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Evidence of increased Hepatitis E virus exposure in Lao villagers with contact to ruminants

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Abstract

Although pigs are the main reservoir, ruminants have also been shown to be susceptible to hepatitis E virus (HEV). We investigated zoonotic transmission of HEV in rural settings of Lao People's Democratic Republic (Lao PDR) where humans are in close contacts with ruminants and where pigs are rare. Villagers with (n = 171, risk group) and without (n = 155, control group) cattle were recruited in seven villages in Vientiane Capital. Owners of pigs were excluded. Blood, as well as information on socio-demographics, animal contact, dietary habits and awareness of zoonoses were collected to assess risk factors. Blood and rectal swabs were collected from cattle (n = 173) and other ruminants (27 goat, 5 buffaloes) to measure anti-HEV antibody and virus prevalence. A similar anti-HEV antibody seroprevalence was found in cattle (6.8%) and other ruminants (8%). HEV RNA was detected in none of the animal rectal swabs and human sera. Anti-HEV IgG seroprevalence was higher in cattle farmers than in the control group (59.1% vs. 43.9%, p = 0.008) and increased significantly with age. Other risk factors included male gender, close contact with cattle and consumption of undercooked meat. We find that HEV is highly endemic in rural Laos and provide first evidence that HEV circulates in free-roaming ruminants with open access to village water sources. Despite some awareness about hygiene, villagers are likely constantly exposed to zoonotic diseases by dietary and lifestyle habits. Cattle farmers had a higher risk of HEV infection than other villagers. Our study highlights the need to raise the awareness of the rural population about water- and food-borne pathogens, and about the role of cattle as a possible source of infection. The knowledge gained on local risk factors and husbandry conditions should guide future awareness raising campaigns and promote appropriate hygienic measures including handwashing and the consumption of safe food and water.

KEYWORDS
cattle, developing countries, Laos, risk analysis, zoonosis

1 | INTRODUCTION

Hepatitis E virus (HEV) infections in humans are mostly mild or asymptomatic, but progression to fulminant or chronic hepatitis occurs in risk groups such as pregnant women, immunocompromised patients and patients with chronic liver disease (Hamid et al., 2002; Kamar, Rostaing, & Izopet, 2013; Patra, Kumar, Trivedi, Puri, & Sarin, 2007). Phylogenetic analyses of complete virus genome sequences identified the four major genotypes that affect humans (Doceul, Bagdassarian, Demange, & Pavio, 2016; Lu, Li, & Hagedorn, 2006; Sridhar, Teng, Chiu, Lau, & Woo, 2017). Differences in geographic distribution and host range between the four HEV genotypes explain their distinct epidemiological characteristics: while genotypes 1 and 2 are restricted to humans and circulate mainly in Asia and Africa, genotypes 3 and 4 have been detected in various mammalian species worldwide. Mainly in developing countries, domestic exposure to fecally contaminated water has been incriminated in HEV outbreaks and large waterborne epidemics in Asia were caused by genotype 1 (Shrestha et al., 2015). In contrast, sporadic cases in both developed and developing countries are mostly associated with contact to animals infected with genotypes 3 and 4. Other sources of infection are contaminated animal products, such as raw meat (Meng, 2013) and possibly milk (Baechlein & Becher, 2017; Drobeniuc et al., 2001; Huang et al., 2016). Pigs are considered the main reservoir of zoonotic HEV (Meng, 2016), but several independent studies provided recent serological and molecular evidence of HEV circulation in cattle and goats (Arankalle et al., 2001; Di Martino et al., 2016; Dong et al., 2011; El-Tras, Tayel, & El-Kady, 2013; Fu et al., 2010; Geng et al., 2011, 2010; Huang et al., 2016; Long et al., 2017; Sanford et al., 2013; Xu et al., 2014; Yan et al., 2016; Zhang et al., 2008). So far, all isolates from cattle clustered with genotype 4 strains, while both genotypes 3 and 4 strains were found in goats (Di Martino et al., 2016; Hu & Ma, 2010; Huang et al., 2016; Long et al., 2017; Xu et al., 2014; Yan et al., 2016). In Lao PDR, HEV strains circulating in the population have not been characterized, despite confirmed endemicity of HEV in humans and pigs (Blacksell et al., 2007; Conlan et al., 2011, 2012; Holt et al., 2016; Syhavong et al., 2010). There is only one report of a HEV genotype 4 closely related to human and porcine strains from other Asian countries and isolated from Lao pigs (Conlan et al., 2011). The role of other host species (e.g., small and large ruminants) in HEV epidemiology remains unclear. As a consequence of the rising demand for meat in Asia (Clonan, Roberts, & Holdsworth, 2016), the number of cattle in Lao PDR increased by 2/3 within the last decade (Steering Committee for the Agricultural Census Agricultural Census Office, 2012), and this land-locked country has become a hub for cattle trade in the Greater Mekong Subregion (Smith et al., 2015). Similarly, goat production more than doubled in the country since 1999 (Steering Committee for the Agricultural Census Agricultural Census Office, 2012). In light of the above reports of HEV circulation among ruminants, we determined the seroprevalence of HEV in different ruminant species and investigated the role of cattle in zoonotic transmission in rural Lao PDR. Among other risk factors, we found that farmers who are exposed to cattle are at a higher risk of contracting HEV than villagers without such contacts.

Impacts

- In rural Lao People's Democratic Republic, animal and human habitats largely overlap. This facilitates the transmission of pathogens of animals, such as Hepatitis E virus (HEV). HEV may cause fulminant and chronic hepatitis in risk groups.
- We find that HEV is highly prevalent in humans and ruminants. Risk factors for HEV infection include contact to cattle, consumption of raw meat and male gender.
- Raising knowledge and awareness for basic biosafety measures (e.g., handwashing with soap after contact with animals, consumption of safe food and water) at a village level is required to prevent animal-human transmission of pathogens.

2 | MATERIALS AND METHODS

2.1 | Study design and sampling

Seven villages with predominant cattle and goat farming were selected in Xaythany district, Vientiane Capital (Figure 1). In 2015, 186 serum samples and 185 rectal swabs were collected from ruminants: 173 cattle, 27 goats and 5 buffaloes.

In addition, 326 healthy villagers (mean age: 48.0 years; age range: 18–85 years) were recruited from the same seven villages in 2016. Although it was initially planned to recruit farmers from whom animal data was available, farmer reluctance and changes in livestock ownership within the two years of sample collection unfortunately complicated the sampling. Nevertheless, 16% of human and animal samples could be matched. In Northern Lao PDR, smallholders of ethnic minority groups play an important role in pig production, whereas in the central provinces, a successful transition from smallholder to medium-sized farms has taken place (Steering Committee for the Agricultural Census Agricultural Census Office, 2012). Consequently, only few farm households keep pigs in the participating villages and these were excluded from the study to reduce the confounding effect of pigs as source of zoonotic HEV. The enrolled villagers were assigned to either the risk (n = 171, mean age: 49.4 years; age range: 18–85 years) or the control group (n = 155, mean age: 45.4 years; age range: 18–84 years) depending on whether they owned ruminants or not. Information on socio-demographics, animal contact, dietary habits and awareness of zoonotic diseases were obtained by questionnaire.

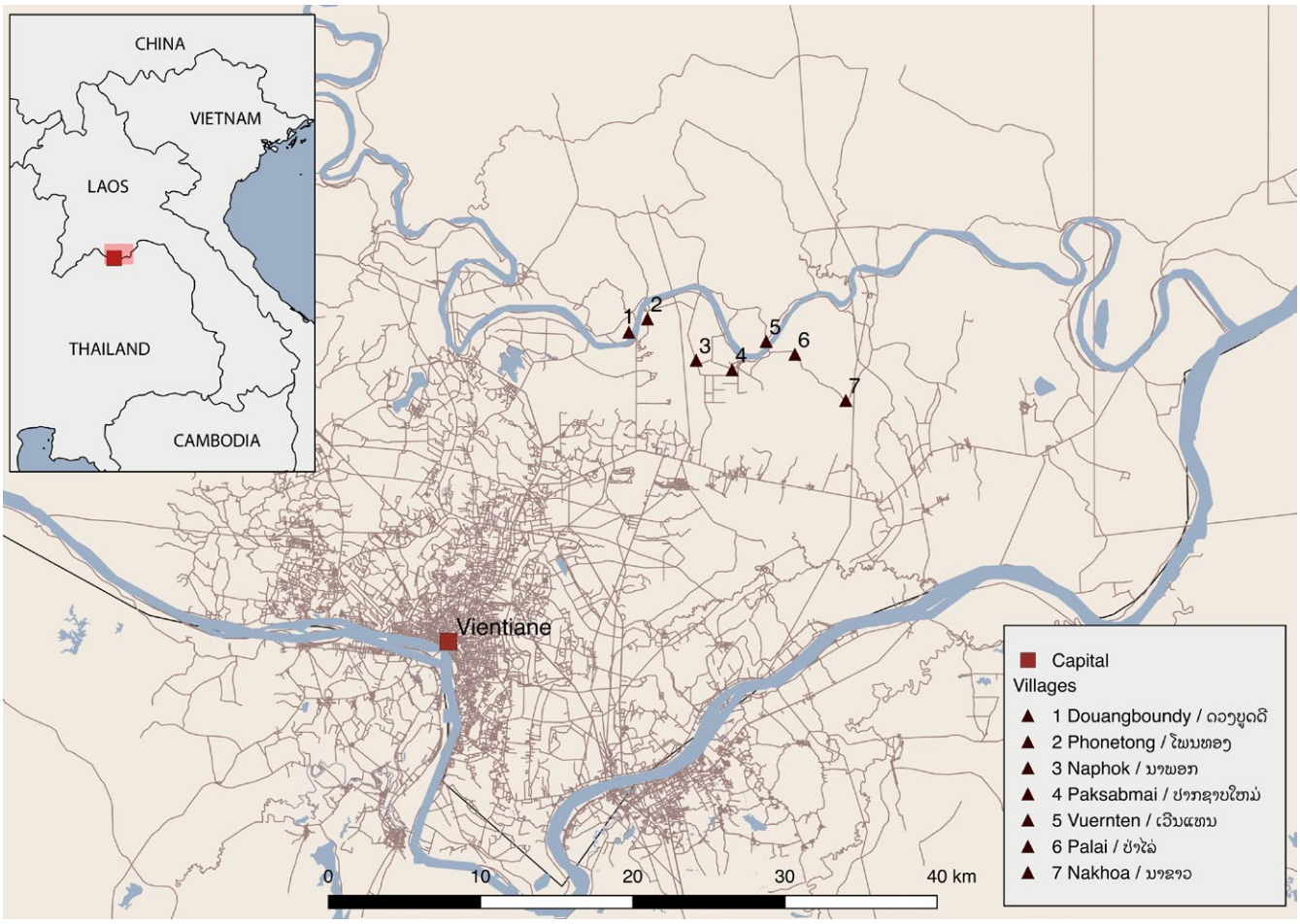

FIGURE 1 Map of Study region. The map was created with QGIS (QGIS Development Team, 2017) using collected GPS-data and OpenStreetMap data (OpenStreetMap contributors, 2017). Projection used: EPSG:3857 – WGS 84/Pseudo-Mercator

Rectal swabs from the ruminants were directly transferred into tubes containing 500 µl of viral transport medium (Medium 199 with 200 U/ml penicillin, 200 mg/ml streptomycin, 2.5 µg/ml fungizone, 1,800 U/ml penicillin G, 2,000 U/ml polymyxin B, 250 µg/ml gentamycin, 60 µg/ml ofloxacin HCL, 200 µg/ml sulfamethoxazole, 0.5% BSA). Blood and personal data were collected from volunteers in accordance with the Declaration of Helsinki. Prior to blood collection, written informed consent was obtained and a unique identifier code assigned to each participant. The study was approved by the local public and animal health stakeholders and ethical approval for the study was granted by the Lao National Ethics Committee (No 012/2016 NIOPH/NECHR). The samples were conserved at +4°C upon collection and during transportation to the Institut Pasteur du Laos, where the samples were finally stored at −80°C.

2.2 | Laboratory testing

Human sera were tested for serological markers of recent or past HEV infection using two commercial ELISA assays (abia HEV IgM and abia HEV IgG, AB Diagnostics, Berlin, Germany). Animal sera were tested with the HEV ELISA 4.0v kit (MP Biomedicals, Eschwege, Germany) allowing the simultaneous detection of specific IgA, IgM and IgG.

To detect also early stages of HEV infection, all human sera and animal rectal swabs were screened for HEV RNA. At least in pigs, the duration of HEV shedding in feces is expected to last longer than HEV viremia (Kasorndorkbua et al., 2004). Thus, rectal swabs—and not sera—were screened by PCR. In humans, both, viraemia and IgM detection are markers for an acute infection (Aggarwal, Kini, Sofat, Naik, & Krawczynski, 2000; Huang et al., 2010; Kamar, Dalton, Abravanel, & Izopet, 2014), justifying the screening of the human sera by PCR and IgM ELISA. RNA was extracted from human sera using the NucleoSpin Virus kit (Macherey-Nagel, Duren, Germany) and from the rectal swabs of the ruminants using the QIAmp viral RNA Minikit (Qiagen, Venlo, The Netherlands), following the manufacturer's instructions. Detection of HEV RNA was performed by a real-time PCR targeting the ORF3 gene of all four HEV genotypes (Jothikumar, Cromeans, Robertson, Meng, & Hill, 2006) with probe modifications (Garson et al., 2012). The Quantitect Probe kit (Qiagen, Venlo, The Netherlands), which contains a ready-to-use mastermix, was used. The final volume was of 25 µl, containing 2 µl of RNA and primers and probe at concentrations of 600 nM and 150 nM, respectively.

2.3 | Data analysis

Descriptive and inferential statistics were performed in R studio (R Core Team, 2016) using the "stats" package. Chi-square test and Fisher's exact test were applied to assess which sociodemographic (e.g., age, gender, level of education), work-related (e.g., ownership of cattle, close contact to cattle) and domestic (e.g., consumption of raw or undercooked meat, drinking of unsafe water) factors increase the odds of HEV infection. The epitab function of the R package epitools (Aragon, 2012) was used to estimate odds ratios (OR) and confidence intervals (95% CI) of the chi square statistics.

Logistic regression with binomial response variable was performed in order to examine which factors including age, gender, cattle ownership and dietary habits affect anti-HEV IgG seropositivity. Predictors, that affected the response variables as revealed by bivariate analyses (significance level less than 0.2), were included in the binomial generalized linear models (GLMs). The best fitted model was selected using the function *stepAIC* of the MASS package (Venables & Ripley, 2002). The GLMs were assessed with binomial error structure and logit link function (Hosmer & Lemeshow, 2000), and the GLMs were fitted using the function *glm* of the R-package "lme4" (Bates, Maechler, Bolker, & Walker, 2015). The best model was selected by comparing the Akaike Information Criterion (AIC) weights for a set of fitted models. The model was tested for overdispersion as well as for multicollinearity by deriving the Variance Inflation Factor using the function vif of the R-package car (Fox & Weisberg, 2011). Non-significant interaction terms with a Variance Inflation Factor above 5 were excluded from the model. The significance of the full model was assessed by likelihood ratio test: the deviance of the full model with that of the null model was compared using the function ANOVA with the argument test set to "Chisq." The same function was applied to obtain the contributing effect of each predictor included in the model. The predictive ability of the model was assessed by calculating the area under the curve (AUC) and predicted probabilities. Finally, 95% confidence intervals were computed for the categorical predictors. Plots and figures were constructed using the packages "ggplot2" (Hadley & Wickham, 2009) and "forestplot" (Gordon & Lumley, 2017).

3 | RESULTS

3.1 | Descriptive statistics of the human and animal cohorts

In total, 326 individuals were recruited in seven villages to explore the risk of HEV infection associated with direct or indirect contact with cattle (Figure 2, Table 1). Although all participants received the same questionnaire, not all questions were answered by every participant.

Two thirds of the participants let their animals roam freely throughout the village (64.8%; 206/318) and many animals had access to river water (36.9%; 117/317; Table 2). Most participants reported to drink safe water (i.e., bottled commercial drinking water or treated water; 89.3%; 291/326), but many reported to consume unsafe food such as undercooked meat (60.8%; 197/324), raw blood (53.1%; 172/324) or inner organs (98.5%; 319/323; Table 1). The risk (reported ruminant ownership) and the control (no reported ruminant ownership) groups did not differ significantly with regard to their dietary habits (Table 1). Cattle ownership was most common among the elderly (glm; z-value: 2.3; $p = 0.023$; Table 1) who were also significantly less likely to consume raw blood than younger villagers (glm; z-value: -4.1; $p < 0.001$). No age-related differences were revealed for other presumed risk behaviours for HEV infection. Men were significantly more likely than women to own cattle (OR:

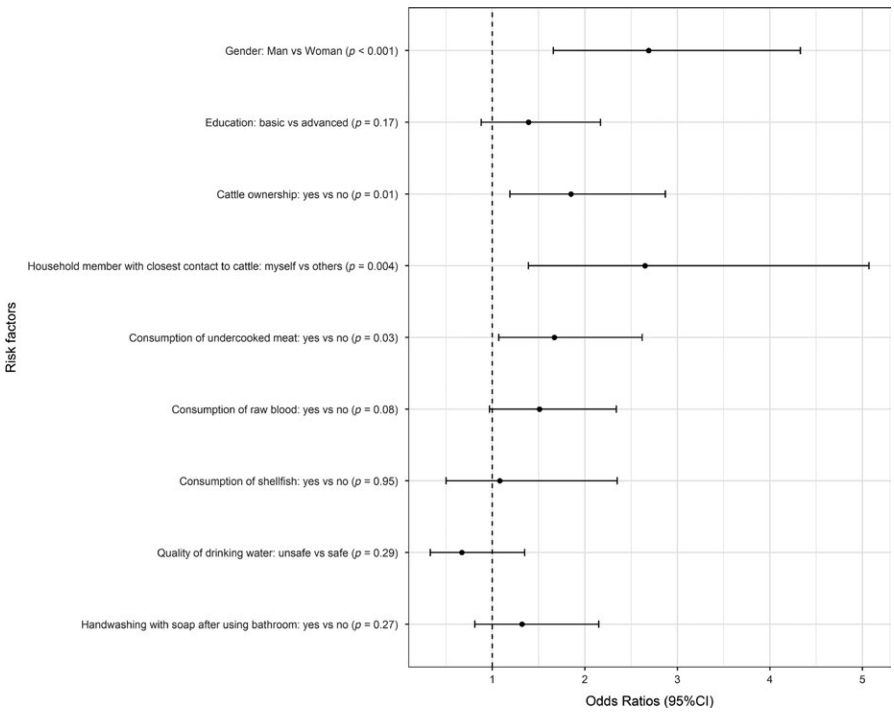

FIGURE 2 Forest plot of the effect of risk factors on anti-HEV IgG antibody seroprevalence. Odds ratios, 95% Confidence intervals and *p*-value as estimated by Chi-square test

TABLE 1 Cohort characteristics and anti-Hepatitis E virus IgG antibody seropositivity

		Anti-Hepatitis E virus IgG seropositivity		
	Total numbers	Complete dataset	Control group	Risk group
Variables	*N* (%)	*n*/*N* (%)	*n*/*N* (%)	*n*/*N* (%)
Total	326 (100)	169/326 (51.8)	68/155 (43.9)	101/171 (59.1)
Gender				
Woman	215 (65.9)	94/215 (43.7)	45/111 (40.5)	49/104 (47.1)
Man	111 (34.1)	75/111 (67.6)	23/44 (52.3)	52/67 (77.6)
Age groups (years)				
1 (18–30)	51 (15.6)	18/51 (35.3)	10/31 (32.3)	8/20 (40)
2 (31–40)	64 (19.6)	26/64 (40.6)	12/38 (31.6)	14/26 (53.9)
3 (41–50)	68 (20.9)	35/68 (51.5)	16/30 (53.3)	19/38 (50)
4 (51–60)	83 (25.5)	46/83 (55.4)	9/29 (31.0)	37/54 (68.5)
5 (61–70)	37 (11.3)	26/37 (70.3)	10/16 (62.5)	16/21 (76.2)
6 (71–85)	23 (7.1)	18/23 (78.3)	11/11 (100)	7/12 (58.3)
Village				
Douangboundy	48 (14.7)	29/48 (60.4)	11/21 (52.4)	18/27 (66.7)
Nakhoa	61 (18.7)	39/61 (63.9)	15/25 (60)	24/36 (66.67)
Naphok	19 (5.8)	11/19 (57.9)	0/0 (0)	11/19 (57.9)
Paksarbmai	60 (18.4)	33/60 (55)	21/43 (48.8)	12/17 (70.6)
Palai	31 (9.5)	16/31 (51.6)	4/7 (57.1)	12/24 (50)
Phonetong	43 (13.2)	19/43 (44.2)	7/23 (30.4)	12/20 (60)
Vuernten	64 (19.6)	22/64 (34.4)	10/36 (27.8)	12/28 (42.9)
Education[a]				
Advanced	128 (39.3)	60/128 (46.9)	22/64 (34.4)	38/64 (59.4)
Basic	198 (60.7)	109/198 (55.1)	46/91 (50.6)	63/107 (58.9)
Cattle ownership				
No (control group)	155 (47.5)	68/155 (43.9)	n.a.	n.a.
Yes (risk group)	171 (52.5)	101/171 (59.1)		
Household member in closest contact to cattle				
Myself	83 (51.2)	59/83 (71.1)	0/0 (0)	59/83 (71.1)
Other	79 (48.8)	38/79 (48.1)	0/0 (0)	38/79 (48.1)
NA	164 (50.3)	72/164 (43.9)	68/155 (43.9)	4/9 (44.4)
Consumption of undercooked meat				
No	127 (39.2)	56/127 (44.1)	29/64 (45.3)	27/63 (42.9)
Yes	197 (60.8)	112/197 (56.9)	38/90 (42.2)	74/107 (69.2)
NA	2 (0.6)	1/2 (50)	1/1 (100)	0/1 (0)
Consumption of internal organs of pigs or cattle				
No	4 (1.2)	1/4 (25)	0/2 (0)	1/2 (50)
Yes	319 (98.8)	168/319 (52.7)	68/152 (44.7)	100/167 (59.9)
NA	3 (0.9)	0/3 (0)	0/1 (0)	0/2 (0)
Consumption of raw blood				
No	152 (46.9)	71/152 (46.7)	29/71 (40.9)	42/81 (51.9)

(Continues)

TABLE 1 (Continued)

		Anti-Hepatitis E virus IgG seropositivity		
	Total numbers	Complete dataset	Control group	Risk group
Variables	*N* (%)	*n/N* (%)	*n/N* (%)	*n/N* (%)
Yes	172 (53.1)	98/172 (56.9)	39/84 (46.4)	59/88 (67.1)
NA	2 (0.6)	0/2 (0)	0/0 (0)	0/2 (0)
Quality of drinking water				
Safe	291 (89.3)	154/291 (52.9)	61/142 (42.9)	93/149 (62.4)
Unsafe	35 (10.7)	15/35 (42.9)	7/13 (53.9)	8/22 (36.4)
Handwashing with soap after using bathroom				
No	90 (27.7)	42/90 (46.7)	21/49 (42.9)	21/41 (51.2)
Yes	235 (72.3)	126/235 (53.6)	46/105 (43.8)	80/130 (61.5)
NA	1 (0.3)	1/1 (100)	1/1 (100)	0/0 (0)

Note. NA: not available; n.a.: not applicable.

[a]Advanced education: participants attending secondary school, professional school or university; basic education: participants attending only primary school or participants without scholastic education.

1.6; 95% CI: 1–2.6; $p = 0.047$), to be the household member with the closest contact to cattle (OR: 6.2; 95% CI: 3–12.5; $p < 0.001$), and to consume undercooked meat (OR: 3.4; 95% CI: 2–5.7; $p < 0.001$) or raw blood (OR: 2.8; 95% CI: 1.7–4.5; $p < 0.001$). In addition, significantly less men than women reported to wash their hands with soap after defecation (OR: 0.5; 95% CI: 0.3–0.8; $p = 0.009$).

Men and women also differed in their educational level and in their knowledge about zoonotic diseases (Table 3). Women had 2 times higher odds than men to have completed primary school only (OR: 2; 95% CI: 1.3–3.2; $p = 0.004$). Women were also less informed about zoonotic diseases and ways of protection: while 77.9% of the men were able to provide a correct example for a protection measure against zoonotic infection, this was the case for only 65.3% of the women. Overall, approximately half of the participants were aware that certain animal diseases can be transmitted to humans (52.1%; 170/326; Table 3) and more than half of the provided examples of zoonotic diseases were correct (64.1%; 84/131) with influenza (61.3%; 49/80), dengue fever (21.3%; 17/80) and anthrax (10%; 8/80) most frequently mentioned. Many villagers were able to correctly state at least one basic preventive measure (69.5%; 130/187) such as avoiding contact with sick animals, boiling water, cooking food, handwashing with soap, cleaning of house and stables, sleeping under mosquito nets and wearing protective equipment. A large majority was afraid of contracting a zoonotic disease (94.1%; 225/239). Only few participants knew that water is a vehicle for zoonotic pathogens (26.9%; 88/326), and could correctly name a zoonotic waterborne disease. About half the participants had heard about HEV before (47.5%; 155/326), but only a minority was aware of the possible transmission routes (18.4%; 26/141) (Table 3). All knowledge-related data can be found in Table 3.

Thus, our data show that there is a considerable knowledge gap about zoonotic diseases in rural Lao PDR and potentially a high risk for zoonotic infections either through direct contact with livestock, or indirectly through consumption of unsafe animal products or the contaminated environment.

TABLE 2 Comparison of participating villages

	Free-roaming animals	Animals with access to river water	Anti-HEV antibody seropositity in ruminants	Anti-HEV antibody seropositity in humans
Villages	*n/N* (%)	*n/N* (%)	*n/N* (%)	*n/N* (%)
Vuernten	29/62 (46.8)	28/62 (45.2)	4/32 (12.5)	22/64 (34.4)
Phonetong	24/42 (57.1)	16/42 (38.1)	3/33 (9.1)	19/43 (44.2)
Palai	24/30 (80)	12/29 (41.4)	2/44 (4.5)	16/31 (51.6)
Paksarbmai	39/58 (67.2)	22/58 (37.9)	0/11 (0)	33/60 (55)
Douangboundy	34/46 (73.9)	22/46 (47.8)	0/37 (0)	29/48 (60.4)
Naphok	15/19 (79)	0/19 (0)	2/9 (22.2)	11/19 (57.9)
Nakhoa	41/61 (67.2)	17/61 (27.9)	2/20 (10.0)	39/61 (63.9)
Total	206/318 (64.8)	117/317 (36.9)	13/186 (7)	169/326 (51.8)

TABLE 3 Awareness and knowledge of zoonotic diseases

Questions	*n*/*N* (%)
Awareness of zoonotic diseases	
Do you know that animals can transmit certain diseases to humans?	
No	156/326 (47.9)
Yes	170/326 (52.1)
Examples provided for such a disease	
Incorrect	40/131 (30.5)
Partially correct	7/131 (5.3)
Correct	84/131 (64.1)
Knowledge on transmission routes	
Do you know that you can get an animal disease through contaminated water?	
No	238/326 (73.0)
Yes	88/326 (26.9)
Examples provided for such a disease	
Incorrect	23/34 (67.7)
Partially correct	2/34 (5.9)
Correct	9/34 (26.5)
Knowledge on prevention measures	
Examples of prevention measure provided	
Incorrect	40/187 (21.4)
Partially correct	17/187 (9.1)
Correct	130/187 (69.5)
Knowledge on Hepatitis E Virus	
Have you ever heard of Hepatitis E Virus	
No	171/326 (52.5)
Yes	155/326 (47.5)
Examples provided for Hepatitis E Virus transmission routes	
Incorrect	115/141 (81.6)
Partially correct	6/141 (4.3)
Correct	20/141 (14.2)

3.2 | Evidence of HEV circulation in villages

Overall, 7.0% (13/186; Table 2) of the ruminants had antibodies against HEV and seropositivity was similar in cattle (6.8%, 11/161) and other ruminants (8%, 2/25; in goats: 1/20; in buffalos: 1/5). Despite serological evidence of HEV circulation, repeated attempts for detecting HEV RNA in animal rectal swabs were not successful.

Overall, 51.8% (169/326; 95% CI: 46.3–57.4; Table 1) of the villagers had anti-HEV IgG and 17.5% (57/326; 95% CI: 13.6–22.1) had anti-HEV IgM antibodies. 12.6% (41/326) of the villagers had antibodies of both immunoglobulin classes and 4.9% (16/326) were positive for IgM antibodies only. HEV RNA was not detected in any human serum by RT-PCR. Anti-HEV antibody prevalence among humans and ruminants varied considerably across villages (Table 2). At a village level, no association between prevalence rates in ruminants and humans could be detected.

3.3 | Risk factors for HEV exposure

Significantly higher anti-HEV IgG seropositivity rates were determined for the risk group compared to the control group (59.1% vs. 43.9%; OR: 1.9; 95% CI: 1.2–2.9; $p = 0.008$; Table 1). Overall, participants reporting to be the household member in closest contact with cattle had nearly 3 times higher odds to be seropositive than participants who did not (71.1% vs. 48.1%; OR: 2.7; 95% CI: 1.4–5.1; $p = 0.004$; Figure 2). Consumption of raw blood, animal organs or unsafe water was not associated with higher anti-HEV IgG seropositivity, and also handwashing with soap after defecation did not significantly reduce the odds of seropositivity (Figure 2). There was also no difference in seropositivity between participants that were aware of the risk of zoonotic diseases or of HEV and those who were not.

Bivariate analyses revealed a significant effect or a trend towards significance ($p < 0.2$) of gender ($p < 0.001$), cattle ownership

(p = 0.01), consumption of raw or undercooked meat (p = 0.03), consumption of raw blood (p = 0.08) and of educational level (p = 0.17) on HEV exposure (Figure 2, Table 1). Hence, these categorical predictors, as well as the covariate "age" (p < 0.001) were included in a GLM analysis. The final model selected can be found in Table 4. The overall model was highly significant (likelihood ratio test: −223.62; χ^2 = 46.97; p < 0.001; AUC = 72%) and the effects of all predictors together could explain 18% (Nagelkerke Pseudo-R-squared) of the variability in the dataset. The model confirmed that the probability of anti-HEV IgG seropositivity increased with age and, men and participants consuming raw blood were more likely to have anti-HEV IgG (adjusted OR: 1.86 and 1.65; p < 0.001 and p = 0.07). The significance of the interaction term (cattle ownership and consumption of raw or undercooked meat; p = 0.02) showed that the effect of cattle ownership on anti-HEV IgG positivity depended significantly on the dietary habits: people owning cattle and eating undercooked meat had significantly higher odds to be seropositive than the controls that consumed no undercooked meat (adjusted OR: 3.26; p = 0.02).

4 | DISCUSSION

The susceptibility of ruminants to certain HEV strains was demonstrated recently by molecular analyses (Hu & Ma, 2010; Huang et al., 2016; Xu et al., 2014; Yan et al., 2016) and there is increasing evidence of a close phylogenetic relationship between human and ruminant HEV strains (Di Martino et al., 2016; Hu & Ma, 2010; Huang et al., 2016; Long et al., 2017; Xu et al., 2014; Yan et al., 2016). Here, we provide first sero-epidemiological evidence that ruminants are a potential source of zoonotic HEV in rural Lao PDR, where human and livestock habitats largely overlap.

With an IgG seroprevalence of 51.8%, HEV is hyperendemic in the rural population at least in Vientiane Capital. In South-East Asia, seroprevalence rates between 8.9% and 77.7% have been reported (Gonwong et al., 2014; Holt et al., 2016; Sa-nguanmoo et al., 2015; Yamada et al., 2015). This wide range certainly reflects regional differences and differing cohort selection criteria, but also disparities in sensitivity and specificity between commercial antibody detection kits (Avellon, Morago, Garcia-Galera del Carmen, Munoz, & Echevarria, 2015; Vollmer, Diekmann, Eberhardt, Knabbe, & Dreier, 2016; Wenzel, Preiss, Schemmerer, Huber, & Jilg, 2013). Currently, there is no gold standard for the detection of anti-HEV antibodies and in particular the results of IgM assays diverge (Vollmer et al., 2016). Surprisingly, our assay detected anti-HEV IgM in 17.5% of the participants. This is very high compared to prevalences of 0.6%–0.9% reported in blood donors using other commercial ELISAs (Guo et al., 2010; Nasrallah et al., 2017). Generally, anti-HEV IgM antibodies become undetectable 4–8 months after acute infection (Huang et al., 2010; Kamar et al., 2014), but this detection period may again depend on the detection assay, as well as the immune response capacity. Nevertheless, our IgM seroprevalence data clearly exceeds biological plausibility and may reflect a cut-off set too low or a poor specificity. Nonspecific reactions do not seem to be uncommon with

TABLE 4 Binomial generalized linear model showing the factors affecting anti-HEV IgG antibody seropositivity

Predictor variables included in model	Summary of model					Contributing effects				
	OR	Estimate	Std. Error	z value	Pr (>|z|)	Df	Deviance	Resid. Df	Resid. Dev	Pr (>Chisq)
Intercept	0.14 (0.05–0.39)	−1.96	0.53	−3.73	<0.001		322	447.25		
Age	1.03 (1.01–1.05)	0.03	0.01	3.57	<0.001	1	19.12	321	428.13	<0.001
Gender (male)	1.86 (1.09–3.20)	0.62	0.27	2.25	0.02	1	11.28	320	416.85	<0.001
Cattle ownership (yes)	0.84 (0.40–1.76)	−0.17	0.38	−0.45	0.65	1	4.88	319	411.98	0.03
Consumption of raw or undercooked meat (yes)	0.67 (0.33–1.38)	−0.40	0.37	−1.08	0.28	1	2.56	318	409.42	0.11
Consumption of raw blood (yes)	1.65 (0.95–2.90)	0.50	0.29	1.76	0.08	1	3.26	317	406.16	0.07
Cattle ownership (yes):Consumption of raw or undercooked meat (yes)	3.26 (1.25–8.52)	1.18	0.49	2.42	0.02	1	5.88	316	400.28	0.02

other commercial assays (Norder et al., 2016). Consequently, the IgM results were ignored in the statistical analysis.

We found 6.8% seropositivity in cattle and 8% in other ruminants. This is comparable with rates reported in these animals elsewhere [1.4%–47% and 0.6%–28.2%; (Arankalle et al., 2001; El-Tras et al., 2013; Fu et al., 2010; Geng et al., 2010; Peralta et al., 2009; Sanford et al., 2013; Vitral et al., 2005; Yan et al., 2016; Yu et al., 2009; Zhang et al., 2008)]. None of the rectal swabs from cattle were positive for HEV RNA by real-time PCR likely because viral loads were too low for detection (Fu et al., 2010) or because animals with acute infection were missed due to the limited size of the dataset. Although genetically characterizing the circulating strains would have provided further evidence, our serology demonstrated that cattle are hosts of HEV in Lao PDR. This is of concern as cattle are susceptible to zoonotic HEV genotype 4 strains present in Lao pigs (Conlan et al., 2011; Hu & Ma, 2010; F. Huang et al., 2016; Xu et al., 2014; Yan et al., 2016). In a follow-up study, patients with acute hepatitis should be screened by PCR to confirm that HEV strains closely related to animal strains affect the human population. In this study, all human sera were negative for HEV RNA probably because samples were collected after the viremic phase that lasts only 3 weeks after the onset of symptoms (Kamar et al., 2014).

Our epidemiological observations nevertheless suggest that direct or indirect zoonotic transmission from cattle occurs: we found a significantly higher HEV IgG seropositivity in cattle farmers (59.1%) than in the control group (43.9%; $p = 0.008$). Our questionnaire revealed that in particular, individuals with the closest contact to cattle had more than twice higher odds to be seropositive than other household members. Men were significantly more likely than women to keep cattle ($p = 0.047$) and to engage in risk-associated activities. This is also reflected by their higher anti-HEV IgG seroprevalence compared to women (67.6% compared to 43.7%). Gender-specific differences in mobility and occupation were shown to lead to differences in HEV exposure (Labrique et al., 2009). In line with previous studies (Drobeniuc et al., 2001; Faber et al., 2012; Lagler et al., 2014), seropositivity increased significantly with age, because of cumulative lifetime exposure to HEV, but also because cattle farming is particularly popular among the elderly.

Interestingly, anti-HEV antibodies were also detected in participants from villages where no antibodies were detected in ruminants. There are several possible explanations for this discrepancy. Firstly, we may have missed evidence for virus circulation due to the limited size of the cattle cohort. Moreover, besides cattle, other susceptible animals, such as other ruminant species (Geng et al., 2011; Vitral et al., 2005; Zeng et al., 2017; Zhang et al., 2008) may be hosts of zoonotic HEV in rural Lao PDR. Indeed, even in our small cohorts of goat and buffaloes, few seropositives were found, suggesting a role also of other ruminants in HEV epidemiology. Besides, the livestock free-roaming throughout the villages and fields, and with unrestricted access to rivers, may contribute considerably to fecal contamination of the village environment and of open waters (e.g., irrigation channels, rivers, ponds). Unsafe water (i.e., piped, well or river water) may be a vehicle of animal HEV strains. In our study, we found no association between consumption of unsafe water and a higher risk of HEV infection. This finding, however, is likely biased: overall, only 10.7% of the participants reported to drink unsafe water, but since unsafe water is commonly used in Lao kitchens, inadvertent ingestion of unsafe water is likely. Contaminated water was also assumed to be the main source of (zoonotic) HEV infection for humans in two other provinces of Lao PDR (Holt et al., 2016) and elsewhere in the world (Guthmann et al., 2006; Shrestha et al., 2015). Among other risky dietary habits, only eating undercooked meat significantly increased the risk for HEV infection. Milk production and consumption are still negligible in Lao PDR (Food & Agriculture Organization, 2005) and were thus not taken into account in this study. In both developing and developed countries, consumption of undercooked pork products is generally recognized as a major source of HEV infection (Berto, Martelli, Grierson, & Banks, 2012; Di Bartolo et al., 2012; Gonwong et al., 2014; Hinjoy et al., 2013; Renou, Afonso, & Pavio, 2014; Sa-nguanmoo et al., 2015). The majority of the participants reported to consume unsafe food, and still only a minority was aware that (zoonotic) diseases can be transmitted via contaminated water. This and the dramatic education and knowledge gap between women and men should be addressed in the future especially since women are mainly involved in food preparation.

5 | CONCLUSION

Our study revealed that HEV is highly endemic in rural Lao PDR, also in villages where mainly ruminants are reared. Cattle farmers have a higher risk of HEV infection than other villagers; the household members in closest contact with cattle having the highest risk. Free-roaming cattle excreting HEV may contaminate water sources and the village environment. Thus, even in villages were pigs are rare, the whole village community is continuously exposed to (zoonotic) HEV due to risky dietary habits, as well as poor sanitation during food preparation and field work, or when attending to domestic animals. Our study highlights the need to improve the protection of cattle farmers and the rural population against HEV, a model for water/food-borne and fecal-orally transmitted pathogens. Future awareness raising campaigns should focus in a broader sense on water- and foodborne diseases, as well as on basic hygienic measures appropriate for rural settings. Applying a community-based participatory approach, protective measures should be promoted at village-level and men should be the main target of such interventions.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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