

ORIGINAL ARTICLE

Belgian Wildlife as Potential Zoonotic Reservoir of Hepatitis E Virus

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Keywords:

hepatitis E virus; wild boar; cervids; Belgium; prevalence; zoonosis

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These results strongly support the high and the low levels of HEV circulation in wild boars and cervids, respectively, in Belgium.

Received for publication June 2, 2015

doi:10.1111/tbed.12435

Summary

Hepatitis E is an acute human liver disease in healthy individuals but may become chronic in immunocompromised patients. It is caused by the hepatitis E virus (HEV) and can have a zoonotic origin, particularly in high-income countries. In this study, 383 sera from wild boars were selected for serology; for virological analyses, 69 sera and 61 livers from young wild boars were used. A total of 189 and 235 sera of, respectively, red deer and roe deer were collected for serological analysis. For virological analyses, 84 and 68 sera and 29 and 27 livers from, respectively, red and roe deer were sampled. An apparent seroprevalence of 34% (95% CI 29.71–39.46) was found in wild boars, of 1% (95% CI 0–2.4) in red deer and 3% (95% CI 0.8–4.2) in roe deer. To assess the ELISA screening prevalence, Western blot (WB) analyses were carried out, a receiver operating characteristic curve analysis was performed and different scenarios with varying ELISA specificities relative to WB were analysed. Seroprevalence remained high whatever the scenario in the wild boar population. In wild boar, 4 of 69 sera and 4 of 61 livers were detected as positive for HEV RNA. All sequences obtained from sera belonged to genotype HEV-3. HEV RNA, belonging to genotype HEV-3, was detected in one of 29 red deer livers. Wild boar can be considered as a host reservoir of the virus in Belgium. However, in contrast to the epidemiological role played by them in other countries, the low prevalence in deer makes these species an unlikely reservoir. This evidence needs further investigation to determine in which situation deer can serve as reservoir. These results also raise the question of the dynamics of HEV infection between wild fauna, domestic pigs and humans.

Introduction

Hepatitis E virus (HEV), belonging to the family *Hepeviridae*, genus *Orthohepevirus*, is a small, non-enveloped virus with a single-stranded, positive sense RNA genome of approximately 7.2 kb (Emerson and Purcell, 2003; Smith et al., 2014). The first human outbreak of hepatitis E

occurred in India during the winter of 1955–1956, and HEV was identified in pigs for the first time in 1997 (Meng et al., 1997). Since then, HEV and HEV-related viruses have been detected in an increasing number of animal species such as wild boar and deer (Thiry et al., 2015). According to a new proposed classification, the *Hepeviridae* family contains two genera, five species (*Orthohepevirus* A, B, C,

D and Piscinehepevirus A) and nine genotypes (Smith et al., 2014). Genotypes HEV-1 and 2 include only human strains, while genotypes HEV-3 and 4 have also been detected in other species, especially in swine. HEV-1 and 2 are mainly present in developing countries (Aggarwal, 2011). In Asia, the Middle East and Africa, HEV is a major aetiological agent responsible for human epidemics of viral hepatitis (Nicand et al., 2009; Purcell and Emerson, 2010). The main transmission route in these regions is faecal–oral via contaminated drinking water (Borgen et al., 2008). In industrialized countries, HEV-3 is the mainly detected genotype and, although real transmission evidences have been poorly documented to date (Tei et al., 2003; Li et al., 2005; Okano et al., 2013; Renou et al., 2014; Riveiro-Barciela et al., 2014), the zoonotic transmission between pigs, wild boars or deer and humans is of particular concern (Teo, 2010; Meng, 2011).

Hepatitis E virus RNA and antibodies have been detected in wild boars in several countries (Meng, 2011). Most HEV strains detected in wild boars globally belong to genotype HEV-3 (Meng, 2010). However, HEV sequences belonging to a new HEV genotype have been detected in wild boars in Japan (Takahashi et al., 2011). In Germany, a HEV seroprevalence of 29.9% of 107 tested sera was reported (Adl-hoch et al., 2009). In the Netherlands, a seroprevalence of 12% of 1029 animals was shown (Rutjes et al., 2010). In France, a HEV seroprevalence of 14% was observed in sera from 421 wild boars (Carpentier et al., 2012). In Italy, an overall seroprevalence of 10.2% (226/2211) was found (Martinelli et al., 2013). In Spain, seroprevalences of 26.5% of 735 wild boars and 57.4% of 108 wild boars were reported in 2012 and 2015, respectively (Boadella et al., 2012; Kukielka et al., 2015). Few studies have been carried out to detect HEV infection in cervids. In Japan, low prevalences were observed in Sika deer which are considered an incidental host for HEV (Matsuura et al., 2007; Yu et al., 2007). In Europe, some studies on red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) showed that these animals could be reservoir hosts (Reuter et al., 2009; Rutjes et al., 2009). In Spain, an apparent seroprevalence of 10.4% was observed on 968 red deer (Boadella et al., 2010). A second study performed in Spain showed a seroprevalence of 12.85% in red deer in 2015 (Kukielka et al., 2015). In Italy, a total of 35 of 251 red deer sera were seropositive (Di Bartolo et al., 2015). However, other studies shows very low or no HEV antibody prevalence in cervids, in the Netherlands, 2 of 38 red deer and 0 of 8 roe deer were seropositive (Rutjes et al., 2010), and in Poland, no antibodies were detected on 118 red deer and 38 roe deer sera (Larska et al., 2015).

In 2003, zoonotic transmission was reported in Japan. Four human cases were linked to the consumption of uncooked wild boar liver and Sika deer meat. The transmission could only be confirmed for the deer as no wild boar

liver remained for testing (Matsuda et al., 2003; Tei et al., 2003). In 2004, another study showed a 99.7% homology between the full genome viral sequences found in wild boar and a deer hunted in the same forest as the Sika deer consumed by the four patients above (Takahashi et al., 2004). Another report from Japan, in 2005, demonstrated transmission of HEV via ingestion of uncooked wild boar meat (Li et al., 2005).

In several European countries, wild boar populations have consistently increased since the 1980s (Massei et al., 2014) and in Belgium the hunting bag statistics (number of wild boars annually harvested) reached a total of more than 25000 heads in 2012 in the southern part of the country (Walloon Region, 16 903 km²). The red and roe deer bag statistics showed lower numbers of around 5300 and 14 400, respectively, in 2012 in the same area (A. Licoppe & R. Walloon, unpublished data). In Belgium, four-fifths of the forests are in the Walloon Region, with a wooded expanse of 4952 km² representing 29% of its total area. Moreover, this region also presents a high human population density (210 people/km²) which could enhance contact possibilities between wildlife and the human population. These data highlight the need on pursuing studies about transmission routes in wildlife and on interspecies transmission opportunities between wildlife and human populations.

This study aims to investigate the presence and the spread of HEV infection in three different wild species in a region with high human population densities, to genotype the strains identified, to compare them to sequences previously detected from humans and swine in Belgium and to assess the potential for contact between wild fauna and pigs in the Walloon Region.

Materials and Methods

Sampling

In the frame of a targeted surveillance programme, wild boars were sampled by the surveillance network of wildlife diseases (Linden et al., 2011) during the hunting season from September 2010 to February 2011. The study was conducted in 31 of the 33 forest districts of the Walloon Region, and a two-stage cluster sampling was realized. Having selected hunting areas in each forest district, animals were then randomly sampled in each hunting area. All animals were necropsied in the field, within 2–3 h after being shot. Individual post-mortem examinations included the determination of sex, age, body weight and body condition. Age was determined on the basis of tooth eruption patterns and weight. Animals were classified as subjuveniles (<6 months old), juveniles (between 6 and 12 months old), subadults (between 1 and 2 years old) and adults (over 2 years old). By applying the binomial law, a sample size of 381 allows the detection of a seroprevalence of 50% with an

accepted error of 5% and a confidence level of 95%. A sampling of 383 sera from wild boars over 6 months belonging to 196 males, 184 females and three indeterminate genders was performed. They were divided into three age groups: 150 adults, 99 subadults and 133 juveniles. For one sample, the age could not be specified. For the virological study, all available samples from the subjuvenile wild boars were used, that is 69 sera and 62 associated livers. In addition, 25 sera from older wild boars (>6 months) were also tested.

Both serum and liver sampling from cervids was also performed by the surveillance network of wildlife diseases during the hunting season from October 2012 to December 2012 in the forest districts of the Walloon Region. The study was conducted in 28 of the 33 forest districts following the same methodology as described for wild boar. One hundred and eighty-nine and 235 sera were sampled for serological analyses in red deer and roe deer, respectively, allowing the theoretical detection of a seroprevalence of 10% and 15% with an accepted error of 5% and a confidence level of 95%. For the virological analyses, 84 red deer and 68 roe deer sera, corresponding to the available samples from animals <1 year old, were tested. Among these animals, 29 and 27 livers of red and roe deer, respectively, were also sampled for the virological study.

Serological analyses

A double antigen sandwich ELISA (das-ELISA) able to detect IgM, IgG and IgA directed against HEV in all animal species was used (HEV ELISA kit 4.0V; MP Biomedicals, Illkirch, France) (Bouwknegt et al., 2008; Hu et al., 2008; Rutjes et al., 2010; Thiry et al., 2014). A total of 383 wild boars, 189 red deer and 235 roe deer samples were tested in duplicate. In addition, 25 samples from specific-pathogen-free (SPF) piglets born in Biosafety Level 3 facilities, and five positive sera from experimental infections of pigs (one against genotype 4 and four against genotype 3 HEV), were tested in duplicate as negative and positive controls, respectively, in a previous study (Thiry et al., 2014).

A Western blot (WB) for the detection of antibodies (Ab) against HEV in humans (recomLine HEV IgG/IgM; Mikrogen Diagnostik, Neuried, Germany) was used as described previously (Thiry et al., 2014). It was adapted for use on wild boar sera by replacing the conjugate of the kit (anti-human IgG – peroxidase) with a conjugate made from a secondary goat polyclonal Ab, reactive against pig IgG and coupled to peroxidase (Abcam, Cambridge, UK) and for cervid sera, using a conjugate made from secondary rabbit polyclonal Ab, reactive against deer IgG and coupled with peroxidase (KPL, Gaithersburg, MD, USA). Tests were performed with conjugates at 1/1000 or 1/500 dilution for wild boar or cervid sera, respectively. Three sera from infected pigs were tested as positive controls, and three sera

from SPF piglets were tested as negative controls. A total of 77, 39 and 51 sera from wild boars, red deer and roe deer, respectively, corresponding to 20% of each species serological sampling, were randomly selected and tested with the IgG WB. Four categories were defined according to the optical density (OD) averages obtained by ELISA. For wild boar, the first category included an OD ranging from 0 to 0.15; the second, an OD from 0.15 to 0.26; the third, an OD from 0.26 to 1; and the fourth, ≥ 1 (Table 1a). For red and roe deer, the first category included an OD ranging from 0 to 0.15, the second an OD from 0.15 to 0.21, the third an OD from 0.21 to 1 and the fourth, ≥ 1 (Table 1b, c). The tests were carried out on a ProfiBlot T48 (TECAN, Mechelen, Belgium).

Virological analyses

To detect HEV RNA in the samples, virological analyses were performed according to the protocol used in a previous study (Thiry et al., 2014). Hepatitis E virus RNA sequences of 302 nucleotides in open reading frame 2 were analysed using BioEdit and MEGA version 6 (Hall, 1999; Tamura et al., 2013), with a set of sequences available from

Table 1. Hepatitis E virus IgG Western blot (WB) results in wild boars (a), red deer (b) and roe deer (c) classified according to four ranges of hepatitis E virus ELISA optical density

| Class* | OD [†] ELISA range | Western blot [‡] | | | Total |
|--------|-----------------------------|---------------------------|------------|----|-------|
| | | + | Borderline | – | |
| (a) | | | | | |
| 1 | [0–0.15] | 9 | 3 | 30 | 42 |
| 2 | [0.15–0.26] | 2 | 1 | 4 | 7 |
| 3 | [0.26–1] | 7 | 0 | 5 | 12 |
| 4 | [1–5] | 14 | 1 | 1 | 16 |
| Total | | 32 | 5 | 40 | 77 |
| (b) | | | | | |
| 1 | [0–0.15] | 1 | 0 | 36 | 37 |
| 2 | [0.15–0.21] | 0 | 0 | 0 | 0 |
| 3 | [0.21–1] | 1 | 0 | 0 | 1 |
| 4 | [1–5] | 0 | 0 | 0 | 0 |
| Total | | 2 | 0 | 36 | 38 |
| (c) | | | | | |
| 1 | [0–0.15] | 3 | 1 | 41 | 45 |
| 2 | [0.15–0.21] | 0 | 0 | 0 | 0 |
| 3 | [0.21–1] | 0 | 0 | 2 | 2 |
| 4 | [1–5] | 1 | 0 | 1 | 2 |
| Total | | 4 | 1 | 44 | 49 |

*Classes 1 and 2: ELISA negative results; classes 3 and 4: ELISA positive results.

[†]Optical density.

[‡]Positive: WB results with a score ≥ 4 ; Borderline: Western blot result with a score of 3; Negative: WB results with a score ≤ 2 . Score were given according to the manufacturer's instructions.

GenBank and sequences previously detected in swine and humans in Belgium (Thiry et al., 2014), to determine genotypes and subtypes according to the classification of Lu et al. (2006). Phylogenetic relationships were inferred using the maximum likelihood method based on the Tamura-Nei substitution model chosen following the BIC and Akaike scores (Tamura and Nei, 1993) and 1000 bootstrap replicates. The tree with the highest log likelihood (-3406.4522) is shown. Bootstrap values are reported at each node. Initial tree(s) for the heuristic search were obtained by applying the neighbour-joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter = 0.5519)]. The analysis involved 44 nucleotide sequences. There were a total of 295 positions in the final data set. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

We deposited eight HEV sequences originating from wild boar and one from red deer in GenBank under Accession no. KP296177 (BeWbS7), KP296178 (BeWbS24), KP296179 (BeWbS67), KP296180 (BeWbS68), KP296181 (BeWbL1), KP296182 (BeWbL24), KP296183 (BeWbL68), KP296184 (BeWbL67) and KR149812 (BeCL48).

Statistical analysis

A 95% confidence interval (CI) of prevalence was estimated using an exact binomial distribution. The receiver operating characteristic (ROC) curve with WB considered as reference test was performed to investigate the optimal cut-off (CO) point of the ELISA. Different scenarios with varying ELISA specificities relative to WB were analysed to measure the impact on HEV seroprevalence in wild boar (Table 2). For the wild boar analyses, a multivariate logistic regression was performed with the following explanatory variables: gender (female as reference), age of animals (adult as reference), density of animals expressed as number of shots/1000 ha of forest (lower density as reference) and area of sampling collection (administrative direction of Arlon as reference). The size of the chosen spatial unit is compatible with the regular spread of wild boar (Prevot and Morelle, 2012). In addition, to assess the collinearity, a backward elimination of variables was performed (Preux et al., 2005). If a variable induced a modification of the odds ratio of more than 20%, this variable was retained in the final model where the interaction was tested. All pairwise interactions between the variables in the final model were examined for significance. Goodness of fit was assessed using the Hosmer–Lemeshow goodness-of-fit test. Statistical analyses were performed using STATA/SE Acad. 12 (Stata Corp., College Station, TX, USA).

Table 2. Estimation of hepatitis E virus seroprevalence in the Belgian wild boar population. Analysis of three scenarios of varying ELISA specificities relative to WB (third to fifth lines). The two-first lines show the parameters estimated for the optimal cut-off obtained by the ROC curve (first line) and the average ELISA cut-off obtained according to the manufacturer instructions (second line)

| Cut-off | Specificity (%) | Sensitivity (%) | Estimated ELISA screening prevalence (%)* | Estimated seroprevalence after confirmation by WB (%)† |
|---------|-----------------|-----------------|---|--|
| 0.30 | 87.50 | 65.63 | 31.59 | 40.40 |
| 0.26 | 85.00 | 65.63 | 34.46 | 40.56 |
| 0.85 | 50.00 | 87.00 | 64.23 | 34.16 |
| 0.15 | 75.00 | 71.88 | 46.48 | 42.01 |
| 0.39 | 90.00 | 59.38 | 27.68 | 40.27 |

*ELISA screening prevalence (%) = (number of positive results in ELISA/number of animals tested by ELISA) × 100.

†Prevalence after confirmation by WB (%) = [(number of true positive animals in ELISA + number of false-negative animals in ELISA)/total number of animals] × 100.

With: Number of true positive animals in ELISA = number of animals positive in ELISA × (number of positive animals both in ELISA and in WB/number of positive animals in ELISA) and number of false-negative animals in ELISA = number of animals negative in ELISA × (number of animals negative in ELISA but positive in WB/number of negative animals in ELISA).

Results

ELISA screening prevalence of HEV infection

The presence of HEV-specific antibodies was demonstrated by ELISA, using the CO value, obtained following the manufacturer's instructions and calculated for each plate. An overall apparent seroprevalence of 34% (95% CI 29.71–39.46) was found in wild boars, of 1% (95% CI 0–2.4) in red deer and 3% (95% CI 0.8–4.2) in roe deer.

Western blot analysis

To assess the ELISA screening prevalence, WB analyses against IgG were performed (Table 1a–c; Fig. 1). In wild boars, 22 of 77 sera showed discrepant results. The three sera from experimentally infected swine were positive; of these, two were infected with genotype 3 and one with genotype 4. The three SPF pig sera were all negative. Discrepant results were also detected for 1 of 38 and 7 of 49 sera in red deer and roe deer, respectively.

The data obtained following the WB analyses in wild boars were used for the conception of a ROC curve analysis with WB as reference test. Due to the extremely low seroprevalences obtained in red and roe deer, no ROC curve was made for these species.

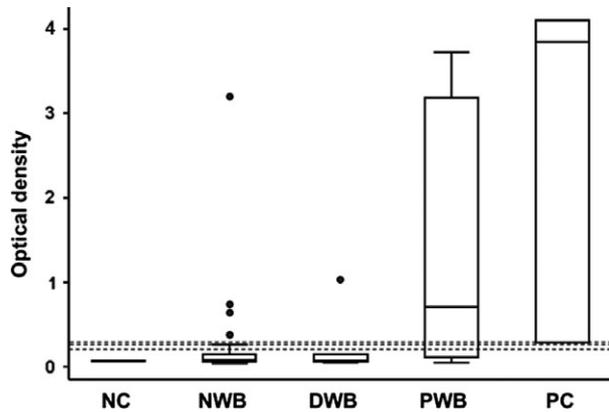


Fig. 1. Box plot. Distribution of wild boar serum samples according to the ELISA OD value classified following the Western blot results. NC = specific-pathogen-free pigs, NWB = negative in Western blot, DWB = doubtful in Western blot, PWB = positive in Western blot, PC = positive control (experimentally infected pigs), --- The three horizontal lines = optimal cut-off of 0.29 estimated by the ROC curve and 95% CI based on the computation of the cut-off according to producer based on the average of the calculated cut-off for nine plates (i.e. 0.26) (95% CI 0.24–0.28).

Virological results and comparison with swine and human strains

To complete the serological investigation in wild boars and cervids and to assess the role of these species in the potential transmission of the disease from wildlife to swine and humans, the different sequences obtained in this study were compared with those observed in swine and humans in the same region. In wild boar, 4 of 69 sera and 4 of 61 livers were detected as positive for HEV RNA. All sequences obtained from sera belonged to HEV genotype HEV-3, with three belonging to subtype 3f and one to 3c according to the classification of Lu et al. (2006). All sequences obtained from the livers were of genotype HEV-3 subtype f. They were compared with the human and swine strains belonging to genotype HEV-3. Most of these sequenced fragments also belonged to subtype 3f (Fig. 2). None of the sera of >6 months old wild boar was positive. HEV RNA was detected in 1 of 29 livers from red deer but not in roe deer. The detected sequence belongs to genotype 3f. No HEV RNA was detected in either red or roe deer sera.

ROC curve analysis in wild boar

According to the methodology used in a previous study (Thiry et al., 2014), seroprevalence results obtained by ELISA screening in wild boar were re-evaluated by a ROC curve analysis using WB as reference test after exclusion of the borderline data (Fig. 3). Different scenarios were analysed in Table 2. Different values of ELISA specificity relative

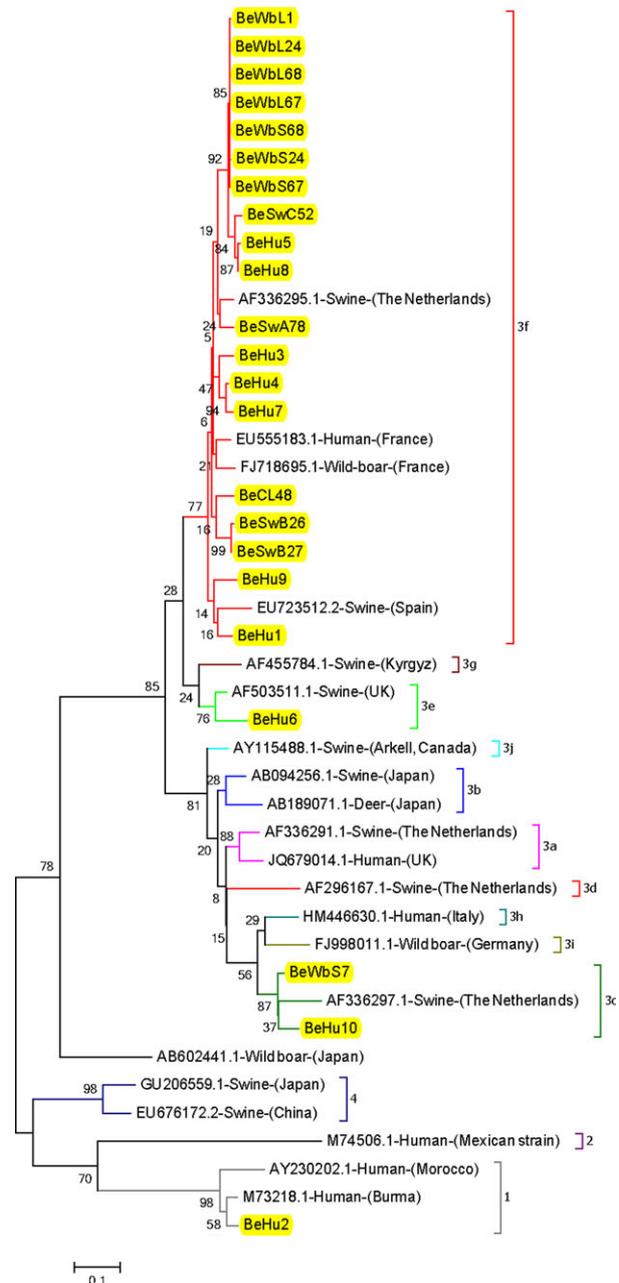


Fig. 2. Phylogenetic relationships on 44 302-bp-long sequences in open reading frame 2. GenBank Accession numbers are shown for each HEV strain used in the phylogenetic analysis. The scale bar indicates nucleotide substitutions per site. Belgian strains are underlined in yellow. BeHu, Belgian human; BeSw, Belgian swine; BeWbL = Belgian Wild boars liver; BeWbS = Belgian Wild boar serum; BeCL = Belgian Cervid Liver).

to WB and of CO (CO obtained by the ROC curve or by the ELISA) were assessed. Seroprevalence remained high whatever the scenario in the wild boar population. Indeed, it ranged from 27% to 64% for the ELISA screening prevalence and from 34% to 42% after confirmation by WB.

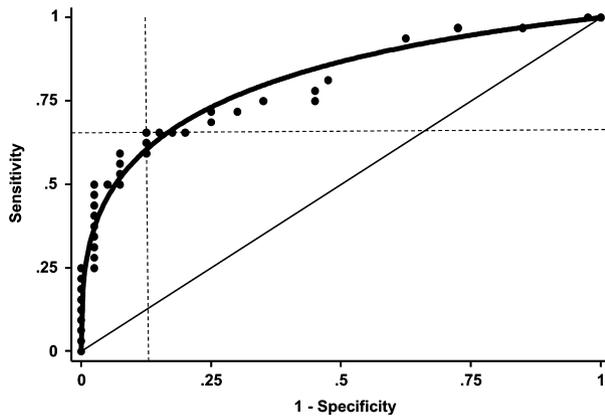


Fig. 3. Receiver operating characteristic curve (ROC curve) of the wild boar ELISA data versus Western blot as reference test after exclusion of the borderline data. Area under curve = 0.8148; standard error (area) = 0.0509. - - : Fitted ROC curve; ● : Observed values of the ROC curve; - - - : Intersection between horizontal and vertical lines is the optimal cut-off (0.30) (based on observed data).

Statistical analysis

Using a multivariate logistic regression and after a backward stepwise procedure, a model with three variables (age, density and direction) was retained. No interaction between variables was observed excluding collinearity between the variable density and the variable administrative direction. Next two different models were tested (age and density versus age and administrative direction), and the second model was retained based on the higher result of the Hosmer–Lemeshow test. A significant effect of age was observed. The number of seropositive juvenile (6 month to 1 year of age) and subadult wild boars was lower (OR = 0.46 with 95% CI: 0.27–0.76, P -value = 0.003 and OR = 0.57 with 95% CI: 0.33–0.99, P -value = 0.047, respectively) than adult animals taken as reference (Table 3). A significant effect of density was also observed. Area with density between 46.5 and 63.2 wild boars by 1000 ha (Fig. 4) contained less seropositive animals (OR = 0.36 with 95% CI: 0.13–0.985, P -value = 0.047) than the reference area with density between 0.3 and 16 wild boars by 1000 ha (Table 3). The Hosmer–Lemeshow test showed that the final model fits well with the data ($\chi^2 = 2.06$, $df = 9$, P -value = 0.96).

Discussion

A high HEV seroprevalence was observed in wild boars in a region where HEV is already known to be well distributed in domestic pigs. The combination of these two facts raises the question of HEV transmission between the domestic and wild population of suids. In wild boars, the HEV

seroprevalence was unevenly distributed between the age groups with a higher prevalence in older animals. Despite the high density of both deer and wild boar populations in the investigated region, a much lower prevalence was observed in cervids than in wild boars, raising the question of interspecies transmission and the main transmission routes for HEV in the considered areas. All strains detected in wild boars, cervids and pigs in the same region belonged to genotype HEV-3, most of them to subtype f.

Despite the high seroprevalence observed in wild boars, 34% (95% CI 29.71–39.46), it appears to be lower than that obtained in domestic pigs in the same region, using the same methodology (Thiry et al., 2014). The free-ranging behaviour of wild boars could induce a more progressive and slower circulation of the virus in this population compared to domestic pigs bred in an intensive production system. A significant difference was observed between HEV seroprevalences with regard to hunting bags, which are regarded as the best approximation to approach wildlife population size estimates (Hagen et al., 2014; Massei et al., 2014). The area with a density between 46.5 and 63.2 wild boars by 1000 ha contained less seropositive animals than the reference area with density between 0.3 and 16 wild boars by 1000 ha. The area with a lower number of seropositive wild boars is close to France where low seroprevalences were observed, especially in the north (7.3%) (Carpentier et al., 2012). The reference density area is close to Germany where a higher seroprevalence was observed (29.9%) (Adlhoeh et al., 2009). These results suggest that HEV seroprevalences in wild boars are linked to forest massifs crossed by national borders. These data support the need for supra-national studies to understand the spreading of the virus in the wild boar populations.

In cervids, a low seroprevalence was observed: 1% and 3% in red and roe deer, respectively. Despite this low seroprevalence, the circulation of the virus was confirmed by the detection of HEV RNA in red deer. Whereas a similar high seroprevalence, to that observed in this study, has been documented in wild boars in other European countries (Adlhoeh et al., 2009; Boadella et al., 2012), the low prevalences in cervids were unexpected. The differences could be explained by the fact that cervids have a more solitary behaviour compared to the social grouping of wild boars caused by their gregarious instinct. Nevertheless, the virus may be transmitted by faeces in a zone where cervids and wild boars interact, such as feeding sites. Indeed, in the Walloon Region, artificial feeding causes spatial aggregation of wild animals and could increase contact between cervids and wild boars (Gregoire et al., 2012). These two hypotheses could offer a better understanding of the virus transmission between these two species and suggest that cervids should be considered more as incidental hosts of HEV.

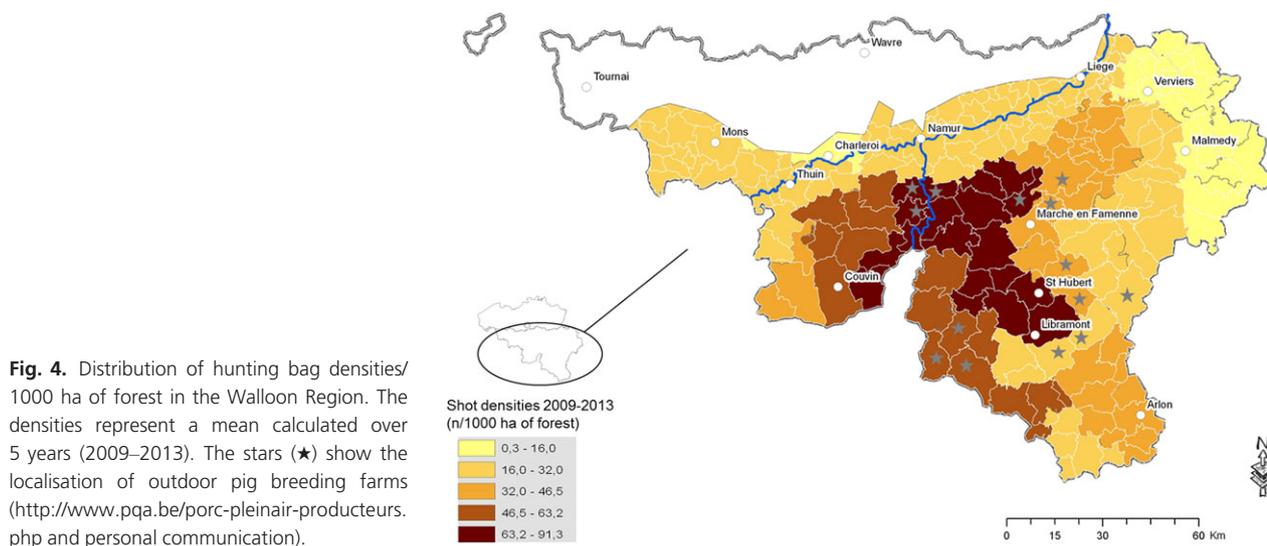


Fig. 4. Distribution of hunting bag densities/1000 ha of forest in the Walloon Region. The densities represent a mean calculated over 5 years (2009–2013). The stars (★) show the localisation of outdoor pig breeding farms (<http://www.pqa.be/porc-pleinair-producteurs.php> and personal communication).

Table 3. Risk factors related to the hepatitis E virus seroprevalence of wild boars using multivariate logistic regression

| Variable | Modalities | OR (95% CI) | P-value |
|--|-------------------------|------------------|---------|
| Age | Adult (>24 months) | Reference | – |
| | Subadult (12–24 months) | 0.57 (0.33–0.99) | 0.047* |
| | Juvenile (6–12 months) | 0.46 (0.27–0.76) | 0.003* |
| Density of animals (shots/1000 ha of forest) | 0.362509–16.031017 | Reference | – |
| | 16.031018–32.039480 | 0.61 (0.25–1.48) | 0.27 |
| | 32.039481–46.560416 | 0.78 (0.32–1.91) | 0.59 |
| | 46.560417–63.278476 | 0.36 (0.13–0.99) | 0.047* |
| | 63.278477–91.321668 | 1.05 (0.44–2.51) | 0.91 |

OR, odds ratio; CI, confidence interval.

*P-value < 0.05.

Due to the differences in wild boar and deer seroprevalences observed between Belgium and other European countries as well as with domestic pigs in Belgium, the ELISA screening prevalence obtained in wild animals was confirmed with a WB adapted to these two species. The analysis of scenarios performed on wild boar data showed that the estimated ELISA screening prevalence remained high and ranged between 27% and 65% whatever the ELISA specificity relative to WB. In cervids, despite the lack of concordance between ELISA and WB (Table 1b,c), the observations of positive results in both tests confirm that the virus circulates in this species despite the observed low seroprevalence. We cannot absolutely rule out a lack of sensitivity of the used ELISA. Discrepant result between ELISA and WB observed for some deer sera show that the serological tools should be refined for further analyses in deer populations. In the case of low seroprevalence, a combination of both ELISA and WB could enhance the sensitivity and the specificity of antibody detection in wildlife.

In the present study, all the viruses identified in wild boar and red deer samples fall into genotype HEV-3. The identified sequences have strong similarities between themselves and those found in pigs and humans in Belgium. This finding is of great importance in terms of human exposure. It raises concerns regarding the high rates of HEV circulation in the wild boar population with the risk of transmission to humans through direct or indirect contact (e.g. hunters) or through contaminated wild boar products (e.g. uncooked meat). Indeed, two cases of transmission from uncooked wild boar meat to humans were already described in Japan (Li et al., 2005; Okano et al., 2013). In Belgium, some outdoor pig breeding sites are located in regions with high densities of wild boars and, therefore, transmission between them cannot be excluded (Fig. 4). Hepatitis E virus transmission was recently evidenced between wild boars, swine and humans in Japan, Spain and France (Li et al., 2005; Renou et al., 2014; Riveiro-Barciela et al., 2014). These data are also supported by the high level of genetic relationships

observed between HEV strains from humans, pigs and wild boars in one region (Widen et al., 2011).

The phylogenetic study was performed on a 302-bp fragment corresponding to the 5' end of ORF2, which is highly conserved among all HEV isolates (Lu et al., 2006). Sequences detected in pigs and human samples mainly cluster with subtype 3f, as did most of the wild boar and red deer strains detected in this study. In wild boar, the sera and liver numbered 24, 67 and 68 belong to the same animals. These data support a zoonotic potential for HEV in Belgium. Further work needs to be carried out to investigate the discrepancy between the high prevalence in pigs and the lower amount of wild boar and red deer infections.

In Walloon Region, more than 20 000 wild boars are harvested annually since 2005 during game activities. As animals are directly eviscerated, hunters are thus directly exposed to blood and organs with risk of transmission of pathogens, including HEV. It is highly recommended for hunters to wear protective gloves for handling and evisceration in field conditions. Even though the consumption of uncooked wild boar meat is uncommon in this region, thorough cooking of meat is an essential recommendation to limit the risk of HEV transmission. In addition, preventive measures such as the setting up of double fences with a height of 1.2 m and maintaining sows indoors during heat (Gregoire et al., 2012) must be adopted by the outdoor pig farmers.

Wild boar can be considered as a host reservoir of HEV in Belgium. However, contrarily to the apparent epidemiological role of deer in other countries, the low prevalence in this species excludes it as a reservoir, establishing deer more as accidental hosts. Further investigations are needed to determine in which epidemiological situation deer can serve as reservoir. These results raise also the question of the dynamics of HEV infection between wildlife, domestic pigs and humans.

Acknowledgements

We are profoundly thankful to Louisa Ludwig for the English proofreading of the manuscript. We also thank Wim Van der Poel and Roland Cariolet for the generous provision of positive and negative control sera. This research was supported by the Belgium Federal Public Service, Health, Food Chain Safety and Environment (Project HEVEA, RF10/6236) and the Walloon Public Service.

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