly used approach for the diagnosis of HEV infection is testing IgM antibodies by ELISA. This approach is widely implementation due to its advantage of being easy to implement at a low cost. In contrast, determination of HEV-RNA requires a more complex procedure and, consequently, an increase in the cost of screening. Nevertheless, the main advantage is that it has a higher specificity than the determination of IgM antibodies with a higher sensitivity during the first days of the onset of symptoms (EASL, 2018; Rivero-Juárez *et al.*, 2020). Thus, European HEV guidelines recommend using a combination of serology and HEV-RNA testing by PCR to diagnose acute HEV infection (EASL, 2018). However, this recommendation is not supported by an evaluation study; consequently, the determination of IgM antibodies remains the only diagnosis approach in the majority of settings (ECDC, 2019). Thus, we aimed to evaluate the accuracy of HEV diagnosis in patients with acute hepatitis, including approaches based on single sampling IgM determination and HEV-RNA detection.

2. Methods

2.1. Population

A prospective study was conducted in 6 reference hospitals from Andalusia (South Spain) between February 2016 and November 2020, including patients with suspicion of HEV infection. These patients were diagnosed with acute hepatitis and were negative for other hepatitis viruses, including serological and molecular markers for hepatitis A virus (IgM antibodies), hepatitis B virus (HBsAg, HBcAb, and viral DNA), hepatitis C virus (IgG antibodies and viral RNA), cytomegalovirus (IgM antibodies) and Epstein-Barr virus (IgM antibodies).

2.2. HEV evaluation

The same serum sample was evaluated for hepatitis E virus infection, including both IgM antibodies and viral RNA. IgM antibodies were evaluated by enzyme immunoassay using the HEV-IgM kit developed by Wantai (Beijing Wantai Biological Pharmacy Enterprise © Ltd, Beijing, China) with an automated procedure (Triturus, Grifols), and positivity was confirmed by immunoblotting (recomLine HEV IgG/IgM®; Mikrogen Diagnostik, Neuried, Germany). Additionally, in all patients IgG antibodies were determined using the the specific Wantai kit (Beijing Wantai Biological Pharmacy Enterprise © Ltd, Beijing, China), also confirming positive results by immunoblotting. For HEV molecular analysis, RNA was extracted from 400 µL of serum using the QIAamp Mini Elute Virus Spin kit (QIAgen, Hilden, Germany) by an automated procedure (QIAcube, QIAgen). The purified RNA was eluted in a 50-µL volume. For RT q-PCR, the QIAgen One-Step PCR Kit (QIAgen, Hilden, Germany) was used for 25 µL of template (50 µL of reaction volume) following a pangenotypic in-house protocol targeting the ORF3 region developed and validated by our group with a detection limit set at 21 IU/mL (Frías *et al.*, 2021). Positive HEV-RNA samples were sequenced by nested RT-PCR targeting the ORF2 region following the procedure described elsewhere (Frías *et al.*, 2021). Subtype assignment and phylogenetic analyses were

performed using the HEVnet genotyping tool (https://www.rivm.nl/mpf/typingtool/hev/) and confirmed by BLAST (Mulder *et al.*, 2019). All sequences were submitted to GenBank (MN62857-MN62867, MT250082, MT250083, MN537838, MN914126, MN914126, MT776550-MT776555, MT859329, and MW143072).

2.3. Statistical analysis

Hepatitis E infection was considered positive in an individual exhibiting positivity for IgM antibodies and/or detectable HEV-RNA following the definition of clinical guidelines (EASL, 2018; Rivero-Juárez *et al.*, 2020). The frequency of HEV genotype distribution was reported.

2.4. Ethical statement

This study was designed and conducted in accordance with the Declaration of Helsinki. The Ethics and Clinical Trials Committee (CEIC) of Andalucia approved the study protocol, obtaining the informed consent of each patient (reference 4535). The SSPA Biobank has coordinated the collection, processing, handling and assignment of the biological samples used in this study in accordance with the standard procedures established for this purpose (agreement S2100110).

3. Results

During the study period, 182 patients with suspicions of HEV infection were included in the study. Of them, 94 (51.6%) were male, and the median age was 49 years (37-56 years). A total of 68 (37.4%; 95% CI, 30.3%-44.4%) patients were diagnosed with HEV infection. Of them, the majority were male (n = 46; 67.6%), and the median age was 47 years (IQR: 37-55 years). Baseline characteristics of patients are shown in Table 1. Three patients were infected by HIV, all of whom had undetectable HIV viral loads and CD4+ cell counts greater than 200 cells/mL.

Table 1. Baseline characteristics of patients with diagnosis of Hepatitis E virus infection.

Characteristic	Value
Male, n (%)	46 (67.6%)
Age (years), median (IQR)	47 (37-55)
Hospital admision, n (%)	8 (11.7%)
Underlying condition, n (%)	
HIV infection	3 (4.4%)
Diabetes mellitus	2 (2.9%)
Chronic Hepatitis B	1 (1.4%)
Pregnancy	1 (1.4%)
Symptoms, n (%)	
Fever	34 (50%)
Digestive †	34 (50%)
Articular pain	23 (33.8%)
Jaundice	21 (30.8%)
Limb pruritus	9 (13.2%)
Analytical parameters, median (IQR)	
ALT (U/L)	131 (36-435)
AST (U/L)	97 (23-396)
GGT (U/L)	115 (35-286)
Total bilirubin (mg/dL)	0.7 (0.6-4.6)

Legend: number of cases [n]; percentage [%]; interquartile range [IQR]; units [U]; liter [L]; milligram [mg], deciliter [dL]. † Digestive symptoms include vomit, diarrhea and abdominal pain.

Forty-three (63.2%) patients showed detectable HEV viral loads and were sequenced. All strains were consistent with genotype 3, and most of them were classified as genotype 3f (90.6%). Only four sequences were consistent with other subtypes, including one classified as genotype 3e (MN628559), another classified as 3m (MT250083), and two cases in which a subtype could not be assigned (MT776550 and MT776555).

Of the 68 cases of HEV infections, 54 out 68 cases (79.4%) were positive for IgM. Of them 29 (42.6%) were positive for both IgM and HEV RNA, and 25 (36.8%) were positive

only for IgM. Finally, 14 (20.6%) patients with detectable HEV RNA exhibited negative IgM antibodies. The accuracy of a hepatitis E virus diagnosis based on single consideration of IgM antibodies or HEV RNA at the same time point is shown in Table 2.

Table 2. Accuracy of a hepatitis E virus diagnosis based on single consideration of IgM antibodies or HEV RNA.

Diagnosis approach	Positive	Negative	AUROC (95% CI)*
Anti HEV IgM	54/68 (79.4%)	14/68 (20.6%)	0.89 (0.83-0.95)
HEV-RNA	43/68 (63.2%)	25/68 (36.8%)	0.81 (0.74-0.88)

Legend: Hepatitis E virus (HEV); IgM antibodies (IgM); Hepatitis E virus ribonucleic acid (HEV-RNA); Area Under the Receiver Operator (AUROC); 95% Confidence Interval (CI). * Presence of Anti HEV IgM or HEV-RNA (any positive) is considered reference approach and include all the cases of HEV infection (68/68, AUROC =1).

Regarding IgG antibodies, 38 out 54 (70.3%) patients with positive IgM antibodies also exhibited positivity to IgG antibodies. None of the patients with detectable HEV RNA showed positive IgG antibodies.

4. Discussion

The European Centers for Disease Control (ECDC) consider optional the determination of HEV-RNA in acute cases, because PCR testing might not be available in all laboratories and settings (ECDC, 2019). Thus, in immunocompetent patients with suspicion of HEV infection, single IgM antibody testing is typically applied (Lhomme *et al.*, 2019). HEV-RNA testing is only applied for diagnosis in immunocompromised subjects given the delay or lack of antibody seroconversion (Kamar *et al.*, 2014). Our study shows that any diagnostic algorithm of HEV infection in patients with acute hepatitis should be based on the determination of both markers (IgM antibodies and HEV-RNA) because the single sampling IgM antibodies determination results in an unacceptable number of false negatives. According to our results, the determination of HEV-RNA should not be limited

to immunosuppressed individuals because a high proportion of cases could be misdiagnosed. In this sense, our study provides evidence that supports the European Association for the Study of the Liver (EASL) recommendation to use both ELISA and PCR as screening approaches in patients with suspicion of HEV infection (EASL, 2018).

The sensitivity and accuracy of a screening approach based on determination of single sampling IgM antibodies will depend on the assay employed (Lhomme *et al.*, 2019). In our study, we used one of the commercial kits with higher sensitivity and specificity and consequently one of the most commonly used kits worldwide (Abravanel *et al.*, 2013). Using this assay, we failed to detect 20% of the confirmed cases of HEV infection by HEV-RNA detection and sequencing in our study. Thus, the sensitivity of this assay could be much lower than previously considered (approximately 80%), which is consistent with previous reports (Avellon *et al.*, 2015; Pas *et al.*, 2013). This point strengthens the fact that HEV-RNA determination should be included in the screening of acute HEV infection.

Our study presents several limitations. First, only patients with acute hepatitis were included. HEV infection can result in extrahepatic manifestations, even in the absence of liver damage (Pischke *et al.*, 2017). Consequently, the determination of both markers in the screening of extrahepatic HEV infection needs to be evaluated. Second, only cases of HEV infection by genotype 3 were detected. Thus, the accuracy of this screening approach needs to be evaluated in other settings where other genotypes are circulating. Thirdly, in the present study we only evaluate HEV RNA in serum, not considering stools. Because the virus is shedding by feces during a long period, the use of both serum and stools could significantly increase the diagnostic value of PCR determination. Nevertheless, we cannot evaluate that because stool is not included in sampling for the screening of acute hepatitis. Fourthly, our study is based on single sampling IgM antibodies determination. Because there is a delay between detectable HEV RNA and IgM seroconversion at early phase of the infection, IgM serial samples testing

could increase the diagnostic value of this screening approach. Finally, these results could vary if other ELISAs and PCR protocols were employed.

5. Conclusions

In conclusion, our study provides evidence that in the diagnostic algorithm of HEV infection in patients with acute hepatitis, the determination of both IgM antibodies and HEV RNA is necessary. The single use of one of these markers could lead to an important proportion of misdiagnosed cases.

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VIII. Publicación 3

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Review

Human Immunodeficiency Virus Infected Patients are Not at Higher Risk for Hepatitis E Virus Infection: A Systematic Review and Meta-Analysis

Pedro Lopez-Lopez, Mario Frias, Angela Camacho, Antonio Rivero and Antonio Rivero-Juarez *

Infectious Diseases Unit, Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Hospital Universitario Reina Sofía de Córdoba, Universidad de Córdoba, 14004 Córdoba, Spain; lopezlopezpedro07@gmail.com (P.L.-L.); mariofriascasas@hotmail.com (M.F.); acamachoespejo@gmail.com (A.C.); ariveror@gmail.com (A.R.)

* Correspondence: arjvet@gmail.com; Tel.: +34-957-213-806; Fax: +34-957-011-885

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Human Immunodeficiency Virus infected patients are not at higher risk for Hepatitis E Virus infection: A systematic review and meta-analysis

1. Introduction

Infection with the hepatitis E virus (HEV) is the most common cause of acute hepatitis worldwide (Kamar *et al.*, 2014). Six major HEV genotypes (1-4, 7 and 8) cause infection in human (Forni *et al.*, 2018). In most cases, HEV infection is asymptomatic and self-limiting (Hoofnagle *et al.*, 2012), although there are clinical contexts in which the course of acute HEV infection has a worse prognosis, for example, in women who are pregnant and patients with underlying chronic liver disease (Krain *et al.*, 2014; Frias *et al.*, 2018). Furthermore, in immunocompromised patients, acute HEV infection can develop into chronic infection, characterized by rapid progression from liver fibrosis to cirrhosis (Neukam *et al.*, 2013).

In patients infected with the human immunodeficiency virus (HIV), the course of HEV infection may involve additional problems to those found in the general population. In immunosuppressed HIV-infected patients, acute HEV infection can progress to a chronic form (Neukam *et al.*, 2013). Furthermore, HIV patients are often co-infected with hepatotropic viruses (B and C), in which case, the course of acute HEV infection can be more severe and lead to progression or decompensation of chronic liver disease (Frias *et al.*, 2018). The question of whether people infected with HIV constitute a population with increased susceptibility to infection by HEV has also been raised. Many studies have evaluated the relationship between HEV and HIV, although the reported findings are controversial. Some studies have found that HIV-infected patients are at greater risk of infection by HEV (Shrestha *et al.*, 2017; Riveiro-Barciela *et al.*, 2013; Rapicetta *et al.*, 2013; Fainboim *et al.*, 1999), while others have found the opposite (Madden *et al.*, 2016; Taha *et al.*, 2015; Junaid *et al.*, 2014; Keane *et al.*, 2012). This is a crucial point in the sense that determining whether HIV-infected patients indeed constitute a population at

risk of HEV infection will influence the decision as to whether to apply prevention and control measures. The aim of this systematic review and meta-analysis therefore was to evaluate whether HIV-infected patients constitute a risk group for HEV infection.

2. Materials and Methods

2.1. Search strategies and selection criteria

This analysis is reported in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Moher *et al.*, 2009) (**Supplementary TableS2**). A systematic literature search of the PubMed database was performed, using a combination of the terms "HEV" and "HIV", for articles on HEV associated with HIV infection published between January 1990 and August 2018. Two independent researchers conducted the search and determined whether the studies were related to the objective of our study based on title and abstract (**Figure 1**).

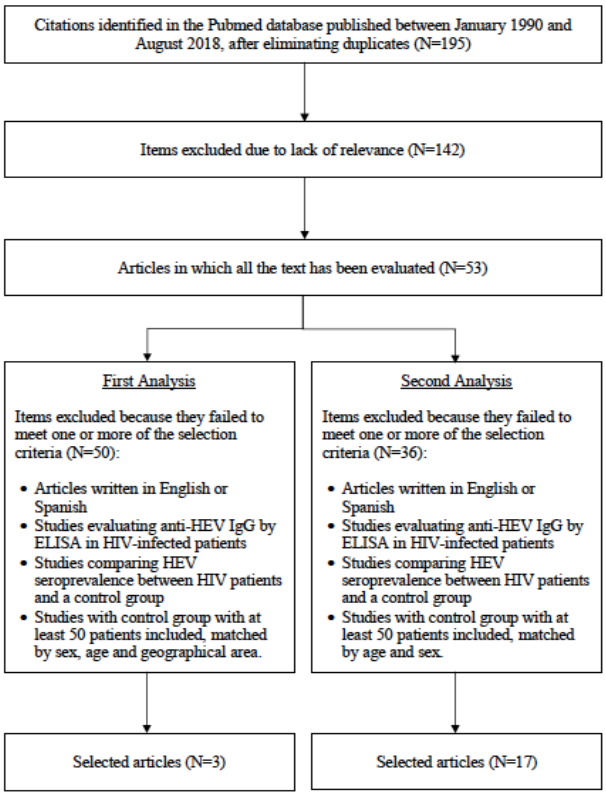

Figure 1. Flow diagram for the selection of studies.

2.2. Selection of studies

The full texts of the selected articles were reviewed. The criteria for inclusion of the studies were as follows: i) articles written in English or Spanish; ii) studies evaluating anti-HEV IgG by ELISA in HIV-infected patients; iii) studies comparing HEV seroprevalence between HIV patients and a control group; and iv) studies with control group with at least 50 patients included, matched by sex, age and geographical area. In addition, a second review was performed with the same criteria excluding matched by age and sex. The two researchers carried out the selection independently. Conflicts over decisions were resolved by discussion or through the involvement of a third investigator (**Figure 1**).

2.3. Data extraction and analysis

The selected studies were read to extract the data. The data of interest extracted were: author, year of publication, country of sampling, definition of the general population, sample size, the number of individuals uninfected and infected by HEV, number of individuals uninfected and infected by HIV, and type of ELISA assay employed. The odds ratio (OR) was calculated to determine the risk of becoming infected by HEV in each group; the data were calculated if they were not expressed in the article. The data were collected by one researcher and confirmed by another. The number of infections was calculated in studies showing only sample size and prevalence of HEV. The data was collected systematically and using a standardized approach.

2.4. Assessment quality of studies

We used The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomized studies in meta-analyses (Wells *et al.*, 2013). This scale uses a 'star system' witch judge three domains with a maximum of 9 point: the selection of the study groups (4 points); the comparability of the groups (1 point); and the ascertainment of either the exposure for case-control (3 points). A study can be awarded a maximum of

one star for each numbered item within the Selection and Exposure categories. A maximum of two stars can be given for Comparability.

2.5. Statistical analysis

To compare HEV infection between HIV-infected individuals and the control groups, the ORs were calculated and 95% confidence intervals (CI) estimated, as well as p values. Heterogeneity between studies was calculated using the I^2 statistic: I^2 > 75% was high heterogeneity and < 25% was low heterogeneity. To obtain the summary measure, the fixed-effect model was used with the inverse-variance weighting method, which considers only intra-study variability, and the random effects model, which also assesses the existence of variability of results between the different studies. Possible publication bias was evaluated using the funnel plot method. The Review Manager (RevMan) [Computer program], version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014, was used for data analysis.

3. Results

3.1. Data recovery and study selection

In the literature search, a total of 195 citations were found in the PubMed database, from which 53 studies were selected that potentially related to the objective of our study. Fifty were subsequently excluded after reading the full text because they failed to meet all the inclusion criteria: 1 article were excluded due to criterion i, 24 due to criterion iii, and 25 articles due to criterion iv (**Supplementary Table S1**). Three papers were finally selected according to the quality evaluated by NOS, which is shown in Table 1. Subsequently, the data were extracted to perform the meta-analysis. The first study conducted by Abravanel *et al.* in France included 900 individuals in a 1:2 ratio of HIV-positive to healthy donors (300 HIV-positive and 600 blood donors) (Abravanel *et al.*, 2017). Of the HIV-infected patients, 116 (38.7%) were positive for anti-HEV IgG, and 284 (41.3%) were healthy donors (Abravanel *et al.*, 2017). The second study conducted by

Boon *et al*. in Uganda included a total of 985 individuals: 491 not infected with HIV identified from a prospective population-based cohort, and 494 HIV-infected. In both populations, a high prevalence of anti-HEV IgG antibodies was found: 46.4% in HIV patients and 47.7% in the control group (Boon *et al.*, 2018). Finally, the third study conducted by Bura *et al*. in Poland included 490 individuals. Of these, 244 were HIV-infected and 246 were blood donors. In the HIV and control groups, a total of 124 (50.8%) and 122 (49.6%) subjects respectively were positive for anti-HEV IgG (Bura *et al.*, 2017). Full details of these studies can be found in Table 1.

In the second review, 17 articles out of the 53 potentially related to the objective of our study were selected. Thirty six articles were excluded after reading the full text because they failed to meet all the inclusion criteria: 1 articles were excluded due to criterion i, 24 due to criterion iii, and 11 articles due to criterion iv (**Supplementary Table S1**).

3.2. Meta-analysis

In the first analysis, a total of 2,375 individuals were included in this meta-analysis: 1,038 of these were HIV-positive patients (43.7%) and 1,337 were healthy donors (56.3%). Of the 1,038 HIV patients included, 469 were positive for anti-HEV IgG antibodies (45.2%) and 569 were negative for anti-HEV IgG (54.8%). In the population of healthy donors, a total of 640 subjects were HEV anti-IgG positive (47.9%) and 697 were negative (52.1%) (**Table 1**).

Table 1. List of included studies in the first meta-analysis, evaluation of quality by NOS, and data extracted for the meta-analysis.

Included studies				NOS for assessing the quality of studies				Extracted data							
								HIV-positive patients			HIV-negative subjects				
Author	Year	Place of study	Tittle	Selection	Comparability	Exposure	Total score	Total patients included	HEV + patients	%	Total subjects included	HEV + patients	%	OR (95% CI)	p value
Abravanel *et al.*	2017	France (Europe)	HEV infection in French HIV-infected patients	****	**	**	8	300	116	38.7	600	284	41.3	0.70 (0.53-0.93)	0.0138
Boon *et al.*	2018	Uganda (Africa)	Hepatitis E Virus Seroprevalence and Correlates of Anti-HEV IgG Antibodies in the Rakai District, Uganda	****	**	**	8	494	229	46.4	491	234	47.7	0.95 (0.74-1.22)	0.6824
Bura *et al.*	2017	Poland (Europe)	Hepatitis E virus IgG seroprevalence in HIV patients and blood donors, west-central Poland	****	**	**	8	244	124	50.8	246	122	49.6	1.05 (0.74-1.50)	0.7861
TOTAL								1038	469	45.2	1337	640	47.9	-	-

Legend: Human immunodeficiency virus (HIV); Hepatitis E virus (HEV); The Newcastle-Ottawa Scale (NOS); odds ratio (OR); 95% confidence interval (95% CI).

The overall OR calculated for HIV patients in the meta-analysis was 0.87 (95% CI: Ç0.74-1.03) for fixed effects (**Figure 2A**), and 0.88 (95% CI: 0.70-1.11) for random effects (**Figure 2B**), with a p value of 0.11 and 0.27, respectively. Heterogeneity was identified as moderate according to the chi-squared test (X^2 = 3.78), the I^2 statistic (I^2 = 47%) and the vis. ual representation of the forest plots for both the fixed-effects and random-effects analyses (**Figures 2A and 2B**). In the random-effects analysis, the Tau^2 statistic was 0.02. The funnel plot showed absence of publication bias (**Figure 3**), with the point cloud distributed symmetrically around the summary measure of the effect.

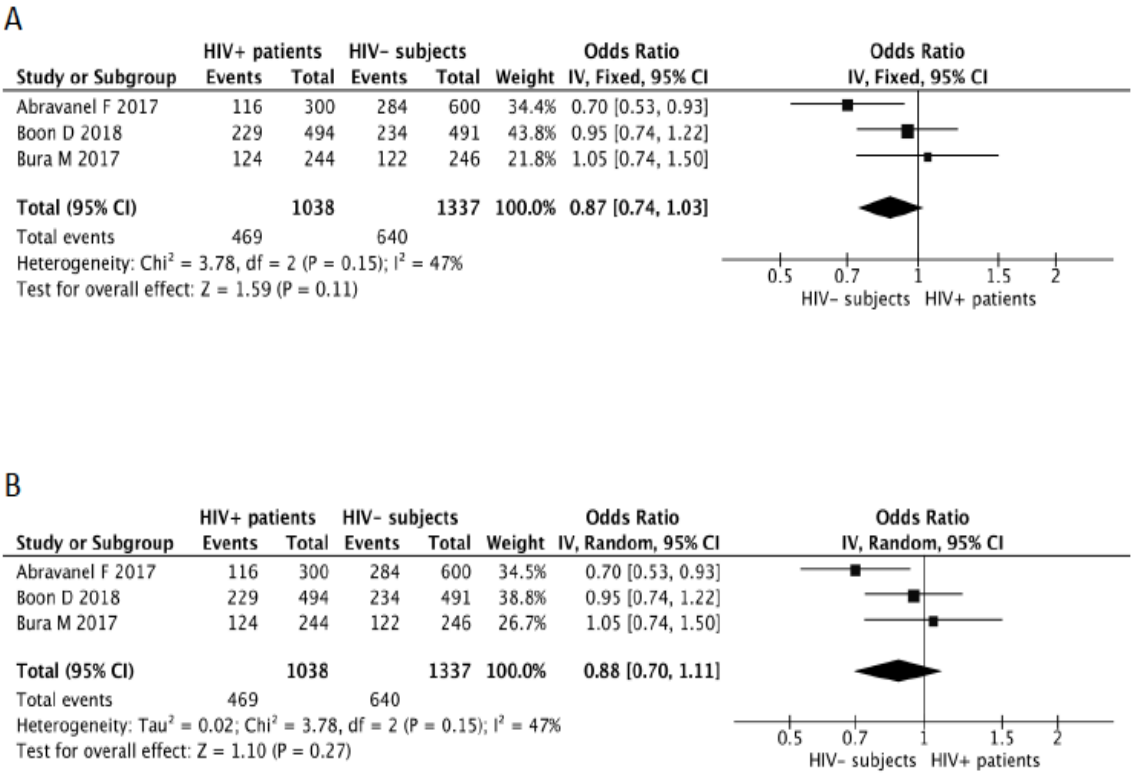

Figure 2. First meta-analysis of the HIV-associated risk of being infected by HEV. Lines represent the OR in meta-analysis and 95% CI, estimated using the inverse variance method in the fixed effects model (A) and the estimate of heterogeneity (I^2, P het) in the random effects model (B).

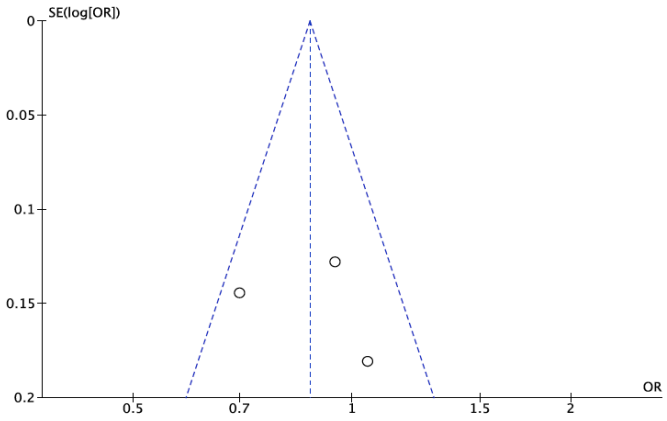

Figure 3. Graphical representation of publication bias by funnel plot of the first meta-analysis. The point cloud is distributed symmetrically around the summary estimate of the effect, indicative of absence of bias.

In the second meta-analysis the OR calculated was 1.07 (95% CI: 0.71-1.61) for random effects with a p value of 0.75. Heterogeneity was identified as severe according to the chi-squared test (X^2 = 176.01), the I^2 statistic (I^2 = 91%) and the visual representation of the forest plots for random-effects (**Figure 4**). The funnel plot did not show absence of publication bias (**Figure 5**), with the point cloud distributed asymmetrically around the summary measure of the effect.

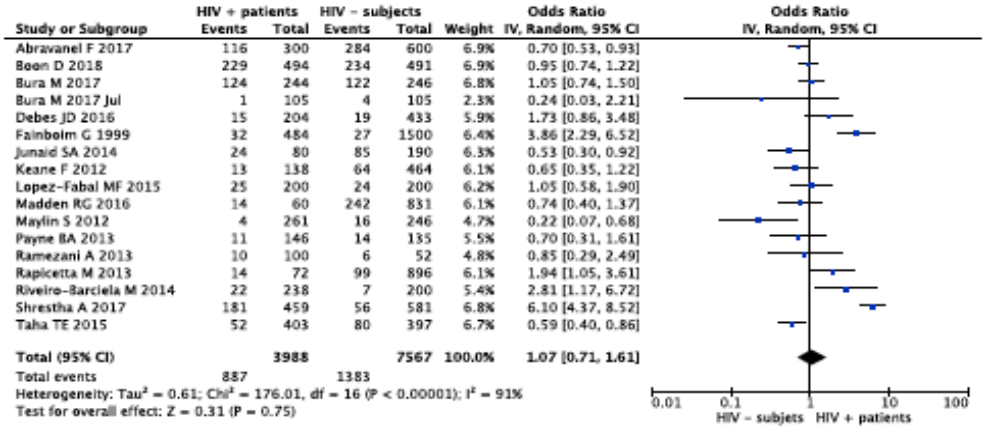

Figure 4. Second meta-analysis of the HIV-associated risk of being infected by HEV. Lines represent the OR in meta-analysis and 95% CI and the estimate of heterogeneity (I^2, P het) in the random effects model.

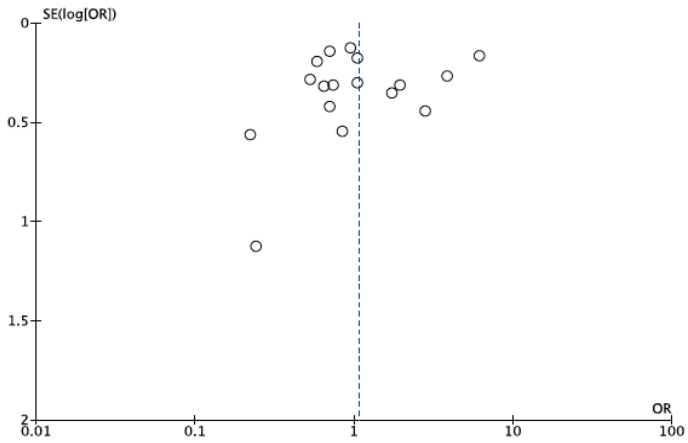

Figure 5. Graphical representation of publication bias by funnel plot of the second meta-analysis. The point cloud is distributed symmetrically around the summary estimate of the effect, and indicates the possible presence of bias.

4. Discussion

The results of our meta-analysis do not show that HIV-infected patients are at an increased risk for HEV infection compared with healthy subjects.

We selected comparative studies in the general population matched by sex, age and area. The reason for eliminating studies that were not sex-matched was that males are at a higher risk of HEV infection than females (Zeng *et al.*, 2017; Pineda *et al.*, 2014). Similarly, age-matched studies were a requirement because some studies have found age to be a risk factor for HEV infection (Rapicetta *et al.*, 2013; Junaid *et al.*, 2014; Christensen *et al.* 2018; Rivero-Juarez *et al.*, 2015), with the population aged 50 years or more being more susceptible to HEV infection. Finally, it was obligatory for studies to be matched by geographical area because this variable has been identified as an important risk factor for HEV infection (Rivero-Juarez *et al.*, 2017; Mansuy *et al.*, 2016; Cleland *et al.*, 2013), and because the genotypes and transmission routes of HEV vary according to geographical area. Studies that did not control for these risk factors could have biased the interpretation of results and so were excluded from the meta-analysis.

Our study selection controlled for all these aspects, which is why the final number of studies included in the meta-analysis is low, with only 3 articles. The meta-analysis included fixed and random effects because the level of heterogeneity was identified as moderate, with no significant differences between the two analyses. It can be interpreted therefore that heterogeneity did not influence our meta-analysis and that there was neither intra-study variability nor variability of results between the different studies. The ORs in both analyses, as well as in the three studies separately, were similar and did not identify HIV as a risk factor to be infected by HEV. Additionally, the second systematic review and the meta-analysis in which more articles were included because we used less restrictive criteria, it was observed that being HIV positive was not a risk factor for HEV infection with an OR similar to the previous meta-analysis. Nevertheless, more studies evaluating the relationship between possible susceptibility in HIV-infected patients and the various HEV genotypes are necessary.

5. Conclusions

The present study does not show that HIV-infected patients are at a higher risk of being infected by HEV compared with HIV-negative individuals. For this reason, despite the possible limitations, there are no arguments that recommend taking additional preventive measures for patients infected with HIV compared with those recommended for the general population.

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7. Supplementary Materials

Supplementary Table S1. List of excluded studies in which all the text has been evaluated.

References	Criteria for exclusion							
	First Review				Second Review			
	i	ii	iii	iv	i	ii	iii	iv*
Abebe *et al.*, 2017				X				X
Bivigou-Mboumba *et al.*, 2017	X		X	X	X		X	X
Bradley-Stewart *et al.*, 2015			X	X			X	X
Bura *et al.*, 2017				X				
Caron *et al.*, 2012			X	X			X	X
Crum-Cianflone *et al.*, 2012				X				X
Đaković Rode *et al.*, 2014			X	X			X	X
Debes *et al.*, 2016				X				
Fainboim *et al.*, 1999				X				
Feldt *et al.*, 2013			X	X			X	X
Ferreira *et al.*, 2018			X	X			X	X
Furukawa *et al.*, 2016			X				X	
Harritshøj *et al.*, 2018				X				X
Hassing *et al.*, 2014			X	X			X	X

Jacobs *et al.*, 2014				X				X
Jardi *et al.*, 2012			X	X			X	X
Joulaei *et al.*, 2015			X				X	
Junaid *et al.*, 2014				X				
Kaba *et al.*, 2011			X	X			X	X
Keane *et al.*, 2012				X				
Kenfak-Foguena *et al.*, 2011				X				X
Lanini *et al.*, 2015			X	X			X	X
López-Fabal *et al.*, 2015				X				
Madden *et al.*, 2016				X				
Mancinelli *et al.*, 2017			X	X			X	X
Mateos-Lindemann *et al.*, 2012				X				X
Mateos-Lindemann *et al.*, 2014				X				X
Maylin *et al.*, 2012				X				
Munné *et al.*, 2014				X				X
Ng *et al.*, 2000			X	X			X	X
Nouhin *et al.*, 2015			X	X			X	X
Payne *et al.*, 2013				X				
Pineda *et al.*, 2014			X	X			X	X
Pischke *et al.*, 2015			X	X			X	X
Pischke *et al.*, 2010			X	X			X	X
Politou *et al.*, 2015			X	X			X	X
Ramezani *et al.*, 2013				X				
Rapicetta *et al.*, 2013				X				
Renou *et al.*, 2010			X	X			X	X
Riveiro-Barciela *et al.*, 2014				X				
Rivero-Juarez *et al.*, 2015			X	X			X	X

Scotto *et al.*, 2014				X				X
Scotto *et al.*, 2015				X				X
Sellier *et al.*, 2011			X	X			X	X
Sherman *et al.*, 2014				X				X
Shrestha *et al.*, 2017				X				
Taha *et al.*, 2015				X				
Yong *et al.*, 2014			X	X			X	X
Zeng *et al.*, 2017			X	X			X	X
Zhou *et al.*, 2018			X	X			X	X

Legend: i) Articles written in English; ii) Studies evaluating anti-HEV IgG by ELISA in HIV-infected patients; iii) Studies comparing HEV seroprevalence between HIV patients and a control group; iv) Studies with a control group matched by sex, age and area.

*Studies with control group matched only by geographical area.

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Supplementary Table S2. PRISMA 2009 Checklist.

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	1-2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	2-3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	-
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	3
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	3
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	3-4
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	4-5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5-6
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5-6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	6

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	6
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	-
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	4,6-7
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	8
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	8
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	9-11
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	7-11
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	9-11
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	-
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	12
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	12-13
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	13
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	13-14

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

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IX. Publicación 4

frontiers in Microbiology

ORIGINAL RESEARCH
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Mutations in the Progesterone Receptor (PROGINS) May Reduce the Symptoms of Acute Hepatitis E and Protect Against Infection

Pedro López-López[1], Antonio Rivero-Juarez[1], Mario Frias[1], Isabel Machuca[1], Javier Caballero-Gómez[1,2], Israel Olivas[1], Angela Camacho[1], María de los Angeles Risalde[1], Ignacio García-Bocanegra[2] and Antonio Rivero[1]*

[1] Infectious Diseases Unit, Hospital Universitario Reina Sofía de Córdoba, Instituto Maimonides de Investigación Biomédica de Córdoba, University of Córdoba, Córdoba, Spain, [2] Department of Animal Health, University of Córdoba, Córdoba, Spain

Mutations in the progesterone receptor (PROGINS) may reduce symptoms of acute hepatitis E and protect against infection

1. Introduction

Progesterone is a steroid hormone with the attributed function of downregulating activity in the immune system (Hall *et al.*, 2017; Jones *et al.*, 2010; Butts *et al.*, 2007). In vitro studies have demonstrated that high levels of progesterone promote downregulation of proinflammatory cytokines and chemokines (Devadas *et al.*, 2018; Arruvito *et al.*, 2008). Consequently, progesterone levels may condition susceptibility to different processes, as well as clinical features and evolution.

Mutations in the progesterone receptor (PR) gene can reduce the activity of the hormone progesterone (Romano *et al.*, 2006; Romano *et al.*, 2007). These mutations in the PR were called PROGINS (Rowe *et al.*, 1995), consist of a 320-bp Alu insertion in intron G and two substitutions, one in exon 4 (V660L) and the other in exon 5 (H770H) (Romano *et al.*, 2007). In the overall population, the frequency of these mutations ranges from 0.07 to 0.26 (Modugno, 2004). Several studies have evaluated the role of PR gene polymorphisms and their associations in different pathologies including malignancies, where PROGINS could be a risk factor for uterine cancer and leiomyomas (Gallegos-Arreola *et al.*, 2015; Yuan *et al.*, 2013; Lee *et al.*, 2010), or reproductive disorders in women that can cause infertility, where carrying the PROGINS gene is a risk factor for developing endometriosis (Silva and Moura , 2016; Costa *et al.*, 2011).

PROGINS has also been observed to influence the activity of the immune system (Lhomme *et al.*, 2016) that can have an impact on the clinical features and evolution of the viral infection. In this context, the PR has been studied in relation to hepatitis E virus (HEV) infection (Debes *et al.*, 2018; Bose *et al.*, 2010), where it has been observed that those with the PROGINS gene may go on to develop a worse clinical course of hepatitis E. The aim of our study was to evaluate the influence of PROGINS on susceptibility and the

clinical course of HEV infection in HIV patients in an area with high prevalence and incidence of hepatitis E.

2. Materials and Methods

2.1. Patients

This study retrospectively included HIV patients evaluated in previous prospective studies of HEV prevalence and incidence carried out in the province of Cordoba (southern Spain) between 2012 through 2014 (Rivero-Juarez *et al.*, 2015; Rivero-Juarez *et al.*, 2017). Patient selection was based on a diagnosis of HEV infection and blood sample available. Three groups of patients were created: i) never infected, defined as IgG- and IgM-seronegative and aviremic; ii) past infection, defined as IgG positive but negative for both IgM and HEV RNA; and iii) recently infected, IgM positive and/or HEV RNA positive. Data concerning the presence of symptoms associated with HEV infection as well as epidemiological and clinical information of each patient were also collected in the recently-infected subgroup. We follow criteria for HEV screen specified in clinical guidelines (Rivero-Juarez *et al.*, 2019; EASL, 2018).

2.2. Variable collection and definition

The main outcome variable was infection with HEV, defined as past or recent infection (primary analysis). The secondary outcome variable was presence of symptoms associated with HEV infection (secondary analysis).

2.3. Anti-HEV IgG/IgM serology and RT-PCR for detection of HEV

ELISA were used for the detection of anti-HEV IgG (Wantai HEV-IgG ELISA®; Beijing Wantai Biological Pharmacy Enterprise© LTD, Beijing, China) and anti-HEV IgM (Wantai HEV-IgM ELISA®; Beijing Wantai Biological Pharmacy Enterprise© LTD, Beijing, China). The ELISAs were undertaken in accordance with instruction provided by the manufacturer

using a cut-off value of >1.1. Specimens with absorbance value to Cut-off ratio between 0.9 and 1.1 are considered borderline. All samples positive and borderline samples for anti-HEV IgG/IgM were confirmed by Western blot analysis (recomBlot HEV IgG/IgM®; Mikrogen Diagnostik GmbH, Neuried, Germany). RT-PCR for HEV RNA was performed on all patients (amplicube HEV®; Mikrogen Diagnostik GmbH, Neuried, Germany).

2.4. Determination of PROGINS

PR genotype was identified from retrospectively collected blood samples stored at -80°C until required for analysis. Genomic DNA was extracted from 200uL of blood using the QIAamp DNA Blood Mini Kit (QIAgen, Hilden, Germany) and an automated procedure (QIAcube, QIAgen, Hilden, Germany). PCR was performed with MyTaq™ DNA Polymerase (Bioline, Meridian Life Science, Memphis, USA), together with the following primers (20 μM) used to detect intron G and identify the PR genotype: forward primer 5'-GCCTCTAAAATGAAAGGCAGAAAG-3' and reverse primer 5'-GTATTTTCTTGCTAAATGTCTG-3' (Agoulnik *et al.*, 2004). The thermal profile was 95ºC for 1 min, followed by 35 cycles at 95ºC 15 s, 60ºC for 15 s and 72ºC 10 s. Electrophoresis was conducted with 10 μL of PCR products, mixed with 1.6μL of (6x) Gel Loading Dye, Blue (New England BioLabs) on 2% agarose gel with 5 μL of ethidium bromide in a volume of 150 mL for 50 minutes at a constant voltage of 90 volts. The Tracklt 100-bp DNA ladder (Invitrogen, Burlington, Ontario, Canada) was used to identify the molecular weight of bands in the agarose gel. Bands were visualized using the Molecular Imager Gel Doc XR System (BioRad, Hercules, CA).

2.5. Genotypic classification of the progesterone receptor

We classified patient genotypes by visualizing the different molecular weight bands in the gel. The 174-bp band corresponds to the wild-type genotype and the 494-bp band to the PROGINS genotype (**Figure 1**). Patients were classified prospectively as i)

homozygous wild-type; ii) homozygous PROGINS; and iii) heterozygous showing both bands. (**Figure 1**).

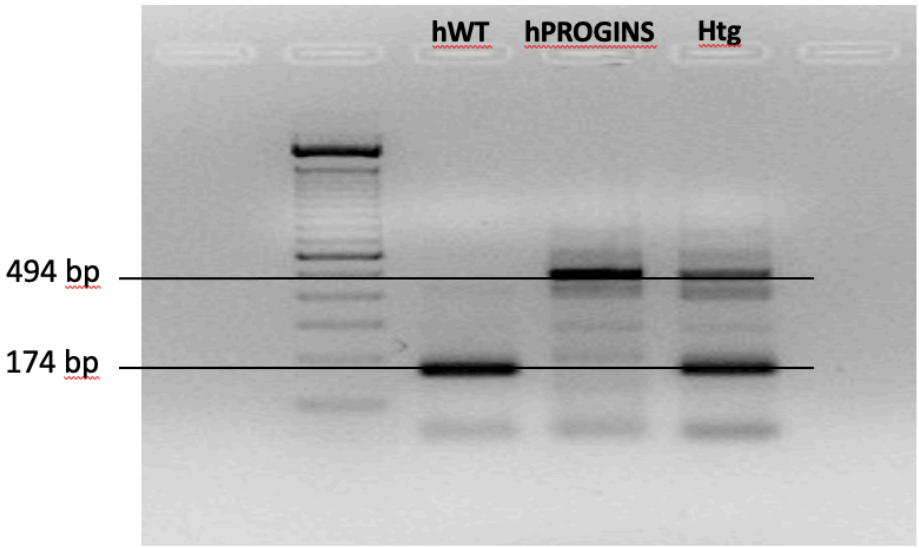

Figure 1. Identification of progesterone receptor genotypes.

Legend: homozygous wild-type (hWT); homozygous PROGINS (hPROGINS); heterozygous (Htg); base pairs (bp).

2.6. Statistical analysis

The prevalence of PR genotypes in the study population was calculated. Categorical variables were expressed as numbers of cases (percentages). Frequencies were compared using the x2 test or Fisher's exact test, and significance was set at a two-tailed p-value of less than 0.05. We use x2 test when expected values of at least 80% of the cells in a contingency 2x2 contingency table of be greater than 5. When these conditions are not verified, we use to compare qualitative variables the Fisher's exact test. We have now included this point in the Statistical analysis section. The following formula was used to calculate allele frequencies of the PR gene: 2*N homozygous + N heterozygous / 2*N total. We first evaluated the proportion of patients who were homozygous for PROGINS or not according to HEV infection (never infected [group i] vs. infected [groups ii and iii]). In patients with recent HEV infection (group iii), we also

compared the proportion of individuals who were PROGINS carriers or not according to the presence of symptoms (symptomatic vs. asymptomatic). Analyses were carried out using SPSS statistical software package version 18.0 (IBM Corporation, Somers, NY, USA).

2.7. Ethics

This study was designed and performed according to the Helsinki Declaration. The local CEIC (Clinical Trial and Ethical Committee) approved the study protocol.

3. Results

3.1. Study population

A total of 311 HIV-infected patients were included in the study: 191 (61.4%) males and 120 (38.6%) females. The distribution of patients according to HEV infection was: i) never infected, 141(45.3%); ii) past infection, 131 (42.1%); and iii) recent infection, 39 (12.6%). In terms of PR genotype, 198 were homozygous wild-type (63.7%); 91 were heterozygous (29.3%); and 22 were homozygous PROGINS (7.1%) (**Table 1**). Allele frequencies were 0.78 for the wild-type and 0.22 for the PROGINS allele which is similar to other studies (Mckenna *et al.*, 1995; Runnebaum *et al.*, 2001). In addition, allelic frequencies to PROGINS genotype were 0.21 and 0.22 in patients infected and never infected by HEV respectively.

Table 1. Prevalence of HEV infection according to progesterone receptor genotype.

Genotype	Never Infected (N=141)	Past infection (N=131)	Recent infection (N=39)	Total (N=311)
Homozygous wild-type	91 (64.5%)	81 (61.8%)	26 (66.7%)	198 (63.7%)
Heterozygous	41 (29.1%)	42 (32.1%)	8 (20.5%)	91 (29.3%)
Homozygous PROGINS	9 (6.4%)	8 (6.1%)	5 (12.8%)	22 (7.1%)

Legend: Hepatitis E Virus (HEV); Number of subjects (N).

3.2. Association between PROGINS and risk for HEV infection

The relationship between homozygous PROGINS and HEV infection in the total population was analyzed (**Table 2**). Among the homozygous PROGINS patients, 9 (40.9%) were never infected, while of the non-homozygous PROGINS patients there were 132 (45.7%) (p = 0.48) (**Table 2**). When patients were classified by sex, an association was found between PROGINS and the never-infected group in females, but not in males (**Table 2**).

Table 2. Comparative analysis of never-infected and HEV-infected patients homozygous for PROGINS in the total population and according to sex.

	Homozygous PROGINS	Never Infected (N=141)	Infected (N=170)	p value
Total	No	132 (45.7%)	157 (54.3%)	0.480
	Yes	9 (40.9%)	13 (59.1%)	
Males	No	50 (28.6%)	125 (71.4%)	0.102
	Yes	3(18.7%)	13 (81.3%)	
Females	No	82 (71.9%)	32 (28.1%)	< 0.001
	Yes	6 (100%)	0 (0%)	

Legend: Hepatitis E Virus (HEV); Number of subjects (N).

3.3. Association between PROGINS and symptomatic HEV infection

Among the 39 patients with recent HEV infection, 23 (59%) were asymptomatic and 16 (41%) showed symptomatic infection. The main symptoms identified in these patients were digestive alterations, nephropathies (chronic renal failure and pyelonephritis), febrile syndrome, hepatic cytolysis and cholestasis. In the overall analysis, none of the homozygous PROGINS patients presented symptoms (**Table 3**).

Table 3. Patients with recent HEV infection: analysis of total population and according to sex.

	Homozygous PROGINS	Asymptomatic (N=23)	Symptomatic (N=16)	p value
Total	No	18 (52.9%)	16 (47.1%)	< 0.001
	Yes	5 (100%)	0 (0%)	
Men	No	13 (48.1%)	14 (51.9%)	< 0.001
	Yes	5 (100%)	0 (0%)	
Women	No	5 (71.4%)	2 (28.6%)	NC*
	Yes	0 (0%)	0 (0%)	

*Not calculable.

Legend: Hepatitis E Virus (HEV); Number of subjects (N).

4. Discussion

The results obtained in the present study point out that the presence of the homozygous PROGINS genotype in women was associated with lower HEV seroprevalence in HIV-infected individuals. Our findings suggest that this genotype reduces susceptibility to HEV infection and is associated with a better clinical course of infection.

The function of the PR is associated with its binding to progesterone, a steroid hormone involved in modulation of the immune system (Jones *et al.*, 2010). Studies suggest that high levels of progesterone may be related to increased susceptibility to infection. Byrne *et al.* observed that women who used injectable progestin-only contraception were more susceptible to HIV infection (Byrne *et al.*, 2016). Furthermore, in vitro studies suggest that progestins could reduce the secretion of proinflammatory cytokines and chemokines and alter their function of attracting inflammatory cells, such as neutrophils and macrophages, and apoptosis of natural killer cells (Arruvito *et al.*, 2008; Huijbregts *et al.*, 2014; Devadas *et al.*, 2018; Preciado-Martínez *et al.*, 2018). At the same time, the role of progesterone in the immune system would also be influenced

by its binding to the receptor. In this context, it has been shown that PROGINS can alter the function of the progesterone hormone (Romano *et al.*, 2006; Romano *et al.*, 2007). There are two different progesterone receptors: the wild-type receptor is assumed to bind normally to progesterone, which means that progesterone levels modulate actions in the immune system naturally, while the PROGINS receptor, which is the one that presents mutations, binds more weakly to progesterone (Romano *et al.*, 2007), thereby reducing progesterone activity regardless of blood hormone levels. Consequently, the relationship between the PROGINS receptor and low progesterone activity could reduce susceptibility to HEV infection.

With respect to the symptomatology, the majority of cases of HEV infection (90%) are generally asymptomatic and self-limiting (Dalton and Seghatchian, 2016). There are however certain risk groups, such as cirrhotic patients, pregnant women and patients with HIV infection, which follow a worse clinical course (Krain *et al.*, 2014, Frias *et al.*, 2018). In the prospective studies from which the population included in this study derived, we prospectively evaluated the presence or absence of signs or symptoms of HEV infection in 39 patients who presented acute infection (Rivero-Juarez *et al.*, 2015; Rivero-Juarez *et al.*, 2017). Our study found an association between PR genotype and the development of symptoms during HEV infection: none of the HEV-infected patients with the homozygous PROGINS genotype presented symptoms.

Two previous studies found the opposite situation, namely that PROGINS mutations could be a risk factor for HEV infection (Bose *et al.*, 2011; Debes *et al.*, 2018). Both these studies focused their analyses on those with PROGINS versus those who were not carriers of this allele, without specifying homozygosity. With reference to our results, our group considered that the patient's genotype should be taken into account to analyze the effect of PROGINS, since heterozygous patients may have modulated responses through the presence of the wild-type allele. On this point, a meta-analysis performed by Pooley *et al*. suggested that the PROGINS gene has a codominant effect (Pooley *et al.*, 2006), while

another study found that the PROGINS allele has a gene dosage effect, so that expression of this gene is greater in individuals who present homozygosity (Wang-Gohrke *et al.*, 2000). In addition, Alter *et al*. observed allelic dosage effects in transient tachypnea of the newborn, in which the PROGINS gene has a protective effect against this disease (Alter *et al.*, 2010).

Our study also observed possible differences between males and females with respect to the effect of homozygous PROGINS genotype on HEV infection. Some studies in this area have identified being male as a risk factor for being infected with HEV (Pineda *et al.*, 2014, Zeng *et al.*, 2017). The differences between male and female sex hormone systems could also explain the differential immunological activity against HEV. According to Ghosh and Klein, the disparity between men and women can be found in the development of immune responses to viral infections (Ghosh and Klein, 2017). Another study found that men have worse outcomes than women in infections such as hepatitis C and B associated with sex hormones (Ruggieri *et al.*, 2018). In our study, it would appear that the homozygous PROGINS genotype effect has more of an impact on women than men in terms of being infected with HEV or developing symptoms, which may explain why being male was identified as a risk factor for HEV infection.

A limitation of our study is the number of patients included. Due to the low frequency of the PROGINS allele in our population, the number of patients homozygous for this allele was relatively low so that we had to assume that allele frequencies were constant in the population in order to perform the statistical analysis.

5. Conclusion

The PROGINS mutation in the PR gene played a protective role against infection with HEV and is associated with subclinical infection in HIV-infected patients, particularly women.

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Risk factors associated with hepatitis E virus in pigs from different production systems

Pedro Lopez-Lopez[a], Maria de los Angeles Risalde[b], Mario Frias[a], Ignacio García-Bocanegra[c], Teresa Brieva[a], Javier Caballero-Gomez[c], Angela Camacho[d], Vicente Fernández-Molera[e], Isabel Machuca[d], Jose Carlos Gomez-Villamandos[c], Antonio Rivero[d], Antonio Rivero-Juarez[a,*]

[a] Instituto Maimonides de Investigación Biomédica de Córdoba (IMIBIC), Avenida Menendez Pidal s/n, 14006, Cordoba, Spain
[b] Animal Pathology Department, Veterinary Science College, Universidad de Córdoba, Cordoba, Spain
[c] Animal Health Department, Veterinary Science College, Campus Universitario de Rabanales, 14014, Cordoba, Spain
[d] Unidad de Enfermedades Infecciosas, Hospital Provincial, Complejo Hospitalario Reina Sofía de Córdoba, Avenida Menendez Pidal s/n, 14006, Cordoba, Spain
[e] Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía, Calle Tabladilla, s/n, 41001, Sevilla, Spain

Risk factors associated with Hepatitis E virus in pigs from different production systems

1. Introduction

The hepatitis E virus (HEV) is widespread throughout the world and is the main cause of acute hepatitis (Hoofnagle *et al.*, 2012). Of the seven genotypes, genotype 3 is the main one found in Europe (Hoofnagle *et al.*, 2012; Kamar *et al.*, 2017) and is spread by zoonotic transmission (Burri *et al.*, 2014). In recent years, the number of cases reported in humans has increased in these countries (Adlhoch *et al.*, 2016; Aspinall *et al.*, 2017). Pigs are considered important reservoirs of HEV and so constitute a major risk of transmission to humans (Lange et al, 2017), either by direct contact or by consuming contaminated pork products that are raw or undercooked (Slot *et al.*, 2017; Riveiro-Barciela *et al.*, 2015). Studies carried out on European pig farms have shown a mean prevalence of anti-HEV antibodies of 35%, ranging between 20% and 95% (Lange *et al.*, 2017; Thiry *et al.*, 2014), and a mean prevalence of active HEV infection of 2.5% (Grierson *et al.*, 2015; Rose *et al.*, 2011).

Once the magnitude of the disease on European pig farms has been estimated, the identification of risk factors associated with HEV infection in these species could help determine contingency strategies to minimize the risk of transmission to humans. The aim of our study was to evaluate risk factors associated with active infection by HEV in pigs bred under different production systems in southern Spain.

2. Materials and methods

2.1. Sampling and population

Twenty-six pig farms in Cordoba province (southern Spain) (37°200 N–38°600 N, 4°010 W–5°570 W) were included in the present study conducted during October and November 2015. The farms were randomly selected, but were representative of the two main pig production systems in Spain: i) intensive (white and Iberian pigs); and ii) extensive

(Iberian pigs.) The number of pigs to be sampled at each farm was determined to ensure a 95% probability of detecting at least one positive animal, assuming a minimum prevalence of 5%.

2.2. Variable collection and definition

A 5 mL whole blood sample without additives was collected from the orbital sinus of all animals. Serum was obtained by centrifugation for 10 min at 400g and stored at -80ºC until analysis. Epidemiological information was obtained by interviewing the farmers directly. Data included age (fattening and sow), breed (white and Iberian), date of sample collection, farm location, type of farming (extensive and intensive), contact with domestic species (sheep, goat, cattle, horse, dogs and cats), contact with wild animals (wild boar and red deer), presence of a sanitary ford and quarantine.

2.3. RT-PCR for detection of HEV

Viral RNA was extracted from 200ul of serum using the commercial QIAamp Mini Elute Virus Spin Kit (QIAgen. Hilden, Germany) and an automated procedure (QIAcube. QIAgen, Hilden, Germany). For diagnosis of HEV infection, RT-PCR was performed using the LightCycler 480 system (Roche. Basel, Switzerland), described elsewhere (Abravanel *et al.*, 2012). For the reaction, the QIAgen OneStep PCR Kit (QIAgen, Hilden, Germany) was used. The primers (20uMol) employed were: sense primer HEV5260 (5´-GGTGGTTTCTGGGGTGAC-3´) and antisense primer HEV5330 (5´-AGGGGTTGGTTGGATGAA-3´). The probe employed (15uMol) was HEV5283 (5´-FAM-TGATTCTCAGCCCTTCGC-TAMRA-3´). The thermal profile was 50ºC for 30 min and 95ºC for 15 min, followed by 45 cycles of 94ºC for 10s, 55ºC for 20s and 72ºC for 60s. An external (in-run) standard curve was applied to calculate HEV viral load using a WHO Standard HEV strain (consistent with genotype 3a) supplied by the Paul-Ehrlich-Institute (code 6329/10).

2.4. Statistical analysis

The prevalence of active HEV infection in the study population was calculated. Prevalence of active infection according to animal and farm characteristics was also calculated. For HEV prevalence, a two-sided, 95% confidence interval (95% CI) was calculated, based on exact binomial distributions. Categorical variables were expressed as number of cases (percentages). Frequencies were compared using the x2 test or Fisher's exact test, and significance was set at a two-tailed p-value of less than 0.05. Bivariate analysis was carried out to discover which variables were related to HEV infection. Multivariate logistic regression analysis included variables related to the outcome variable in bivariate analysis with a p-value of less than 0.2 or a possible association. Analyses were carried out using the SPSS statistical software package version 18.0 (IBM Corporation, Somers, NY, USA).

3. Results

3.1. Population

A total of 1,040 pigs from 26 farms were included in this study. Figure 1 shows the geographical distribution of the farms included. Twenty sows and 20 fattening pigs were randomly selected from each herd according to age; a total of 520 were fattening pigs and 520 were sows.

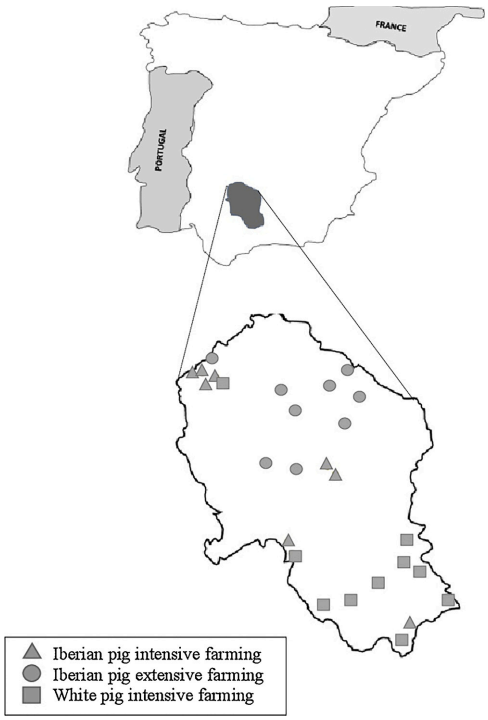

Figure 1. Distribution and type of farms.

3.2. Global prevalence and risk factors

One hundred and seventy-two pigs were infected by HEV, meaning an individual prevalence of 16.5% (95% CI: 14.4%-18.9%). Results of the descriptive analysis are shown in Table 1. There was a significantly higher prevalence of HEV among pigs on extensive farms than among those bred on intensive farms (23.9% vs. 12.6%; p <0.001). The prevalence of HEV infection was lower among pigs on farms with a quarantine period compared to those without quarantine (10.6% vs. 20.8%; p =0.006). Farms that used sanitary ford showed a lower prevalence of HEV than those that did not use them (12.7% vs. 33.8%; p <0.001). Among animals in contact with other domestic animals, the prevalence of HEV infection was 24.5%, compared with 3.9% in animals without such contact (p <0.001). Age, breed, and contact with wildlife animals were not identified as factors associated with HEV infection. In multivariate analysis, independent risk factors associated with HEV infection were: extensive farming, absence of sanitary ford, no quarantine period, and contact with domestic animals (**Table 1**).

Table 1. Prevalence of HEV infection according to risk factors and OR univariate and multivariate analysis.

Factor	Condition	Prevalence			Univariate analysis		Multivariate analysis	
		N	Positive HEV	% (95% CI)	OR (95% CI)	p value	OR (95% CI)	p value
Age	Sow	520	81	15.6 (12.7-19.0)	1	0.404	1	1.000
	Fattening	520	91	17.5 (14.5-21.0)	1.150 (0.828-1.595)		1.000 (0.613-1.630)	
Breed	White	400	62	15.5 (12.3-19.4)	1	0.476	-	-
	Iberian	640	110	17.2 (14.5-20.3)	1.131 (0,805-1.589)		-	
Farming	Intensive	680	86	12.6 (10.3-15.4)	1	<0.001	1	0.040
	Extensive	360	86	23.9 (19.8-28.6)	2.168 (1.556-3.020)		2.239 (1.036-4.837)	
Sanitary ford	Yes	560	71	12.7 (10.2-15.7)	1	<0.001	1	0.001
	No	240	81	33.8 (28.1-40.0)	3.509 (2.435-5.056)		3.597 (1.649-7.850)	
Quarantine period	Yes	160	17	10.6 (6.7-16.4)	1	0.006	1	0.002

	No	400	83	20.8 (17.1-25.0)	2.202 (1.261-3.848)		2.723 (1.450-5.112)	
Contact with domestic species	Yes	600	147	24.5 (21.2-28.1)	7.936 (4.222-14.914)	<0.001	3.893 (1.453-10.431)	0.007
	No	280	11	3.9 (2.1-7.0)	1		1	
Contact with wildlife	Yes	160	32	20.0 (14.5-26.9)	1.179 (0.765-1.816)	0.456	-	-
	No	720	126	17.5 (14.9-20.5)	1		-	

Legend: number of cases (N); hepatitis E virus (HEV); 95% confidence interval (95% CI); odds ratio (OR).

3.3. Extensive farming, absence of biosecurity measures and contact with domestic animals as independent risk factors for HEV infection: multivariate analysis

We included all variables identified in the bivariate analysis, as well as age, in a logistic regression multivariate analysis. In this analysis, extensive farming (OR=2.239; $p < 0.04$), absence of biosecurity measures, such absence of sanitary ford (OR=3.597; $p < 0.001$) and no quarantine period (OR=2.723; $p < 0.002$), as well as contact with domestic animals (OR=3.893; $p < 0.007$) as possible source of crossspecies transmission of HEV, were identified as independent risk factors associated with HEV infection (**Table 2**).

Table 2. Multivariate analysis.

Factor	Condition	β	OR	95% CI	p value
Age	Sow	0	1	0.613-1.630	1.000
	Fattening		1.000		
Farming system	Intensive	0.806	1	1.036-4.837	0.040
	Extensive		2.239		

Sanitary ford	Yes	1.208	1	1.649-7.850	0.001
	No		3.597		
Quarantine period	Yes	1.002	1	1.450-5.112	0.002
	No		2.723		
Contact with domestic species	Yes	1.359	3.893	1.453-10.431	0.007
	No		1		

Legend: number of cases (N); hepatitis E virus (HEV); 95% confidence interval (95% CI); odds ratio (OR). Constant β = -4.26

4. Discussion

HEV infection would be expected to be more prevalent in pigs reared in intensive farming conditions, due to overcrowding and the degree of direct contact between the animals, although this has not been observed in previous studies. Nevertheless, there is some disagreement between studies that have compared different pig production systems. A study conducted in the Netherlands of pigs raised under extensive and intensive farming systems found similar HEV seroprevalence, suggesting that exposure to HEV in both systems was comparable and that access to outdoor areas was not a risk factor for HEV infection (Rutjes *et al.*, 2014). On the other hand, Jori *et al.* performed a study in Corsica (France) and found significantly higher seropositivity in domestic pigs bred outdoors compared with those bred on closed farms (Jori *et al.*, 2016). In our study, there was a higher prevalence of HEV infection on extensive compared with intensive farms, and extensive farming was, therefore, implicated as a risk factor for HEV infection in Mediterranean ecosystems.

The higher prevalence of infection under extensive farming conditions observed in our study could have been due to contact with other animal species or by environmental contamination, including waterborne, that could be potential reservoirs for HEV transmission (Burri *et al.*, 2014; Baez *et al.*, 2017). Likewise, our study found a strong association between contact with domestic animal species and the prevalence of HEV, which could therefore be a potential source of transmission. It was shown that sheep, goats (Long *et al.*, 2017), cows (Huang *et al.*, 2016) and horses (Saad *et al.*, 2007), and possibly

cat and dogs (Zeng *et al.*, 2017) could be a risk factors for transmitting the virus to pigs and humans. Our study suggests that contact between domestic animals and pigs could be an important source of HEV infection on pig farms. On the other hand, wildlife animals, such as wild boar and red deer, are also well known reservoirs of the HEV (Prpić *et al.*, 2015; Kukielka *et al.*, 2016; Schlosser *et al.*, 2014), although our study did not identify the presence of wildlife species as a risk factor for HEV infection. In a previous study performed by our group, we suggest that the prevalence of HEV in boars may depend on the season (October-February) (Rivero-Juarez *et al.*, 2018). Our study period coincided with the hunting season, so that contact with wild boars may have been a risk factor for transmitting HEV to domestic pigs, since it is at the same time that pigs on extensive farms are moved outdoors. This interface should also be studied.

Biosecurity measures are important for reducing or eliminating infection on farms. These include fencing, the disinfection of vehicles, use of boots or clothing to visit the farm, control programs for rodents, among others (Casal *et al.*, 2007; Simon-Grifé *et al.*, 2013). In our study, hygiene and sanitary measures, in particular the absence of sanitary ford and no quarantine period, were identified as the main factors associated with the prevalence of HEV infection. The usefulness of implementing these measures has been widely described in connection with containing other viral diseases. In this context, an evaluative study of porcine reproductive and respiratory syndrome virus (PRRSV) infection was performed by Fablet *et al.* in Western France, who reported that a long quarantine period between 40-60 days of isolation reduced the risk of transmission of this viral infection (Fablet *et al.*, 2016). Quarantine prevents or reduces the spread of infection due to the inclusion of new infected pigs to the herd during the incubation period. Further longitudinal studies should be performed to assess specifically the impact of implementing these measures on reducing HEV infection.

There are several limitations to the present study. First, the number of farms of different systems included was relatively low. Second, with respect to the variables

selected, it would be very difficult to identify and record every variable with a possible association with HEV infection and also to determine and quantify the multiple modifications that certain variables could undergo over time, such as contact with domestic or wild animals, whose presence will vary depending on many other factors, such as season and transfer to other locations. Third, domestic species have been analyzed together, so we cannot identify which animals have been most important in the transmission of HEV to pigs. Fourthly, in this study we did not evaluate the prevalence of other infections shown to have a synergistic effect with HEV, such as porcine circovirus-2 (PCV2) or PRRSV (Yang *et al.*, 2015; Salines *et al.*, 2015).

5. Conclusions

Our study shows evidence that pigs extensively reared are at greater risk of being infected by HEV than those bred on intensive farms. Contact between pigs and other domestic animals could be a source of infection. Farms without sanitary fords or quarantine periods were shown to be risk factors affecting the prevalence of HEV infection in pigs. These results emphasize the fact that the use of control measures could reduce the risk of HEV infection in pigs and consequently minimize the risk of zoonotic transmission.

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XI. Conclusiones/Conclusions

Conclusiones

1. Nuestro ensayo pudo detectar todos los genotipos descritos del género *Orthohepevirus* A y mostró un mayor rendimiento que los kits comerciales. Por tanto, nuestro ensayo puede suponer una mejora significativa en el diagnóstico molecular de la infección por el VHE con importantes implicaciones clínicas y epidemiológicas.

Artículo: "Development and clinical validation of a pangenotypic PCR- based assay for the detection and quantification of hepatitis E virus (*Orthopevirus* A genus)". Journal of Clinical Microbiology (2021) (*Autores con igual contribución).

2. Nuestro estudio proporciona evidencia de que en el algoritmo diagnóstico de la infección por VHE en pacientes con hepatitis aguda es necesaria la determinación tanto de anticuerpos IgM como de ARN de VHE. En este sentido, el uso único de uno de estos marcadores podría dar lugar a una proporción importante de casos mal diagnosticados.

Artículo: "Limited Value of Single Sampling for IgM Antibody Determination as a Diagnostic Approach for Acute Hepatitis E Virus Infection". Microbiology Spectrum (2021).

3. Nuestro estudio no muestra que los pacientes infectados por el VIH tengan mayor riesgo de infección por el VHE en comparación con los individuos VIH negativos. Por ello, a pesar de las posibles limitaciones, no existen argumentos que recomienden tomar medidas preventivas adicionales para los pacientes infectados por el VIH en comparación con las recomendadas para la población general.

Artículo: "Human Immunodeficiency Virus Infected Patients are Not at Higher Risk for Hepatitis E Virus Infection: A Systematic Review and Meta-Analysis". Microorganisms (2019).

4. La mutación PROGINS en el gen RP juega un papel protector contra la infección por VHE, y está asociada con la infección subclínica en pacientes infectados por VIH, particularmente mujeres.

Artículo: “Mutations in the Progesterone Receptor (PROGINS) May Reduce the Symptoms of Acute Hepatitis E and Protect Against Infection”. Frontiers in Microbiology (2019).

5. Nuestro estudio muestra evidencia de que los cerdos criados en extensivo tienen un mayor riesgo de infectarse por el VHE que los criados en intensivo. Se demostró que no usar vado sanitario o período de cuarentena en las explotaciones son factores de riesgo de infección para el VHE en los cerdos. Además, el contacto entre cerdos y otros animales domésticos puede ser una fuente de infección.

Artículo: “Risk factors associated with hepatitis E virus in pigs from different production systems”. Veterinary Microbiology (2018).

Conclusions

1. Our assay was able to detect all described genotypes of the genus *Orthohepevirus* A and showed higher performance than commercial kits. Therefore, our assay may represent a significant improvement in the molecular diagnosis of HEV infection with important clinical and epidemiological implications.

Article: "Development and clinical validation of a pangenotypic PCR- based assay for the detection and quantification of hepatitis E virus (*Orthopevirus* A genus)". Journal of Clinical Microbiology (2021) (*Autores con igual contribución).

2. Our study provides evidence that in the diagnostic algorithm of HEV infection in patients with acute hepatitis, the determination of both IgM antibodies and HEV RNA is necessary. In this regard, the use of only one of these markers could result in a significant proportion of misdiagnosed cases.

Article: "Limited Value of Single Sampling for IgM Antibody Determination as a Diagnostic Approach for Acute Hepatitis E Virus Infection". Microbiology Spectrum (2021).

3. Our study does not show that HIV-infected patients are at increased risk of HEV infection compared to HIV-negative individuals. Thus, despite possible limitations, there is no argument to recommend taking additional preventive measures for HIV-infected patients compared to those recommended for the general population.

Article: "Human Immunodeficiency Virus Infected Patients are Not at Higher Risk for Hepatitis E Virus Infection: A Systematic Review and Meta-Analysis". Microorganisms (2019).

4. The PROGINS mutation in the RP gene plays a protective role against HEV infection, and is associated with subclinical infection in HIV-infected patients, particularly women.

Article: “Mutations in the Progesterone Receptor (PROGINS) May Reduce the Symptoms of Acute Hepatitis E and Protect Against Infection”. Frontiers in Microbiology (2019).

5. Our study shows evidence that extensively raised pigs are at higher risk of HEV infection than intensively raised pigs. Not using a sanitary fence or quarantine period on farms was shown to be a risk factor for EHV infection in pigs. In addition, contact between pigs and other domestic animals can be a source of infection.

Article: “Risk factors associated with hepatitis E virus in pigs from different production systems”. Veterinary Microbiology (2018).

XII. ANEXOS

ANEXO I: OTRAS PUBLICACIONES DERIVADAS DEL TRABAJO DEL DOCTORANDO

1. Caballero-Gómez J, Rivero-Juarez A, Jurado-Tarifa E, Jiménez-Martín D, Jiménez-Ruiz E, Castro-Scholten S, Ulrich RG, López-López P, Rivero A, García-Bocanegra I. Serological and molecular survey of hepatitis E virus in cats and dogs in Spain. TRANSBOUNDARY AND EMERGING DISEASES. 2021 Dec 24. doi:10.1111/tbed.14437. PMID: 34951935. IF (JCR) 5.005 (4/146) Q1 / T1 / D1 Science Edition-VETERINARY SCIENCES.

2. Rivero-Juárez A, Dashti A, Santín M, Köster PC, López-López P, Risalde MA, García-Bocanegra I, Gómez-Villamandos JC, Caballero-Gómez J, Frías M, Bailo B, Ortega S, Muadica AS, Calero-Bernal R, González-Barrio D, Rivero A, Briz V, Carmena D. Diarrhoea-causing enteric protist species in intensively and extensively raised pigs (Sus scrofa domesticus) in Southern Spain. Part II: Association with Hepatitis E virus susceptibility. TRANSBOUNDARY AND EMERGING DISEASES. 2021 Nov 30. doi:10.1111/tbed.14408. PMID: 34850588. IF (JCR) 5.005 (4/146) Q1 / T1 / D1 Science Edition-VETERINARY SCIENCES.

3. Dashti A, Rivero-Juárez A, Santín M, George NS, Köster PC, López-López P, Risalde MA, García-Bocanegra I, Gómez-Villamandos JC, Caballero-Gómez J, Frías M, Bailo B, Ortega S, Muadica AS, Calero-Bernal R, González-Barrio D, Rivero A, Briz V, Carmena D. Diarrhoea-causing enteric protist species in intensively and extensively raised pigs (Sus scrofa domesticus) in Southern Spain. Part I: Prevalence and genetic diversity. TRANSBOUNDARY AND EMERGING DISEASES. 2021 Nov 9. doi:10.1111/tbed.14388. PMID: 34755463. IF (JCR) 5.005 (4/146) Q1 / T1 / D1 Science Edition-VETERINARY SCIENCES.

4. Rivero-Juarez A, Risalde M., Gortázar C, Lopez-Lopez P, Barasona JA, Frias M, Caballero-Gomez J, de la Fuente J, Rivero A. Detection of hepatitis E virus in Hyalomma lusitanicum ticks feeding on wild boars. FRONTIERS IN MICROBIOLOGY. 2021 Jul 9;12:692147. doi:10.3389/fmicb.2021.692147 PMID: 34305854. IF (JCR) 5.640 (28/136) Q1/T1 MICROBIOLOGY.

5. Casares-Jimenez M, Lopez-Lopez P, Caballero-Gomez J, Frias M, Perez-Hernando B, Oluremi AS, Risalde MA, Ruiz-Caceres I, Opaleye OO, Garcia-Bocanegra I, Rivero-Juarez A, Rivero A. Global molecular diversity of Hepatitis E virus in wild boar and domestic pig. One Health. 2021 Aug 9;13:100304. doi:10.1016/j.onehlt.2021.100304. PMID: 34466650. IF (JCR) 3.800 (58/203) Q2/T1 PUBLIC, ENVIRONMENTAL & OCCUPATIONAL HEALTH.

6. Fatawou Modiyinji A, Rivero-Juarez A, Lopez-Lopez P, Amougou Atsama M, Gwladys Monamele C, Nola M., Rivero A, Njouom R. First molecular characterization of the hepatitis E virus in humans in Cameroon: Confirmation of the HEV outbreak in Touboro, North-Cameroon. JOURNAL MEDICINE OF VIROLOGY. 2021 Jun;93(6):4018-4022. doi:10.1002/jmv.26277 PMID: 32639604 IF (JCR) 2.327 (27/39) Q3/T3 Science Edition-VIROLOGY.

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Rivero A. Evaluation of a non-invasive screening approach to determine hepatitis E virus status of pig farms. VETERINATY RECORD. 2020. 187 (7):272. doi:10.1136/vr.105840 PMID: 32576682 IF (JCR) 2.695 (25/146) Q1 / T1 Science Edition-VETERINARY SCIENCES.

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Infected Patients. FRONTIERS IN MICROBIOLOGY. 2019 Jun 26;10:1425. doi:10.3389/fmicb.2019.01425 PMID: 31297100 IF (JCR) 5.640 (28/136) Q1 / T1 MICROBIOLOGY.

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hepatitis E virus RNA in saliva for diagnosis of acute infection. ZOONOSES AND PUBLIC HEALTH. 2018. 65 (5) 584-588. doi:10.1111/zph.12472 PMID: 29659194 IF (JCR) 2.702 (24/146) Q1 / T1 Science Edition-VETERINARY SCIENCES

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28. Frias M, Rivero-Juarez A, Tellez F, Perez-Perez M, Camacho A, Machuca I, Lorenzo-Moncada S, Lopez-Lopez P, Rivero A. Spontaneous clearance of chronic hepatitis C is rare in HIV-infected patients after effective use of combination antiretroviral therapy. PLOS ONE. 2017. 12(5):-e0177141. doi:10.1371/journal.pone.0177141 PMID: 28472191 IF (JCR) 3.240 (26/72) Q2 / T2 Science Edition-MULTIDISCIPLINARY SCIENCES

ANEXO II: COMUNICACIONES A CONGRESOS INTERNACIONALES Y NACIONALES DERIVADOS DEL PROYECTO DE TESIS DOCTORAL

A. Internacionales

1. Limited value of IgM antibody determination as single screening approach for hepatitis E virus. 31st European Congress of Clinical Microbiology and Infectious Diseases. 09/07/2021-12/07/2021. European Society of Clinical Microbiology and Infectious Diseases. A. Rivero-Juarez; P. López-López; J.A. Pineda; J.C. Alados; A. Fuentes-Lopez; E. Ramirez-Arellano; C. Freyre; A. B. Perez; M. Frias; A. Rivero.

2. Hepatitis E rabbit genotype infection in HIV-infected patients. 27th Conference on Retroviruses and Opportunistic Infections. Boston, Estados Unidos de América. 08/03/2020-11/03/2020. IAS-USA. A. Rivero-Juárez; M. Frías; P. López-López; J. Berenguer; F. García; J. Macías; Begoña Alcaraz; A. Castro; J. Caballero-Gomez; A. Rivero.

3. Subtipo emergente del virus de la hepatitis E en España. 37èmes Rencontres du Groupe d'Etudes sur l'Eco-pathologie de la Faune Sauvage de Montagne. Etroubles, Francia. 13/06/2019-16/06/2019. Groupe d'Etudes sur l'Eco-pathologie de la Faune Sauvage de Montagne. J. Caballero-Gómez; A. Rivero-Juarez; S. Jiménez-Ruiz; P. López-López; M.A. Risalde; D. Cano-Terriza; M. Frias; J.A. Barasona; A. Rivero; I. García-Bocanegra.

4. Parsonage-Turner syndrome associated with hepatitis E infection. 29th European Congress of Clinical Microbiology & Infectious Diseases. Amsterdam, Holanda. 13/04/2019-16/04/2019. European Society of Clinical Microbiology and Infectious Diseases. C. Mendoza López; P. López López; M. Frias Casas; A. Rivero-Juarez; R.Benito.

5. Prevalence of hepatitis E virus infection in wild boar in Mediterranean eco-system: Can microRNAs act as biomarkers of chronic infections? Reunión de Ungulados Silvestres Ibéricos (RUSI). Granada, Andalucía, España. 04/10/2018-06/10/2018. Grupo de Investigación RNM118 Especies Cinegéticas y Plagas. J. Caballero-Gómez; J.J. Garrido; M.A.

Risalde; S. Zaldívar-López; A. Martínez-Padilla; P. López-López; D. Cano-Terriza; S. Jiménez-Ruiz; I. Zafra; I. García-Bocanegra.

6. Circulación del virus de la hepatitis E y papel regulador de los microARNs en jabalíes infectados de forma natural en el sur de España. 36èmes rencontres du Groupe d'Etudes sur l'Eco-pathologie de la Faune Sauvage de Montagne (GEEFSM). Orlu, Francia. 13/09/2018-16/09/2018. Groupe d'Etudes sur l'Eco-pathologie de la Faune Sauvage de Montagne (GEEFSM). Caballero-Gómez J.; Garrido J. J.; Risalde M. A.; Zaldívar-López S.; Martínez-Padilla A.; López-López P.; Cano-Terriza D.; Jiménez-Ruiz S.; Zafra I.; García-Bocanegra I.

7. Hepatitis E virus in sympatric cattle and wild ungulates in the Doñana National Park, Spain. I Hepatitis E Workshop: Paradigma of a food-borne zoonotic emerging disease in Europe. Madrid, Comunidad de Madrid, España. 04/06/2018-05/06/2018. Centro VISAVET, Universidad Complutense de Madrid. J. Caballero; I. García-Bocanegra; S. Jiménez-Ruíz; J. Vicente; A. Rivero; M.A. Risalde; J.A. Barasona; P. López-López; A. Rivero-Juárez.

8. HLA-B, HLA-C and KIR Improve the Predictive Value of IL28B for Hepatitis C Spontaneous Clearance. 16th European AIDS Conference. Milán, Lombardia, Italia. 25/10/2017-27/10/2017. European AIDS Clinical Society. A. Rivero-Juarez; M. Frias; D. Rodriguez-Cano; P. López-López; A. Camacho; T. Brieva; I. Machuca; M.A. Risalde; A. Rivero.

9. Sofosbuvir/ledispavir as cholesterol-increase risk factor in HIV subjects. Conference on retroviruses and opportunistic infections (CROI) 2017. Boston, Estados Unidos de América. 13/02/2017-17/02/2017. IAS-USA. M. Frías; A. Rivero-Juárez; F. Cuenca-López; A. Camacho; D. Rodríguez-Cano; P. López-López; T. Brieva; A. Rivero.

B. Nacionales (solo los de autor principal)

1. Prevalencia del virus de la Hepatitis E en alimentos de origen porcino. 3er Congreso

Andaluz de Salud Pública. Córdoba, Andalucía, España. 23/09/2021-24/09/2021. Consejo Andaluz de Colegios Oficiales de Veterinarios. P. López López; M. Casares Jiménez; J. Caballero Gómez; A. Martín Gómez; J. Martínez Blasco; I. Agulló Ros; I.García Bocanegra; J.C. Gómez Villamandos; A. Rivero Juárez; M. Risalde.

2. Cinética de anticuerpos del VHE en pacientes VIH cirróticos. 6º Congreso Nacional del Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la SEIMC. 21/09/2021-23/09/2021. Entidad organizadora: Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). P. López López; M. Frías; G. García Delgado; I. Machuca; J. Caballero Gómez; M. Casares Delgado; M.A. Risalde; I. Ruíz Cáceres; I. García Bocanegra; I. Zafra Soto; I. Pérez Valero; L. Ruíz Torres; J.C. Gómez Villamandos; Á. Camacho; A. Rivero Juárez; A. Rivero.

3. Incidencia de la infección por el virus de la hepatitis E en pacientes trasplantados renales. 6º Congreso Nacional del Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la SEIMC. 21/09/2021- 23/09/2021. Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). P. López López; A. Páez Vega; A. Rivero Juárez; S. Cantisán; C. Facundo Molas; M. Sunyer; R. Escudero Sánchez; J.R. Yuste Ara; S. Rodríguez; R. Rodríguez Álvarez; D. Redondo; C. González Rico; I. Los Arcos; M. López Oliva; P. Ruiz Esteban; J.M. Aguado; J. De La Torre Cisneros.

4. Seroreversión de anticuerpos IGG frente al virus de la hepatitis E en pacientes trasplantados renales. 6º Congreso Nacional del Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la SEIMC. 21/09/2021-23/09/2021. Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). P. López López; A. Páez Vega; S. Cantisán; A. Rivero Juárez; I. Silva Torres; M. Sunyer; J. Fortún; J.R. Yuste Ara; M. Palomo; R. Rodríguez Álvarez; D. Redondo; C. González Rico; I. Los Arcos; M. López Oliva; D. Hernández; M. Fernández Ruiz; J. De La Torre Cisneros.

5. ¿Tienen los pacientes VIH mayor riesgo de infección por VHE? Resultados de un

metaanálisis. XI Congreso Nacional GeSIDA y XIII Reunión Docente de la Red de Investigación de SIDA (RIS). Toledo, Castilla-La Mancha, España. 10/12/2019-13/12/2019. Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica. P López López; M Frías; A Camacho; I Zafra Soto; L Ruiz Torres; I Ruiz Cáceres; V Muñoz Moreno; J Caballero Gómez; L Milla Serrano; A Rivero Juarez; Antonio Rivero.

6. Identificación y clasificación de cepas del VHE circulantes en España. XXI Congreso de la Sociedad Andaluza de Enfermedades Infecciosas. Sevilla, Andalucía, España. 21/11/2019-23/11/2019. Sociedad Andaluza de Enfermedades Infecciosas (SAEI). P López López; M Frías; F Téllez; E Ramírez de Arellano; F Gracias; P Rincón; I Zafra Soto; C Freyre; MJ Ríos; M Fernández; A Rivero Juárez.

7. Los pacientes VIH infectados no tienen mayor riesgo de infección por el virus de la hepatitis E: resultados de un metaanálisis. XXI Congreso de la Sociedad Andaluza de Enfermedades Infecciosas. Sevilla, Andalucía, España. 21/11/2019-23/11/2019. Sociedad Andaluza de Enfermedades Infecciosas (SAEI). P López López; M Frías; A Camacho; I Zafra Soto; L Ruiz Torres; I Ruiz Cáceres; V Muñoz Moreno; J Caballero Gómez; L Milla Serrano; A Rivero Juarez; Antonio Rivero.

8. Identificación y clasificación de cepas del VHE circulantes en Andalucía: Estudio GEHEP-0017. V Congreso Nacional del Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). Cáceres, Extremadura, España. 26/09/2019-28/09/2019. Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). P López López; M Frías; F Téllez; E Ramírez de Arellano; F Gracias; P Rincón; I Zafra Soto; C Freyre; MJ Ríos; M Fernández; A Rivero Juárez.

9. Los pacientes VIH infectados no tienen mayor riesgo de infección por el virus de la hepatitis E: resultados de un metaanálisis. V Congreso Nacional del Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la Sociedad Española de Enfermedades Infecciosas y

Microbiología Clínica (SEIMC). Cáceres, Extremadura, España. 26/09/2019-28/09/2019. Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC). P López López; M Frías; A Camacho; I Zafra Soto; L Ruiz Torres; I Ruiz Cáceres; V Muñoz Moreno; J Caballero Gómez; L Milla Serrano; A Rivero Juarez; Antonio Rivero.

10. HIV-infected patients are not at higher risk for HEV infection: A systematic review and meta-analysis. X Jornadas de Jóvenes Investigadores del Instituto Maimónides de Investigación Biomédica de Córdoba. Córdoba, Andalucía, España. 16/05/2019-17/05/2019. Instituto Maimónides de Investigación Biomédica de Córdoba. Lopez-Lopez P.; Frias M.; Camacho A.; Rivero A.; Caballero-Gómez J.; Milla-Serrano L.; Rivero-Juarez A.

11. Efecto de las mutaciones del receptor de la progesterona (RP) en la susceptibilidad y curso clínico de la infección por el VHE. XX Congreso de la Sociedad Andaluza de Enfermedades Infecciosas (SAEI). Jerez de la Frontera, Andalucía, España. 29/11/2018-01/12/2018. Sociedad Andaluza de Enfermedades Infecciosas (SAEI). P López López; I Zafra; L Ruiz Torres; M Frias; T Brieva; I Reyes Torres; I Machuca; A Camacho; A Rivero; A Rivero Juarez.

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13. La tasa de infección por el virus de la hepatitis E en donantes de sangre asintomáticos sugiere la aplicación de cribado universal. IV Congreso Nacional del Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la SEIMC. Córdoba, Andalucía, España. 27/09/2018-29/09/2018. Sociedad Española de Enfermedades Infecciosas y Mircrobiologia

Clinica. P López López; I Zafra Soto; L Ruiz Torres; M Frías Casas; T Brieva Herrero; I Reyes Torres; I Machuca Sánchez; A Camacho Espejo; A Rivero Román; A Rivero Juárez.

14. Resultados iniciales de la implantación de un programa de cribado de la hepatitis e en pacientes con hepatitis aguda. XIX Congreso de la Sociedad Andaluza de Enfermedades Infecciosas (SAEI). Granada, Andalucía, España. 14/12/2017-16/12/2017. Sociedad Andaluza de Enfermedades Infecciosas (SAEI). P López-López; I Zafra; MA Risalde; M Frías; I Machuca; L Ruiz-Torres; A Camacho; T Brieva; A Rivero; A Rivero-Juárez.

15. Diagnóstico de infección aguda por Hepatitis E genotipo 3 mediante detección viral en saliva. III Congreso Nacional del Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la SEIMC. Sevilla, Andalucía, España. 28/09/2017-30/09/2017. Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica. P. López-López; I. Zafra-Soto; L. Ruiz-Torres; M. Frías; M.A. Risalde; T. Brieva; I. Machuca; B. Marmesat; A. Camacho; A. Martínez-Peinado; A. Rivero; A. Rivero-Juárez.

16. Prevalencia e incidencia de la infección por el virus de la Hepatitis E en pacientes con fallo renal crónico en pre y post trasplante. III Congreso Nacional del Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la SEIMC. Sevilla, Andalucía, España. 28/09/2017-30/09/2017. Sociedad Española de Enfermedades Infecciosas y Mircrobiologia Clinica. P López-López; R Aguado; M Frias; I Zafra; A Paez-Vega; C Rodelo-Haard; A Salinas; A Rodríguez-Benot; M Suñer; JR Yuste; M Montejo; AM Fernández; P Muñoz; C Facundo; S Cantisán; A Rivero; J Torre-Cisneros; A Rivero-Juárez.

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18. Transmisión del virus de la Hepatitis E por transplante hepático: Estudio HepE-Tx. III Congreso Nacional del Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la SEIMC. Sevilla, Andalucía, España. 28/09/2017-30/09/2017. Sociedad Española de Enfermedades Infecciosas y Mircrobiologia Clinica. P López-López; R Aguado; M Sánchez-Frías; M Frias; I Zafra; A Paez-Vega; A Salinas; S Cantisán; M Medina; M De la Mata; A Rivero; J Torre-Cisneros; A Rivero-Juárez.

19. Prevalencia del virus de la hepatitis E en caballos de Andalucía: Estudio HEPEHORSE. XVIII Congreso de la Sociedad Andaluza de Enfermedades Infecciosas (SAEI). Córdoba, Andalucía, España. 24/11/2016-26/11/2016. Sociedad Andaluza de Enfermedades Infecciosas (SAEI). P López-López; I García-Bocanegra; M Frías; MA Risalde; I Zafra; D Ruíz; A Martínez-Peinado; L Ruiz-Torres; T Brieva; I Machuca; A Camacho; A Rivero; JC Gomez-Villamandos; A Rivero-Juárez. 17 - S1,ISSN 1576-3129

20. Prevalencia del virus de la hepatitis E en cerdos de la provincia de córdoba: estudio HEPEPORK. XVIII Congreso de la Sociedad Andaluza de Enfermedades Infecciosas (SAEI). Córdoba, Andalucía, España. 24/11/2016-26/11/2016. Sociedad Andaluza de Enfermedades Infecciosas (SAEI). P López-López; MA Risalde; M Frías; I García-Bocanegra; I Zafra; D Ruíz; A Martínez-Peinado; T Brieva; I Machuca; A Camacho; A Rivero; JC Gomez-Villamandos; A Rivero-Juárez. 17 - S1, ISSN 1576-3129

21. Prevalencia del virus de la hepatitis E en jabalíes de la provincia de Córdoba: Estudio HEPEBOAR. XVIII Congreso de la Sociedad Andaluza de Enfermedades Infecciosas (SAEI). Córdoba, Andalucía, España. 24/11/2016-26/11/2016. Sociedad Andaluza de Enfermedades Infecciosas (SAEI). P López-López; MA Risalde; M Frías; I García-Bocanegra; I Zafra; D Ruíz; A Martínez-Peinado; L Ruiz-Torres; T Brieva; I Machuca; A Camacho; A Rivero; JC Gomez-Villamandos; A Rivero-Juárez. 17 - S1, ISSN 1576-3129

ANEXO III: CAPITULOS DE LIBRO DERIVADOS DEL PROYECTO DE TESIS DOCTORAL

Camacho Espejo A., Frías Casas M., López López P., Rivero Juárez A. Capítulo 8: Coinfección por VHC y otros virus hepatotropos: actualización 2020. TAFICS SL. VIH & TAR. Novedades 2021 (231-270)

ANEXO IV: PROYECTOS DE INVESTIGACIÓN FINANCIADOS EN CONVOCATORIA PÚBLICA COMPETITIVA EN LOS QUE PARTICIPA EL DOCTORANDO

1. Título: Impacto de la infección aguda por Virus de Hepatitis E en pacientes infectados por el VIH

Investigador principal: Antonio Rivero Juárez

Entidad financiadora: Instituto de Salud Carlos III

Cód. según financiadora: PI16/01297

Fecha de inicio-fin: 01/01/2017 - 30/06/2020

Cuantía total: 92.565 €

2. Título: RED ESPAÑOLA DE INVESTIGACIÓN EN SIDA (RIS)

Investigador principal: Antonio Rivero Román

Entidad financiadora: Instituto de Salud Carlos III

Cód. según financiadora: RD16/0025/0034

Fecha de inicio-fin: 02/02/2017 - 31/12/2021

Cuantía total: 143.000 €

3. Título: Infectividad Del Virus De La Hepatitis E Detectado En Alimentos De Origen Porcino Utilizando Modelos Animales Y Cultivos Celulares (Estudio Hepefood)

Investigador principal: María de los Ángeles Risalde Moya

Entidad financiadora: Consejería de Salud, Junta de Andalucía

Cód. según financiadora: PI-0287-2019

Fecha de inicio-fin: 01/01/2020 - 31/12/2022

Cuantía total: 65.009 €

4. Título: Trazabilidad epidemiológica y microbiológica de la infección por el virus de la Hepatitis E: Estudio TrazHE

Investigador principal: Antonio Rivero Juárez

Entidad financiadora: Instituto de Salud Carlos III

Cód. según financiadora: PI19/00864

Fecha de inicio-fin: 01/01/2020 - 31/12/2022

Cuantía total: 68.970 €

5. Título: Evaluación de la infección por *Orthohepevirus* C como causa emergente de enfermedad de origen zoonótico

Investigador principal: Antonio Rivero Román

Entidad financiadora: Instituto de Salud Carlos III

Cód. según financiadora: PI21/00793

Fecha de inicio-fin: 01/09/2021 – 01/09/2024

Cuantía total: 90.750 €

ANEXO V: ESTANCIA EN CENTRO INTERNACIONAL DURANTE EL PERIODO PREDOCTORAL

Centro: Department of Veterinary Sciences, Abel Salazar Institute of Biomedical Sciences (ICBAS), Faculty of Pharmacy, Laboratory of Microbiology, Department of Biological Sciences, Epidemiology Research Unit (EPIUnit), Instituto de Saúde Pública da Universidade do Porto, Porto, Portugal.

Duración: 4 meses. (11 de enero al 15 de mayo de 2021)

Responsable: D.V.M., M.Sc., PhD. João Rodrigo Mesquita Contacto: jrmesquita@icbas.up.pt. jmesquita@outlook.com

Dirección: Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal.

ANEXO VI: PUBLICACIONES DERIVADAS DEL TRABAJO DEL DOCTORANDO DURANTE LA ESTANCIA EN CENTO INTERNACIONAL

Moraes D, Lopez-Lopez P, Palmeira JD, Torres RT, Rivero-Juarez A, Dutra V, Nascimento M, Mesquita JR. Screening for hepatitis E virus genotype 3 in red deer (Cervus elaphus) and fallow deer (Dama dama), Portugal, 2018-2020. TRANSBOUNDARY AND EMERGING DISEASES. 2021 Dec 16. doi:10.1111/tbed.14427. PMID: 34913605. IF (JCR) 5.005 (4/146) Q1 / T1 / D1 Science Edition-VETERINARY SCIENCES.

ANEXO VII: PREMIOS DE INVESTIGACION DERIVADOS DEL PROYECTO DE TESIS DOCTORAL

1. Premio a la mejor comunicación en el III Congreso Nacional del Grupo de Estudiode las Hepatitis Víricas de la SEIMC. “Diagnóstico de Infección Aguda por Hepatitis E mediante detección viral en saliva”, Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (GEHEP/SEIMC). Septiembre de 2017.

2. Premio a las mejores comunicaciones en el XXI CONGRESO DE LA SOCIENDAD ANDALUZA DE ENFERMEDADES INFECCIOSAS. "Identificación yclasificación de cepas del VHE circulantes en España", Sociedad Andaluza de Enfermedades Infecciosas (SAEI). Noviembre de 2019.

3. Premio al reconocimiento a la mejor comunicación presentada en el III Congreso Andaluz de Salud Pública Veterinaria. “Prevalencia del virus de la Hepatitis E en alimentos de origen porcino”. Septiembre de 2021.