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Molecular detection of Hepatitis E virus in rivers in the Philippines

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27 **Abstract**

28 To understand the HEV-pollution status in the environment in the Philippines, a total
29 of 12 water samples were collected from rivers in Manila City for detection of HEV
30 RNA. Three out of 12 samples were positive for HEV RNA indicating that HEV is
31 circulating in the Philippines. Phylogenetic analysis classified all of the HEV sequences
32 into genotype 3.

33

Hepatitis E virus (HEV) is a positive-sense single-stranded RNA virus that belongs to the genus *Hepevirus* in the family *Hepeviridae* [1, 2, 3]. HEV is the causative agent of acute or fulminant hepatitis E, primarily transmitted by the fecal-oral route[4]. The relatively high mortality rate in HEV-infected pregnant women, up to 28%, is unique among hepatitis viruses [1, 5]. Hepatitis E is a zoonotic disease, with swine, wild boars and wild deer serving as the reservoir for human infections [6, 7, 8]. Four genotypes of HEV (G1-G4) have been detected in humans and G3 and G4 HEV are responsible for sporadic and autochthonous infections in both humans and other animal species worldwide [9, 10, 11, 12].

HEV is a public health concern in many Asia and Africa countries where sanitation conditions are insufficient [13, 14]. Large waterborne outbreaks with high attack rates among young adults have been described in regions characterized by poor sanitary conditions in countries such China, Indian, Somalia, and Uganda[15]. However, there have been no reports of HEV infection in the Philippines. Neither information about hepatitis E patients, nor HEV infection in animals has been reported, and no sequence data has been deposited from this country. There is also no report of the HEV-pollution status of the environmental sewage water. With the hypothesis that environmental water samples may reflect the prevalence of HEV circulation, we examined river water samples to investigate HEV in the environment in one of the most densely populated cities in the world Manila City, a metropolitan area in the Philippines with over 10 million residents.

A total of 12 water samples were collected from rivers that run through Manila City. Six sampling sites were selected (Fig. 1). Sampling sites 1 to 3 were in the Pasig River, sites 4 and 5 were in the Paranaque River, and site 6 was in the Las Pinas River. These rivers receive the wastewater from the residents nearby. Water samples were drawn at all locations during both the dry season (2012.12.23) and the wet season (2013.07.23), and were named

D1 to D6 and W1 to W6, respectively. The water samples were kept at 4°C during transport.

The concentration and purification of these water samples was carried out as described previously[16]. Briefly, 500 mL of water was collected from each sampling site, and centrifuged at 3,000 rpm for 30 min at 4°C. Then 2.5 mM $MgCl_2$ was added to the supernatant to a final concentration of 0.05 mM. The pH value was adjusted to 3.5. The solution was filtered through a 0.45-μm mixed cellulose ester membrane filter (Merck Millipore Japan) by a positive-pressure pump. Absorbents on the filter were then eluted with 10 mL of 3% beef extract solution by ultrasonication, three times. The solution was centrifuged at 12,000 rpm for 30 min, and the supernatant was stored at −80°C until RNA extraction.

The RNA was extracted using the MagNA Pure LC Total Nucleic Acid isolation kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations. Reverse transcription (RT) was performed with a high-capacity cDNA reverse transcription kit (ABI Applied Biosystems) at 25 °C for 10 min, 37 °C for 120 min followed by 85 °C for 5 min in a 20-μl reaction mixture containing 1 μl reverse transcriptase, 2 μl of the random primer, 1 μl RNase inhibitor, 2 μl RT buffer, 0.8 μl 10-mM deoxynucleoside triphosphates, 8 μl RNA and 5.2 μl distilled water. A nested reverse transcription polymerase Chain Reaction (RT-PCR) analysis was performed to amplify a portion of the ORF2 genome, based on the method described previously[17].

By RT-PCR, three samples (W4, W5 and W6) out of the 12 water samples were positive for HEV RNA. Excluding the primer sequences, the length of the nested RT-PCR products was 338 nucleotides corresponding to nt 5959-6296 in the ORF2 of the Myanmar strain (D10330). PCR products were purified using the QIAquick PCR purification kit (Qiagen) and cloned into TA cloning vector pCR2.1 (Invitrogen). Each of 20 clones was sequenced. The clones with the same nucleotide sequence were

counted as one strain. Finally, 21 HEV strains were obtained (GenBank accession nos. KF546257-KF546277), of which five strains were isolated from W4, 10 strains from W5, and six strains from W6. Phylogenetic analysis indicated that all 21 strains were G3 HEV. With the exception of strain W5-13, the other 20 strains' sequences belonged to sub-genotype 3a[18], separated into four clusters (Cluster1 to 4) with nucleotides sequence identities of 89.6%-99.7% (Fig. 2). In cluster 1, the sequences of three strains isolated from W6 were close to that of HEV strain EF530663 (isolated from a patient in Hungary) with nucleotide sequence identities of 92.3% to 92.6%. The nucleotide sequences of all nine of the strains in cluster 2 detected from W5 were close to that of a Japan swine HEV strain (AB094215) with identities of 91.1%-92.6%. Cluster 3 contained six strains three from W4 and three from W6. Their sequences were close to that of AB671098, isolated from a Japanese donor, with nucleotide sequence identities of 93.5%-94.4%. Cluster 4 comprised two strains from W4, with sequences close to Japan strain AB 807429 (identities of 91.7%-92.0%). The strain W5-13 dose not belong to any known sub-genotype and shares identities of 84.0%-84.3%, 90.2%-91.7%, 85.5%-88.2% and 83.7%-84.0%, with the Philippines HEV strains in clusters 1 to 4, respectively. The strain W5-13 thus constitutes a new sub-genotype of G3 HEV.

A BLAST analysis showed that the nucleotide sequence identities between these HEV strains detected in the Philippines and other HEV strains that have been published in GenBank were lower than 94.4%, indicating that area-specific HEV strains are circulating in the Philippines. All 21 of the HEV strains we detected in the river water were collected during the wet season, suggesting that the wet season presents a higher risk of individuals in the area contracting HEV infections.

The results of this study beg the question, what is the source of HEV detected in the Manila City rivers? Because no epidemiological information about HEV in the Philippines is currently available, for human patients, animal outbreaks, or genetic sequences, it is difficult to speculate about the sources of HEV. However, since the HEV

115 is primarily transmitted by the fecal-oral route, HEV might be present in rivers
116 containing human or animal stool. In this study, all of the HEV strains were detected
117 from sampling sits 4-6, located in the Paranaque River and the Las Pinas River. None of
118 the water samples from the Pasig River (sampling sits 1-3) were found to be HEV RNA
119 positive. The Paranaque River and the Las Pinas River are considerably smaller than the
120 Pasig River, and flow through a residential area having high population density. The
121 degree of wastewater pollution is higher for sampling sits 4-6 than for sampling sits 1-3.
122 All of the HEV detected in the river water samples belonged to G3. Genotype 3 HEV
123 can be isolated not only from infected humans but is known to be zoonotic and has also
124 been isolated from domestic swine, and wild boars, wild deer, mongoose, and rabbits [6, 7,
125 9, 11, 19, 20]. The rivers were probably contaminated with HEV by human or animal
126 excrement, or both.
127 In conclusion, we have detectedand here report HEV in the Philippines for the first
128 time, and showed that G3 HEV in particular is circulating in the rivers of Manila City.
129 In order to fully elucidate and address the HEV infection situation in the Philippines, it
130 will be necessary to collect and analyze hepatitis patients' information and investigate
131 the prevalence of HEV infection in swine and wild animals in these areas.
132

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222 **Figure legends**

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224 **Fig. 1.** Map of Manila City and the sampling sites. Yellow circles indicate the sampling
225 sites. Each river is labeled with an arrow.

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227 **Fig. 2.** Phylogenetic analysis of HEV isolated from river water samples in Manila City,
228 the Philippines. Nucleic acid sequence alignment was performed using Clustal X 1.81
229 (www.clustal.org). The genetic distance was calculated by Kimura's two-parameter
230 method. A phylogenetic tree with 1,000 bootstrap replicates was generated by the
231 neighbor-joining method based on the partial genome (338nt) of HEV ORF2 of the
232 genotypes 1-4 and avian HEV isolates. The scale bar indicates nucleotide substitutions
233 per site.

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Fig.1

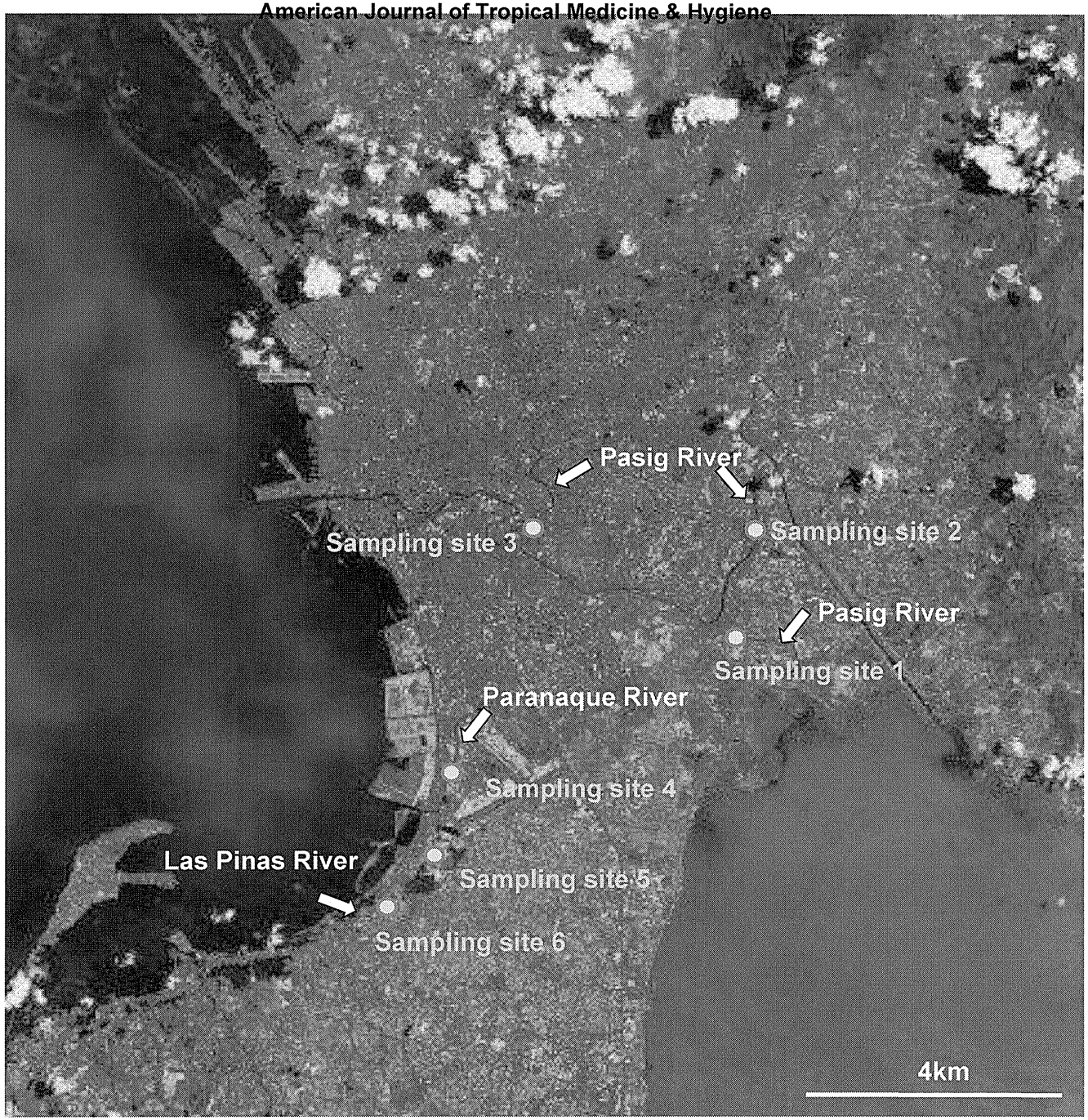
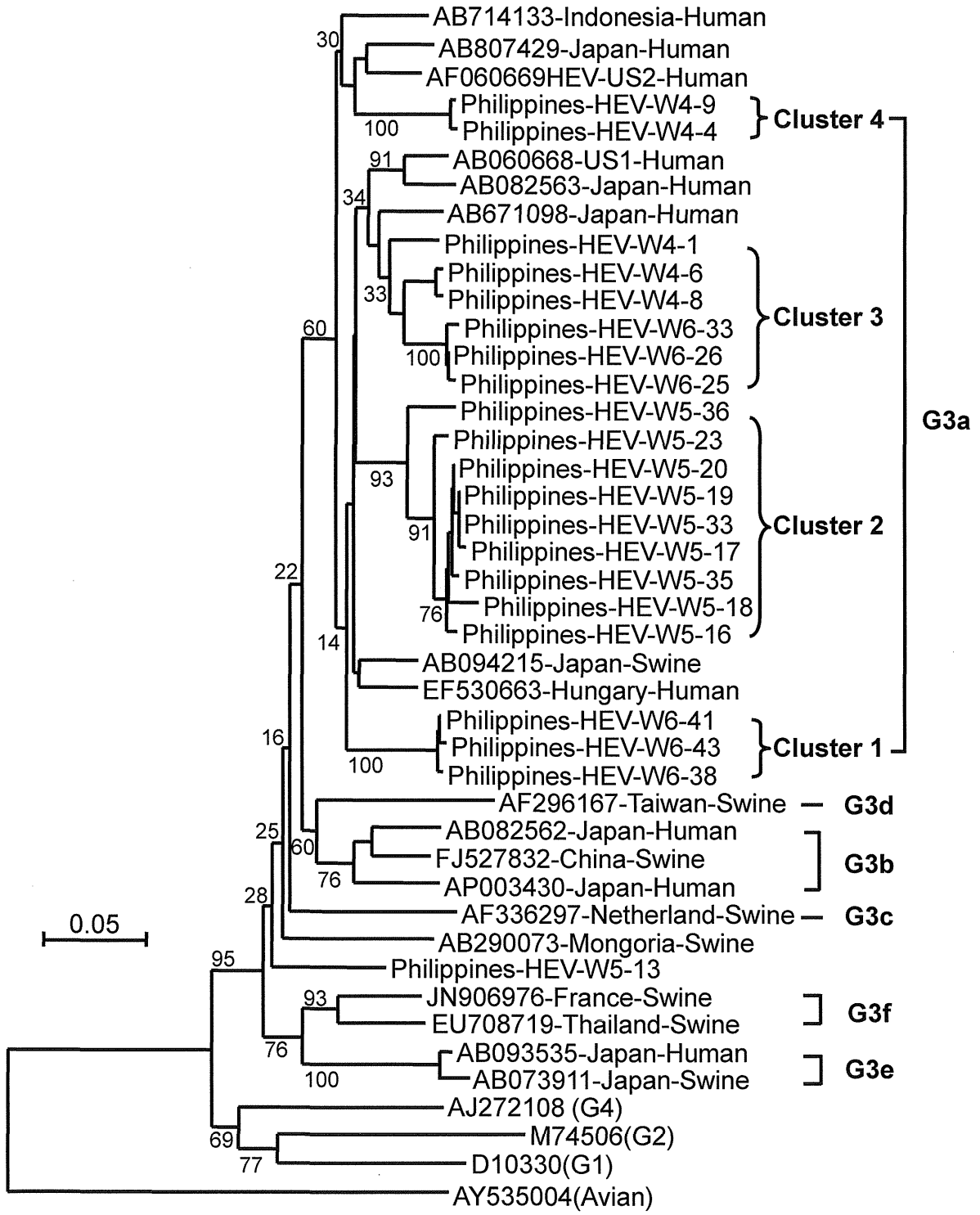

Pasig River
Sampling site 3
Sampling site 2
Pasig River
Sampling site 1
Paranaque River
Sampling site 4
Las Pinas River
Sampling site 5
Sampling site 6
4km

Fig.2


AB714133-Indonesia-Human
AB807429-Japan-Human
AF060669HEV-US2-Human
Philippines-HEV-W4-9
Philippines-HEV-W4-4
Cluster 4
AB060668-US1-Human
AB082563-Japan-Human
AB671098-Japan-Human
Philippines-HEV-W4-1
Philippines-HEV-W4-6
Philippines-HEV-W4-8
Philippines-HEV-W6-33
Philippines-HEV-W6-26
Philippines-HEV-W6-25
Cluster 3
Philippines-HEV-W5-36
Philippines-HEV-W5-23
Philippines-HEV-W5-20
Philippines-HEV-W5-19
Philippines-HEV-W5-33
Philippines-HEV-W5-17
Philippines-HEV-W5-35
Philippines-HEV-W5-18
Philippines-HEV-W5-16
Cluster 2
G3a
AB094215-Japan-Swine
EF530663-Hungary-Human
Philippines-HEV-W6-41
Philippines-HEV-W6-43
Philippines-HEV-W6-38
Cluster 1
AF296167-Taiwan-Swine
G3d
AB082562-Japan-Human
FJ527832-China-Swine
AP003430-Japan-Human
G3b
AF336297-Netherland-Swine
G3c
AB290073-Mongoria-Swine
Philippines-HEV-W5-13
JN906976-France-Swine
EU708719-Thailand-Swine
G3f
AB093535-Japan-Human
AB073911-Japan-Swine
G3e
AJ272108 (G4)
M74506(G2)
D10330(G1)
AY535004(Avian)
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