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Detection of Hepatitis E Virus Infections in Wild Boars in Southwest Germany Using a Stepwise Laboratory Diagnostic Approach

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Abstract: Hepatitis E virus (HEV) is the main cause of enterically transmitted hepatitis in humans worldwide. Among HEV, genotypes 3 and 4 are considered zoonotic agents associated with domestic pigs and wild boars, showing an increasing trend in Europe. The aim of this study is to contribute data on the prevalence of HEV in wild boars in Southwest Germany and to present a time and cost-effective two-step laboratory diagnostic approach for serological monitoring of blood samples. This method uses enzyme-linked immunosorbent assay (ELISA), followed by testing for HEV RNA by reverse transcription real-time PCR (RT-qPCR). A total of 2295 blood samples were collected in 234 municipalities in 12 counties in the period from 2016 to 2020. There was an overall seroprevalence of 10.8%, ranging from 3.6% to 17.5% per county and 7.5% to 14% per year. Retesting of these blood samples for HEV RNA revealed 15.7% viremic wild boars originating from 30 municipalities in 11 counties. Viremic wild boars were found in seven regional clusters, including 84% of the animals that tested positive for HEV. Seropositive animals <1 year of age were significantly more likely to be viremic than those >1 year. Further characterization of HEV RNA resulted in the identification of genotype 3. Altogether, serological monitoring of the blood samples, complemented by successive and targeted investigations into the presence of HEV RNA based on blood samples, provide reliable information on the seroprevalence and virus load in wild boars, which proved to be a relevant and persistent sylvatic reservoir for HEV.

Keywords: hepatitis E virus genotype 3; ELISA; RT-qPCR; seroprevalence; wild boar; sylvatic reservoir; viremia; zoonosis



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1. Introduction

Hepatitis E virus (HEV) is the pathogen agent of the most frequent enterically transmitted type of hepatitis in humans in the world [1–3]. A ten-fold increase in the incidence rate of HEV infections in humans has been observed within the last decade, raising concerns about a looming health risk [4–6]. HEV, belonging to Orthohepevirus A of the Hepeviridae family, is one of the relevant hepatitis virus species for humans, livestock and wild game worldwide [7]. Among the eight genotypes described so far, genotypes 1 and 2 infect humans exclusively and are considered to be water-borne infections transmitted by the faecal-oral route. HEV-5 and HEV-6 have been isolated from wild boars only, HEV-7 and HEV-8 from camelids, with only one report of an HEV-7 isolate from a transplant patient [7,8]. However, HEV belonging to genotype 3 (HEV-3) and 4 (HEV-4) are zoonotic agents which can induce autochthonous, food-borne infections causing acute and chronic hepatitis in humans [2,9–12], but silent infections in pigs [13]. Domestic pigs and wild boars are considered to be the natural host and major reservoir for HEV-3 [3,14] throughout Europe. Molecular studies on HEV have shown a ubiquitous distribution of this HEV genotype in domestic pigs and wild boars [6,7,15–17]. HEV-3 and HEV-4 have been detected in

the liver, muscle and blood of infected animals worldwide [13,18–20]. Therefore, the main risk associated with becoming infected with these HEV genotypes is the consumption of raw or undercooked pork [6,21–23] and wild boar products [4,13,17–19,24,25].

High prevalence rates of HEV in domestic pigs and wild boars have been demonstrated in countries throughout Europe, with a rising trend over the last decade [6]. This situation could have been caused by the transmission of HEV between wild boars and domestic pigs [7], as the prevalence of HEV in wild boars depends on its population, which has significantly increased within the last decade [13].

Data on the prevalence of HEV for Germany and neighboring nations are available for about the last decade. The most recent data on the prevalence of HEV in wild boars in Germany are provided by a study from 2017 [18], reporting seroprevalence values of 24% and 11% for detection of HEV by PCR. Studies conducted in the Netherlands [26], Poland [13] and the Czech Republic [27] revealed seroprevalence values of 12%, 49% and 9% and detection of HEV RNA in 8%, 12% and 7% of wild boars, respectively. Studies on the seroprevalence of HEV in wild boars in France [28] and Switzerland [29] yielded values of 14% and 13%, respectively. The generalization of HEV in wild boars emphasizes the relevance of this zoonotic pathogen in the wild boar population in central Europe.

Wildlife habitats of wild boars and thus also zoonotic pathogen agents have come in closer contact with urban habitats and human settlements. It is therefore essential to address this issue by collecting and analyzing current monitoring data on the occurrence of HEV in wild boars.

The aim of the present study is to contribute current data on the occurrence of HEV in wild boars in Southwest Germany and to present suitable monitoring tools and procedures.

2. Materials and Methods

2.1. Sample Collections

Within the framework of the mandatory national monitoring program for classical and African swine fever [30], a total of 2295 blood samples from wild boars were sent to our institute during a five-year period from 2016 to 2020. The samples originated from 234 hunting grounds in municipal districts (municipalities) of 12 counties, in the administrative district of Stuttgart, in the federal state of Baden-Wuerttemberg (Germany). The number of samples per county was determined by the competent authority and was representative of the respective regional wild boar population. The age of the hunted wild boars was determined by the hunters on the basis of tooth eruption and notified on an accompanying questionnaire. All samples were analyzed for seroprevalence using enzyme-linked immunosorbent assay (ELISA). To obtain more information about the viremic state of the animals, selected samples were retested by reverse transcription real-time PCR (RT-qPCR).

2.2. Serological Analyses

The sera were obtained by centrifuging ($1410 \times g$ for 8 min) of the blood samples and were subsequently stored at $-20\text{ }^{\circ}\text{C}$ until testing. Antibody testing was carried out using a commercially available hepatitis E ELISA for pigs and other species, based on genotype 3 (ID Screen[®] Hepatitis E Indirect Multi-species, IDvet, Grabels, France), according to the manufacturer's instructions. This ELISA detects IgG antibodies using a protein A/G horseradish peroxidase mixture. According to the manufacturer's instructions, sera were considered positive for sample/positive control values $\geq 70\%$, questionable for $>60\%$ and $<70\%$ and negative for $\leq 60\%$.

2.3. RNA Extraction and RT-qPCR

All serological positive sera were tested for HEV RNA using RT-qPCR in pools, including a maximum of five samples. Additionally, 386 seronegative sera were tested using RT-qPCR in pools in the same way. These seronegative samples originated from

19 municipalities where positive sera had also been previously found. All positive pooled samples were split into individual samples and re-tested using RT-qPCR.

HEV RNA was extracted using the MagMAX™ CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. RNA was detected by RT-qPCR with 50 cycles according to Jothikumar et al. (2006) [31], using bacteriophage MS2 as an internal process control [32].

2.4. HEV RNA Genotyping

Genotyping of HEV RNA was performed on sera revealing a Cq value below 30 with RT-qPCR. PCR products for genotyping of partial ORF1 region by DNA sequencing were obtained using the reverse transcriptase PCR Kit qScript™ XLT 1-Step RT-PCR (Quanta bio, VWR, Bruchsal, Germany) and the primers described for a nested RT-PCR (HEV.ORF1_F1/HEV.ORF1_R1 and HEV.ORF1_F2/HEV.ORF1_R2) by Vina-Rodriguez et al. (2015) [33]. These primers had been chosen to match the majority of European and German HEV sequences in order to correspond to the neutral subtype scheme proposed by Lu et al. (2006) [16].

The PCR products generated by the nested PCR (Accustart™ GelTrack™ PCR Super Mix, Quanta bio, VWR, Bruchsal, Germany) were cut out of the agarose gel, and the purified DNA (QIAquick Gel Extraction Kit, Qiagen, Hilden, Germany) was sequenced on demand (Microsynth, Balgach, Switzerland). Sequence data were evaluated using the freely accessible online platform Hepatitis E Virus Genotyping Tool version 0.1 of the National Institute for Public Health and the Environment (Ministry of Health, Welfare and Sport) in the Netherlands (available online: <https://www.rivm.nl/mpf/typingtool/hev/>, accessed 10 December 2021) in order to determine the HEV genotype.

2.5. Statistical Analyses

Fisher's exact test was carried out using the program OpenEpi version 3.01 two-by-two table, 95% confidence interval, available online: <http://www.openepi.com>, accessed on 13 October 2021) for the calculation of *p*-values and odds Ratios (OR) [34]. *p*-values < 0.05 were considered significant. Questionable results in ELISA were excluded from data analyses due to evaluations based on unambiguous results only.

3. Results

3.1. Serological and RT-qPCR Studies

In our study, sera originating from wild boars were first tested for antibodies against HEV. Sera that proved seropositive were re-tested in a follow-up study for HEV RNA using RT-qPCR to evaluate the viremic state.

A total of 2295 sera were available from wild boars in 234 municipalities of 12 counties located in the federal state of Baden-Wuerttemberg in Southwest Germany. These had been taken over a time period of five years from 2016 to 2020.

The serological examinations carried out by ELISA revealed 249 positive (10.8%), 9 questionable (0.4%) and 2037 negative (88.8%) results (Table 1). The proportion of questionable results was negligible and therefore not further considered. The seropositive samples originated from 96 municipalities in all of the 12 counties. The annual seroprevalence rate lay in the range of 7.5% to 14%, and the prevalence of HEV carrying wild boars was between 9.1% and 22.2% over the 5 year test period (Table 1). The seroprevalence per county ranged from 3.6% to 17.5%, and the prevalence of seropositive and HEV RNA positive wild boars from 0% to 25.8% (Figure 1). Statistical analyses did not reveal significant differences in seroprevalences or detection of HEV specific RNA between the years of the test period.

Table 1. Detection of antibodies against hepatitis E virus (HEV) by enzyme-linked immunosorbent assay (ELISA) and HEV RNA in previously seropositivity tested wild boar sera using reverse transcription real-time PCR (RT-qPCR) in the time period from 2016 to 2020.

Year	HEV ELISA				HEV RT-qPCR		
	Negative	Questionable	Positive	Total	Negative	Positive	Total
2016	135 (86%)	0 (0%)	22 (14%)	157 (6.8%)	20 (90.9%)	2 (9.1%)	22 (8.8%)
2017	476 (89.1%)	1 (0.2%)	57 (10.7%)	534 (23.3%)	50 (87.7%)	7 (12.3%)	57 (22.9%)
2018	630 (85.7%)	5 (0.7%)	100 (13.6%)	735 (32%)	81 (81%)	19 (19%)	100 (40.2%)
2019	577 (91.7%)	0 (0%)	52 (8.3%)	629 (27.4%)	45 (86.5%)	7 (13.5%)	52 (20.9%)
2020	219 (91.3%)	3 (1.2%)	18 (7.5%)	240 (10.5%)	14 (77.8%)	4 (22.2%)	18 (7.2%)
Total	2037 (88.8%)	9 (0.4%)	249 (10.8%)	2295 (100%)	210 (84.3%)	39 (15.7%)	249 (100%)

The serological screening was followed by testing all seropositive sera in pools comprising up to five samples using HEV RT-qPCR. This selective testing resulted in 39 RT-qPCR positive sera (15.7%), which had been taken from wild boars in 30 different municipalities located in 11 counties. In Figure 1, the seropositive and PCR positive sera are assigned to the counties of the administrative district of Stuttgart.

In addition, 386 seronegative sera were also tested by RT-qPCR. These samples originated from 19 municipalities where seropositive samples were also found. Due to the geographical proximity, these animals were chosen on the assumption that their risk of infection with HEV would be comparable to the risk to animals with seropositive results. Only five samples in this group tested positive for HEV RNA (1.3%). A comparison of the serological results with detection of HEV RNA showed a highly significant correlation between serological and RT-qPCR positive results ($p \leq 0.0000001$, two-tailed, OR 14.15, 5.49–36.45). In detail, 15.7% (39 of 249) of the sera tested positive by ELISA also showed positive results in RT-qPCR, while only 1.3% (5 of 386) of the serologically negative sera tested positive for HEV in RT-qPCR (Table 2).

Table 2. Comparison of the results obtained from examinations of wild boar sera tested for antibodies against HEV using ELISA and for HEV RNA by RT-qPCR.

		Positive ¹	HEV RT-qPCR Negative	Total
HEV ELISA	Positive	39 (15.7%)	210 (84.3%)	249 (39.2%)
	Negative	5 (1.3%)	381 (98.7%)	386 (60.8%)
	Total	44 (6.9%)	591 (93.1%)	635 (100%)

¹ Cq values of the RT-qPCR ranged from 26.3 to 40.0, except for three samples yielding Cq values of >40 (40.6, 40.7, 44.0).

Analyses on the basis of municipalities revealed that HEV RNA positive wild boars were found in 7 regional clusters (3–8 animals per cluster), including 84% (37 of 44) of all animals tested positive for HEV. Further, two clusters contained two animals, and in three municipalities only one animal was HEV positive, respectively. The hunting grounds forming geographical clusters were about 10 km apart from each other (Figure 1).

In addition, an analysis on the age of animals that had been tested for HEV RNA by RT-qPCR was conducted. This comparative evaluation showed a significantly greater proportion of sera positive for HEV in RT-qPCR taken from animals <1 year than from animals >1 year ($p = 0.0006726$ (one-tailed), 0.001345 (two-tailed), OR 3.52, 1.65–7.52) (Table 3).

Table 3. Detection of HEV RNA using RT-qPCR in samples from seropositive (ELISA) wild boars <1 year in comparison to sera of animals >1 year.

Age (Years)	HEV RT-qPCR		Total
	Positive	Negative	
<1	26 (24.5%)	80 (75.5%)	106 (44.9%)
>1	11 (8.5%)	119 (91.5%)	130 (55.1%)
Total	37 (15.7%)	199 (84.3%)	236 (100%) ¹

¹ Total amount of samples 236 instead of 249 because 13 animals had no details on age.

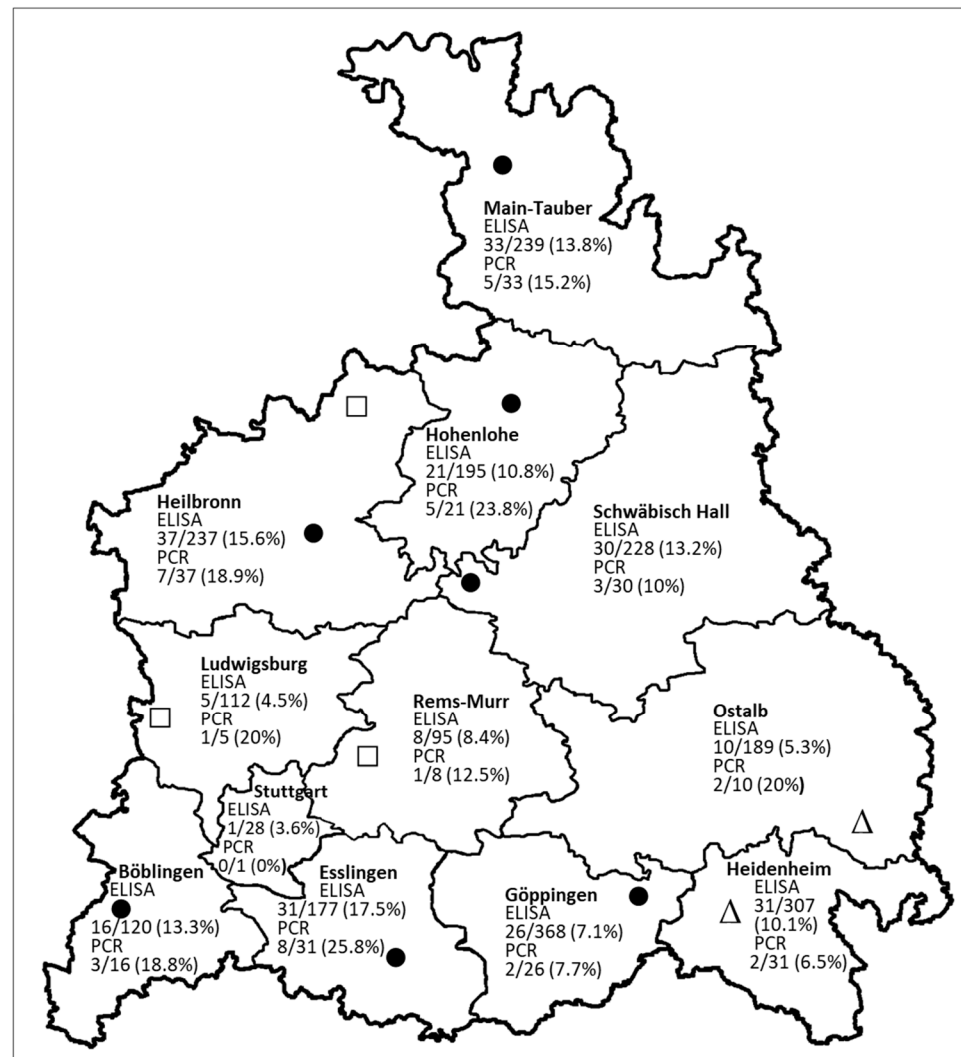


Figure 1. Geographical allocation of results from examinations on antibodies against HEV using ELISA and detection of HEV RNA in seropositive blood samples using RT-qPCR. The blood samples were taken from wild boars in the 12 counties of the administrative district of Stuttgart located in Southwest Germany during the hunting seasons 2016–2020. ● Clusters of HEV RNA positive wild boars consisting of three to eight animals △ Clusters of HEV RNA positive wild boars consisting of two animals □ Single HEV RNA positive wild boars.

3.2. Genotyping

Genotyping of HEV based on partial sequencing of the ORF1 region proved to be successful for all five sera that had shown C_q values <30 in the RT-qPCR. In detail, two sera were suitable for the generation of HEV DNA in sufficient quantities for sequencing when employing the first PCR. However, amplification of HEV DNA suitable for sequencing

could be achieved with all five sera when using the nested PCR (second PCR). All five sequenced nucleic acid amplification products obtained by an ORF1 nested PCR (GenBank accession numbers OL829969 to OL829973) could be identified as genotype 3 with the help of the freely accessible online platform Hepatitis E Virus Genotyping Tool of the National Institute for Public Health and the Environment in the Netherlands. Thus, HEV-3 could be identified in five wild boar sera originating from five municipalities in four different regional clusters located in the counties Göppingen, Heilbronn, Hohenlohekreis, and Main-Tauber-Kreis (2×) (Figure 1).

4. Discussion

HEV-3 is an emerging zoonotic agent of porcine origin in industrialized countries that poses a health hazard to humans worldwide [1–3]. The number of autochthonous cases of HEV detected in European countries has increased in the last decade, with the majority of cases being reported in France, Germany and the UK [6].

Consistent monitoring studies on HEV based on powerful tools that can provide current data for assessing active circulation of zoonotic agents are therefore of pivotal importance [35].

In the event of viral infections, studies based on antibody testing use well-established screening techniques as a first step, followed by further investigations focusing on the direct detection and characterization of the pathogen, supported by sensitive molecular methods [36]. This is also possible for comprehensive, long-term monitoring studies based on serum samples stored over a five year period or even longer [15]. When implementing this procedure, one central issue is the choice of samples. The swine fever monitoring program provides blood samples for serological and molecular testing in a statistically representative amount and distribution. However, although testing for HEV in tissue specimens, especially in the liver, would be advantageous, it would also mean increased effort for the hunters, possibly resulting in reduced numbers of sampled animals. Nevertheless, comparative studies conclude that blood samples are appropriate for the detection of antibodies against HEV as well as HEV RNA [18,37–39], even after long storage [15]. Altogether, this procedure simplifies the collection of samples in the field and the storage of sample material.

In our study, we were able to detect antibodies against HEV in wild boars with annual fluctuations ranging from 7.5% to 14% in the counties tested during the five year period from 2016 to 2020. Previous studies conducted in Germany confirm these data for southern federal states, revealing seroprevalence values of 10% [24]. In comparison, seroprevalence values of 33% have been reported in northern federal states of Germany [24,40].

In a subsequent step, serologically positive serum samples were selected and tested for HEV RNA. These examinations detected viremic animals in 39 of 249 (15.7%) seropositive wild boars. It is striking that the number of RT-qPCR and serologically positive sera diverge between consecutive years or adjacent counties. However, we could not prove significant annual differences for the seroprevalences and prevalence of HEV. Spancerniene et al. (2016) [41] could not detect year-dependent differences of seroprevalences within a three year period in Lithuania either. In contrast, a comparative study recently conducted in Germany, using data from two consecutive hunting seasons in 2013/2014 and 2014/2015, revealed significantly different proportions of seropositive animals, represented by 27% and 52%, respectively [18]. Analyses on the basis of municipalities reveal that 84% of HEV RNA positive wild boars clustered regionally in various municipalities. Considering the different regional origins of samples, a previous study performed in different counties of the federal state of Saxony-Anhalt (Germany) revealed statistically significant geographical clusters of seropositive animals. The authors conclude that the detection of regional clusters is presumably related to high and strongly growing wild boar densities promoting the risk of HEV transmission between the animals and leading to increased HEV prevalence rates [40]. Likewise, Adlhoch et al. (2009) [24] observed different seroprevalence values of wild boars when comparing federal states in southwestern and northeastern Germany, revealing

10% and 37%, respectively. Other studies also provide data on significantly differing seroprevalence for adjacent regions in Bulgaria (10–73%) [42] and Spain (0–64%) [37]. For Poland, a wide range of seroprevalences from 5–88% [43], 10–72% [13], and 8–40% [44] has been reported. In contrast to these countries, differences in seroprevalence data in Lithuania were reported to be less pronounced but were at a consistently high level [41].

On the whole, it can be concluded that HEV is highly prevalent among wild boars, which present a natural, sylvatic reservoir for this virus [15,18]. This is all the more true because the easy transmission of HEV via the faecal-oral route is intensified by the increasingly high wild boar population densities. This situation leads to stable, persistent virus reservoirs in domestic pigs and wild boars [33,45].

In our study, HEV was significantly more frequently detected by RT-qPCR in seropositive sera (15.7%) than in seronegative sera (1.3%) taken in the same counties. Similar results verifying a positive correlation between seropositive wild boars and detection of HEV RNA in serum or liver have been previously published [13]. In this study, HEV RNA was mainly found in the faeces of seropositive animals due to efficient virus replication in the intestinal epithelial cells in the early stage of infection. In another study, 82% of seropositive sera were also positive for HEV RNA [13]. In contrast, De Deus et al. (2008) [37] and Dorn-In et al. (2017) [44] report no correlation between detection of IgG antibodies and HEV RNA in blood. However, de Deus et al. (2008) [37] note that the proportion of positive IgM animals was significantly higher amongst RT-PCR positive animals. Moreover, Dorn-In et al. (2017) [44] present data with 50% HEV RNA positive results in seropositive sera compared to 21% in seronegative sera, what we believe could be interpreted as a significant difference. A likely explanation is that a successful, simultaneous detection of HEV-antibodies and HEV RNA can only be made within a limited time frame, during the early stage of viremia and with sufficient seroconversion.

In further investigations considering the age of the wild boars, we were able to prove a significantly higher probability for detection of HEV RNA in sera taken from animals younger than one year compared to older animals. Other studies also confirm a positive correlation between positive results of juvenile wild boars (<1 year) in HEV PCR compared to older animals (>1 year) [37,46,47]. It appears plausible that animals living in an environment with a high virus load go through an HEV infection early in life, resulting in future immunity against this virus. However, studies conducted by Adlhoch et al. (2009) [24] and Rivero-Juarez et al. (2018) [48] could not detect an age-related viral load in wild boar sera. It should be noted that our study might be biased due to the serological pre-selection of the sera.

These results show that testing of seropositive sera for HEV RNA represents a reliable approach for the detection of wild boars in their viremic phase. This is especially valid when seropositive sera of wild boars younger than one year are tested.

This method of serological screening examinations in a first step followed by subsequent testing for HEV RNA in a follow-up study was also implemented by Seminati et al. (2008) [39]. These researchers also report finding the largest proportion of HEV viremic pigs that excrete viruses via faeces among the seropositive and younger animals. Results corroborating seroconversion in artificially or naturally HEV infected pigs have been presented by several researchers, without statements on statistical calculations, however [49–51]. Furthermore, comparative studies on the detection of HEV RNA in the liver, which represents the target organ for HEV, and in serum positively screened wild boars showed almost identical results [18,52]. This makes serum a reliable sample matrix for the detection of HEV in infected wild boars based on antibody pre-testing.

Further studies on genotyping of HEV RNA lead to the identification of HEV genotype 3 in all five serum samples tested. HEV-3 is considered the prevalent genotype in wild boars worldwide and is recognized as an important zoonotic viral agent [7,16]. Samples yielding Cq values in RT-qPCR <30 proved suitable for providing sufficient quantities of HEV nucleic acid suitable for successful DNA sequencing. Likewise, other researchers

have come to similar conclusions and recommended samples with C_q values <31 in qPCR for reliable success in whole genome sequencing (WGS) [53].

5. Conclusions

The present study shows that wild boars are a persistent, natural and sylvatic reservoir for the zoonotic agent HEV, also in southwestern Germany, located in central Europe. Monitoring programs are essential elements for the assessment of current and future HEV prevalence. This is all the more true because wildlife habitats and human settlements increasingly overlap. The establishment of consecutive laboratory diagnostic steps based on blood samples has turned out to be a suitable, timely and cost-effective procedure for comprehensive monitoring. Serological testing of monitoring samples using ELISA gives reliable information about the prevalence of HEV. Subsequent testing of seropositive pool samples of wild boars younger than one year by RT-qPCR allows an estimation of the viral load in the tested population and gives an indication of possible infection pressure in the region.

Further monitoring studies based on blood samples should be conducted on the prevalence of HEV in wild boars in order to provide assessment data on the risk for foodborne HEV infections in humans.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article. The data presented in this study are available in Detection of Hepatitis E Virus Infections in Wild Boars in Southwest Germany Using a Stepwise Laboratory Diagnostic Approach.

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