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BRIEF REPORT

Prevalence of hepatitis E virus among swine and humans in two different ethnic communities in Indonesia

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Abstract The purpose of this study was to investigate the prevalence of hepatitis E virus (HEV) infection in swine and humans in different environments in Java and Bali, Indonesia. The prevalence of anti-HEV antibodies in people over 20 years old living in communities in Bali was significantly higher than that in Java. While 68.8% and 90.0% of swine in Bali were anti-HEV positive at 1 and 2 months of age, respectively, swine in Java were at significantly lower risk of HEV infection by the age of 2 months. Our present data suggest that substantial differences in swine-breeding conditions and human living environments affect the rate of HEV infection in humans and swine.

Hepatitis E virus (HEV), a causative agent of acute hepatitis in humans, is a small non-enveloped single-stranded positive-sense RNA virus. HEV has been classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [5].

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HEV is usually transmitted by the fecal-oral route from contaminated water in developing countries where sanitation is suboptimal, so that outbreaks of HEV infection in many endemic regions occur especially in association with flooding and heavy rains [4]. It has also been reported that HEV is transmitted to humans through the consumption of uncooked or undercooked pork and viscera [15, 16]. In addition, the risk of HEV infection has been found to increase in association with occupational contact with swine and swine excrement [6, 17]. Thus, HEV infection has been recognized as a form of zoonosis between swine and human in both developed and developing countries [13, 14, 18]. In other words, exposure to swine constitutes a potential risk factor of HEV infection. HEV can be divided into at least four genotypes, with genotypes 1 and 2 considered to be transmitted to humans via the fecal-oral route, and genotype 3 and 4 considered to be zoonosis.

In Indonesia, three major HEV outbreaks have been documented [2, 3, 12]. In addition, several studies conducted in Bali have reported the prevalence of anti-HEV antibodies in both swine and humans [15, 16]. However, few studies of HEV infection in Indonesia have focused on the zoonotic aspect, including the prevalence of HEV in swine-farm workers. The purpose of this study was to investigate the prevalence of HEV infection in both swine and humans on the islands of Java and Bali, Indonesia, which are two geologically and religiously distinct communities with different swine breeding conditions, and to assess the risk factors by investigating the zoonotic aspects of HEV infection.

Serum samples were collected from 54 healthy individuals, including 42 "community people" (non-swine-farm workers) (mean age: 35.1 ± 10.7 years; 11 males and 31 females) and 12 swine-farm workers (mean age: 45.5 ± 12.0 years; 10 males and 2 females), in Tulungagung

(population: 65,262), a rural area of Java. Also, serum samples were collected from 158 apparently healthy individuals, including 94 community people (mean age: 31.3 ± 17.2 years; 33 males and 61 females) and 64 swine-farm workers (mean age: 32.5 ± 15.4 years; 21 males and 43 females) who visited *Puskesmas* (a community health center) in Mengwi (population: 43,805), a rural area of Bali, for a private health check, and 41 children (mean age: 5.7 ± 3.9 years; 23 males and 18 females) who visited the pediatric outpatient department of Sanglah Hospital in Denpasar, Bali. A total of 199 individuals in Bali thus participated in this study. Serum samples were also collected from 89 swine in Tulungagung, Java, and 119 landrace swine in Mengwi, Bali. Swine-breeding conditions in the two communities are completely different. In Tulungagung, swine are bred at a clean swine farm that is isolated and far from the human community. In Mengwi, on the other hand, swine are raised as domestic animals within the human community, which is a custom unique to Bali. The two communities also differ in terms of religion: the vast majority of the population of Java is Muslim, but that of Bali is Hindu. Both swine and human samples were collected from March 2008 to February 2010. Demographic information was obtained from a questionnaire. Written informed consent was obtained from each individual and the study protocol was reviewed and approved by the ethics committees of Kobe University in Japan and Airlangga University in Indonesia.

To detect IgG-class anti-HEV antibodies in human sera, an enzyme-linked immunosorbent assay (ELISA) was carried out with a commercial kit (IgG anti-HEV antibodies EIA, Institute of Immunology, Tokyo, Japan). To detect anti-HEV antibodies in swine sera, we used the HEV-antigen-coated microplates supplied with the commercial ELISA kit that was used for detecting human antibodies and peroxidase-conjugated rabbit anti-swine IgG (Cappel, Ohio, USA) as the secondary antibody [13], together with positive and negative controls for swine (Cosmic Corporation, Tokyo, Japan). To minimize false positives and to confirm the positive prevalence of anti-HEV antibodies in human and swine sera, a species-independent double-antigen sandwich ELISA (HEV ELISA 4.0v; MP Biomedicals Asia Pacific Pte Ltd., Singapore) was used, which enables specific and efficient detection of swine antibodies against HEV [7, 11]. The HEV ELISA 4.0v utilizes a proprietary recombinant antigen, which is highly conserved between different HEV strains. The recombinant protein pET2.1, which consists of open reading frame (ORF) 2 fragment and a six-histidine tag, was expressed in *Escherichia coli* [7]. In this study, human and swine sera were considered positive for anti-HEV antibody if positive results were obtained with the two different assay kits.

All 208 anti-HEV positive swine sera and 25 human sera were tested for HEV-RNA by reverse transcriptase (RT)-polymerase chain reaction (PCR). Total RNA was extracted from 200 µl of serum using TRIzol-LS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT and first-round amplification were carried out with a one-step RT-PCR kit using ORF2 nested primers as described previously [10].

The amplified fragments were sequenced directly using a Big Dye Deoxy Terminator Cycle Sequencing Kit with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Reference sequences were then retrieved from the DDBJ/EMBL/GenBank database and, after the nucleotide sequences had been aligned with Clustal X software, a phylogenetic tree was constructed by the neighbor-joining (NJ) method using 1,000 replicates of bootstrap resampling. These analyses were carried out with the Molecular Evolutionary Genetics Analysis (MEGA) software program (available at http://www.megasoftware.net). HEV genotypes were assigned as described previously [15].

The chi-square test or Fisher's exact test for categorical variables was used for statistical analysis. A P value <0.05 was considered significant.

Among community people, the prevalence of anti-HEV-IgG was 4.8% (2/42) in Java and 13.3% (18/135) in Bali when tested by using a commercial kit (IgG anti-HEV antibodies EIA). On the other hand, when the species-independent double-antigen sandwich ELISA was used, the anti-HEV prevalence was nil (0/42) in Java and 8.1% (11/135) in Bali. Similarly, among swine farm workers, the prevalence of anti-HEV determined by the former kit was 16.7% (2/12) in Java and 21.9% (14/64) in Bali, while the prevalence determined by the latter was 16.7% (2/12) in Java and 18.8% (12/64) in Bali. We consider the samples that tested positive by both methods in this study to be true positives. We thus conclude that the prevalence of anti-HEV antibodies in community people was 8.1% (11/135) in Bali and nil (0/42) in Java, and that the prevalence in swine-farm workers was 18.8% (12/64) in Bali and 16.7% (2/12) in Java (Table 1). When community people over 20 years old were compared, the prevalence of anti-HEV antibodies in Bali (13.6%; 9/66) was significantly higher than that in Java (0/42) (P=0.01). It should also be noted that, in Bali, there was a statistically significant difference (P=0.028) in the prevalence of anti-HEV antibodies between the age groups of 0-19 years (2.9%; 2/69) and over 20 years (13.6%; 9/66). Anti-HEV antibodies were not detected in children less than 10 years old (n=32). The prevalence of anti-HEV antibodies among swine workers in Java was 16.7% (2/12). This prevalence was significantly higher (P=0.046) than that observed in community people of the same age group in the same area (0/42), although the number of samples tested was small. In Bali,

Table 1 Prevalence of anti-HEV antibodies in humans in two ethnically different areas in Indonesia

	Age (yr)	Java	Bali
Community people	0–19	nd[a]	2/69 (2.9%)[c]
	≥20	0/42 (0%)[b]	9/66 (13.6%)[b,c]
	Total	0/42 (0%)	11/135 (8.1%)
Swine-farm workers	0–19	nd[a]	3/18 (16.7%)
	≥20	2/12 (16.7%)	9/46 (19.6%)
	Total	2/12 (16.7%)	12/64 (18.8%)

[a] Not done

[b] $P = 0.01$

[c] $P = 0.028$

the prevalence was 16.7% (3/18) and 19.6% (9/46) among swine workers less than 20 and over 20 years old, respectively (Table 1).

The IgG class assay detected an overall positive anti-HEV antibody rate of 94.4% (84/89) in farm swine in Java and 96.7% (115/119) in Bali, while the HEV-specific assay detected corresponding rates of 61.8% (55/89) in Java and 82.4% (98/119) in Bali. The final positive rate for anti-HEV antibody in swine in this study was considered to be 61.8% (55/89) in Java and 82.4% (98/119) in Bali. While 68.8% and 90.0% of swine in Bali were anti-HEV positive at 1 and 2 months of age, respectively, swine in Java were at significantly lower risk of infection (Table 2).

HEV-RNA was detected in one 3-month-old swine among the 89 tested in Java (1.2%), and in one 2-month-old swine among the 119 tested in Bali (0.9%) (Table 2). HEV-RNA was not detected in any of the anti-HEV-positive human sera tested. Nucleotide sequences of the ORF of two HEV strains (BP53 from Bali and TP42 from Java) were determined and compared with those of known swine HEV strains of genotypes 1 to 4. Both of the HEV-RNA-positive piglets were positive for anti-HEV antibodies. Phylogenetic analysis revealed that both BP53 (AB541112) and TP42 (AB541111) belonged to genotype 4 (Fig. 1). The strain BP53 (AB541112) was related most closely to the strains from Bali (AB298178), with 96.5% nucleotide sequence identity and 100% amino acid sequence identity (data not shown). On the other hand, the isolate TP42 proved to be distinct from the cluster of reference strains for Bali (Fig. 1), and showed a similar distance from a Balinese strain (AB298178), the BP53 isolate found in Bali in this study, and a Chinese strain (AJ344192), with nucleotide sequence identities of 87.5%, 87.6% and 86.3%, respectively.

In this study, we selected two distinct communities to investigate whether swine-breeding conditions influenced the rate of HEV infection and the presence of anti-HEV antibodies in both swine and humans. Interestingly, swine in Bali showed a high prevalence of anti-HEV antibodies in all age groups, even at the age of one month, although no HEV infection was detected in one-month-old swine in Java. It is possible that this difference is due to different breeding conditions. Piglets in Bali are kept together with older swine from an earlier age, while swine of different age groups are isolated from each other in separate barns in Java. In the natural course of HEV infection in swine, they commonly remain uninfected until the age of approximately 2 months because of protection by anti-HEV in the milk of the mother, especially in the colostrum [8]. In this study, HEV-RNA was detected in only two sera from swine aged 2 to 3 months, and this result suggests that the duration of HEV viremia is very short. In this connection, a significant delay in the onset of both seroconversion and viremia in piglets with maternal antibodies has been reported [8]. The substantial difference in the rate of HEV infection between these two communities may thus largely be attributable to the different swine-breeding environment.

We found that HEV infection among community people over 20 years old was more prevalent in Bali (13.6%) than in Java (0%). The custom of the Balinese residents, who are mostly Hindu, to consume uncooked or undercooked swine meat and fresh swine blood appears to increase the risk of HEV transmission and at least partly explains the high prevalence of anti-HEV antibodies in this group. On the other hand, the majority of inhabitants of Java are Muslims who avoid swine meat for religious reasons, and their eating habits are likely to be associated with the low

Table 2 Prevalence of anti-HEV antibodies and HEV-RNA in swine in two ethnically different areas in Indonesia

Age (months)	Java		Bali	
	Anti-HEV antibodies	HEV-RNA	Anti-HEV antibodies	HEV-RNA
1	0/9 (0%)[a]	–	11/16 (68.8%)[a]	–
2	8/22 (36.4%)[b]	–	18/20 (90.0%)[b]	1
3	16/19 (84.2%)	1	17/21 (81.0%)	–
4	14/18 (77.8%)	–	20/21 (95.2%)	–
5	17/21 (81.0%)	–	18/20 (85.7%)	–
6	nd[d]	–	14/20 (70.0%)	–
Total	55/89 (61.8%)[c]	1/89 (1.2%)	98/119 (82.4%)[c]	1/119 (0.9%)

[a] $P = 0.001$

[b] $P = 0.0004$

[c] $P = 0.0009$

[d] Not done

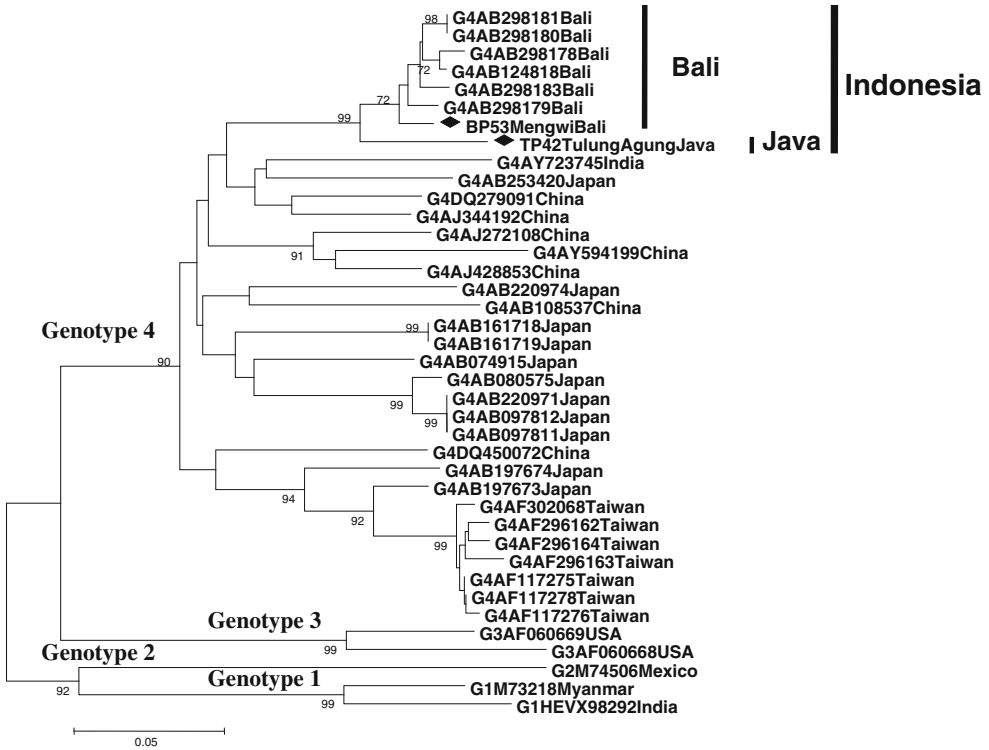

Fig. 1 Phylogenetic tree constructed by the neighbor-joining method based on the partial nucleotide sequence of the ORF2 region of 39 HEV reference strains. Reference isolates from the database are indicated with their accession numbers, and the country of origin is indicated for all strains. The number inside the tree indicates the bootstrap reliability. Strains of genotypes 1, 2 and 3 were used as outgroups. Vertical bars in the phylogenetic tree indicate the clusters consisting of the strains obtained from Bali and Java, Indonesia

prevalence of anti-HEV antibodies. The major difference in HEV infection rates between adults over and under 20 in Bali can also be partly explained by a higher rate of consumption of pork by adults.

The risk for HEV infection is reportedly related to close contact with animals and/or animal waste, e.g., cleaning barns [4, 16]. The prevalence of anti-HEV antibody is higher in swine-farm workers (16.7%) than in community people (0%) in Tulungagung, Java, where community people, perhaps for religious reasons, are much less likely to come into contact with swine and pork than swine farm workers. Although the number of samples from both groups in this study was too small to reach a definitive conclusion, it is conceivable that close contact with swine and/or their waste as well as the habit of consuming uncooked or undercooked swine meat might be a risk factor for HEV infection, as observed in another comparison between community people in Java and those in Bali.

A large number of serological assays have been developed for HEV, but it has been demonstrated that there is a significant lack of concordance between many of these assays, especially those for swine [1, 9, 11]. In this study, we used two different ELISA assays for the detection of anti-HEV antibodies to minimize false positives, one being a modified ELISA kit for human IgG, and the other a species-independent ELISA for the detection of anti-HEV antibodies of the IgG, IgM and IgA classes [7]. The anti-HEV antibody detected in this study was considered positive if positive results were obtained with the two different assay kits. Our findings also demonstrate that different assays differ greatly in their sensitivity, and therefore the modified IgG class ELISA kit for swine needs to be supplemented with at least one other assay, such as a species-independent assay (HEV-specific assay kit) for a more accurate identification of the prevalence of anti-HEV antibodies in swine.

To the best of our knowledge, this is the first study to determine the prevalence of anti-HEV antibodies in swine and HEV-RNA positivity and nucleotide sequence in swine in Java. Genetic analysis revealed that the swine isolates from Java (TP42) and Bali (BP53) belonged to HEV genotype 4, which includes a previously identified strain from Bali. The isolate from Java (TP42), however, was genetically somewhat different from those of the Bali cluster, suggesting that the HEV strain in Java belongs to a subclassification divergent from the lineage of Balinese strains. Further investigations with more strains are needed to elucidate the genetic position of the strains from Java.

In conclusion, the presence of anti-HEV antibodies in swine is relatively common, and the substantial difference

in HEV infection in swine at an earlier stage of life between two communities may depend on their different breeding conditions.

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