Journal of Viral Hepatitis, 2007, **14,** 435–445 doi:10.1111/j.1365-2893.2006.00801.x

Evaluation of human (genotype 1) and swine (genotype 4)-ORF2-based ELISAs for anti-HEV IgM and IgG detection in an endemic country and search for type 4 human HEV infections

V. A. Arankalle, K. S. Lole, T. M. Deshmukh, L. P. Chobe and S. S. Gandhe *Hepatitis Division, National Institute of Virology, Pune, India*

Received March 2006; accepted for publication May 2006

SUMMARY. Open reading frame 2 proteins (ORF2) from swine (genotype 4, S-ORF2) and human (genotype 1, H-ORF2) hepatitis E virus (HEV) having 91.4% identity at amino acid level were expressed using baculovirus expression system. Comparison of ELISAs based on the two proteins yielded identical results when sequential serum samples from monkeys and pigs experimentally infected with genotypes 1 and 4 HEV, respectively, were tested. Samples from patients (n = 258) suffering from non-A, non-B hepatitis during outbreaks of the disease and 180 sera from apparently healthy children were screened by H-ORF2-, S-ORF2-based ELISAs and Genelabs ELISA, a widely used commercial test for HEV diagnosis. Specificity of all three tests in detecting IgM and IgG antibodies in healthy children was comparable. Excellent correlation was noted in detecting both IgM (98.7% concordance) and IgG (97.7% concordance) anti-HEV antibodies when H-ORF2 and S-ORF2 ELISAs were compared. When compared with Genelabs ELISA, both H-ORF2 and S-ORF2 ELISAs identified 34 and 18 additional positives, respectively, in IgM and IgG anti-HEV tests showing comparatively less sensitivity of the commercial assay. The concordance of Genelabs ELISA in IgM detection was 86.4% and 85.6%, respectively, with H-ORF2 and S-ORF2 ELISAs. The concordance between Genelabs ELISA and H-ORF2 decreased further to 73.6% when 129 human samples from recent HEV epidemics (2002–2004) were tested for IgM. Similar results were obtained when sequential samples from 11 hepatitis E patients were examined. Screening of serum samples from 137 sporadic non-A, non-B hepatitis cases further confirmed the superiority of the H-ORF2 and S-ORF2 ELISAs. All 36/137 HEV-RNA-positive samples from sporadic cases belonged to genotype 1 confirming absence/rarity of type 4 human infections. H-ORF2 and S-ORF2 antigens were swappable in ELISAs for detecting both genotypes 1 and 4 HEV infections.

Keywords: ELISA, genotype 1, genotype 4, HEV, ORF2 protein

INTRODUCTION

Hepatitis E is an important public health problem in developing countries [21]. It is a common cause of large waterborne epidemics and sporadic hepatitis in adults in India [1,2]. The disease is usually self-limiting with high mortality (20%) among pregnant women, especially in the third trimester [16]. Hepatitis E virus (HEV) is a 27- to 32-nm diameter nonenveloped RNA virus with a single-stranded, positive sense genome, approximately 7.2 kb in size containing partially overlapping open reading frames (ORFs). ORF1 encodes nonstructural protein while ORF2 encodes capsid protein [24] and ORF3 encodes cytoskeleton-associated phosphoprotein [30]. Several immunoreactive domains have been identified in ORF2 and 3 proteins [14,15,29]. Immunoassays in ELISA and western blot formats were developed using recombinant ORF2/ORF3 proteins individually or in combination or with synthetic peptides representing these regions [9,11,25].

Different strains of HEV infecting mammals worldwide are divided into four genotypes [23]. Swine and human HEVs from the United States (genotype 3) [18], Taiwan (genotype 4) [13] and Japan (genotypes 3 and 4) [19,20] were shown to belong to the same genotype with remarkable nucleotide identity among strains from the same geographic region. In India, human isolates representing epidemic and sporadic cases (1976–2005) and swine isolates (1985–2000) belong to different genotypes, i.e. genotype 1 and genotype 4, respectively [7,8]. Although we were able to detect type 4 HEV infections in animals using genotype-1-based ELISA [7,8], it was thought logical to express Indian swine HEV ORF2 protein, develop a recombinant-protein-based ELISA and compare

Abbreviations: ORF2, open reading frame 2 proteins; HEV, hepatitis E virus; ORFs, open reading frames; HBsAg, hepatitis B surface antigen; aa, amino acids; OD, optical density; PBS, phosphate-buffered saline..

Correspondence: Dr Vidya A. Arankalle, Hepatitis Division, National Institute of Virology, 20-A, Dr Ambedkar Road, Pune, India 411001. E-mail: varankalle@yahoo.com

the performance of ELISAs based on genotype 1 (human HEV) and genotype 4 (swine HEV) proteins, especially with respect to identification of type 4 HEV infections in humans. In addition, the usefulness of IgM-anti-HEV and IgG-anti-HEV-human-ORF2 ELISAs when compared with the commercially available, widely used Genelabs ELISAs in HEV diagnosis and surveillance, respectively, in an endemic country was also evaluated.

MATERIALS AND METHODS

Cloning and expression of genotype 1 and genotype 4 ORF2 proteins in baculovirus expression system

Complete ORF2 gene (1980 nt) from HEV-RNA-positive 10% stool suspension of a human sample (PM2000, genotype 1) collected during an outbreak in Lonawala, western India (2000) and ORF2 gene (2022 nt) of swine HEV (genotype 4) from 10% stool suspension of a sample obtained from a pig experimentally infected with Indian swine HEV (genotype 4) were amplified by RT-PCR. The amplified genes were cloned downstream of the polyhedrin promoter into the baculovirus vector, pFASTBAC-1 (Invitrogen, Life Technologies, Carlsbad, CA, USA). Complete sequences of the ORF2 genes from both the strands were determined to get consensus sequences using Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Samples were analysed on automated sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). DH10MaxBAC cells were transformed with the pFASTBAC-ORF2 plasmids to get recombinant baculovirus in the form of closed circular DNA (bacmid). *Spodoptera frugiperda* (Sf9) cells were transfected with recombinant bacmids using cationic lipid Cellfectin (Life Technologies, Invitrogen). The resultant baculovirus isolates were screened for the ORF2 inserts and for protein expression in Sf9 cells.

SDS–PAGE and western blot analysis: Monolayers of Sf9 cells (about 9×10^5 cells/well in 6-well plate) were infected and harvested after different time intervals (24–120 h). Cell pellets as well as supernatants were analysed on 10% SDS–PAGE. Gels were either stained directly or blotted onto nitrocellulose membrane. Blots were blocked with phosphate-buffered saline (PBS), pH 7.4 containing 5% nonfat milk powder O/N at 4°C. Anti-HEV-IgM-positive serum at 1:50 dilution was used as a primary antibody. Secondary antibody was anti-human-IgM antibody conjugated to horseradish peroxidase. Blots were developed in diaminobenzidine and H_2O_2. All incubations were carried out in 2.5% nonfat milk powder solution in PBS and washing with PBS containing 0.05% Tween 20.

ELISA for anti-hepatitis E virus antibody detection

Sf9 cells (2×10^6) infected with ORF2 recombinant baculovirus (10 PFU/cell) were harvested at 96 h post-infection, cell pellets were resuspended in 200 μL of PBS containing 0.1% NP40 supplemented with fresh phenyl methyl sulfonyl fluoride (PMSF; final concentration, 0.5 mM) and vortexed vigorously. Two hundred microlitres of cell lysate were diluted to 10 mL with 50 mM carbonate buffer (pH 9.5); one Maxisorp microtitre 96-well plate (Nunc, Rosekilde, Denmark) was coated (100 μL/well) and incubated at 37°C for 2 h. The plate was blocked with PBS containing 10% donor calf serum, 0.5% Tween 20 and 0.5% gelatin. Serum samples were diluted 1:100 in blocking solution and added to the coated assay plates (100 μL/well). Horseradish peroxidase conjugated anti-human IgM (Sigma chemicals, St Louis, MO, USA) were used as detector antibody. The enzymatic reaction with substrate (*O*-phenylenediamine and urea peroxide) was stopped by the addition of 4 N H_2SO_4. Human samples known to be positive and negative for anti-HEV IgG or IgM were included in respective assay plates. The cut-off values for anti-HEV IgM and IgG were calculated as mean optical density (OD) values for three negative controls × 3. Samples showing OD values ≥ cut-off values were considered to be anti-HEV IgM/IgG positive.

Commercial ELISA

Performance of H-ORF2 and S-ORF2 ELISAs was compared with the widely used commercial kit from Genelabs Diagnostics, Singapore for anti-HEV IgM (kit ref. no. 21160-096T) and IgG detection (kit ref. no. 21150-096T). The assay was performed according to the instructions provided by the manufacturers.

Serum samples

Serum samples collected from clinically and biochemically confirmed acute viral hepatitis patients from various epidemics were screened for hepatitis B surface antigen (HBsAg) (Surase B-96, TMB Kit; General Biologicals Corp., HSIN Chu Taiwan) and IgM antibodies to hepatitis A virus (HAV; Hepavase MA-96, TMB Kit). All HBsAg positives were tested for the presence of IgM antibodies to hepatitis B core antigen (IgM-anti-HBc) with CORZYME-M test (Abbott laboratories, Chicago, IL, USA). Patients negative for IgM-anti-HAV and IgM-anti-HBc were considered as probable epidemic hepatitis E patients. In the present study, all the serum samples were stored at −20° C until use. These included

Human sera:

1. Acute viral hepatitis patients representing 17 epidemics of hepatitis E from two states in western India (Maharashtra and Gujarat) over the period 1976–2001 ($n = 258$). The samples were collected from 1 to 13 weeks after the onset of symptoms. These were thawed at least twice before screening during the present study.

2. Acute viral hepatitis patients representing five outbreaks of hepatitis E from the state of Maharashtra during 2002–2004 ($n = 129$). The samples were collected within 2 weeks of the onset of symptoms. These were thawed for the first time before screening during the present study.
3. Four sequential samples from 11 hepatitis E patients taken during an epidemic of the disease in 2004.
4. Sera from 180 school children between the ages 7 and 15 years [6].
5. Blood samples from 137 clinically and biochemically confirmed sporadic acute viral hepatitis non-A, non-B patients investigated during August 2003 to July 2004 were examined. Aliquots of all serum samples were stored immediately at −70°C.

Animal sera:

1. Sequential serum samples from 25 rhesus monkeys infected experimentally with epidemic and sporadic genotype 1 HEV isolates from India [3].
2. Sequential serum samples from two pigs experimentally infected with genotype 4 Indian swine HEV [7].
3. Serum sample of pigs from different areas of Pune ($n = 54$) [7].

PCR, sequencing and phylogenetic analysis

Blood samples from 137 clinically and biochemically confirmed sporadic acute viral hepatitis non-A, non-B patients investigated during August 2003 to July 2004 were tested for the presence of HEV RNA employing TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) according to the previously described method [7]. A 416-nt fragment from ORF1 region of HEV was amplified using primers: external sense – F1: 5′-TGAGAATGATTTCTCTGAGTTTG-3′, external antisense – R1: 5′-ATGTTATTCATTCCACCCG-3′, internal sense – F2: 5′-ATACCGTCTGGAACATGGC-3′, internal antisense – R2: 5′-AGCATCCCAATCAGGTTATG-3′, which could detect both types 1 and 4 HEV RNA. The PCR products were purified using Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced. Multiple alignment of nucleotide sequences was carried out using Clustal X, v.1.83: http://www.igbmc.u-strasbg.fr. Phylogenetic analysis based on partial ORF1 gene sequences (300 nt) was carried out employing MEGA 2.1 software [17]. For analysis in MEGA, the Jukes–Cantor distance model was utilized employing the neighbour-joining algorithm. The reliability of different phylogenetic groupings was evaluated using the bootstrap test, with 1000 bootstrap replications, available in MEGA.

The designations, countries and GenBank accession numbers of the full genome sequences employed for analysis in the present study were as follows: *Genotype 1:* BUR-86, Burma (D10330); BUR-82, Burma (M73218); MOR-03, Morocco (AY230202); *Genotype 2:* MEX-86, Mexico (M74506); *Genotype 3:* Arkell, Canada (AY115488); US-1 and US-2, USA (AF060668–69); US-SW, USA (AF082843); *Genotype 4:* JSN-Sap-FH, Japan (AB091395); swJ13-1, Japan (AB097811); CCC220, China (AB108537); INDPIG4, India (AY723745).

The 36 partial ORF1 sequences reported here have been deposited with GenBank accession no. DQ453923 to DQ453958.

Statistical methods

Statistical analysis was carried out using SPSS version 11.0. McNemar test was used to compare the prevalence of anti-HEV IgM and anti-HEV IgG in acute hepatitis E patients and school children and anti-HEV IgG in pig and monkey sera when tested using both the antigens. Concordance between the OD values or S/CO ratios of the two tests were measured using Pearson's correlation coefficient and Spearman's rank correlation coefficient.

RESULTS

RT-PCR of ORF2 gene and cloning

Complete ORF2 genes of type 1 HEV (H-ORF2) and type 4 HEV (S-ORF2) were amplified by RT-PCR and the products were T and A overhangs of plasmid (TA) cloned. Orientations of the inserts were checked with restriction enzymes and genes were further cloned into vector pFASTBAC-1. The clones were sequenced completely and compared with representative sequences from GenBank. When compared with strain SAR-55 (type 1, human isolate) [25], H-ORF2 [1980 nt, 660 amino acids (aa), GenBank accession no. DQ459342] showed 94% nucleotide homology; while at aa level, the homology was 99.5%. The swine ORF2 (2022 nt, 674 aa, GenBank accession no. AY723745) showed 87.1% and 86.7% nucleotide identity with T1 and SWJ13-1 (human and swine isolates of type 4, respectively) [19,27], while at aa level the identity appeared to be much higher (96–98.8%). H-ORF2 protein had a cleavable hydrophobic signal peptide at the extreme amino terminus of the protein and possible cleavage site between 19 and 20 aa (predicted by PSORT II) with a final product of 641 aa. S-ORF2 protein showed cleavage site between aa 33 and 34 with predicted product of 641 aa. Percent aa identity of H-ORF2 and S-ORF2 proteins was 91.4% (Fig. 1), while identity at nucleotide level was 79.5%.

Hepatitis E virus ORF2 protein expression in insect cells

Sf9 cells were infected with HEV ORF2 recombinant baculovirus and temporal expression of ORF2 protein was studied. Cells and media harvested at different time intervals after infection (24–120 h) were analysed by SDS–PAGE and western blot. Both H-ORF2- and S-ORF2-recombinant-baculovirus-infected Sf9 cells showed multiple immunoreactive proteins, which were mostly cell associated. Among those,

```
PM2000  ---------- ----MRPRPI LLLFLMFLPM LPAPPPGQPS GRRRGRRSGG SGGGFWGDRV
SAR-55  ---------- --AT...... ...L...... .......... .......... ..........
INDPIG4 MNNMSFCSVH GDAT..S.AL .F.L.VL... .....A.... D......... A.........

PM2000  DSQPFAIPYI HPTNPFAPDV TAAAGAGPRV RQPARPLGSA WRDQAQRPAA ASRRRPTTAG
SAR-55  .......... .......... .......... .......... .......... ..........
INDPIG4 ......L... .......S.I ST.....A.P .......... ....S..... PA...SAP..

PM2000  AAPLTAVAPA HDTPPVPDVD SRGAILRRQY NLSTSPLTSS VATGTNLVLY AAPLSPLLPL
SAR-55  .......... .......... .......... .......... .......... ..........
INDPIG4 .S....T... P..A...... .......... .........T I......... ..........

PM2000  QDGTNTHIMA TEASNYAQYR VARATIRYRP LVPNAVGGYA ISISFWPQTT TTPTSVDMNS
SAR-55  .......... .......... .......... .......... .......... ..........
INDPIG4 .......... .......... .V........ .......... .......... ..........

PM2000  ITSTDVRILV QPGIASELVI PSERLHYRNQ GWRSVETSGV AEEEATSGLV MLCIHGSPVN
SAR-55  .......... .......... .......... .......... .......... ..........
INDPIG4 .......... .......... .......... .......... .......... ..........

PM2000  SYTNTPYTGA LGLLDFALEL EFRNLTPGNT NTRVSRYSST ARHRLRRGAD GTAELTTTAA
SAR-55  .......... .......... .......... .......... .......... ..........
INDPIG4 .......... .......... .......... .........S ...K...... ..........

PM2000  TRFMKDLYFT STNGVGEIGR GIALTLFNLA DTLLGGLPTE LISSAGGQLF YSRPVVSANG
SAR-55  .......... .......... .......... .......... .......... ..........
INDPIG4 .......H.. G......V.. .......... .......... .......... ..........

PM2000  EPTVKLYTSV ENAQQDKGIA IPHDIDLGES RVVIQDYDHQ HEQDRPTPSP APSRPFSVLR
SAR-55  .......... .......... .......... ........N. .......... ..........
INDPIG4 .......... .......... .......... ........N. .......... ..........

PM2000  ANDVLWLSLT AAEYDQSTYG SSTGPVYVSD SVTLVNVATG AQAVARSLDW TKVTLDGRPL
SAR-55  .......... .......... .......... .......... .......... ..........
INDPIG4 .......... .V....T... ...N.M.... T..F...... ..G.S..... S.........

PM2000  STIQQYSKTF FVLPLRGKLS FWEAGTTKAG YPYNYNTTAS DQLLVENAAG HRVAISTYTT
SAR-55  .......... .......... .......... .......... .......... ..........
INDPIG4 T......... .......... .......... ......A... ..I.I..... ...C......

PM2000  SLGAGPVSIS AVAVLAPHSA LALLEDTLDY PARAHTFDDF CPECRPLGLQ GCAFQSTVAE
SAR-55  .......... .........V .......M.. .......... .......... ..........
INDPIG4 N..S...... ..G....... ..V....T.. .......... .....A.... ..........

PM2000   LQRLKMKVGK TREL*----- --------
SAR-55   .......... .....FICLC PPSFCCLF
#INDPIG4 .......... ...Y.----- --------
```

Fig. 1 Alignment of the deduced sequences of the amino acids (aa) of Indian swine (674 aa) and human HEV ORF2 (660 aa) proteins. Identical aa are indicated by dots and dashes represent deletions.

72, 55 and 35 kDa proteins were prominent in both recombinants, suggesting similar processing of both proteins in the insect cells (Fig. 2). The protein synthesis peaked by 96 h post-infection and it declined gradually. Similar protein pattern was seen in the media also, but the protein concentrations were extremely low. These results confirmed the earlier reported observations by Robinson *et al.* [22] and indicated the proteolytic cleavages of 72 kDa protein in Sf9 cells.

IgM and IgG-anti-hepatitis E virus antibody detection

Table 1 presents comparison of performance of three ELISAs in detecting IgM and IgG-anti-HEV antibodies among acute viral hepatitis patients examined during epidemics of hepatitis E (1982–2001) and apparently healthy children. Specificity of all ELISAs in detecting IgM antibodies in healthy children was comparable; only one sample being positive in H-ORF2 ELISA. In patients category, 182 samples were positive and 38 samples were negative in all three assays. Thus, 220/258 sera gave identical results. However, H-ORF2 and S-ORF2 antigens identified 34 additional samples with overall concordance of 98.4%. The concordance of Genelabs ELISA was 86.4% (H-ORF2) and 85.6% (S-ORF2). ORF2-based ELISAs showed 97.4% and Genelabs ELISA showed 90.3% (H-ORF2) and 92% (S-ORF2) concordance in IgG-anti-HEV detection in patients. Among healthy children,

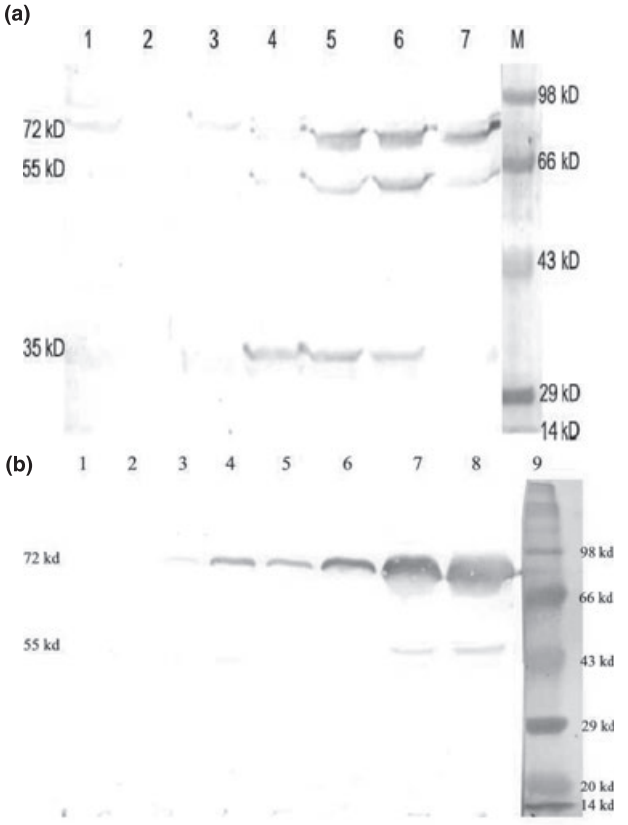

Fig. 2 Immunoblot analysis of SF9 cells infected with recombinant baculovirus expressing: (a) H-ORF2 protein with IgM-anti-HEV antibodies in human serum. Lanes: 1, control SF9 cells; 2, empty; 3–7, cell lysates obtained at 48, 72, 96,120, 144 h post-infection; 8, mol. wt. marker. (b) S-ORF2 protein with IgM-anti-HEV antibodies in human serum. Lanes: 1, control SF9 cells; 2, empty; 3–8, cell lysates obtained at 48, 72, 96,120, 144, 168 h post-infection; 9, mol. wt. marker.

concordance for IgG-anti-HEV positivity were 95.5% (Genelabs/H-ORF2), 94.3% (Genelabs/H-ORF2) and 98.8% (H-ORF2/S-ORF2).

As repeated freezing/thawing may affect ELISA reactivity, 129 samples collected during epidemics of hepatitis E (2002–2004) were tested in ELISA, immediately stored and thawed for the first time, for comparing with Genelabs assay. Of these, 79 samples were positive in both tests, 16 were negative in both tests, whereas 29 and 5 samples were positive by H-ORF2 and Genelabs assays, respectively. The concordance between two tests was 73.6%.

Detection of anti-hepatitis E virus antibodies in sequential samples

Sequential blood samples collected from 11 hepatitis E patients during an epidemic of the disease in 2004 were screened by H-ORF2 and Genelabs ELISAs for IgM-anti-HEV antibody detection (Table 2). The first sample collected 3–19 days after the onset of the disease was positive for all the 11 patients by both the tests. The number of IgM-anti-HEV positives among the subsequent follow-up samples was 11, 10 and 7 in H-ORF2 ELISA, while only 7, 2 and 3 samples could be identified as anti-HEV IgM positives in Genelabs ELISA. No sample was H-ORF2 ELISA negative, Genelabs ELISA positive.

Seroconversion in monkeys and pigs experimentally infected with hepatitis E virus

Serial serum samples from 25 rhesus monkeys experimentally infected with human HEV (genotype 1) were screened by H-ORF2 ELISA. These samples were earlier tested using SAR-55-based recombinant ORF2 antigen (Dr Purcell, NIH, Bethesda, MD, USA). All the pre-infection samples were negative for IgG-anti-HEV antibodies, whereas seroconversion was recorded on the same day in all 25 monkeys by both ORF2 antigens.

Sequential samples of a representative monkey from the above group (Fig. 3a) and an experimentally infected pig (with swine HEV, genotype 4) (Fig. 3b) were parallely screened with H-ORF2 and S-ORF2 antigens. The reactivity in ELISA was almost identical regardless of the antigen. With homologous antigen, slightly higher values were obtained for some samples. Pearson's correlation coefficient was significantly high for swine ($r = 0.975$) and monkey samples ($r = 0.943$). The concordance of the results deduced by H-ORF2 and S-ORF2 antigens was 96.4% for swine samples and 100% for monkey samples.

Fifty-four swine serum samples collected from different areas of Pune were screened for IgG-anti-HEV using H-ORF2 and S-ORF2 antigens. Forty-eight samples were positive and six were negative in both ELISAs, with 100% concordance.

IgM-anti-hepatitis E virus and hepatitis E virus RNA positivity in sporadic acute viral hepatitis patients

In order to assess relationship of HEV-RNA and ELISA positivity, sera from 137 sporadic non-A, non-B hepatitis patients were tested by nested RT-PCR and three ELISAs (H-ORF2-, S-ORF2-based ELISAs and Genelabs ELISA) (Table 3). Of these, 25 and 66, respectively, were positive and negative in all the four assays. Nineteen samples were positive in all the three ELISAs but HEV RNA negative. Five HEV RNA positives were negative in all the three ELISAs. However, six HEV-RNA-positive samples, which were positive in H-ORF2 and S-ORF2 ELISAs, were negative in Genelabs ELISA. Eleven HEV-RNA-negative samples were positive in ORF2-based ELISAs but were negative in Genlabs ELISA, whereas three Genelabs ELISA positives (HEV RNA negative) were negative in other two ORF2-based ELISAs. Thus, of the 36 HEV-RNA-positive patients, both the ORF2-based ELISAs could identify 31 as HEV infections (86.1%), whereas Genelabs ELISA detected 25 (69.4%) patients as hepatitis E.

Table 1 IgM and IgG-anti-HEV reactivity in H-ORF2, S-ORF2 and Genelabs ELISAs

	Epidemic patients		Healthy children	
ELISA reactivity	IgM-anti-HEV	IgG anti-HEV	IgM-anti-HEV	IgG-anti-HEV
H-ORF2 positive S-ORF2 positive Genelabs positive	182	199	0	4
H-ORF2 negative S-ORF2 negative Genelabs negative	38	15	179	164
H-ORF2 positive S-ORF2 positive Genelabs negative	34	18	0	6
H-ORF2 positive S-ORF2 negative Genelabs positive	0	0	0	0
H-ORF2 negative S-ORF2 positive Genelabs positive	2	1	0	0
H-ORF2 positive S-ORF2 negative Genelabs negative	1	4	1	0
H-ORF2 negative S-ORF2 positive Genelabs negative	1	1	0	2
H-ORF2 negative S-ORF2 negative Genelabs positive	0	0	0	2
Concordances				
H-ORF2/S-ORF2	98.4	97.4	99.4	98.8
H-ORF2/Genelabs	85.6	90.3	99.4	95.5
S-ORF2/Genelabs	86.4	92.01	100	94.3

H-ORF2, ELISA based on genotype 1 ORF2 protein; S-ORF2, ELISA based on genotype 4 ORF2 protein.

Determination of hepatitis E virus genotype of acute sporadic viral hepatitis samples

Phylogenetic analysis of partial ORF1 sequence (300 nt) of 36 HEV-RNA-positive samples showed clustering of all the samples in genotype 1 (Fig. 4).

DISCUSSION

Hepatitis E is an important cause of sporadic as well as epidemic hepatitis in both urban and rural India demanding availability of sensitive and specific assay systems. In the absence of a suitable *in vivo* or *in vitro* HEV culture system to obtain large amounts of the antigens, attempts have been made to develop immunoassays using either synthetic peptides or recombinant proteins or mixture of both. So far, ORF2 protein expressed in SF9 cells has been shown to be of optimal diagnostic value.

To address certain research questions in the past, we used ELISA developed by the NIH, USA, based on the SAR-55 strain-ORF2 expressed in baculovirus system (26). Although this ELISA was found to be satisfactory in serological identification of HEV infections, no commercial assay was made available using this protein. Commercially available ELISA from Genelabs has been the method of choice in India.

Another issue has been documentation of circulation of distinct genotypes in humans (genotype 1, 1976–2005) and swine (genotype 4, 1985–2000) from India [4,7,8 and our unpublished data]. This was in contrast to reports from several countries including the USA, Taiwan and Japan, wherein same genotype was shown to be prevalent in humans and pigs. Sequence homology between human and swine strains was reported to be very high postulating zoonotic spread of the virus. Although we were able to detect type 4 infections in pigs using genotype-1-based ELISA [7,8], it was thought to be important to examine whether the use of type 4 reagents would lead to the detection of type 4 HEV infections in humans, raising the possibility of zoonotic spread of the virus. Thus, our aim was twofold: (i) to develop recombinant-protein-based ELISA using Indian strain of

Table 2 IgM-anti-HEV positivity in sequential samples from hepatitis E patients determined by H-ORF2 and Genelabs ELISAs

Patient no.	Reactivity (post-onset day of sample collection) and S/CO ratios in H-ORF2/Genelabs ELISAs			
1	+/+ (11)	+/+ (25)	−/− (40)	−/− (61)
	2.26/3.29	1.49/1.01	0.8/0.67	0.57/0.46
2	+/+ (13)	+/+ (27)	+/+ (42)	± (63)
	6.15/4.53	3.31/2.2	1.22/1.01	1.0/0.54
3	+/+ (11)	± (25)	± (48)	+/+ (69)
	7.09/1.21	5.44/0.8	2.36/0.44	2.07/1.07
4	+/+ (19)	± (33)	± (40)	± (61)
	9.98/1.08	8.24/0.67	5.47/0.54	3.54/0.8
5	+/+ (11)	± (25)	± (32)	NA
	5.58/1.01	2.38/0.45	1.12/0.23	
6	+/+ (3)	+/+ (17)	± (34)	−/− (65)
	5/1.1	3.69/1.47	2.05/0.44	0.99/0.3
7	+/+ (5)	+/+ (19)	± (35)	−/− (56)
	3.55/1.24	2.66/1.4	1.05/0.48	0.06/0.47
8	+/+ (6)	+/+ (20)	± (35)	± (56)
	5.3/2.74	3.3/1.09	2.09/0.47	2.02/0.29
9	+/+ (11)	± (25)	± (40)	± (61)
	4.12/1.47	2.94/0.78	1.86/0.6	1.25/0.33
10	+/+ (3)	+/+ (17)	+/+ (32)	+/+ (53)
	8.61/7.64	6.94/4.92	4.64/2.64	3.67/1.91
11	+/+ (3)	+/+ (17)	± (32)	+/+ (53)
	7.17/4.91	3.61/1.79	2.2/0.97	1.3/1.12

S/CO, OD for sample/cut-off value for the test; H-ORF2, ELISA based on genotype 1 ORF2 protein.

human HEV and compare with the widely used, commercially available Genelabs ELISA in an endemic country and (ii) to compare the performance of human and swine HEV-based ELISAs in detecting HEV infections. These aspects mainly deal with IgM-anti-HEV antibodies that are of diagnostic value. In addition, comparison of the performance of Genelabs and human/swine HEV ORF2-based ELISAs in detecting IgG antibodies useful in surveillance was also considered worthwhile.

For the initial evaluation, a large number of sera collected from the patients of acute viral hepatitis during the epidemics of hepatitis E (earlier identified as non-A, non-B) over a period of 17 years were screened ($n = 258$, Table 1). This was considered the best option, as most non-A, non-B hepatitis patients during single source, waterborne outbreaks would be suffering from hepatitis E. For the assessment of specificity, sera from 180 children were screened. This age group was selected because exposure to HEV was earlier shown by us to be minimum, adults exhibiting higher prevalence of anti-HEV [6]. Excellent specificity of all the three assays for IgM-anti-HEV detection was evident. However, Genelabs ELISA was considerably less sensitive in detecting IgM-anti-HEV positives in epidemic patients, with concordance of 85.6% (H-ORF2 ELISA) and 86.4% (S-ORF2 ELISA). This observation was further confirmed by testing sequential serum samples from 11 hepatitis E patients (Table 2). Although initial sample was positive by both the tests, detection by Genelabs assay declined over time. These results clearly show that negativity in Genelabs assay may not always be indicative of non-E infection in an endemic country.

A comparison for the detection of IgG-anti-HEV antibodies showed similar results; concordance with the Genelabs assay being 90.3% (H-ORF2) and 92% (S-ORF2) in the epidemic situation and 95.5% and 94.3%, respectively, among healthy children. These differences cannot be attributed to viral genotypes, as the comparison was made during epidemics of the disease confirmed to be due to genotype 1. Importantly, use of either genotype-1- or genotype-4-based ELISA was equally efficient in detecting both IgM and IgG antibodies. Testing of sequential samples collected from experimentally infected monkey (genotype 1) or swine (genotype 4) (Fig. 3a,b) and naturally infected swine yielded identical results in both ELISAs.

We therefore conclude that either of these antigens can be employed in ELISA for the detection of both IgM and IgG-anti-HEV antibodies against genotypes 1 and 4 HEV. As a result of the unavailability of sera against types 2 and 3 HEV, similar observations could not be extended to these genotypes. In this connection, it is important to note that swine HEV ORF2 (genotype 3, USA) and human HEV ORF2 (SAR-55, genotype 1, Pakistan) were interchangeable in ELISA with respect to their ability to detect anti-HEV [10].

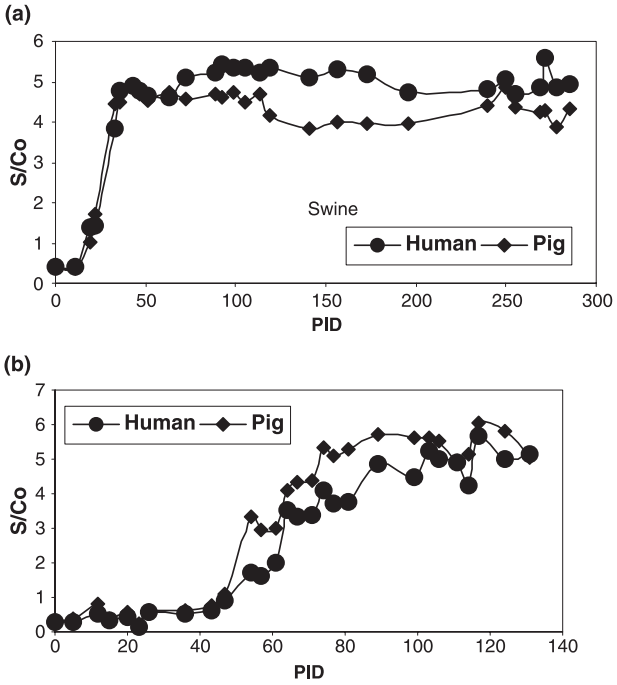

Fig. 3 Comparison of HEV H-ORF2- and S-ORF2-based ELISAs in detecting IgG-anti-HEV antibodies in (a) rhesus monkey infected experimentally with genotype 1 human HEV and (b) pig infected with genotype 4 swine HEV. S/CO values indicate ratio of OD values in ELISA and the cut-off value for the test. PID denoted days after infection with the respective viruses.

Both the proteins were expressed using recombinant baculovirus system. Usefulness of ORF2 protein expressed in this system has already been proved [12,26]. Indian swine HEV ORF2 encodes 674 aa, whereas the human HEV encodes a protein of 660 aa (Fig. 1). However, a predicted hydrophobic signal peptide cleavage site in ORF2 protein of swine isolate eliminates additional 14 aa at the amino terminus resulting in identical sizes of the mature proteins of both human and swine HEV-ORF2 (641 aa). Immunoblot analysis showed a major protein of 72 kDa and an ~55 kDa protein reacting with human IgM (Fig. 2a,b). It may be pertinent to note here that swine and human HEV ORF2 used for the expression of proteins differed by 79.5% at nucleotide and 91.4% at aa levels (and aa substitutions at 57 positions) did not affect immunoreactivity suggesting that these aa may not be involved in determining immunoreactivity (Fig. 1).

We evaluated Equipar ELISA (Equipar Srl, Saronno (va), Italy), which uses synthetic antigens from ORF2 and ORF3 regions for IgM-anti-HEV detection from a small number of acute viral hepatitis patients ($n = 88$, data not shown). H-ORF2 ELISA picked up additional 14 positives demonstrating significantly less sensitivity of the commercial ELISA with concordance of 81.8%.

Table 3 HEV RNA positivity and IgM-anti-HEV reactivity in sporadic non-A, non-B acute viral hepatitis patients

ELISA reactivity	No. of patients
S-ORF2 positive	25
H-ORF2 positive	
Genelabs positive	
HEV RNA positive	
S-ORF2 negative	66
H-ORF2 negative	
Genelabs negative	
HEV RNA negative	
S-ORF2 negative	5
H-ORF2 negative	
Genelabs negative	
HEV RNA positive	
S-ORF2 positive	19
H-ORF2 positive	
Genelabs positive	
HEV RNA negative	
S-ORF2 positive	6
H-ORF2 positive	
Genelabs negative	
HEV RNA positive	
S-ORF2 positive	11
H-ORF2 positive	
Genelabs negative	
HEV RNA negative	
S-ORF2 positive	2
H-ORF2 negative	
Genelabs negative	
HEV RNA negative	
S-ORF2 negative	3
H-ORF2 negative	
Genelabs positive	
HEV RNA negative	

H-ORF2, ELISA based on genotype 1 ORF2 protein; S-ORF2, ELISA based on genotype 4 ORF2 protein.

As the probability of detecting type 4 HEV infections would be higher in sporadic setting when compared with common source waterborne epidemics caused by type 1 HEV, we screened 137 sporadic non-A, non-B patients by all the three assays for IgM-anti-HEV detection and by nested RT-PCR for the detection of HEV RNA. The results confirmed superiority of ORF2-protein-based assays in detecting HEV RNA positives as well as IgM-anti-HEV alone positives. Interestingly, none of the 36 HEV RNA positives belonged to genotype 4, all being genotype 1 HEV. These results clearly show that type 4 HEV infections in humans are absent or too rare and are at variance with a study from China [28]. Four overlapping regions of Chinese type 4 HEV ORF2 and the entire ORF3 were expressed in *Escherichia coli* as fusion proteins. Evaluation of 41 Genelabs ELISA positives with the

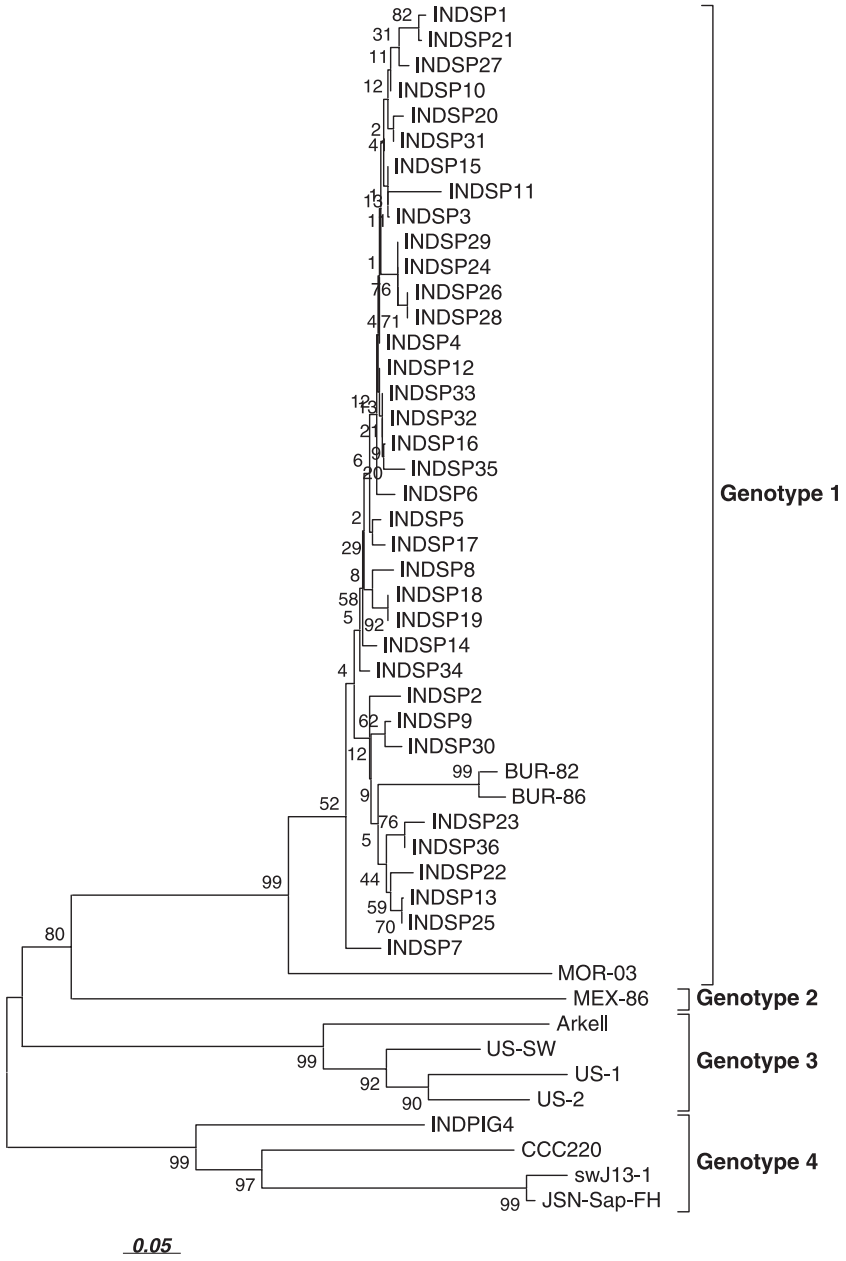

Fig. 4 Phylogenetic tree depicting genotypic status of 36 acute sporadic non-A, non-B hepatitis (INDSP1–INDSP36) human isolates on the basis of partial ORF1 (300 nt) sequences. Refer Material and Methods section for abbreviations used and GenBank accession numbers for the reference sequences.

ELISAs based on the individual five recombinant polypeptides showed differential sensitivity, the best being detection of 40/41 positives. In addition, 10/39 non-A to non-E patients scored positive by type 4 ELISA. Of the 10 anti-HEV positives, 6 were HEV-RNA positive, 2 and 4 were genotypes 1 and 4, respectively. These results probably reflect higher sensitivity of type 4 polypeptide-based ELISA when compared with Genelabs ELISA rather than genotype-based additional detection of type 4 HEV infections.

In conclusion, ELISAs based on the use of recombinant ORF2 proteins from types 1 and 4 HEV expressed in insect cells can be efficiently used to detect infections with type 1 as well as type 4 viruses. Although specificity of Genelabs ELISA was comparable with recombinant ORF2-based ELISAs, sensitivity was markedly less in IgM-anti-HEV detection. This will pose problems in identifying hepatitis E cases, especially if samples are collected late in the course of the disease (≥5 weeks from the onset of the disease), a scenario not very uncommon in India. It has been shown earlier that up to 3 weeks after the onset of symptoms, about 80% of the patients were IgM-anti-HEV positive, declining to 21.8% at the end of 12th week [2]. There is a need for commercial availability of more sensitive and equally specific serological test(s) for HEV diagnosis to avoid under-diagnosis of hepatitis E and overestimation of non-A to non-E cases. Although type 4 HEV continues to circulate in pigs from India since at

least 1985, human infections with this genotype are absent/too rare.

ACKNOWLEDGEMENTS

The authors are grateful to Dr A.C. Mishra, Director, National Institute of Virology, for the critical evaluation of the manuscript. Statistical assistance of Mr A.M. Walimbe and technical assistance of Mr S.S. Ranawade, Ms N.C. Thorat and Mr P.B. Jawalkar are gratefully acknowledged. We thank Dr M.S. Chadha, Assistant Director, Hepatitis Division, for her help in collecting some of the blood samples used for analysis.

REFERENCES

1 Arankalle VA, Chobe LP, Jha J *et al.* Etiology of acute sporadic non-A, non-B hepatitis in western India. *J Med Virol* 1993; 40: 121–125.
2 Arankalle VA, Chadha MS, Tsarev SA *et al.* Seroepidemiology of water-borne hepatitis in India and evidence for a new enterically transmitted hepatitis agent. *Proc Natl Acad Sci USA* 1994; 91: 3428–3432.
3 Arankalle VA, Chadha MS, Chobe LP, Nair R, Banerjee K. Cross-challenge studies in rhesus monkeys employing different Indian isolates of hepatitis E virus. *J Med Virol* 1995; 46: 358–360.
4 Arankalle VA, Paranjape S, Emerson SU, Purcell RH, Walimbe AM. Phylogenetic analysis of hepatitis E virus isolates from India (1976–1993). *J Gen Virol* 1999; 80: 1691–1700.
5 Arankalle VA, Joshi MV, Kulkarni AM *et al.* Prevalence of anti-HEV antibodies in different Indian animal species. *J Viral Hepat* 2001; 8: 223–227.
6 Arankalle VA, Chadha MS, Chitambar SD, Walimbe AM, Chobe LP, Gandhe SS. Changing epidemiology of hepatitis A and hepatitis E in urban and rural India (1982–1998). *J Viral Hepat* 2001; 8: 293–303.
7 Arankalle VA, Chobe LP, Joshi MV, Chadha MS, Kundu B, Walimbe AM. Human and swine hepatitis E viruses from western India belong to different genotypes. *J Hepatol* 2002; 36: 417–425.
8 Arankalle VA, Chobe LP, Walimbe AM, Yergolkar PN, Jacob GP. Swine HEV infection in south India and phylogenetic analysis (1985–1999). *J Med Virol* 2003; 69: 391–396.
9 Dawson GJ, Chau KH, Cabal CM, Yarbough PO, Reyes GR, Mushahwar IK. Solid-phase enzyme-linked immunosorbent assay for hepatitis E virus IgG and IgM antibodies utilizing recombinant antigens and synthetic peptides. *J Virol Methods* 1992; 38: 175–186.
10 Engle RE, Yu C, Emerson SU, Meng XJ, Purcell RH. Hepatitis E virus (HEV) capsid antigens derived from viruses of human and swine origin are equally efficient for detecting anti-HEV by enzyme immunoassay. *J Clin Microbiol* 2002; 40: 4576–4580.
11 Favorov MO, Khudyakov YE, Mast EE. IgM and IgG antibodies to hepatitis E virus (HEV) detected by an enzyme immunoassay based on an HEV-specific artificial recombinant mosaic protein. *J Med Virol* 1996; 50: 50–58.
12 He J, Tam AW, Yarbough PO, Reyes GR, Carl R. Expression and diagnostic utility of hepatitis E virus putative structural proteins expressed in insect cells. *J Clin Microbiol* 1993; 31: 2167–2173.
13 Hsieh SY, Meng XJ, Wu YH. Identity of a novel swine hepatitis E virus in Taiwan forming a monophyletic group with Taiwan isolates of human hepatitis E virus. *J Clin Microbiol* 1999; 37: 3828–3834.
14 Kaur M, Hyams KC, Purdy MA. Human linear B-cell epitopes encoded by the hepatitis E virus include determinants in the RNA-dependent RNA polymerase. *Proc Natl Acad Sci USA* 1992; 89: 3855–3858.
15 Khudyakov YE, Favorov MO, Jue DL, Hine TK, Fields HA. Immunodominant antigenic regions in a structural protein of the hepatitis E virus. *Virology* 1994; 198: 390–393.
16 Khuroo MS, Tell MR, Skidmore S, Sofi MA, Khuroo MI. Incidence and severity of viral hepatitis in pregnancy. *Am J Med* 1981; 70: 252–255.
17 Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* 2001; 17: 1244–1245.
18 Meng XJ, Purcell RH, Halbur PG. A novel virus in swine is closely related to the human hepatitis E virus. *Proc Natl Acad Sci USA* 1997; 94: 9860–9865.
19 Nishizawa T, Takahashi M, Mizuo H, Miyajima H, Gotanda Y, Okamoto H. Characterization of Japanese swine and human hepatitis E virus isolates of genotype IV with 99% identity over the entire genome. *J Gen Virol* 2003; 84: 1245–1251.
20 Okamoto H, Takahashi M, Nishizawa T, Fukai K, Muramatsu U, Yoshikawa A. Analysis of the complete genome of indigenous swine hepatitis E virus isolated in Japan. *Biochem Biophys Res Commun* 2001; 289: 929–936.
21 Purcell RH, Emerson SU. Hepatitis E virus. In: Fields BN, Knipe DM, Howley PM *et al.* eds Fields Virology, 4th edn. Philadelphia: Lippincott Raven Publishers, 2001: 3051–3061.
22 Robinson RA, Burgess WH, Emerson SU. Structural characterization of recombinant hepatitis E virus ORF2 proteins in baculovirus-infected insect cells. *Protein Expr Purif* 1998; 12: 75–84.
23 Schlauder GG, Mushahwar IK. Genetic heterogeneity of hepatitis E virus. *J Med Virol* 2001; 65: 282–292.
24 Tam AW, Smith MM, Guerra ME. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* 1991; 185: 120–131.
25 Tsarev SA, Emerson SU, Reyes GR. Characterization of a prototype strain of hepatitis E virus. *Proc Natl Acad Sci USA* 1992; 89: 559–563.
26 Tsarev SA, Tsareva TS, Emerson SU. ELISA for antibody to hepatitis E virus (HEV) based on complete open-reading frame-2 protein expressed in insect cells: identification of HEV infection in primates. *J Infect Dis* 1993; 168: 369–378.
27 Wang Y, Ling R, Erker JC. A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis. *J Gen Virol* 1999; 80: 169–177.

28 Wang Y, Zhang H, Li Z. Detection of sporadic cases of hepatitis E virus (HEV) infection in China using immunoassays based on recombinant open reading frame 2 and 3 polypeptides from HEV genotype 4. *J Clin Microbiol* 2001; 39: 4370–4379.
29 Yarbough PO, Tam AW, Fry KE. Hepatitis E virus: identification of type-common epitopes. *J Virol* 1991; 65: 5790–5797.
30 Zafrullah M. The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. *J Virol* 1997; 71: 9045–9053.