

## Seroprevalence of hepatitis E virus in Lithuanian domestic pigs and wildlife

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### Abstract

Hepatitis E is an important public health concern disease that causes acute hepatitis in humans with different hepatitis E virus strains reported among domestic and wild animals. In Lithuania, seroprevalence studies on animals considered to be possible virus reservoirs, particularly in wildlife species, had never been investigated before. In order to assess the actual distribution of the virus in the domestic pig and wildlife species, serum samples originating from different geographic areas of Lithuania were tested for the presence of antibodies to the virus using enzyme-linked immunosorbent assay. The study results proved that hepatitis E virus infection is prevalent among domestic pigs, wild boar, moose, and roe deer in Lithuania, with the overall prevalence of IgG antibodies against hepatitis E virus being 43.75%, 57.05%, 11.76%, and 1.20%, respectively. Significantly higher ( $P < 0.01$ ) seroprevalence values of 53.66% and 80% were observed in weaned pigs and adult wild boar. Herds with 15 001–30 000 pigs were 2.4 times more likely ( $P < 0.01$ ) to test positive for hepatitis E virus antibodies (70.18%), than herds with  $\leq 500$  pigs (21.11%). The differences in seroprevalence rates between domestic pigs (from 25 to 66.67%) and wild boar (from 42.86 to 73.33%) in all 10 counties were not significant ( $P > 0.05$ ). Estimated low seroprevalence rate in moose, roe deer and absence of antibodies in red deer suggest that such wildlife species are accidental hosts of hepatitis E virus or at least hepatitis E virus is not present in Lithuanian cervides.

*HEV, sow, wild boar, cervidae, serum, zoonosis*

According to data reported by the World Health Organization (2015), in humans there are estimated 20 million cases of hepatitis E infections, over 3 million symptomatic cases of hepatitis E, and 56 600 hepatitis E-related deaths every year. Therefore, hepatitis E is considered an emerging zoonotic disease in many developing and industrialized countries. The aetiological agent of the disease is hepatitis E virus (HEV) that affects multiple hosts. The HEV is unique among known hepatitis (A, B, C, D) viruses because domestic pigs, wild boar, deer, and other animal species are considered to be possible reservoirs of HEV. Up to date, at least seven genotypes of HEV have been reported, and five of them have been described in humans (HEV-1 and HEV-2 anthroponotic, HEV-3 and HEV-4 zoonotic, HEV-7 zoonotic potential is still being questioned) (Roth et al. 2016). In contrast to humans, pigs and wild boar infected by HEV do not show obvious clinical manifestations.

The consumption of raw or undercooked contaminated meat of domestic pig, wild boar or deer and also seafood receiving sewage sludge or direct contact with infected animals have been identified as modes of transmission linked to autochthonous sporadic cases of acute hepatitis E in humans (Pavio et al. 2010; Kaba et al. 2013). Moreover, increased distribution and availability of game meat poses a major risk for foodborne transmission of HEV worldwide. In addition,

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hunters or human populations with occupational exposure (veterinarians, slaughterhouse workers, and farmers) have been found to be associated with a higher prevalence of anti-HEV antibodies compared to normal blood donors (Mansuy et al 2008).

It is well known that wild boar harbor many important infectious agents that are transmissible to domestic pigs and other animal species, including humans. Moreover, wild boar have shown a notable increase in the population density throughout Europe and the USA over the past decades (Schielke et al. 2009). Changes of human habitation to suburban areas owing to growing world populations, increased use of lands for agricultural purposes, and deforestation have all increased chances of contact exposure of wild boar to humans and domestic animals, including pigs. Close contact between wild boar and humans not only means a larger number of hosts available for the transmission of the disease, but also a higher contact rate between hosts (Ruiz-Fons et al. 2008).

Clearly, the higher the prevalence among animals, the greater is the risk of transmission to humans. Therefore, in order to assess the actual distribution of HEV in the domestic pigs and wildlife species, sera from different geographic areas of Lithuania were tested for the presence of the HEV using a commercially available indirect enzyme-linked immunosorbent assay (ELISA) kit.

### Materials and Methods

#### Domestic pig sampling

A total of 384 blood samples from domestic pigs were obtained between 2014 and 2015, from 9 different geographic areas in Lithuania. Samples were collected from selected farms ( $n = 155$ , located in counties mentioned in Fig. 1) in the context of official surveillance programs of infectious diseases by veterinarians. Information on samples was systematically collected on farms. Domestic pig age classification was as follows: weaned pigs ( $n = 82$ ), pigs for fattening until slaughter ( $n = 200$ ) and sows  $> 1$  year ( $n = 102$ ).

#### Wildlife sampling

A total of 312 blood samples were obtained between 2014 and 2015 from wild boar located in 10 different geographic areas in Lithuania. Samples were randomly collected from wild boar shot during the hunting seasons by hunters. They were categorized on the basis of age (teeth method) and location of death for most samples. Samples were divided in the following age distribution: piglets, up to 1 year ( $n = 150$ ), yearling, 2<sup>nd</sup> year ( $n = 104$ ), subadult, 3<sup>rd</sup> year ( $n = 38$ ) and adult,  $> 4^{\text{th}}$  year ( $n = 20$ ).

In addition, serum samples from moose ( $n = 34$ ), roe deer ( $n = 166$ ), red deer ( $n = 108$ ), and European bison ( $n = 3$ ) collected between 2014 and 2015 were also available for serological analysis, with no estimation of the animals' age.

Since wild animals were killed during permitted hunting seasons, according to the Lithuanian legislation no ethical approval was needed.

#### Blood and detection of HEV-specific antibodies by ELISA

Blood samples obtained from wildlife were gathered from the hearts or thoracic cavity of the hunted animals during necropsies. All animal blood samples were centrifuged immediately after arrival at the laboratory, and aliquots of serum were frozen at  $-20^{\circ}\text{C}$  until further analysis. HEV-specific antibodies (anti-HEV immunoglobulin IgG) were detected by a commercially available ID Screen Hepatitis E multi species indirect ELISA (IDvet, France) suited for use in swine and other species according to the manufacturer's instructions. HEV genotype 3 capsid antigen for HEV antibody detection was used. The microplate was read and recorded the optical density (O.D) at 450 nm. The results were interpreted by calculating the S/P percentage = (corrected O.D. of sample / corrected O.D. of positive control)  $\times 100$ . The samples presenting  $S/P \geq 70\%$  were considered as positive,  $S/P$  between 60–70 as doubtful, and  $S/P \leq 60\%$  as negative.

#### Statistical analysis

Descriptive statistics were prepared using Microsoft Excel 2007 and results were presented as percentage. For comparison of the prevalence between different groups, Fisher's exact test was applied. Differences were considered significant at  $P \leq 0.05$ . To determine the correlation between the presence of HEV in different animal species and herd size, odds ratios (OR) and their corresponding 95% confidence intervals (CI) were calculated using binary logistic regression analysis. Pearson Chi square test was used to establish association between serological results and different risk factors (animal species, herd size) considered in this study. Spearman correlation test was applied to assess the relationship between wild boar population density and seropositivity. The densities of wild boar were calculated from data obtained using official reports (years 2012–2015) of the Ministry of Environment of the Republic of Lithuania (ME 2012–2015).

Table 1. HEV seroprevalence in domestic pigs and wildlife samples in Lithuania during 2014–2015.

Animal species	Number of tested / positive serum samples	%	95 CI, %	OR	P-value
Domestic pig ( <i>Sus scrofa domestica</i> )	384/168	43.75	38.9–48.7	0.78	< 0.05
Wild boar ( <i>Sus scrofa</i> )	312/178	57.05	51.5–62.4	1.33	< 0.05
Moose ( <i>Alces alces</i> )	34/4	11.76	4.7–26.6	0.13	< 0.001
Roe deer ( <i>Capreolus capreolus</i> )	166/2	1.20	0.3–4.3	0.01	< 0.001
Red deer ( <i>Cervus elaphus</i> )	108/0	0.00	0–3.4	0.00	< 0.001
European bison ( <i>Bison bonasus</i> )	3/0	0.00	0–56.1	0.00	

HEV - hepatitis E virus; CI - confidence interval; OR - odds ratio

## Results

Serological analysis (Table 1) showed that 168 out of 384 (43.75%; 95% CI: 38.9–48.7;  $P < 0.05$ ) domestic pigs, 178 out of 312 (57.05%; 95% CI: 51.5–62.4) wild boar, 4 out of 34 (11.76%; 95% CI: 4.7–26.6) moose, and 2 out of 166 (1.20%; 95% CI: 0.3–4.3) roe deer samples were positive for anti-HEV IgG antibodies. All 108 red deer and 3 European bison samples were negative for anti-HEV IgG antibodies.

Significant association was observed with regard to animal species. Roe deer accounted for the lowest seroprevalence (1.2%) while the highest seroprevalence was detected among wild boar (57.05%). Wild boar were recorded as the main important animal species to be at risk for HEV positivity (57.05%, OR = 1.33), followed by domestic pigs (43.75%,

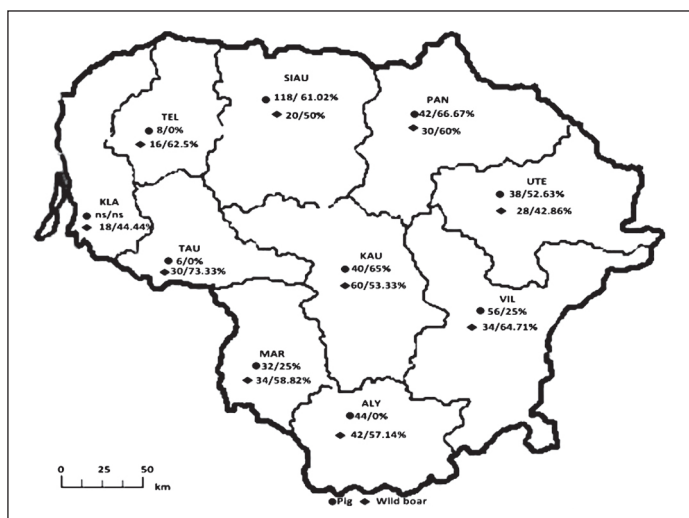


Fig. 1. Hepatitis E virus (HEV) seroprevalence distribution in domestic pig and wild boar samples in different Lithuanian counties during 2014–2015.

Bold letters represent different counties and are abbreviated as follows: ALY, Alytus; MAR, Marijampole; VIL, Vilnius; KAU, Kaunas; TAU, Taurage; KLA, Kaipeda; TEL, Telsiai; SIAU, Siauliai; PAN, Panevezys; UTE, Utena. The first number indicates the number of all investigated serum samples, the second number (after the slash) indicates the detected HEV prevalence rate (%) out of all investigated samples in this study; ns - no sample available

OR = 0.78). We performed a binary logistic regression analysis and obtained that seroprevalence rate was significantly associated with affected animal species ( $P < 0.05$ , and  $< 0.001$ ) for domestic pig and wild boar, and for moose, roe deer and red deer, respectively.

The HEV prevalence detected in different counties of Lithuania where positive samples were detected ranged from 25.00% (95% CI: 13.66–36.34) to 66.67% (95% CI: 52.41–80.93) for domestic pigs and from 42.86 (95% CI: 24.53–61.19) to 73.33% (95% CI: 57.51–89.15) for tested wild boar. The highest percentages of seropositive domestic pigs (66.67%) and wild boar (73.33%) were observed in Panevezys and Taurage, respectively (Fig. 1). The differences in seroprevalence rates between domestic pigs or wild boar in all 10 counties were not significant ( $P > 0.05$ ).

HEV was determined in all age groups of tested domestic pigs and wild boar. However, relatively higher seroprevalence values were noticed in weaned pigs (53.66%) and in adult (80.00%) wild boar (Fig. 2). Weaned pigs presented the highest seroprevalence and this value was significantly ( $P < 0.01$ ) more frequent than in pigs for fattening (35%). Moreover, sows (52.94%) were more likely to test positive ( $P < 0.01$ ) for HEV antibodies than pigs for fattening, however, seroprevalence differences between weaned pigs (53.66%) and sows (52.94%) were not significant ( $P > 0.05$ ).

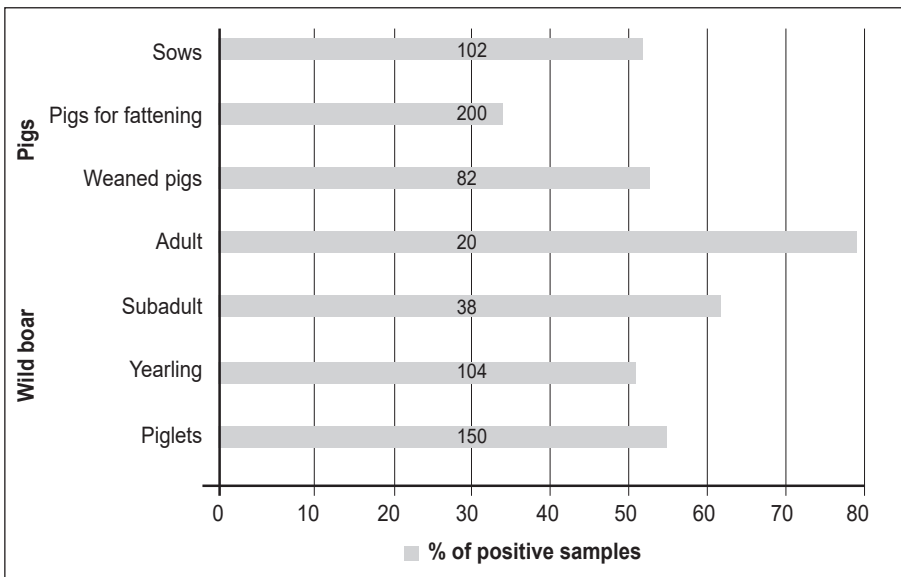


Fig. 2. The prevalence of hepatitis E virus in sera samples of pigs and wild boar displayed by age groups. Numerals inside columns indicate the total number of tested samples in different age groups.

Detection rates of piglets, yearlings, subadults, and adults in wild boar were 56.00%, 51.92%, 63.16%, and 80.00% respectively. However, no significant differences between wild boar age groups were observed. Only adult wild boar seroprevalence value was significantly higher ( $P < 0.05$ ) compared to piglets and yearlings.

The impact of herd size on the prevalence of HEV was examined by classification based on the approximate number of pigs in the herd (Table 2). In total, antibodies against HEV were detected in 168 pigs herds samples (43.75%; 95% CI: 38.9–48.7) in 68 farms (44.74%, data not shown). Of the 384 herds, 170 included 5 001 or more pigs and were classified as large

Table 2. Seroprevalence of HEV in pig farms according to the herd size 2014–2015.

Size of pigs herds (approximate number of pigs)	Number of tested / positive herds samples	%	95 CI, %	OR	<i>P</i> -value
1–500	180/38	21.11	15.8–27.6	0.3	< 0.001
1000–5000	34/18	52.94	36.7–68.5	1.1	
5001–10 000	32/14	43.75	28.2–60.7	0.8	
10 001–15 000	24/18	75.00	55.1–88	3.0	< 0.05
15 001–30 000	114/80	70.18	61.2–77.8	2.4	< 0.001
Total	384/168	43.75	38.9–48.7	0.8	< 0.05

HEV - hepatitis E virus; CI - confidence interval; OR - odds ratio

Table 3. Prevalence of HEV in wild boar 2013–2015.

Year	Number of wild boar	Total forest land area (ha)	Population density of wild boar (animals/km <sup>2</sup> )	Number of tested/ positive wild boar samples (%)	95 CI, %
2015	27 497	2 179 900	1.26	80/42 (52.50%)	41.56–63.44
2014	22 325	2 177 000	1.03	232/136 (58.62%)	52.28–64.96
2013	61 795	2 174 000	2.84	140/83 (59.23%)*	51.09–67.37
Total	111 617	6 530 900	1.71	452/261 (57.74%)	51.56–62.54

HEV - hepatitis E virus; CI - confidence interval.

\* - Data obtained from sera of wild boar shot in 2013 (the authors, unpublished)

herds; 214 of them included < 5 000 pigs and were classified as small herds. Results of the present study revealed that larger herds were more likely to be seropositive than small herds. The likelihood of presence of HEV antibodies in large herds of 10 001–15 000 (75.00%, OR = 3.0, 95% CI: 55.1–88,  $P < 0.05$ ) and 15 001–30 000 (70.18%, OR = 2.4, 95% CI: 61.2–77.8,  $P < 0.001$ ) pigs is  $\times 3$  and  $\times 2.4$  higher, respectively, compared to other herd size groups.

Wild board population density was assessed by dividing the total population size by the total forest land area. During the period 2013–2015 the density of wild boar population decreased (Table 3). Since 2014 a significant decline in density was observed, as a result of control measures (intensive hunting efforts due to outbreaks of African swine fever). Although the population density in 2015 (1.26 animals/km<sup>2</sup>) increased compared to the population density in 2014 (1.03 animals/ km<sup>2</sup>), we detected higher HEV seroprevalence in 2014 (58.62%, 95% CI: 52.28–64.96) compared to seroprevalence in 2015 (52.50%, CI: 41.56–63.44). The relationship between wild boar population density and seropositivity suggests that there is moderate positive correlation but inconclusive evidence about the significance of this association ( $r_s = 0.5$ ,  $P = 0.667$ ).

## Discussion

Nowadays, hepatitis E is common not just in developing countries with inadequate water supply and environmental sanitation, but more autochthonous sporadic cases are diagnosed also in industrialized countries (Mansuy et al. 2008; Kaba et al. 2013), possibly mediated by the international trade of pigs, migration of wild animals, human contact with animals and their biological material, etc.

The majority of published studies indicate that zoonotic human infection can presumably be caused by consumption of raw or undercooked contaminated meat from the domestic

pig, wild boar, or deer, or via close contact with infected animals. Also distinct HEV-like viruses were identified in the rat, rabbit, ferret, mink, fox, bat and moose, and a distantly related agent was even described in salmonid fish (Johné et al. 2014).

Diagnosis of HEV is usually determined serologically by detection of the presence of IgG or IgM antibodies. It is important to note that differences in sensitivities of anti-HEV IgG tests must be taken into account when interpreting published HEV seroprevalences, because estimates differ significantly depending on the assay used, the geographical region, and the type of study cohort (Hartl et al. 2016).

Wild boar are indigenous and one of the more intensively hunted ungulate species in many countries, including Lithuania. Nevertheless, wild boar population has been expanding throughout Europe during the last 40 years (Ruiz-Fons et al. 2008). Increased consumption of game meat or viscera from wild boar and cervids (moose, roe deer, red deer) has become more popular, thus posing an increased risk of HEV transmission to humans. In, our study, samples taken from domestic pigs, wild boar, moose, roe deer and red deer, all potential HEV reservoirs used in the food chain were examined for the presence of HEV antibodies for the first time in Lithuania. Obtained results indicated that HEV is highly prevalent in Lithuanian domestic pigs and wild boar with a mean seroprevalence rate of 43.75% and 57.05%, respectively. The seroprevalence rate in Lithuanian pigs nearly corroborates study reports from Canada (Yoo et al. 2001), Spain (Seminati et al. 2008) and Switzerland (Burri et al. 2014) where 38–59.5% of tested serum samples had antibodies against HEV. In contrast, a study from the UK indicated that 92.8% of 640 pigs sampled at slaughter were seropositive to HEV (Tedder 2014). Approximately 62% and 73% of serum from domestic pigs were positive for anti-HEV IgG antibodies in Estonia (Ivanova et al. 2015) and Denmark (Kaba et al. 2013), respectively.

The relatively high detected HEV seroprevalence rate (57.05%) in Lithuanian wild boar serum was similar to previously published data from Italy (Mazzei et al. 2015a) that revealed seroprevalence of 56.2% in the wild boar population. A Polish study determined 44.4% of HEV antibodies in the serum of wild boar from 11 different Polish provinces (Larska et al. 2015), while a Spanish study (de Deus et al. 2008) found an overall seroprevalence of 42.7% in wild boar population from South Central Spain, whereas IgG seroprevalence ranged from 0% to 63.3% depending on geographical region. On the other hand, overall seroprevalence in wild boar population in Switzerland and Croatia was reported to be 12.5% (Burri et al. 2014) and 12.3% (Prpic et al. 2015) of all tested samples, respectively.

Moreover, few studies have been carried out to evaluate HEV prevalence in the cervids or other wildlife animals. In Spain, an apparent seroprevalence of 10.4% was observed in 968 red deer (Boadella et al. 2010), in Italy – 5.6% of 54 red deer (Mazzei et al. 2015b), and in Belgium – 1% of 189 red deer (Thiry et al. 2015). The HEV specific antibodies were detected in serum of 5% of 38 red deer and none of 8 roe deer studied in Netherlands (Rutjes et al. 2010); whereas in Poland, no antibodies were detected in 118 red deer and 38 roe deer serum samples (Larska et al. 2015). We have estimated a 1.2% HEV seroprevalence in Lithuanian roe deer and this result was partially in line with Thiry et al. (2015) who determined 3% of 235 roe deer to be seropositive in Belgium. We suppose that lower seroprevalence in roe deer in Lithuania may suggest that this species may be an accidental host of HEV or HEV does not actively circulate in Lithuanian roe deer population. The present study also revealed that none of the 108 studied red deer were positive for HEV antibodies, matching the data from the neighbouring Poland (Larska et al. 2015). Lack of scientific studies on seroprevalence in moose and European bison makes comparisons difficult. In Sweden, 19% (231/43) of moose were seropositive for HEV antibodies (Lin et al. 2015). We noticed seroprevalence of 11.76% in moose and no positive samples of 3 European bison. Meanwhile, in the United States where bison meat

is increasingly consumed, 4.6% of domesticated bison (*Bison bison*) were positive for HEV antibodies (Dong et al. 2011). Notably, the absence of seropositivity in European bison samples is not significant due to the small sample size, but the results are in line with a report from Poland where HEV antibodies were not found in any of the 68 European bison (*Bison bonasus*) tested (Larska et al. 2015). To sum up, much lower prevalence was observed in the cervids compared to wild boar. The differences could be explained by the fact that cervids have a more solitary behaviour compared to the social grouping of wild boar (Thiry et al. 2015).

Our seroprevalence studies have indicated that HEV is present in all age groups of domestic pigs. Antibodies against HEV were detected in 53.66%, 35.00%, and 52.94% of weaned pigs, pigs for fattening and sow serum, respectively. Weaned pigs presented the highest seroprevalence and this value was significantly ( $P < 0.01$ ) more frequent than in pigs for fattening. In particular, the seroprevalence was significantly higher ( $P < 0.01$ ) in sows compared to pigs for fattening. Results of the age distribution of the HEV infection in domestic pig population varies in different countries. Berto et al. (2012) determined that the prevalence in pigs for fattening ranged between 8% and 73% in six different EU countries. The highest seroprevalence of 70.6% and the lowest seroprevalence of 12.1% were noted in Italy, in sows and weaners, respectively (Mazzei et al. 2015b). In contrast, a study in Croatia reported the highest prevalence of HEV in the age class of 1–4 week suckling piglets (Prpic et al. 2015). Our study results support the notion by Pavio et al. (2010) that wide variations in within-herd seroprevalence would appear to be the norm in HEV-infected herds.

Different seroprevalence rates have also been observed in different age classes of wild boar. Adult wild boar (80.0%) presented the highest seroprevalence ( $P < 0.05$ ) rate compared to piglet or yearling wild boar. This age group fact is in line with the results reported from Switzerland, where the highest prevalence of 22.5% was also detected among adult wild boar (Burri et al. 2014). Significantly higher seroprevalence in adult wild boar could be explained by abundant artificial feeding. The artificial feeding of wild boar has been practiced in Lithuania for many years as supplementation during winter time, as a dissuasive measure aimed to reduce crop damage by wild boar or an attractive measure during the hunting season. Artificial feeding causes a special aggregation of wild boar and increases the contact among animals. The adult wild boar have more chances of contact with HEV-infected feed or faeces in the artificial feeding grounds compared to piglets, yearlings or subadult wild boar.

The seroprevalence of HEV on pig farms compared to the herd size showed the likelihood of presence of HEV antibodies in large herds of 10 001–15 000 and 15 001–30 000 pigs is  $\times 3$  and  $\times 2.4$  higher, respectively, compared to other herd size groups. This finding could be explained by the fact that on large farms, more frequent contact between pigs and their faeces can occur, leading to repeated HEV infection and consequently a higher prevalence rate.

In this study we also evaluated HEV seroprevalence compared to the density of wild boar in 2013–2015. According to the official reports of the Ministry of the Environment of the Republic of Lithuania (ME 2012–2015), the number of wild boar changed from 61 795 in 2013 to 22 325 in 2014, and 27 497 in 2015.

The different seroprevalence rates of HEV detected in this study during 2013–2015 may suggest an influence of wild boar density to the presence of HEV among Lithuanian wild boar. Similarly to our results, the highest percentages of seropositive wild boar of 88.2% and 84.2% were detected in two provinces in Poland, which corresponded to the highest densities (1.6 and 1.2 animals/km<sup>2</sup>) of wild boar population, respectively (Larska et al. 2015).

To summarize, we can state that HEV is present in Lithuanian domestic pig and wild boar populations and could pose a potential zoonotic threat for human health in all

10 Lithuanian counties. The estimated low HEV seroprevalence rate in roe deer and moose, and no detected antibodies in red deer suggest that such wildlife species are accidental hosts of HEV or HEV is not present in the Lithuanian cervides.

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