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Serological and virological survey of hepatitis E virus (HEV) in animal reservoirs from Uruguay reveals elevated prevalences and a very close phylogenetic relationship between swine and human strains

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ABSTRACT

Hepatitis E virus (HEV) infection is an issue of public health concern in high-income and non-endemic countries. Increasing evidence supports the hypothesis of a zoonotic route as the main mode of infection in this epidemiological setting, since the transmission of genotypes HEV-3 and HEV-4 from reservoirs to humans has been demonstrated.

In America, studies have confirmed the circulation of HEV in pig herds but the zoonotic role of wild boars has never been evaluated.

Uruguay has a high burden of HEV- associated acute hepatitis, and a close phylogenetic relationship was observed among human HEV-3 strains and European isolates detected in swine. However in this context, swine herds have never been surveyed.

Herein is reported a survey of HEV in swine herds, pigs at slaughter-house and free-living wild boar populations.

Two-hundred and twenty sera and 150 liver tissue samples from domestic pigs, and 140 sera from wild boars were tested for HEV by ELISA and PCR-based approaches.

All tested swine farms resulted seropositive with an overall rate of 46.8%. In turn, 22.1% of the wild boars had anti-HEV antibodies. HEV RNA was detected in 16.6% and 9.3% of liver samples from slaughter-age pigs and adult wild boars sera, respectively. Three strains from domestic pig were also amplified by nested-PCR approaches. By contrast, none of the positive samples obtained from wild boars could be confirmed by nested-PCR.

Phylogenetic analysis revealed a very high nucleotide identity among swine strains and sequences obtained from humans in Uruguay.

Results showed that HEV is widely distributed among swine herds in Uruguay. Additionally, this study evidences for the first time in the American continent that wild boar populations are a reservoir for HEV, though its zoonotic role remains to be elucidated. Altogether, data presented here suggest a high zoonotic risk of HEV transmission from swine to humans.

1. Introduction

Hepatitis E virus (HEV) is a major etiological agent of acute hepatitis, responsible for outbreaks and large epidemics associated with water-borne transmission in many endemic regions from Asia and Africa (Khuroo, 1991; Rein et al., 2012). In high-income and non-endemic countries, HEV infection occurs as sporadic autochthonous cases, and moderated-elevated rates of anti-HEV IgG antibodies and RNA detection have been reported in selected populations. Seroprevalence rates as high as 39.1% were identified In Southern France, while HEV

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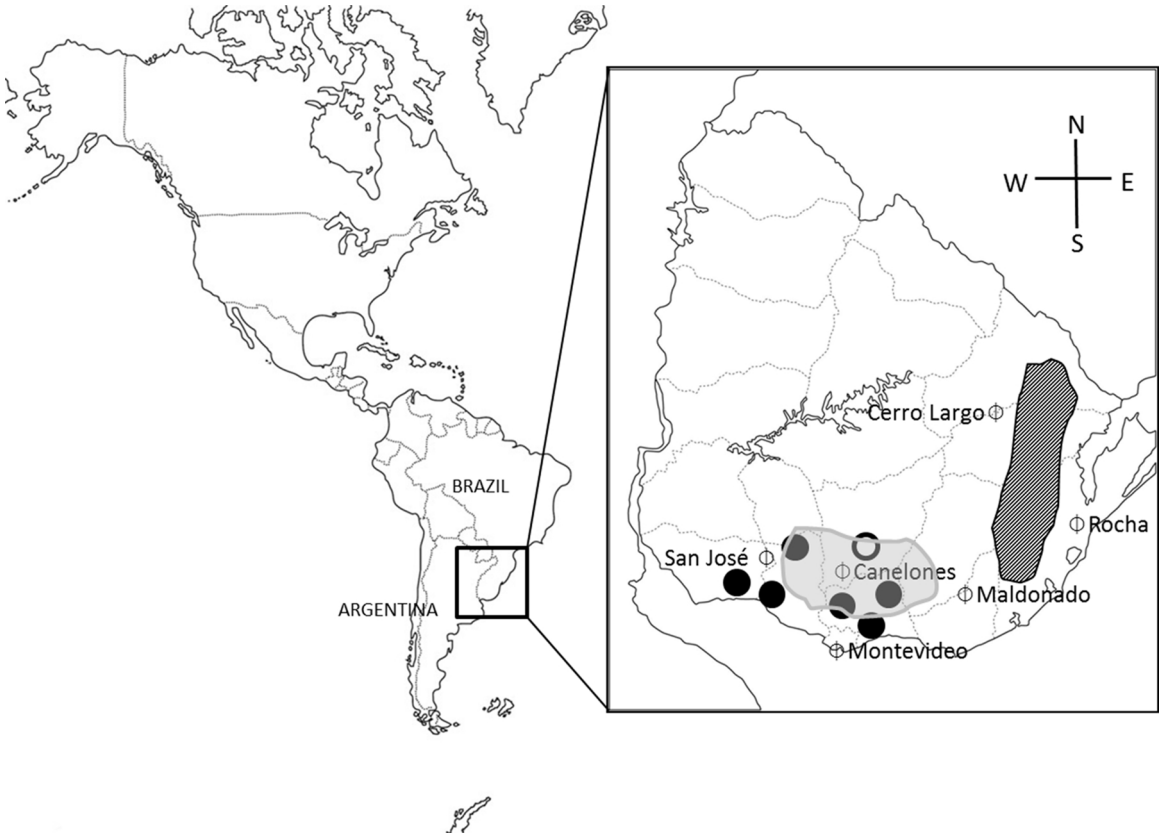

Fig. 1. Map of Uruguay depicting geographic location of domestic pig farms and hunting areas of wild boars. Serum samples from pigs were collected in Montevideo, Canelones y San José departments (full circles), where more than 90% of the farms are located. White circle indicates location of the abattoir where animals from 15 farms were slaughtered (grey zone indicates region of derivation of these 15 herds). Hunting area of free living wild boar populations covering regions of Maldonado, Rocha and Cerro Largo departments is indicated (spotted area).

RNA could be detected in 1/591 donations. Blood donor populations from Germany and USA, in turn, exhibit more moderate seroprevalences rates (5.9 and 9.5%, respectively) (Kumar et al., 2012; Donnelly et al., 2017). Commonly, the source of HEV infection in this epidemiological setting remains unclear, but increasing evidences strongly support the hypothesis of a zoonotic transmission of this virus (Meng, 2003; Christensen et al., 2008; Lewis et al., 2010). HEV infection is frequently asymptomatic but fulminant hepatic failure can occur in pregnant women and patients with underlying chronic liver disease (Patra et al., 2007; Rein et al., 2012). Unknown aspects of HEV infection have been recently uncovered, such as the possibility of the disease to become chronic in transplanted patients and immunocompromised individuals (Aggarwal et al., 2016). Extra-hepatic manifestations of hepatitis E can also occur (Kumar et al., 2012; Mirazo et al., 2014a).

HEV belongs to the *Hepeviridae* family, which has been recently proposed to be divided into two genera: *Orthohepevirus* and *Piscihepevirus* (Smith et al., 2014). *Orthohepevirus* genus is in turn divided into four species: *Orthohepevirus A*, *B*, *C* and *D*. HEV is a small non-enveloped particle with a size of 27–32 nm with a single stranded positive-sense RNA genome of 7,2 Kb that encodes three overlapping open reading frames (ORFs): ORF1, ORF2 and ORF3 (Emerson and Purcell, 2006).

HEV strains from human and animal reservoirs belong to *Orthohepevirus A* species and are classified into 8 phylogenetically distinct genotypes (HEV-1 to HEV-8) (Smith et al., 2014; Sridhar et al., 2017). HEV-1 and HEV-2 have a limited host range and are thought to be restricted to humans. By contrast, HEV-3 and HEV-4 have a broader host range and can infect across species barriers (Arankalle et al., 2006; Doceul et al., 2016). Humans, pigs, wild boars and deers are the main reservoir of the zoonotic genotypes (Dalton et al., 2013; Pavio et al., 2015; Meng, 2016). HEV-1, HEV-2, HEV-3 and HEV-4 are further divided into several subtypes (Lu et al., 2006; Smith et al., 2016). HEV-5 and HEV-6 were recently identified in Japanese wild boars, though they were never detected in humans (Sato et al., 2011; Takahashi et al., 2011). HEV-7 and HEV-8 in turn, were detected in camels (Sridhar et al., 2017) and HEV-7 was also identified in a patient with acute hepatitis who regularly consumed camel milk (Lee et al., 2016).

Like human HEV strains, the horizontal transmission route in swine is fecal-oral, since feces from infected pigs contain large amount of infectious virus (Meng, 2010). HEV is widespread in swine herds and specific antibodies have been detected in domestic pigs in regions where HEV is not endemic (Grierson et al., 2015; Ricci et al., 2017).

The zoonotic transmission of HEV-3 and HEV-4 from swine, wild boar and deer to humans through consumption of contaminated meat products has been proven (Doceul et al., 2016). In fact, molecular analyses exhibit high nucleotide identity between strains of human and pig origin (Bouquet et al., 2012).

In Latin America, the epidemiological features and the basic modes of transmission of HEV are still missing (Echevarría et al., 2013). The few reports on waterborne outbreaks, and the accumulated evidence of HEV circulation among humans and swine livestock, reinforce the role of domestic pigs in the transmission of HEV (Echevarría et al., 2013; Fierro et al., 2016). Serological and molecular reports consistently confirm HEV-3 circulation in pig herds from Latin American countries and phylogenetic comparisons have shown highly variable degree of relatedness among human and swine strains (Cooper et al., 2005; Vitral et al., 2005; Munné et al., 2006; Paiva et al., 2007; Kase et al., 2008; Dell'Amico et al., 2011). On the other hand, and despite its wide geographical distribution along the American continent, the role of wild boars in the transmission of HEV have never been reported in these regions.

Uruguay has a high burden of HEV-associated viral hepatitis and the

number of confirmed cases has been constantly increasing since the first report in 2009 (Mirazo et al., 2011, 2014b). HEV-3 is the most prevalent genotype among humans and the strains comprise a monophyletic cluster that has been recently proposed to be very closely related to European swine strains, particularly from Germany (Mirazo et al., 2016).

Here we conducted for the first time a serological and molecular survey of swine herds, pigs at slaughter and free-living wild boar populations to investigate the HEV distribution in these animal reservoirs from Uruguay, the extent of infection and its zoonotic risk to human population.

2. Material and methods

2.1. Sampling strategy

Serum samples from pigs were collected in the period 2012–2016 from 8 different medium and small sized-farms located in the South and South-West regions of the country (San José, Montevideo and Canelones departments), where 90% of the pig −farms are located (Fig. 1). Taking into account the relatively low sensitivity of sera for HEV RNA detection and the short viremia (Di Bartolo et al., 2011), domestic pig liver samples were further chosen for molecular analyses. Liver tissue samples, collected in a 1-year period, were obtained in an official abattoir, and belonged to animals from15 different farms located in San José and Canelones departments. In all cases, origin, name of the farm, and type of farming were registered.

In 1982, the wild boar was officially declared as a free-hunting animal (Decree 463/982) in Uruguay, and since then several groups of hunters had organized hunting trips and festivals in endemic areas. The sampling of wild boar was performed mainly in summer months, and was thus driven, by practical reasons, by the schedule of the hunting groups and the few wild boar festivals held along the country. Blood specimens from free-living wild boars were collected from hunted animals in 3 hunting locations in the East of Uruguay (Maldonado, Cerro Largo and Rocha Departments) during January and April of two consecutive years (2013 and 2014). All wild boar samples were obtained from certified hunters, trained for sample collection by official technicians from the Ministerio de Ganadería, Agricultura and Pesca (MGAP) of Uruguay.

2.2. Domestic pig serum samples

A total of 220 serum samples were collected from 8 (N°1-8) (Table 1) medium and small-sized commercial farms. Relevant data including sex and age was recorded in each case. Veterinary procedures were approved by the Ethical Committee resolution N° CBA_02356_013.

Table 1
Serological survey of HEV in domestic pigs and wild boars from Uruguay.

Reservoir	Test	HEV + Farm	HEV + (N)	Age Category	% (N)	HEV + (N)
Domestic Pig	ELISA	N°1	50% (5)			
		N°2	35% (14)	Young (< 6months)	45.6 (98)	33.6% (33)
		N°3	35.5% (16)			
		N°4	80% (20)			
		N°5	50% (10)	Slaughter-age (> 6 months)	55.4 (122)	57.4% (70)*
		N°6	45% (18)			
		N°7	40% (10)			
		N°8	60% (10)			
Wild boar	ELISA	–	21.1% (31)	–	–	–

N, amount of animals.
* p-value < 0.05.

According to age, animals were categorized as young (< 6 months) and slaughter-age (> 6 months), each group representing a 44.6% and 55.4% of the total, respectively (Table 1). All farms had semi-open breeding system (outdoor access with fenced field). Blood samples were extracted by venipuncture by trained veterinary personnel, and kept at 4 °C. Serum was separated by centrifugation at 4000 rpm and stored at −20 °C.

2.3. Domestic pig liver samples

One hundred and fifty liver samples were obtained from adult animals (slaughter-age) at one single abattoir. The 15 commercial farms (N°9-23) from which slaughtered animals came from held a median of 110 animals per herd, and according to the type of farming they were closed (93%) and semi-open breeding system (7%). Ten per cent of the animals were sampled per farm and relevant data was recorded. A small fragment of 1 cm^3 was cut on three faces of the liver with sterile scalpel, and the pieces of each animal were pooled, transported on dry ice and stored at −70 °C until processing.

2.4. Wild boar serum samples

Blood specimens were obtained from dead adult animals hunted by sport hunting groups certified by MGAP. Five milliliters of blood were collected from 140 individuals by heart puncture with sterile syringe and store at 4 °C. Serum was separated by centrifugation at 4000 rpm and stored at −20 °C.

2.5. HEV ELISA testing

Detection of anti-HEV IgG antibodies in samples was performed using 10 µl of serum by using ID Screen® Hepatitis E Indirect Multi-species kit (IDVet, France), according to the manufacturer's specifications. This kit was developed by using a recombinant HEV-3 Capsid antigen (ORF2-coded). For each tested sample, the OD (optical density)/cut-off ratio was calculated.

2.6. RNA extraction, real time–PCR (RT-qPCR) and nested-PCR

RNA extraction was performed from 140 µl of wild boar serum with QIAmp Viral RNA mini kit (QIAGEN, USA) and from 0.25 g of domestic pig liver tissue specimens with TRIzol® reagent (ThermoFisher Scientific, USA), according to manufacturer's specifications. The purified RNA was stored at −70° until use.

Reverse transcription was performed in 20 µl volume with random hexamer primers (Life Technologies, USA) and Revert Aid™ (ThermoFisher Scientific, USA) enzyme, following the supplier's recommendations. Integrity of RNA and absence of PCR inhibitors in liver tissue samples was evaluated by amplifying a region within the beta actin gene (Silva-Benítez et al., 2015). For HEV RNA detection, samples were subjected to a quantitative real time PCR (RT-qPCR) targeting a region within the viral ORF2, adapted from Jothikumar et al. (2006). Additionally, and in order to perform the phylogenetic analyses, the 5'end of the ORF1 was amplified by nested-PCR as previously reported (Mirazo et al., 2013). All samples from domestic pig liver tissue and wild boar sera were tested by both PCR approaches. A plasmid containing the complete genome of the HEV-1 Hyderabad strain kindly provided by Dr. Shahid Jameel was used as positive control. Several no template control (NTC) were included in each stage. PCR products obtained from nested-PCR positive samples were cloned into pJET Vector (ThermoFisher Scientific, USA) and 5 positive clones for each sample were sequenced.

2.7. Sequencing and phylogenetic analysis

The plasmids containing the amplified region were purified with

NucleoSpin® Plasmid (Macherey-Nagel, Germany) and sequenced directly in both directions by Macrogen automatic sequencing service, Korea. Sequence analysis was performed with Clustal W software and identity matrices were constructed by BioEdit v7.0.5 software. Phylogenetic tree was reconstructed by the neighbor-joining method with Tamura-Nei as the substitution method by using Molecular Evolutionary Genetics Analysis (MEGA) v6.0 software. Reference sequences of each subtype of HEV-3 (Lu et al., 2006; Smith et al., 2016) and of HEV-1, HEV-4 and HEV-2 were included in the analysis. The substitution model that best fitted the data was obtained with ModelTest tool. Bootstrap values for providing significant evidence for phylogenetic grouping were determined with 1000 resampling of the datasets.

2.8. Statistical analysis

With the aim to compare the frequency of swine anti-HEV IgG, animals were divided in two age categories: < 6 months and > 6 months. Chi-square and unpaired F (Kruskal-Wallis) tests were applied using STATA (Intercooled Stata 6.0, Texas, USA). A p value < 0.05 was considered statistically significant.

3. Results

3.1. HEV seroprevalence in domestic pig herds and wild boars

All the farms were seropositive for HEV (Table 1) with an overall prevalence of specific anti-HEV IgG antibodies of 46.8% (103/220). A higher prevalence was observed among slaughter-age animals (57.3%, 70/122) in comparison with young animals (33.6%, 33/98) ($p < 0.05$) (Table 1). Anti HEV seroprevalence in adult wild boars was 22.1% (31/140). No significant differences in the OD (optical density)/cut-off ratio were observed among the farms or wild boar populations from different regions (data not shown).

3.2. HEV RNA detection and genotyping

3.2.1. Pig liver samples

Molecular analysis by RT-qPCR of 150 liver samples from domestic pigs obtained from 15 different farms showed a global infection rate of 16.6% (25/150). Range of Ct values of RNA amplification for these positive samples were 22.5–29. Farms 9, 11, 12, 21 and 23 had infected animals, and in three of the HEV positive samples a successful amplification by nested-PCR was achieved (Table 2). Beta actin gene was amplified from all the liver samples.

Table 2
Molecular survey of HEV in domestic pigs and wild boars from Uruguay.

Sample	Test	HEV + Farm	HEV + (N)	Identified Genotype (N)
Domestic Pig liver tissue[a]	nested-PCR[b]	N° 9	2% (2)	n/d
	RT-qPCR[c]	N°11	4% (7)	HEV-3 (1)
		N°12	5.1% (9)	HEV-3 (1)
		N°21	3% (5)	HEV-3 (1)
		N°23	2.5% (2)	n/d
Wild boar serum	nested-PCR RT-qPCR	–	9.3% (13)	n/d

n/d not determined.
N, amount of animals.
[a] Animals from several farms were obtained in the same slaughter-house.
[b] Reverse transcription – nested PCR.
[c] Reverse transcription quantitative real time PCR.

3.2.2. Wild boar serum samples

Molecular survey by RT-qPCR of the collected wild boar sera resulted in 9.3% positive samples (13/140), with Ct values ranging from 28.7 to 29.5. None of these positive samples could be amplified by end point nested-PCR.

3.3. Phylogenetic analyses

Sequence analysis of the 287-bp region within the viral 5′ORF1 amplified from the three swine strains revealed a high percentage of nucleotide identity (88–98.1%) with HEV-3 sequences. In addition, these 3 samples, detected in different periods during 2015, were very closely related among them (mean 98.5 and 99% at the nucleotide and amino acidic level, respectively). The phylogenetic reconstruction clustered these swine strains with a high statistical support with the monophyletic cluster of sequences obtained from human cases of hepatitis E reported in Uruguay (Fig. 2). Nucleotide identities percentages among swine and human strains ranged from 97 to 98.1%. According to the HEV subtype classification (Lu et al., 2006; Smith et al., 2016), swine strains belonged to 3i subtype and were phylogenetically related to a HEV strain detected in a wild boar in Germany (GenBank accession number FJ748530) (Schielke et al., 2009).

Sequences from five clones of each sample were identical and corresponding strains were designated SwUy1, SwUy2 and SwUy3. Sequences were deposited in GenBank with the accession numbers MF590059, MF590060 and MF590061.

4. Discussion

Little is known regarding the molecular epidemiology and modes of transmission of HEV infection in non-endemic developing or high-income regions. In the last decade the number of acute sporadic and autochthonous hepatitis E cases, mainly involving HEV-3 strains, have increased dramatically. Additionally, unexpected moderate/high seroprevalence rates among the general population in these regions have been estimated, which implies a high exposition to HEV (Aspinall et al., 2017). Uruguay has reported the circulation and molecular characterization of HEV-3 strains isolated from human cases, which were found to be phylogenetically related to swine and wild boar strains from Europe (Mirazo et al., 2013, 2016). This, together with the recently reported cases of hepatitis E associated to HEV-1 (Mirazo et al., 2014b), suggests that the epidemiology of this infection in Uruguay seems to be complex and needs to be further investigated. In this context, no information is available on circulation of HEV in animal reservoirs. This study described for the first time occurrence of HEV infection in domestic pigs and free-living wild boar populations.

The serological survey performed herein suggests that HEV is widely spread among domestic pigs in the country, since all tested herds were seropositive with an overall seroprevalence of 46.8%. Of note, this rate is comparable with the few reports performed in the region and with most of the studies from high-income countries (dos Santos et al., 2009; Echevarría et al., 2013; Clemente-Casares et al., 2016). By contrast, among slaughter-aged animals this rate is lower than that reported by dos Santos et al. from Brazil (88.4%). The differences in the antibody rates may indeed be affected by the type of ELISA kit and the HEV antigen used in each study. Significant differences in the capabilities of the currently available commercial kits have been reported (Zhang et al., 2011). Circulation of HEV in pigs occurs at the end of the nursery or the beginning of the fattening period but virus can be detected at all ages, and it is widely accepted that breeding sows can play a role as HEV reservoirs and can transmit the virus to sucking piglets (Salines et al., 2017). Anti-HEV antibodies were found in both age-categories included in this study and exposure of this reservoir to HEV seems to be higher in slaughter-aged pigs, with generally elevated seroprevalence rates. In fact, this difference in the detection rates among age-categories has been previously reported (Wibawa et al., 2004; Vitral et al., 2005).

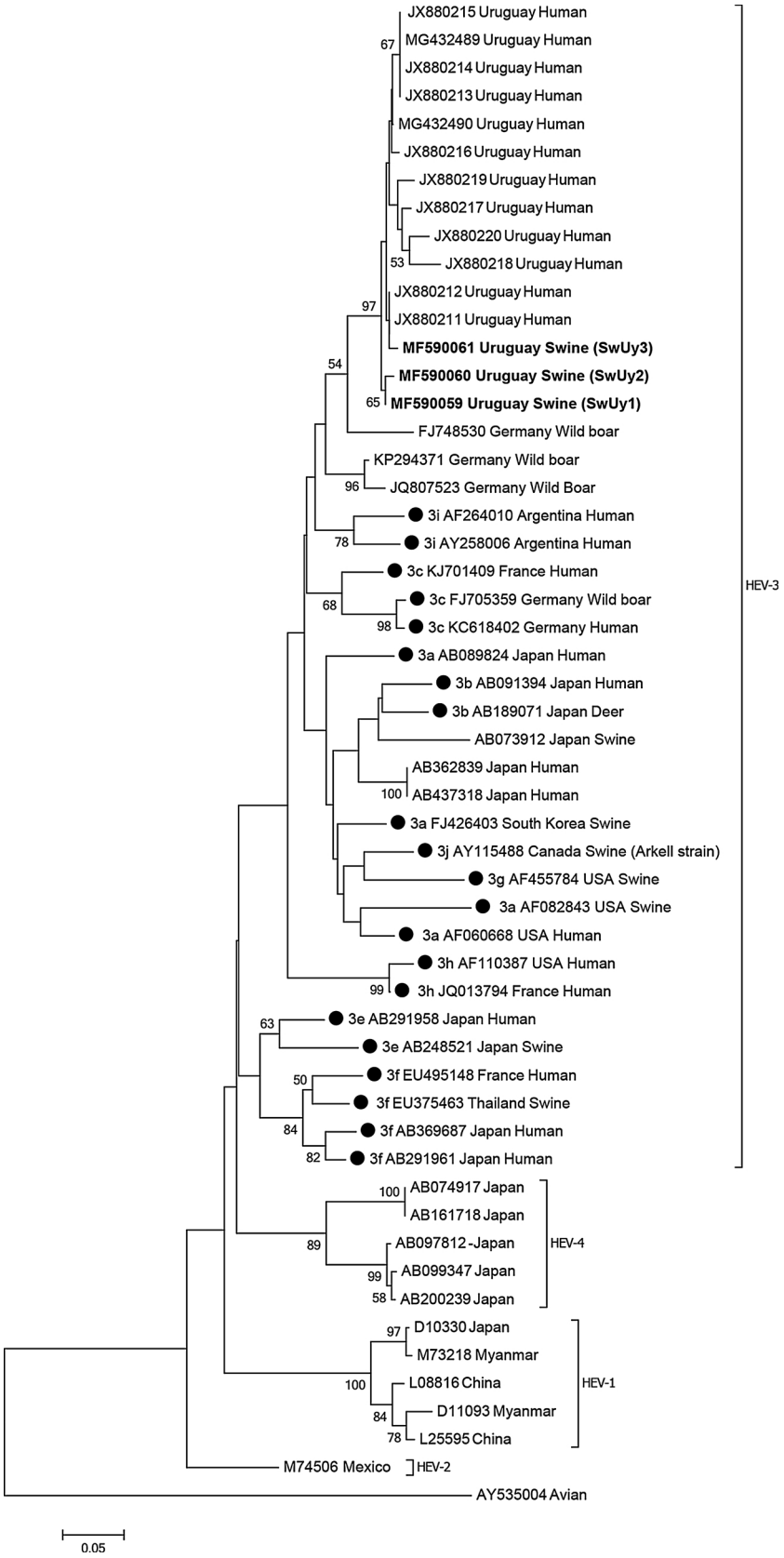

Fig. 2. Phylogenetic tree based on the partial 287-nt region within the ORF1 (nucleotide position 50–336 in the reference strain US1, Genbank accession number AF060668). Tree was generated by using the neighbor-joining algorithm using Tamura-Nei as the best substitution model as tested by ModelTest v3.7 tool. The robustness of the trees was determined by bootstrap with 1000 replicates. Swine Uruguayan sequences are shown in bold. References sequences of each subtype of HEV-3 as defined by Lu et al. (2006) and Smith et al. (2016) are marked with full dark circles.

HEV history in Uruguay is thought to be very recent (Mirazo et al., 2016). Furthermore, seroprevalence of HEV in blood donor population was about 1% at the end of the 90's (Cruells et al., 1997). Thus, the unexpected high seroprevalence of HEV in swine has at least two plausible explanations: the first one is that the HEV rapidly emerged in the country with an explosive and wide expansion among swine herds; and the second could be due to extensive changes in herd management and farming system, which implied an increase in the rate of zoonotic

transmission events to humans. This last explanation is the less convincing since the semi-open breeding system in Uruguay has been applied virtually unchanged during the last 50 years, and most (90%) of the officially registered farms keep it nowadays (DICOSE Declaración Jurada, 2015). However, the elevated seroprevalences may be also due to a high sensitivity of the ELISA kit used in this work. In fact, it has been recently shown that Capsid antigens are, also for in-house systems, highly specific (more than ORF3-coded protein), which may give relatively elevated antibodies rates (Dremsek et al., 2013). Viral testing results from domestic pig liver samples are in agreement with this notion that HEV is widely spread in Uruguay. RNA detection in liver content in animals from slaughter-house represents a clear risk of entrance of HEV into the food chain, since about 16% of the liver samples contained HEV RNA. This rate is lower than that previously reported by de Souza in Brazil (up to 27% of positive livers was observed), though the sample size was much smaller in that study. By contrast, HEV RNA could only be detected in 1.7% of the liver samples in a study performed in a different region from Brazil (Gardinali et al., 2012).

From a methodological point of view, RT-qPCR seemed to be more sensitive in the detection of HEV in liver tissues, since not all positive samples could be amplified by the end point PCR and in all cases RNA extraction was successful, as tested by beta actin gene amplification. This may probably due to the viral load, since many of the HEV containing liver samples had Ct values very close to 30, considered as the cut off value (Jothikumar et al., 2006). Swine strains detected in this study exhibited a very high nucleotide and amino acid identity with the monophyletic cluster of HEV-3 strains detected in human cases from Uruguay (Mirazo et al., 2013). Very similar, if not identical, HEV sequences have been identified from events of zoonotic transmission from wild boar, pigs and deers to humans (Doceul et al., 2016). This close phylogenetic relationship among human and swine strains from Uruguay suggests that domestic pig infection implies an associated risk for human infection, and that a zoonotic transmission might have been one plausible route of infection in these human cases from Uruguay, since the source of infection was never identified (Mirazo et al., 2013). HEV infection through consumption of pork and wild boar meat, as well as the burden of HEV infected derived products and its associated risk to human health, have been extensively documented (Miyashita et al., 2012; Szabo et al., 2015; Rivero-Juarez et al., 2017). Furthermore, this zoonotic route seems to be the main mode of transmission of HEV in the developed world and non-endemic regions (Pavio et al., 2015). In Uruguay, pork meat and derivatives (liver containing sausages, liver patés), both locally produced and imported from European countries, are heavily consumed as daily diet. Thus, this dietary habit may indeed represent a risk factor for HEV infection, as suggested by Slot et al. (2017).

Seroprevalence of HEV in wild boars was comparable to that observed in high-income European countries, where they were extensively investigated (Adlhoch et al., 2009; Clemente-Casares et al., 2016; Thiry et al., 2017a). Results suggest that these animals might serve as an important HEV potential reservoir in Uruguay but also for other South American countries, taking into account the absence of geographical barriers among them, which leads to border crossing events and a wide spread of the wild populations (Garcia et al., 2011). The RNA detection in about 10% of the wild boars supports this hypothesis. Furthermore, HEV molecular identification in wild boars was performed from serum, as the only available biological specimen. These results indicate that infection rates in those animals may be underestimated, since RNA testing from serum sample is not the most reliable method for HEV detection. Due to the short viremia, HEV is only transiently detected in blood, and both liver tissue or bile and, particularly, fecal samples, where virus shedding is longer might offer better possibilities to detect and amplify viral RNA (Di Bartolo et al., 2011; Backer et al., 2012; Salines et al., 2017). The low HEV viral load in the sera from wild boars (with Ct values near the cut off) may explain the absence of positive samples amplified by conventional PCR and the lacking of HEV sequences. This limiting condition impairs the interpretation of at what extent the infection of wild boars affects the viral genetic burden in domestic pigs, and hence their zoonotic potential. In fact, it has been recently evidenced that zoonotic strains could be transmitted via the natural fecal-oral route of infection between wild boar and pigs (Thiry et al., 2017b).

This study has two main limitations. First, though the sample size tested for serological and molecular analysis were small, it is representative of the whole country since more than 90% of the pig farms in Uruguay are located in the sampling area (South and South-West of Uruguay). And second, the scant hunting groups with official licenses that are currently operating in Uruguay impeded the access to a higher number of wild boars for serological and molecular testing. To mitigate this limitation, wild boars specimens were collected in areas with the higher population density in Uruguay (Garcia et al., 2011).

5. Conclusions

In summary, here is reported the widely distributed circulation of HEV among domestic pig herds in Uruguay, with high seroprevalence and infection rates. Genetic characterization of the strains demonstrated a very high nucleotide identity with HEV strains detected in acute human cases reported in Uruguay, with an associated potential zoonotic risk. In addition, we described for the first time in the Americas the circulation of HEV in free-living wild boar populations and its role as an active reservoir for infection. Further studies are needed to fully characterize at the molecular level these domestic pig and wild boar strains, and to estimate the extent of infection in order to minimize the risk of human exposure and the impact in public health.

Conflict of interest statement

None

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