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LETTER

First Serological Evidence on Endemicity of HEV Infection in Wild Boar (*Sus scrofa*) Populations from Portugal

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Dear Editor,

Increasing number of hepatitis E virus (HEV) infection has been described in industrialized countries, with infection occurring as a sporadic disease, usually associated with travel to endemic regions or small outbreaks due to ingestion of contaminated food or water. More recently some cases were found to be associated with zoonotic transmission, particularly through consumption of uncooked deer, wild boar or pig meat, or through occupational contact with faeces or excrements from infected animals (Berto *et al.* 2012; Carpentier *et al.* 2012; Ruggeri *et al.* 2013). Portugal has a long history of hunting, with hunted animals being used for human consumption as meat or local products, some of them consumed raw. The evaluation of HEV infection in those animals is, therefore, crucial to assess the potential risk of its zoonotic transmission to humans.

Despite HEV infection is well characterized in Portuguese pigs (Berto *et al.* 2012), the information on the prevalence of HEV infection in wild boars is rather scarce, and limited to genome detection as marker for ongoing infection (Mesquita *et al.* 2014), without information on seroprevalence rates, indicating the true frequency of HEV infection in these animals. In face of this, the present study intends to characterize HEV infection in hunted wild boars from two different regions of Portugal, comprising the evaluation of markers for both past and/or ongoing infection.

Wild boar (*Sus scrofa*) samples were collected during the 2013 hunting season, between January and February, at two different Portuguese districts (districts of Portalegre and Santarém) with common hunting habits (Fig. 1). Taking into account the geographic localization and environment barriers, each wild boar population was considered epidemiologically independent. Fifteen animals were sampled in the district of Portalegre, and 14 in the district of Santarém. Sample collection was performed post-mortem, immediately after the opening of the wild boar's bodies. Blood and stool samples were collected from all animals, and bile samples were obtained from 27 wild boars.

The presence of HEV antibodies were evaluated through a double antigen sandwich enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (HEV ELISA 4.0v, MP Diagnostics, Sipaco, Portugal). This ELISA kit uses a recombinant HEV ORF2 capsid antigen, highly conserved between different HEV strains, to detect the presence of all the immunoglobulins (IgG, IgM and IgA) in serum from swine and other animal species. All serum samples were tested, in duplicate, according to the manufacturer's instructions, at a dilution of 1/5. A positive result was considered whenever OD value were superior to the calculated Cut-off value (Cut-Off value = mean of the negative control plus 0.200). Four of the 29 tested serum samples (14%) revealed the presence of specific HEV antibodies (Table 1), 3 of them were from animals hunted in Santarém district, and the other one from an animal hunted in Portalegre district. Nevertheless, no significant differences in HEV seroprevalence were observed between wild boars from the different districts studied (Fisher exact

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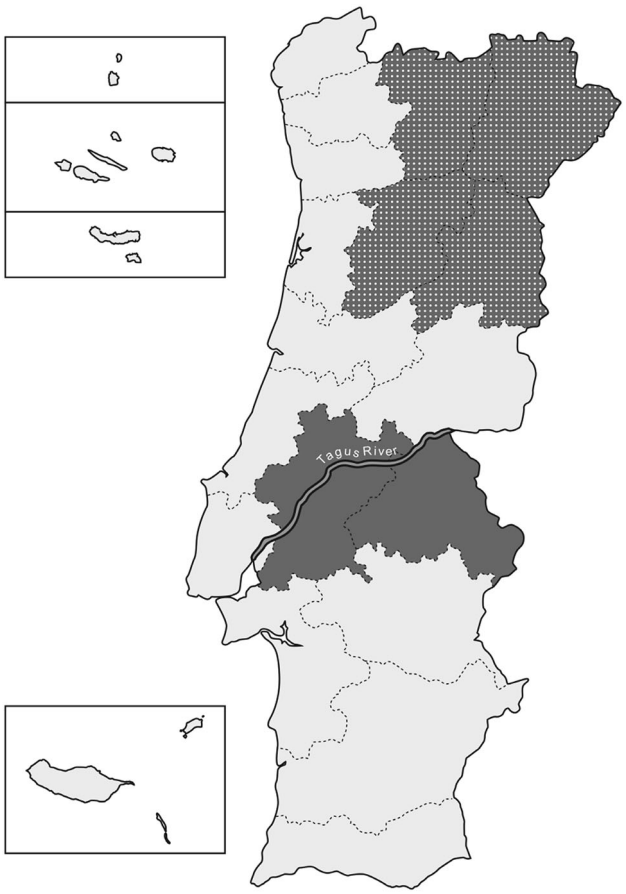

Fig. 1 Map of Portugal, showing the districts of hunted wild boars evaluated in the present study (dark grey), and those investigated in the study of Mesquita *et al.* (2014) (dark grey with dots).

test, $P = 0.3$). The kit enables the detection of all the immunoglobulins against HEV, without discrimination between the classes of Ig present. Therefore, the positive results obtained for the 4 studied animals only means that specific HEV Ig was present, and that those animals are immunized, without information in what type of Ig were responsible for the obtained positive results. Therefore, once no discrimination among the Ig classes could be done, it is not possible to infer if the obtained positive results are due to current, recent or past infection. Rather, we can only conclude that 14% of the studied animals have experienced, sometime, HEV infection.

Here reported HEV seroprevalence in Portuguese wild boar is similar to that reported in other European countries, such as France (Carpentier *et al.* 2012), Estonia (Ivanova *et al.* 2015), Sweden (Roth *et al.* 2016), Germany (Oliveira-Filho *et al.* 2014), Netherlands (Rutjes *et al.* 2010) and our neighbour country, Spain (Risalde *et al.* 2017). Nevertheless, differences in seroprevalence rates among studies need to be interpreted within the context of the geographic area, the population density, and the ecological differences in each wild boar population studied.

Active HEV infection was also screened in hunted animals through the evaluation of HEV genome in bile and stool samples. Both sample types of each animal were screened for HEV genome, in order to enhance the chance of detection of acutely infected animals. Nucleic acid extraction was performed with QIAamp® viral RNA Mini Kit (Qiagen®, Isaza, Portugal) in both sample types. All nucleic acid extracts were evaluated for the presence of HEV RNA through two different qRT-PCR protocols (a TaqMan® based and a SYBR® Green based chemistry protocols, respectively), in order to compare the sensitivity and specificity between them. A set of previously described primers, targeting the ORF2 region of HEV genome, covering all HEV genotypes, was used in both protocols (Rolfe *et al.* 2010). For TaqMan protocol, a specific described probe for the amplified ORF2 region of HEV genome was used (Rolfe *et al.* 2010; Mokhtari *et al.* 2013), and amplification was carried out at the following temperatures: 50 °C for 30 min, 95 °C for 2 min, and 45 cycles of 95 °C for 15 s and of 60 °C for 1 min. For SYBR® Green protocol the following temperature conditions were used: 50 °C for 10 min, 95 °C for 5 min and 45 cycles of 95 °C for 10 s and of 60 °C for 30 s. The comparison of positive controls used in all amplification reactions revealed lower C_T values for TaqMan protocol than for SYBR® Green protocol, confirming the higher sensitivity expected for the first protocol.

Although, suspicious positive result (very low C_T values with unusual amplification curves) were obtained for 4 bile samples with SYBR® Green protocol. The duplicate analysis of these four samples through the TaqMan protocol revealed undetectable HEV RNA. Considering that the same set of primers and the same equipment were used for both protocols, and once SYBR® Green protocol uses a molecule able to link any dsDNA fragment to detect amplified products, in spite of a specific probe for the amplified region of HEV genome used in TaqMan protocol, despite no sequence characterization was performed,

Table 1 Evidence of past and/or ongoing HEV infection in wild boars hunted in two districts (Santarém and Portalegre) from Portugal.

Hunting district	Santarém (n = 14)	Portalegre (n = 15)	Total (n = 29)
Evidence of past infection [Seroprevalence (%)]	3 (21%)	1 (6%)	4 (14%)
Evidence of ongoing infection [HEV RNA (%)]	0 (0%)	0 (0%)	0 (0%)

those four bile samples were considered as having undetectable HEV genome, and SYBR® Green fluorescence observed was considered as a false positive result, probably due to unspecific detection. All the remaining 23 bile samples also did not reveal detectable HEV genome through SYBR® Green protocol. As initially expected, the protocol using the TaqMan® chemistry, apart from higher sensitivity, also revealed higher specificity for HEV genome detection, when compared to SYBR® Green protocol, hence, we strongly suggest the utilization of probe based qPCR amplification protocols for its detection in biological samples from wild boars.

The absence of detectable HEV genome from all the evaluated samples suggest that, apart from 14% of the studied population have been infected with HEV (as revealed by serological analysis), resulting in an immunized wild boar population, at the moment of hunt, none of the evaluated wild animals seems to be actively infected with HEV. Similar low frequencies of HEV genome detection in wild boars were also reported in other countries, such as Italy (Di Profio *et al.* 2016), Sweden (Roth *et al.* 2016), France (Kaba *et al.* 2010) and Estonia (Ivanova *et al.* 2015). Nevertheless, other study carried out in Northeast Portuguese wild boars, revealed higher values of HEV genome prevalence (25%) (Mesquita *et al.* 2014) (Fig. 1). Such different results could be due to differences in the biology and ecology of the wild boar populations investigated, once studied animals were not from the same Portuguese geographic regions. It is also possible that Tagus River could work as an important physical barrier to the contact between wild boar populations from the two studies, and consequently to the spread of the virus between them (Ferreira *et al.* 2009). Despite no active HEV infection was demonstrated for the hunted animals included in the present study, serological analysis revealed, for the first time, the endemicity of HEV infection in wild boars populations from Portugal. The detection of specific HEV antibodies in 14% of the studied animals supports the hypothesis of this species as reservoir for zoonotic spread of HEV.

Significantly higher anti-HEV seroprevalence has been observed in forestry workers, when compared to controls workers, which is assigned to hunting activities, and direct contact to wild boar body fluids (Carpentier *et al.* 2012; Dremsek *et al.* 2012). Further, a recent Portuguese study revealed a significantly higher prevalence of anti-HEV IgG in workers occupationally exposed to swine compared to general population (Teixeira *et al.* 2017). The endemicity observed in studied wild boar populations evaluated in the present study give strength to such hypothesis, and points this wild species as a source of HEV infection to humans with close contact to them. Another recent Portuguese study revealed a very low frequency of HEV seroprevalence in Portuguese children (Oliveira *et al.* 2017), when compared to adult population (Teixeira *et al.* 2017), revealing that this infection is acquired late in life, which is in accordance with the hypothesis of wild boar as a source of HEV infection to humans, specially hunters or individuals who manipulate the carcasses of hunted animals, mainly adult individuals.

The data presented in this study reinforce the importance of including HEV in national and regional surveillance programs for wild animal diseases, like other more commonly known zoonotic agents, as well as to the awareness for thorough cooking all wild boar products and to improve education of occupationally exposed people in order to prevent HEV infection.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement All institutional and national guidelines for experiments in animal samples were followed.

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