Serological evidence of hepatitis E virus infection in pigs from Northern Bulgaria

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Summary
The purpose of the present study was to investigate pigs in Northern Bulgaria for serological evidence of hepatitis E virus (HEV). Sera from 225 individuals from three industrial farms were tested for anti-HEV IgG antibodies. The overall HEV seroprevalence was 36% (81/225); weaners 6.8% (5/74); fattening pigs 38.7% (29/75) and in sows 61.8% (47/76). Compared to weaners, HEV positivity was higher in fattening pigs and sows: OR = 8.70 (95% CI: 3.14-24.12) and OR = 22.37 (95% CI: 8.07-61.96), respectively. These data confirm that HEV is endemic in pigs throughout Bulgaria, and can be a Public Health problem due to the transmission of HEV to humans through the consumption of pork meat and pork products.

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus (ICTV 2018, Purdy *et al.* 2017). Since the first report of its genomic sequence (Tam *et al.* 1991), strains have been widely detected, not only in humans, but also in a great number of animal species. HEV is classified into *Hepeviridae* family which has recently been divided in two genera: *Ortohepevirus* and *Piscihepevirus* (ICTV 2018). *Ortohepevirus* includes four species: *Orthohepevirus A* (Genotypes: HEV-1 – HEV-8), *Orthohepevirus B* (Avian HEV Genotype 2), *Orthohepevirus C* (Germany Rat HEV, Vietnam Rat HEV and Ferret HEV) and *Orthohepevirus D* (Germany Bat HEV) (ICTV 2018, Pepovich *et al.* 2019). Genus *Piscihepevirus* has only one species – *Piscihepevirus A*, and one genotype – Cutthroat Trout HEV (ICTV 2018).

The number of laboratory-confirmed cases of hepatitis E in Europe has dramatically increased in recent years, from 514 in 2005 to 5,617 cases in 2015 (ECDC 2017). In total, 28 deaths associated with HEV infection were reported from five countries between 2005 and 2015 (ECDC 2017). These were almost all locally acquired and were thought to be porcine enzoonotic infections: the main route of infection is considered to be consumption of infected pig meat. Recent estimates suggest that there may be at least two million human infections with HEV in Europe every year, most of which are asymptomatic (EASL 2018).

In Bulgaria, the first evidence of human HEV infection was reported in 4 patients in 1995 (Teoharov *et al.* 1995). Afterwards many other cases of human HEV infection were recorded in Bulgarian citizens (Baymakova *et al.* 2016, Bruni *et al.* 2018, Cella *et al.* 2019). Baymakova and colleagues reported serological evidence of anti-HEV IgM and anti-HEV IgG antibodies in 20 patients out of 806 patients showing acute viral hepatitis (Baymakova *et al.* 2016). Bruni and colleagues found 103 HEV IgM positive serum samples collected from hospitalized patients with acute hepatitis from all over Bulgaria (Bruni *et al.* 2018). The same authors performed phylogenetic analysis (n = 64 patients) and genotyped HEV-1 in 2% of the cases (1/64), HEV-3e subtype in 62% (39/63), HEV-3f subtype in 24% (15/63), HEV-3c subtype in 13% (8/63), and HEV-3hi subtype in 2% (1/63). A recent analysis of 2,257 cases of human hepatitis in Bulgaria (1995 to 2018) showed that 13.1% were caused by HEV, predominantly genotype 3 (Baymakova *et al.* 2019). The first preliminary data for swine HEV infection in Bulgaria were published in 2018 (Pishmisheva *et al.* 2018), showing an overall seroprevalence of anti-HEV antibodies of 40% (34/85). In 2019, a detailed seroprevalence study of HEV infection in pigs from Southern Bulgaria (Tsachev *et al.* 2019), documented an overall HEV seroprevalence of 60.3% (217/360).

The aim of our study was to investigate pigs in Northern Bulgaria for serological evidence of HEV, in order to obtain a complete nationwide picture of viral exposure in this important primary host. This could indeed amplify the transmission of HEV to humans through the consumption of pork meat and derivative products.

Two hundred and twenty-five pigs (n = 225) from three commercial farrow to finish pig farms of Northern Bulgaria (Goliyamo Vranovo, Nikolovo and Pleven, 38,000, 15,000 and 700 heads of pigs, respectively) were enrolled (Figure 1). Pigs included in the study were divided into three age groups: weaners (age: 30-100 days), fattening pigs (101-160 days) and sows (> 365 days). Pigs showed no clinical signs at sampling time point. The sex (weaners and fattening pigs) was not recorded. The enrolled pigs were randomly selected for sampling. The collection of the samples was planned in context of farm capacity.

Swine blood samples (up to 5 mL per individual) were taken by puncture of the *sinus ophthalmicus*. Blood collection tubes without anticoagulant were kept at room temperature (20 °C) until clot retractionwas visible. Then they were centrifuged at 1,500 g for ten minutes, and the serum was separated and stored at - 20 °C until testing.

The serum samples were tested for HEV antibodies in the Laboratory of Infectious Diseases, Faculty of Veterinary Medicine, Trakia University, Stara

Figure 1. *Geographic distribution of HEV infection in pigs from Bulgaria.* The data from the current paper are shown in grey. The investigated farms were located in the Northern Bulgarian plains (approximately 43°40′N and 43°84′N Latitude, 24°62′E and 25°95′E Longitude); the climate is continental (mean annual temperature 10-12 ºC, precipitation approximately 630 mm/m^2). Data from our previous study in Southern Bulgaria are shown in black, for comparison (Tsachev *et al.* 2019).

Zagora, Bulgaria. A commercial enzyme-linked immunosorbent assay (ELISA, PrioCHECK HEV Ab porcine, Mikrogen GmbH, Neuried, Germany) was used, according to the manufacturer's instructions. The PrioCHECK HEV Ab porcine is a diagnostic test for detection of HEV-specific antibodies in porcine serum and meat juice samples. A microtiter plate is coated with recombinant HEV antigen of the Open Reading Frame 2 (ORF2) and ORF3 of genotypes HEV-1 and HEV-3. The test has 91.0% sensitivity and 94.1% specificity. The cut-off, as well as positive, negative and borderlie results were calculated as described by the manufacturer. Borderline results were repeated and those remaining in the borderline range were considered negative.

HEV positive results among different swine age groups and farms were compared by using the Chi-square test. Binary logistic regression was used to evaluate the risk of positive results according to age group. Statistical analysis was performed by SPSS Statistics 19.0 (IBM Corp., Armonk, New York, USA). A P-value < 0.05 was considered statistically significant.

The study was approved by the Ethics Committee in Animal Experimentation and Animal Welfare at Trakia University, Stara Zagora (Bulgaria) and was conducted according to the ethical principles of animal experimentation, adopted by the Bulgarian Ministry of Agriculture, Food and Forestry.

Anti-HEV IgGs were detected in 81 (36%) of the 225 tested sera (Table I). The overall seropositivity in weaners, fattening pigs and sows was 6.8% (5/74), 38.7% (29/75) and 61.8% (47/76), respectively (Table II). The highest HEV seropositivity in weaners was found in Goliyamo Vranovo (16.7%; 5/30). The highest HEV seropositivity in fattening pigs and sows was documented in Nikolovo – 96.7% (29/30) and 80% (24/30), respectively. The overall prevalence of anti-HEV antibodies in each farm was: Goliyamo Vranovo 31.1% (28/90); Nikolovo 58.9% (53/90); and Pleven – 0% (0/45) (Figure 1). There were significant differences in HEV seropositivity between pig age and farms (Table I).

To estimate the risk for HEV seropositivity, the odds ratio (OR) in different age groups was performed by binary logistic regression. The OR of anti-HEV antibodies occurrence in fattening pigs and sows was determined comparing to group weaners (Table II). We found that the odds of HEV infection was nearly 8-times higher in fattening pigs and 22-times higher in sows than in weaners.

The prevalence of HEV-antibodies in both pigs and humans is quite variable between and within countries. This variability may, at least in part, be influenced by the study design, diagnostics methods and tested population. Various laboratory tests also influenced the final results (Krumbholz *et al.* 2013). Nevertheless, it seems that many pigs worldwide are infected with HEV and represent zoonotic risk for transmission to humans. The seroprevalence in our study (36%; 81/225) is similar to data found in several other countries, including Taiwan (37.1%; 102/275) (Hsieh *et al.* 1999); Serbia (34.6%; 109/315) (Lupulovic *et al.* 2010); USA (34.5%; 29/84) (Withers *et al.* 2002); Croatia (32.9%; 469/1,424) (Jemersic *et al.* 2017); South Korea (40.7%; 57/140) (Meng *et al.* 1999); France (31%; 2,035/6,565) (Rose *et al.* 2011); Thailand (30.7%; 23/75) (Meng *et al.* 1999); Romania (42.7%; 65/145) (Savuta *et al.* 2007) and India (42.9%; 122/284) (Arankalle *et al.* 2002).

Sows had the highest seroprevalence (61.8%) in our study. Martinelli and colleagues found 70.6% HEV-prevalence in sows, also the highest seroprevalence in their study (Martinelli *et al.* 2011), whereas Danish investigation presented 73.2% positive sows for HEV-IgG (Breum *et al.* 2010). The results of our study showed an age-dependent seroprevalence (OR = 8.70, p < 0.001; OR = 22.37,

Table I. *Seroprevalence of HEV infection according to category groups in pigs from Northern Bulgaria.*

Age groups	Age, days	Investigated pigs, n	HEV positive, n (%)	Chi-square	Df	P-value
Goliyamo Vranovo						
Weaners	30-100	30	5 (16.7)	45.52	2	< 0.001
Fattening pigs	101-160	30	0 (0.0)			
Sows	> 365	30	23 (76.7)			
Nikolovo						
Weaners	30-100	30	0 (0.0)	66.18	2	< 0.001
Fattening pigs	101-160	30	29 (96.7)			
Sows	> 365	30	24 (80.0)			
Pleven						
Weaners	30-100	14	0 (0.0)	NA	NA	NA
Fattening pigs	101-160	15	0 (0.0)			
Sows	> 365	16	0 (0.0)			

HEV = Hepatitis E virus; df = degrees of freedom; NA = not applicable.

Table II. *Logistic regression showing the relationship between HEV positive pigs and age.*

Age groups	Investigated pigs, n	HEV positive, n (%)	PE	SE	P-value	OR	95% CI
Weaners	74	5 (6.8)	NA	NA	NA	1.00	NA
Fattening pigs	75	29 (38.7)	2.16	0.52	< 0.001	8.70	3.14-24.12
Sows	76	47 (61.8)	3.11	0.52	< 0.001	22.37	8.07-61.96

HEV = Hepatitis E virus; PE = Parameter estimate; SE = Standard error; OR = Odds ratio; CI = Confidence interval; NA = not applicable.

$p < 0.001$). Similar observations have been reported in other studies (Breum *et al.* 2010). HEV infection occurs in all age groups of pigs and it seems most likely that infection occurs in nursery and fattening periods (Pavio *et al.* 2010). Most pigs become infected at 6-8 weeks of age, while virus in feces peaks at 12-14 weeks and declines at 20-22 weeks (Pavio *et al.* 2010). HEV-IgGs appear at 8-9 weeks, increase in frequency, and approximately all infected pigs at 22-24 weeks have HEV-IgG (Pavio *et al.* 2010).

The established HEV seroprevalence in pigs from Northern Bulgaria is lower (mean 36%, and 38.7% for fattening) than that reported from Southern Bulgaria (mean 60.3%, and 75.8% for fattening) (Tsachev *et al.* 2019). There is a number of possible explanations for this observation, relating to geographical differences in pig farm density, husbandry conditions, model of pig farming, animal contact with the environment, sewage systems and water facilities. In the present study, the involved farms were typical industrial farms with low and moderate density of animals, longer farming for fattening and small herds. These factors may have contributed to the lower HEV seroprevalence in pigs from Northern Bulgaria, compared to pigs from Southern Bulgaria.

Our study shows that many pigs in Northern Bulgaria have been exposed to HEV, but the frequency of exposure is somewhat lower than that documented in Southern Bulgaria. These data confirm that HEV is endemic in pigs throughout Bulgaria, and so it is a nationwide Public Health concern. In addition, compared to other European countries, our results are similar to those of some Southern European countries such as France (31%) (Rose *et al.* 2011), Croatia (32.9%) (Jemersic *et al.* 2017), Serbia (34.6%) (Lupulovic *et al.* 2010) and Romania (42.7%) (Savuta *et al.* 2007), and differed greatly from the results of Continental and Northern Europe such as Switzerland (58.1%) (Burri *et al.* 2014), Germany (68.6%) (Wacheck *et al.* 2012), Finland (86.3%) (Kantala 2017) and Norway (90%) (Lange *et al.* 2017). In conclusion, we think that the present study complemented the knowledge about HEV infection both nationally and regionally.

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