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Phylogeny and spatiotemporal dynamics of hepatitis E virus infections in wild boar and deer from six areas of Germany during 2013–2017

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Abstract

The hepatitis E virus (HEV) can cause acute and chronic hepatitis in humans. Infections with the zoonotic HEV genotype 3, which can be transmitted from infected wild boar and deer to humans, are increasingly detected in Europe. To investigate the spatiotemporal HEV infection dynamics in wild animal populations, a study involving 3572 samples of wild boar and three deer species from six different geographic areas in Germany over a 4-year period was conducted. The HEV-specific antibody detection rates increased between 2013–2014 and 2016–2017 in wild boar from 9.5% to 22.8%, and decreased in deer from 1.1% to 0.2%. At the same time, HEV-RNA detection rates increased in wild boar from 2.8% to 13.3% and in deer from 0.7% to 4.2%. Marked differences were recorded between the investigated areas, with constantly high detection rates in one area and new HEV introductions followed by increasing detection rates in others. Molecular typing identified HEV subtypes 3c, 3f, 3i and a putative new subtype related to Italian wild boar strains. In areas, where sufficient numbers of positive samples were available for further analysis, a specific subtype dominated over the whole observation period. Phylogenetic analysis confirmed the close relationship between strains from the same area and identified closely related human strains from

Germany. The results suggest that the HEV infection dynamics in wild animals is dependent on the particular geographical area where area-specific dominant strains circulate over a long period. The virus can spread from wild boar, which represent the main wild animal reservoir, to deer, and generally from wild animals to humans.

KEYWORDS
deer, hepatitis E virus, infection dynamics, phylogeny, wild boar, zoonosis

1 | INTRODUCTION

Infections with the hepatitis E virus (HEV) can cause hepatitis in humans. Worldwide, HEV infections account for an estimated 3.3 million symptomatic cases and 44,000 deaths per year (WHO, 2021). In several European countries, a steep increase of clinical hepatitis E cases has been noticed during the last years (Adlhoch et al., 2016), for example in Germany, from 222 notified cases in 2010 to 3252 in 2020 (Robert Koch-Institut, 2021). Besides acute hepatitis, chronic HEV infections potentially leading to fatal liver cirrhosis are increasingly found in transplant patients and extrahepatic disease manifestations have also been described (Velavan et al., 2021). However, serological population surveys indicate that subclinical HEV infections are also common (Faber et al., 2012, 2018).

HEV has a single-stranded RNA genome with a size of approximately 7.2 kbp, which harbours three open reading frames (ORFs) (Pallerla et al., 2020). ORF1 encodes a non-structural polyprotein, which contains several functional domains, for example, for an RNA-dependent RNA polymerase (RdRp). The most important human-pathogenic HEV strains are classified into genotypes 1 to 4 of the species *Orthohepevirus A* within the family *Hepeviridae*. These genotypes can be further assigned to subtypes according to their degree of sequence identity with defined subtype reference strains (Smith et al., 2020). Genotypes 1 and 2 exclusively infect humans and are mainly transmitted by faecally contaminated drinking water (Pallerla et al., 2020). They are mainly found in low- and middle-income countries in Asia and Africa, where they can cause large hepatitis outbreaks. In contrast, genotypes 3 and 4 are zoonotic and can be transmitted by contact to infected animals or by ingestion of contaminated meat products (Velavan et al., 2021). Genotype 4 is mainly confined to China, whereas genotype 3 is distributed worldwide and represents the most important HEV genotype in Europe and Germany (Pallerla et al., 2020).

The main animal reservoirs of genotype 3 are domestic pigs and wild boar, but other animal species like deer have also been shown to be HEV-infected and to transmit the virus to humans (Pavio et al., 2017; Spahr et al., 2018). Foodborne virus transmission from wild animals to humans has been demonstrated in case studies, where the same HEV strains were identified in patients and in food residues from wild boar liver (Matsuda et al., 2003), wild boar meat (Masuda et al., 2005), or deer meat (Tei et al., 2003). Moreover, serological studies on hunters and forest workers indicate higher seroprevalences of HEV-specific antibodies as compared to control groups (Dremsek et al., 2012; Pavio et al., 2017). In line with an assumed HEV-transmission by direct contact to wild boar, wearing gloves has been identified as a protective measure leading to lower HEV seropositivity in hunters (Baumann-Popczyk et al., 2017; Schielke et al., 2015).

The presence of HEV in wild boar has been described in several studies from different countries, reporting detection rates of 1.6%–57.4% for HEV-specific antibodies and 0%–68.2% for HEV-RNA (Pavio et al., 2017). Various factors have been suggested to explain the large variation of detection rates, for example, geographical differences, age, season, population density or ecology (de Deus et al., 2008; Jori et al., 2016; Kaba et al., 2010; Martinelli et al., 2015; Rivero-Juarez et al., 2018; Schielke et al., 2009, 2015; Serracca et al., 2015). Yet, the sample size and investigated time frames in these studies were often small and different sample types as well as different detection methods were used making interpretation and comparison of the data difficult. A large number of different HEV genotype 3 subtypes has been identified in wild animals, which often showed close phylogenetic relationship to human clinical strains indicating their zoonotic potential (Pavio et al., 2017; Schielke et al., 2009; Weigand et al., 2018). However, only very few studies on long-term determinants of the seroprevalence of HEV-specific antibodies in wild boar are available (Barroso et al., 2021), and little is known about the dynamics of strain transmission and molecular phylogenetics in wild boar populations of different areas or during longer time frames.

The majority of published studies of HEV infection of wild animals focused on wild boar, whereas virus transmission to other animal species like deer have only scarcely been investigated. Generally, the reported common detection rates of 0%–14% for HEV-specific antibodies and 0%–10% for HEV-RNA in deer species are considerably lower than in wild boar (Pavio et al., 2017). Hence, it still remains controversial if deer represent a true reservoir for HEV (van der Poel, 2014) or if spillover infections from wild boar can explain the HEV-positive animals (Anheyer-Behmenburg et al., 2017).

To investigate the spatial and temporal dynamics of HEV infection in wild animal populations in more detail, a large sample derived from six different geographic areas in Germany over a 4-year period was tested in this study for the presence of HEV-specific antibodies and HEV-RNA. The data were analysed to identify factors explaining the observed differences in prevalence and to unravel the infection dynamics in the different areas, age groups and between animal species. Genotyping data were used to assess the diversity of circulating strains and to clarify, if a persistence of dominant strains can be

observed within the distinct populations or if continuous strain fluctuations are more common. A detailed phylogenetic comparison of the detected strains was carried out to determine their distinct relationships and further investigate their zoonotic potential. The study provides new insights into the epidemiology and transmission dynamics of HEV in wild animals, which might serve as a prerequisite for developing approaches to prevent virus transmission from wild animals to humans in future.

2 | MATERIALS AND METHODS

2.1 | Study population and sampling

A total of 3572 wild animals from six different areas in Germany, which included a subset of 415 animals already published previously (Anheyer-Behmenburg et al., 2017), were included in the study. All sampled animals belonged to free-ranging populations living in military training areas of the German armed forces and were collected during regular hunting events within the hunting season (October to January). A map showing the location of the sampling areas is presented in the Supplementary Data S1 (see also Figure 5) and further characteristics of the areas including percentage of forest, green area, the number of shot wild boar per km^2 and year, as well as dominant deer species are indicated in Supplementary Data S2. In three areas (designated P, B, J), samples were collected during four hunting seasons (2013–2014 to 2016–2017), whereas in additional three areas (designated S, H, O) samples from three hunting seasons (2014–2015 to 2016–2017) were available. In total, 1961 wild boar (*Sus scrofa*), 559 roe deer (*Capreolus capreolus*), 736 red deer (*Cervus elaphus*) and 316 fallow deer (*Dama dama*) were tested (more details in Supplementary Data S3). A liver and a serum sample (if both were available) were separately taken from each animal immediately after the battue and stored at −80°C until further analysis. Data on species, sex, age group, area and date of sampling were recorded. Species classification was based on visual inspection, age classification resulted from teeth status and dental formula, conducted by experienced hunters and veterinarians.

2.2 | Detection of HEV-specific antibodies and HEV-RNA

Samples were analysed for the presence of HEV-specific antibodies and HEV-RNA as described in detail by Anheyer-Behmenburg et al. (2017). Serum samples were tested for anti-HEV antibodies by an indirect ELISA based on a recombinant HEV genotype 3 capsid protein and an anti-multispecies IgG–horseradish peroxidase conjugate (ID Screen Hepatitis E Indirect; ID Vet, Grabels, France) according to the manufacturer's instructions.

Liver samples were preferentially analysed for HEV-RNA; however, if a liver sample was not available from an individual animal, the respective serum sample was used. Liver samples were homogenized in a Tissue Lyser (QIAGEN, Hilden, Germany) followed by additional homogenization by a QIA-Shredder column (QIAGEN). RNA was extracted from the liver homogenate using the QIAsymphony RNA kit and from serum samples using the QIAsymphony DSP Virus/Pathogen Kit in a QIAsymphony device according to the manufacturer's instructions. An HEV-specific RT-qPCR was performed using primers and probe described by Jothikumar et al. (2006) together with the QuantiTect Probe RT-PCR Kit (QIAGEN, Hilden, Germany).

2.3 | Sequencing and sequence analysis

HEV-RNA-positive samples were further characterized by amplification of a 331 bp (280 bp excluding primer sequences) long fragment of the RdRp region using nested RT-PCR (Anheyer-Behmenburg et al., 2017; Johne et al., 2010), followed by Sanger sequencing by a commercial provider (Eurofins GmbH, Ebersberg, Germany). Resulting sequences were submitted to GenBank (accession numbers OK076723–OK076784 and OK135143). Genotyping was performed with a fasta36 similarity search (Pearson, 2016) using the HEV subtype reference sequences (Smith et al., 2020) as database. In addition, all sequences were aligned with HEV sequences from human cases in Germany supplied by the National Consultant Laboratory (accession numbers MZ814768, MZ814791, MZ814871 and MZ814777) using MAFFT 7 (Katoh & Standley, 2013). Alignments were processed with RAxML (Stamatakis, 2014) and the phylogenetic tree was calculated with the maximum likelihood method, bootstrap of 1000 replicates and rabbit HEV as an outgroup. Trees were visualized with FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Per cent identity matrixes were calculated using R and the ape package function dist.dna with model = 'raw' (R Core Team, 2020; RStudio Team, 2019; Paradis & Schliep, 2019).

2.4 | Statistical analysis

Univariate and multivariate analyses were performed to assess factors associated with HEV-RNA positivity in wild boar. The following factors (independent variables) were included in the analyses: hunting area (area B, area H, area S, area J, area O), hunting season (2013–2014, 2014–2015, 2015–2016, 2016–2017), hunting month (October, November, December, January), age group (juvenile: <1 year, subadult: 1 to <2 years, adult: from 2 years), sex (female, male). Area P was not included in the analyses, as no HEV-RNA-positive wild boar were found due to the small sample. After chi-square independence tests (univariate analysis), adjusted *p* values were calculated to assess factors (hunting season, hunting area, age, group, sex, month) associated with HEV-RNA positivity in wild boar. The adjusted *p* values were compared with the Bonferroni-corrected significance level, and pairwise comparisons according to Bonferroni post hoc tests were implemented ('pairwiseNominalIndependence' from the R package rcompanion; Mangiafico, 2022). For multivariate analysis, logistic regression models based on binomial distribution with logit link were applied. As first model we calculated a mixed model with hunting area as a

random variable and the other variables as fixed factors with glmer function from lme4 package (Bates et al., 2015). In the second step, this was compared to a model with fixed effects (with glm function in R) to better compute natural time- or age-related effects. To measure the goodness of fit for model selection the regression models, predictors were excluded stepwise (backward selection) based on the Akaike information criteria (AIC) to obtain the most suitable model. The Adjusted Odds Ratio (AOR) was calculated with 95% CI. A two-tailed p value of <.05 was considered statistically significant. Details on the applied model for multivariate analysis are shown in Supplementary Data S4. All data were analysed with the statistical software R version 4.02 (R Core Team, 2020) and IBM SPSS Statistics for Windows, Version 21 (IBM Corp., Armonk, NY, USA). Figures were created with Microsoft® Excel 2016 and IBM SPSS Statistics for Windows, Version 26 (IBM Corp., Armonk, NY, USA). HEV-specific antibody detection rates/HEV-RNA detection rates on bars were given with exact 95% CI according to Collett (2002).

3 | RESULTS

3.1 | Mean detection rates of anti-HEV antibodies and HEV-RNA and general trends

Samples from 3572 wild animals were collected from six areas in Germany during a 4-year period of hunting seasons. Details on the areas and the study population are presented in Supplementary Data S1–S3 (for the location of the areas, see also Figure 5). In total, mean detection rates of 12% for HEV-specific antibodies and 6.1% for HEV-RNA were determined. For the different animal species, mean detection rates of 20.9% and 9.6% for wild boar and 0.4% and 1.9% for the deer (all deer species summarized) were calculated for HEV-specific antibodies and HEV-RNA, respectively. Analysis of the detection rates according to the hunting seasons showed increasing trends of detection rates for both HEV-specific antibodies (Figure 1a) and for HEV-RNA (Figure 1b). In detail, the HEV-specific antibody detection rates increased between 2013–2014 and 2016–2017 in wild boar from 9.5% to 22.8% and slightly decreased in deer from 1.1% to 0.2%. At the same time, the HEV-RNA detection rate increased in wild boar from 2.8% to 13.3% and in deer from 0.7% to 4.2%. In the years 2014–2015, 2015–2016 as well as 2016–2017, the HEV-RNA detection rate in wild boar was significantly higher than in the other three species. No correlation between species according to the HEV-RNA detection rate was observed in 2013–2014. Details on the detection rates according to the distinct animal species are shown in Figure 1b and in Supplementary Data S5.

3.2 | Variation in detection rates by month and age group in wild boar

As shown, detection rates in deer were very low; therefore, detailed analyses were carried out for wild boar data only. The HEV-RNA detection rates in wild boar were assessed monthly per hunting season and an increasing trend was observed in January for most of the hunting seasons (Figure 1c). In line with this observation, significantly ($p < .001$) more HEV-RNA-positive animals were only found in January as compared to the other months, regardless of the hunting season, in the univariate analysis (Table 1). The detection rates according to age groups of wild boar are shown in Figure 2. Whereas the HEV-RNA detection rate decreased ($p < .001$, Table 1, univariate analysis) with higher age (12.5% in juveniles to 4.9% in adults), the HEV-specific antibody detection rate increased with age (16.9% in juveniles to 24.3% in adults).

3.3 | Regional variations of detection rates in wild boar

The detection rates in wild boar according to the hunting areas show considerable variations (Figure 3). For HEV-specific antibodies, the mean detection rates over the whole time-period varied between 10.2% in area B and 37.5% in area O. Moreover, different trends in temporal variation were found (Figure 3a). For HEV-RNA, the mean detection rates over the whole time-period varied between 0% in area P and 2.1% in area H to 16.4% in area S and 19.1% in area J. In areas H, S and B, HEV-RNA-positive wild boar were only detected in the last 2 or 3 years of the study, albeit with an increasing trend over time (Figure 3b). In contrast, HEV-RNA-positive animals were detected in all hunting seasons in area J, with some variations in the detection rate. Looking at the hunting areas independently of the hunting season, significantly more HEV-RNA-positive wild boar occurred in area J and S (Table 1, univariate analysis).

3.4 | Factors associated with HEV-RNA positivity in wild boar – multivariate analysis

For the multivariate analysis of factors associated with HEV-RNA positivity in wild boar, a mixed model and a model with fixed effects were applied. As both models showed comparable results in terms of effects (odds ratio) and p values, only the results of the fixed model are shown in Table 1. It is evident, that the hunting seasons 2016–2017 ($p < .001$) and 2015–2016 ($p < .05$ in multivariate analysis), the month January ($p < .05$ in multivariate analysis), the juvenile age group ($p < .001$) and the region S and J ($p < .001$) are significantly associated with a high detection rate. In contrast, there was no significant correlation between sex and the detection rate.

3.5 | Time-dependent detection of HEV-RNA in different wild animal species

In order to represent the infection dynamics between wild boar and deer, the HEV-RNA detection rates in the different areas were compared over time with regard to the animal species (Figure 4). For area J, HEV RNA was detected in wild boar and deer rather consistently

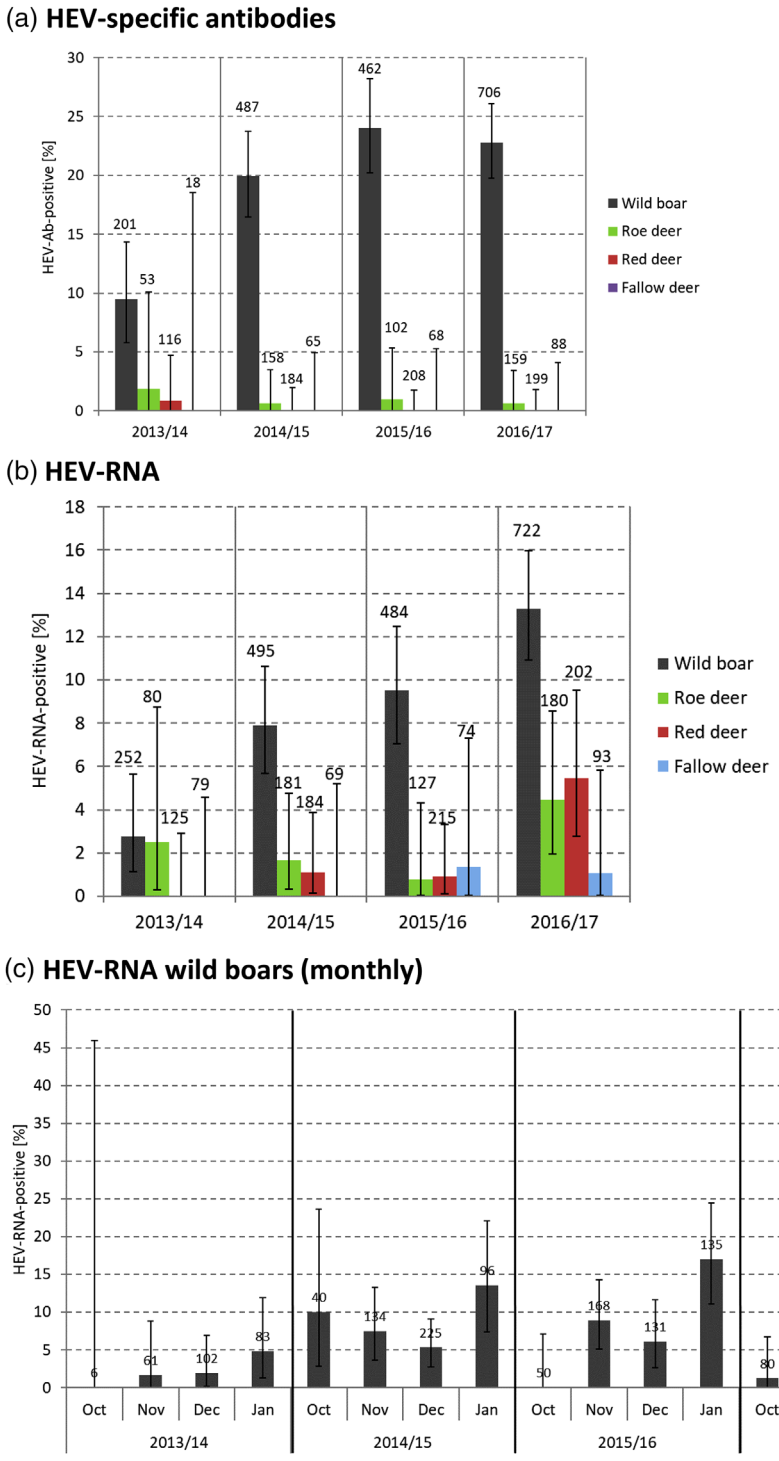

FIGURE 1 Detection of HEV-specific antibodies and HEV-RNA between 2013 and 2017 according to hunting seasons. (a) Positive rate of HEV-specific antibodies according to hunting season and animal species. (b) Positive rate of HEV-RNA according to hunting season and animal species. (c) Monthly positive rate of HEV-RNA in wild boar during the hunting seasons. The total numbers of tested animals (small numbers above the columns) and the confidence intervals are indicated

during all four analysed hunting seasons. In area P, only two positive animals (fallow deer) were identified, making further data analysis difficult. In the remaining areas, the first HEV-RNA detection in deer species occurred 1 year (areas H, B, O) or 2 years (area S) after the first detection in wild boar.

3.6 | Subtyping of HEV-positive samples

All HEV-RNA-positive samples (n = 216) from wild boar and deer were subjected to RT-PCR amplifying a fragment of the RdRp genome region, which was sequenced and used for HEV subtyping. By this, 128

TABLE 1 Factors associated with HEV-RNA positivity in wild boar as determined by univariate and multivariate analysis

	N = 1953		Univariate analysis		Multivariate analysis	
Characteristics	**Total in group**	**Positive (%)**	p_{adjust} [a]	**Letter** [b]	**AOR (95% CI)**	***p***
Hunting season						
2013–2014	252	7 (2.8)	<**.001**	a	Ref.	
2014–2015	495	39 (7.9)	.1	b	2.2 (0.8–7.7)	.18
2015–2016	484	46 (9.5)	.9	bc	3.0 (1.1–10.5)	**.04**
2016–2017	722	96 (13.3)	<**.001**	c	6.6 (2.6–22.5)	<**.001**
Corrected alpha[§]			.0062			
Month						
October	176	5 (2.8)	<**.001**	a	Ref.	
November	541	40 (7.4)	<**.001**	a	1.4 (0.5–4.4)	.58
December	730	62 (8.5)	.2	a	2.5 (0.9–8.2)	.10
January	506	81 (16)	<**.001**	b	4.1 (1.4–13.9)	<**.05**
Corrected alpha[§]			.0062			
Age group (years)						
Juvenile (<1)	879	110 (12.5)	<**.001**	a	2.8 (1.7–5.0)	<**.001**
Sub-adult (1 to <2)	486	33 (6.8)	<**.001**	b	1.4 (0.8–2.7)	.28
Adult (from 2)	366	18 (4.9)	<**.001**	b	Ref.	
Corrected alpha[§]			.0083			
Sex						
Female	855	59 (6.9)	.1	–	Ref.	
Male	638	59 (9.2)			1.2 (0.8–1.7)	.44
Hunting area						
Area B	823	38 (4.6)	<**.001**	a	Ref.	
Aera H	284	6 (2.1)	<**.001**	a	0.8 (0.3–1.9)	.6
Area S	116	19 (16.4)	<**.001**	b	10.0 (4.7–21.2)	<**.001**
Area J	618	118 (19.1	<**.001**	b	4.9 (3.2–7.6)	<**.001**
Area O	96	7 (7.3)	.41	ab	3.8 (1.4–9.6)	.09
Corrected alpha[§]			.005			

[a] p_{adjust}: Bonferroni-adjusted *p* values.
[b] Different letters indicate statistical significance in pairwise comparisons performed with Bonferroni post hoc tests. Same letters between groups indicate no significant difference.
[§] Bonferroni corrected significance level.
Ref., reference category; AOR, adjusted odds ratio. Significant differences are highlighted in bold.

samples could be subtyped. In total, 17 samples were assigned to subtype 3c, 10 to subtype 3f and 11 to subtype 3i. In addition, 90 samples could not be assigned to an established subtype, but all showed high sequence identity to a previously published wild boar strain from Italy (GenBank accession number MF959764), which presumably represents a novel HEV-3 subtype.

3.7 | Spatial and temporal distribution of subtypes

An analysis of the geographical distribution of the determined subtypes by assigning them to their hunting areas identified marked differences (Figure 5). Whereas subtype 3i and the putative novel MF959764-like subtype were exclusively found in areas S, O and J (located in the North-East of Germany), subtypes 3c and 3f were mainly found in area B (located in the North-West of Germany). Within area B, a further sub-regional distribution of subtype 3c in the Northern part and subtype 3f in the Southern part of the area was evident (data not shown). Despite this general subtype distribution, a low number of subtype 3c and 3f strains were also identified in areas O and J, respectively. For analysis of the distribution of subtypes over time, the number of identified strains of the subtypes per hunting season and area is shown in Figure 6. It is evident that in areas J and S, one main distinct subtype can be identified, which is persistently present in all analysed hunting seasons. In both areas, other subtypes are only detected in very low numbers and only in one hunting season. Analysis of the data from the

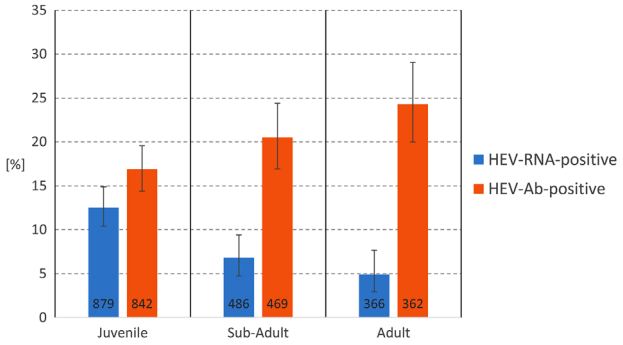

FIGURE 2 Detection of HEV-specific antibodies (Ab) and HEV-RNA in different age groups of wild boar. The total numbers of tested animals (small numbers in the columns) and the confidence intervals are indicated

other areas is difficult as either subtype data are only available for one hunting season (area B) or the sample number is too low (area O). More details on the distribution of subtypes are available in Supplementary Data S6, which also shows that the same subtypes are found in wild boar and deer from the same area.

3.8 | Phylogenetic analysis and relationship with human strains

To analyse the relationship between the strains, a phylogenetic tree was constructed based on the determined sequences (Figure 7). By inclusion of the HEV genotype 3 subtype reference sequences, a clear clustering of the strains according to their previously identified subtype is evident. Sequences also cluster according to the

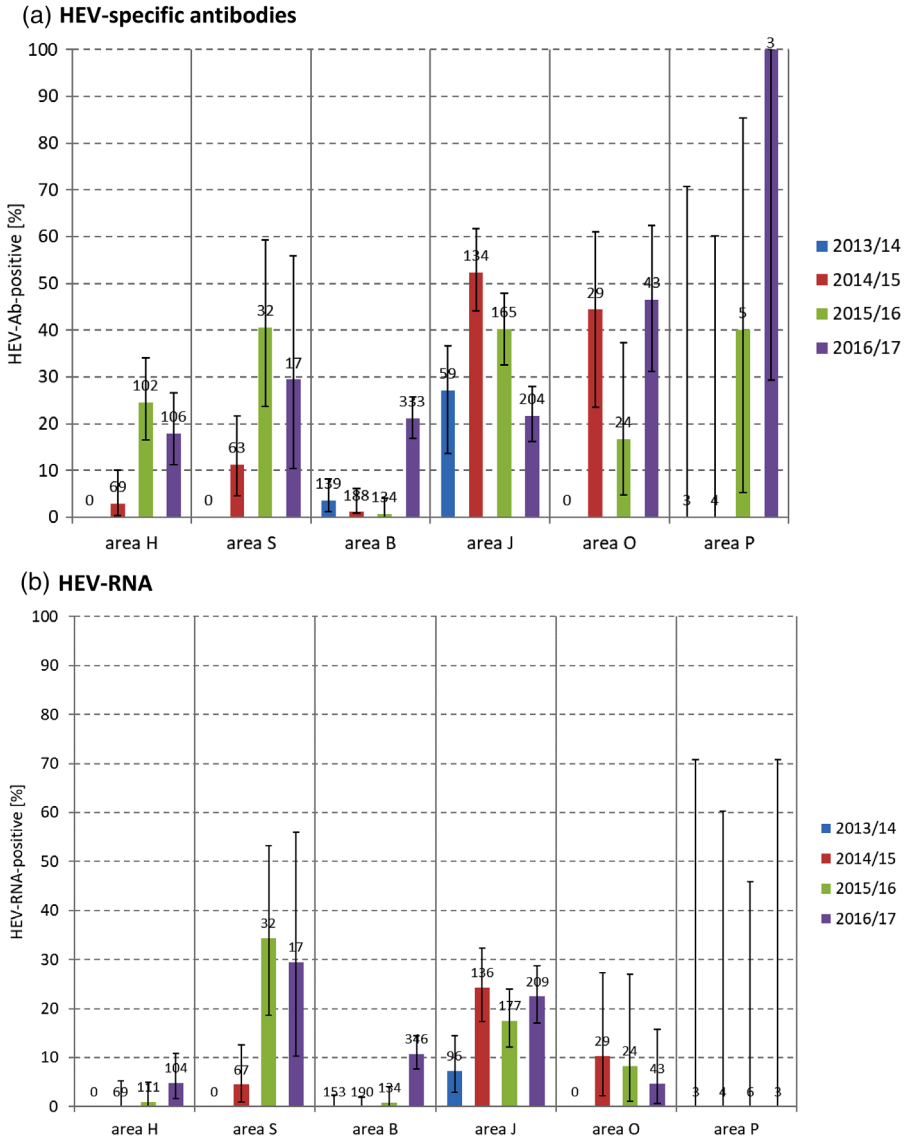

FIGURE 3 Detection of HEV-specific antibodies and HEV-RNA in wild boar according to area and hunting season. (a) Positive rate of HEV-specific antibodies. (b) Positive rate of HEV-RNA. The total numbers of tested animals (small numbers above the columns) and the confidence intervals are indicated

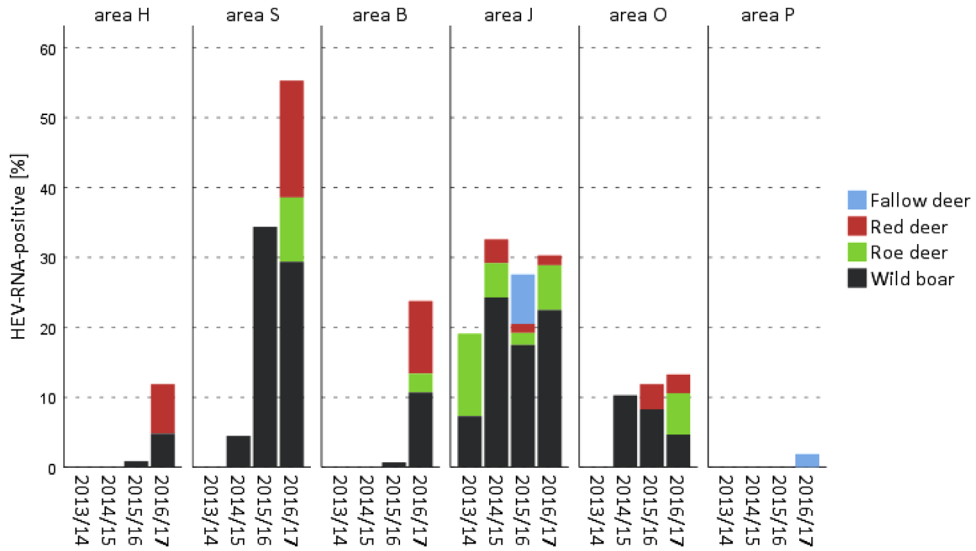

FIGURE 4 Detection of HEV-RNA in different animal species according to area and hunting season. The positive rate is shown

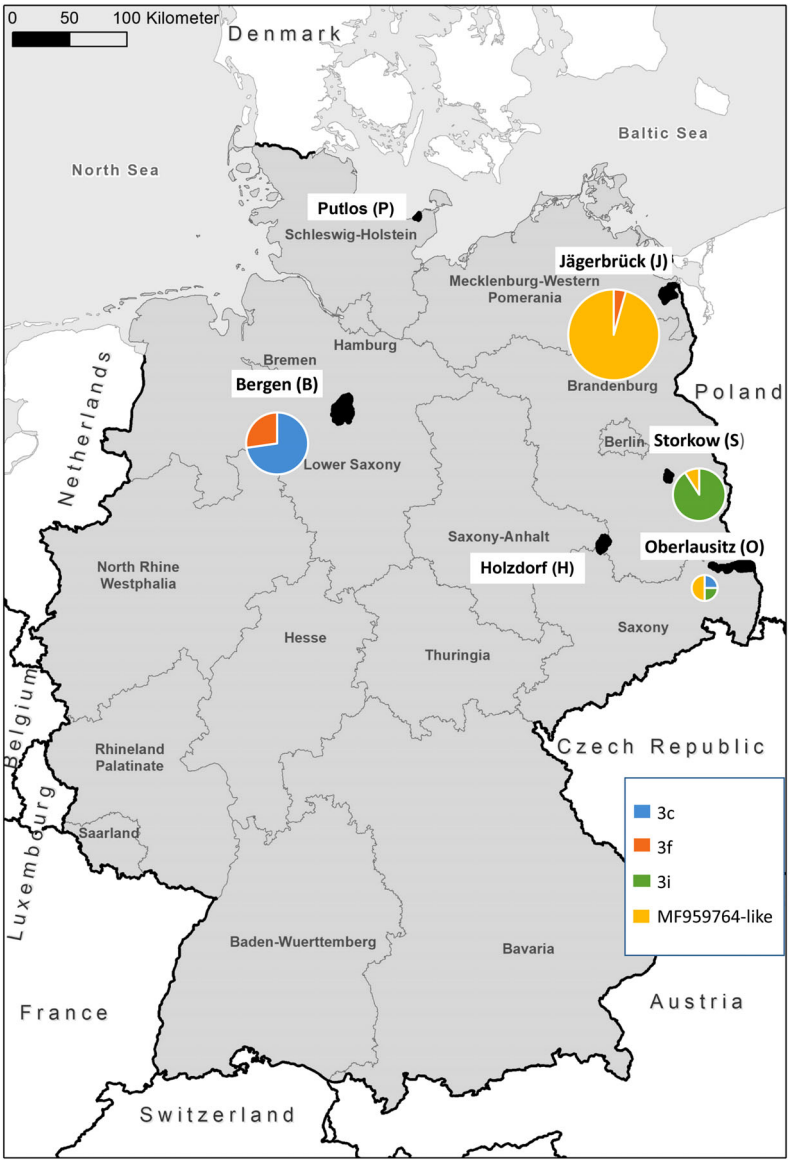

FIGURE 5 Geographical distribution of HEV subtypes at the different hunting areas in Germany. The circles indicate the proportion of the subtypes compared to all identified subtypes of an area. The diameter of the circles corresponds to the number of subtyped strains in the area, not in scale. Black areas – hunting areas. Grey – Federal states. White – surrounding countries. The map was provided by the Bundeswehr Geoinformation Service

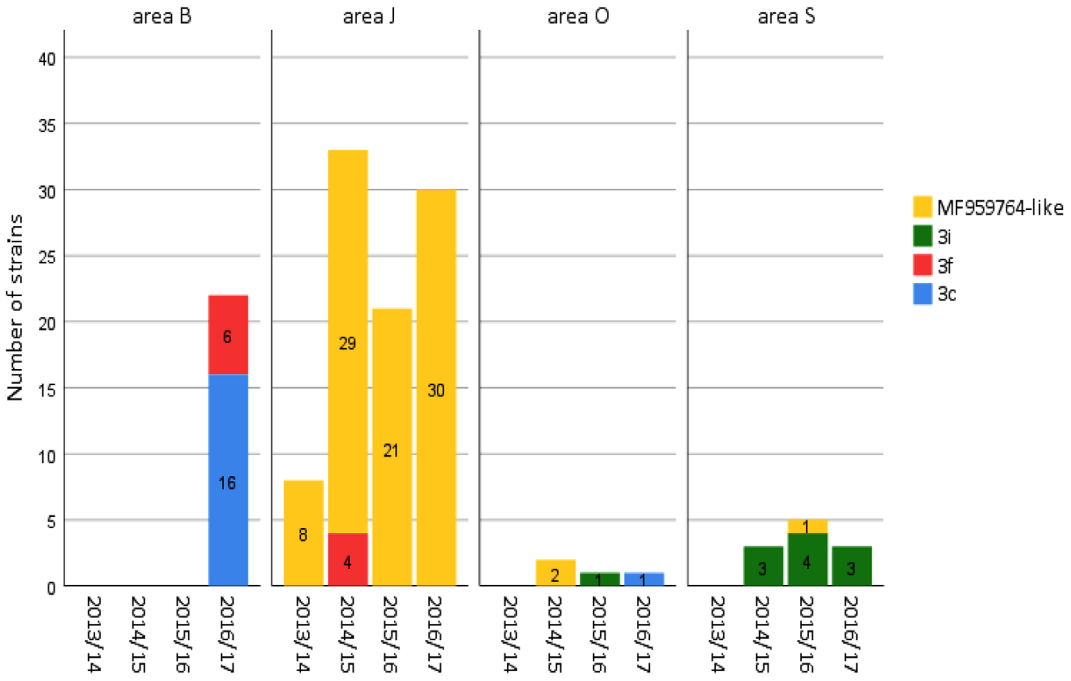

FIGURE 6 Time- and area-dependent detection of HEV subtypes. The numbers of identified strains of a distinct subtype are indicated (small numbers in the columns)

hunting area, and strains of wild boar and deer from the same area show close phylogenetic relationship. Inclusion of sequences derived from human patients from Germany shows that for each subtype there is a human strain related to the wild animal-derived strains. The nucleotide sequence identities between the clinical human strains and all wild animal strains ranged between 81.9% and 97.9%, with the highest similarities for the strains of subtypes 3c and 3f.

4 | DISCUSSION

Wild boar and deer are known as the main wild animal species carrying HEV genotype 3, with the potential to transmit the zoonotic virus to humans. Here, we present a large study involving >3000 animals and investigating the HEV infection in several different hunting areas over a time-period of four hunting seasons. As a result, mean detection rates of 20.9% for HEV-specific antibodies and 9.6% for HEV-RNA were determined for wild boar. Generally, this confirms the important reservoir role of wild boar and the potentially high risk of HEV transmission from wild boar to humans. Previous studies from Germany reported detection rates of 11.5%–45.0% for HEV-specific antibodies and 3.8%–68.2% HEV-RNA in wild boar (Adlhoch et al., 2009; Anheyer-Behmenburg et al., 2017; Weigand et al., 2018). The wide range of reported detection rates may be explained by different sample types and assays used in each study, as well as the examination of different wild boar populations. In addition, the number of samples and investigated areas is usually small in these studies, so sampling bias and existing regional differences may strongly influence the results.

In contrast to wild boar, the determined detection rates in deer were low in our study (0.4% HEV-specific antibodies, 1.9% HEV-RNA). This is in line with previously published detection rates for different deer species in Germany, ranging from 0% to 6.8% for HEV-specific antibodies and from 0% to 6.6% for HEV-RNA (Anheyer-Behmenburg et al., 2017; Neumann et al., 2016; Trojnar et al., 2020). These low detection rates might indicate a lower risk for deer regarding the transmission of HEV to humans. In our study, the first detection of HEV-RNA generally occurred at least 1 year earlier in wild boar compared to deer, which might indicate spillover infections from wild boar to deer (Figure 4). In addition, strains from wild boar and deer from the same area were rather identical, suggesting interspecies transmission events between wild boar and deer (Figure 7). Moreover, our previous study including quantitative data showed that the mean amount of HEV-RNA was significantly lower in deer than in wild boar (Anheyer-Behmenburg et al., 2017). These results can be interpreted as an argument against a role of deer as a true reservoir, where HEV would circulate independently of wild boar infections. However, as cases of HEV transmissions from deer to humans have already been described (Tei et al., 2003), infection-preventive measures for handling deer meat and offal are still warranted.

Our study showed a generally increasing trend of HEV infection markers in wild animals as well as an increase in the number of HEV-positive areas within the investigated time period between 2013 and 2017. As this is the first study investigating HEV infections in wild animals in Germany for a longer time period, the data cannot be compared directly to other studies. Interestingly, the notified human cases also increased from 459 to 2951 during the same time period (Robert Koch-Institut, 2021). However, other factors like increasing

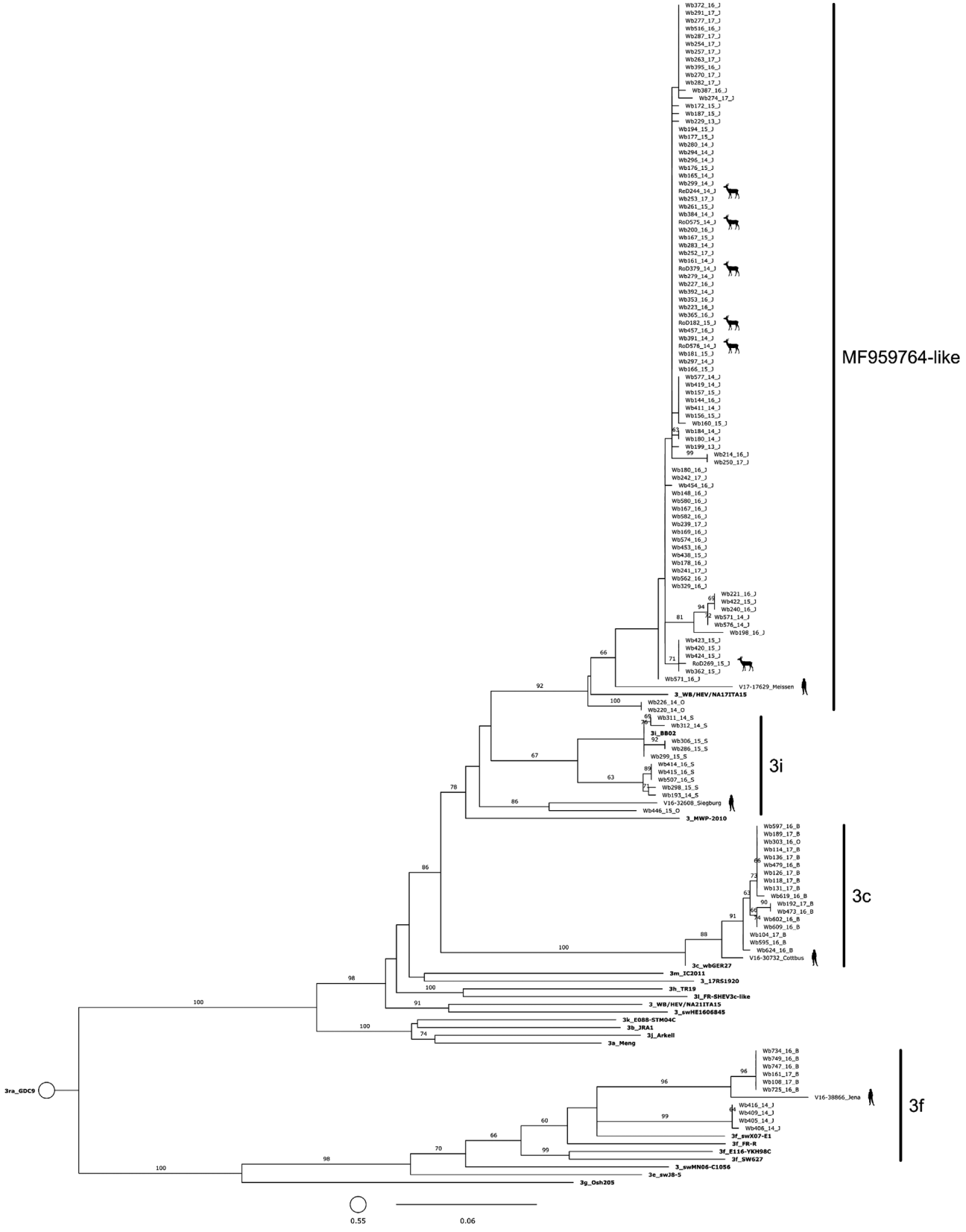

FIGURE 7 Phylogenetic relationship of detected HEV strains with subtype reference strains and with human patient strains from Germany. The phylogenetic tree is based on a 280 bp sequence of the RNA-dependent RNA polymerase region. The assigned subtypes are indicated on the right. Subtype reference strains are shown in bold face. Strains derived from deer and human are indicated with pictograms. Strains are designated with the animal species (Wb – wild boar, RoD – roe deer, ReD – red deer), animal number, year and area letter. Branches with a bootstrap >60 are labelled. The tree is scaled in nucleotide substitutions per site

awareness of physicians or the use of more sensitive diagnostic tests may also explain the increase in notified cases, since several prevalence studies on HEV-specific antibodies in the human population in Germany show decreasing trends (Faber et al., 2018; Mahrt et al., 2018; Wenzel et al., 2014). Further surveillance in animals and humans is necessary in order to clarify if there is a general increasing trend in HEV infections.

To better understand the factors influencing HEV-positive rates in wild boar as the main wild animal reservoir, uni- and multivariate analyses were performed. The sex of the animals was not significantly associated with HEV-positive rates, which is in line with other studies, although (not significantly) more HEV infections in males have previously been found (de Deus et al., 2008; Martinelli et al., 2015; Serracca et al., 2015). In contrast, our study found a significant association between HEV infection markers and age, with higher RNA detection rates in young animals and higher antibody prevalence in older animals (Figure 2). Other studies reported no or only non-significant differences between age groups of wild boar (de Deus et al., 2008; Martinelli et al., 2015; Schielke et al., 2009; Serracca et al., 2015). The inclusion of higher animal numbers and the systematic sampling approach may explain the increased significance of age in our study. Studies in domestic pigs showed a similar age-dependency, with significantly higher infection rates in young animals and higher antibody prevalences in older animals (de Deus et al., 2008; Martinelli et al., 2015; McCreary et al., 2008).

An analysis of detection rates in wild boar according to month of sampling indicated that an increasing trend of HEV-RNA positivity was found in January. This is in contrast to the findings of a study from Spain, where the highest detection rates were found in October and November, which thereafter declined (Rivero-Juarez et al., 2018). Further studies are needed to confirm potential seasonal patterns of HEV infection in wild boar and to clarify if regional differences or differences related to climate or different peaks of mating can explain the observed discrepancies between the studies.

One of the most important identified factors for HEV-positivity rates in wild boar was the hunting area. Whereas areas J and S showed high infection rates, area P had no RNA detection (although with low sample numbers) and the other areas showed low and mostly increasing infection rates over time (Figure 3b). The consistently high RNA and antibody detection rates in area J indicate a persistent virus circulation in this area, whereas areas H and B seem to represent examples of new virus introductions into the area followed by virus spread. The large ranges of previously reported detection rates in smaller studies (Anheyer-Behmenburg et al., 2017; Adlhoch et al., 2009; Weigand et al., 2018) may therefore be interpreted as snapshots of specific timepoints during more complex infection dynamics. It is worth noting that we did not observe that the virus was detected in one area and disappeared the following year. Therefore, further spread of infection into new wild animal populations can be concluded for the investigated areas and time period.

A more detailed characterization of the strains identified the circulation of four distinct subtypes. Beside subtypes 3c, 3f and 3i, which have been previously described in humans or animals in Germany (Anheyer-Behmenburg et al., 2017; Schemmerer et al., 2019; Wang et al., 2018), a putatively new subtype was identified. It was most closely related to strains from wild boar reported recently from Italy (de Sabato et al., 2020). According to Smith et al. (2020), those strains may represent a novel subtype, but more sequences from unrelated sources are necessary to confirm this classification. Here, we identified this subtype in 90 samples, which underlines its prominent role in wild animals in Germany. It has to be mentioned that in our previous study, these strains were subsumed under the designation 3ci (Anheyer-Behmenburg et al., 2017). Therefore, reanalysis of existing sequence data may generally be necessary in order to assess the distribution and origin of this putative novel subtype.

The design of our study allowed for the first time an analysis of the spatiotemporal changes in HEV subtype occurrence over a longer timeperiod in several distinct areas in parallel. Analysis of the geographic distribution of subtypes revealed a very diverse picture, with different main subtypes occurring in different hunting areas (Figure 5). In addition, in the areas J and S (representing the only areas with sufficiently available data for this type of analysis), these subtype constellations were very stable over the entire study period (Figure 6). It might therefore be concluded, that a dominant subtype had itself established in these areas, and sporadic introductions of other subtypes did not result in their further spread. The distinct reasons for these observations are not known so far. A permanent transmission of the dominant subtype through frequent contacts between individuals of the same wild boar population, but only sporadic contacts with subtypes introduced by migrating wild boar may be one. In contrast, three different subtypes were detected in three consecutive hunting seasons in area O, which might indicate three independent virus introductions into this area. However, the low sample size available from this area limits the significance of this finding. Generally, further surveillance in subsequent hunting seasons including analysis of more sequences from the areas is needed to better understand the transmission dynamics of HEV strains in wild animals.

A more detailed phylogenetic analysis of the identified HEV sequences showed a very close relationship between sequences from samples originating in the same hunting area, confirming the hypothesis of a main area-specific circulating variant. Inclusion of HEV sequences from German patients showed that a phylogenetic relationship between human pathogenic strains and strains circulating in wild animals exists. Especially for the subtypes 3c and 3f, a high degree of sequence identity between wild boar and human patient strains was evident. Unfortunately, no further information is available on the patients, so possible exposure to wildlife in the respective areas could not be further investigated. However, the close relationship between wild boar strains and human strains, which has also been described in previous studies (Schielke et al., 2009; Weigand et al., 2018), indicates a high risk of transmissibility of the strains of wild boar to humans.

In conclusion, wild animals represent a constant source of HEV, and increasing HEV detection rates in multiple areas during the study period might indicate an increasing risk of virus transmissions to

humans. Wild boar represent the main wild animal reservoir, but deer species might also be infected by spillover infections and therefore represent potential sources of infection. A strong age-dependency of wild boar infections was identified, with young animals showing the highest infection rates. High variations in infection rates of wild boar were found between the different hunting areas, including no infections in one area, several areas with recent virus introduction followed by an efficient virus spread as well as one area with continuously high infection rates. Circulating subtypes were manifold and markedly differently distributed among the certain hunting areas. Dominant subtypes were present in some distinct areas, which persisted throughout the whole study period. The identification of closely related strains in human patients highlights the zoonotic potential of the identified strains from wild animals. In summary, the spatiotemporal HEV infection dynamics in wild animal populations appears to be complex. However, taking into account that control or eradication of virus infections in wild animals is difficult, efforts should be increased to understand the specific mechanisms of HEV persistence and infection dynamics in wild animal populations. The gained knowledge may be used to elaborate concepts for better prevention of HEV transmission from wild animals to domestic animals and to humans.

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AUTHOR CONTRIBUTIONS

Conceptualization: U.S., R.J., A.B., G.K. Investigation: U.S., S.B., H.A.-B., A.B., K.S.-L., K.S. Data and analysis: A.M., M.S., J.J.W, A.B., K.S.-L., C. M.-G., U.S. Writing—original draft preparation: A.M., U.S., R.J. Writing—review and editing: M.S., J.J.W, K.S.-L, K.S., C.K., U.S., S.B., H.A.-B., A.B., C.M.-G., R.J. Funding acquisition: G.K., R.J., C.K., U.S. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted in the journal's author guidelines page, have been adhered to. Samples used in this study were those submitted to the Central Institute of the Bundeswehr Medical Service Kiel, Germany, for HEV diagnosis. Ethical approval was not required.

DATA AVAILABILITY STATEMENT

Data are available in Supplementary Data and additional data can be retrieved upon request from Ulrich Schotte (email: Ulrich-Schotte@bundeswehr.org).

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