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Epidemiological survey and risk factors associated with *Paslahepevirus balayani* in equines in Europe

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ABSTRACT

Paslahepevirus balayani (HEV) is an important emerging zoonotic virus in Europe. Although domestic pigs and wild boar are the main reservoirs of this pathogen, susceptibility to this virus has been confirmed in a growing number of animal species, including equines. However, their role in the epidemiology of this virus remains poorly understood. Our aim was to assess HEV circulation and identify potential risk factors associated with exposure in equid species in different European countries. A total of 596 equines, including 496 horses, 63 donkeys and 37 mules/hinnies bred in four European countries (Spain, Italy, United Kingdom and Ireland) were sampled. Thirty-three animals (5.5%; 95%CI: 3.7–7.4) had anti-HEV antibodies. Seropositivity was found in 4.6% of horses, 11.1% of donkeys and 8.1% of mules/hinnies tested. By country, 6.3%, 5.4%, 5.0% and 4.0% of the equines sampled in Spain, Italy, United Kingdom and Ireland, respectively, were seropositive, respectively. Statistical analysis showed that "species" and "drinking water from ponds and streams" were potential risk factors associated with HEV seropositivity in equines in Europe. HEV RNA was not detected in any (0.0%; 95%CI: 0.0–1.8) of the 202 equines tested. Our results provide evidence of a low, spatially homogeneous and widespread viral circulation that is not equal across species in equid populations in the European countries analyzed and indicate that these species appear to play a limited role in the epidemiology of this virus. Further studies are required to elucidate the differences in seroprevalence between donkeys, mules/hinnies and horses and to determine the risk of zoonotic transmission of this pathogen from equid species.

1. Introduction

Hepatitis E virus, formally known as *Paslahepevirus balayani* (HEV; family *Hepeviridae*), is the main cause of acute viral hepatitis in humans worldwide. Eight different genotypes (HEV-1 to HEV-8) have been confirmed to date, of which HEV-1 to HEV-4 are the most important from the point of view of public health. HEV-1 and HEV-2 affect only humans, mainly in developing countries, whereas HEV-3 and HEV-4 are zoonotic and are frequently reported in high-income regions, including Europe. In Europe, the number of autochthonous cases of this genotype has increased considerably in recent years [1–3], with most infections being acquired through consumption of animal products or the contact with infected animals [2,4]. Domestic pigs (*Sus scrofa domesticus*) and wild boar (*Sus scrofa*) are considered the main reservoirs of HEV, but viral circulation has been confirmed in a growing number of mammal species over the last two decades [5].

Throughout history, equines have been closely associated with humans not only as a common source of food, but also as working

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animals used for transport, agriculture and also for recreation. Previous studies have shown that these species, which are found worldwide, may be potential sources of zoonotic pathogens, including HEV [6–8]. However, even though the European Food Safety Authority has highlighted the need to develop surveillance programs for HEV in equines [9], there is very little information on the role of these species in the worldwide epidemiology of this pathogen worldwide. In this context, the aims of the present study were to assess HEV circulation and to identify potential risk factors associated with viral exposure in equids in different European countries.

2. Material and methods

A cross-sectional study was conducted in different European countries between 2013 and 2021. Blood samples from 496 horses (*Equus ferus caballus*) were collected in four countries of Europe: Spain ($n = 200$; including 100 from Andalusia (southern Spain) and 100 from Catalonia (northeastern Spain)), Italy ($n = 100$), United Kingdom (n = 100) and Ireland ($n = 96$). The total sample size was calculated as 498 horses, assuming a prevalence of 50% with a 97.5% confidence level (97.5%CI) and a desired precision of ±5% [10]. Whenever possible, at least 99 horses from each country were sampled to detect exposure with a 95% probability and a minimum within-country seroprevalence of 3%. In addition, samples from donkeys (*Equus africanus asinus*) ($n = 63$: 34 from Spain, 27 from Italy, 2 from Ireland) and two equid hybrids, mules/hinnies (*E. africanus* × *ferus*) ($n = 37$: 34 from Spain, 2 from Italy, 1 from Ireland) were also gathered during the study period using convenience sampling.

Blood samples were collected by puncture of the jugular vein and sera were obtained by centrifugation at 400*g* for 10 min and kept frozen at −20 °C until laboratory analysis. Individual animal and herd epidemiological information was collected for each animal whenever possible (Table 2). The presence of anti-HEV antibodies was assessed using the commercial double-antigen multispecies sandwich HEV ELISA 4.0 (MP Diagnostics, Illkirch, France), following the manufacturer's instructions. This assay uses the highly conserved recombinant ET2.1 protein of the HEV capsid to coat the plates [11], and detects total antibodies (IgG, IgM and IgA) to the virus in serum from a wide range of animals, including perissodactyls [12].

In parallel, a subset of approximately one third of the sampled animals (165 horses, 24 donkeys and 13 mules/hinnies) was randomly selected for the molecular analyses, including six seropositive and 196 seronegative samples. Viral nucleic acids were from 400 µl of pooled serum (100 µl per animal) using the QIAmp MinElute Virus Spin kit (QIAGEN, Hilden, Germany). The presence of HEV RNA was determined using three different RT-PCR assays in parallel (Table 1). A real-time RT-PCR that is able to detect all HEV genotypes was performed, using 10 µl of RNA template and the One Step PrimeScript III RT-PCR Kit (Takara Bio, Shiga, Japan) [13]. Two nested broad-spectrum RT-PCRs for the detection of hepevirus were also performed [14,15]. For these two assays, the first and second PCR rounds were performed using the One Step PrimeScript III RT-PCR kit (Takara Bio, Shiga, Japan), and the 2× premixed solution containing Taq DNA polymerase, dNTPs and reaction buffer (Promega, Madison, WI, USA), respectively. The nested RT-PCRs amplicons were examined on 1.5% agarose gel stained with RedSafe™ Nucleic Acid Staining solution (iNtRON Biotechnology, Seongnam, Korea).

3. Statistical analysis

The prevalence of antibodies and the prevalence of viremic animals to HEV were estimated from the ratio of ELISA- or RT-PCR- positive animals, respectively, to the total number of analyzed animals, with a 95%CI. The continuous variable "intra-farm equid count" was categorized using the 33rd and 66th percentiles as cut-off points. Associations between seroprevalence to HEV and explanatory variables were analyzed using the Pearson's chi-square or Fisher's exact tests. Variables with $p < 0.10$ in bivariate analysis were included for further analysis. Collinearity between pairs of variables was tested by Spearman's Rho. Finally, a multivariable analysis was performed using a generalized estimating equations (GEE) model. The number of seropositive animals was assumed to follow a binomial distribution and "municipalities" was defined as the subject variable. Variables with p values <0.05 in the GEE model were considered statistically significant. Statistical analyses were performed using SPSS 25.0 software (Statistical Package for Social Sciences, Inc., Chicago, IL, USA).

4. Results and discussion

Since the first description in animals in 1997 [16], the host range of HEV has expanded considerably [17]. Since then, the susceptibility of equines to HEV has been confirmed [7] and contact with horses has been suggested as a risk factor for human HEV infection [18]. Nevertheless, only a small number of studies have assessed exposure to this zoonotic pathogen in equines worldwide [19]. To the best of our knowledge, this is the first large-scale study to assess HEV exposure in domestic equines in Europe. A total of 33 (5.5%; 95%CI: 3.7–7.4) of the 596 equids analyzed showed antibodies against HEV. By species, the frequencies of seropositivity in horses, donkeys and mules/hinnies were 4.6%, 11.1% and 8.1%, respectively (Table 2), confirming that these three equine species are naturally exposed to this zoonotic virus in Europe. The seroprevalence detected in horses (4.6%) is lower than that previously found in this equine species in other parts of the world, including serosurveys carried out in China (16.3%; 8/49) [20], Korea (12.4%; 35/283) [21] and Egypt (13.0%; 26/200) [22]. In contrast, the seropositivity observed in donkeys (11.1%) was similar to that detected in the only large study conducted in this species to date (Rui et al., 2019), whose authors found that 49 (12.2%) of the 401 donkeys sampled in China had anti-HEV antibodies. Consistent with this, one of the three donkeys tested from a zoo in Germany was exposed to HEV [23]. Besides horses and donkeys, exposure to this virus has been shown in other equine species, such as Przewalski's horse (*Equus caballus przewalski*) and the Somali wild ass (*Equus africanus somaliensis*) [12,23]. The presence of HEV antibodies in mules in our study is consistent with the

Table 1
Molecular assays and list of primers and probe used in the present study.

PCR	Oligonucleotide	Sequence (5′-3′)	Reference
Real-time RT-PCR	FWD-Pangenotypic	RGTRGTTTCTGGGGTGAC	[13]
	RVS-Pangenotypic	AKGGRTTGGTTGGRTGA	
	Probe-Pangenotypic	TGAYTCYCARCCCTTCGC-TAMRA	
Broad spectrum nested RT-PCR	HEV-cs	TCGCGCATCACMTTYTTCCARAA	[14]
	HEV-cas	GCCATGTTCCAGACDGTRTTCCA	
	HEV-csn	TGTGCTCTGTTTGGCCCNTGGTTYC†G	
	HEV-casn	CCAGGCTCACCRGARTGYTTCTTCCA	
Broad spectrum heminested RT-PCR	HEV-F4228	ACYTTYTGTGCYYTITTTGGTCCITGGTT	[15]
	HEV-R4598	GCCATGTTCCAGAYGGTGTTCCA	
	HEV-R4565	CCGGGTTCRCCIGAGTGTTTCTTCCA	

Table 2
Distribution of *Paslahepevirus balayani* seroprevalence in equid species by category and results of bivariate analysis.

Variable	Categories	No. positives/ Overall*	Seroprevalence (%)	*P*
Species	Donkey	7/63	11.1	0.083
	Horse	23/496	4.6	
	Mule/hinny	3/37	8.1	
Age	Geriatric	7/134	5.2	0.663
	Adult	13/252	5.2	
	Foal	7/92	7.6	
Sex	Female	19/327	5.8	0.463
	Male	14/265	5.3	
Breed	Pure	19/312	6.1	0.352
	Crossed	8/168	4.8	
Activity	Sport	8/163	4.9	0.730
	Milk production	1/23	4.3	
	Work	3/43	7.0	
	Breeder	9/120	7.5	
	Family leisure	7/174	4.0	
Country	Ireland	4/99	4.0	0.846
	Italy	7/129	5.4	
	Spain	17/268	6.3	
	United Kingdom	5/100	5.0	
Breed	Pure	19/312	6.1	0.352
	Crossed	8/168	4.8	
Sampling year	2013	5/74	6.8	0.819
	2015	0/9	0.0	
	2016	0/14	0.0	
	2017	2/66	3.0	
	2018	2/27	7.4	
	2019	17/270	6.3	
	2021	6/121	5.0	
Intra-farm equid count	<18	8/158	5.1	0.861
	18–100	8/159	5.0	
	>100	8/152	5.3	
Density of ticks	High	5/88	5.7	0.243
	Low	2/15	13.3	
	Absence	9/226	4.0	
Shelter (autumn-winter)	Outdoor	10/188	5.3	0.754
	Indoor	11/240	4.6	
	Indoor/ Outdoor	3/41	7.3	
	Outdoor	17/292	5.8	
Shelter (spring-winter)	Indoor	4/131	3.1	0.442
	Indoor/ Outdoor	3/46	6.5	
Presence of dogs	Yes	15/258	5.8	0.409
	No	6/130	4.6	
Presence of cats	Yes	13/232	5.6	0.470
	No	10/159	6.3	
Presence of domestic ruminants	Yes	4/44	9.0	0.241
	No	8/161	5.0	
Presence of poultry	Yes	6/93	6.5	0.434
	No	12/226	5.3	
Contact with horses outside the farm	Yes	4/84	4.8	0.561
	No	14/267	5.2	
Contact with wildlife	Yes	14/242	5.8	0.519
	No	10/224	4.5	
Tap water to drink	Yes	12/250	4.8	0.340
	No	16/266	6.0	
Well water to drink	Yes	7/132	5.3	0.572
	No	21/384	5.5	
Water from ponds and streams to drink	Yes	4/30	13.3	0.071
	No	24/486	4.9	
Cleaning of facilities	Yes	20/419	4.8	0.246
	No	4/50	8.0	
Disinfection of facilities	Yes	9/124	7.3	0.154
	No	15/344	4.4	
Deworming program	Yes	23/449	5.1	0.520
	No	2/51	3.9	
Rodent control	Yes	13/259	5.0	0.509
	No	11/204	5.4	

* Missing values omitted.

previous detection of HEV RNA in this hybrid species in Spain [7] and confirms susceptibility to this virus.

Seropositive equids were detected in all four countries analyzed, with seroprevalences ranging from 4.0% (4/99) in Ireland to 6.3% (17/268) in Spain (Table 2 and Fig. 1), and in 27 (15.4%) of the 175 municipalities sampled. Seropositivity was also found in five of the seven years sampled. These results suggest widespread and homogeneous circulation of HEV in equids in Europe during the last decade. Notably, antibodies against HEV were detected in a yearling foal sampled in Ireland in 2019, which suggests circulation of this virus during that year. However, since the ELISA can detect IgG, IgM and IgA and the exact age of this animal was unknown, the presence of maternal antibodies cannot be ruled out.

The GEE model showed that "species" was a potential risk factor associated with HEV seropositivity (Table 3). The risk of exposure to HEV was 2.1 times higher in donkeys than in horses ($p < 0.001$), which could be associated with a higher genetic susceptibility to HEV infection in donkeys or unequal exposure of the two species to the virus in Spain, where the highest seropositivity was found in donkeys. The high environmental stability of the virus [24] and the frequent handling of donkeys in extensive systems in this region may explain the higher risk of contact with this virus. In any case, further studies including a larger number of donkeys from the different countries analyzed are needed to confirm this hypothesis.

Significantly higher seroprevalence was found in equids that used ponds and streams (13.3%) for drinking compared to those that did not have access to these water sources (4.9%) ($p < 0.001$) (Table 3). HEV has been detected in a wide variety of water sources [25]. Although HEV-1 and HEV-2 are responsible for waterborne outbreaks in humans in developing countries, contaminated water has also been suggested as a source of infections by zoonotic HEV genotypes, not only in humans [26,27] but also in other mammal species, such as domestic pigs. Walachowski et al. [28] observed a significantly higher prevalence among swine that drank water from springs or shallow wells than in those whose water sources were tap water or deep wells. Similarly, Holt et al. [29] found that pigs on farms where manure ended up in water sources were more likely to be exposed to HEV. Our results support the hypothesis that untreated or undertreated water may be a source of HEV infection in mammals, but further studies are warranted to assess the role of environmental waters in the eco-epidemiology of HEV [30].

Apart from domestic pigs, and to a lesser extent non-human primates (NHPs), the course of HEV infection in other animal species has scarcely been studied. Experimental studies in these species have shown limited HEV viraemia lasting between one and four weeks after infection [31,32]. In our study, none (0.0%; 95% CI: 0.0–1.8) of the 202 serum samples tested were positive for HEV RNA, which suggests absence of active circulation of HEV during the study period. However, given that the ELISA detects total antibodies and the presence of IgM in seropositive animals cannot be discarded, a recent exposure cannot be ruled out. Absence of circulation or a low prevalence of active HEV infection has also been reported in horses from Korea (0%; 0/397), Spain (0.4%; 3/692), China (2.0%; 1/49) and Egypt (4.0%; 4/100) [7,20–22], in donkeys from Spain (1.2%; 1/86) and China (4.2%; 17/401) [7,33], and in mules from Spain (3.6%; 3/83) [7]. Nevertheless, the high homology between HEV strains detected in humans and equines in previous studies suggests a potential risk of zoonotic transmission to humans [7,12,20,33].

In conclusion, the seropositivity found in the present study provides evidence of natural exposure to HEV, but low, spatially homogeneous and widespread viral circulation that is not equal across species in different equid populations in Europe. Our results suggest that consumption of contaminated water could be a potential source of HEV in mammals. Serological and molecular results indicate that equines

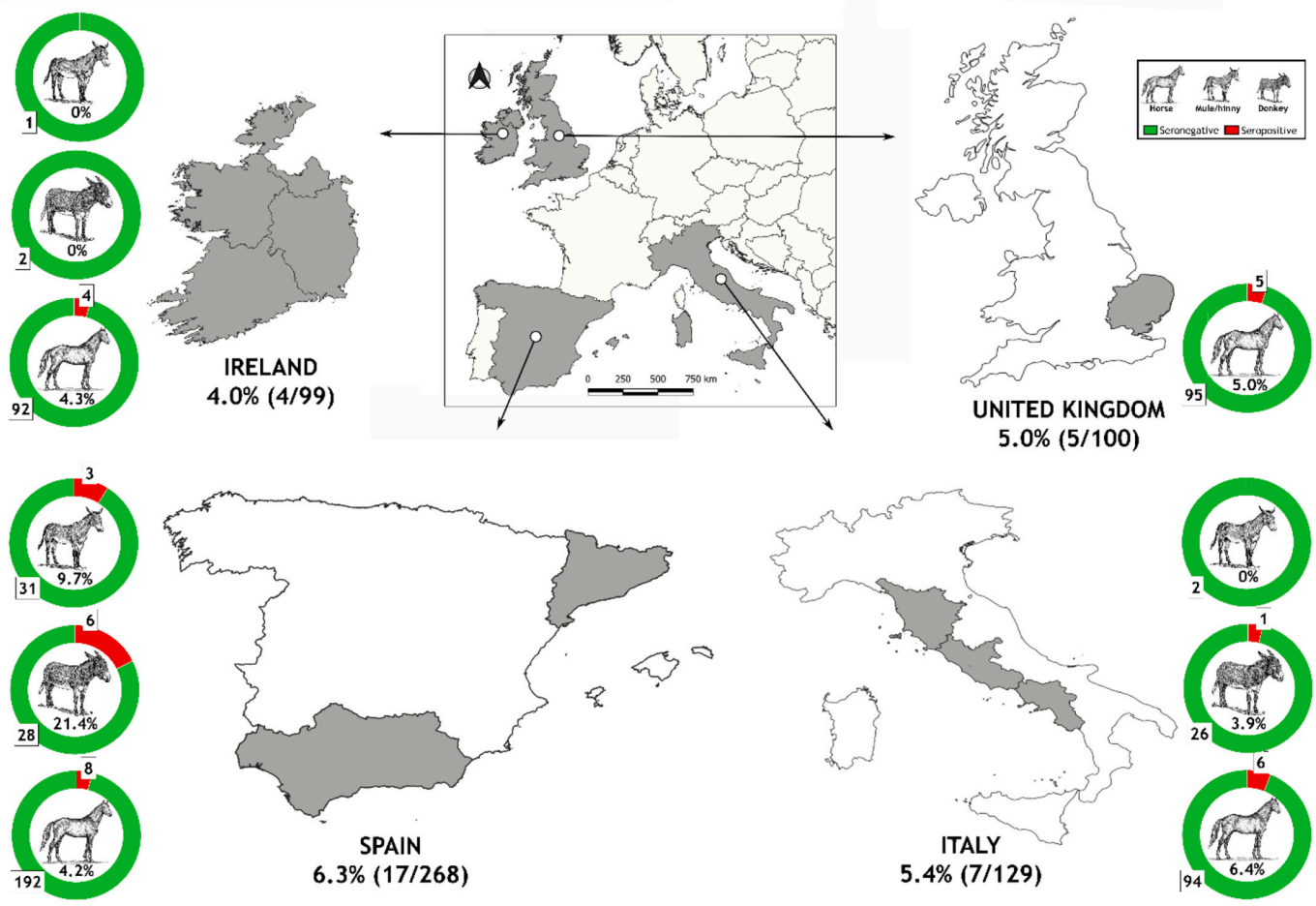

Fig. 1. Seropositivity of *Paslahepevirus balayani* by equid species in different European countries.

Table 3
Results of the generalized estimating equation analysis of potential risk factors associated with HEV exposure in equid species in different European countries.

Variable	Categories	β	*P*	OR (95% CI)
Species	Mules/ hinnies	1.046	0.057	2.9 (0.9–8.4)
	Donkeys	0.746	<0.001	2.1 (1.7–2.6)
	Horses	[a]	[a]	[a]
Water from ponds and streams to drink	Yes	1.073	<0.001	2.9 (2.0–4.3)
	No	[a]	[a]	[a]

[a]Reference category.

appear to play a limited role in the epidemiology of this virus. Further studies are required to determine differences in HEV exposure among donkeys, mules and horses and to determine the risk of zoonotic transmission of this pathogen from equid species.

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Ethical statement

Horse samples were obtained within the official Epidemiological Surveillance System Programs and from specimens subjected to medical check-ups or surgical interventions during the study period. Animal handling and sampling were performed by qualified and trained veterinarians following European (Directive 86/609/CEE). Therefore, no ethical approval was necessary.

Declaration of Competing Interest

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

Data availability

The data that support the findings of this study are available from the authors upon reasonable request.

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