Biological and immunological characteristics of hepatitis E virus-like particles based on the crystal structure

Tetsuo Yamashita[a,1], Yoshio Mori[a,1], Naoyuki Miyazaki[b,c], R. Holland Cheng[c], Masato Yoshimura[d], Hideaki Unno[e], Ryoichi Shima[a], Kohji Moriishi[a], Tomitake Tsukihara[b], Tian Cheng Li[f], Naokazu Takeda[f], Tatsuo Miyamura[f], and Yoshiharu Matsuura[a,2]

[a]Department of Molecular Virology, Research Institute for Microbial Diseases and [b]Department of Protein Crystallography, Research Institute for Protein Research, Osaka University, Osaka 565-0871, Japan; [c]Department of Molecular and Cellular Biology, University of California, Davis, CA 95616; [d]National Synchrotron Radiation Research Center, 101 Hsin-Ann Road, Hsinchu Science Park, Hsinchu 30076, Taiwan; [e]Department of Applied Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki 852-8521, Japan; and [f]Department of Virology II, National Institute of Infectious Diseases, Tokyo 208-0011, Japan

Edited by Michael G. Rossmann, Purdue University, West Lafayette, IN, and approved June 8, 2009 (received for review April 3, 2009)

Hepatitis E virus (HEV) is a causative agent of acute hepatitis. The crystal structure of HEV-like particles (HEV-LP) consisting of capsid protein was determined at 3.5-Å resolution. The capsid protein exhibited a quite different folding at the protruding and middle domains from the members of the families of *Caliciviridae* and *Tombusviridae*, while the shell domain shared the common folding. Tyr-288 at the 5-fold axis plays key roles in the assembly of HEV-LP, and aromatic amino acid residues are well conserved among the structurally related viruses. Mutational analyses indicated that the protruding domain is involved in the binding to the cells susceptive to HEV infection and has some neutralization epitopes. These structural and biological findings are important for understanding the molecular mechanisms of assembly and entry of HEV and also provide clues in the development of preventive and prophylactic measures for hepatitis E.

capsid | HEV | VLP

Hepatitis E is an acute viral hepatitis caused by infection with hepatitis E virus (HEV) that is transmitted primarily by a fecal-oral route (1, 2). Numerous epidemic and sporadic cases have occurred in developing countries of Asia, the Middle East, and North Africa, where sanitary conditions are not well-maintained. Hepatitis E affects predominantly young adults, and HEV infection in pregnancy is one of the risk factors for severe disease and death (3). Recent epidemiological studies show that significant prevalence of HEV and anti-HEV antibody is found in humans and several animals worldwide, even in developed countries (4–8).

HEV is the sole member of the genus *Hepevirus* within the family *Hepeviridae* and has a 7.2-kb positive-sense RNA genome (9). Five major genotypes have been identified so far (2). The viruses in the genotypes 1 and 2 are maintained among only humans, while those in the genotypes 3 and 4 are found in pigs or wild animals (4–6). However, infections of human with genotypes 3 and 4 via zoonotic transmission or blood transfusion were reported in the developed countries, such as Japan and the United States (7, 8, 10), suggesting that hepatitis E caused by infection with genotypes 3 and 4 of HEV is an important emerging infectious disease. The viruses in the genotype 5 are of avian origin and are thought to be uninfectious to humans (11). The genomic RNA contains three ORFs (ORFs) encoding nonstructural proteins (ORF1), the viral capsid protein composed of 660 amino acids (ORF2) and a small phosphorylated protein of unidentified function (ORF3) (1, 9). The viral capsid protein induces neutralizing antibodies by its immunization (12–15) or during the course of infection (16, 17). A typical signal sequence at the N terminus and 3 potential *N*-glycosylation sites (Asn-X-Ser/Thr) are well-conserved in the capsid protein derived from all mammalian genotypes (18, 19), but the glycosylation status of this protein is still controversial and the biological significance of the modification in the viral life cycle remains unknown. Although propagation of HEV in the cell culture systems reported in earlier studies was not efficient (20–23), Tanaka et al. succeeded in the establishment of a persistent infection system of HEV genotype 3 in human hepatoma (PLC/PRF/5) and human carcinomic alveolar epithelial (A549) cell lines (24). However, sufficient amounts of viral particles cannot be obtained for studies of the structure, life cycle, and pathogenesis of HEV.

Electron microscopy of human stool specimens showed that HEV is a nonenveloped spherical particle with a diameter of approximately 320 Å (25). As an alternative to in vitro propagation of HEV, the baculovirus expression system opens the prospect of studying HEV capsid assembly, since HEV-like particles (HEV-LP) with protruding spikes on the surface can be formed in insect cells infected with a recombinant baculovirus expressing the capsid protein of a genotype 1 strain (26–28). Cryo-electron microscopic (cryoEM) analysis has revealed that HEV-LP is a $T = 1$ icosahedral particle composed of 60 copies of truncated products of ORF2 (27, 28). The HEV-LP appeared to be empty due to a lack of significant density containing RNA inside and was 270 Å in diameter (26–28), which is smaller than the diameter of the native virions. However, the HEV-LP retained the antigenicity and capsid formation of the native HEV particles.

The crystal structures of the recombinant or native $T = 3$ viral particles derived from structurally related mammalian and plant viruses, such as recombinant Norwalk virus (rNV; PDB accession code 1IHM) (29), San Miguel sea lion virus (SMSV; PDB accession code 2GH8) (30), the members of the family *Caliciviridae*, and Carnation mottle virus (CARMV; PDB accession code 1OPO) (31), a member of the family *Tombusviridae*, have

Author contributions: T.Y., Y. Mori, T.T., T.C.L., N.T., T.M., and Y. Matsuura designed research; T.Y., Y. Mori, R.S., K.M., T.C.L., N.T., and Y. Matsuura performed research; T.Y., Y. Mori, N.M., R.H.C., M.Y., and H.U. analyzed data; and T.Y., Y. Mori, and Y. Matsuura wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2ZTN).

[1]T.Y. and Y. Mori contributed equally to this work.

[2]To whom correspondence should be addressed at: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita-shi, Osaka 565-0871, Japan. E-mail: matsuura@biken.osaka-u.ac.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/0903699106/DCSupplemental.

been determined at resolutions of 3.4 Å, 3.2 Å, and 3.2 Å, respectively. In this study, to understand the structural basis on HEV, we solved the crystal structure of HEV-LP derived from a genotype 3 strain at 3.5-Å resolution and found differences in the folding of the capsid protein among these viruses. On the other hand, we found several structural similarities of shell formation. In particular, it was revealed that aromatic amino acids (Tyr-288 in the case of HEV-LP) at the 5-fold axis play a crucial role in the hydrophobic interaction required for particle formation and are well conserved among these viruses. Furthermore, mutational analyses depicted the putative cellular receptor-binding regions and epitopes for neutralizing of binding (NOB) antibodies on the 3D structure of HEV-LP. The availability of the 3D structure of HEV-LP at high resolution will provide valuable information not only for analyses of the entry and assembly of HEV, but also for the development of a vaccine for hepatitis E.

Results

Preparation of HEV-LP of a Genotype 3. Upon infection with a recombinant baculovirus possessing a genome of the truncated capsid protein (amino acids 112–608) from a genotype 3 strain under the control of polyhedrin promoter, a large amount of HEV-LP was secreted into the culture supernatant as described in the case of HEV-LP of genotype 1 strain (26–28). The purified HEV-LP of genotype 3 was used for further structural and biological analyses.

Overall Structure of HEV-LP. The crystal structure of HEV-LP derived from the genotype 3 strain was determined at 3.5-Å resolution by the molecular replacement method by using a cryoEM map of HEV-LP derived from the genotype 1 strain (27, 28) as an initial phasing model (Fig. 1*A*). As shown in the previous papers (27, 28), HEV-LP shows a $T = 1$ icosahedral symmetry with an external diameter of 270 Å. This particle is composed of 60 subunits of the truncated capsid proteins, forming the icosahedral 2-, 3-, and 5-fold axes. It has 30 protrusions at the 2-fold axis of the surface with large depressions at the 3- and 5-fold axes.

Structure of the HEV Capsid Protein. The truncated HEV capsid protein has 3 definite domains designated as S (shell), M (middle), and P (protruding) composed of the amino acid residues 129–319, 320–455, and 456–606, respectively (Fig. 1*B*). Because the N- and C-terminally truncated capsid proteins were used for the characterization, the typical signal sequence (amino acids 1–22) and following arginine-rich domain (amino acids 23–111) and the C-terminal domain removed by cleavage in insect cells (amino acids 609–660) were not determined in this study. Additionally, the amino acid residues 112–128, 486–487, 555–560, and 607–608 were disordered in this study. The S domain, which forms an internal scaffold structure of the particle, folds into a classical anti-parallel jelly roll-like β-sandwich structure with 8 β-strands (designated as B to I) and 4 short α-helices that are conserved among many viral capsids (Fig. 1*B* and Fig. S1) (29–33). The M domain, which is one of the characteristic domains, has a twisted anti-parallel β-barrel structure composed of 6 β-strands and 4 short α-helices. This domain is tightly associated with the S domain and located on the surface around the icosahedral 3-fold axis (Fig. 1*A* and *B*). The M and P domains are linked with a long proline-rich hinge (amino acids 445–467). Previous studies on the structures of rNV (29) and SMSV (30) revealed that the P domains of the viruses are composed of 2 subdomains, P1 and P2, and the P2 subdomain is located as a large protrusion of the P1 subdomain (Fig. S1). In contrast, the P domain of HEV-LP is composed of a single individual domain forming a twisted anti-parallel β-sheets structure (Fig. 1*B* and Fig. S1), demonstrating that the capsid protein

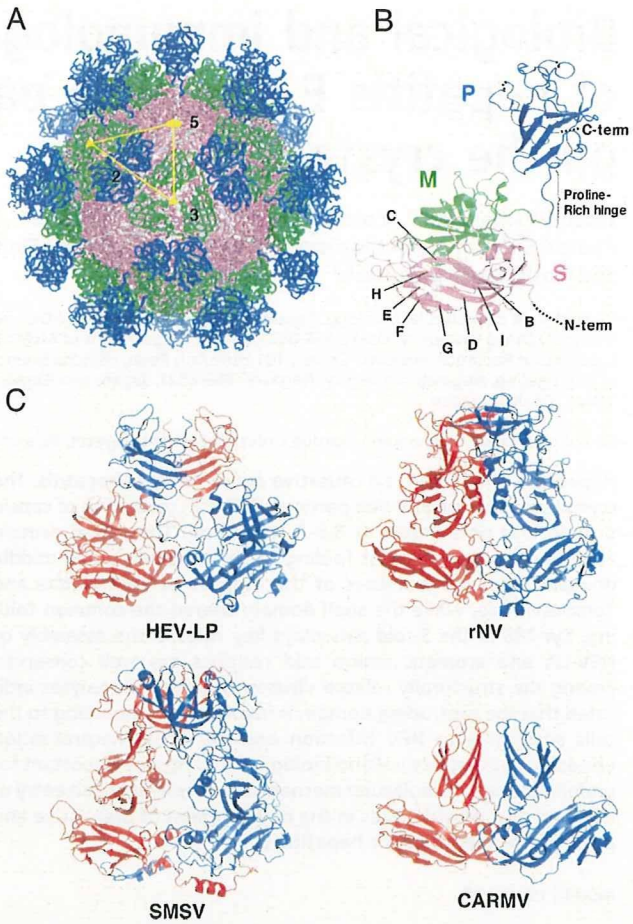

Fig. 1. Crystal structure of HEV-LP and comparison of capsid protein dimers of HEV-LP, rNV, SMSV, and CARMV. The S, M, and P domains of the HEV capsid protein are indicated by pink, green, and blue, respectively. (*A*) HEV-LP is composed of sixty capsid subunits forming icosahedral 2-, 3-, and 5-fold axes and indicating a $T = 1$ symmetry. (*B*) The ribbon diagram of a capsid subunit of HEV-LP (PDB accession code: 2ZTN) shows P, M, and S domains at the top, middle, and bottom, respectively. The disordered regions are shown with dashed lines. The S domain shows a jerry roll-like β-barrel structure conserved in some viruses. The conserved anti-parallel β-strands are indicated (B to I). (*C*) The ribbon diagrams of crystal structures of capsid protein dimers of HEV-LP and those of rNV (PDB accession code 1IHM), SMSV (PDB accession code 2GH8), and CARMV (PDB accession code 1OPO) are indicated. Each capsid protein monomer is colored in red and blue.

of HEV-LP has a significantly different fold from those of caliciviruses, except for the S domain. Although we have no evidence of glycosylation of HEV-LP prepared in insect cells, the HEV capsid protein has 3 potential *N*-glycosylation sites, Asn-137-Leu-Ser, Asn-310-Leu-Thr and Asn-562-Thr-Thr (19). In the dimer structure, the former 2 sites are mapped on the horizontal surface of the S domain, as shown in Fig. S2*A*. However, Asn-137 and Asn-310 are located in the interfaces of the pentamer and trimer structures, respectively (Fig. S2*B* and *C*), suggesting that, if it occurs at all, *N*-glycosylation in these sites may inhibit assembly of HEV-LP. Indeed, Graff et al. (18) reported that HEV carrying mutations in Asn-137 or Asn-310 to Glu lost infectivity to cells or rhesus macaques due to a defect in the virion assembly. On the other hand, Asn-562 is mapped in the central region in the top of the P dimer and exposed in the surface of HEV-LP.

The Dimer Structure at the 2-Fold Axis. It is noteworthy that the HEV-LP dimer at the icosahedral 2-fold axis shows a crossing

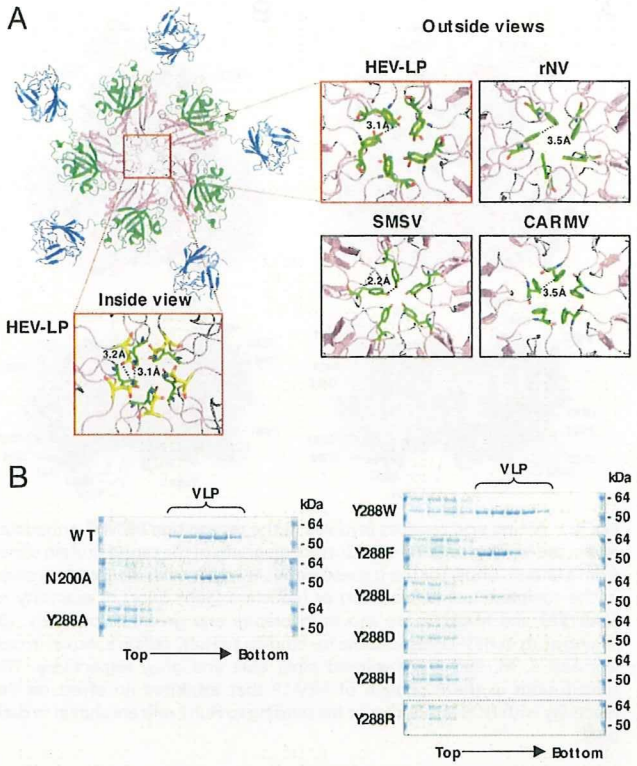

Fig. 2. Interaction of capsid protein subunits of HEV-LP around the 5-fold axis. (*A*) The pentamer of the capsid protein of HEV-LP. The close-up surface diagram of the 5-fold axis showed from outside and inside of HEV-LP. Amino acid residues Asn-200 and Tyr-288 are shown in yellow and green, respectively. The close-up surface diagram of the 5-fold axis showed from outside of rNV, SMSV, and CARMV. The aromatic amino acids Phe-118 of rNV, Tyr-330 of SMSV, and Phe-145 of CARMV are indicated in green. The deduced interacting atoms are connected with dashed lines, and the distances are indicated. (*B*) Sucrose density fractionation assay using the wild-type or mutant capsid proteins (53 kDa) in which the amino acids composing the 5-fold axis were substituted. The capsid protein composing HEV-LP was found in the 5–9th fractions from the top, while that which failed to form particles was found in the top fractions. The molecular mass of approximately 64 kDa was a non-specific protein.

topology of the P versus M and S domains, while that of the other viruses with protrusions at the 2-fold axis, containing rNV, SMSV, and CARMV, exhibits a parallel topology of each domain (Fig. 1*C*). The flexibility of the long proline-rich hinge region between the M and P domains allows this unique topology of HEV-LP. The P domain of HEV-LP interacts with not only the P domain but also the M domain of the counterpart to stabilize the dimer structure. Despite these topological differences, the overall structure of the protrusion dimeric structure at the 2-fold axis is similar to that of rNV and SMSV. The disordered residues 486–487 and 555–560 are located in the apical region of the protrusion, suggesting that this region is flexible to take advantage of the interaction with other molecules.

Five-Fold Axis Packaging. The inter-molecule-interface of the capsid pentamer at the icosahedral 5-fold axis is composed of only S domains, and these interaction regions are narrower than those of the dimer and trimer at the 2-fold and 3-fold axes, respectively (Fig. 2*A*), suggesting that the pentamer formation is a key step of HEV-LP assembly. There are 4 loops between the β-sheets in the S domain, designated as loops B–C (amino acids 139–152), D–E (amino acids 196–206), F–G (amino acids 236–241), and H–I (amino acids 281–296), around the center of the pentamer structure. Among them, the loops B–C and F–G are not in close proximity to the next subunits, suggesting they are not implicated in the inter-molecular interaction. In contrast, loops D–E and H–I do interact with the next subunits. In particular, the side chains of Asn-200 and Tyr-288 in loops D–E and H–I, respectively, interact with those of the next subunits, from which they are separated by a distance of approximately 3.2 Å, filling in the central pore (Fig. 2*A*). These observations led us to hypothesize that these amino acid residues are important for assembly and stability of the particles. To examine this hypothesis, we constructed 2 mutant capsid proteins in which Asn-200 was replaced with alanine (N200A) or Tyr-288 was replaced with alanine (Y288A), and the effect of these mutations on the particle formation was determined by a density-fractionation assay (Fig. 2*B*). Comparative amounts of the mutant proteins to the wild-type capsid were expressed and released into the supernatants of cells infected with the recombinant baculoviruses. N200A but not Y288A formed VLP as the wild-type, indicating that Tyr-288 plays a more crucial role in particle formation than Asn-200. The aromatic amino acids, Phe-118, Tyr-330, and Phe-145, are also found in the icosahedral 5-fold axis of rNV, SMSV, and CARMV, respectively (Fig. 2*A*). To examine the role of the aromatic side chain in Tyr-288 in the particle formation, a series of mutants in which Tyr-288 was replaced with tryptophan, phenylalanine, leucine, asparatic acid, histidine, or arginine (Y288W, Y288F, Y288L, Y288D, Y288H, or Y288R) were generated. All of them were expressed and released into the culture medium, as well as was the wild type. The mutants with aromatic amino acids, Y288W and Y288F, were able to form HEV-LP, whereas other mutants produced no or very few particles (Fig. 2*B*). These results suggest that the aromatic side chain of Tyr-288 plays a crucial role in the HEV-LP formation by shutting off the central pore of the pentamer, and that the aromatic amino acids in the positions corresponding to Tyr-288 of HEV are functionally conserved among the structurally related viruses.

Binding of HEV-LP to Cultured Cells. The early steps of HEV entry remain unclear because of the lack of a robust cell culture system for HEV, despite recent progress in the in vitro propagation of HEV in the cell lines PLC/PRF/5 and A549 (24). HEV-LP was able to bind to several cell lines, including PLC/PRF/5 and A549 cells, but not to mouse myeloma P3 × 63Ag8U.1 (P3U1) cells (Fig. S3), suggesting that a binding assay using HEV-LP is useful to examine the first step of receptor-binding of HEV to the target cells. Among the cell lines examined, the human hepatoma cell line Huh7, exhibited a greater ability to bind to HEV-LP than the cell lines PLC/PRF/5 and A549. Therefore, Huh7 cells were used for the following binding experiments of HEV-LP.

Three-Dimensional Mapping of Epitopes for NOB Antibodies. We examined the ability of the 10 newly produced anti-HEV-LP monoclonal antibodies to inhibit the binding of HEV-LP to Huh7 cells (Fig. 3*A*). Two of the monoclonal antibodies, MAB1323 and MAB272, exhibited NOB of HEV-LP to Huh7 cells and recognized the P domain by immunoblotting using the GST (GST)-fused HEV capsid proteins (Fig. S4). However, further truncation of the C-terminal 28 or N-terminal 24 amino acids from the GST-fused P domain abrogated the binding with the antibodies, indicating that it is difficult to determine the epitopes of the antibodies in more detail using a series of truncated mutants of the P domain. A competitive enzyme-linked immunosorbent assay (ELISA) suggested that MAB1323, MAB272, and MAB161, but not MAB358, which was used as a detector in the binding assay, recognized the same or adjacent epitopes (Fig. S5). The P domains of rNV and feline calicivirus were suggested to be involved in the binding to the receptor molecules (34–36), and we therefore hypothesized that the P

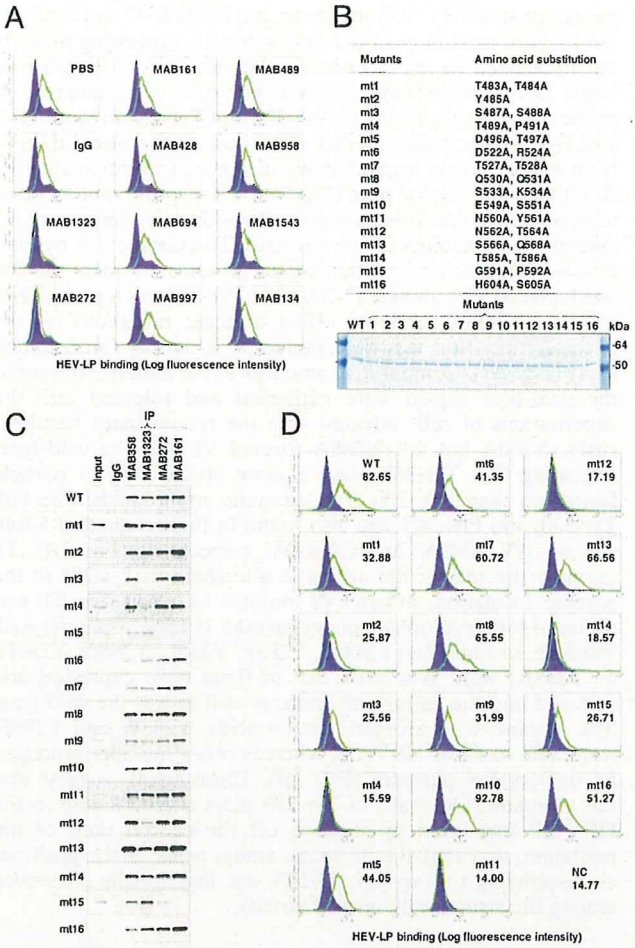

Fig. 3. Characterization of monoclonal antibodies and mutant HEV-LPs. (*A*) Neutralization of binding (NOB) of HEV-LP to Huh7 cells by monoclonal antibodies to HEV-LP. After preincubation of HEV-LP (10 μg/mL) with each of the monoclonal antibodies (20 μg/mL) for 1 h at 37°C, the mixture was inoculated into Huh7 cells and incubated for 1 h at 4°C. HEV-LP (lined area) bound to cells was detected by flow cytometry. The filled area indicates mock-incubated cells. (*B*) Construction of HEV-LP mutants. Sixteen HEV-LP mutants, in which the surface amino acid residues of the P domain were substituted, were constructed. The protein bands of 100 ng each of the purified wild-type and mutant HEV-LPs were visualized by Coomassie brilliant blue staining after SDS/PAGE. (*C*) Reactivities of NOB antibodies with the mutant HEV-LPs. Immunoprecipitation analyses of a series of HEV-LPs by NOB (MAB1323 and MAB272) or non-NOB antibodies (MAB358 and MAB161). Immunoprecipitated HEV-LPs were detected by an anti-HEV capsid rabbit polyclonal antibody. (*D*) Binding capability of the mutant HEV-LPs to Huh7 cells. Wild-type or mutant HEV-LPs (10 μg/mL) were incubated with Huh7 cells for 1 h at 4°C, and then HEV-LP (lined area) bound to cells was detected by flow cytometry. The filled area indicates mock-incubated cells. The MFI is shown in each panel.

domain of HEV-LP might also be involved in the cell binding. To examine this possibility, we prepared 16 HEV-LP mutants in which 1 or 2 amino acid residues at the surface of the P domain were substituted (Fig. 3*B*). The density fractionation assay indicated that all of the mutant proteins formed HEV-LP in the manner of the wild-type capsid protein. MAB358, which recognized an epitope on the M domain (Fig. S4), was capable of precipitating all of the mutants (Fig. 3*C*). MAB1323 exhibited no interaction with mt3 and a weak precipitation of mt4 and mt12. Both MAB272 and MAB161 exhibited no or weak precipitation of mt5 and mt15, whereas MAB272 but not MAB161 exhibited

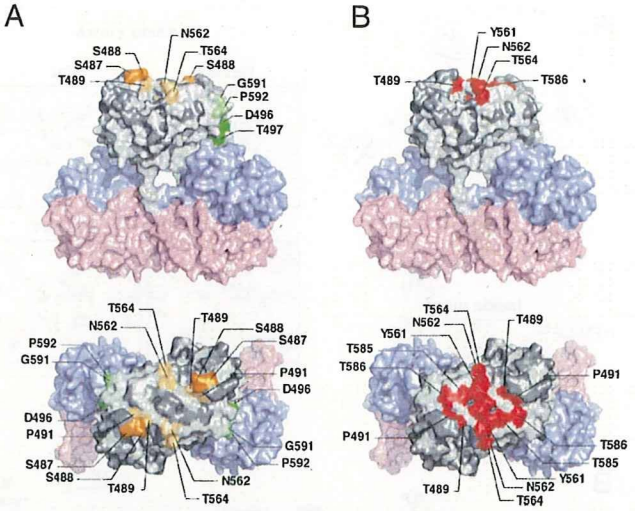

Fig. 4. Amino acid residues involved in the recognition by NOB antibodies and in the binding to Huh7 cells. Surface diagrams of the capsid protein dimer from a lateral (*Upper*) or top (*Lower*) view. (*A*) Amino acids in HEV-LP involved in the complete loss (deep color) or reduction (light color) of reactivity to MAB1323 and MAB272 are shown in orange and green, respectively. (*B*) Amino acids in HEV-LP responsible for binding to Huh7 cells are shown in red. Domains S, M, and P are colored pink, blue and gray, respectively. The substitutions in the P domain of HEV-LP that exhibited no effect on the reactivity with NOB antibodies or the binding to Huh7 cells are shown in dark gray.

NOB of HEV-LP to Huh7 cells (Fig. 3*A* and *C*). The substituted amino acids of these mutants are illustrated in the 3D structure of the capsid dimer (Fig. 4*A*), and these results suggest that the NOB antibodies MAB1323 and MAB272 recognize the peripheral region of the apical surface (orange) and the horizontal region (green) of the P domain above the M domain at the 3-fold axis, respectively.

Three-Dimensional Mapping of a Region Crucial for Binding to the Target Cells. To determine the region important for binding to the cell surface, the mutant HEV-LPs substituted into the P domain were also used in the assay of binding to Huh7 cells (Fig. 3*D*). The wild-type HEV-LP bound to Huh7 cells with a geographic mean fluorescence intensity (MFI) of 82.65. Among the mutants examined, mt4, mt11, mt12, and mt14 exhibited significantly low MFI values of less than 20. Similar results were obtained using A549 cells (Fig. S6). The amino acid residues required for cell binding were mapped in the central flexible region of the apical surface as shown in Fig. 4*B* (red). This region is partially overlapped with epitopes of MAB1323 (Fig. 4*A*) and other neutralizing antibodies reported by Schofield et al. (16) as shown in Fig. S7. These results suggested that the apical center region of the P domain is involved in the association with not-yet-identified cellular receptor(s).

Discussion

The expression of the truncated HEV capsid protein (amino acids 112–608) in insect cells resulted in assembly of HEV-LP, which retains an antigenicity similar to that of the native HEV particles (26, 37). This particle with a $T = 1$ symmetry has a diameter of 270 Å, which is smaller than the 320-Å diameter of the native virion detected in the fecal specimens of patients (25). It has been reported that the interior cavity of HEV-LP is too small to accommodate a viral RNA of 7.8 kb in length (28) and that the particles show no evidence of nucleotide contents (26, 28). Therefore, native HEV particles are sug-

Table 1. Data collection and processing statistics for HEV-LP

Data collection	
Space group	$P2_12_12_1$
Cell dimensions	
a, *b*, *c*, Å	336.8, 349.4, 359.5
X-ray wavelength, Å	1.0000
Resolution, Å	50–3.55 (3.68–3.55)
R_{merge}*	0.131 (0.498)
$I/\sigma I$	9.8 (3.2)
Completeness, %	99.9 (99.8)
Redundancy	5.6 (5.2)
Refinement	
Resolution range, Å	20–3.56
No. reflections	494,466
R_{work}/R_{free}	30.5/30.9
No. atoms	
Protein	215,400
B factors	
Protein	94.9
rmsd	
Bond length, Å	0.009
Bond angle, °	1.355

Values in square brackets refer to the highest-resolution shell.
*$R_{merge} = \Sigma_{hkl}\Sigma_i|I(hkl)_i - \langle I(hkl)\rangle|/\Sigma_{hkl}I(hkl)$, where $I(hkl)_i$ is the *i*th measurement of the intensity of reflection hkl and $\langle I(hkl)\rangle$ is the mean intensity of reflection *hkl*.

gested to be composed of a larger number and/or a larger size of capsid proteins than HEV-LP. In some cases of plant viruses with a $T = 3$ symmetry, the capsid proteins assembled into particles with a $T = 1$ symmetry by deletion of the N-terminal basic region (38, 39) or amino acid substitutions either in the N-terminal region or in the linker domain between the N-terminal region and S domain (39), suggesting that the N-terminal basic region plays an important role in switching of the transition from $T = 3$ to $T = 1$ symmetry. In addition, expression of the NV capsid protein in insect cells resulted in production of not only $T = 3$ large particles but also small particles thought to have the $T = 1$ symmetry (40). Based on many similarities of the capsid structures and their packaging of structurally related viruses, the native HEV particles are suggested to possess a $T = 3$ surface lattice. The flexibility of the proline-rich hinge linking the M and P domains could allow the capsid protein dimer to switch conformations between the A/B and C/C subunits found in $T = 3$ viruses. Although structure of the native HEV may be slightly different from that of the HEV-LP, the data obtained in this study by using HEV-LP should provide useful information to understand the structure of viral particle, life cycle, and pathogenesis of HEV. The S domain shares the jellyroll fold with some other icosahedral viruses (29–33). It was found that the capsid proteins with substitutions of Tyr-288 positioned at the center of the pentamer structure built in interS domain-interaction failed to assemble into HEV-LP. Alignment analysis of amino acid sequences using data available in GeneBank showed that Tyr-288 is completely conserved within 5 genotypes of HEV. Furthermore, residues corresponding to Tyr-288 of the HEV capsid protein are found in the structures of rNV (Phe-118), SMSV (Tyr-330), and CARMV (Phe-145), although the positions of these aromatic residues are different. Tyr-288 of HEV and Tyr-330 of SMSV located in the H-I loop and Phe-110 of rNV in the D–E loop are exposed at the outside surface of the particles, whereas Phe-145 of CARMV located in the D–E loop is exposed at the interior of the particle. These data suggest that the aromatic side chains of these residues are involved in hydrophobic interactions with those of the next subunits, assuring stable assembly of the particles. During entry into cells, rearrangement of the virion structure is required for release of the genome from the shell. However, the entry and uncoating mechanisms of HEV remain unknown. Because the center of the pentamer is the thinnest region of the particle and takes a channel-like structure (28), this region might also be important for uncoating and release of the viral RNA. It has been proposed that the 5-fold axis of poliovirus is involved in the genomic RNA translocation via conformational change of the virion initiated by binding to the receptor molecules (41, 42).

The first step in viral entry into a target cell is binding to the cellular receptors. The human hepatoma PLC/PRF/5 and lung epithelial A549 cell lines, which are highly susceptible to persistent HEV-infection (24), are likely to express functional HEV receptors on the cell surface. However, HEV-LP had reduced binding to these cells compared to the other cell lines examined. Therefore, the human hepatoma cell line Huh7, which also exhibited a susceptibility to HEV infection (13, 18) and readily bound to HEV-LP, was mainly used in this study. It has been reported that the P domains of noroviruses and the feline calicivirus were involved in the binding to the putative receptors, histo-blood antigens (35, 36) and the feline junctional adhesion molecule (34), respectively. The peptide of the HEV capsid protein (amino acids 368–606), which consists of a part of the M and an entire P domain, was shown to be capable of binding to several cell lines (13), suggesting that the P domain of HEV is also involved in the binding to the cell receptors. Indeed, the mutational analyses in this study indicated that the central flexible region of the top of the P domain of HEV-LP plays a crucial role for binding to Huh7 and A549 cells. This is consistent with a recent study by Graff et al. in which an N562Q mutant of HEV lost infectivity to culture cells and rhesus macaques despite the production of viral particles (18). Interestingly, a possible *N*-glycosylation site, Asn-562-Thr-Thr, is mapped in this region. *N*-glycosylation is an unusual posttranslational modification for nonenveloped viruses, except for rotaviruses (43). The mutant capsid mt12, which has substitutions of Asn-562 and Thr-564 to alanine, exhibited the same migration as the wild-type protein in SDS/PAGE, suggesting that the HEV-LP produced in insect cells was not glycosylated at Asn-562. Lack of *N*-glycosylation in the capsid protein has also been reported in mammalian cells infected with HEV (18), whereas some portion of the capsid protein was glycosylated and transported to the cell surface upon overexpression in mammalian cells (19). *N*-glycosylation of the HEV capsid at Asn-562 may have a negative effect on the receptor-binding, whereas it may play a positive role in other functions, including pathogenesis. The biological significance of the glycosylation of HEV capsid protein remains to be studied.

Although there is currently a lack of sensitive and reliable assays to determine the neutralizing activity of anti-HEV antibodies, the assay of NOB of HEV-LP binding to the target cells is thought to be a suitable alternative method. Measurement of the reactivity of a panel of mutant HEV-LPs revealed that the epitopes of MAB1323 and MAB272 antibodies are mapped in the peripheral region of the apical surface and the horizontal region of the P domain dimer, respectively. These results further support the notion that the P domain of HEV-LP is important for the binding to cells. MAB1323 is suggested to directly inhibit the interaction between HEV-LP and cellular receptors through binding to the apical surface, whereas MAB272 may have an allosteric effect, inducing conformational change of the P domain through binding to the horizontal region. A number of monoclonal antibodies are capable of neutralizing in vitro and in vivo infection of HEV (12–17), and many of them recognize conformational epitopes

of the capsid protein, as seen in the MAB1323 and MAB272 antibodies prepared in this study. Monoclonal antibodies against linear epitopes located in amino acids 578–607 of a genotype 1 capsid protein (16) were overlapped with a part of the putative receptor-binding domain and the epitope of MAB272, supporting the data of the present study. On the other hand, monoclonal antibodies against the linear epitopes located in amino acids 423–438 and amino acids 423–443 in the M domain of a genotype 1 capsid protein neutralized binding of a peptide derived from the capsid protein to cells and HEV-infection (13), suggesting the importance of the M domain in the binding step.

In summary, we have determined the crystal structure of HEV-LP produced in insect cells and demonstrated its structural characteristics in comparison with the structurally related animal and plant viruses. This study will provide useful information for elucidation of the molecular mechanisms of HEV-life cycles and for the development of prophylactic and therapeutic measures for hepatitis E.

Materials and Methods

Expression, Purification, and Crystallization of HEV-LP. The recombinant baculovirus encoding the ORF2 of the HEV genotype 3, 2712 strain was expressed in insect cells. HEV-LP was purified as described previously (28) and crystallized by the hanging-drop vapor-diffusion method. Details are reported in *SI Materials and Methods*.

Data Collection and Phase Determination. x-ray diffraction data were collected at 100 K on beamlines BL17A at the Photon Factory (KEK). The statistics of X-ray diffraction data collection are summarized in Table 1. The solved 3D structure of HEV-LP was submitted to the Protein Data Bank under the PDB accession code of 2ZTN. Details are reported in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank H. Murase for her secretarial work and the staff of SPring-8 BL44XU beamline and synchrotron beamline NW-17A of the Photon Factory, High Energy Accelerator Research Organization for their assistance with the data collection. This work was supported in part by grants-in-aid from the Research and Development Program for New Bio-industry Initiatives of Bio-oriented Technology Research Advancement Institution (BRAIN) and the Foundation for Research Collaboration Center on Emerging and Re-emerging Infections.

1. Panda SK, Thakral D, Rehman S (2007) Hepatitis E virus. *Rev Med Virol* 17:151–180.
2. Purcell RH, Emerson SU (2008) Hepatitis E: An emerging awareness of an old disease. *J Hepatol* 48:494–503.
3. Navaneethan U, Al Mohajer M, Shata MT (2008) Hepatitis E and pregnancy: Understanding the pathogenesis. *Liver Int* 28:1190–1199.
4. Meng XJ, *et al.* (1997) A novel virus in swine is closely related to the human hepatitis E virus. *Proc Natl Acad Sci USA* 94:9860–9865.
5. Sonoda H, *et al.* (2004) Prevalence of hepatitis E virus (HEV) Infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan. *J Clin Microbiol* 42:5371–5374.
6. Okamoto H (2007) Genetic variability and evolution of hepatitis E virus. *Virus Res* 127:216–228.
7. Li TC, *et al.* (2005) Hepatitis E virus transmission from wild boar meat. *Emerg Infect Dis* 11:1958–1960.
8. Yazaki Y, *et al.* (2003) Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. *J Gen Virol* 84:2351–2357.
9. Tam AW, *et al.* (1991) Hepatitis E virus (HEV): Molecular cloning and sequencing of the full-length viral genome. *Virology* 185:120–131.
10. Matsubayashi K, *et al.* (2004) Transfusion-transmitted hepatitis E caused by apparently indigenous hepatitis E virus strain in Hokkaido, Japan. *Transfusion* 44:934–940.
11. Huang FF, *et al.* (2004) Determination and analysis of the complete genomic sequence of avian hepatitis E virus (avian HEV) and attempts to infect rhesus monkeys with avian HEV. *J Gen Virol* 85:1609–1618.
12. Emerson SU, *et al.* (2006) Putative neutralization epitopes and broad cross-genotype neutralization of Hepatitis E virus confirmed by a quantitative cell-culture assay. *J Gen Virol* 87:697–704.
13. He S, *et al.* (2008) Putative receptor-binding sites of hepatitis E virus. *J Gen Virol* 89:245–249.
14. Meng J, *et al.* (2001) Identification and characterization of the neutralization epitope(s) of the hepatitis E virus. *Virology* 288:203–211.
15. Takahashi M, *et al.* (2008) Production of monoclonal antibodies against hepatitis E virus capsid protein and evaluation of their neutralizing activity in a cell culture system. *Arch Virol* 153:657–666.
16. Schofield DJ, Glamann J, Emerson SU, Purcell RH (2000) Identification by phage display and characterization of two neutralizing chimpanzee monoclonal antibodies to the hepatitis E virus capsid protein. *J Virol* 74:5548–5555.
17. Schofield DJ, Purcell RH, Nguyen HT, Emerson SU (2003) Monoclonal antibodies that neutralize HEV recognize an antigenic site at the carboxyterminus of an ORF2 protein vaccine. *Vaccine* 22:257–267.
18. Graff J, *et al.* (2008) Mutations within potential glycosylation sites in the capsid protein of hepatitis E virus prevent the formation of infectious virus particles. *J Virol* 82:1185–1194.
19. Zafrullah M, Ozdener MH, Kumar R, Panda SK, Jameel S (1999) Mutational analysis of glycosylation, membrane translocation, and cell surface expression of the hepatitis E virus ORF2 protein. *J Virol* 73:4074–4082.
20. Huang R, *et al.* (1999) Cell culture of sporadic hepatitis E virus in China. *Clin Diagn Lab Immunol* 6:729–733.
21. Kazachkov Yu A, *et al.* (1992) Hepatitis E virus in cultivated cells. *Arch Virol* 127:399–402.
22. Meng J, Dubreuil P, Pillot J (1997) A new PCR-based seroneutralization assay in cell culture for diagnosis of hepatitis E. *J Clin Microbiol* 35:1373–1377.
23. Tam AW, *et al.* (1997) In vitro infection and replication of hepatitis E virus in primary cynomolgus macaque hepatocytes. *Virology* 238:94–102.
24. Tanaka T, Takahashi M, Kusano E, Okamoto H (2007) Development and evaluation of an efficient cell-culture system for Hepatitis E virus. *J Gen Virol* 88:903–911.
25. Bradley D, *et al.* (1988) Aetiological agent of enterically transmitted non-A, non-B hepatitis. *J Gen Virol* 69:731–738.
26. Li TC, *et al.* (1997) Expression and self-assembly of empty virus-like particles of hepatitis E virus. *J Virol* 71:7207–7213.
27. Li TC, *et al.* (2005) Essential elements of the capsid protein for self-assembly into empty virus-like particles of hepatitis E virus. *J Virol* 79:12999–13006.
28. Xing L, *et al.* (1999) Recombinant hepatitis E capsid protein self-assembles into a dual-domain T = 1 particle presenting native virus epitopes. *Virology* 265:35–45.
29. Prasad BV, *et al.* (1999) X-ray crystallographic structure of the Norwalk virus capsid. *Science* 286:287–290.
30. Chen R, Neill JD, Estes MK, Prasad BV (2006) X-ray structure of a native calicivirus: Structural insights into antigenic diversity and host specificity. *Proc Natl Acad Sci USA* 103:8048–8053.
31. Morgunova E, *et al.* (1994) The atomic structure of Carnation Mottle Virus capsid protein. *FEBS Lett* 338:267–271.
32. Hogle JM, Chow M, Filman DJ (1985) Three-dimensional structure of poliovirus at 2.9 A resolution. *Science* 229:1358–1365.
33. Tsao J, *et al.* (1991) The three-dimensional structure of canine parvovirus and its functional implications. *Science* 251:1456–1464.
34. Bhella D, Gatherer D, Chaudhry Y, Pink R, Goodfellow IG (2008) Structural insights into calicivirus attachment and uncoating. *J Virol* 82:8051–8058.
35. Bu W, *et al.* (2008) Structural basis for the receptor binding specificity of Norwalk virus. *J Virol* 82:5340–5347.
36. Choi JM, Hutson AM, Estes MK, Prasad BV (2008) Atomic resolution structural characterization of recognition of histo-blood group antigens by Norwalk virus. *Proc Natl Acad Sci USA* 105:9175–9180.
37. Li TC, *et al.* (2004) Protection of cynomolgus monkeys against HEV infection by oral administration of recombinant hepatitis E virus-like particles. *Vaccine* 22:370–377.
38. Hsu C, *et al.* (2006) Characterization of polymorphism displayed by the coat protein mutants of tomato bushy stunt virus. *Virology* 349:222–229.
39. Kakani K, Reade R, Katpally U, Smith T, Rochon D (2008) Induction of particle polymorphism by cucumber necrosis virus coat protein mutants in vivo. *J Virol* 82:1547–1557.
40. White LJ, Hardy ME, Estes MK (1997) Biochemical characterization of a smaller form of recombinant Norwalk virus capsids assembled in insect cells. *J Virol* 71:8066–8072.
41. Belnap DM, *et al.* (2000) Molecular tectonic model of virus structural transitions: The putative cell entry states of poliovirus. *J Virol* 74:1342–1354.
42. Bubeck D, Filman DJ, Hogle JM (2005) Cryo-electron microscopy reconstruction of a poliovirus-receptor-membrane complex. *Nat Struct Mol Biol* 12:615–618.
43. Jayaram H, Estes MK, Prasad BV (2004) Emerging themes in rotavirus cell entry, genome organization, transcription and replication. *Virus Res* 101:67–81.

doi:10.1111/j.1440-1746.2008.05677.x

HEPATOLOGY

Detection of hepatitis E virus RNA and genotype in Bangladesh

Masahiko Sugitani,* Akinori Tamura,[†,‡] Yohko K Shimizu,[†] Aleemuzzaman Sheikh,* Noriko Kinukawa,* Kazufumi Shimizu,[†] Mitsuhiko Moriyama,[‡] Kazuo Komiyama,[§] Tian-Cheng Li,[¶] Naokazu Takeda,[¶] Yasuyuki Arakawa,[‡,††] Koyu Suzuki,[*,‡‡] Shamsuddin M Ishaque,[§§] Projesh K Roy,[§§] ASMA Raihan[§§] and Mahmud Hasan[§§]

Departments of *Pathology and [†]Immunology and Microbiology, [‡]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Nihon University School of Medicine, [§]Department of Pathology, Nihon University School of Dentistry, [¶]Virology II, National Institute of Infectious Diseases, [††]Akiru Municipal Medical Center, [‡‡]Diagnostic Pathology, St. Luke's International Hospital, Tokyo, Japan; and [§§]Department of Gastroenterology, Internal Medicine, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh

Key words
Bangladesh, genotype, hepatitis E virus (HEV), HEV RNA, IgM specific anti-HEV.

Accepted for publication 25 August 2008.

Correspondence
Dr Masahiko Sugitani, Department of Pathology, Nihon University School of Medicine, 30-1 Oyaguchi-Kamimachi, Itabashi-ku, Tokyo 173-8610, Japan. Email: sugitani@med.nihon-u.ac.jp

Abstract

Background/Aims: Hepatitis E virus (HEV) in Bangladesh has not been adequately documented. We report HEV RNA and genotype detection in Bangladesh.

Methods: In total, 82 samples were used; 36 sporadic acute hepatitis (AH), 12 fulminant hepatitis (FH), 14 chronic liver disease (CLD) and 20 from an apparently healthy population (HP) positive for both immunoglobulin (Ig) M and IgG specific anti-HEV antibodies (anti-HEV). The male/female ratio was 61/21, ages 12–67 (mean 30.4) years. RNA was extracted, transcribed to cDNA and amplified in nt 6345–6490 (ORF2) of HEV. Nucleic and amino acid sequences were determined. Homology comparison between Bangladesh clones and other representative HEV clones and phylogenetic tree analyses were done. Relations between HEV RNA-positivity and clinical factors were analyzed.

Results: HEV RNA was positive in 9/36 (25.0%) of AH cases, 4/12 (33.3%) FH, 3/14 (21.4%) CLD and 0/20 (0%) HP samples; total 16/82 (19.5%). Four factors correlated significantly with HEV RNA-positivity (Mann-Whitney U test); alanine aminotransferase (ALT) ($P = 0.0229$), aspartate aminotransferase (AST) ($P = 0.0448$), and titers of IgG ($P = 0.0208$) and IgM ($P = 0.0095$) specific anti-HEV. The 16 HEV clones were divided mainly into two groups, A and B, including six different cDNA sub-groups.

Conclusion: HEV RNA was found in sporadic AH and FH and sub-clinical CLD cases, but not in HP. HEV RNA-positivity was significantly related to values of ALT and AST and titers of IgG and IgM specific anti-HEV, with IgM specific anti-HEV showing the most significant relationship. All clones were genotype I, which is prevalent in South Asia.

Introduction

Hepatitis E virus (HEV) is a single-stranded, approximately 32 to 34 nm in diameter, positive sense, 7.2 kb RNA virus. Hepatitis E virus infection causes both endemic and sporadic types of acute hepatitis (AH) and fulminant hepatitis (FH) transmitted via the oral-fecal route. Outbreaks of acute hepatitis E are common, mainly in developing countries with inadequate environmental sanitation, with contaminated drinking water considered to be the main source of infection. The first documented outbreak of acute hepatitis E was in New Delhi[1,2] in 1955. Subsequently, similar outbreaks were reported worldwide, for example in Southeastern[3] and Central[4] Asia, China,[5] Africa[6] and Mexico.[7] Furthermore, hepatitis E was recognized as being the major etiological agent for acute hepatitis and acute liver failure in endemic regions.[8] Though numerous endemic or sporadic types of acute hepatitis E have been reported in India,[9] Pakistan,[10] Nepal[11] and Burma,[12] the state of hepatitis E in Bangladesh has not been adequately documented. As there are many rivers in Bangladesh, floods are common during the rainy season. Therefore, waterborne hepatitis has long been suspected to exist in Bangladesh. Our previous report[13] on the etiologies of Bangladesh FH and a report[14] describing a peacekeeping soldier who suffered from hepatitis E while traveling in Bangladesh, support the assumption that waterborne hepatitis E does exist in this area. Additionally, infection with hepatitis A virus (HAV), which is also transmitted via the oral-fecal route, occurs in 98% of the population by the age of 15 in Bangladesh.[13] Given the life long immunity to HAV, hepatitis A was not considered to be significant in Bangladeshi adults.

Table 1 Summary of parameters assessed in cases positive for both IgG and IgM specific anti-HEV

	AH	FH	CLD	HP	Total
Number of Cases	36	12	14	20	82
Male/Female	24/12	8/4	11/3	18/2	61/21
Age (mean, years)	17–67 (29.8)	22–60 (32.8)	12–60 (31.7)	16–62 (29.2)	12–67 (30.4)
ALT (mean, U/L)	23–1648 (176)	25–679 (169)	14–806 (130)	17–53 (31)	14–1648 (132.5)
AST (mean, U/L)	8–513 (63)	14–748 (129)	4–330 (45)	9–27 (16)	4–748 (58.1)
Total Bilirubin (mean, mg/dL)	0.3–31.2 (6.4)	0.8–19.5 (8.5)	0.3–6.0 (1.9)	0.2–1.0 (0.4)	0.2–31.2 (4.5)
Titer of IgG specific anti-HEV (mean)	0.506–3.613 (2.942)	0.769–3.691 (3.048)	1.242–3.742 (2.563)	0.512–3.535 (2.234)	0.506–3.742 (2.720)
Titer of IgM specific anti-HEV (mean)	0.184–2.911 (1.712)	0.209–3.338 (2.116)	0.136–1.375 (0.596)	0.124–2.356 (0.468)	0.124–3.338 (1.277)

AH, acute hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CLD, chronic liver disease; FH, fulminant hepatitis; HEV, hepatitis E virus; HP, healthy population; IgG, immunoglobulin G; IgM, immunoglobulin M.

We obtained sera positive for both immunoglobulin (Ig) G and IgM specific anti-HEV antibodies (anti-HEV), as demonstrated by ELISA,[15] from patients with sporadic AH, FH, chronic liver diseases (CLD) and an apparently healthy population (HP) in Bangladesh. The reason why we examined anti-HEV in sera from the HP group was to assess subclinical HEV infection in Bangladesh. We measured anti-HEV in sera also from subjects with CLD. First, we wanted to determine whether or not HEV co-infection exists in sera from chronically HBV- and/or HCV-infected Bangladeshi subjects. Second, we wanted to obtain HEV-infected sera for the purpose of HEV cloning. Our next step was to investigate HEV RNA in the above sera and statistically analyze the relationships between HEV RNA-positivity and clinical parameters. We then determined nucleic and amino acid sequences and the HEV genotype prevalent in Bangladesh.

Materials and methods

Background of the study

Sera from patients with clinically diagnosed sporadic AH or FH and with CLD were collected at the Department of Gastroenterology, Internal Medicine, Bangabandhu Sheikh Mujib (BSM) Medical University (formerly the Institute of Post Graduate Medicine and Research) Hospital, Dhaka, Bangladesh, while the HP subjects were selected randomly from various walks of life of Dhaka, Bangladesh, in 1995 and 1996. Study subjects with AH, FH, CLD and HP were enrolled based on the following criteria. Inclusion criteria for sporadic AH were a typical prodrome of viral hepatitis from history, persistence or progressive jaundice of less than 6 months duration, and biochemical evidence of hepatocellular necrosis or injury. The FH criteria were presence of a typical prodrome of viral hepatitis from history and clinical observation of jaundice, biochemical evidence of severe hepatocellular injury and the presence of hepatic encephalopathy within 8 weeks of the development of jaundice. Criteria for CLD including chronic hepatitis (CH), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) were persistence of viral markers of HBV, HCV or HDV for more than 6 months. Other CLD criteria were as follows: CH, persistent or intermittent jaundice for more than 6 months and history of acute hepatitis 6 or more months previously with persistence of prodromal symptoms of acute hepatitis; LC, clinical features suggestive of liver cirrhosis based on symptoms, signs, physical examination and laboratory findings; HCC, a space occupying lesion in the liver on ultrasonographic examination, elevated alpha-fetoprotein level and no other primary malignancy anywhere in the body. The study did not require separating CH, LC and HCC, therefore, the combined group of CLD was used. Criteria for the HP were to be unrelated to the patients included in this study though living in a similar hygienic environment, and to be apparently healthy and have no past history of jaundice, liver dysfunction, blood transfusion, or exposure to alcohol or other hepatotoxic drugs.

Sera were immediately frozen at BSM Medical University in Dhaka, packed in dry ice, and then transported to Japan for examination. Out of the total of 549 samples from AH, FH, CLD and HP subjects, 97 (40 AH, 14 FH, 23 CLD and 20 HP) were positive for both IgG and IgM specific anti-HEV. Among the 97 subjects, 15 samples were not suitable for the present examination due to inadequate sample volumes.

Samples

Samples from 82 subjects in total, 36 AH, 12 FH, 14 CLD and 20 HP, were used in this study. The male/female ratio was 61/21, the age range was 12–67 (mean 30.4) years, and ranges for IgG and IgM specific anti-HEV titers were 0.506–3.742 (mean 2.720) and 0.124–3.338 (mean 1.277), respectively. Positive cut-off titers for IgG and IgM specific anti-HEV were over 0.150 and 0.110, respectively.[15] Sera from AH and FH subjects examined in this study had not always been collected at the peak of disease severity. All 14 CLD subjects were positive for HBV surface antigen, and four were also positive for HCV RNA in sera. Sex, age, values of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin, as well as titers of IgG and IgM specific anti-HEV, in the AH, FH, CLD and HP groups, are summarized in Table 1. All serum samples were collected after obtaining informed consent and appropriate approval.

Assays for IgG and IgM specific anti-HEV

IgG- and IgM-specific anti-HEV antibodies (anti-HEV) were determined by in-house enzyme-linked immunosorbent assay

Journal of Gastroenterology and Hepatology **24** (2009) 599–604

(ELISA).[15] In brief, a recombinant antigen of empty virus like particles (VLPs) of HEV was used for measurement of anti-HEV. These VLPs possess antigenicity similar to that of authentic HEV particles and are highly sensitive for detecting HEV-specific IgM and IgG antibodies. Serum samples were added to microplate wells and the reaction procedures were performed in the ordinary way. Finally, the absorbance at 492 nm was measured. Sera from hepatitis patients, previously confirmed by serological tests as well as reverse transcriptase polymerase chain reaction (RT-PCR) to be positive for hepatitis E viral infection, served as positive controls. Sera from healthy Japanese individuals, negative for both anti-HEV IgM and IgG, served as negative controls.

Detection of HEV RNA and sequencing

RNA was extracted from the sera of AH, FH, CH and HP subjects in Bangladesh using TRIzol LS (Invitrogen Corporation, Carlsbad, CA, USA) by a modification of the acid guanidinium thiocyanate-phenol-chloroform method,[16] and transcribed to complementary DNA (cDNA) using Super Script II reverse transcriptase (Invitrogen) by incubation at 42°C for 60 min. The cDNA was amplified by AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) using primers designed to amplify nucleotides 6369–6466 (length of amplified cDNA: 98 bp excluding primers) from the 5′ end of the open reading frame of HEV. Nucleic acid sequences of sense and anti-sense primers for the first PCR were GCC GAC AGA ATT GAT TTC GTC GGC (nucleotides 6295–6318) and GTC CTG CTC ATG TTG GTT GTC ATA (6496–6473), those for the second PCR CCG TCG TCT CAG CCA ATG GCG AGC (6345–6368) and CTC ATG TTG GTT GTC ATA ATC CTG (6490–6467), respectively. Nested PCR was performed as follows. Samples were incubated for denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 30 s. The first denaturation was performed for 9 min, the last extension for 7 min. The first and second PCR were carried out with 35 cycles each. One tenth of the volume of the first PCR products was used for the second PCR. The second PCR products were analyzed by electrophoresis on a 3% agarose gel, stained with ethidium bromide and examined under UV light.

DNA was extracted and purified and nucleic acid sequences were determined using an automated DNA sequencer (Applied Biosystems) in both directions in all HEV cDNA-positive samples. The amino acid sequence was then analyzed on the basis of the nucleic acid sequence. Comparisons of cDNA homology between Bangladesh clones and other representative HEV isolates were carried out. The isolate names and GenBank accession numbers of the reported sequences were as follows: M73218 from Burma, AF185822 and M80581 from Pakistan, X99441, X98292 and AF076239 from India, M94177, D11093 and AJ272108 from China, AF051830 from Nepal, AY230202 from Morocco, M74506 from Mexico and AF060668 from the United States. Phylogenetic trees were constructed by the neighbor-joining method,[17] based on the above partial nucleotide sequence in the ORF2 region of HEV. The final tree was drawn using DNASIS Pro software (Hitachi Software Engineering Co., Ltd., Tokyo, Japan).

Statistical analysis

Relationships between HEV RNA-positivity and clinical parameters were statistically analyzed. For the gender and clinical diagnosis analyses, the chi-square test was used, and for those of age, values of ALT, AST and total bilirubin and IgG and IgM specific anti-HEV titers, the Mann-Whitney U test was used. Concomitant infection was frequently found in AH and FH. These infections were assessed in 27 subjects (75.0% of 36) in the AH and 5 (41.7% of 12) in the FH group. Therefore, relationships between HEV RNA-positivity and concomitant infection were analyzed, using the chi-square test, only in the AH and FH groups. A P-value below 0.05 was considered significant.

Furthermore, two cluster groups of HEV clones were identified and the relationships of these clusters to clinical diagnoses were also statistically analyzed using the chi-square test.

Results

Ratios of the number of subjects positive for HEV RNA to those examined were 9/36 (25.0%) in the AH, 4/12 (33.3%) in the FH, 3/14 (21.4%) in the CLD and 0/20 (0%) in the HP group; 16/82 (19.5%) in total. The male/female ratios for HEV RNA-positive subjects were 7/2, 3/1 and 2/1 in the AH, FH and CLD groups, respectively; 12/4 in total. The age ranges of HEV RNA-positive subjects were 17–67 years (mean 35.8), 22–50 years (mean 31.2) and 18–60 years (mean 36.7) in the AH, FH and CLD groups, respectively, and that for all subjects was 17–67 years (mean 34.9). On one hand, there were no significant relationships between HEV RNA-positivity and either gender or clinical diagnosis (chi-square test), nor between HEV RNA-positivity and age (Mann-Whitney U test). On the other hand, significant correlations were identified between HEV RNA-positivity and ALT and AST values ($P = 0.0229$ and $P = 0.0448$, respectively, Mann-Whitney U test), while total bilirubin was not related to HEV RNA positivity.

Titers of IgG and IgM specific anti-HEV in HEV RNA-positive subjects were 0.506–3.742 (mean 2.998) and 0.192–3.338 (mean 1.994), respectively. In HEV RNA-positive subjects, mean titers of IgG specific anti-HEV in the AH, FH and CLD groups were 2.852, 3.477 and 2.798, respectively, whereas those of IgM specific anti-HEV were 1.996, 2.810 and 0.636, respectively. Significant correlations between these IgG and IgM titers and HEV RNA-positivity were demonstrated ($P = 0.0208$ and $P = 0.0095$, respectively, Mann-Whitney U test).

Concomitant infections with HEV and other hepatitis viruses were detected in 6 of 9 samples from HEV RNA-positive subjects with AH but in none of the four from HEV RNA-positive subjects with FH. Among the six AH cases, HEV was super-imposed on HBV carrier status in five, and one case with HCV infection was also a HBV carrier. On the other hand, concomitant infections were found in 21 of 27 samples from HEV RNA-negative subjects with AH, and in 5 of 8 samples from HEV RNA-negative subjects with FH. The ratio of concomitant infection with hepatitis viruses was high in both the HEV RNA-positive and the HEV RNA-negative group. There were no significant correlations between HEV RNA-positivity and concomitant infection with either AH or FH (chi-square test).

Nucleic and amino acid sequences of 16 samples are shown in Tables 2 and 3, respectively. Changes in nucleic acids showed a scattered distribution. Six subgroups (A1, A2, A3, A4, B1 and B2) of HEV clones from Bangladesh were found by nucleic acid analysis, and were classifiable into two main (A and B) groups. However, only two groups with minor changes were identified by

Table 2 Nucleic acid sequence and number of Bangladesh HEV clones found in this study

Clone	Nucleic acid sequence	Number of clones
A1	5′ CAACTGTTAAGCTGTATACATCTGTAGAGAATGCTCAGCAGGATAAGGG TATTGCAATCCCGCATGACATTGATCTTGGAGAATCTCGTGTGGTTATT 3′	6
A2	5′ ----------T---------- 3′	5
A3	5′ ----------T----------G-- 3′	1
A4	5′ ----------G---------- 3′	1
B1	5′ -G-----G-----A----------T---------- ---C----------T-----C--C----------A------ 3′	2
B2	5′ -G---------- ---C----------T-----C--C----------A------ 3′	1

Table 3 Reduced amino acid sequence and number of Bangladesh HEV clones found in this study

Clone	Reduced amino acid sequence	Number of clones
A1	TVKLYTSVENAQQDKGIAIPHDIDLGESRVVI	6
A2	----------V	5
A3	----------V	1
A4	----------	1
B1	----------	2
B2	----------	1

amino acid analysis (Table 3) and were not related to the A and B groups demonstrated by nucleic acid analysis. Homology comparisons between Bangladesh clones and other representative HEV clones with the same DNA sequence areas are summarized in Table 4. Bangladesh clones showed high homology with South Asian, Southeast Asian and Chinese clones of genotype I in comparison to those of genotype II, III and IV clones. Therefore, Bangladesh clones were recognized as belonging to genotype I, in terms of the nucleic acid sequence, rather than the other genotypes. The phylogenetic tree (Fig. 1) supports these results.

There were no significant associations between these two clusters of HEV clones (Groups A and B) and clinical diagnoses (chi-square test).

Discussion

In Bangladesh, HEV infection, serologically indicated by positivity for both IgG and IgM specific anti-HEV, was detected in adolescents and adults of both genders. In this study, HEV RNA was detected in 9 (25.0%) of 36 samples positive for both IgG and IgM specific anti-HEV in sporadic AH, in 4 of 12 (33.3%) FH, and in 3 of 14 (21.4%) CLD, but in none of the 20 sera positive for both antibodies from HP subjects with subclinical HEV infection. HEV RNA detection ratios were in the order of symptom severity for these three diseases. HEV RNA was reportedly detected in sera from AH and FH cases in an endemic HEV infection area, while co-existence of HEV infection in CLD was shown, at the nucleic acid level, to be rare. None of the HP samples in this study were positive for HEV RNA, though all samples came from an endemic HEV infection area. Mitsui *et al.*[18] reported subclinical HEV infection in healthy individuals with evidence of transient viremia using periodically collected sera. Examination using sera collected only once, rather than periodically, might not be adequate to detect HEV RNA in HP. Mean titers of IgM specific anti-HEV in the AH, FH, CLD and HP groups were 1.712, 2.116, 0.596 and 0.468, respectively. That of the HP group was lower than those of the AH and FH groups, both of which had marked clinically symptomatic HEV infection. However, these titers did not differ significantly between the CLD and HP groups. The reason for no HEV RNA-positive cases being detected in the HP group was unclear, though a few samples examined in this study did have high titers of IgM specific anti-HEV. These findings might be attributable to HEV RNA disappearing much earlier than IgM specific anti-HEV in HP sera. HEV is considered to persist longer in stool than in sera. Therefore, if examinations were performed using stool collected at the same time as sera, from subjects with HP, HEV RNA might be detectable in HP. Other possibilities include a cross-reactive or a somewhat more remote infection affecting the anti-HEV results. Finally, HEV RNA-positivity was significantly related to four parameters, values of ALT and AST and titers of IgG and IgM specific anti-HEV. Of these four parameters, the *P*-value for IgM specific anti-HEV was by far the smallest, below 0.01, indicating the IgM specific anti-HEV titer to be the parameter with the most significant relationship to HEV RNA-positivity. The duration of anti-HEV IgM-positivity in sera is about 100 days according to Li *et al.*,[15] while that of HEV RNA is 40 days at the longest according to Takahashi *et al.*[19] Serum positive for IgM specific anti-HEV is thus not always positive for HEV RNA. Therefore, the time elapsed between HEV infection and blood collection was considered to be a significant factor in HEV RNA detection. Concomitant infection is characteristic of areas of endemic HEV infection such as South Asia, in contrast to industrialized countries. Therefore, relationships between HEV RNA-positivity and concomitant infection in AH and FH cases are of interest in areas of endemic HEV infection, though no significant correlations with either AH or FH were detected using the chi-square test in this study. Further studies are required to investigate HEV and concomitant infections.

Table 4 Homology percentages within Bangladesh clones, and between Bangladesh clones and previously reported HEV clones. Figures in parentheses are GenBank accession numbers

		Bangladesh clones					
		A1	A2	A3	A4	B1	B2
Bangladesh clones	A1	100					
	A2	98.97	100				
	A3	97.95	98.97	100			
	A4	98.97	97.95	96.93	100		
	B1	90.81	89.79	88.77	90.81	100	
	B2	93.98	92.85	91.83	92.85	96.93	100
Genotype I	Burma B1 (M73218)	95.91	94.89	93.87	94.89	92.85	95.91
	Pakistan Abb-2B (AF185822)	95.91	94.89	93.87	94.89	92.85	95.91
	India 2 Madras (X99441)	95.91	94.89	93.87	94.89	90.81	93.87
	Nepal TK1592 (AF051830)	94.89	93.87	92.85	93.87	95.91	98.97
	China HeBei (M94177)	93.87	92.85	91.83	92.85	92.85	95.91
	India hev037 (X98292)	93.87	92.85	91.83	92.85	90.81	93.87
	India Hyderabad (AF076239)	93.87	92.85	91.83	92.85	92.85	95.91
	China Uigh179 (D11093)	92.85	91.83	90.81	91.83	90.81	93.87
	Pakistan SAR-55 (M80581)	91.83	90.81	89.79	92.85	91.83	93.87
	Morocco (AY230202)	89.79	88.77	87.75	88.77	87.75	89.79
Genotypes II, III & IV	Mexico (M74506) Genotype II	84.69	83.67	82.65	85.71	81.63	80.61
	US1 (AF060668) Genotype III	76.53	75.51	74.48	76.53	75.51	78.57
	China (AJ272108) Genotype IV	77.55	78.57	77.55	77.55	79.59	73.46

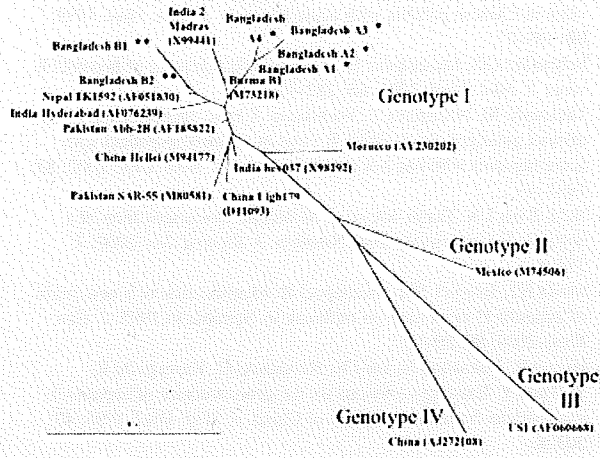

Figure 1 Phylogenetic tree analyses: * and ** indicate Bangladesh HEV clones, groups A and B, respectively. Isolates classified as genotype I are shown in the upper left area, those of genotypes II, III and IV in the lower right area. GenBank accession numbers are in parentheses. The bar in the lower left area indicates genetic distance.

Six different subgroups, that is clones with minor changes, were detected in 16 HEV Bangladesh clones by nucleic acid analysis. However, only two groups were identified by amino acid analysis, indicating most nucleic acid changes to be silent in these clones. Several HEV genotype nomenclatures[20–22] have been proposed based on the phylogenetic relationships of various HEV isolates. In this study, the widely recognized genotype I-IV system[22] was used. All six distinct clones found in this study were classified as genotype I, the most prevalent HEV genotype in South Asia. According to homology comparisons and phylogenetic tree analysis of the six clones, Bangladesh Group A (A1, A2, A3 and A4) clones were similar to the clone groups of Burma (M73218) and India (X99441), while the Group B (B1 and B2) clones resembled those of Nepal (AF051830) and India (AF076239) in genotype I. Thus, Bangladesh HEV isolates in this study were divided into two groups (A and B). The possibility was raised that two distinct HEV origins within genotype I are prevalent in Bangladesh. However, the nucleic acid sequences analyzed in this study were not particularly long. Therefore, this finding is not conclusive. However, different genotypes or subgenotypes were reportedly found in the same area, for example, genotypes III and IV in Japan,[23] genotypes I and II in Namibia[24,25] and genotypes I and IV in China.[21,26] Therefore, further studies of the genetic diversity of Bangladesh HEV are anticipated. Most Bangladesh Group A clones were derived from AH and FH patient sera, whereas Group B clones were mainly derived from CLD sera. In view of the clinical diagnosis, the severities of disease symptoms in the two groups apparently differed. However, there were no statistically significant relations between isolate clusters and clinical diagnosis.

We have presented herein six partial sequences of Bangladesh HEV clones, for comparison with the many HEV isolates identified in other countries. To our knowledge, this is the first study focusing on the analysis of previously unknown Bangladesh HEV isolates.

Acknowledgements

We thank Professor Toru Ariga, MD, Department of Medical Care-Welfare Engineering Major, Graduate School of Science and Technology, Nihon University, for supporting our Bangladesh hepatitis project, and Dr Hiroshi Matsumura, Division of Gastroenterology

and Hepatology, Department of Internal Medicine, Nihon University School of Medicine, for his invaluable assistance with the statistical analysis.

This work was supported by Interdisciplinary Global Joint Research Grants for Nihon University in 1995 and 1996 (DC95-003), and a General Joint Research Grant from Nihon University in 1999, Tokyo, Japan.

References

1 Gupta DN, Smetana HF. The histopathology of viral hepatitis as seen in the Delhi epidemic (1955–56). *Indian J. Med. Res.* 1957; **45** (Suppl.): 101–13.
2 Sarin SK, Kumar M, Hepatitis E, Hepatitis E. In: Boyer TD, Wright TL, Manns MP, eds. *Zakim and Boyer's Hepatology: A Text Book of Liver Disease*, 5th edn. Philadelphia, PA: Saunders Elsevier, 2006; 693–724.
3 Corwin AL, Khiem HB, Clayson ET *et al.* A waterborne outbreak of hepatitis E virus transmission in southwestern Vietnam. *Am. J. Trop. Med. Hyg.* 1996; **54**: 559–62.
4 Albetkova A, Drobeniuc J, Yashina T *et al.* Characterization of hepatitis E virus from outbreak and sporadic cases in Turkmenistan. *J. Med. Virol.* 2007; **79**: 1696–702.
5 Aye TT, Uchida T, Ma XZ *et al.* Complete nucleotide sequence of a hepatitis E virus isolated from the Xinjiang epidemic (1986–1988) of China. *Nucleic Acids Res.* 1992; **20**: 3512.
6 Belabbes EH, Bouguermouh A, Benatallah A, Illoul G. Epidemic non-A, non-B viral hepatitis in Algeria: strong evidence for its spreading by water. *J. Med. Virol.* 1985; **16**: 257–63.
7 Velazquez O, Stetler HC, Avila C *et al.* Epidemic transmission of enterically transmitted non-A, non-B hepatitis in Mexico, 1986–1987. *JAMA* 1990; **263**: 3281–5.
8 Acharya SK, Panda SK. Hepatitis E virus: epidemiology, diagnosis, pathology and prevention. *Trop. Gastroenterol.* 2006; **27**: 63–8.
9 Das K, Agarwal A, Andrew R, Frosner GG, Kar P. Role of hepatitis E and other hepatotropic virus in aetiology of sporadic acute viral hepatitis: a hospital based study from urban Delhi. *Eur. J. Epidemiol.* 2000; **16**: 937–40.
10 Ticehurst J, Popkin TJ, Bryan JP *et al.* Association of hepatitis E virus with an outbreak of hepatitis in Pakistan: serologic responses and pattern of virus excretion. *J. Med. Virol.* 1992; **36**: 84–92.
11 Shrestha SM, Shrestha S, Tsuda F *et al.* Molecular investigation of hepatitis E virus infection in patients with acute hepatitis in Kathmandu, Nepal. *J. Med. Virol.* 2003; **69**: 207–14.
12 Myint H, Soe MM, Khin T, Myint T-M, Tin KM. A clinical and epidemiological study of an epidemic of non-A non-B hepatitis in Rangoon. *Am. J. Trop. Med. Hyg.* 1985; **34**: 1183–9.
13 Sheikh A, Sugitani M, Kinukawa N *et al.* Hepatitis E virus infection in fulminant hepatitis patients and an apparently healthy population in Bangladesh. *Am. J. Trop. Med. Hyg.* 2002; **66**: 721–4.
14 Drabick JJ, Gambel JM, Gouvea VS *et al.* A cluster of acute hepatitis E infection in United Nations Bangladeshi peacekeepers in Haiti. *Am. J. Trop. Med. Hyg.* 1997; **57**: 449–54.
15 Li TC, Zhang J, Shinzawa H *et al.* Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. *J. Med. Virol.* 2000; **62**: 327–33.
16 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 1987; **162**: 156–9.
17 Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 1987; **4**: 406–25.
18 Mitsui T, Tsukamoto Y, Suzuki S *et al.* Serological and molecular studies on subclinical hepatitis E virus infection using periodic serum samples obtained from healthy individuals. *J. Med. Virol.* 2005; **76**: 526–33.
19 Takahashi M, Kusakai S, Mizuo H *et al.* Simultaneous detection of immunoglobulin A (IgA) and IgM antibodies against hepatitis E virus (HEV) is highly specific for diagnosis of acute HEV infection. *J. Clin. Microbiol.* 2005; **43**: 49–56.
20 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**: 1691–700.
21 Wang Y, Ling R, Erker JC *et al.* A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis. *J. Gen. Virol.* 1999; **80**: 169–77.
22 Schlauder GG, Mushahwar IK. Genetic heterogeneity of hepatitis E virus. *J. Med. Virol.* 2001; **65**: 282–92.
23 Takahashi M, Nishizawa T, Yoshikawa A *et al.* Identification of two distinct genotypes of hepatitis E virus in a Japanese patient with acute hepatitis who had not traveled abroad. *J. Gen. Virol.* 2002; **83**: 1931–40.
24 He J, Binn LN, Tsarev SA *et al.* Molecular characterization of a hepatitis E virus isolate from Namibia. *J. Biomed. Sci.* 2000; **7**: 334–8.
25 Maila HT, Bowyer SM, Swanepoel R. Identification of a new strain of hepatitis E virus from an outbreak in Namibia in 1995. *J. Gen. Virol.* 2004; **85**: 89–95.
26 Huang R, Nakazono N, Ishii K, Kawamata O, Kawaguchi R, Tsukada Y. Existing variations on the gene structure of hepatitis E virus strains from some regions of China. *J. Med. Virol.* 1995; **47**: 303–8.

Annals of Tropical Medicine & Parasitology, Vol. 103, No. 4, 343–350 (2009)

Sero-epidemiology of sporadic acute hepatitis in Bangladesh: high prevalences of infection with type-B, type-E and multiple types of hepatitis virus

M. SUGITANI[*], A. SHEIKH[*], K. SUZUKI[*,†], N. KINUKAWA[*], M. MORIYAMA[‡], Y. ARAKAWA[‡,§], K. KOMIYAMA[¶], T.-C. LI[**], N. TAKEDA[**], S. M. ISHAQUE[††], P. K. ROY[††], A. S. M. A. RAIHAN[††] and M. HASAN[††]

[*]*Department of Pathology, School of Medicine, Nihon University, 30-1 Oyaguchi-Kamimachi, Itabashi-ku, Tokyo 173-8610, Japan*
[†]*Department of Diagnostic Pathology, Saint Luke's International Hospital, 9-1 Akashicyo, Cyuo-ku, Tokyo 104-8560, Japan*
[‡]*Division of Gastroenterology and Hepatology, Department of Internal Medicine, School of Medicine, Nihon University, 30-1 Oyaguchi-Kamimachi, Itabashi-ku, Tokyo 173-8610, Japan*
[§]*Akiru Municipal Medical Center, 78-1 Hikida, Akiruno-shi, Tokyo 197-0834, Japan*
[¶]*Department of Pathology, School of Dentistry, Nihon University, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan*
[**]*Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan*
[††]*Department of Gastroenterology, Internal Medicine, Bangabandhu Sheikh Mujib Medical University, Shahbag, Dhaka – 1000, Bangladesh*

Received 2 March 2009, Revised 16 April 2009, Accepted 20 April 2009

In a recent investigation of hepatitis in Bangladesh, the sera from 74 adult patients (aged 15–67 years) who had been clinically diagnosed as cases of sporadic acute hepatitis were collected at various hospitals in and around Dhaka. Five cases were positive for IgM antibody against the hepatitis A virus and 30 were positive both for the surface antigen of the hepatitis B virus (HBV) and for IgM antibody against the HBV core (HBc). The six cases found positive for antibodies against the hepatitis D virus were all also positive for the HBV surface antigen but negative for anti-HBc IgM. Thirteen patients harboured hepatitis C virus RNA and 29 were positive for IgM antibody against the hepatitis E virus (HEV). There were 14 non-A-to-E subjects, whose illness was of unknown aetiology. Of the 83 infections with hepatitis viruses detected in the other 60 patients, 6%, 36%, 16%, 7% and 35% were of types A, B, C, D and E, respectively. Each of 28 of the patients (47% of those confirmed to have viral hepatitis) had concomitant infection with more than one type of hepatitis virus. The predominance of HBV and HEV infections and the high prevalence of multiple infection seen among these Bangladeshi cases have not been observed among hepatitis cases in developed countries.

Current serological assays allow the viruses that can cause human hepatitis to be separated into at least five main types. The general prevalences of infection with each type of hepatitis virus have been clarified by recent world-wide research, although the relevant data are lacking for some developing countries, where endemic-type acute viral hepatitis is a major problem. The endemic types of hepatitis, A and E, have well characterised geographical distributions, with high prevalences in most

Reprint requests to: M. Sugitani.
E-mail: sugitani@med.nihon-u.ac.jp; fax: +81 3 3972 8830.

DOI: 10.1179/136485909X435120

developing countries such as those of South–east Asia, South Asia, China, the Middle East, Africa and parts of America (Gupta and Smetana, 1957; Belabbes *et al.*, 1985; Myint *et al.*, 1985; Velazquez *et al.*, 1990; Aye *et al.*, 1992; Shapiro and Margolis 1993; Krawczynski *et al.*, 2001). Aetiological analyses of acute hepatitis of the sporadic type have been conducted in most industrialized countries, and there have been quite a few similar studies in developing countries (Al-Knawy *et al.*, 1997; Gunaid *et al.*, 1997; Chadha *et al.*, 2003; Tsatsralt-Od *et al.*, 2006). It is not possible to determine the type of hepatitis virus causing human illness from the clinical features alone. Although, for cases of acute or aggressive hepatitis, it is important to identify the specific viral type so that the optimal therapy can be offered and an accurate prognosis can be made, it is sometimes difficult to perform the necessary tests in developing countries. In Bangladesh, for example, the limited resources, and the relatively high costs of the appropriate serology, make aetiological analyses difficult in many hospitals.

Although diseases of the gastro–intestinal tract and hepato–biliary system are very common in Bangladesh — accounting for approximately 25% of patients at medical-college hospitals (Hasan and Khan, 1997) — there has been surprisingly little research on hepatitis in the country. The main aim of the present study was to use a mixture of serology and molecular biology to determine the types of hepatis virus causing sporadic acute hepatitis in and around the capital city of Dhaka.

MATERIALS AND METHODS

Subjects

Between November 1995 and October 1996, sera from 74 adult patients with sporadic, non-endemic, acute hepatitis were collected at various hospitals in and around Dhaka and stored, frozen at −20°C, at the Hospital of Bangabandhu Sheikh Mujib Medical University (BSMMU), in Dhaka. In November 1996, these sera were transported on dry ice to Nihon University in Tokyo and there stored at −80°C until they could be tested further.

The study subjects had to show a typical prodrome of viral hepatitis, with a history of persistent or progressive jaundice of <6 months' duration, and biochemical evidence of hepatocellular necrosis or injury. Patients who had suffered with jaundice or shown any viral markers for >6 months, those who had developed ascites and/or encephalopathy, and those showing clinical, endoscopic or ultrasonographic evidence of established cirrhosis of the liver or bile-duct obstruction were excluded. Not all of the sera investigated were obtained during the peak stages of the patients' illnesses.

The ages of the sample donors ranged from 15–67 years (mean=29.5 years), with 11 (five males), 27 (21 males), 24 (21 males), nine (six males), zero and three (two males) in their 2nd, 3rd, 4th, 5th, 6th and 7th decades, respectively. There were 55 male donors (74%) and 19 female (26%).

Serum samples were collected after obtaining informed consent, and the study protocol was approved by the Ethics Committee of Nihon University's School of Medicine.

Serology and Molecular Biology

Commercial kits were used, according to their manufacturers' instructions, to detect evidence of infection with hepatitis viruses of types A (HAV), B (HBV), C (HCV) and D (HDV). Whole-immunoglobulin and IgM-specific anti-HAV, HBV surface antigen (HBsAg), IgM antibody against the HBV core antigen (anti-HBc) and anti-HBc in 200-fold-diluted sera were each investigated in radio-immuno-assays from Dainabot (Tokyo), who also made the enzyme-immuno-assay used to detect anti-HDV antibody. The concentration of HCV RNA in each serum was explored using the

relevant Amplicore kit (Roche Diagnostics, Basel, Switzerland), while titres of IgG and IgM antibody against hepatitis E virus were determined using the ELISA described by Li *et al.* (2000), which uses a recombinant antigen based on empty virus-like particles of HEV.

Only the sera found negative for anti-HBc IgM and positive for HBsAg were tested for anti-HBc after being diluted 200-fold, and only the sera found positive for HBsAg or anti-HBc IgM were assayed for anti-HDV.

Statistical Analysis

Unpaired Student's *t*-tests were used to explore the significance of the relationships between donor age, the results for anti-HAV IgM, HBsAg, HCV RNA, anti-HDV and anti-HEV IgM and the seroprevalence of 'multiple' infection (i.e. infection with more than one type of hepatitis virus). The significance of the relationships between gender and each of these factors was similarly explored in χ^2 tests. A *P*-value of <0.05 was considered indicative of a statistically significant difference.

RESULTS

In terms of the evidence of infection with hepatitis viruses A–E, 15 different patterns were recognised among the 74 serum donors (Table 1). Five cases were positive for anti-HAV IgM and were classified as having acute hepatitis A. All five were, however, also positive for other hepatitis viral markers, indicating their co-infection with two or more types of hepatitis virus. The remaining 69 cases (93%) were positive for anti-HAV immunoglobulin.

Thirty of the serum donors were each positive for both HBsAg and anti-HBc IgM and they were classified as cases of acute hepatitis B. Among these 30, 13 were also positive for other markers of viral hepatitis.

Another 14 cases were positive for HBsAg but negative for anti-HBc IgM. When diluted 200-fold, the sera from all of these cases showed the high inhibition titres (>95%) that indicated that they had high levels of anti-HBc (Wada *et al.*, 1980), but all 14 sera also harboured markers of hepatitis viruses other than type B (i.e. anti-HAV IgM, HCV RNA, anti-HDV, and/or anti-HEV IgM). The cause of the acute hepatitis in these 14 cases was recorded as infection with hepatitis viruses other than type B, acutely superimposed on HBV carrier status. None of the patients investigated was found negative for HBsAg but positive for anti-HBc IgM.

Thirteen cases had HCV RNA in their sera but nine of these 13 cases were also positive for one or more markers of non-C viral hepatitis. The aetiology of the acute hepatitis seen in these 13 cases was difficult to determine. As all 74 patients investigated had been clinically diagnosed as cases of sporadic acute hepatitis, the four found positive only for HCV RNA were considered to be suffering from acute hepatitis C. The nine cases found positive for both HCV RNA and one or more markers of non-C hepatitis were considered to be possible (but unconfirmed) cases of acute infection with HCV.

Sera from six cases were positive for both anti-HDV and HBsAg and showed high inhibition titres (>95%) when diluted 200-fold and tested for anti-HBc, although they were negative for anti-HBc IgM. Two (of the six) cases were also both positive for the markers of non-B and non-D viral hepatitis. Since all of the subjects had been clinically diagnosed as having sporadic acute hepatitis, acute infection with HDV was considered to be confirmed in four of these six cases and possible but unconfirmed in the other two cases. Evidence of HDV infection was found in six (43%) of the 14 cases with HBV carrier status.

Overall, 29 of the 74 cases were found ELISA-positive for anti-HEV IgM, and all 29 (but none of the other patients investigated) were found positive for anti-HEV IgG. These 29 were considered to have

TABLE 1. *The serological results for the 74, clinically diagnosed, Bangladeshi cases of sporadic acute hepatitis*

Result of the assay for:								
HBsAg	Anti-HBc IgM	Anti-HBc in 200-fold diluted sera	Anti-HAV IgM	HCV RNA	Anti-HDV	Anti-HEV IgM	No. of cases	Possible diagnoses
+	+	ND	−	−	−	−	17	Acute hepatitis B
+	+	ND	+	−	−	−	2	Acute hepatitis A and B
+	+	ND	−	−	−	+	7	Acute hepatitis B and E
+	+	ND	−	+	−	−	2	Acute hepatitis B and C, or acute hepatitis B superimposed on HCV carrier status
+	+	ND	−	+	−	+	2	Acute hepatitis B, C and E, or acute hepatitis B and E superimposed on HCV carrier status
+	−	+*	+	+	−	+	2	Acute hepatitis A, C and E superimposed on HBV carrier status, or acute hepatitis A and E superimposed on carrier status for both HBV and HCV
+	−	+*	−	+	−	−	2	Acute hepatitis C superimposed on HBV carrier status
+	−	+*	−	−	+	−	4	Acute hepatitis D superimposed on HBV carrier status
+	−	+*	−	−	−	+	4	Acute hepatitis E superimposed on HBV carrier status
+	−	+*	−	−	+	+	1	Acute hepatitis D and E superimposed on HBV carrier status, or acute hepatitis E superimposed on carrier status for both HBV and HDV
+	−	+*	−	+	+	+	1	Acute hepatitis C, D and E superimposed on HBV carrier status, acute hepatitis D and E superimposed on carrier status for both HBV and HCV, acute hepatitis C and E superimposed on carrier status for both HBV and HDV, or acute hepatitis E superimposed on carrier status for HBV, HCV and HDV
−	−	ND	+	−	ND	+	1	Acute hepatitis A and E
−	−	ND	−	+	ND	−	4	Acute hepatitis C
−	−	ND	−	−	ND	+	11	Acute hepatitis E
−	−	ND	−	−	ND	−	14	Acute hepatitis with cause other than hepatitis viruses A–E

HBsAg, Surface antigen of the hepatitis B virus; HBc, core of the hepatitis B virus; HAV, hepatitis A virus; HCV, hepatitis C virus; HDV, hepatitis D virus; HEV, hepatitis E virus; ND, not determined; HBV, hepatitis B virus.
*High inhibition titre (>95%).

acute hepatitis E, although 18 of them were also positive for at least one marker of non-E viral hepatitis.

Fourteen of the 74 subjects investigated in the present study were negative for all of the markers of viral hepatitis investigated except anti-HAV immunoglobulins (all 14) and anti-HEV IgG (eight cases) and were therefore classified as cases of acute non-A-to-E hepatitis.

The prevalences of apparent infection with hepatitis viruses of types A to E among the other 60 subjects are shown in Table 2. Of these 60 subjects, 28 cases (47%) appeared to be co-infected with two, three or four types of hepatitis virus.

None of the links between the carriage of anti-HAV IgM, HBsAg, HCV RNA, anti-HDV or anti-HEV IgM and multiple infection, donor age or donor gender appeared to be statistically significant ($P>0.05$ for each).

DISCUSSION

Two characteristic findings were revealed by this study. First, HBV and HEV infections were the most common and equally significant aetiologies of clinically diagnosed cases of acute sporadic viral hepatitis in Bangladesh, each of these viruses accounting for about half of the cases in which the probable cause was identified (Table 2). Acharya and Panda (2006) reported that HEV was the major aetiological agent for acute hepatitis and acute liver failure in endemic regions, and Purcell and Emerson (2008) found hepatitis E to be either the most important or the second most important cause of acute clinical hepatitis in adults throughout Asia, the Middle East and Africa. Although all the subjects of the present study came from Dhaka or its environs, the seroprevalence of HEV infection recorded among them is very similar to the values previously reported among Bangladeshi adults with fulminant hepatitis (Sheik *et al.*, 2002; Mahtab *et al.*, 2008), and similar to the corresponding values recorded in India, Nepal and other HEV-endemic areas (Coursaget *et al.*, 1998; Shrestha *et al.*, 2003; Acharya and Panda, 2006). That HEV infection is a major cause of the acute and fulminant hepatitis seen in Bangladesh probably reflects the often-inadequate levels of public sanitation and hygiene in the country, as well as the frequency of natural disasters such as typhoons and flooding, which is much higher in Bangladesh than in most industrialized countries.

As evidence of acute HEV infection, anti-HEV antibodies were investigated in the present study, instead of HEV RNA, since infected humans remain seropositive for anti-HEV IgM for about 100 days (Li *et al.*, 2000) but harbour detectable HEV RNA in their sera for no more than 40 days (Takahashi *et al.*, 2005). The HEV-infected sera detected in the present study were subsequently used for the isolation of HEV clones. When the nucleotide and amino-acid sequences of these clones were

TABLE 2. *Final diagnoses for the 60 patients showing evidence of infection with hepatitis viruses A, B, C, D and/or E (including the 28 who were each found positive for two to four types of hepatitis virus)*

	Diagnosis				
	Acute infection with HAV	Acute infection with HBV	Possible acute infection with HCV	Possible acute infection with HDV	Acute infection with HEV
No. and (%) of the 60 cases	5 (8)	30 (50)	13 (22)	6 (10)	29 (48)
% of the 83 infections detected	6	36	16	7	35

HAV, Hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis D virus; HEV, hepatitis E virus.

analysed, genotype I was found to be the prevalent form of HEV circulating in Bangladesh (Sugitani *et al.*, 2009).

In contrast to the relative commonness of HBV and HEV observed in the present study, acute infection with HAV (as indicated by the presence of anti-HAV IgM in the serum) — another virus transmitted via the faecal–oral route — appeared rare. It seems that HAV has little significance in the aetiology of sporadic acute hepatitis among Bangladeshi adults. Acute HAV infection is, however, considered to be prevalent in childhood in Bangladesh (Sheik *et al.*, 2002), and such early infection probably produces lifelong immunity to re-infection. This would explain why almost all (93%) of the adult subjects of the present study carried antibodies against HAV.

Given the present results, in which HBV accounted for about half of the cases in which the probable cause was identified (Table 2), it is surprising that Mahtab *et al.* (2007) concluded that HBV accounted for only 10%–35% of the acute hepatitis cases seen in Bangladesh. Differences in the sensitivities of the methods used to detect HBV-related antigens and antibodies may account for this apparent discrepancy. Another possible explanation is that the present results were affected by a gender bias, with most (74%) of the samples analysed in the present study coming from men. Further investigations are clearly necessary to elucidate the true state of HBV infection in Bangladesh.

The superimposition of HBV carrier status on infection with hepatitis viruses of types A, C, D or E was frequently detected in the present study, being recorded in 14 (23%) of the cases in which the probable cause was identified. Infection with HBV is considered to be moderately endemic in Bangladesh (Mahtab *et al.*, 2007). In Taiwan, a country considered to be hyperendemic for HBV, Chu *et al.* (1999) found the prevalence of HBsAg carrier status among 334 cases of sporadic acute hepatitis to be even higher (53.0%).

In the present study, the measurement of the serum concentrations of HBV DNA would probably have been advantageous, especially for the HBV-positive cases who were co-infected with other types of hepatitis virus, including those suspected to have HBV carrier status. Unfortunately, however, this was not possible because the serum volumes available were inadequate.

After HBV and HEV, the third most significant infectious agent in sporadic acute viral hepatitis among Bangladeshi adults appears to be HCV. Although HDV infection appeared to have little role in the poor health of the present subjects, HDV can replicate only in HBV-infected individuals and the prevalence of HDV infection among the 14 cases considered to have HBV carrier status was quite high, at 43%. This value is markedly higher than the seroprevalences of HDV infection detected, by Zaki *et al.* (2003), among other Bangladeshi carriers of HBsAg, whether those carriers were symptomatic or not (21.8%–25.6%). As all the samples used in the present study were collected from clinically established cases of sporadic acute hepatitis, however, a simple comparison of the present data with those collected by Zaki *et al.* (2003) might be inappropriate. The number of subjects with HBV carrier status in the present study was also small. Further investigations of HDV prevalence and estimation of the total number of HBV-infected subjects in Bangladesh remain necessary.

In the present study, the hepatitis seen in 14 subjects could not be attributed to hepatitis viruses of types A–E. These subjects may have been suffering from other viral or bacterial infections or the effects of drugs, auto-immunity, toxicity, ischaemia, shock etc. (Alcohol abuse was an unlikely cause of their illness because the ingestion of alcohol goes against the religious views of most Bangladeshis.)

A notable result of the present study was the high frequency of co-infection with two to four types of hepatitis virus. Although rare in the industrialized world,

such multiple infection is commonly seen among cases of acute and/or fulminant hepatitis in other areas where HBV and/or HEV are endemic (Wu *et al.*, 1994; Arora *et al.*, 1996; Coursaget *et al.*, 1998), where it might play an important role in the aetiology of sporadic acute viral hepatitis.

ACKNOWLEDGEMENTS. The authors thank Dr A. M. Prince (Chairman of the Hepatitis Research Foundation in Poughkeepsie, NY), for his critical review of the manuscript, Professor T. Ariga (Graduate School of Science and Technology, Nihon University), for supporting our Bangladeshi hepatitis project, and Dr H. Matsumura (Nihon University School of Medicine), for his invaluable assistance with the statistical analysis. This work was supported financially by Interdisciplinary Global Joint Research grants awarded in 1995 and 1996 (DC95-003), and a General Joint Research grant awarded in 1999, all to Nihon University.

REFERENCES

Acharya, S. K. & Panda, S. K. (2006). Hepatitis E virus: epidemiology, diagnosis, pathology and prevention. *Tropical Gastroenterology*, **27**, 63–68.

Al-Knawy, B., El-Mekki, A. A. & Yarbough, P. O. (1997). The role of hepatitis E virus infection among patients with acute viral hepatitis in southern Saudi Arabia. *Annals of Saudi Medicine*, **1**, 32–34.

Arora, N. K., Nanda, S. K., Gulati, S., Ansari, I. H., Chawla, M. K., Gupta, S. D. & Panda, S. K. (1996). Acute viral hepatitis types E, A, and B singly and in combination in acute liver failure in children in north India. *Journal of Medical Virology*, **48**, 215–221.

Aye, T. T., Uchida, T., Ma. X. Z., Iida, F., Shikata, T., Zhuang, H. & Win, K. M. (1992). Complete nucleotide sequence of a hepatitis E virus isolated from the Xinjiang epidemic (1986–1988) of China. *Nucleic Acids Research*, **20**, 3512.

Belabbes, E. H., Bouguermouh, A., Benatallah, A. & Illoul, G. (1985). Epidemic non-A, non-B viral hepatitis in Algeria: strong evidence for its spreading by water. *Journal of Medical Virology*, **16**, 257–263.

Chadha, M. S., Walimbe, A. M., Chobe, L. P. & Arankalle, V. A. (2003). Comparison of etiology of sporadic acute and fulminant viral hepatitis in hospitalized patients in Pune, India during 1978–81 and 1994–97. *Indian Journal of Gastroenterology*, **22**, 11–15.

Chu, C. M., Lin, S. M., Hsieh, S. Y., Yeh, C. T., Lin, D. Y., Sheen, I. S. & Liaw, Y. F. (1999). Etiology of sporadic acute viral hepatitis in Taiwan: the role of hepatitis C virus, hepatitis E virus and GB virus-C/hepatitis G virus in an endemic area of hepatitis A and B. *Journal of Medical Virology*, **58**, 154–159.

Coursaget, P., Buisson, Y., N'Gawara, M. N., van Cuyck-Gandre, H. & Roue, R. (1998). Role of hepatitis E virus in sporadic cases of acute and fulminant hepatitis in an endemic area (Chad). *American Journal of Tropical Medicine and Hygiene*, **58**, 330–334.

Gunaid, A. A., Nasher, T. M., El-Guneid, A. M., Hill, M., Dayton, R., Pal, A., Skidmore, S. J., Coleman, J. C. & Murray-Lyon, I. M. (1997). Acute sporadic hepatitis in the Republic of Yemen. *Journal of Medical Virology*, **51**, 64–66.

Gupta, D. N. & Smetana, H. F. (1957). The histopathology of viral hepatitis as seen in the Delhi epidemic (1955–56). *Indian Journal of Medical Research*, **45** (Suppl.), 101–113.

Hasan, M. & Khan, A. A. (1997). Development of gastroenterology in Bangladesh. *Journal of Gastroenterology and Hepatology*, **12**, S13–S14.

Krawczynski, K., Kamili, S. & Aggarwal, R. (2001). Global epidemiology and medical aspects of hepatitis E. *Forum (Genoa, Italy)*, **11**, 166–179.

Li, T. C., Zhang, J., Shinzawa, H., Ishibashi, M., Sata, M., Mast, E. E., Kim, K., Miyamura, T. & Takeda, N. (2000). Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. *Journal of Medical Virology*, **62**, 327–333.

Mahtab, M. A., Rahman, S., Khan, M., Kamal, M., Mamun, A. A. & Karim, M. F. (2007). Viral load speaks little about toll on liver. *Hepatobiliary and Pancreatic Diseases International*, **6**, 483–486.

Mahtab, M. A., Rahman, S., Khan, M., Mamun, A. A. & Afroz, S. (2008). Etiology of fulminant hepatic failure: experience from a tertiary hospital in Bangladesh. *Hepatobiliary and Pancreatic Diseases International*, **7**, 161–164

Myint, H., Soe, M. M., Khin, T., Myint, T.-M. & Tin, K. M. (1985). A clinical and epidemiological study of an epidemic of non-A non-B hepatitis in Rangoon. *American Journal of Tropical Medicine and Hygiene*, **34**, 1183–1189.

Purcell, R. H. & Emerson, S. U. (2008). Hepatitis E: an emerging awareness of an old disease. *Journal of Hepatology*, **48**, 494–503.

Shapiro, C. N. & Margolis, H. S. (1993). Worldwide epidemiology of hepatitis A virus infection. *Journal of Hepatology*, **18** (Suppl. 2), S11–S14.

Sheikh, A., Sugitani, M., Kinukawa, N., Moriyama, M., Arakawa, Y., Komiyama, K., Li, T. C., Takeda, N., Ishaque, S. M., Hasan, M. & Suzuki, K. (2002).

Hepatitis E virus infection in fulminant hepatitis patients and an apparently healthy population in Bangladesh. *American Journal of Tropical Medicine and Hygiene*, **66**, 721–724.

Shrestha, S. M., Shrestha, S., Tsuda, F., Nishizawa, T., Gotanda, Y., Takeda, N., Okamoto, H. (2003). Molecular investigation of hepatitis E virus infection in patients with acute hepatitis in Kathmandu, Nepal. *Journal of Medical Virology*, **69**, 207–214.

Sugitani, M., Tamura, A., Shimizu, Y. K., Sheikh, A., Kinukawa, N., Shimizu, K., Moriyama, M., Komiyama, K., Li, T.-C., Takeda, T., Arakawa, Y., Suzuki, K., Ishaque, S. M., Roy, P. K., Raihan, A. S. M. A. & Hasan, M. (2009). Detection of hepatitis E virus RNA and genotype in Bangladesh. *Journal of Gastroenterology and Hepatology*, **24**, 599–604.

Takahashi, M., Kusakai, S., Mizuo, H., Suzuki, K., Fujimura, K., Masuko, K., Sugai, Y., Aikawa, T., Nishizawa, T. & Okamoto, H. (2005). Simultaneous detection of immunoglobulin A (IgA) and IgM antibodies against hepatitis E virus (HEV) is highly specific for diagnosis of acute HEV infection. *Journal of Clinical Microbiology*, **43**, 49–56.

Tsatsralt-Od, B., Takahashi, M., Endo, K., Buyankhuu, O., Baatarkhuu, O., Nishizawa, T. & Okamoto, H. (2006). Infection with hepatitis A, B, C, and delta viruses among patients with acute hepatitis in Mongolia. *Journal of Medical Virology*, **78**, 542–550.

Velazquez, O., Stetler, H. C., Avila, C., Ornelas, G., Alvarez, C., Hadler, S. C., Bradley, D. W. & Sepulveda, J. (1990). Epidemic transmission of enterically transmitted non-A, non-B hepatitis in Mexico, 1986–1987. *Journal of the American Medical Association*, **263**, 3281–3285.

Wada, T., Akahonai, Y., Kaido, I., Sato, S., Ishikawa, K., Hirasawa, T., Okuda, K., Kotouda, K, Iwama, S., Suzuki, H., Iino, S., Endo, R., Shikata, T., Kanda, Y., Arakawa, Y., Furuta, S., Kiyosawa, K., Akahane, K., Kawahara, K., Takahashi, Y., Fukuda, Y., Sano, M., Nakano, H., Imura H., Ito, K., Yamamoto, S., Kuroki, T., Nagashima H., Yumoto, Y., Suo, T., Kato, S., Ohta, Y., Ohno, N., Tanikawa K., Abe, H., Sata, M., Yano, M. & Mayumi, M. (1980). Study on the measurement of serum anti-HBc antibody by RIA (CORAB). *Japanese Journal of Medicine and Pharmaceutical Science*, **3**, 105–110.

Wu, J. C., Chen, C. L., Hou, M. C., Chen, T. Z., Lee, S. D. & Lo, K. J. (1994). Multiple viral infection as the most common cause of fulminant and subfulminant viral hepatitis in an area endemic for hepatitis B: application and limitations of the polymerase chain reaction. *Hepatology*, **19**, 836–840.

Zaki, H., Darmstadt, G. L., Baten, A., Ahsan, C. R. & Saha, S. K. (2003). Seroepidemiology of hepatitis B and delta virus infections in Bangladesh. *Journal of Tropical Pediatrics*, **49**, 371–374.