

Differences in hepatitis E virus (HEV) presence in naturally infected seropositive domestic pigs and wild boars - an indication of wild boars having an important role in HEV epidemiology

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ABSTRACT

Hepatitis E (HE) is an emerging public health problem in developed countries worldwide. Nowadays, it is known that the hepatitis E virus (HEV) has zoonotic potential, whereas domestic pigs and wild boars are recognized as the true reservoirs of the disease. Therefore, the aim of our study was to define the seroprevalence and viral persistence of HEV in pigs and wild boars in Croatia. In total, 1424 samples originating from domestic pigs from 14 counties, and 1000 samples of wild boar origin, collected from 16 counties, were tested during 2016 for the presence of HEV antibodies using a commercially available enzyme linked immunosorbent assay (ELISA). All positive samples, as well as samples originating from in-contact domestic pigs and wild boars, were further tested for the presence of HEV RNA using a real time RT-PCR protocol, followed by a nested RT-PCR protocol as a confirmatory test. In 11 of 14 counties, 469 seropositive domestic pigs were found with an overall seroprevalence of 32.94% (95% CI 30.55-35.42), ranging from 8.33% to 60.00%, depending on

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their geographical origin. Seropositive wild boars were found in six out of 16 counties, showing an overall seroprevalence of 31.10% (95%CI 28.31-34.04%), ranging from 7.70% to 50.60%. The highest seroprevalence was found in the Vukovar-Srijem and Osijek-Baranja Counties (North-East Croatia) where pig breeding dominates and the highest density of wild boars is recorded. While no positive HEV RNA samples were detected in tested domestic pigs, seventeen serologically positive wild boars (11.33%; 95%CI 7.19-17.14) younger than one year of age were found to be HEV RNA positive, indicating chronic infection and possible prolonged virus spread into the environment. The detected viral presence despite the immune response indicates that wild boars have a key role in HEV epidemiology.

Key words: hepatitis E, domestic pig, wild boar, seroprevalence, viraemia

Introduction

Hepatitis E (HE) is an enterically transmitted viral infection mostly with an acute self-limiting course. It is endemic in the developing countries of Asia, Africa and Central America, with an occasional epidemic episode. HE is also becoming a public health problem in the developed countries of Europe, Asia and USA, where the number of sporadic cases of autochthonous infection is increasing (KAMAR et al., 2008; TEO, 2010; AGGARWAL and JAMEEL, 2011; MOAL et al., 2014; LAPA et al., 2015). The causative agent of HE is a small (27-34 nm) non-enveloped single-stranded virus, with a linear positive-sense RNA of approximately 7.2 kb in length (TAM et al., 1991; EMERSON and PURCELL, 2003). According to genetic diversity, HE virus (HEV) is classified in the family *Hepeviridae* consisting of two genera, *Orthohepevirus* (with species *A*, *B*, *C* and *D*) and *Piscihepevirus* containing one known isolate derived from a trout (SMITH et al., 2014). Even though contaminated water is the main source of HEV infection, zoonotic potential has been recognized for the genotypes HEV-3 and HEV-4, members of *Orthohepevirus A* (RODRIGUEZ-LAZARO et al., 2012). Recently these genotypes have been derived from numerous mammal species and humans. Even so, only members of the *Suidae* family are currently recognized as true reservoirs of HEV, presenting a potential public health risk. HEV has been detected in the tonsils, spleen, lymph nodes, lung tissue, muscles, kidneys, liver, gallbladder mucosa, as well as epithelia of the digestive and urinary system of infected pigs (WILLIAMS et al., 2001; de DEUS et al., 2008; BOUWKNEGT et al., 2009; PAVIO et al., 2010). However, the infection in pigs remains asymptomatic (HALBUR et al., 2001).

In 2015, according to the data of the Croatian Agriculture Agency and the Ministry of Agriculture, the Croatian domestic pig population was 1,398,556 and there were 52,000 wild boars, estimated on the basis of the annual hunting bag. However, the distribution of pig breeding varies largely on county level, where 98% of all farms are located in Northern Croatia where the natural habitat of wild boars is also located. Since 2009 HEV RNA has been consecutively derived from swine and wild boars in Croatia and all strains found have been proven to be members of genotype HEV-3 (PRPIĆ et al., 2015). Detection of HEV in pigs and wild boars was followed by the recognition of first autochthonous cases of HE in patients showing signs of hepatic disorders (ČIVLJAK et al., 2013; ĐAKOVIĆ

RODE et al., 2014). This study shows the results of seroprevalence and HEV detection in domestic pigs and wild boars from different counties in Croatia, especially areas where pig breeding dominates and where HEV RNA was previously recognized (Northern region). The aim was to fulfill epidemiological data and gain a better understanding of HEV infection in domestic pigs and wild boars and their significance as potential reservoirs of HE infection.

Materials and methods

Sample collection and preparation. Swine and wild boar samples were collected from January up until the end of December 2016, in accordance with an ongoing classical swine fever monitoring program (Program of the Veterinary and Food Safety Directorate of the Ministry of Agriculture). Samples were randomly chosen taking into account the sample quality and geographical origin (based on the estimated population size per county, with a 5- or 10% prevalence and a 95% or 90% probability) (Table 1). In total, 1424 sera samples from domestic pigs from 14 counties and 1000 sera samples of wild boar from 16 counties (collected on the spot by official veterinarians, by venepuncture of the v. jugularis or in shot wild boars by educated hunters directly from the heart) were serologically tested.

Sera were prepared from blood samples by centrifugation at 1000g for 15 min. The supernatants were decanted into sterile test tubes and stored at -80 °C until further testing.

Detection of specific anti-HEV antibodies. A commercially available enzyme linked immunosorbent assay (ELISA) kit (PrioCHECK HEV Ab porcine, Prionics AG, Switzerland) was used for detection of specific anti-HEV antibodies of swine origin towards genotypes HEV-1 and HEV-3. All results above or equal to the cut-off value (the mean optical density calculated at 450 nm of the cut off control multiplied by 1.2) were considered positive, as recommended by the manufacturer. The optical densities were read by an automatic ELISA processor, ETI-Max 3000 (DiaSorin, Italy).

Detection of viral RNA. All serologically positive samples, as well as samples originating from in-contact animals collected from the same farm, or in the case of wild boars, from the same hunting ground, were further tested for the presence of HEV RNA. In total, 670 domestic pig samples were tested within 352 test reactions, singularly or pooled. Pooled samples contained three to five samples per pool following the criteria that the pooled samples were collected the same day from pigs belonging to the same husbandry and age group, and that they derived the same ELISA result (positive or negative). Regarding wild boar sera, samples were tested singularly, however for PCR procedures, only ELISA positive samples with high quality and no signs of degradation (representative samples) were tested (150 samples), taking into account the inclusion of representative sera samples from each hunting ground and wild boar pack. Viral RNA

was extracted using a MagMAX Pathogen RNA/DNA kit (Thermo Fisher Scientific, Waltham, MA, USA) from 50 μ L of each sera sample according to the producer's instructions. For nested PCR, reverse transcription was performed (1Step RT PCR Kit, highQu, Kraichtal, Germany) in a GeneAmp PCR System 9700 machine (Applied Biosystems, Carlsbad, California, USA). To identify HEV RNA carriers, a real time RT-PCR protocol (JOTHIKUMAR et al., 2006) was carried out for detecting highly conserved fragments within ORF3. The amplification was done in a Stratagene, Mx3005Pro using commercially available kits - 1Step RT qPCR Probe ROX L kit (highQu, Kraichtal, Germany) according to the producer's instructions. All positive samples were re-tested for confirmation by a nested RT-PCR protocol (VAN DER POEL et al., 2001) for detecting variable fragments within ORF1. The amplification procedure for nested PCR (ALLin Red Taq Masternix, highQu, Kraichtal, Germany) was as follows: denaturation at 95 °C for 1 min; incubation at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 45 s (40 cycles); and incubation at 72 °C for 7min. PCR products were separated by agarose gel electrophoresis in 1.5% agarose gel stained with a fluorescent dye (GelStar™ Nucleic Acid Gel Stain 10,000X, Lonza, Walkersville, Maryland, USA) and visualized by UV transillumination. As a positive control, sequences of previously detected fragments of HEV RNA genotype 3 were used (derived from positive swine sera). Negative controls were aliquots of ultra-pure water.

Statistics. The lower and upper limits of the 95% confidence interval (CI) for a proportion were calculated. To define the differences in the occurrence of hepatitis E in Croatia in domestic pigs and wild boars, the Chi-square test was used. $P \leq 0.05$ was considered statistically significant.

Results

Serology results. In domestic pigs, 469 sera out of 1424 tested samples were positive for the presence of specific anti-HEV antibodies. The seroprevalence found was 32.94% (Table 1) with a 95% confidence interval (CI) of 30.55-35.42%. Positive animals were detected in 11 of the 14 counties included in the study (Fig. 1), with the seroprevalence ranging from 8.33% to 60.00%. Only in three Counties were no positive samples found (Karlovac, Krapina-Zagorje and Virovitica-Podravina), probably due to the limited number of samples received ($\Sigma 54$). The highest seroprevalence was determined in Osijek-Baranja, (60%, 95% CI of 44.6-73.65%) and Vukovar Srijem County (50.11%, 95%CI of 45.61-54.61%), respectively.

In six of the 16 counties (Fig. 1) included in the study, 31.10% positive wild boars were found showing seroprevalence of (95%CI 28.31-34.04%), ranging from 7.70% (95%CI 1.37-33.31) to 50.60% (95%CI of 45.28-55.88%). The highest seroprevalence was detected in the Vukovar-Srijem (50.60%, 95%CI of 45.28-55.88%) and Osijek-Baranja Counties (43.18%, 95%CI of 29.68-57.77%), respectively.

Table 1. Results of antibody and HEV RNA testing of sera originating from domestic pigs and wild boars

County	Number of antibody tested /positive/negative samples by ELISA		Seroprevalence (%) and 95% confidence interval (CI) in parenthesis		Tested/positive/negative PCR results (%) and 95% confidence interval (CI)	
	Domestic pigs	Wild boars	Domestic pigs	Wild boars	Domestic pigs	Wild boars
Bjelovar Bilogora	6/2/4	14/0/14	33.33 (9.7-70.00)	0	6/0/6	0
Brod Posavina	88/12/76	105/25/80	13.64 (7.98-22.34)	23.81 (16.68-32.79)	16/0/16 (*3 pools)	41/13/28 (31.71%, 19.57-46.99)
Dubrovnik Neretva	n.d.	1/0/1	n.d.	0	n.d.	13/1/12 (7.69%, 1.37-33.31)
Karlovac	30/0/30	20/3/17	0	15.00 (5.24-36.04)	0	42/1/41 (2.38%, 0.42-12.32)
Koprivnica Križevci	24/4/20	4/0/4	16.67 (6.68-35.86)	0	4/0/4	3/0/3
Krapina Zagorje	4/0/4	n.d.	0	0	0	n.d.
Lika Senj	12/1/11	1/0/1	8.33 (1.49-35.38)	0	3/0/3	1/0/1
Međimurje	n.d.	1/0/1	n.d.	0	n.d.	49/2/47 (3.92%, 1.13-13.71)
Osijek Baranja	40/24/16	44/19/25	60.00 (44.6-73.65)	43.18 (29.68-57.77)	28/0/28 (*4 pools)	0
Požega Slavonia	25/4/21	1/0/1	16.00 (6.4-34.65)	0	8/0/8	0
Sisak Moslavina	537/150/387	429/92/337	27.93 (24.3-31.87)	21.45 (17.83-25.58)	275/0/275 (*78 singular and *70 pools)	0
Split Dalmatia	8/4/4	12/0/12	50.00 (21.50-78.48)	0	8/0/8	0
Šibenik Knin	n.d.	7/0/7	n.d.	0	n.d.	0
Varaždin	31/4/27	1/0/1	12.90 (0.51-28.85)	0	7/0/7	0
Virovitica Podravina	20/0/20	9/0/9	0	0	0	0

n.d. no data; * Number of pooled reactions per County; **Number of singularly tested sera samples per County

Table 1. Results of antibody and HEV RNA testing of sera originating from domestic pigs and wild boars (continued)

County	Number of antibody tested /positive/negative samples by ELISA		Seroprevalence (%) and 95% confidence interval (CI) in parenthesis		Tested/positive/negative PCR results (%) and 95% confidence interval (CI)	
	Domestic pigs	Wild boars	Domestic pigs	Wild boars	Domestic pigs	Wild boars
Vukovar Srijem	471/236/235	338/171/167	50.11 (45.61-54.61)	50.60 (45.28-55.88)	252/0/252 (**88 singular and *63 pools)	0
Zagreb	128/28/100	13/1/12	21.88 (15.59-29.80)	7.70 (1.37-33.31)	63/0/63 (*10 pools)	1/0/1
TOTAL	1424/469/955	1000/311/689	32.94 (30.55-35.42)	31.10 (28.31-34.04)	670/0/670 (*352 PCR reactions)	150/17/133 (11.33%, 7.19-17.14)

n.d. no data; * Number of pooled reactions per County; **Number of singularly tested sera samples per County

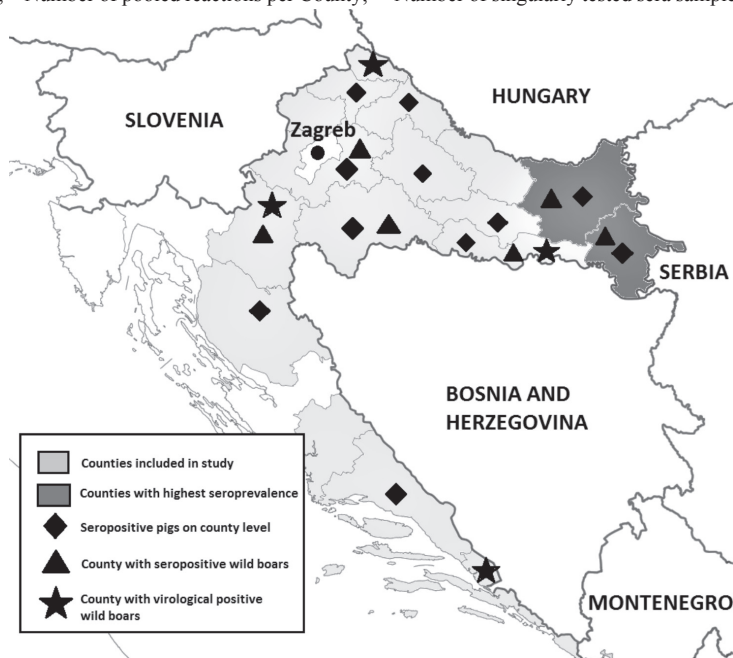


Fig. 1. Distribution of Counties included in the study (grey areas); Osijek-Baranja and Vukovar-Srijem Counties (black areas) where the highest seroprevalence was determined in domestic pigs and wild boars; serologically positive domestic pigs (diamond) and wild boars (triangle) per county in Croatia. The stars represent wild boars found to be HEV RNA positive in four counties.

No significant statistical difference ($P = 0.365$) was detected when the results from domestic pigs and wild boars were compared. However, a statistical significance ($P < 0.0001$) was detected when positive results found in the Osijek-Baranja and Vukovar-Srijem Counties were compared to the results obtained in all other investigated counties, regardless of species (domestic pig or wild boar).

PCR results. All tested samples originating from domestic pigs were negative for the presence of HEV RNA, regardless of the detected seropositivity.

HEV RNA positive wild boars were positive by both PCR protocols used, and all had been previously found to be seropositive. HEV RNA was detected in 17 of the 150 tested samples (Table 1) in four investigated Counties (Brod Posavina, Dubrovnik Neretva, Karlovac and Međimurje). The RNA prevalence was 11.33% (95%CI 7.19-17.14%). The most positive HEV RNA wild boars were detected in the Brod-Posavina County ($\Sigma 13$), two in Međimurje County, and one each in the Dubrovnik-Neretva and Karlovac Counties. In total, ten wild boars up to 6 months of age were HEV RNA positive, as well as antibody positive, while 7 were 6 months to one year of age (according to the data provided by hunters, and estimated by tooth growth and wear). No HEV RNA was detected in adult animals. Regarding sex, 10 animals were males and 7 females, showing no statistical significance in regards to the results and the sex of wild boars ($P = 0.845$).

Discussion

The incidence and recognition of autochthonous hepatitis E in developed countries is increasing and becoming a serious public health problem worldwide. In Europe, the prevalence of HEV RNA and anti-HEV antibodies in the general population and blood donors is highly variable (LAPA et al., 2015). In Croatia, autochthonous HE was recognized in 2012 (ČIVLJAK et al., 2013). The seroprevalence in tested non A-C hepatitis patients with hepatic disorders was 10.70%, (ĐAKOVIĆ RODE et al., 2014), and 5.6% in asymptomatic individuals (VILIBIĆ-ČAVLEK et al., 2016).

To gain a better understanding of the farm-to-table risk in Croatia, a survey of HEV RNA presence in domestic pigs and wild boars was carried out from 2009 and revealed a prevalence of 24.50% and 12.30%, respectively (JEMERŠIĆ et al., 2010; PRPIĆ et al., 2015). All isolates belonged to genotype HEV-3 (PRPIĆ et al., 2015). In our present study we supplemented the epidemiological data with the first results of HEV seroprevalence in domestic pigs and wild boars from 14 and 16 counties in Croatia (Table 1), respectively. Our results show an overall seroprevalence of 32.94% and 31.10% in domestic pigs and wild boars. Both results correspond to results previously reported in European countries (BURRI et al., 2014; ANITA et al., 2014; MONTAGNARO et al., 2015; THIRY et al., 2015; MAZZEI et al., 2015). The majority of samples tested within our study were collected from the northern regions of Croatia, where HEV RNA prevalence in pigs and wild boars

was previously detected (PRPIĆ et al., 2015). Our study reveals a statistically significant ($P < 0.0001$) higher seroprevalence detected in domestic pigs and wild boars from Osijek-Baranja and Vukovar-Srijem Counties than from all other counties included in the study. These two are neighboring counties in the North East of Croatia with a high density of both, pigs (in Osijek-Baranja 883.70/10 km and in Vukovar-Srijem 951.60/10 km) and wild boars (in Osijek-Baranja 15.10/10 km and in Vukovar-Srijem 11.00/10 km), suggesting that high animal density increases the spread of infection. Since these counties neighbor with other European countries, such as Hungary and Serbia where the presence of HEV was previously confirmed (FORGACH et al., 2010; LUPULOVIC et al., 2010), there is a possibility of transboundary HEV transmission and spread through wild boar migration. Furthermore, we detected the spread of infection into HEV naive counties with no previous HEV RNA findings (Požega-Slavonia County in domestic pigs and Karlovac, Osijek-Baranja and Zagreb Counties in wild boars), indicating the circulation of HEV among pigs, probably due to trade and pig movement, and/or migration of wild boars. In the Karlovac County HEV RNA was detected in one seropositive wild boar, indicating active infection. The same finding was observed in the Dubrovnik-Neretva County where hepatitis E infection was previously detected only in humans (ĐAKOVIĆ-RODE et al., 2014). Therefore, the spread of HEV by wild boars into HEV naive territories may be more progressive than by domestic pigs, especially if the pigs are bred in an intensive production system with high biosecurity standards. However, in some areas of Croatia pigs are kept traditionally in an open breeding system with minimal biosecurity measures implemented, allowing indirect contact with wild boars and *vice versa*. Interspecies HEV transmission was previously confirmed in Croatia from a finding of highly related strains of HEV in pigs and wild boars (PRPIĆ et al., 2015). Our results additionally support this thesis, since positive wild boars were only detected in areas where a high seroprevalence in domestic pigs was found (Table 1). Even so, the transmission route between pigs and wild boars needs to be further investigated, since a high titer of the virus must be present to allow fecal-oral infection (BOUWKNEGT et al., 2009).

Serologically positive samples, including samples originating from in-contact pigs and in-contact wild boars, were further tested for the presence of HEV RNA. We also tested in-contact animals to ensure the detection of recently infected pigs, since viraemia in pigs declines when specific anti-HEV antibodies elevate (HUANG et al., 2010), and the virus may be spread among different age groups by indirect and direct contact (WU et al., 2002). Interestingly, no HEV RNA positive sera were recorded in seropositive or seronegative domestic pigs, despite the previous findings reported by PRPIĆ et al. (2015). This could be due to the fact that the youngest pigs included in this study (less than 6 months of age) were older than 2-4 months of age when the highest peak of viraemia is expected (WU et al., 2002; TAKAHASHI et al., 2005; de DEUS et al., 2008). However, other factors that may influence the results must be taken into account, such as the sensitivity

of the method used, even though this protocol is considered highly sensitive and can detect four GE copies of genotype 3 derived from pigs (JOTHIKUMAR et al., 2006). On the other hand, HEV RNA positive samples (11.33%) were recorded in tested wild boars, even though seven of the 17 animals were older than 6 months of age. Since all HEV RNA positive wild boars, regardless of age, were also positive for specific anti-HEV antibodies, we assume that the differences exist in the viral dynamics of post HE infection in domestic pigs *versus* wild boars. Our study involved samples of wild boars with no pathological findings, hunted in their natural habitat. The prolonged viraemia found in 17 wild boars also demonstrates the possibility of prolonged viral shedding. A study by SCHLOSSER et al. (2015) revealed that chronic HE may occur in wild boars in controlled conditions, allowing the transmission of the virus by the oral-fecal route to domestic pigs. The viraemia recorded lasted continuously for over five months, nearly until the end of the trial, even though anti-HEV antibodies developed. Our results confirm the parallel existence of viraemia and anti-HEV antibodies in naturally infected wild boars, and highlight the fact that wild boars may be persistent reservoirs of HEV and as such represent not only a risk for the pig industry, but also may play an important role in the epidemiology of HEV infection in Europe. The possibility of prolonged viraemia in wild boars emphasizes the need for further investigations, mostly regarding the duration of viraemia in natural conditions. This is not included in our study because the sampling was conducted from shot wild boars, but this also stresses the importance of implementing adequate prevention measures, such as the use of protective gloves (SCHIELKE et al., 2015), especially when handling wild boar carcasses.

Conclusions

The seroprevalence of HEV infection found in domestic pigs (32.94%) and wild boars (31.10%) in Croatia corresponds to results reported in other European countries. However, the findings of new cases of HEV infection in pigs and wild boars in previously naive counties indicate the further spread of the disease, probably due to trade and pig movement and/or migration of wild boars. The highest seroprevalence was detected in the counties with the highest density of domestic pigs and wild boars (Vukovar-Srijem and Osijek-Baranja), implying that animal density is also an important risk factor for viral spread. HEV RNA was not detected in sera samples of domestic pigs in 2016, even though a high seroprevalence was found. Most likely this is the result of the age of the pigs included in the study that was over four to five months of age when anti-HEV antibodies are formed and viraemia declines. Our results reveal that differences exist in the viral dynamics of post HE infection in domestic pigs *versus* wild boars. HEV RNA was detected in seventeen wild boars (11.33%) that were also shown to be ELISA antibody positive. Seven of them were over six month of age, indicating prolonged viraemia and opening the question of their role in the epidemiology of HEV infection.

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SAŽETAK

Hepatitis E (HE) rastući je javnozdravstveni problem razvijenih država svijeta. Danas je poznato da je uzročnik HE virus koji pokazuje zoonotski potencijal, te su domaća i divlja svinja prepoznate kao rezervoari infekcije. Cilj našeg istraživanja bio je utvrditi seroprevalenciju i opstojnost virusa hepatitisa E (HEV) u domaćih i divljih svinja u Republici Hrvatskoj. Stoga su tijekom 2016. godine pretražena 1424 uzorka podrijetlom od domaćih svinja iz 14 županija i 1000 uzoraka podrijetlom od divljih svinja iz 16 županija komercijalnim imunoenzimnim kompletom (ELISA) za dokazivanje specifičnih protutijela za HEV. Pozitivni uzorci, te negativni uzorci koji su sakupljeni od svinja koje su bile u kontaktu sa serološki pozitivnim svinjama, dodatno su bili pretraženi na prisutnost RNK HEV lančanom reakcijom polimerazom u stvarnom vremenu i umreženom lančanom reakcijom koja se koristila kao potvrdni test. U 11 od 14 županija utvrđeno je 469 serološki pozitivnih domaćih svinja, tj. prevalencija je iznosila 32,94% (95 % CI 30,55-35,42), a kretala se od 8,33 % do 60,00 % ovisno o geografskom podrijetlu svinja. Serološki pozitivne divlje svinje nađene su u šest od 16 županija uz seroprevalenciju od 31,10 % (95 % CI 28,31-34,04 %), a koja je iznosila od 7,70 % do 50,60 %. Najviša seroprevalencija utvrđena je u Vukovarsko-srijemskoj i Osječko-baranjskoj županiji u kojima je svinjogojstvo dominantna grana stočarstva i gdje je visoka gustoća nastanjenih divljih svinja. Dok u serološki pozitivnih domaćih svinja nije utvrđena prisutnost HEV RNK, u 17 (11,33 %; 95 % CI 7,19-17,14) serološki pozitivnih divljih svinja nađena je HEV RNK i to u divljih svinja mlađih od jedne godine starosti što upućuje na moguću kroničnu infekciju i posljedično prolongirano izlučivanje virusa u okoliš. Dokazani HEV u serološki pozitivnih divljih svinja upućuje na moguću ključnu ulogu divljih svinja u HEV epidemiologiji.

Ključne riječi: hepatitis E, domaće svinje, divlje svinje, seroprevalencija, viremija
