

Prevalence and phylogenetic analysis of hepatitis E virus in pigs in Vietnam

Hu Suk Lee (✉ h.s.lee@cgiar.org)

International Livestock Research Institute <https://orcid.org/0000-0002-8731-9836>

Duy Tung Dao

National Institute of Veterinary Research

Vuong Nghia Bui

National Institute of Veterinary Research

Anh Ngoc Bui

National Institute of Veterinary Research

Duy Thanh Le

National Institute of Veterinary Research

Hung Nguyen-Viet

International Livestock Research Institute

Delia Grace Randolph

International Livestock Research Institute

Krishna K. Thakur

UPEI

Katsuro Hagiwara (✉ k-hagi@rakuno.ac.jp)

Rakuno Gakuen University

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Abstract

Background: Hepatitis E virus (HEV) is one of the important zoonotic diseases with a worldwide distribution. The main objective of this study was to assess the sero-prevalence and phylogenetic analysis of HEV in Vietnam. Pig blood and fecal pooled samples were used to determine the prevalence of HEV. We evaluated the true prevalence (TP) of HEV from apparent prevalence (AP) by taking into account the sensitivity and specificity of diagnostic tests using a Bayesian approach. For phylogenetic analysis, the data compared with worldwide HEV reference strains illustrating all eight genotypes (G1-G8) which were identified in previous study.

Results: A total of 475 sera and 250 fecal pooled samples were collected at slaughterhouses and pig farms from five provinces, in Viet Nam. Overall, the AP of HEV was 58.53% (95% CI: 53.95-62.70) while the TP was slightly higher (59.96%, 95% credible interval: 54.49-65.41). In terms of pooled samples, overall, the AP (13.38%, 95% credible interval: 9.89-17.30) of HEV was much higher than TP (6.80%, 95%CI: 4.01-10.66). One strain in Hanoi, two strains in Dak Lak, seven strains in An Giang, four strains in Son La and two strains in Nghe An were isolated. The phylogenetic tree demonstrated that 19 Vietnamese strains were clustered into HEV 3 and 4.

Conclusions: This study provided evidence that HEV is circulating in domestic pigs in Vietnam. From a public health perspective, it is very important to raise public awareness for high-risk groups (e.g. slaughterhouse workers, pig traders, farmers and market sellers) who have more opportunities to come in contact with pig and contaminated meats.

Background

Hepatitis E virus (HEV) is one of the important zoonotic diseases with a worldwide distribution, and it is commonly reported in Asia, Africa and Latin America [1]. Annually, 20 million HEV infections are reported around the world, with cases in South and Southeast Asia accounting for 60.6% or the total and deaths 64.7% [1, 2]. The virus has been classified into the *Orthohepevirus* genus within the *Hepeviridae* family [3]. Currently, at least eight genotypes of HEV have been identified [4, 5]. Five genotypes (HEV 1-4 and 7) are transmitted from human to human primarily via the fecal-oral route due to fecal contamination of drinking water [1, 6]. In general, these genotypes are commonly circulating in the areas with poor sanitation and low socio-economic status. Genotypes 3 and 4 have been considered a foodborne zoonotic disease that is commonly transmitted to humans by ingestion of raw or undercooked meat products (e.g. pig, wild boar and deer), drinking of animal milk (e.g. camels) and via direct exposure to animal feces [6-8].

Previous studies have isolated HEV 3 and 4 from pigs in the United States, the United Kingdom, France, South Korea, China and Japan [9-14]. Phylogenetic study provided evidence that the swine-origin HEV and human HEV isolates had a close association, suggesting that pigs play an important role in the transmission of the virus to humans [15]. In Southeast Asia, some studies revealed the transmission of HEV 3 and 4 from animals to humans providing evidence of circulating HEV in pigs [16-18].

In Vietnam, the first HEV outbreak in humans was reported in 1994 along the river bordering Cambodia in the southwestern part [19]. One study showed that HEV is circulating in pigs (prevalence: 19.1% in fecal samples and 8.2% in rectal samples) while humans showed 31.7% seroprevalence [20]. In addition, a recent study in Vietnam found that pig farmers, slaughterhouse workers and pork meat retailers had high seroprevalence compared to unexposed groups [21]. Also, it showed that HEV was detected from liver tissues in pigs.

To our knowledge, no studies have been conducted to evaluate the true prevalence (TP) of HEV in pigs in Vietnam. TP is the proportion of animals which have a disease in the population while apparent prevalence (AP) is the proportion of animals testing positive by a diagnostic test. If sensitivity and specificity of diagnostic test are less than 100%, estimated prevalence is biased. We therefore used a Bayesian analysis to estimate the TP from AP. The main objective of this study was to assess the TP and phylogenetic analysis of HEV in pigs from 5 provinces in Vietnam.

Results

Estimated true prevalence of HEV

A total of 475 sera samples were collected at slaughterhouses from five provinces. Overall, the AP of HEV was 58.53% (95% CI: 53.95-62.70) while the TP was slightly higher (59.96%, 95% credible interval: 54.49-65.41) (Table 1). Dak Lak had the highest prevalence followed by Nghe An and Son La whereas An Giang and Hanoi showed the lowest prevalence. Overall, the TPs were slightly higher than the APs except for An Giang and Hanoi provinces. Brooks-Gelman-Rubin (BGR) plots displayed that all chains converged for TPs (S1 Fig). A total of 250 pooled samples were collected at farm level. Overall, the TP was estimated at 13.38% (95% credible interval: 9.89-17.30) which was much higher than AP (6.8%, 95% CI: 4.01-10.66%) (Table 2). An Giang had the highest prevalence followed by Dak Lak while Hanoi showed the lowest prevalence. There were no statistically significant differences in prevalence between female (79.10%, 95% CI: 73.74-83.81) and male (79.23, 95% CI: 73.06-84.54) pigs.

Phylogenetics analysis of HEV

The 348bp HEV ORF2 nucleotide sequence was successfully obtained from fecal samples. A total of 19 Vietnamese strains were isolated in samples, including 1 strain in Hanoi, 5 strains in Dak Lak, 7 strains in An Giang, 4 strains in Son La and 2 strains in Nghe An (Fig. 2). All 19 nucleotide sequences of HEV ORF2 have been deposited on GenBank (accession number MT670024–MT670042). The phylogenetic tree demonstrated that 19 Vietnamese strains were clustered into HEV 3 and 4 among 8 genotypes. Genotype 3 is dominant including 17 strains divided into two sub-genotypes 3 (15 strains belonged to sub-genotype 3a and 2 strains clustered into sub-genotype 3d). Two remaining strains were identified from Son La province were clustered into HEV 4, sub-genotype 4b. These strains showed a close relationship with the strain isolated from a monkey in China.

Based on the host phylogenetic tree, it is assumed that HEVs are human in origin (Fig. 3). Humans are the main reservoir that spread the virus to other animals including swine, wild boar, goat, and camel. However, humans can also be infected with HEVs from swine and camel. Swine play an important role in transmitting the HEVs to mongoose, monkey, wild boar and in contaminating the environment (seawater). In addition, the host phylogenetic tree indicated the HEVs can be transmitted from goats to sheep and from wild boar to deer.

Discussion

A large national study was implemented to evaluate the prevalence and molecular characteristics of HEV in pigs across Viet Nam. Overall, our anti-HEV IgG prevalence (approximately 58%) was very similar to other studies in Germany (47%), Italy (50.2%), Japan (56%), Philippines (50.3%) and Laos (51.2%) [22–26]. Some studies showed a higher seroprevalence of IgG as pigs grow older [27, 28]. However, we were not able to evaluate this because sera samples were obtained from fattening pigs (6-8 months old and weighing 60-120 kg) at slaughterhouses. Interestingly, TPs of three provinces (Dak Lak, Nghe An and Son La) was estimated more than 80%, suggesting a high potential zoonotic transmission to humans. Overall, the RNA-positive rate (6.8%) in pooled fecal samples was very similar to other studies in Laos (11.5%), Philippines (7.4%) and Thailand (1.27-2.9%) [18, 22, 29, 30].

Previous studies have provided evidence that there was an epidemiological association between consumption of pork meat and HEV cases [31, 32]. In Vietnam, most of the /farmers in rural areas raise pigs in backyards with poor sanitary conditions. They have more opportunities to come in contact with pigs and pig waste. In addition, it is very common that local people consume uncooked pork, livers meats, sausages, unwashed vegetables and insufficiently treated drinking water. Therefore, it might be possible that people who are exposed to pig and pork meats are at an increased risk of HEV transmission [33]. Further studies are needed to evaluate whether this exposure route poses a public health concern as well as to better understand the human behavior for preventing HEV infections.

The Bayesian maximum clade credibility (MCC) host discrete traits tree provided information about the transmission of HEVs between humans, swine and other animals. Humans are the main reservoir/ maintenance host of HEV but pigs are a well-known as a reservoir of HEV that transmit the virus to humans. In addition, the host phylogenetic tree indicated that HEV can be transmitted from camel to humans [8]. This tree showed that HEV genotypes 3 and 4 are considered zoonotic viruses, and the new HEV genotype 7 can be transmitted from camel to humans. Therefore, HEVs are now recognized as an emerging zoonotic agent.

We found that both genotypes HEV 3 and 4 were detected in domestic pigs across the country which was in line with other Asian countries [34–36]. Most of them (15/19) were classified into the existing genotype 3 which is the commonly detected subtype in Asia (including Japan, South Korea and Philippines) [22, 37, 38]. The two remaining Vietnamese HEV strains that were isolated in An Giang, were clustered into sub-genotype 3d with Cambodia and Taiwan strains. The HEV 4 (sub-genotype 4b) was only detected in Son La province which is on the borders with Laos [18, 34, 39]. Therefore, it might provide evidence that this type is circulating in these areas. In Son La province, the percentage of minorities (e.g. Thai and H'Mong) is relatively higher than areas in Vietnam (the majority ethnic group is Kinh, accounting for 85.7% of the population) [40]. In general, ethnic minorities have lower education levels and incomes than the Kinh group, which has led to limited awareness of hygiene and food safety in Vietnam [41]. In addition, other studies have suggested that people with high education had a better perception of food safety in comparison with those with low education [42, 43]. Ethnic minorities may benefit from efforts to enhance public awareness of food safety and disease prevention. Further epidemiological study needs to be conducted to establish a transmission link between pigs and humans in Son La province. Also, one recent study showed that the HEV seroprevalence was higher among individuals occupationally exposed to pig and pork products [21]. Therefore, the human health impact of HEV should be properly defined to establish appropriate interventions.

The main limitation of our study was that our blood and fecal samples may not be representative because blood samples were only collected at slaughterhouses and were disproportionately collected depending on the number of pigs at each farm. Large and medium scale farmers were not willing to participate (mainly for biosecurity reasons) in our study. Although, our pig samples were not representative of the general population, we think that our findings provide valuable information on the epidemiology of HEV in Vietnam. It could be possible that the TP of HEV may be under/overestimated due to a random error with our measuring facilities. In addition, our prior estimates for the sensitivity and specificity of the ELISA test were obtained from our experiment and therefore, we are not clear which estimates are suitable for the Vietnamese

context. We conducted some simulations to explore what happens with N=80, 70, 60, etc. out of 95 samples. There was a large discrepancy between the AP and TP as the positive cases decreased. The main reason was that when data (likelihood) is weak, posterior estimates are strongly influenced by priors [44].

A Bayesian method provides a chance to integrate prior information with observed data, which is useful for estimating values for both prevalence and diagnostic characteristics of tests [45]. If a diagnostic test has less than 100% sensitivity and specificity, the estimated prevalence would be biased. Therefore, a Bayesian analysis was utilized to estimate the TP from AP. Overall, Bayesian models are very sensitive depending on the priors, so we used a Jeffreys prior to minimize the impact of prior to the posterior distribution as there was no prior information for HEV prevalence in the study sites. This study was the first attempt to estimate the TP for HEV in Vietnam and demonstrates how a Bayesian approach can be utilized to better estimate the prevalence of diseases.

Conclusion

This study provided evidence that HEV is circulating in domestic pigs in Vietnam. Further study is necessary to evaluate the possible transmission from pigs and environmental risk factors to humans. From a public health perspective, it is very important to raise public awareness for high-risk groups (e.g. slaughterhouse workers, pig traders, farmers and market sellers) who may have more opportunities to come in contact with pig and contaminated meats. Therefore, a national surveillance system and practical guidelines for proper handling of meat products needs to be established for disease control and prevention.

Methods

Study locations and sampling

Pig blood samples from previous cross-sectional survey [46] were used to estimate the sero-prevalence of HEV at the National Institute of Veterinary Research (NIVR) in Hanoi. Sera samples from fattening pigs (6-8 months old and weighing 60-120 kg) were randomly collected from the jugular vein at slaughterhouses in five provinces (An Giang, Dak Lak, Hanoi, Nghe An, Son La) (Fig. 1). For our study, 95 sera samples were randomly selected from each province (385 samples / province). Fecal pooled samples were collected in 25 farms/province from 2018 to 2019. Two fecal pooled samples of a minimum of 2g were collected in a sterile plastic container at each farm and were kept at -20 °C until transportation. This study was approved by the Hanoi Medical University Institutional Review Board (IRB: no. 00003121), Vietnam

Laboratory analysis

Serum examination: Anti-HEV antibodies were detected by enzyme-linked immunosorbent assay (ELISA) using anti-Pig IgG antibody–HRP

(Bethyl Laboratories Inc. TX. USA) conjugate as a secondary antibody. The Index value $\frac{\text{Sample}-\text{negative control}}{\text{Positive}-\text{negative control}} \times 100$ was calculated from the optical density (OD) value obtained by ELISA. The cut off value was determined at 0.295. As a result, the sensitivity and specificity for the test were 0.909 and 0.9 respectively.

RNA extraction and detection of HEV by RT-PCR: A portion of fecal samples was suspended with PBS and centrifuged at 5000 rpm for 5 min, and 250 ul of the supernatant was used for RNA extraction. The RNA was extracted using the RNA purification kits via the Direct-zol™ (Zymo Research, CA. USA) method. One-step RT-PCR using HEV ORF2 as a target region was performed using the extracted RNA as a template. The QIAGEN One-Step RT-PCR Kit (QIAGEN) was used for RT-PCR master mix using 5 µl of RNA as a template, and 10 µM each primers Her F1: 5'-AATTATGCYCATCGRTTG-3' and Her R1: 5'-CCCTTRTCYTGCTGMGCATTCTC-3' [9]. The reaction conditions were reverse transcription at 50 °C for 30 minutes, and then at 95 °C for 15 minutes. As a PCR cycle, denaturation was performed at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute for 40 cycles. Next, nested-PCR was performed using the product obtained by RT-PCR as a template. A total of 10 µM primers (Her F2: 5'-GTWATGCTYTCATWCATGGCT-3' and Her R2: 5'-AGCCGACGAAATCAATTCTGTC-3') and TaKaRa ExTaq (Takara Bio Inc, Japan) were used. The reaction conditions were: preheating at 95 °C for 2 minutes, 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds as one amplification cycle, followed by extension at 72 °C for 5 minutes. The product of nested-PCR was electrophoresed on a 1.5% agarose gel, and an amplified band of HEV-RNA was detected. The expected amplified band (348bp) was sequenced after purification.

DNA sequencing: Two PCR products from each DNA sample were sequenced in both directions using the same nested PCR primers (Her F2, Her R2) by ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosciences, Foster City, CA, USA). The sequences were analyzed by 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). The consensus sequences were generated from two bidirectional repeats of each sample by software GENETYX Ver.13.0.3 (GENETYX Corp., Tokyo, Japan).

Data analysis

We evaluated the true prevalence (TP) of HEV from apparent prevalence (AP) by taking into account the sensitivity and specificity of diagnostic tests using a Bayesian approach [47]. For sera samples, the prior distribution for sensitivity [fixed (0.909)] and specificity [fixed (0.9)] were estimated from the ELISA experiments. For the TP, a non-informative Jeffreys prior [$\text{beta} = (0.5, 0.5)$] was used to minimize the influence on the posterior [48]. For fecal pooled samples, the prior distribution for sensitivity [fixed (0.95)] and specificity [fixed (100%)] were estimated from the previous research [9] experiments. Markov Chain Monte Carlo (MCMC) sampling was implemented by JAGS through the rjags package using the in "truePrev" function in the package prevalence in R [49, 50]. The first 1,000 samples of the three MCMC chains were discarded as a burn-in period and the following 10,000 iterations were used for posterior inference. The AP was calculated with a 95% Clopper-Pearson/Exact confidence interval (CI). The TP was estimated based on the posterior median value with a 95% credible interval. The statistical significance of the differences between prevalence estimates was assessed by examining the overlap of the respective credible intervals. The outputs from the three chains were visually evaluated using MCMC trace-plots, posterior density distribution plots, Brooks-Gelman-Rubin (BGR) plots, and auto-correlation plots using the CODA package [51]. All data were imported into Microsoft Excel 2016 and analyzed using R (version 3.6.2). QGIS (Quantum GIS Development Team 2018. QGIS version number 3.8.3) was used to create the map.

Phylogenetic analysis

The 348bp HEV ORF2 nucleotide sequences obtained in this study were aligned using CLUSTALW multiple alignments in BioEdit version 7.2.5. The data compared with worldwide HEV reference strains including all eight genotypes (G1-G8) which were identified in previous study [52]. In addition, phylogenetic analyses were conducted using the Maximum Likelihood (ML) method by MEGA X software [53] with parameter settings of 1,000 bootstrap replicates, and the best fit model GTR + G+I.

The Bayesian maximum clade credibility (MCC) host discrete traits tree was constructed by using software BEAST v1.8.4 (<http://tree.bio.ed.ac.uk/software/beast/>). The strict clock and the best fit GTR +G+I nucleotide substitution model with a constant population size coalescent tree prior were used. The MCMC was run at 50,000,000 generations and sampled at every 5,000 generations. The effective sample size (ESSs) of the analysis was checked by software Tracer v1.6 (<http://tree.bio.ed.ac.uk/software/tracer/>). The MMC host discrete traits output tree was generated by using TreeAnnotator v1.10.4 (<http://tree.bio.ed.ac.uk/software/beast/>) afterburn 10% of the first trees. The host phylogenetic tree was reconstructed by software FigTree v1.4.3 (<http://tree.bio.ed.ac.uk/>).

Abbreviations

HEV: Hepatitis E virus; NIVR: National institute of veterinary research; ELISA: Enzyme-linked immunosorbent assay; OD: Optical density; RT-PCR: Real time – polymerase chain reaction; TP: true prevalence; AP: apparent prevalence; MCMC: Markov Chain Monte Carlo; BGR: Brooks-Gelman-Rubin; MCC: maximum clade credibility; ESS: effective sample size

Declarations

Ethical approval and consent to participate

This study was approved by the Institutional Review Board (IRB) of Hanoi University of Public Health (HUPH), Vietnam (Reference Number IRB/018-186/DD-YTCC. Verbal informed consent was obtained from all owners to collect samples from the pigs. The IRB approved verbal informed consent as most of the farmers were either illiterate or semi-literate. This consent form was dated and signed by the researchers indicating that "I have read and explained this consent form to the participant before receiving the participant's consent, and the participant had knowledge of its contents and appeared to understand it".

Consent for publication

Not applicable.

Availability of data and materials

All datasets supporting our findings are available from the corresponding author on reasonable request.

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Authors' contributions

Conceived and designed the experiments: HSL, HNV, and KH. Performed the experiments: HSL, DTD, VBN, ANB, DTL, and KH. Analyzed the data: HSL, DTD, KKT, and KH. Wrote the paper: HSL, DTD, SDX, HNV, DG, KKT, and KH. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests

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Tables

Table 1. Apparent prevalence with 95% CI^a and true prevalence with 95% credible interval for hepatitis E virus in sera samples in pig slaughterhouses

Province (no.)	Positive samples	AP (%) with 95% CI ^a	TP (%) ^b with 95% credible interval
An Giang (95)	20	21.05 (13.36-30.62)	13.49 (3.86-24.27)
Dak Lak (95)	86	90.53 (82.78-95.58)	97.73 (91.19-99.99)
Hanoi (95)	26	27.37 (18.72-37.48)	21.53 (11.18-32.88)
Nghe An (95)	75	78.95 (69.38-86.64)	85.26 (74.51-94.76)
Son La (95)	71	74.74 (64.78-83.09)	80.22 (68.73-90.19)
Total (475)	278	58.53 (53.95-62.70)	59.96 (54.49-65.41)

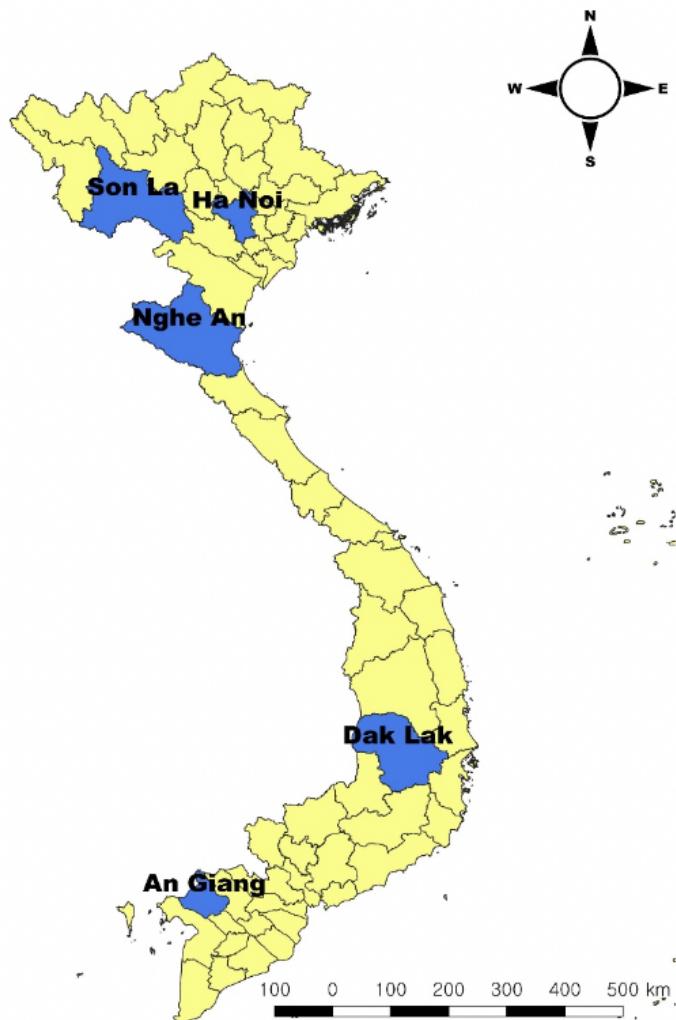
^a CI: confidence interval; ^b Median value was recorded

Table 2. Apparent prevalence with 95% CI^a and true prevalence with 95% credible interval for hepatitis E virus in fecal pooled samples in pig farms

Province (no.)	Positive pooled samples	AP (%) with 95% CI ^a	TP (%) ^a with 95% credible interval
An Giang (50)	6	12.00 (4.53-24.31)	19.89 (11.31-30.17)
Dak Lak (50)	3	6.00 (1.25-16.55)	12.76 (5.80-21.71)
Hanoi (50)	1	2.00 (0.05-10.65)	7.05.41 (1.56-15.19)
Nghe An (50)	3	6.00 (1.25-16.55)	12.76 (5.80-21.71)
Son La (50)	4	8.00 (2.22-19.23)	15.24 (7.65-24.66)
Total (250)	17	6.80 (4.01-10.66)	13.38 (9.89-17.30)

^a CI: confidence interval; ^b Median value was recorded

Figures



*This map was created by our own

Figure 1

Five sampling provinces for HEV in pigs in slaughterhouses and farms

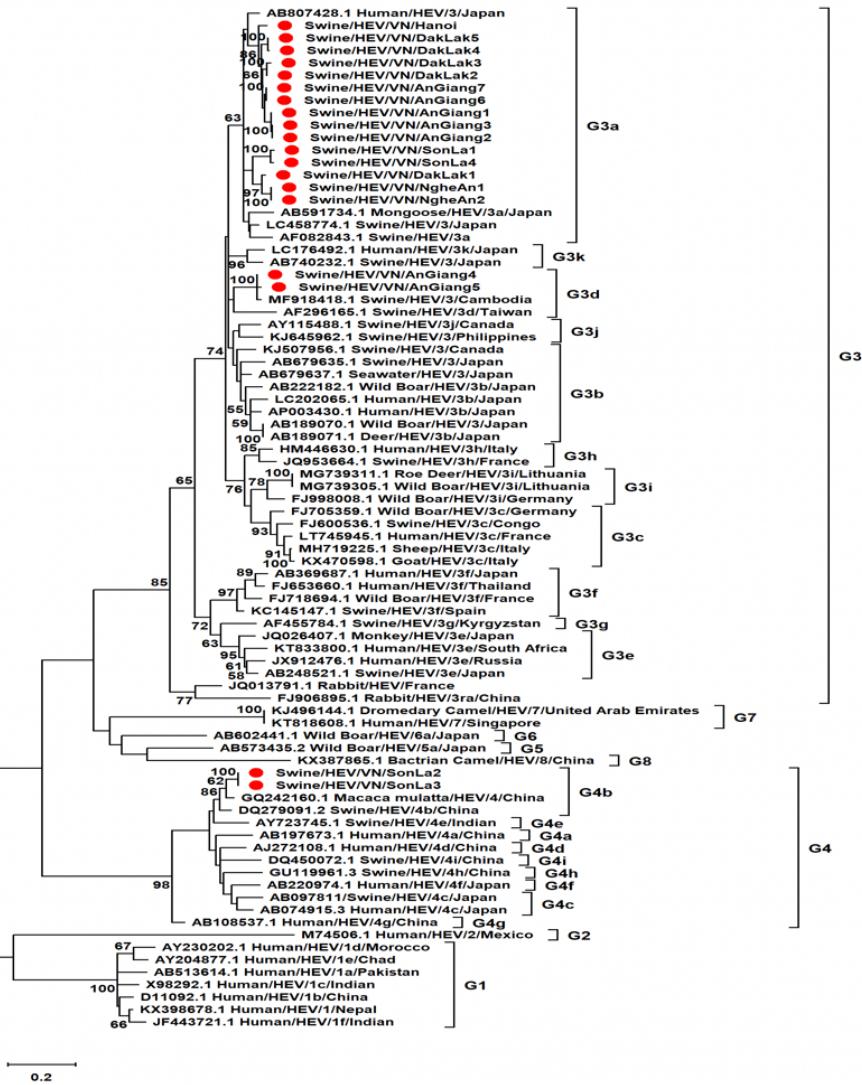


Figure 2

Maximum-likelihood tree constructed for 348bp HEV ORF2 nucleotide sequences (nt position 6022-6369) of 19 Vietnamese strains (indicated by red dot), and reference strains obtained from the GenBank database. Genotype G1-G8 are indicated on the right of the figure. The bar at the bottom of the figure denotes evolutionary distance.

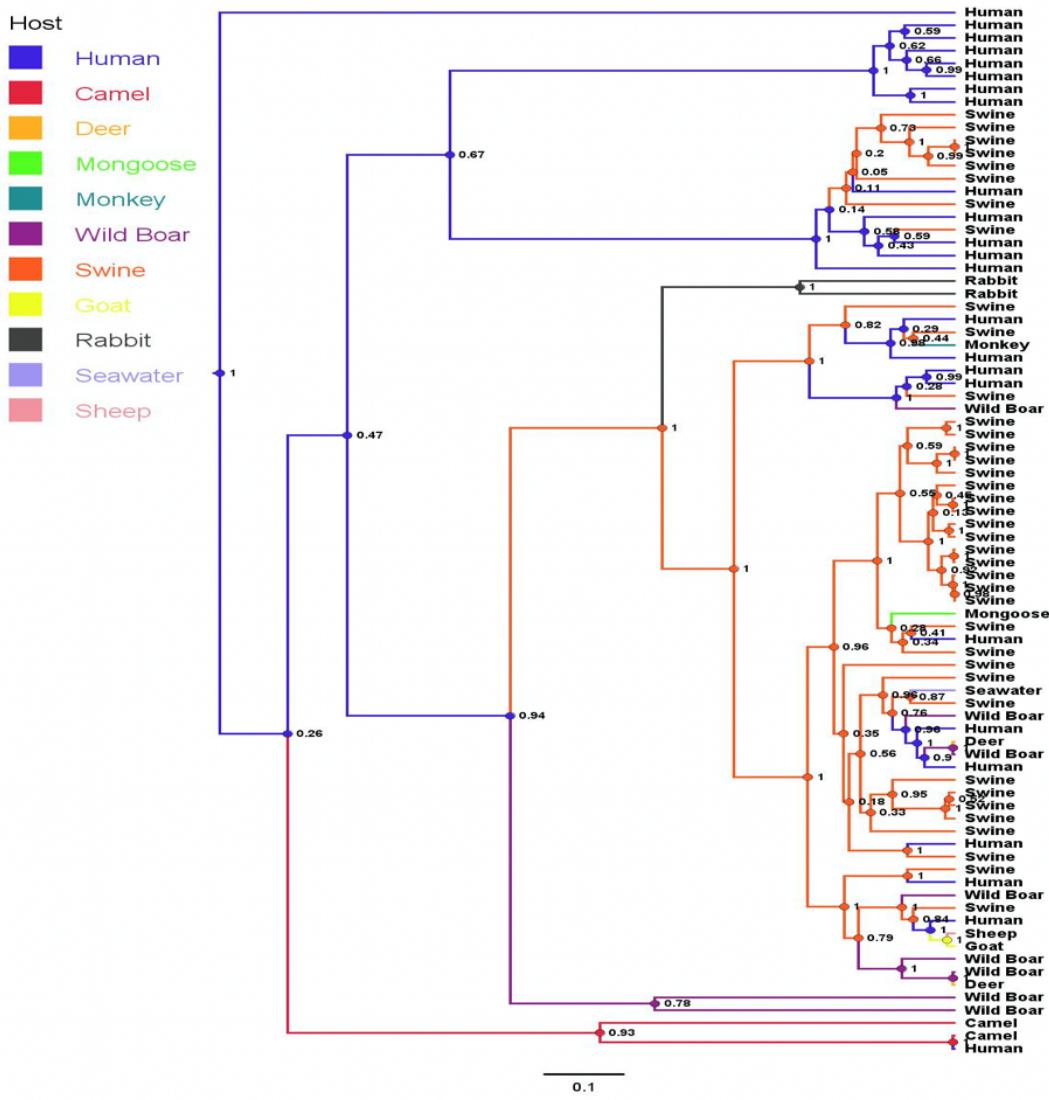


Figure 3

The Bayesian maximum clade credibility (MCC) host discrete traits tree for 348bp HEV ORF2 nucleotide sequences (nt position 6022-6369) of 19 Vietnamese strains, and reference strains obtained from the GenBank database. The phylogenetic host tree indicated the transmission between HEV hosts, the host at node indicated the ancestor of sub-group and the number at the node indicated the posterior probability. The bar at the bottom of the figure denotes evolutionary distance.

Supplementary Files

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