Vol. 33, No. 12

Purification of a Baculovirus-Expressed Hepatitis E Virus Structural Protein and Utility in an Enzyme-Linked Immunosorbent Assay

JUNKUN HE,[1] WEI-MEI CHING,[1] PATRICE YARBOUGH,[2] HUI WANG,[2] AND MITCHELL CARL[3]*

Viral and Rickettsial Diseases Program, Naval Medical Research Institute, Bethesda, Maryland[1]; Genelabs Inc., Redwood City, California[2]; and Department of Internal Medicine, Infectious Disease Division, Naval Medical Center San Diego, San Diego, California[3]

We report on the purification of the full-length structural protein encoded by open reading frame 2 (ORF-2) of hepatitis E virus. The ORF-2 protein, expressed in Sf9 cells by using a recombinant baculovirus vector system, was successfully purified to homogeneity. Gel electrophoresis of the purified ORF-2 protein showed a single polypeptide of 75 kDa by Coomassie blue staining and by Western blot (immunoblot) analysis. We demonstrated that the partially purified ORF-2 protein could be used successfully in a sensitive and specific enzyme-linked immunosorbent assay for the detection of antibodies to hepatitis E virus.

Hepatitis E virus (HEV) is a major cause of enterically transmitted non-A, non-B hepatitis (2). Enterically transmitted non-A, non-B hepatitis was first documented in New Delhi, India, in 1955 (24). HEV is largely waterborne, and epidemics have been reported from many countries, such as India (11, 12, 19, 25), Pakistan (5), Bangladesh (7), Nepal (10), Burma (14, 18), Africa (3, 17), Mexico (21), the People's Republic of China (4, 9, 28), the former Soviet Union (22), and Europe (13). The highest attack rate of HEV occurs among young- to middle-aged adults, with the highest case fatality rate being among pregnant women (15). Because of the lack of an easily performed, sensitive, and rapid diagnostic test for HEV infection, the true worldwide extent of the disease is unknown. The cDNAs derived from four isolates (from Burma, Mexico, the People's Republic of China, and Pakistan) have been cloned and fully sequenced (1, 8, 20, 22). On the basis of the cDNA sequences of the Burmese strain and the Pakistani strain, open reading frame 2 (ORF-2) and ORF-3 structural proteins of HEV have been expressed in *Escherichia coli* and Sf9 cells (6, 16, 23). Both have been used in diagnostic Western blot (immunoblot) assays (6, 16). We report here the purification of the full-length, non-fusion protein encoded by ORF-2 of HEV (Burmese strain) in Sf9 cells by using a baculovirus expression system as well as the use of this protein as an antigen for the detection of human antibodies to HEV in an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Cells and virus. Sf9 cells were obtained from the American Type Culture Collection (Rockville, Md.). Sf9 cells were cultured in monolayer flasks with Hink's TnM-FH insect medium (JRH Biosciences, Lenexa, Kans.) supplemented with 10% fetal bovine serum. The medium also contained 1% penicillin-Streptomycin-amphotericin B (Fungizone) solution (JRH Biosciences). The wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and transfer vector pBlueBac 2 were obtained from Invitrogen Co. (San Diego, Calif.).

Construction of recombinant baculovirus. The recombinant baculovirus (pJKH1) was described previously (6). Briefly, the full-length ORF-2 fragment with flanking *Xba*I restriction endonuclease cleavage sites was generated by

PCR. The amplified DNA fragment was subcloned into the baculovirus transfer vector pBlueBac 2 at a compatible *Nhe*I site. Recombinant transfer vector and wild-type AcNPV DNAs were used to cotransfect Sf9 cells in order to generate recombinant viruses by homologous recombination. After plaque purification, a recombinant virus (pJKH1) containing the coding sequences of the ORF-2 protein was selected.

Production of antigen. Sf9 cells were grown in T-150 tissue culture flasks at 27°C to confluency and were collected by scraping; this was followed by centrifugation at $1,000 \times g$ for 5 min. The Sf9 cells pellets were suspended in fresh medium and were seeded into the flasks. To optimize the maximum expression and minimum degradation of ORF-2 protein expressed in Sf9 cells, a time course experiment was carried out. Sf9 cells were infected with recombinant virus (pJKH1) at multiplicities of infection of 5, 10, 20, 50, and 100 and were incubated at 27°C for 0, 24, 36, 48, 72, and 96 h. One milliliter of the cell suspension for each sample was centrifuged at $16,000 \times g$ for 5 min. Supernatants were concentrated 10- to 15-fold by using a Centricon P-30 filter (Amicon, Beverly, Mass.). Sodium dodecyl sulfate (SDS) and Triton X-100 were added to the concentrated supernatant to final concentrations of 2 and 1%, respectively, and the mixture was boiled for 3 min. Cell pellets were resuspended in a 2% SDS and a 1% Triton X-100 solution and boiled for 3 min. Uninfected Sf9 cells and Sf9 cells infected with wild-type baculovirus were treated identically and were used as controls. All samples were fractionated on SDS–10% polyacrylamide gels and were analyzed by Western blot analysis.

Solubilization of Sf9 cells. All purification steps, unless stated otherwise, were carried out at 4°C. All chemical materials were purchased from Sigma (St. Louis, Mo.). Protease inhibitors were purchased from Boehringer Mannheim Corp. (Indianapolis, Ind.). A 500-ml culture of Sf9 cells infected with recombinant baculovirus (pJKH1) at a multiplicity of infection of 10 was harvested by centrifugation at $5,000 \times g$ for 10 min, and the cell pellet was resuspended in 20 ml of homogenization buffer (1 mM Tris-HCl [pH 8.0]) containing protease inhibitors (antitrypsin, 100 μg; pA-PMS, 40 μg; aprotinin, 2 μg; bestain, 8 μg; chymostatin, 100 μg; E 64, 10 μg; EDTA, 1,000 μg; leupeptin, 1 μg; pepstatin, 20 μg; phosphoramidon, 200 μg; phenylmethylsulfonyl fluoride, 10 μg). The cell pellet suspension was homogenized by a motor-driven homogenizer through five cycles, with 2 min of homogenization and 5 min of cooling on ice for each cycle. The homogenized solution was clarified by centrifugation at $12,000 \times g$ for 15 min, and the cell debris was discarded. The supernatant was collected for further processing.

Ammonium sulfate precipitation. Solid ammonium sulfate was added to the supernatant to reach 5, 10, 15, 20, and 30% in different aliquots of the sample. The proteins were allowed to precipitate at 4°C for 1 h at each concentration. After centrifugation at $12,000 \times g$ for 20 min, the precipitate was resuspended in TE buffer (10 mM Tris, 1 mM EDTA) containing 1 M urea until it was fully dissolved and was dialyzed against TE buffer. Resuspended protein solutions were concentrated and desalted with a Centricon P-30 filter (Amicon). Samples from both pellets and supernatants were fractionated on SDS–10% polyacrylamide gels and were analyzed by Coomassie blue staining and Western blotting to determine the optimal ammonium sulfate concentration for the precipitation of expressed ORF-2 protein.

Ion-exchange column chromatography. The preparation of the CM-Sepharose column was carried out as described by the manufacturer (Pharmacia, Uppsala, Sweden). The precipitate, from 20% saturation of ammonium sulfate at 4°C, was dissolved in TE buffer containing 1 M urea. For the large-scale preparation a

* Corresponding author. Mailing address: Department of Internal Medicine, Infectious Disease Division, Naval Medical Center San Diego, San Diego, CA 92134-5000. Phone: (619) 532-6102. Fax: (619) 532-8798.

CM-Sepharose column (4 by 80 cm; Pharmacia) was used. After sample loading, the column was washed with the equilibrating buffer (25 mM tricine [pH 8.0], 1 mM EDTA) until the absorbance of the eluent at 280 nM reached the baseline. The bound proteins were eluted with a linear salt gradient from 0 to 1 M NaCl at a rate of 2 ml/min for 1 h. Fractions of 4 ml were collected every 2 min. The presence of the ORF-2 protein in the collected fractions was monitored by an immuno-dot blot assay. A total of 50 μl from each fraction collected during ion-exchange chromatography was spotted onto nitrocellulose paper with a microsample filtration manifold (Schleicher & Schuell, Inc., Keene, N.H.). The immunoblot analysis was performed as described previously (6). The fractions containing the ORF-2 protein were pooled, diluted fourfold with equilibrating buffer, and then used for immunoaffinity chromatography.

Affinity column chromatography. CNBr-activated Sepharose 4B resin was purchased from Pharmacia. The coupling of a murine immunoglobulin M (IgM) monoclonal antibody, BT9 (27), which is specific for the HEV ORF-2 protein (26), to activated agarose beads was carried out as described by the manufacturer (Pharmacia), with some modifications. CNBr-activated Sepharose beads (Pharmacia) were washed with 1 mM HCl (1 g, 200 ml); this was followed by washing with 0.5 M NaCl in 0.1 M NaHCO$_3$ (pH 8.3) at 0 to 4°C. In order to determine the coupling efficiency the adsorbances of the monoclonal antibody preparations were compared before and after coupling. BT9 was coupled to the washed beads at a concentration of 2.5 mg/ml of gel at 4°C overnight with gentle shaking. The antibody-conjugated beads were washed four times with washing buffer containing 25 mM tricine (pH 8.0)–1 mM EDTA at 4°C, and the remaining active group was blocked with 1 mM Tris-HCl (pH 8.0) at 4°C overnight with gentle shaking. The antibody-bound beads were further washed with three washing cycles of one bed volume of alternating buffers 0.1 M acetate–0.5 M NaCl (pH 4) and 0.1 M Tris-HCl–0.5 M NaCl (pH 8.0) for each cycle. Finally, the beads were equilibrated with equilibrating buffer, packed into a column (1 by 8 cm), equilibrated with the same buffer, and stored at 4°C for further use.

The corresponding fractions from the CM-Sepharose column were combined, diluted with an equal volume of equilibration buffer, and slowly applied to the affinity column (0.1 ml/5 min). The column was washed with equilibrating buffer until the optical density at 280 nm (OD$_{280}$) of the eluent matched the OD$_{280}$ of the equilibrating buffer. The column was washed again with 10 bed volumes of equilibrating buffer containing 0.2 M NaCl, and then the ORF-2 protein was eluted with equilibrating buffer containing 1 M NaCl and 1 M urea. The eluted protein was dialyzed against three to four changes of water at 4°C for 24 h. After dialysis, the protein was lyophilized and the pellet was suspended in sterile water (0.5 mg/ml). The final preparation was analyzed on an SDS–10% polyacrylamide gel, and its purity was assessed by Coomassie blue staining and Western blotting. The protein concentration was determined by using the Micro BCA Protein Assay Reagent kit (Pierce, Rockford, Ill.).

ELISA. Secondary antibodies, goat anti-human IgM and IgG, were purchased from Bio-Rad. Partially purified ORF-2 protein was eluted from a CM-Sepharose column. Each well of 96-well flat-bottom microtiter plates (Corning Cell Wells, Corning, N.Y.) was coated with 56 ng of ORF-2 protein in 120 μl of phosphate-buffered saline (PBS) at 4°C overnight. Unbound antigen was removed, and the wells were blocked with blocking solution (100 μl of PBS solution containing 1% sodium-caseinate, 0.05% sodium azide, and 0.05% Tween 20) at room temperature for 1 h. The blocking solution was removed, and the wells were washed four times with washing solution. The wells were incubated with various dilutions of serum in 100 μl of buffer in duplicate at room temperature for 1.5 h. The plates were washed again as described above, and then a 1:1,000 dilution of horseradish peroxidase conjugated goat anti-human IgM or IgG antibody was added to each well (100 μl per well in washing solution). The plates were incubated at room temperature for 1 h and were washed as described above. A total of 100 μl of ABTS peroxidase substrate solution and peroxidase solution (KPI, Gaithersburg, Md.) were added to each well. The reaction was allowed to proceed for 20 min at 37°C. The absorbance of each well was measured at OD$_{405}$ (UV max Kinetic Micro plates reader, Molecular Devices, Menlo Park, Calif.). Additional control antigens from the Sf9 cell pellet, from Sf9 cells infected with wild-type AcNPV, and from negative control sera were tested at the same time. Assays were performed in duplicate, and the means were determined after subtracting the OD$_{405}$ of the wells containing Sf9 cells infected with wild-type AcNPV and patient sera from wells containing ORF-2 protein and patient sera.

Statistical analysis. Groups of ODs were compared for statistical significance by the Student t test.

RESULTS

Expression of recombinant ORF-2 protein. From time course experiments, the maximum expression of ORF-2 recombinant protein occurred after 48 h with a multiplicity of infection of 10. These conditions were used for the large-scale production of ORF-2 protein. The ORF-2 protein was not detected in the culture supernatant (data not shown).

Purification of the recombinant protein. The isoelectric point of ORF-2 was predicated to be 10.3 according to the
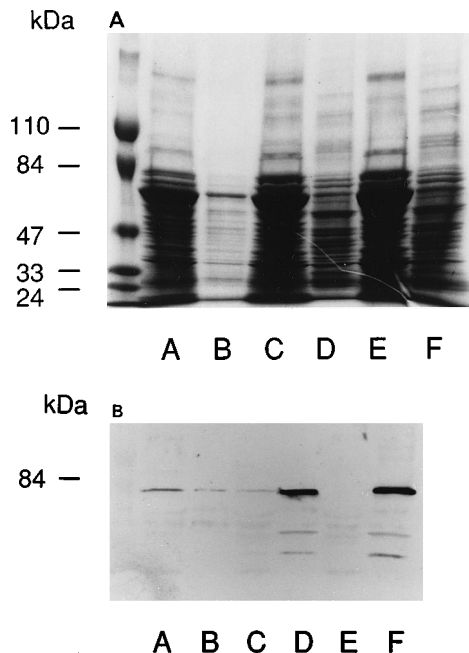

FIG. 1. (A) Coomassie blue staining analysis of ammonium sulfate supernatant (lanes A, C, and E) and precipitate (lanes B, D, and F) at different saturations. Lanes: A and B, 5% ammonium sulfate; C and D, 10% ammonium sulfate; E and F, 20% ammonium sulfate. (B) Immunoblot analysis of ammonium sulfate supernatant (lanes A, C, and E) and precipitate (lanes B, D, and F) at different saturations. Lanes: A and B, 5% ammonium sulfate; C and D, 10% ammonium sulfate; E and F, 20% ammonium sulfate.

deduced amino acid sequence. Therefore, at pH 8.3 ORF-2 should be positively charged and should not be adsorbed to an anion-exchange column. The results were consistent with this prediction. ORF-2 was not retained by the DEAE column at pH 8.3. Therefore, a CM-Sepharose (cation exchanger) column was used for purification. Various concentrations of ammonium sulfate were tested to determine the optimum conditions for precipitation of the ORF-2 protein. A concentration of 20% ammonium sulfate saturation appeared to precipitate the maximum amount of protein (Fig. 1A and B, lanes F). In a typical experiment, 140 mg of total protein was obtained from 500 ml of cell culture. Precipitation with 20% saturation of ammonium sulfate left 60% of unwanted proteins in the supernatant. A total of 560 μg of partially purified ORF-2 protein was recovered from the CM-Sepharose column, and 60 μg of purified ORF-2 protein could be obtained from the affinity column. Samples from different steps of purification were fractionated by SDS–10% polyacrylamide gel electrophoresis (PAGE) and were analyzed by Coomassie blue staining and Western blot assay (Fig. 2A and B). Figure 2A (lane B) represents the protein precipitate of 20% saturation of ammonium sulfate. Figure 2A (lane C) represents the pooled fractions from the CM-Sepharose column. Figure 2A (lane D) represents the ORF-2 eluted from the affinity column. The purified ORF-2 protein (Fig. 2A, lane D) appeared as a single band with a molecular mass of 75 kDa in Coomassie blue-stained gels, with no protein contaminants being detectable. No other degraded ORF-2 protein could be detected in the purified protein by Western blot assay (Fig. 2B, lane D).

ELISA. Usually between 800- and 1,000-fold dilutions of serum resulted in the highest ratio of specific to nonspecific signals (data not shown). Eighteen Sudanese serum samples with detectable IgM anti-HEV and nine Sudanese serum sam-
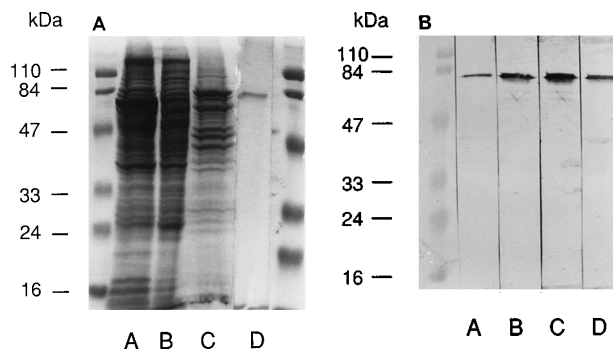
FIG. 2. (A) Coomassie blue-stained SDS-PAGE patterns at different purification steps of the ORF-2 protein. (A) Total proteins from homogenized solution. (B) Ammonium sulfate (20%) precipitate. (C) Fractions containing ORF-2 protein pooled from the CM-Sepharose column. (D) Purified ORF-2 protein from the affinity column. (B) Western blot pattern at different purification steps of the ORF-2 protein. (A) Total proteins from homogenized solution. (B) Ammonium sulfate (20%) precipitate. (C) Fractions containing ORF-2 protein pooled from the CM-Sepharose column. (D) Purified ORF-2 protein from the affinity column.

ples with no detectable IgM anti-HEV (as determined by Western blot assay) were used for ELISA analysis. The OD_{405} values for these 27 serum samples are given in Fig. 3. The OD_{405} values were significantly higher for sera containing IgM anti-HEV (as determined by Western blot analysis) than for sera which showed no anti-HEV IgM by Western blot analysis ($P = 0.0001$; two-tailed Student t test). The results obtained from the ELISA with the partially purified ORF-2 protein used as the antigen correlated 100% with the results obtained from Western blot analysis. Acute-phase sera from 9 patients with hepatitis A virus infection, 7 patients with hepatitis B virus infection, 10 patients with hepatitis C virus infection, and 4 patients with both hepatitis A and hepatitis B virus infections were tested for the presence of anti-HEV IgM by ELISA analysis. The OD_{405} values for these sera were not significantly different from those obtained with sera with no detectable anti-HEV IgM, as determined by Western blot analysis (data not shown).

DISCUSSION

Several observations suggested that the ORF-2 protein expressed in Sf9 cells was not properly folded. First, the ORF-2 protein was extremely sensitive to protease digestion. If protease inhibitors were not included during protein purification, the recombinant protein was totally degraded during the homogenization step (data not shown). Second, this protein precipitated from the homogenization supernatant at a very low ammonium sulfate concentration. Only 20% saturation was enough to precipitate almost all ORF-2 protein from the cell lysate. Normally, proteins that precipitate at less than 25% saturation are generally particulate, preaggregate, very high molecular weight proteins or proteins which are not properly folded and nonnative. Third, the precipitate obtained in this way was difficult to dissolve. Urea at a concentration of 1 M had to be included in the buffer for the total recovery of the ORF-2 protein from the precipitate. One great advantage of precipitating this recombinant protein at such a low concentration of salt was that the preparation was free of protease contaminants after this step. Most proteases are not precipitated at 20% saturation. Therefore, no protease inhibitors were needed in the subsequent procedures. The complete adsorption of ORF-2 onto CM-Sepharose column at pH 8.3

indicated that the pI of the expressed protein is at least 9.3, if not higher. Possible posttranslational modification did not alter the pI value significantly from the predicted value of 10.3. The unfolded nature of the expressed protein indicated that the monoclonal antibody used for affinity purification was against a linear epitope. High titers of IgM and IgG in patient sera against presumed linear epitopes were observed in ELISA experiments in which the presumably improperly folded protein was used as the antigen.

The present study demonstrated that the ELISA described here can reliably detect IgM antibodies directed against HEV. Although limited growth of HEV in a cell culture system has recently been reported (9), sufficient quantities of natural proteins are not readily available. In a typical experiment, 0.56 mg of the partially purified ORF-2 protein can be obtained from a 500 ml of Sf9 cell culture medium. A total of 56 ng of this protein is enough for the diagnosis of HEV in one serum sample. This means that we can screen 10,000 serum samples with only 500 ml of cell culture medium. This is a very economical way to produce an antigen for diagnostic purposes.

The molecular mass of the ORF-2 protein expressed in Sf9 cells was 75 kDa; this is slightly larger than the predicted size for the entire ORF-2 protein (71 kDa). The size difference may be due to the inaccuracy of the estimated molecular mass from SDS-PAGE. The cloned PCR products used in the present
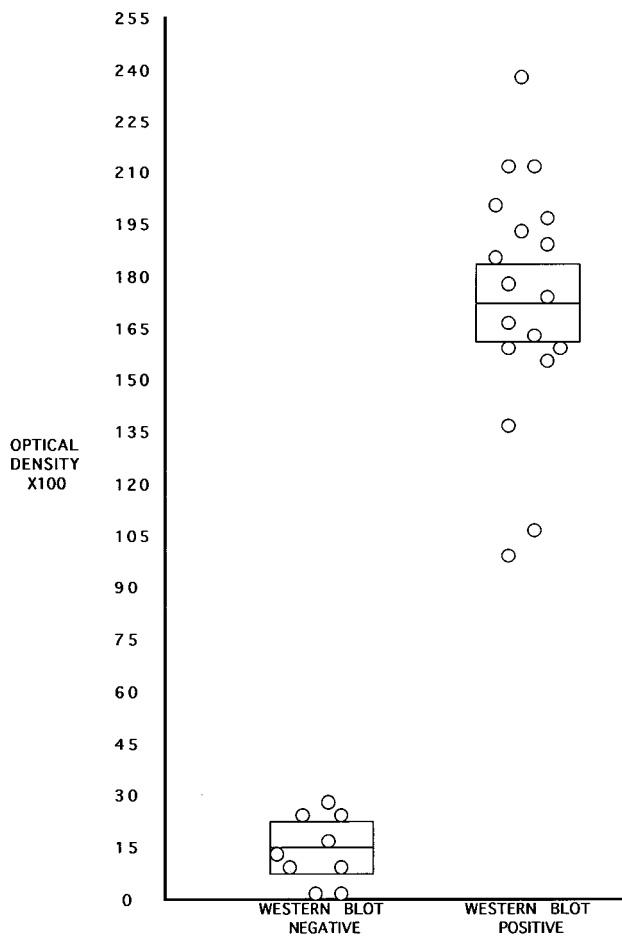

FIG. 3. ELISA for anti-HEV IgM comparing Sudanese sera with or without anti-HEV IgM determined by Western blot assay. The middle horizontal line of each box represents the mean of the datum points for that group, and the outer horizontal lines represents the mean ± standard error of the mean.

study to generate the recombinant baculovirus-expressed ORF-2 protein were derived from the HEV Burma strain. Since there appears to be some divergence in the nucleotide sequences of HEV isolates from different geographical regions (1, 8, 20, 22), there may also be differential antibody responses against these isolates. We are using this Burma strain-derived ORF-2 protein to screen sera from patients with acute non-A, non-B hepatitis from other geographical locations to determine whether this antigen can be universally used for the diagnosis of HEV infection or whether it will be necessary to express recombinant ORF-2 proteins derived from other isolates.

ACKNOWLEDGMENTS

REFERENCES

1. **Aye, T. T., T. Uchida, X.-Z. Ma, F. Iida, T. Shikata, H. Zhuang, and K. M. Win.** 1992. Complete nucleotide sequence of a hepatitis E virus isolated from the Xinjiang epidemic (1986–1988) of China. Nucleic Acids Res. **20:**3512.
2. **Balayan, M. S., A. G. Andiaparidze, S. S. Savinskaya, E. S. Ketiladze, D. M. Braginsky, A. P. Savinov, and V. F. Poleschuk.** 1981. Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. Intervirology **20:**23–31.
3. **Bellabes, H., A. Benatallah, and A. Bourguermouh.** 1984. Non-a/non-B epidemic viral hepatitis in Algeria: strong evidence for its water spread, p. 637. *In* G. N. Vyas, J. C. Dienstag, and J. H. Hoofnagle (ed.), Viral hepatitis and liver disease. Grune and Stratton, New York.
4. **Cao, X. Y., X. Z. Ma, Y. Z. Liu, X. M. Jin, Q. Gao, H. J. Dong, H. Zhuang, C. B. Liu, and G. M. Wang.** 1991. Epidemiological and etiological studies on enterically transmitted non-A, non-B hepatitis in the south part of Xinjiang, p. 297–312. *In* T. Shikata, R. H. Purcell, and T. Uchida (ed.), Viral Hepatitis C, D and E. Elsevier, Amsterdam.
5. **De Cock, K. M., D. W. Bradley, N. L. Sandford, S. Govindrajan, J. E. Maynard, and A. G. Redeker.** 1987. Epidemic non-A, non-B hepatitis in patients from Pakistan. Ann. Intern. Med. **106:**227–230.
6. **He, J., A. W. Tam, P. O. Yarbough, G. R. Reyes, and M. Carl.** 1993. Expression and diagnostic utility of hepatitis E virus putative structural proteins expressed in insect cells. J. Clin. Microbiol. **31:**2167–2173.
7. **Hlady, W. G., M. N. Islam, M. A. Wahab, S. D. Johnson, A. Waiz, and K. Z. Krawozynski.** 1990. Enterically transmitted non-A, non-B hepatitis associated with an outbreak in Dhaka: epidemiology and public health implications. Trop. Doctor **20:**15–17.
8. **Huang, C.-C., D. Nguyen, J. Fernandez, K. Y. Yun, K. E. Fry, D. W. Bradley, A. W. Tam, and G. R. Reyes.** 1992. Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology **191:**550–558.
9. **Huang, R. T., D. R. Li, J. Wei, X. R. Huang, X. T. Yuan, and X. Tian.** 1992. Isolation and identification of hepatitis E virus in Xinjiang, China. J. Gen. Virol. **73:**1143–1148.
10. **Kane, M. A., D. W. Bradley, S. M. Shrestha, J. E. Maynard, E. H. Cook, R. P. Mishra, and D. D. Joshi.** 1984. Epidemic non-A, non-B hepatitis in Nepal: recovery of a possible etiologic agent and transmission studies in marmosets. JAMA **252:**3140–3145.
11. **Khuroo, M. S.** 1980. Study of an epidemic of non-A, non-B hepatitis. Possibility of another human hepatitis virus distinct from post-transfusion non-A, non-B type. Am. J. Med. **68:**818–824.
12. **Khuroo, M. S., W. Duermeyer, S. A. Zargar, M. A. Ahanger, and M. A. Shah.** 1983. Acute sporadic non-A, non-B hepatitis in India. Am. J. Epidemiol. **118:**360–364.
13. **Margulies, A., J. Bernuau, M. S. Balayan, A. G. Andjaparidze, R. Dubois, A. Goudeau, B. Rueff, and J.-P. Benhamou.** 1987. Non-A, non-B fulminant viral hepatitis in France in returnees from Asia and Africa. Digest. Dis. Sci. **32:**1151–1154.
14. **Myint, H., M. M. Soe, T. Khin, T. M. Mint, and K. M. Tin.** 1985. A clinical and epidemiological study of an epidemic of non-A, non-B hepatitis in Rangoon. Am. J. Trop. Med. Hyg. **34:**1183–1189.
15. **Purcell, R. H., and J. R. Ticehurst.** 1988. Enterically transmitted non-A, non-B hepatitis: epidemiology and clinical characteristics, p. 131–137. *In* A. J. Zuckerman (ed.), Viral hepatitis and liver disease. Alan R. Liss, Inc., New York.
16. **Purdy, M. A., K. A. McCaustland, K. Krawczynski, A. Tam, M. J. Beach, N. C. Tassopoulos, G. R. Reyes, and D. W. Bradley.** 1992. Expression of a hepatitis E virus (HEV)-trp E fusion protein containing epitopes recognized by antibodies in sera from human cases and experimentally infected primates. Arch. Virol. **123:**335–349.
17. **Sarthou, J. L., A. Budkowska, M. D. Sharma, M. Lhuillier, and J. C. Pillot.** 1986. Characterization of an antigen-antibody system associated with epidemic non-A, non-B hepatitis in West Africa and experimental transmission of an infectious agent to primates. Ann. Inst. Pasteur Virol. **137E:**225–232.
18. **Shwe, S., and M. M. Soe.** 1985. Epidemiological criteria as indication of non-A, non-B hepatitis in a community. Lancet **ii:**828.
19. **Sreenivasan, M. A., V. A. Arankalle, S. Sehgal, and K. M. Pavri.** 1984. Non-A, non-B epidemic hepatitis: visualization of virus-like particles in the stool by immune electron microscopy. J. Gen. Virol. **65:**1005–1007.
20. **Tam, A. W., M. M. Smith, M. E. Guerra, C.-C. Huang, D. W. Bradley, K. E. Fry, and G. R. Reyes.** 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology **185:**120–131.
21. **Tavera, C., O. Velazquez, C. Avila, G. Ornelas, C. Alvarez, and J. Sepulveda.** 1987. Enterically transmitted non-A, non-B hepatitis--Mexico. JAMA **258:**2036–2037.
22. **Tsarev, S. A., S. U. Emerson, G. R. Reyes, T. S. Tsareva, L. J. Legters, I. A. Malik, M. Iqbal, and R. H. Purcell.** 1992. Characterization of a prototype strain of hepatitis E virus. Proc. Natl. Acad. Sci. USA **89:**559–563.
23. **Tsarev, S. A., T. S. Tsareva, S. U. Emerson, A. Z. Kapikian, J. Ticehurst, W. London, and R. H. Purcell.** 1993. ELISA for antibody to hepatitis E virus (HEV) based on complete open-reading-frame-2 protein expressed in insect cells: identification of HEV infection in primates. J. Infect. Dis. **168:**369–378.
24. **Viswanathan, R.** 1957. Infectious hepatitis in Delhi (1955–1956): a critical study; epidemiology. Indian J. Med. Res. **45**(Suppl.)**:**1–30.
25. **Wong, D. C., R. H. Purcell, M. A. Sreenivasan, S. R. Prasad, and K. M. Pavri.** 1980. Epidemic hepatitis in India: evidence for a non-A, non-B hepatitis virus aetiology. Lancet **ii:**876–879.
26. **Yarbough, P. O. (Genelabs Inc.).** 1992. Personal communication.
27. **Yarbough, P. O., et al.** Unpublished data.
28. **Zhuang, H., X. Y. Cao, C. B. Liu, and G. M. Wang.** 1991. Enterically transmitted non-A, non-B hepatitis in China, p. 277–285. *In* T. Shikata, R. H. Purcell, and T. Uchida (ed.), Viral hepatitis C, D, and E. Elsevier, Amsterdam.