Microbiology and Immunology

Microbiol Immunol 2013; 57: 692–703
doi: 10.1111/1348-0421.12083

ORIGINAL ARTICLE

Hepatitis E virus infection in two different regions of Indonesia with identification of swine HEV genotype 3

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ABSTRACT

Hepatitis E is an emerging disease with a high incidence globally. Few data are available on hepatitis E virus (HEV) infection in Indonesia. To obtain molecular information on HEV infection in two regions of Indonesia with different customs and swine breeding conditions, serum samples from 137 swine farm workers, 100 blood donors and 100 swine (27 fecal samples also obtained) in Yogyakarta (Central Java) and from 12 and 64 swine farm workers, 42 and 135 local residents and 89 and 119 swine in Tulungagung (East Java) and Mengwi (Bali), respectively, from our previous study, were compared. Serological tests for anti-HEV antibodies by ELISA, HEV-RNA detection by RT-PCR and phylogenetic analysis were performed. The total prevalence of anti-HEV antibodies in humans was higher in Bali (11.6%) than in Java (5.1%; $P = 0.015$). No significant differences in anti-HEV prevalence among swine farm workers and local residents in Java were found. The finding of swine HEV genotype 3 in specimens from Yogyakarta and genotype 4 from Tulungagung and Bali is somewhat different from other reports. We suggest other factors in addition to close contact with swine might play an important role in HEV transmission of non-endemic/related custom groups. To the best of our knowledge, this is the first report on swine HEV genotype 3 in Indonesia.

Key words hepatitis E virus, Indonesia, zoonosis transmission.

Hepatitis E virus was first identified in 1983 in a study of human volunteers aimed at pinpointing the causative agent of a large, acute, icteric, self-limiting, enteric, non-A, non-B, hepatitis infection outbreak in India (1). A non-enveloped virus particle of approximately 27 nm in diameter (range, 27–34 nm) was visualized by immune electron microscopy of a mixture of acute phase stool and convalescent serum from a patient in that study. This virus was successfully inoculated into a *Cynomolgus macaque* and was cloned in 1990 (2). HEV is now classified in the genus *Hepevirus* and is the only member of the *Hepeviridae* family. It has an approximately 7.2 kb long, polyadenylated, single-stranded RNA genome, with short 5′ and 3′ untranslated regions, and three ORFs (3).

Genomic sequence analysis has classified HEV isolates from humans and other mammals into four genotypes with at least 24 subgenotypes (1a–1e, 2a–2b, 3a–3j, and 4a–4g) (4). Each HEV genotype appears to have a distinct and, in some cases, overlapping geographical distribution. Regions with low standards of sanitation, which promotes viral transmission, have the highest rates of HEV infection. Studies have shown that HEV epidemics

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Received 4 March 2013; revised 22 June 2013; accepted 4 July 2013.

List of Abbreviations: HEV, hepatitis E virus; ORF, open reading frame.

predominantly occur in regions where fecal contamination of drinking water is common (5). Generally, these outbreaks and sporadic water-borne infections are caused by genotypes 1 and 2. However, sporadic cases of hepatitis E in patients without a history of traveling to endemic countries have also been reported; these are frequently caused by genotype 3 and 4. These autochthonous infections are believed to be caused by zoonotic HEV or foodborne infections related to swine; swine handlers are known to be at higher risk of infection (6–9).

Three major outbreaks of hepatitis E have been documented in Indonesia and a few studies conducted in Bali have reported the prevalence of anti-HEV antibodies in both swine and apparently healthy people. Only one study has reported a case of acute hepatitis E in Indonesia (10–14) (Fig. 1). In our previous study, we tested HEV status in swine farm workers from two different ethnic communities, albeit with a limited number of samples (15). This study represents our ongoing investigation of the prevalence of HEV infection in swine and humans from multiple areas in Java and Bali, Indonesia. Java and Bali are two geologically distinct regions with different customs and swine breeding conditions. In this study, we have focused on the molecular aspects of the partially conserved nucleotide sequences of HEV strains among Indonesian isolates.

MATERIALS AND METHODS

Sample collection

In the Java region, because swine farms are located far from housing and have relatively good hygiene systems, the local residents do not have close associations or contact with swine. However, in Bali swine farms are located near houses and swine are often bred as domestic animals in back yards; thus, residents of Bali have close associations and contact with swine. In July 2011, serum samples from 137 swine workers and 100 human blood donors were collected from the Yogyakarta district (center of Java). In addition, 73 swine serum samples and randomly-chosen fecal samples from 27 of those swine were also obtained. This material was to complement our previous data obtained from serum of 12 and 64 swine farm workers and 42 and 135 local residents of Tulungagung (East Java) and Mengwi (Bali), respectively, plus serum samples from 89 swine in Tulungagung (East Java) and 119 swine in Mengwi (Bali) (15). (Table 1, Fig. 1). The fecal samples were immediately suspended in 10% (w/v) PBS (0.01 M, pH 7.2–7.4) and then clarified by centrifugation at 12,000 *g* for 10 min. The clarified material and serum samples were stored at −80°C until further analysis.

To determine the relationships between age and community setting, serological data for several age

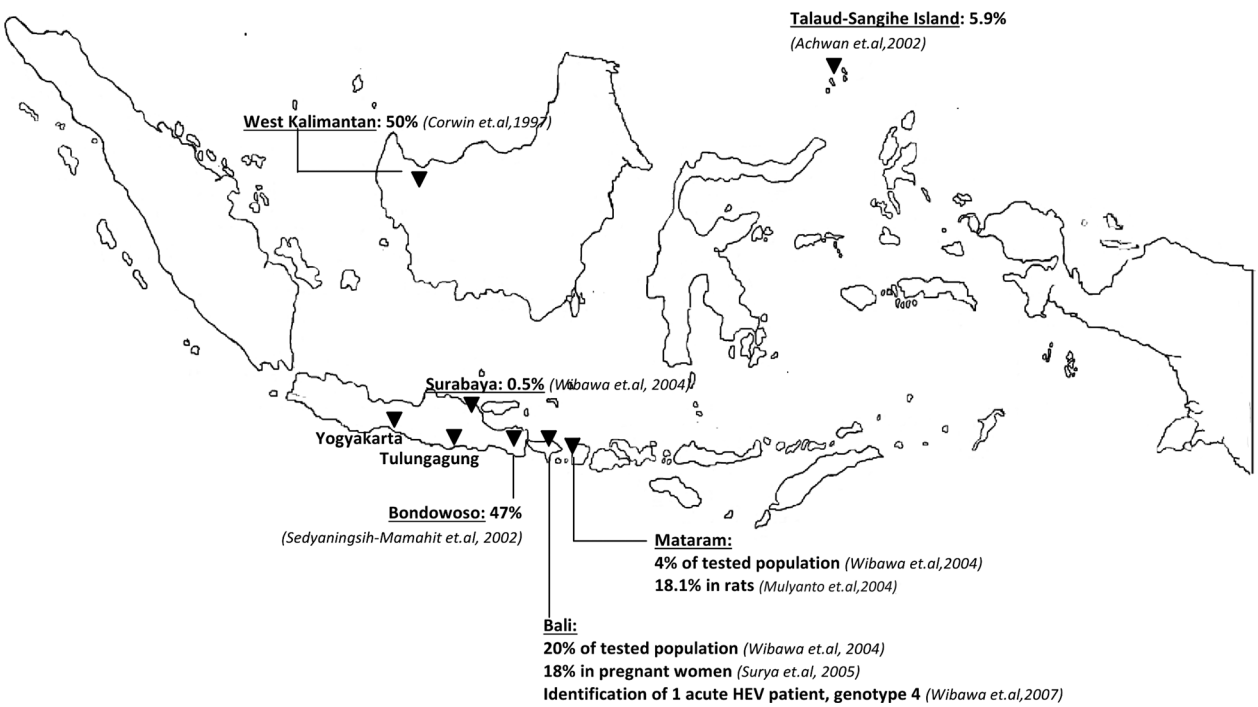


Fig. 1. Map of Indonesia showing locations of previous studies on HEV and the anti-HEV IgG prevalence reported by them.

Table 1. Characteristics of samples from different regions in Indonesia

	Java		Bali	
	Yogyakarta	Tulungagung	Mengwi	Denpasar
Swine workers	n = 137	n = 12	n = 64	
	90 male; 47 female	10 male; 2 female	21 male; 43 female	
	47.5 ± 11.6 years	45.5 ± 12 years	32.5 ± 15.4 years	
Local residents	n = 100	n = 42	n = 94	n = 41
	94 male; 6 female	11 male; 31 female	33 male; 61 female	23 male; 18 female
	31.7 ± 10.3 years	35.1 ± 10.7 years	31.3 ± 17.2 years	5.7 ± 3.9 years
Swine sera	n = 73	n = 89	n = 119	
Swine feces	n = 27			

Age is shown as mean ± SD.

groups (0–14, 15–40, and >40 years) were also analyzed. These age groups were based on the results of a previous study (16). The study protocol was approved by the Medical and Health Ethics Committees of Gadjah Mada University, Airlangga University (Indonesia) and Kobe University (Japan). Informed consent was obtained from all human volunteers.

Serological marker testing

Serum samples were tested for HEV antibody using a species-independent, double-antigen, sandwich enzyme-linked immunosorbent assay (ELISA; MPD HEV ELISA 4.0v; MP Biomedicals Asia Pacific Pte, Singapore) that is able to detect the presence of specific antibodies including IgG, IgM and IgA. Originally recommended for veterinary purposes, it is now commonly used worldwide for both veterinary and human studies. Human test samples with optical density values equal to or greater than a cut off value of 0.4543 were considered anti-HEV antibody positive; for swine samples the cut-off value was 0.2543.

Detection of viral RNA

In this study, HEV infection was serologically screened for by ELISA, which can detect both acute and past infection. HEV-RNA is usually detected only in the acute phase. Subsequently, all 249 anti-HEV-positive swine and human sera were tested for HEV-RNA by RT-PCR. Viral RNA was extracted from 140 μL of serum using a QIAamp Viral RNA Kit (Qiagen GmbH, Hilden, Germany). The extracted RNA was reverse-transcribed to cDNA using SuperScript III RT (Invitrogen, Carlsbad, CA, USA) with an antisense primer (HE040; 5′-CCC TTR TCC TGC TGA GCR TTC TC-3′ [R = A or G]) specific for the HEV ORF2 region. The transcribed cDNA was used to amplify HEV-RNA by nested PCR. In the first round, the primer pair HE044 (sense: 5′-CCA GGH TGG CGY TCK GTT GAG AC-3′ [H = A, T or C; YT or C; and KG or T] and HE040 was used. HE110-2 (sense primer, a mixture of three sequences: 5′-GYT CKG TTG AGA CCT CYG GGG T-3′, 5′-GYT CKG TTG AGA CCA CGG GYG T-3′ and 5′-GYT CKG TTG AGA CCT CTG GTG T-3′) and HE041 were used in the second round (ORF2 PCR). The PCR amplification was performed as previously described (17) and involved 35 cycles in the first round (denaturation at 94°C for 30 s [with an additional 2 min in the first cycle], annealing at 55°C for 30 s, extension at 72°C for 75 s [an additional 7 min was used in the first cycle]). The same conditions were used for the 25 cycles in the second round, except that the extension step lasted 60 s.

Amplification with nested PCR was also performed to confirm the presence of HEV-RNA targeting a region of ORF1 with primer sets consisting of HE090 (sense: 5′-GCA GAC CAC RTA TGT GGT CGA YGC C-3′) and HE094 (antisense: 5′-TGG CGG RTA TGT GGT CGA YGC C-3′) for the first round, and HE092 (sense: 5′-TGT GGT CGA YGC CAT GGA GGC CCA-3′) and HE095 (antisense: 5′-CCR TCR AAR CAG TAA GTS CGG TC-3′ [S = G or C]) (17).

Determination of genotyping by phylogenetic analysis

The amplified PCR products from the second round were sequenced directly by dideoxy sequencing using a Taq Dye Deoxy Terminator Cycle Sequencing Kit with a 3100-Avant genetic analyzer (Applied Biosystems, Foster, CA, USA). The designations and accession numbers of the full-length reference sequences representing the different genotypes for analysis of HEV ORF1 and ORF2 were retrieved from GenBank and are as follows: AF082843, AF060668, AF060669, FJ426403, AB0898241, D10330, AY204877, M74506, AP003430, AB091394, AB236320, AY115488, EU375463, AB290312, AB074915, AB074917, AB108537, EU676172, DQ279091, AB220972, AB298180,

AB298183, AB298178, AB298179, AB124818, AB097811, AB099347, AJ272108, AY594199, AB082558, AF324502, AF324503, EU723512, EU723516, AF455784, AB369691, AB222183 and AB248522. The sequences were aligned using ClustalX software. The neighbor-joining method was used to construct the phylogenetic trees. The analyses were carried out using Molecular Evolutionary Genetics Analysis software (18).

Statistical analysis

Categorical variables were compared using χ^2 test or Fisher's exact test. Results with P values of <0.05 were considered statistically significant. SPSS/PASW Statistics Software version 18.0 (SPSS, Chicago, IL, USA) was used for all analyses.

RESULTS

Prevalence of anti-hepatitis E virus antibody seropositivity among swine farm workers and local residents in Java and Bali

After combining results of samples from Yogyakarta and Tulungagung as all from Java, a total of 5.1% of participants were anti-HEV antibody positive, comprising 10/149 (6.7%) swine farm workers, and 5/142 (3.5%) local residents. The total prevalence of anti-HEV in humans in Bali was 11.6%. Of 64 workers and 135 local residents from Bali, 12 (18.8%) and 11 (8.1%), respectively, were anti-HEV antibody positive (15). The overall prevalence of anti-HEV antibody seropositivity in Bali was significantly higher than that in Java ($P = 0.015$). Significantly more swine farm workers from Bali than workers from Java were anti-HEV antibody seropositive ($P = 0.013$). Although the prevalence of HEV antibody seropositivity in local residents from Bali was also higher than that in Java, this difference was not statistically significant. The rate of anti-HEV antibody seropositivity was also not significantly different between local residents and swine farm workers in Java. The overall prevalence of anti-HEV seropositivity in those aged 15–40 years in Bali was higher than that in other generations in Bali, and also than in the same age group in Java ($P = 0.002$; Table 2). In this study, no HEV-RNA was detected in any human sample.

Prevalence of anti-hepatitis E virus antibody seropositivity among swine in Java and Bali

In all, 281 swine sera were obtained from different regions in Java and Bali. The age of the swine ranged from 1 to 6 months. After combining the Yogyakarta and Tulungagung samples as all from Java, the prevalence of anti-HEV antibody seropositivity in 1- and 2-month-old swine was found to be 5.2% (1/19) and 43.3% (13/30), respectively. Eleven of 16 (68.8%) 1-month-old swine and 18/20 (90%) 2-month-old swine in Bali were positive for anti-HEV antibodies (15). There were significant differences between Java and Bali in the rates of anti-HEV antibody seropositivity, seropositivity in the 1- and 2-month-old swine being higher in Bali than in Java ($P < 0.001$ and $P = 0.01$). The prevalence rates in 3- to 6-month-old swine did not differ significantly between Java and Bali, nor did the overall prevalence rates of anti-HEV antibody seropositivity in swine from the two regions (Table 3).

Identification of hepatitis E virus-RNA in swine sera

Swine HEV-RNA was identified in sera by nested RT-PCR. In this study, two new HEV RNA isolates were identified from 3-month-old swine and one from

Table 2. Total prevalence of anti-HEV antibodies in humans from two different communities in Indonesia (Java and Bali)

	Javanese individuals ($n = 291$)	Balinese individuals ($n = 199$)	*P*-value
Sex			
Male	11/206 (5.3%)	8/76 (10.5%)	NS
Female	4/85 (4.7%)	15/123 (12.2%)	NS
Age (years)			
0–14	0/0	3/79 (3.8%)	n/a
15–40	8/153 (5.2%)	13/76 (17.1%)	0.002
≥40	7/138 (5.1%)	7/44 (15.9%)	NS
Local residents	5/142 (3.5%)*	11/135 (8.1%)†	NS
Swine farm workers	10/149 (6.7%)*	12/64 (18.8%)†	0.013
Overall prevalence	15/291 (5.1%)	23/199 (11.6%)	0.015

n/a, not analyzed; NS, not significant.

*, comparison between local residents and swine farm workers in Java ($P = 0.291$). †, comparison between local residents and swine farm workers in Bali ($P = 0.035$).

Table 3. Prevalence of HEV antibody seropositivity and detected HEV-RNA in swine from different breeding settings in Java and Bali according to age

		Java ($n = 162$)	Bali ($n = 119$)*	*P*-value
Age (months)	1	**1/19 (5.2%)**	**11/16 (68.8%)**	**<0.0001**
		0/19 (0%)	*0/16 (0%)*	
	2	**13/30 (43.3%)**	**18/20 (90.0%)**	**0.01**
		0/30 (0%)	*1/20 (5%)*	
	3	**36/40 (90%)**	**17/21 (80.9%)**	**NS**
		3/40 (7.5%)	*0/21 (0%)*	
	4	**25/29 (86.2%)**	**20/21(95.2%)**	**NS**
		0/29 (0%)	*0/21 (0%)*	
	5	**29/33 (87.9%)**	**18/21 (85.7%)**	**NS**
		1/33 (3.1%)	*0/21(0%)*	
	6	**10/11 (90.9%)**	**14/20 (70.0%)**	**NS**
		0/11 (0%)	*0/20 (0%)*	
Overall prevalence		**114/162 (70.3%)**	**97/119 (81.5%)**	**NS**
		4/162 (2.4%)	*1/119 (0.8%)**	

Upper row indicates prevalence of HEV antibody seropositivity in bold, bottom row indicates HEV-RNA detection in italics.
*From Utsumi *et al*. (15). NS, not significant.

a 5-month-old pig, all from Yogyakarta. The Java (Yogyakarta) strains were reported to GenBank and given the accession numbers AB714131 for YKSB26, AB714132 for YKSB51 and AB714133 for YKSB52. Nucleotide sequences of the two previously detected isolates from Tulungagung and Mengwi (Bali) strains had previously been reported under the accession numbers AB541111 and AB541112 (15).

Genetic analysis of Java and Bali isolates of swine hepatitis E virus

The five HEV isolates from swine sera were genetically analyzed by comparing their conserved ORF2 with those of other swine and human isolates. Phylogenetic analysis showed that the Tulungagung (East Java) and Mengwi (Bali) strains showed strong associations with other Bali isolates of genotype 4, the nucleotide differences being 7.2–8% and 2.2–4.1%, respectively (Table 3, Fig. 2). The YKSB26, YKSB51, YKSB52, YKSF2, YKSF23, YKSF24 and YKSF25 isolates from Yogyakarta (center of Java) formed a distinct branch of genotype 3 and showed strong associations with a USA human isolate (US2; nucleotide similarity, 90.3–91.4%), a Korean swine strain (swKOR1; nucleotide similarity, 88.5–93.2%), and a human isolate from a Japanese patient with acute hepatitis (HE-JA10; nucleotide similarity, 89.3–91.5%; Fig. 3). When the amino acid sequence of part of ORF 2 was compared with reported HEV strains, the Yogyakarta strains were found to have 99.2–100% identity. The Tulungagung strain had 96.6% similarity, whereas the Mengwi strain showed 100% amino acid similarity with other Bali isolates. Alignment of the amino acid sequences are shown in Figure 4.

Identification and confirmation of swine hepatitis E virus-RNA in swine feces

Twenty-seven fecal samples were collected in Java for this study. HEV-RNA was detected in four (14.8%) of these samples. The Java strains from swine feces YKSF2, YKSF23, YKSF24 and YKSF25, were reported to GenBank and given accession numbers AB714127, AB714128, AB714129 and AB714130, respectively. The YKSF23, YKSF24 and YKSF25 strains were isolated from 2-month-old pigs, whereas the YKSF2 isolate was from a 4-month-old pig. Based on ORF2, all the fecal strains were identified as HEV genotype 3, and their genetic similarity with serum samples was 96.2–99.7% (Table 4). This genotype was also confirmed based on ORF1 (Fig. 3), genetic similarity to the closest nucleotide sequences being 88.5–92.5% (Table 5). Amino acid comparison with other genotype 3 strains showed similarity of 97.8–100%. The comparison of amino acid sequence alignment of part of ORF 1 can be seen in Figure 4.

DISCUSSION

In this study, the overall prevalence of anti-HEV antibodies in humans, regardless of whether they were swine farm workers or community members, is consistent with previous data that showed seropositivity for anti-HEV antibodies was much higher in Bali than in Java

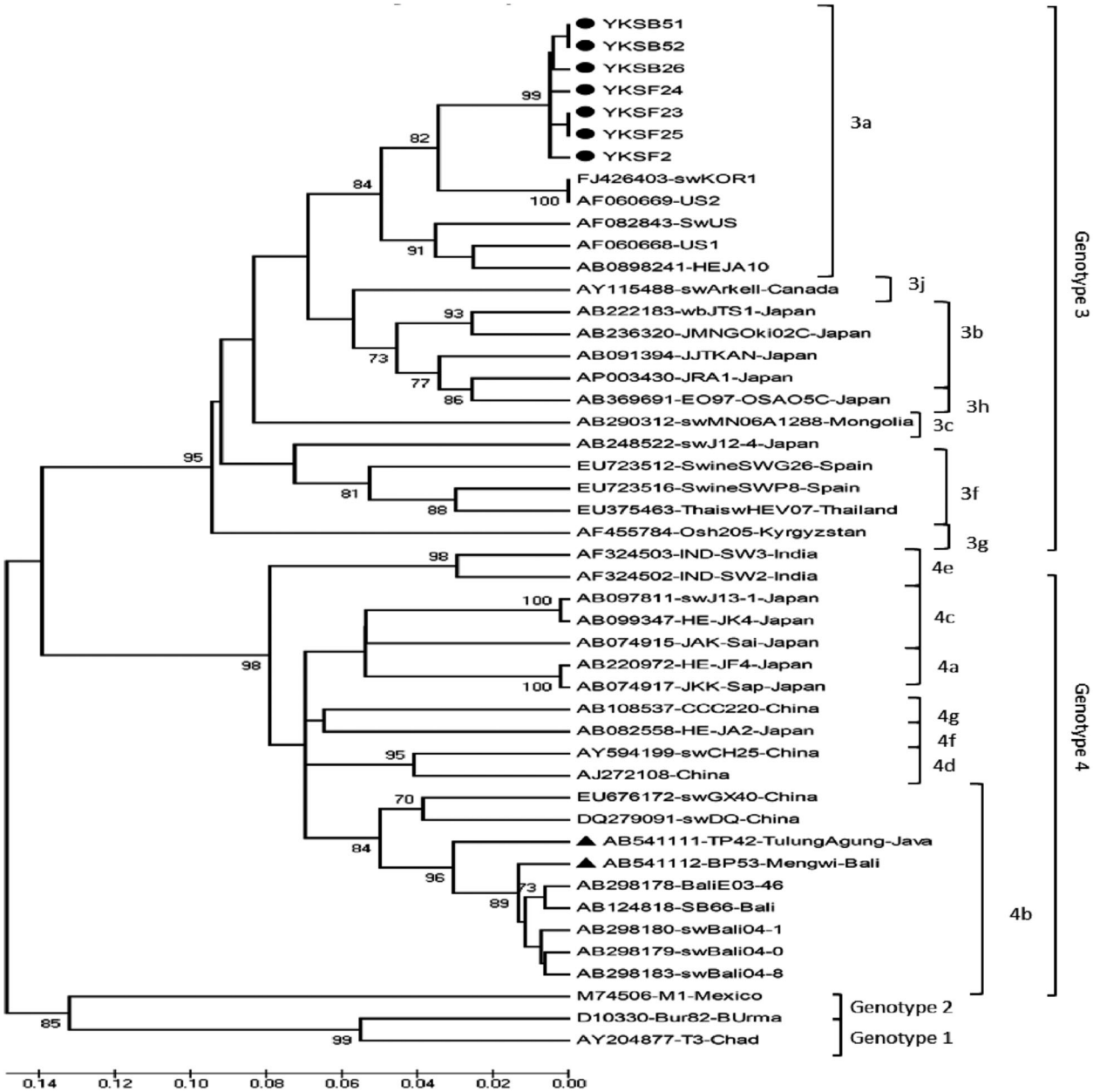


Fig. 2. Phylogenetic tree analysis of the 460 bp ORF2 of hepatitis E virus genotypes 1–4. The Yogyakarta strains isolated in this study are indicated by solid circles and the Tulungagung and Mengwi strains by solid triangles. The bootstrap consensus tree inferred from 1000 replicates is indicated for each branch. The evolutionary distances were computed using the maximum composite likelihood method.

($P = 0.015$). In this study, even though anti-HEV antibody seropositivity was higher in the 15–40-year age group than in the other age groups, this difference was not statistically significant in either Bali or Java. However, people aged 15–40 years in Bali were more likely to be positive for anti-HEV antibody than were individuals in the same age group in Java ($P = 0.002$). This is possible because Balinese individuals are known to associate closely with pigs in their daily lives, because they raise pigs as domestic animals and use them in religious ceremonies. They also commonly consume raw pig viscera and fresh blood mixed with vegetables (14). The swine farm workers in Bali also had a higher prevalence of anti-HEV antibodies than did workers in Java. The breeding conditions in Bali are more traditional than those in Java. In Bali, pigs are raised near houses as domestic

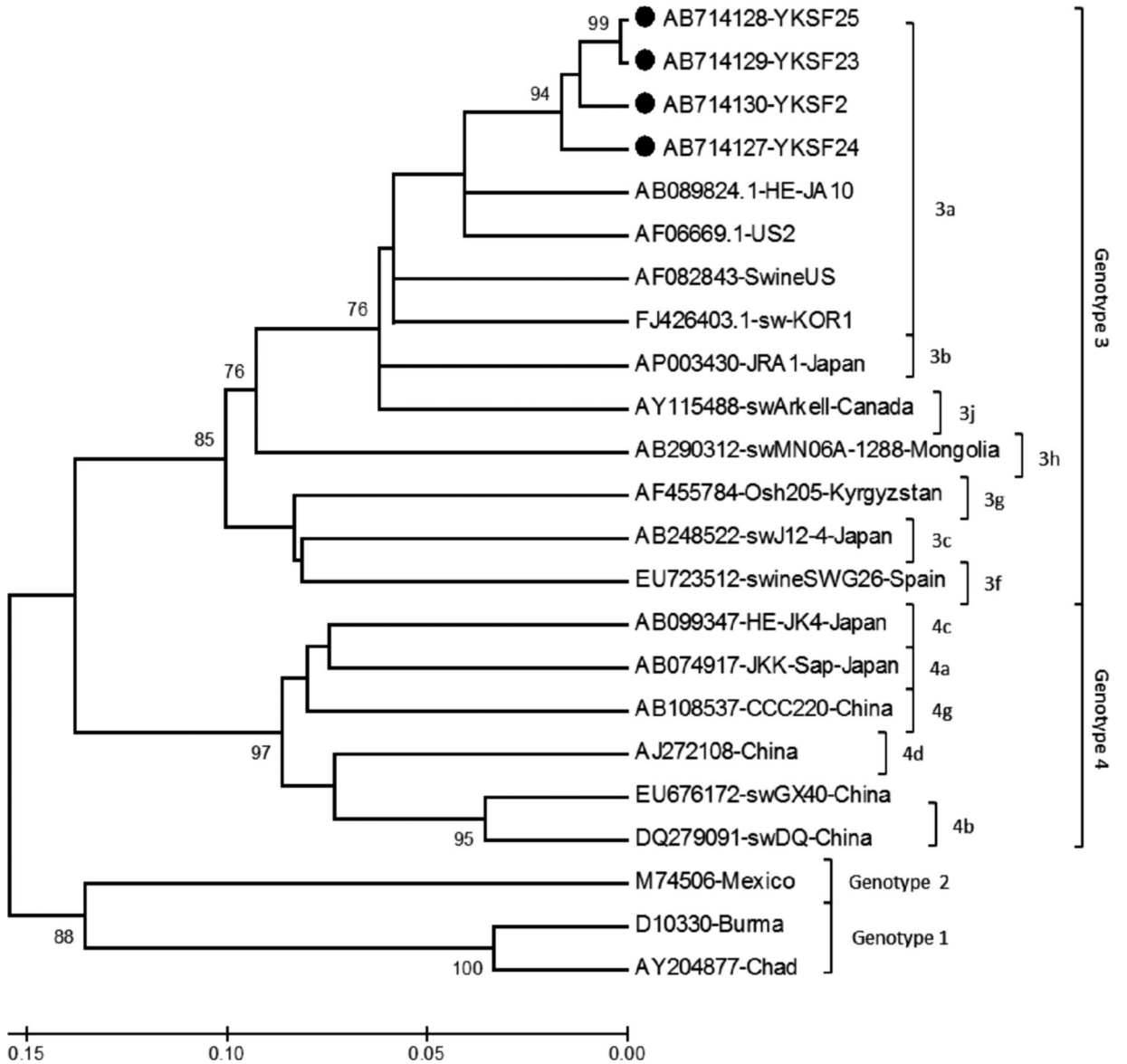
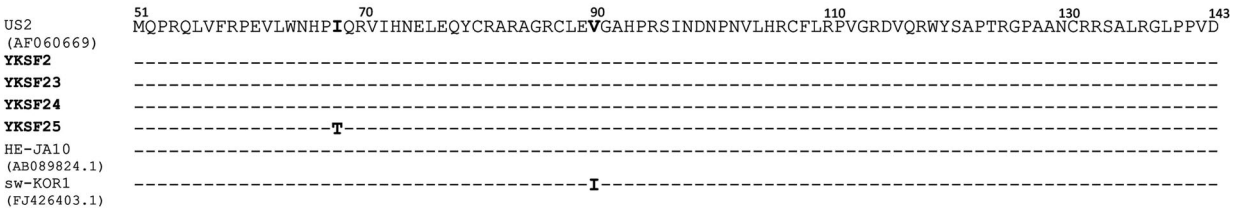


Fig. 3. Phylogenetic tree analysis of the 540 bp ORF1 of hepatitis E virus genotypes 1–4. The Yogyakarta strains isolated in this study are indicated by solid circles. The bootstrap consensus tree inferred from 1000 replicates is indicated for each branch. The evolutionary distances were computed using the maximum composite-likelihood method.

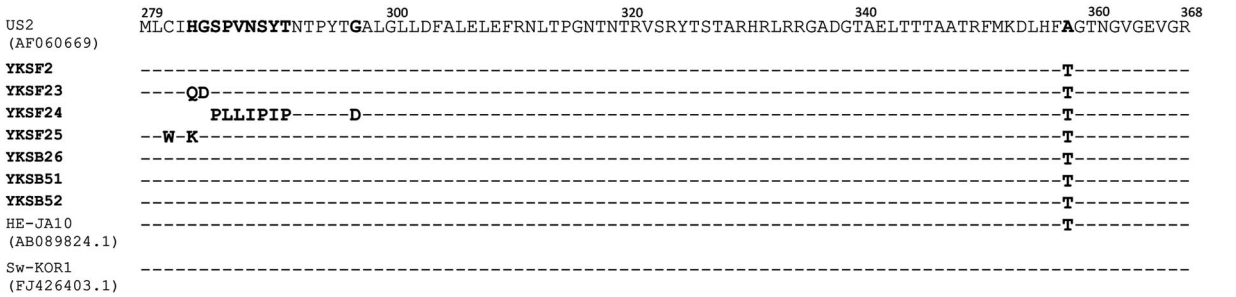
animals whereas the pig farms in Java are usually isolated from the population and have better sanitation systems. As a consequence, farm workers in Bali have more direct contact with pigs, which could be responsible for the higher prevalence of anti-HEV antibodies among swine farm workers in Bali than in Java. The prevalence of anti-HEV antibody seropositivity among local residents tended to be higher in Bali than in Java; however, this difference was not significant. Even though Javanese people do not have traditions or customs of close contact with swine like Balinese people, the prevalence of anti-HEV antibodies among swine farm workers and local residents in Java was not significantly different ($P = 0.291$).

Serologic surveys have revealed that hepatitis E has high prevalence globally. There is high seroprevalence not only in countries like Egypt where hepatitis E is known to be endemic, but also in developed countries (5, 19, 20). Despite identification of anti-HEV antibodies and HEV-RNA in feces and sera from many mammalian hosts other than swine, clear evidence for animal to human transmission has been derived from the isolation of identical viruses from uncooked meat that infected people have eaten and

a Alignment of amino acid sequences of the Yogyakarta samples compared with other genotype 3 isolates in the partially conserved ORF 1 Region



b Alignment of amino acid sequences of the Yogyakarta samples compared with other genotype 3 isolates in the partially conserved ORF 2 Region



c Alignment of amino acid sequences of the Tulungagung and Mengwi, Bali samples compared with other genotype 4 isolates in the partially conserved ORF 2 Region

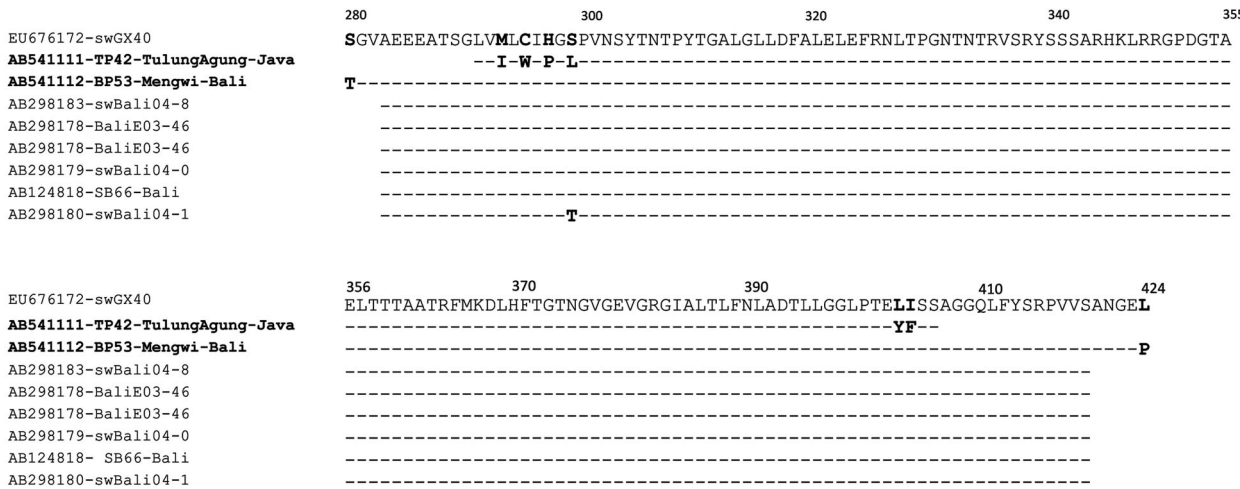


Fig. 4. Alignment of amino acid sequences of the Yogyakarta, Tulungagung and Mengwi (Bali) samples compared with other genotype 3 and 4 isolates. The consensus sequences are shown at the top of each part figure. Samples from this study are indicated in bold.

remnant frozen meat (8, 21). So far, there is no evidence that any route of transmission other than consumption of uncooked meat from domestic animals is a source of HEV infection in the Javanese community; this practice causes interspecies infections similar to that with swine.

In our study, the prevalence of anti-HEV antibodies in Java was relatively high (5.2%) compared with other societies, such as those of Mataram and Surabaya, in which there is no close association with swine. Mataram and Surabaya, where the prevalence rates were 4% and 0.5%, respectively, have similar backgrounds to Java (Fig. 1) (14). All samples from local residents in Java that were positive for anti-HEV antibodies were from the Yogyakarta district. In Yogyakarta, even though the farms are centralized in one area and isolated from the population, the pens are located along a small river into which the waste drains. Although people near the

Table 4. Nucleotide sequence similarity rates for the ORF2 of swine HEV from Yogyakarta, Tulungagung and Mengwi isolates

Yogyakarta isolates	YKSF2 (%)	YKSF 23 (%)	YKSF 24 (%)	YKSF 25 (%)	YKSB 26 (%)	YKSB 51 (%)	YKSB 52 (%)	TP42 (%)	BP53 (%)
AB714128-YKSF23 (Indonesia)	98.7								
AB714129-YKSF24 (Indonesia)	96.8	97.0							
AB714130-YKSF25 (Indonesia)	99.2	99.5	96.5						
AB714131-YKSB26 (Indonesia)	98.9	98.9	96.2	98.7					
AB714132-YKSB51 (Indonesia)	99.7	99.5	96.8	99.2	99.5				
AB714133-YKSB52 (Indonesia)	99.2	98.9	97.3	98.4	98.6	99.2			
AF082843-SwUS (US)	89.8	90.0	88.8	89.5	89.6	89.8	89.4	75.9	78.7
AF060668-US1 (US)	90.5	91.4	87.7	90.4	90.5	89.3	91.0	76.7	78.4
AF060669-US2 (US)	91.1	91.4	90.3	91.1	91.0	91.1	91.0	76.1	79.5
FJ426403-sw-KOR1 (Korea)	91.4	92.5	88.5	91.3	90.8	90.4	93.2	76.7	79.9
AB089824.1-HE-JA10 (Japan)	91.5	91.0	89.3	91.5	91.5	91.0	91.0	76.1	79.5

Tulungagung and Mengwi isolates	TP42	BP53	SwBaliE04-1	SwBaliE04-8	SwBali 03-46	swBali E04-0	SB-66
AB541112-BP53 (Indonesia)	92.0	100.0					
AB298180-SwBaliE04-1 (Indonesia)	92.2	96.6	100.0				
AB298183-SwBaliE04-8 (Indonesia)	92.8	96.8	97.3	100.0			
AB298178-SwBali03-46 (Indonesia)	92.8	96.6	98.1	97.3	100.0		
AB298179-swBaliE04-0 (Indonesia)	92.0	97.8	97.8	97.6	97.8	100.0	
AB124818-SB66 (Indonesia)	92.8	95.9	97.3	97.1	98.3	97.1	100.0
EU676172-swGX40 (China)	88.5	91.5	90.8	89.8	90.3	89.5	90.8
DQ279091-SwDQ (China)	86.2	89.4	89.3	89.3	89.1	88.6	89.3
AB220972-HE-JF4 (Japan)	85.3	89.1	87.3	86.9	87.4	85.9	87.1

Table 5. Nucleotide sequence similarity rates for the ORF1 of swine HEV from Yogyakarta isolates

	YKSF2 (%)	YKSF23 (%)	YKSF24 (%)	YKSF25 (%)
YKSF23	98.5	100.0		
YKSF24	96.8	97.2	100.0	
YKSF25	98.2	99.6	97.5	100.0
AF060669-US2 (USA)	92.4	92.5	90.9	92.1
FJ426403-sw-KOR1 (Korea)	90.7	90.2	89.6	88.5
AB0898241-HE-JA10 (Japan)	92.4	92.2	91.4	92.4

farming area claim they never use the water for daily use, this river flows across towns near houses and untreated water is used to fertilize crops. The residents of Yogyakarta generally show adequate awareness of relatively healthy living habits. They tend to drink water from a well or tap water and regularly boil the water before consumption. However, further studies are needed to determine whether the relatively high prevalence of anti-HEV antibodies in Yogyakarta district, Java is caused by consumption of contaminated river water or by contact with other hosts such as domestic or wild animals. For example, Mulyanto *et al*. reported hepatitis E virus variants in wild rats (*Rattus rattus*) in Indonesia (22). Hereafter, it will be important to investigate the possibility that humans can be infected with HEV via other host animals besides swine. In this study, no HEV-RNA was detected in any human samples. Unlike hepatitis A, in which a small dose of infectious inoculum can cause fulminant hepatitis, higher doses of inoculum are needed for HEV infection to cause acute clinical manifestations. Thus most HEV infections have a clinically silent course. The specific mechanism leading to different clinical outcomes of HEV infection is not fully understood. In addition to the viral factors that have been mentioned, host factors such as stage of liver disease, pregnancy or distinct genetic polymorphisms play a role in determining the course of HEV infection (23).

In this study, the overall prevalence of anti-HEV antibody was not significantly different between swine from Java and Bali. However, there were significant differences in the prevalence rates in 1- and 2-month-old swine, the prevalence in Bali being 68.7% and 90%,

respectively, whereas in Java it was 5.2% and 43.3%, respectively ($P < 0.001$; $P = 0.01$, respectively). These findings are consistent with those of our previous study (14). In swine, the prevalence of anti-HEV antibodies increases with age and remains high until the age of slaughter (usually 6–8 months). Analysis of anti-HEV antibody seropositivity by age group shows that swine aged 3–5 months contribute most to the overall rate (data not shown). Newborn piglets are likely to acquire passive immunity (IgG) through their mothers' colostrum. A previous study showed piglets to be positive for anti-HEV IgG in the absence of infectious markers such as anti-HEV IgM, HEV antigen, and RNA; however, the seropositivity was transient, lasting for approximately 60 days (24). Therefore it is acceptable to assume that the presence of anti-HEV antibody in older piglets is the result of HEV infection and not from their mothers' colostrum. HEV infection mostly occurs at approximately 2-month of age (25). In Bali, piglets are kept with older swine, whereas swine in Java are grouped in cages according to their age. Because the swine in Bali are mixed from birth, the risk of infection at an early age is much greater than that in Java. Fecal–oral transmission is believed to be the natural route of HEV infection among swine. Direct and repeated contact among large numbers of swine in the same pen would increase the likelihood of repeated exposure to swine excreting high titers of HEV in feces (26). This may explain why the prevalence of anti-HEV antibodies was high in all age groups of swine.

Hepatitis E virus-RNA is typically isolated from fecal and serum samples from 2- to 4-month-old swine, albeit with varying prevalence rates (27). In this study, the Java and Bali isolates were detected in 2- to 5-month-old swine, the serum and fecal detection rates being 1.6% and 14.8%, respectively. Aside from the high detection rate of HEV-RNA in this study, it is also much easier to amplify ORFs from fecal samples. Serum samples were only positive for the ORF2 region whereas fecal samples were positive for both ORF1 and ORF2. Even though we tested only a small number of fecal samples, our data support the results of other studies suggesting that fecal samples are more suitable for detecting HEV RNA.

Genotype 3 is mainly found in the USA, Japan and Europe, whereas genotype 4 is mainly found in Asia, including China, Japan and India (4). The present study provides evidence that multiple genotypes of swine HEV are circulating in Indonesia. The seven newly isolated swine HEV strains from Yogyakarta (center of Java) belonged to genotype 3, their closest relationship being to the US2 and HE-JA10 isolates identified from USA and Japanese patients with acute hepatitis and with a swine isolate from Korea (28–30) (Figs 1,2). Meanwhile, the Tulungagung (East Java) and Mengwi (Bali) isolates were classified as genotype 4: they showed the closest nucleotide similarity with other Bali isolates (10, 14) (Fig. 1). HEV from a patient with acute hepatitis E in Bali was reportedly genotype 4 and was identical to the strain found from swine (14). The identification of genotype 3 in Indonesia indicates that this genotype may be a cause of HEV infection of humans, especially in the Yogyakarta region. Therefore, this information will hopefully raise awareness in interested groups and clinicians, particularly those who work with hepatitis patients in Indonesia.

Phylogenetic tree analysis showed that all the Yogyakarta isolates form a cluster divergent from the branch of the USA, Korean and Japanese isolates, which were grouped into subgenotype 3a, with a bootstrap value of 99%. Based on a global genotyping study of HEV isolates (4), the nucleotide differences among isolates belonging to subgenotype 3a are as high as 0.7–6.0%. Meanwhile, the nucleotide differences between the Yogyakarta (center of Java) isolates and the US2 strain are as high as 8.6–9.7%. This suggests that the Yogyakarta strain is likely to be independent of the other strains in subgenotype 3a. Similarly, the genotype 4 isolates from Tulungagung (East Java) and Mengwi (Bali), along with other Bali isolates, are clustered in a divergent branch from swDQ and swGX40 of subgenotype 4b from China, to which they showed the closest nucleotide similarity, with a bootstrap value of 96%. However, the Bali cluster and the TP42 isolate from Tulungagung (East Java) are genetically different, suggesting that the swine HEV genotype 4 strain in Java belongs to a subclassification divergent from the lineage of Balinese strains (14). These results demonstrate the variety of isolates belonging to genotype 4 that are circulating in Indonesia, and also suggest that these isolates may be indigenous to Indonesia.

So far, no international consensus on the classifications of HEV has been developed. One of the most widely accepted studies on the classification of genotypes and subgenotypes of HEV was published in 2006 (4). However, since then further studies describing identification of new HEV strains that could not be classified have been published (31). Therefore, a new universal system for classifying HEV based on a consensus of molecular experts will be particularly important to avoid overlapping definitions of HEV subgenotypes in the future.

In conclusion, we have shown that regions with local customs involving close contact with swine have a higher prevalence of anti-HEV seropositivity in humans. However, we did not identify significant differences in the prevalence of anti-HEV antibodies between swine farm workers and local residents in a population that does not associate closely with swine. Therefore, it is important

to identify routes of HEV transmission other than close and direct contact with swine. Swine isolates from Indonesia belonging to genotypes 3 and 4 are phylogenetically different from previously reported HEV strains. To the best of our knowledge, this is the first report of swine HEV belonging to genotype 3 in Indonesia.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Teguh Triyono, Professor Dr. Ir. Endang Baliarti and Dr Ir. I. Gede Suparta Budisatria for their advice and help with sample collection. This study was supported by a Grant-in-Aid from the Japan Initiative for Global Research Network on Infectious Disease (J-GRID) program from the Ministry of Education, Culture, Sports, Science and Technology, Japan and a SATREPS Grant from Japan Science and Technology Agency and Japan International Cooperation Agency.

DISCLOSURE

The authors have no conflicts of interest to declare.

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