Classification of human and zoonotic group hepatitis E virus (HEV) using antigen detection

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Abstract Hepatitis E virus (HEV) is one of the major pathogens that cause acute viral hepatitis. The human (genotypes 1 and 2) and zoonotic (genotypes 3 and 4) groups of HEV present different epidemiology and clinical features. In this study, we developed a classification method for rapidly classifying HEV into human or zoonotic groups that combines a general antigen test with a zoonotic group-specific antigen test. Evaluation of serial samples from HEV-infected rhesus monkeys indicated that HEV antigen-positive samples can be classified using the antigen-based classification method. The antigen-based classification method was evaluated further on 55 genotyped samples from acute hepatitis E patients, including 9 human and 46 zoonotic groups. The novel method was completely consistent with the sequencing results: 9/9 for the human groups (100%, 95% confidence interval [CI] 66.4–100%) and 46/46 for the zoonotic groups (100%, 95% CI 92.3–100%). This method was also successfully used for the clustering of some samples that could not be clustered by

sequencing. Compared with the sequencing-based method, this method is less time-consuming, less expensive, and less technically complex and is therefore ideal for large numbers of samples. In conclusion, this study provides a convenient and sensitive method for classifying different groups of HEV, and it has potentially important public health applications, especially in underdeveloped areas that cannot afford the high cost of nucleic acid testing.

Keywords Hepatitis E virus · Diagnostic classification · Antigen detection · Zoonotic group · ELISA

Introduction

Hepatitis E, an emerging disease in many developing countries, is one of the major types of acute viral hepatitis worldwide. The hepatitis E virus (HEV), which is the causal agent of hepatitis E, was first detected in China in 1986 during an outbreak in Xinjiang Province in which 120,000 patients were infected (Aye et al. 1992). In China, > 25% of acute viral hepatitis cases are due to HEV (Chandra et al. 2008). In the rural communities of southern China, the overall seroprevalence of HEV is 43% (range 25–66%) (Li et al. 2006). The number of newly reported clinical cases of hepatitis E increased from 15,541 cases in 2005 to 27,922 cases in 2016 according to the annual reports of the National Health and Family Planning Commission of the People's Republic of China (<http://www.moh.gov.cn/jkj/s3578/201304/bb759f23a98a4ff680611f711182bf6a.shtml>, <http://www.moh.gov.cn/jkj/s3578/201702/38ca5990f8a54ddf9ca6308fec406157.shtml>). HEV is a nonenveloped virus with a single-stranded RNA genome (Emerson and Purcell 2003). The 7.2-kb viral genome contains three open reading frames (ORFs) (Kamar et al. 2012), of which ORF2 exclusively encodes a major viral capsid protein.

Gui-Ping Wen and Zi-Min Tang contributed equally to this work.

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Human HEVs are classified into four different genotypes (Hoofnagle et al. 2012; Kamar et al. 2012; Krain et al. 2014; Purcell and Emerson 2008), and these four HEV genotypes are further divided into two major groups based on epidemiology and clinical features: the human (genotypes 1 and 2) and the zoonotic (genotypes 3 and 4) groups (Hoofnagle et al. 2012). The human group of HEV has been implicated in large outbreaks in developing countries (Kamar et al. 2012) and commonly spreads through contaminated drinking water (Hoofnagle et al. 2012). Human HEV is restricted to humans with severe acute hepatitis and leads to high mortality among pregnancies (Kamar et al. 2014). The zoonotic group of HEV, with animal reservoirs, has been detected in other mammalian hosts beyond primates, especially in swine. Since their first identification in swine in 1997 (Meng et al. 1997), zoonotic group HEVs have been found in almost all countries and regions where swine are served as a main source of meat. These zoonotic HEVs are responsible for sporadic cases of autochthonous acute hepatitis E in these countries and regions (Dalton et al. 2008). Zoonotic group HEV infections proceed asymptotically in immunocompetent individuals, but these infections may be prolonged or chronic, and severe or fatal HEV infections have been observed in immunosuppressed patients (Galante et al. 2015; Geng et al. 2014; Gerolami et al. 2008; Kamar et al. 2012; Kamar et al. 2008). Human and zoonotic group HEVs have been observed in mixed epidemics in many developing countries, including China, Mexico, and Thailand (Cooper et al. 2005; Zhu et al. 2010). The identification of different HEV groups will play important roles in public health and the determination of clinical interventions.

Although four major genotypes of HEV are recognized, they share a single serotype (Emerson et al. 2006; Engle et al. 2002). No genotype-specific serologic test has been developed because of the similar antibody responses among HEV infections of different genotypes (Aggarwal 2013; Kamar et al. 2014). Currently, genotyping based on reverse transcription PCR (RT-PCR), sequencing, and multiple sequence alignment is the only method that discriminates each group and genotype of HEV (Schlauder and Mushahwar 2001; Smith et al. 2013; Wang et al. 2014; Zhai et al. 2006). However, the utility of this method is questionable because of the requirement for cumbersome procedures and specialized equipment and the ease of contamination. In this study, we seek to develop a novel classification method based on antigen testing for rapidly classifying HEV into human or zoonotic groups.

Materials and methods

Protein and virus stains

HEV capsid recombinant particle protein p239 (amino acids 368–606) and dimer protein E2 (amino acids 394–606) were

recombinantly expressed as previously reported (Li et al. 2005; Zhang et al. 2005b). The virus sources were stool samples containing the genotype 1 virus (strain Xinjiang), genotype 3 virus (strain JRC-HE3), and genotype 4 virus (strain Ch-S-1) suspended in 10% weight vs. volume (w/v) phosphate-buffered saline (PBS).

Monoclonal antibodies

Monoclonal antibodies (mAbs) were raised against recombinant particle protein p239 (amino acids 368–606) and recombinant dimer protein E2 (amino acids 394–606) using a standard murine mAb preparation protocol (Zhang et al. 2005a). For screening, the binding activity of culture medium from the cell clones was evaluated by indirect ELISA, whereby the microplate was coated with recombinant HEV capsid protein (100 ng per well), and the bound antibody was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (DAKO, Glostrup, Denmark). Cell clones producing mAbs with differential binding activity to different group proteins were preserved for further assessment. The binding activity and virus-capturing capacity of the mAbs were determined as previously described (Zhao et al. 2015). MAb 6E9, which had binding activity for only the zoonotic group recombinant capsid proteins and had the capacity to capture zoonotic group virus (Fig. S1), was screened out.

HEV RNA detection and genotype analysis

Total RNA was extracted from a 50- μ L aliquot of each stool or serum sample. A broadly reactive one-step quantitative real-time reverse transcription (RT) PCR assay that detects all four genotypes of HEV with high specificity was used to determine the copy number of HEV RNA as previously reported (Jothikumar et al. 2006). The copy number determination of all four genotypes of HEV was performed in a single reaction (Jothikumar et al. 2006). Real-time RT-PCR was performed using a CFX96TM Real-Time System and a C1000TM thermal cycler device (Bio-Rad, CA, USA) using a commercial One-Step RT-PCR Kit (Genmagbio, Beijing, China) in accordance with the manufacturer's instructions. The 25- μ L reaction mixture contained 5 μ L of RNA, 0.2 μ L of GenMagScript RT Enzyme Mix (Genmagbio, Beijing, China), 12.5 μ L of 2 $\times$ One-Step RT-PCR reaction buffer (Genmagbio, Beijing, China) and primers and probe at concentrations of 200 nM. The forward and reverse primers were JVHEVF: 5'-GGTGGTTTCTGGGGTGAC-3' and JVHEVR: 5'-AGGGGTTGGTTGGATGAA-3', respectively, and the probe was JVHEVP: 5'-TGATTCTCAGCCCTTCGC-3'. Reverse transcription was performed at 50 °C (30 min) followed by preliminary denaturation at 95 °C (15 min), and DNA was then amplified with 40 cycles at 95 °C for 15 s, 55 °C for 45 s, and 72 °C for 15 s. Plasmids

exhibiting known potency in viral copy number were serially diluted and used to generate standard curves for HEV RNA.

The HEV RNA-positive samples identified using real-time RT-PCR were further analyzed for HEV genotype via nested RT-PCR, DNA sequencing, and phylogenetic analysis. Briefly, 10 μ L of extracted nucleic acid was used in the nested RT-PCR. Reverse transcription was performed in a 25- μ L reaction with 5 U of AMV reverse transcriptase, 0.2 μ L of RNasin, and 2.5 μ M reverse primer of the first-round PCR at 42 °C for 90 min. Five microliters of the cDNA was added in the first round of PCR, which was performed with an outer primer pair consisting of YK1-1 (5'-CAGGGRATATCYGCRTGGAG -3', nt 4291–4310) and YK1-2 (5'-GCAACCTTRACRACATCAGG -3', nt 4888–4907). A 5- μ L aliquot of the first-round PCR product was then used in the second-round PCR, which was performed with an inner primer pair consisting of YK2-1 (5'-TGGTTCCGYGCTATTGARA -3', nt 4339–4358) and YK2-2 (5'-GCTACAACMACACCAGCATA -3', nt 4849–4868). The PCR cycling conditions for both rounds consisted of 35 cycles of amplification at 94 °C for 30 s, annealing for 30 s at 53 °C, and extension for 40 s at 72 °C. After DNA sequencing, the sequences were aligned with corresponding regions of available reference sequences in the GenBank database. Phylogenetic analysis was performed using a neighbor-joining algorithm in Mega 5 software.

Detection protocol for the general HEV antigen test and the z-specific antigen test

Sandwich ELISA using mAb 12F12 as the capture Ab was performed as previously reported (Wen et al. 2015) and is defined as the general antigen test in this study. The general antigen test had the same detection capacity for different HEV genotypes. A new sandwich ELISA with detection capacity specifically for genotypes 3 and 4 viruses was established by coating 96-well microtiter plates with mAb 6E9 and using HRP-conjugated mAb no. 4 for detection. This sandwich ELISA was named the zoonotic group-specific HEV antigen test (abbreviated as the z-specific antigen test). The protocol for the z-specific antigen test was the same as that for the general antigen test. Briefly, a 50- μ L aliquot of a serum or stool specimen was added to the wells. After incubation at 37 °C for 60 min, a 100- μ L aliquot of no. 4-HRP solution was added directly to each well without washing and was incubated at 37 °C for 30 min. After five rinses with wash buffer (PBS containing 0.5% (w/v) Tween 20 PBST), 100 μ L of tetramethylbenzidine (TMB) substrate solution was added to the wells and incubated at 37 °C for 15 min. The optical density (OD) was determined using a microplate reader (Autobio, Zhengzhou, China) at 450 nm with a reference wavelength of 620 nm. The detection capacity of the z-specific antigen test was evaluated using purified recombinant

capsid protein and virus, as described previously (Wen et al. 2015), except that only the results from genotype 3 and 4 samples were used to fit the curve of the z-specific antigen test. The recombinant capsid E2 protein of four genotypes was serially twofold diluted and simultaneously tested using the general antigen test and the z-specific antigen test. Owing to the infrequency of reported cases and geographic limitations of genotype 2 HEV viruses, the evaluation was performed only on genotypes 1, 3, and 4 viruses. Two fecal samples rich in HEV genotypes 1, 3, and 4 ($> 4 \times 10^5$ copies/mL) were evaluated in parallel. After preliminary evaluations and adjustments, all six specimens were serially twofold diluted. The serially diluted specimens were then simultaneously tested using real-time RT-PCR and both the general antigen test and the z-specific antigen test. Curves were fitted for nonlinear regression using GraphPad Prism software as follows: (log [agonist] vs. response – variable slope). OD values of < 0.105 were defined as negative for both antigen tests for the serially diluted samples.

Analysis of serial samples from rhesus monkeys challenged with HEV genotype 1, 3, or 4

Three rhesus monkeys were challenged with HEV genotype 1, 3, or 4. Serial stool and serum samples were collected as previously described (Zhang et al. 2003). Stool samples were suspended in 10% (w/v) PBS and then directly used for HEV antigen and RNA detection. Samples were assayed for the HEV genome by real-time RT-PCR and for the HEV antigen by sandwich ELISA as described above. Anti-HEV antibody (including anti-HEV IgM and anti-HEV IgG) and alanine aminotransferase (ALT) levels in the serum samples were determined using commercial kits (Wantai Biopharm, Beijing, China) in accordance with the manufacturer's instructions. A normal ALT level was defined as < 40 U/L. The results of the HEV antigen and anti-HEV antibody assays were recorded as positive to negative (P/N) ratios. The mean value of the baseline sera from three monkeys was used as the negative control. If the mean value of the baseline sera from the three monkeys was < 0.05 , then 0.05 was used as the negative control. A P/N value ≥ 2.1 was defined as positive.

Collection of serum samples used in this study

Sera from HEV-infected patients were included in this study. The HEV-infected patients were identified by real-time RT-PCR (Jothikumar et al. 2006) and the general antigen test (Wen et al. 2015) via the methods described above. Five samples that were confirmed to possess genotype 1 HEV were from Biratnagar, Nepal; the remaining four genotype 1 samples were collected in Dongtai, Jiangsu Province, China. Forty-two genotype 4 serum samples from HEV-infected patients were also collected in Dongtai, Jiangsu Province, China.

Four genotype 3 samples of HEV-infected patients from the HEV panel (Diamex, Heidelberg, Germany) were included in the diagnostic performance evaluation of the classification method. Additionally, 27 more un-genotyped samples from HEV-infected patients were also collected in Dongtai, Jiangsu Province, China. These 27 samples were confirmed as HEV RNA-positive by real-time RT-PCR and HEV antigen-positive by general antigen test but were genotyped unsuccessfully.

A total of 479 HEV-excluded (normal ALT levels, anti-HEV antibody-negative, and HEV RNA-negative) volunteer blood donor serum samples were also collected in the Xiamen blood center. Before the z-specific antigen test was performed using clinical serum samples, the cut-off value of the z-specific antigen test was determined using these 479 HEV-excluded samples. The cut-off value of the z-specific antigen test was set as the mean plus four standard deviations, equal to 0.12 (supplemental material Fig. S2), and the previously reported cut-off value (0.12) of the general antigen test was used in this study. These 479 samples were also used to evaluate the diagnostic performance of the classification method.

Statistical analysis

The statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) ver. 17.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software ver. 6.

Results

Establishment of a classification method based on an antigen test

Using mAb 6E9, which specifically recognizes zoonotic group HEV (the characterization of 6E9 is shown in supplemental material Fig. S1), as the capture antibody and the reported mAb no. 4 (Zhang et al. 2006) as the detection antibody, a sandwich ELISA method for zoonotic HEV-specific detection was utilized in this study (abbreviated as the z-specific antigen test). The detection capacity of the z-specific antigen test was evaluated using serial dilutions of purified recombinant capsid protein (Fig. 1a) and of the virus (Fig. 1c). A previously reported sandwich ELISA that uses mAb 12F12 as the capture Ab and mAb no. 4 as the detection antibody with the same detection capacity for all genotypes was used as a control (abbreviated as the general antigen test) (Wen et al. 2015) (Fig. 1b and Fig. 1d). Owing to the infrequency of reported cases and geographic limitations of genotype 2 HEV viruses, the evaluation was performed only on genotypes 1, 3, and 4 viruses. As shown in Fig. 1, the z-specific antigen test had the same detection capacity for only zoonotic group HEV capsid E2 proteins and viruses (Fig. 1a

and Fig. 1c), while the general antigen had the same detection capacity for all HEV capsid E2 proteins and viruses (Fig. 1b and Fig. 1d). This finding indicated that the z-specific antigen test was specific for zoonotic group HEV antigen but not human group HEV. The z-specific antigen test had detection capacity for genotypes 3 and 4 viruses similar to that of the general antigen test (with a linear detection range equivalent to 9.9×10^3 to 1.1×10^6 copies/mL of virions) (Fig. 1c). The general antigen test exhibited a linear detection range equivalent to 8.5×10^3 to 1.0×10^6 copies/mL of virions) (Fig. 1d), which was similar to a previous report (Wen et al. 2015). In the previous study (Wen et al. 2015), a general antigen test with a similar detectable range was shown to be an efficient hepatitis E diagnostic method.

Combining the general antigen test and this novel z-specific antigen test, a potential method for rapidly classifying HEV into human or zoonotic groups was established. Each sample was simultaneously evaluated with both of the antigen tests. If positive results were observed in both the z-specific and general antigen tests, the sample would be identified as originating from a zoonotic group HEV infection. By contrast, samples that were positive solely in the general antigen test (z-specific antigen test: negative) represented a human group HEV infection. As a result, all of the diluted samples that were confirmed as HEV antigen-positive by the general antigen test can be classified by the classification method based on the antigen test.

Diagnostic performance of the antigen-based classification method on serial samples from rhesus monkeys challenged with HEV genotype 1, 3, or 4

To analyze the diagnostic performance of the classification method based on the antigen tests, the z-specific antigen test and the general antigen test were used to simultaneous test serial samples from monkeys challenged with HEV genotypes 1, 3, and 4 (Fig. 2). The duration of HEV viremia identified by HEV RNA detection was concomitant with the period of HEV antigen positivity identified with the general antigen test, from 7 days post inoculation (d.p.i.) to 38 d.p.i. The samples collected from HEV genotypes 3- and 4-infected rhesus monkeys between the viremia period showed positive responses for both antigen tests, while the samples collected from HEV genotype 1-infected rhesus monkeys within this period showed single positivity for the general antigen. These results indicated that the serial HEV antigen-positive samples from the HEV genotypes 1-, 3-, and 4-infected rhesus monkeys can be classified by the classification method based on the antigen test. The results from the stool samples also showed that the serial HEV antigen-positive samples can be classified by the classification method based on the antigen test (Fig. S3). These results suggested that the

Fig. 1 Calibration curves for the z-specific antigen test (**a** and **c**) and the general antigen test (**b** and **d**) based on recombinant capsid protein (upper panel) and virus (lower panel). The curves were fitted for nonlinear regression using GraphPad Prism software ver. 6. Only zoonotic group samples were used to fit the calibration curve of the z-specific antigen test. The z-specific antigen test had similar detection capacity for genotype 3 and 4 samples, but not for genotype 1 samples. By contrast, the

general antigen test had the same detection capacities for different viral genotypes. E2(1): genotype 1 E2; E2(2): genotype 2 E2; E2(3): genotype 3 E2; E2(4): genotype 4 E2. Genotype 1, pink, sample 1: solid circles, sample 2: open circles; genotype 3, blue, sample 1: solid squares, sample 2: open squares; genotype 4, purple, sample 1: solid triangles, sample 2: open triangles

antigen-based classification method could be used for HEV classification in serum samples collected as early as 7 d.p.i. and in stool samples collected as early as 5 to 6 d.p.i.

Diagnostic performance of the classification method based on the antigen test in clinical samples from acute hepatitis E patients

To further evaluate the antigen-based classification method, 55 genotyped HEV-positive samples from acute hepatitis E patients and 479 HEV-excluded samples from blood donors were included. The 55 genotyped HEV-positive samples were collected 3–41 (9 ± 8) days after onset. Among the 55 genotyped HEV-positive sera, 9 samples were identified as human group HEV (genotype 1), and the remaining 46 samples were identified as zoonotic group HEV (including 42 genotype 4

samples and 4 genotype 3 samples) using the classification method based on sequencing. As shown in Fig. 3, the 9 human group HEV samples showed single-positive responses in the general antigen test, and the 46 zoonotic HEV samples showed positive responses in both antigen tests, indicating that all 55 of these genotyped HEV-positive samples were successfully classified with the antigen-based classification method, consistent with the sequencing results. The correct clustering rates of the antigen-based classification method were 100% for both the human (9/9, 95% confidence interval [CI] 66.4–100%) and the zoonotic (46/46, 95% CI 92.3–100%) group HEV (Table 1). All 479 HEV-excluded samples from blood donors in the Xiamen blood center showed negative results in each antigen test (Fig. 3). These results suggested that the antigen-based classification method could be used for HEV classification in samples collected as early as 3 days after onset.

Fig. 2 Diagnostic performance of the classification method based on the z-specific antigen and general antigen tests on serial sera collected from rhesus monkeys infected with genotype 1 (upper panel), genotype 3 (middle panel), or genotype 4 (lower panel) HEV. The results of the HEV antigen and anti-HEV antibody (including anti-HEV IgM and anti-HEV IgG) tests are shown as positive to negative (P/N) ratios. A P/N value ≥ 2.1 was defined as positive. The filled and open boxes above each panel show the ALT and anti-HEV IgM/IgG results, respectively. Filled boxes indicate anti-HEV IgM/IgG positivity or abnormal ALT levels, and open boxes indicate anti-HEV IgM/IgG negativity or normal ALT levels

The novel method was further used for the evaluation of 27 serum samples that were confirmed as HEV RNA-positive by real-time RT-PCR but did not present clear sequence and genotype information because the RNA levels in these samples were too low for sequencing. All of these samples were identified as HEV antigen-positive by the general antigen test. Of these 27 samples, 25 were successfully clustered with the zoonotic group HEV antigens, and the remaining 2 samples were clustered with human group HEV antigens (Fig. 4). The results suggested that the novel classification method, which combines the general HEV antigen test with the z-specific antigen test, appeared to be a convenient and reliable method for classifying HEV diagnoses into different groups.

Discussion

As one cause of acute viral hepatitis, HEV has been divided into two groups and four genotypes (Hoofnagle et al. 2012). The epidemiological patterns and pathogenic impacts of the two groups of HEV differ considerably (Hoofnagle et al. 2012; Vollmer et al. 2016). Human group HEV can cause large outbreaks (Kamar et al. 2012) and lead to approximately 20% mortality among pregnancy in developing countries (Khuroo and Kamili 2003; Khuroo et al. 1981). Zoonotic group HEV is responsible for sporadic cases and causes chronic infections in immunosuppressed individuals (Galante et al. 2015; Geng et al. 2014; Gerolami et al. 2008;

Fig. 3 Diagnostic performance of the classification method based on the z-specific antigen and general antigen tests on clinical serum specimens. The dotted lines represent the cut-off value of the antigen test. The full lines represent the means and standard deviations (SD) of the OD values of the antigen tests. A total of 479 HEV-excluded samples, 9 samples from genotype 1 HEV-infected patients, 4 samples from genotype 3 HEV-infected patients, and 42 samples from genotype 4 HEV-infected patients were included in the diagnostic performance analysis of the antigen-based classification method. HEV-excluded: gray; Human group HEV: genotype 1, pink; Zoonotic group HEV: genotype 3, green, genotype 4, blue. Z-specific antigen test: solid circles; general antigen test: open circles

Kamar et al. 2012; Kamar et al. 2008). In many developing countries, both human group HEVs and zoonotic HEVs have been observed (Cooper et al. 2005; Zhu et al. 2010). Altogether, HEV classification will provide important information for the public health warning and the determination of clinical interventions.

Because all genotype HEV share a single serotype, no genotype-specific serologic test has been developed (Aggarwal 2013; Kamar et al. 2014). Current methods for distinguishing these two groups of HEV is based on the

Table 1 Comparison of the antigen-based classification method with sequencing in confirmed acute hepatitis E cases

Sequencing	Antigen-based classification method			Correct clustering rate for the antigen-based classification method (%) (95% CI)
	Human group	Zoonotic group	Total	
Human group HEV (genotype 1, $n = 9$)	9	0	9	100 (66.4–100)
Zoonotic group HEV (genotype 3, $n = 4$ and genotype 4, $n = 42$)	0	46	46	100 (92.3–100)
Total	9	46	55	100 (93.5–100)

Fig. 4 Diagnostic performance of the antigen-based classification method in confirmed but unsuccessfully genotyped acute hepatitis E cases ($n = 27$). The dotted lines represent the cut-off values of the general and z-specific antigen tests

amplification of the complete genome or different partial genomic regions of different sizes, sequencing, and phylogenetic analyses (Schlauder and Mushahwar 2001; Smith et al. 2013; Wang et al. 2014; Zhai et al. 2006). These methods can classify into genotypes and even subtypes. While these methods require a long detection period (days), a trained operator, and specialized equipment. And these methods are RNA dependent and HEV RNA may degrade during the transportation and storage (Wen et al. 2015). The use of immunoassays based on antigen detection could also be implemented to aid in the differentiation of zoonotic from human group infections.

In this study, mAb 6E9, which specifically recognizes the zoonotic group HEVs, was obtained. The z-specific antigen test was established using mAb 6E9 as the capture antibody and the reported mAb no. 4 (Zhang et al. 2006) as the detection antibody. We developed an antigen-based method for rapidly classifying HEV into human or zoonotic group that combines the previously reported general HEV antigen test (Wen et al. 2015) with this new z-specific antigen test. Evaluation of serial samples from HEV-infected rhesus monkeys indicated that HEV antigen-positive samples collected as early as 7 d.p.i. (serum) and 5 to 6 d.p.i. (stool) can be classified by the antigen-based classification method. Fifty-five genotyped HEV-positive samples collected 3–41 (9 ± 8) days after onset from acute hepatitis E patients (including 9 genotype 1 samples, 4 genotype 3 samples, and 42 genotype 4 samples) were further used to evaluate the antigen-based classification method's diagnostic performance. Among these sera, all 55 samples (100%, 95% CI 93.5–100%) were group-differentiable by the antigen-based classification method, with results that were consistent with the sequencing results. These results suggested that the antigen-based classification method could be used for HEV classification in samples collected as early as 3 days after onset. Moreover, the antigen-based

classification method was successfully used to cluster 27 ungenotyped HEV-positive samples from acute hepatitis E patients that contained low HEV RNA levels and could not be clustered by sequencing, possibly attributable to the degradation of HEV RNA during the storage and transportation steps, or because the novel method had better sensitivity than the genotyping method based on sequencing. This result suggested that the clustering method based on antigen tests was a more powerful tool than sequencing/genotyping for the study of RNA viruses such as HEV, the nucleic acids of which are unstable during the collection, transportation, and long-term storage processes.

Because the major epidemic strain of HEV in China is genotype 4 (Chan et al. 2017; Zhang et al. 2015), genotype 1 is prevalent in Nepal, India, Burma, Bangladesh, genotype 2 HEV has so far been found in patients in Mexico, Chad, and Nigeria, and genotype 3 HEV has been identified in developed countries, such as UK, Japan, USA, Spain (Dalton et al. 2008), only 9 genotype 1 samples, 4 genotype 3 samples, and none genotype 2 samples from acute HE patients were included in the evaluation of the antigen-based classification method. The low sensitivity of the classic genotyping method also limited the collection of samples with clear genotype information. The limit of human group sample collection also led to that the lower limit of the 95% CI of correct clustering rates for human group detection of this novel method was only 66.4%. The performance of this method should be investigated in more specimens of each genotype from other geographical areas. However, the results shown in this study suggested that the novel classification method based on the antigen test was efficient and convenient for rapid clustering.

Although some common mAbs and human group HEV or zoonotic group HEV-specific mAbs have been obtained (Riddell et al. 2000; Wen et al. 2016), research on HEV antigen tests has rarely been reported and has focused on general antigen tests (Wen et al. 2015; Zhang et al. 2006). No genotyping or classification antigen test methods have been reported. This study established an antigen-based HEV classification method based on the reported general HEV antigen test (Wen et al. 2015) and a novel antibody that specifically recognized zoonotic group HEV. The new method can be completed within 3 h, whereas sequencing-based classification method takes at least 48 h to complete HEV classification, including RNA extraction, reverse transcription, amplification, sequencing, and multiple sequence alignment analysis. Moreover, the cost of the novel method is one-sixth the cost of the classic genotyping method based on RT-PCR and sequencing. Importantly, the operators of the novel method do not need be well-trained, whereas the classic method based on sequencing requires well-trained operators and expensive instruments, the costs of which are hard to estimate. Therefore, the antigen-based classification method is less time-consuming, less expensive, and less technically complex and therefore

is ideal for testing a large number of samples in developing countries, where HEVs often cause outbreaks and epidemics. In addition to hepatitis E, antigen-based methods have been used for hepatitis B virus (HBV) genotyping in previous studies (Song et al. 2015; Usuda et al. 1999). A one-step lateral flow immunoassay that could complete HBV genotyping within 20 min was developed in a previous study (Song et al. 2015). Similar to HBV genotyping, some lateral flow devices for HEV classification can be developed in future studies using the mAbs reported in this study, which will help reduce the time and cost of testing and the requirements for operators and equipment.

In summary, an antigen-based classification method was established and provided a convenient and sensitive method for classifying different groups of HEV. Compared with the current methods based on sequencing technology, this method could facilitate large-scale molecular epidemiological studies of HEV. The method may have important public health applications, especially in developing and resource-limited regions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were according to the Independent Ethics Committee approval obtained from the medical ethics committee of the School of Public Health, Xiamen University. The animal experiments were designed in accordance with the “Guidelines for Experimental Animal Welfare and Ethical Treatment” of the Ministry of Science and Technology of the People’s Republic of China. Animal usage for the study was approved by the Committee on the Ethical Use of Animals of Xiamen University. The experiments were performed based on the Laboratory Animal Center guidelines for the use of laboratory animals at Xiamen University. All efforts were made to ensure that animal suffering was minimized.

Informed consent Informed consent was obtained from all participants included in the study.

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