Title: Hepatitis E virus is prevalent in the pig population of Lao People's Democratic Republic and evidence exists for homogeneity with Chinese Genotype 4 human isolates

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PII: S1567-1348(11)00128-6
DOI: doi:10.1016/j.meegid.2011.04.022
Reference: MEEGID 995

To appear in: *Infection, Genetics and Evolution*

Received date: 31-1-2011
Revised date: 6-4-2011
Accepted date: 19-4-2011

Please cite this article as: Conlan, J.V., Jarman, R.G., Vongxay, K., Chinnawirotpisan, P., Melendrez, M.C., Fenwick, S., Thompson, R.C.A., Blacksell, S.D., Hepatitis E virus is prevalent in the pig population of Lao People's Democratic Republic and evidence exists for homogeneity with Chinese Genotype 4 human isolates, *Infection, Genetics and Evolution* (2010), doi:10.1016/j.meegid.2011.04.022

Highlights

- 11.6% of pigs had detectable Hepatitis E virus RNA in Lao PDR
- 43.5% of village pig herds were infected.
- All isolates were phylogenetically classified within genotype 4
- Phylogenetic clustering with other Asian human genotype 4 HEV isolates

Hepatitis E virus is prevalent in the pig population of Lao People's Democratic Republic and evidence exists for homogeneity with Chinese Genotype 4 human isolates

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Keywords: Hepatitis E virus; swine; zoonosis; Lao PDR; RNA stabilising buffer

Abstract

The objective of this study was to determine the prevalence and genotypic range of Hepatitis E virus (HEV) in the pig population of northern Lao People's Democratic Republic (PDR). We collected 181 faecal samples from indigenous-breed pigs ≤6 months of age and the faeces was stored in RNA stabilisation buffer due to cold-chain and transport limitations. Twenty-one (11.6%) pigs had detectable HEV RNA and 43.5% of village pig herds were infected. Based on a 240 base pair-nucleotide sequence flanking the junction of open reading frames 1, 2 and 3 (ORF1, ORF2 and ORF3) the isolates were phylogenetically classified within genotype 4. Phylogenetic analyses revealed distinct genetic groupings of the Lao HEV isolates and two groups clustered with human and pig HEV isolates from China. This was the first study to demonstrate genotype 4 HEV in Lao PDR and indicates pigs are a potential reservoir for human HEV infection.

1. Introduction

Hepatitis E virus (HEV) typically causes a self-limiting acute viral hepatitis followed by recovery and on occasion a fulminant hepatitis develops (Aggarwal and Naik, 2009). It is the causative agent of large-scale and sporadic acute hepatitis outbreaks worldwide with endemicity primarily centred in regions with poor sanitation and hygiene; encompassing large areas of Asia, Africa and central and south America (Aggarwal and Naik, 2009; Emerson and Purcell, 2003). Faecal-oral transmission is the predominant route of HEV infection and contaminated drinking water is the most common cause of epidemic human disease (Aggarwal and Naik, 2009; Emerson and Purcell, 2003; Meng, 2009b). Consumption of raw shellfish and uncooked meat and viscera from HEV infected animals are also important sources of human disease and result in sporadic cases (Meng, 2009a, b).

Hepatitis E virus is a non-enveloped, single-stranded positive sense RNA virus of the family *Hepeviridae*, genus *Hepevirus* and is comprised of four distinct genotypes (Emerson and Purcell, 2003; Lu et al., 2006). Genotypes 1 and 2 are primarily associated with humans and account for the majority of HEV infections worldwide, including epidemics (Aggarwal and Naik, 2009). Genotype 3 and 4 HEV have a zoonotic origin (Aggarwal and Naik, 2009; Meng, 2009a) with a diverse animal host range (Lu et al., 2006; Okamoto, 2007). However, pigs are an especially important reservoir of genotype 3 and 4 HEV and a common source of human HEV infection (Lu et al., 2006).

The majority of human HEV infection in Asia can be attributed to genotype 1 virus (Aggarwal and Naik, 2009; Wei et al., 2006) with sporadic acute disease associated with genotypes 3 and 4 (Aggarwal and Naik, 2009; Fu et al., 2010). However, recent evidence from China indicates that genotype 4 HEV is emerging as the predominant cause of human

disease (Li et al., 2006; Zhang et al., 2010). All pig HEV infections in Asia have been attributed to genotype 3 or 4 (Meng, 2009a) with a single genotype 1-like virus isolated from a pig in Cambodia (Caron et al., 2006), but this finding has not yet been verified (Meng, 2009b). Genotype 3 HEV is prevalent in pig populations of Cambodia and Thailand (Caron et al., 2006; Cooper et al., 2005) and Genotype 4 is prevalent in pigs of southern China (Ji et al., 2008). Furthermore, genotype 4 HEV has been associated with shellfish and human disease in northern Vietnam (Koizumi et al., 2004)

In the Lao People's Democratic Republic (Lao PDR), anti-HEV antibodies are prevalent in 16-18% of the general human population (Corwin et al., 1999; Syhavong et al., 2010) and 2-4% of hospital admitted hepatitis cases have been attributed to HEV infection (Corwin et al., 1999; Syhavong et al., 2010). In a sero-epidemiological study of the Lao pig population, anti-HEV antibodies were detected in 51% of slaughter-pigs and 15% of village-surveyed pigs, with a peak sero-prevalence in 7-9 month-old animals (Blacksell et al., 2007). Neither pig nor human HEV isolates have been genetically typed in the Lao PDR and the link between pig and human infections have not been examined. This study describes the detection and molecular epidemiology of HEV in pigs in northern Lao PDR.

2. Materials and methods

2.1 Ethics statement

The research protocols were reviewed and approved by the Murdoch University Animal Ethics Committee (project number: R2108/07) prior to commencing the study. The Lao Department of Livestock and Fisheries does not, at this time, have an ethics committee to review and approve scientific research protocols involving animal subjects.

2.2 Study site

The study was conducted in four provinces in northern Lao PDR, Luangprabang, Oudomxay, Xiengkhuang and Huaphanh, where >90% of pigs are produced in the smallholder sector using traditional low-input production practices. One district in each province was selected for inclusion in the study (Figure 1).

2.3 Faecal samples and storage

Faecal samples were collected from 181 pigs ≤6 months of age in the dry season from January to March 2009 and stored in RNAlater® (Ambion, USA). Approximately 1 g of faecal material was added to 5 ml of RNAlater® and mixed thoroughly with a clean wooden stick. Data on age, breed, sex and village origin were collected for each animal. Samples were stored at 4 °C in the field and at -85 °C upon return to the central laboratory in Vientiane Capital. Samples were shipped on dry ice to the Armed Forces Research Institute for Medical Sciences (AFRIMS), Bangkok, Thailand, for molecular analysis.

2.4 Statistical analysis

Data were entered into a spreadsheet (Microsoft® Excel®, Microsoft Corporation, USA) and analysis was performed using Stata/IC version 10 (Stata Corporation, USA). HEV prevalence

was calculated as the proportion of animals that had detectable HEV RNA in the sample population. The Fisher's exact test was used to explore associations between infection status and sex, province and age category. The effect of sample storage time at 4 °C on HEV RNA detection was tested by Kruskal-Wallis test for non-parametric difference of means. Associations were considered significant if $P \leq 0.05$.

2.5 Polymerase chain reaction, sequencing and phylogenetic analysis

Virus RNA was extracted from stool suspension using QIAamp viral RNA mini kit (QIAGEN, Germany) according to the manufacturer's instruction. The RNA was tested by nested RT-PCR using primers flanking the junction of open reading frame (ORF) 1, 2 and 3 with external primers F2782 (5'-GGDCTBGTTCATAACCTGAT -3') and R2783 (5'-GGTTGGTTGGATGAATATAGG-3') and internal primers F2781 (5'-GTTCATAACCTGATWGGYATGCT-3') and R2784 (5'-GGATTGCGAAGGGCTGAGAATCA -3'). Briefly, genomic RNA was converted to cDNA using specific primer R2783 with the AMV-RT (Promega, USA) according to the manufacturer's instruction. The first and second round PCR were amplified by using AmpliTaq DNA polymerase (Applied Biosystems, USA) with the external and internal primer sets, respectively. Cycling conditions included 35 amplification cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. After electrophoresis in a 1.5% agarose gel stained with ethidium bromide, the expected 310 bp bands of the second round PCR product were visualized on a UV transiluminator. The PCR amplified DNA fragments were purified using QIAquick PCR purification kit and the QIAquick gel extraction kit (QIAGEN, Germany) according to the manufacturer's instructions.

Sequencing reactions were performed using the DYEnamic ET dye terminator sequencing kit (GE Healthcare, UK) according to the manufacturer's instruction, with sequencing primers. The sequencing products were cleaned by standard precipitation before sequencing on a MegaBACE 500 automated DNA sequencer (GE Healthcare, UK). Overlapping nucleic acid sequences were combined for analysis and edited with the aid of Sequencher software (Gene Code Corp., USA). The 21 Lao isolates were combined with 20 known HEV swine and human sequences obtained from GenBank (Figure 3); including 10 human HEV strains (Genotype and GenBank Accession numbers in parenthesis) from Burma (genotype 1; M73218), Mexico (genotype 2; M74506), China (genotype 4; AB108537, AJ272108, AB197673, AB369688), Japan (genotype 4; AB253420, AB097812, AB291966), South Korea (genotype 4; FJ763142), and 10 pig HEV strains, including Thailand (genotype 3; EU375463), China (genotype 4; EU676172, GU119960, EF570133, AY594199, FJ610232, GU361892, GU206559), Japan (genotype 4; AB097811) and India (genotype 4; AY723745). Sequences were aligned using MUSCLE v2.1 (Edgar, 2004) and a phylogenetic tree was constructed using PhyML v3.0 (Guindon and Gascuel, 2003) under the model GTR+G+I determined using jModelTest (Podsada, 2008) and with aLRT, SH-like support (indicated at tree nodes). Pairwise analysis was performed in CLC Main Workbench (CLC bio, Denmark) and Fisher's exact test was used to detect significant evidence of heterogeneity with respect to host.

3. Results

3.1 Detection and epidemiology of HEV in pigs

One hundred and eighty-one faecal samples were collected from pigs ≤6 months old (median: 3 months old), stored in RNAlater® and kept at 4 °C in the field before storage at -85 °C in the laboratory. The median storage time at 4 °C was 11 days (range: 1 – 44). Twenty-one samples (11.6%) had detectable HEV RNA in the faeces and no significant effect of storage time on HEV RNA PCR-amplification was observed (*P*=0.420). Samples were collected from pigs from 95 households in 23 villages (Table 1). There was a significant difference in observed prevalence between the 4 provinces (*P*=0.012) as no pigs sampled in Huaphanh province had detectable HEV RNA (Table 1). Age-stratified prevalence peaked at 15.8% (3/19) in 1-2 month old pigs (Figure 2) but no significant difference (*P*=0.514) in HEV infection was observed for piglet age due to the small sample size. There was no significant association between HEV infection status and the sex of pigs (*P*=0.831).

3.2 Genetic analysis of HEV isolates from viremic pigs

Sequence analysis of the 240-bp fragment indicated that all 21 Lao HEV isolates belonged to genotype 4 (Figure 3) and were 89.6-100% identical to each other. When Lao sequences were compared to known human and pig HEV isolates obtained from GenBank, they had 88.3-97.1% sequence homology with genotype 4 strains, 80.0-83.3% homology with Thai genotype 3 HEV (EU375463), 80.8-84.2% homology with Burmese genotype 1 HEV (M73218) and 79.6-83.3% homology with Mexican genotype 2 HEV (M74506). Genotype 4 sequences from both human and swine HEV isolates appeared to cluster indiscriminately within the phylogenetic tree (Figure 3) and no significant clustering for host was evident (*P*=0.392).

Genetic variability was observed within the 21 Lao HEV isolates and we tentatively assigned them into four apparent groups and a lone genetically distinct isolate (Figure 3). *Group 1* was comprised of four isolates from a single litter in Xiengkuang province (HQ541429-HQ541432) and a piglet in Oudomxay province (HQ541425) and had 100% sequence homology. *Group 1* could not be classified to the subtype level as there was 96.3% sequence homology with subtype 4b swine HEV isolated in Guangxi province in southern China (GenBank accession number: EU676172) (Zhang et al., 2010), 95.4% homology with subtype 4g human HEV isolated in Jilin province in northeast China (AB108537) (Lu et al., 2006) and 95.0% homology with subtype 4d swine HEV isolated in Jiangxi province in southeast China (AY594199) (Lu et al., 2006). A single isolate from Oudomxay province (HQ541423) had 95.0% sequence homology with subtype 4b swine HEV (EU676172) and 94.2% homology with subtype 4c swine HEV isolated in Hokkaido, Japan (AB097811) (Lu et al., 2006). *Group 2* was comprised of two isolates, one each from Luangprabang (HQ541412) and Oudomxay (HQ541427) provinces and had 99.6% sequence homology. *Group 2* isolates had 96.7-97.1% sequence homology with subtype 4a human HEV isolated from a Japanese patient who travelled to Shaanxi province in central China (AB197673) and subtype 4a swine HEV isolated in Xinjiang province in far northwest China (GU119960), giving us confidence that these isolates can be classified as subtype 4a. *Group 3* was comprised of isolates from two litters in a village in Luangprabang province (HQ541414-HQ541417; HQ541418-HQ541419), from a piglet in another village in Luangprabang province (HQ541413) and a piglet from Xiengkhuang province (HQ541428) and had 98.3-100% sequence homology. *Group 4* was comprised of five isolates from five different litters and three villages in Oudomxay province (HQ541420-HQ541422, HQ541424, HQ541426) and had 94.2-98.3% sequence homology. *Group 3* and *4* Lao isolates did not cluster with other Asian HEV strains (88.3-93.8% homology) and were not classified at the subtype level.

The sequence data for the Lao HEV isolates were deposited on GenBank, accession numbers: HQ541412 – HQ541432.

4. Discussion

We report for the first time genotype 4 HEV in the pig population of northern Lao PDR and that almost half of the village pig herds were infected. This finding has epidemiological significance since the growing body of evidence indicates possible geographical partitioning of genotypes 3 and 4 in the Mekong sub-region of Southeast Asia. To our knowledge, only genotype 3 has been isolated from pigs in Cambodia and Thailand (Caron et al., 2006; Cooper et al., 2005) and genotype 4 from humans in Vietnam (Hijikata et al., 2002; Koizumi et al., 2004) and pigs in Lao PDR. The Lao and Vietnamese HEV isolates originate from northern regions and to date no data on the genotypes of HEV circulating in the southern regions of these countries are available. Wide reaching studies are required to confirm geographical partitioning, however, classical swine fever virus, which is endemic throughout the region, provides a precedent for genotypic partitioning in the Mekong region (Blacksell et al., 2005; Blacksell et al., 2004).

The success of this study was dependent on the ability to store and transport faecal samples in a manner that prevented HEV RNA degradation. We used the proprietary RNA stabilisation buffer RNAlater® to preserve HEV RNA for subsequent molecular analysis. RNA stabilisation buffers have been used to preserve RNA in faecal samples in studies involving rotavirus (Whittier et al., 2004) and avian influenza virus (Forster et al., 2008), but to our knowledge this is the first report using RNAlater® to preserve HEV in faeces. Our study clearly demonstrates proof-of-principle and further studies to validate the protocol and determine analytical and diagnostic performance characteristics are warranted. Hepatitis E virus could be detected in faeces stored at 4 °C for 41 days, indicating the usefulness of this method in conducting an epidemiological survey in a hot, tropical country with poor quality infrastructure and cold chains. The most significant drawback of RNAlater® is the

incompatibility with virus isolation (Forster et al., 2008) and dual sampling will be needed if live virus manipulations are required. Since our study was primarily a genotyping study, RNAlater® preservation served our purpose.

Previous studies in Lao PDR indicate that tropical monsoon weather patterns may influence the epidemiology of swine HEV (Blacksell et al., 2007), such that peak seroprevalence corresponds with the wet season months May to September (Conlan et al 2010, In prep). The timing of this present survey coincided with the dry season and the observed prevalence of 11.6% may be indicative of the baseline from which epidemic transmission can occur during the wet season. However, longitudinal studies are needed to determine seasonal influence on virus transmission in Lao PDR; a study in Eastern China demonstrated little seasonal influence on transmission (Lu et al., 2009), but rainfall patterns and pig production practices are vastly different.

The age-stratified HEV RNA positivity data suggested that pigs were infected at a young age with peak prevalence observed in 1-2 month old piglets. This finding is consistent with experimental studies showing piglets start to shed virus from 1 month of age (Kanai et al., 2010) after the waning of IgA material antibodies by 3 weeks of age (de Deus et al., 2008). Piglets can shed virus for up to 4 months post infection (Kanai et al., 2010) providing a continued source of environmental contamination and enabling HEV to remain endemic over the dry season months, November to March.

It is important to note the limitations of interpreting sequence data from a 240-bp fragment; nevertheless, genetic variability was evident amongst the Lao HEV isolates. Interestingly, four genetically distinct isolates were detected in Oudomxay province and all but those

belonging to *Group* 4 clustered with other Asian isolates, and could reflect the movement of people and animals in a region at the junction of five countries (Figure 1). *Groups 1*, *2* and *3* were comprised of isolates from different provinces and provide evidence for HEV dispersal in Lao PDR through pig trade and or human movements. All but three of the Lao isolates fell externally in the phylogenetic tree when compared to the other Asian genotype 4 isolates included in the analysis. The data provides evidence that *Groups 3* and *4* HEV isolates from Lao PDR were unique and possibly geographically partitioned, whereas the remaining isolates were related to Chinese genotype 4 HEV isolates from humans and pigs.

Host related factors are generally thought to be closely associated with the severity of HEV infection (Okamoto, 2007), however, recent studies indicate that genotype 4 HEV was more commonly associated with severe and fulminant hepatitis compared to genotype 3 HEV (Mizuo et al., 2005; Okamoto, 2007). Severe and fulminant hepatitis was reported in 36% and 8% of cases, respectively, for genotype 4 HEV infection compared to no severe or fulminant hepatitis observed in genotype 3 HEV infections (Okamoto, 2007). This finding could have important implications for high-risk groups in northern Lao PDR considering we exclusively found genotype 4 HEV and that genotype 4 HEV is emerging as the dominant cause of human HEV disease in southern and eastern China (Li et al., 2006; Zhang et al., 2010).

The results of the present study showed that genotype 4 HEV was prevalent in Lao PDR and that almost half the herds were infected. The high pig and herd level prevalence in the Lao pig population provides an abundant reservoir of virus for human HEV disease in rural communities of northern Lao PDR. Research will be required to understand the cause of human HEV disease and the use of a validated protocol using RNA stabilisation buffer could provide a suitable means of achieving this objective. Our study provides the first data set from

Lao PDR that can be used to examine the source of human HEV disease. In addition, further work will be required to determine if the dry season prevalence we observed was baseline and a source of epidemic transmission in the monsoonal wet season.

Disclaimer

The opinions or assertions contained herein are the private views of the authors, and not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense (RGJ, PC, MCM).

Acknowledgements

We wish to acknowledge the contributions made by district, provincial and national government staff from the Lao Department of Livestock and Fisheries, Ministry of Agriculture and Forestry, whose contribution was critical to the conduct of this study. In particular, we acknowledge Mr. Lapinh Phithacthep, Ms. Vilaywan Soukvilay, Ms Manivanh Phouarawan and Ms Vilayphet Viravong from the National Animal Health Centre.

This work was funded by the Australian Centre for International Agricultural Research (project number AH2006/161), and JVC was supported by a Murdoch University Research Studentship (MURS) award.

Conflict of interest statement

The authors have declared that no competing interests exist.

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Table and Figures legend

Table 1. Detection of HEV RNA in faeces of pigs in northern Lao PDR

Figure 1. Map of study sites in Lao PDR. 1, Xay district, Oudomxay province; 2, Xieng Ngeun district, Luangprabang province; 3, Paek district, Xiengkhuang province; and 4, Viengxay district, Huaphanh province

Figure 2. Proportion of pigs HEV RNA positive by age-category

Figure 3. Phylogenetic tree constructed by the maximum-likelihood method (GTR+G+I, model) based on 41 sequences and 240 nucleotides flanking the junction of open reading frames 1, 2 and 3 (ORF1-3) of Hepatitis E virus (HEV). Twenty-one HEV isolates from Lao pigs are listed with the province of origin, compared to human and swine sequences obtained from GenBank. Genotypes are denoted on the tree and genetically distinct Lao groups are enclosed in brackets

Table 1. Detection of HEV RNA in faeces of pigs in northern Lao PDR.

Province	Number of villages	Number of households	Number of pigs	Median age (months)	Age range (months)	Proportion pigs HEV positive (95%CI)	HEV positive villages (%)	HEV positive households (%)
Xiengkhuang	6	20	39	3	1.5 - 4.5	12.8 (1.8 - 23.8)	2 (33.3)	2 (10.0)
Huaphanh	5	26	45	3	2.0 - 5.0	0.0 (0.0 - 0.0)	0 (0.0)	0 (0.0)
Oudomxay	6	30	46	4	1.5 - 6.0	17.4 (6.0 - 28.8)	5 (83.3)	8 (26.7)
Luangprabang	6	19	51	4	2.0 - 6.0	15.7 (5.4 - 26.0)	3 (50.0)	3 (15.8)
Total	23	95	181	3	1.5 - 6.0	11.6 (6.9 - 16.3)	10 (43.5)	13 (13.7)


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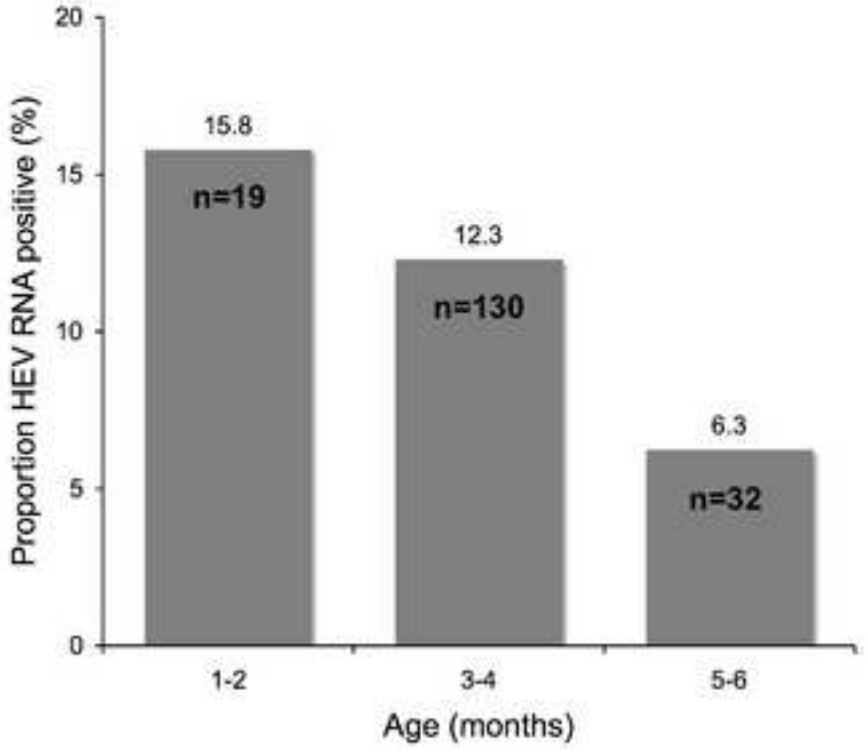
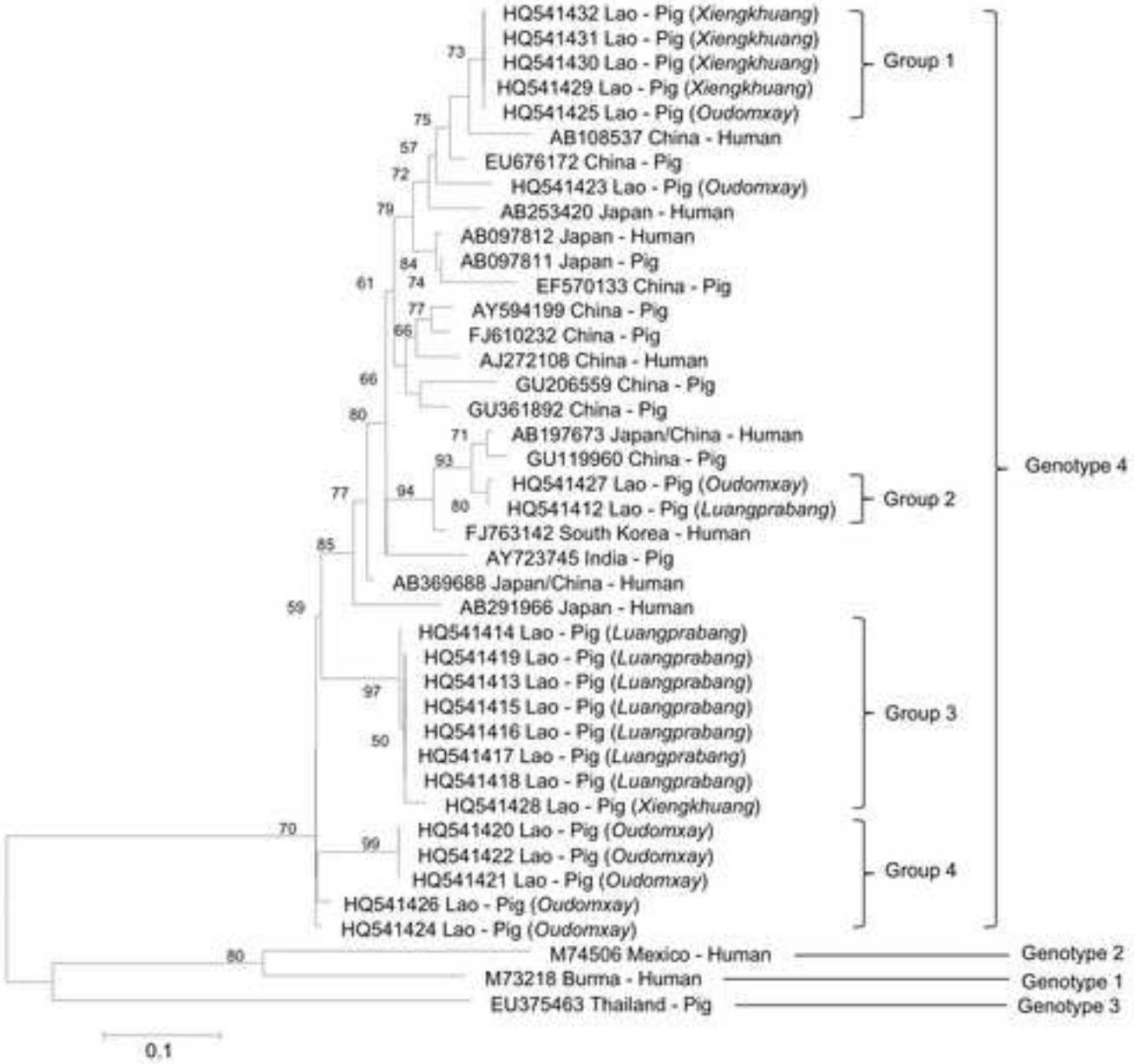
3
4


Proportion HEV RNA positive (%)
20
15
10
5
0
15.8
n=19
12.3
n=130
6.3
n=32
1-2
3-4
5-6
Age (months)


HQ541432 Lao - Pig (Xiengkhuang)
HQ541431 Lao - Pig (Xiengkhuang)
HQ541430 Lao - Pig (Xiengkhuang)
HQ541429 Lao - Pig (Xiengkhuang)
HQ541425 Lao - Pig (Oudomxay)
AB108537 China - Human
EU676172 China - Pig
HQ541423 Lao - Pig (Oudomxay)
AB253420 Japan - Human
AB097812 Japan - Human
AB097811 Japan - Pig
EF570133 China - Pig
AY594199 China - Pig
FJ610232 China - Pig
AJ272108 China - Human
GU206559 China - Pig
GU361892 China - Pig
AB197673 Japan/China - Human
GU119960 China - Pig
HQ541427 Lao - Pig (Oudomxay)
HQ541412 Lao - Pig (Luangprabang)
FJ763142 South Korea - Human
AY723745 India - Pig
AB369688 Japan/China - Human
AB291966 Japan - Human
HQ541414 Lao - Pig (Luangprabang)
HQ541419 Lao - Pig (Luangprabang)
HQ541413 Lao - Pig (Luangprabang)
HQ541415 Lao - Pig (Luangprabang)
HQ541416 Lao - Pig (Luangprabang)
HQ541417 Lao - Pig (Luangprabang)
HQ541418 Lao - Pig (Luangprabang)
HQ541428 Lao - Pig (Xiengkhuang)
HQ541420 Lao - Pig (Oudomxay)
HQ541422 Lao - Pig (Oudomxay)
HQ541421 Lao - Pig (Oudomxay)
HQ541426 Lao - Pig (Oudomxay)
HQ541424 Lao - Pig (Oudomxay)
M74506 Mexico - Human
M73218 Burma - Human
EU375463 Thailand - Pig
Group 1
Group 2
Group 3
Group 4
Genotype 4
Genotype 2
Genotype 1
Genotype 3
0.1