APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1993, p. 2558–2562
0099-2240/93/082558-05$02.00/0

Vol. 59, No. 8

Detection of Hepatitis E Virus in Raw and Treated Wastewater with the Polymerase Chain Reaction

N. JOTHIKUMAR,[1] K. APARNA,[2] S. KAMATCHIAMMAL,[1] R. PAULMURUGAN,[1] S. SARAVANADEVI,[1] AND P. KHANNA[3]*

National Environmental Engineering Research Institute, Madras Zonal Laboratory, Council of Scientific and Industrial Research Complex, Taramani, Madras-113,[1] National Institute of Immunology, New Delhi-110 067,[2] and National Environmental Engineering Research Institute, Nagpur-440 020,[3] India

Received 10 November 1992/Accepted 7 June 1993

The main objective of this study was to determine the applicability of the polymerase chain reaction (PCR) to detection of hepatitis E virus (HEV) in sewage treatment plants and establishment of the prevalence of hepatitis viral diseases in a population. Epidemics of HEV infection because of inadequate public sanitation have been reported in several developing countries. A procedure for concentration of HEV in sewage samples through adsorption to membrane filters, elution with urea-arginine phosphate buffer, and subsequent reconcentration with magnesium chloride enabled us to concentrate HEV to volumes in the microliter range. HEV-specific cDNA was prepared by reverse transcription of the total RNA extracted from samples. Specific DNA amplification by PCR in combination with slot blot hybridization was used to demonstrate the presence of HEV in sewage samples from the inlets and outlets of three sewage treatment plants. The assay was specific for HEV, and a 240-bp amplified product was visualized by ethidium bromide fluorescence. Sewage samples adjusted to pH 5.0 for adsorption of viruses to membrane filters were PCR positive, while samples adjusted to pH 3.5 were PCR negative.

Non-A, non-B hepatitis E virus (HEV) has been responsible for sporadic waterborne epidemics of hepatitis, especially in developing countries and results in high mortality among pregnant women (2, 6, 9, 13). HEV is a 27- to 34-nm nonenveloped particle containing a polyadenylated positive-strand RNA genome of about 7.6 kb and belongs to the *Calciviridae* family (8, 11). Outbreaks of HEV infection have occurred periodically and were first noticed in New Delhi, India, in 1955 following contamination of drinking water. In that outbreak, 29,000 cases of hepatitis were reported (12). In 1991, an HEV outbreak involved about 79,000 people affected by jaundice in Kanpur, India (10).

There is a lack of standard and reliable techniques for isolation of HEV from environmental samples. We have recently reported the use of a membrane filter-based adsorption and elution method for concentrating viruses from water samples (3, 4) with subsequent assay of the concentrated material by polymerase chain reaction (PCR) for detection of enteroviruses (5). The sensitivity of this method encouraged its application to sewage samples for concentration of HEV. This report describes the concentration of HEV in sewage samples and subsequent amplification of the viral nucleic acid by PCR without growing the isolated viruses in cell culture.

MATERIALS AND METHODS

Sewage samples. Sewage samples (500 ml each) were collected every month both at the influent and at the final effluent of three sewage treatment plants, viz., Nesapakkam, Kodungaiyur, and Koyambedu, in Madras City, India, between July and September 1992. The wastewater loads at the three plants are approximately 18 million liters/day in Nesapakkam, 25 million liters/day in Kodungaiyur, and 26 million liters/day in Koyambedu. Wastewater treatment at these plants is performed by activated sludge, single-stage trickling filtration, and two-stage trickling filtration, respectively.

Concentration of HEV in sewage samples. Sample volume was restricted to 500 ml because processing of volumes greater than 500 ml caused clogging of the filters that were used for virus concentration. Preliminary experiments were conducted to study the influence of pH on the recovery of HEV from sewage samples. For these tests, a 500-ml raw sewage sample was blended for 5 min. Coarse material was removed through centrifugation in a 250-ml tube at 3,000 rpm for 20 min. The supernatant was supplemented with $MgCl_2 \cdot 6H_2O$ to a final concentration of 1,200 mg/liter. One of each pair of samples was adjusted to pH 3.5, and the other was adjusted to pH 5.0 to study the effect of pH on virus recovery. Both of the paired samples were then filtered through membrane filters (142-mm-diameter Millipore AP20 filter followed and then through a 142-mm-diameter 0.45-μm-pore-size Millipore filter).

Secondary concentration of viruses from large-volume filter eluates to microliter volumes. Subsequent elution of viruses from the Millipore filters was performed with 100 ml of urea (1.5 M)-arginine (0.02 M)-phosphate (0.008 M) buffer (U-APB). Reconcentration of this primary eluate was performed by addition of 1 ml of $MgCl_2 \cdot 6H_2O$ (1 M) to the eluate. A precipitate which subsequently formed after stirring of the eluate was recovered by centrifugation at 5,000 rpm for 30 min and dissolved in 4 ml of McIlvaines buffer (pH 5.0).

Further concentration of viruses to microliter volumes from the McIlvaines buffer (pH 5.0) was accomplished by passing the solution through smaller-diameter (47-mm) Millipore membrane filters (0.45-μm pore size) for adsorption of viruses, elution of that filter with 5 ml of U-APB, and further reconcentration by adding 100 μl of 1 M $MgCl_2 \cdot 6H_2O$ to the 5 ml of eluate. The resulting precipitate was collected though centrifugation at 7,000 rpm for 30 min, and the precipitate was then dissolved in 400 μl of McIlvaines buffer (pH 5.0) and transferred to a 2-ml Eppendorf centrifuge tube. Sam-

* Corresponding author.

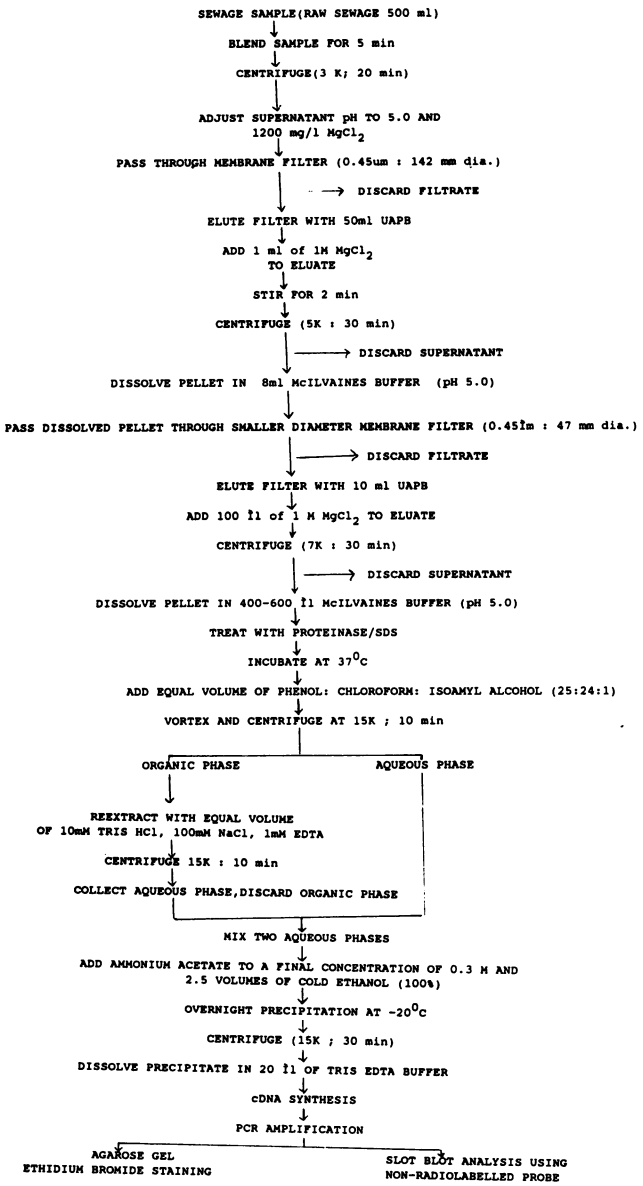

FIG. 1. Procedure for concentration, amplification, and detection of HEV from sewage samples. Íl, microliters.

ples (400 to 600 µl) were treated with sodium dodecyl sulfate (SDS) and proteinase K at final concentrations of 0.5% and 5 mg/ml, respectively, and incubated at 37°C for 90 min. This was followed by phenol-chloroform-isoamyl alcohol extraction of nucleic acid. The procedure is summarized in Fig. 1.

Enterovirus enumeration. Poliovirus 1 was chosen as the model virus for this study for optimization of the experimental protocol because of the advantage of easy quantitation by plaque assay since HEV cannot be cultured in any cell lines under laboratory conditions.

Raw sewage which contained 10.9×10^2 PFU/liter was heat treated at 85°C for 1 h and cooled to room temperature. A known poliovirus 1 stock (12.23×10^3 PFU/ml) was added to the heat-treated sewage (500 ml) and stirred for 30 min at room temperature. The coarse material was removed by centrifugation, and the supernatant was adjusted to pH 3.5 and supplemented with $MgCl_2 \cdot 6H_2O$ to a final concentration of 1,200 mg/liter and concentrated to 4 ml of McIlvaines buffer as summarized in Fig. 1. A 0.5-ml volume of that sample treated with antibiotics was inoculated onto BGMK cell monolayer-containing bottles and overlaid with agar overlay medium for the plaque assay. Counts were expressed as PFU per liter. Percent recovery was calculated to assess the sensitivity of the concentration procedure in terms of the virus concentration in the original sample.

Primers. Sense and antisense synthetic oligonucleotide primers corresponding to the nucleotide sequence of the putative HEV polymerase gene (11) were used in reverse transcription (RT) PCRs for amplification of the viral genome. The primers used were antisense primer 5′ TTCAA CTTCA AG(A)CC ACAGCC 3′ (1000 to 1019) and sense primer 5′ GCGTG GATCT(C) TGCAGGCC 3′ (780 to 797). Clone ET1.1, containing the putative HEV polymerase gene (11), served as a positive control.

RT. The RT mixture contained 10 µl of 1× reverse transcriptase buffer containing a 10 µM concentration of each deoxynucleoside triphosphate (dNTP), 0.4 µmol of the antisense primer, 20 µl of a heat-denatured and quick-chilled test sample containing RNA, and 20 U of avian myeloblastosis virus reverse transcriptase. The reaction mixture was incubated at 37°C for 75 min, the reaction was stopped by heating at 96°C for 2 min, and the mixture was cooled on ice.

PCR. PCR was done with the same RT mixture tube by addition of 50 µl of 1× PCR buffer containing a 200 µM concentration of each dNTP, 0.4 µmol of each of the sense and antisense primers, and 2 U of *Taq* polymerase (AmpliTaq; Perkin Elmer Cetus). The samples were overlaid with 100 µl of mineral oil to prevent evaporation. Amplification was performed with 30 cycles in a thermocycler (Coy Corporation). The cycle involved denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 1 min. The final extension step was done at 72°C for 10 min. Amplified DNA was analyzed by agarose gel electrophoresis. Reaction tubes with all of the components of the PCR except the RNA template served as negative controls in each set of experiments.

Specificity of primers. To confirm that the HEV primers did not cross-react with enteroviruses isolated from sewage samples, control experiments were performed. Sewage sample concentrates (PCR negative for HEV) were inoculated onto monolayers of BGMK cells and overlaid with maintenance medium. Each sample was passaged one additional time through monolayers of BGMK cells and observed for a cytopathic effect. Cells were frozen and thawed three times, and 100 µl of the cell lysate was boiled for 2 min and chilled with ice immediately. Twenty microliters of the cell lysate was used for cDNA synthesis with HEV primers, which was followed by PCR amplification. Another 20-µl cell lysate sample was used for cDNA synthesis with an enteroviral primer, which was also followed by PCR as described previously (5), and this resulted in an amplification product of the predicted length of 154 nucleotides.

Labelling of the DNA probe. Slot blot hybridization was performed to determine the specificity of the PCR-amplified products. A vector containing ET-1.1 insert DNA was labelled with digoxigenin 11-UTP as specified by the manufacturer (Boehringer Mannheim).

DNA hybridization. PCR product samples (20 µl) were denatured by being boiled for 10 min and then immediately cooled. A nitrocellulose membrane filter was soaked in distilled water and then in 20× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) before application of a

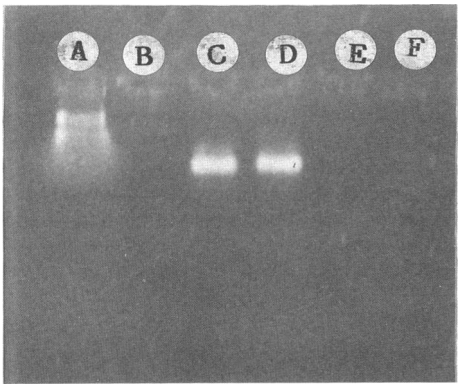

FIG. 2. Agarose gel electrophoresis of PCR-amplified products. Lanes: A, molecular weight markers (*Hin*fI-digested pBR322); B, cDNA synthesis followed by PCR amplification of nucleic acid extracted from cells infected with sewage samples using HEV primers; C, cDNA synthesis followed by PCR amplification of nucleic acid extracted from cells infected with sewage samples using enterovirus primers; D, positive control with poliovirus 1 using enterovirus primers; E, negative control PCR tube containing all of the components of the PCR for enterovirus except template RNA; F, negative control PCR tube containing all of the components of the PCR for HEV except the template.

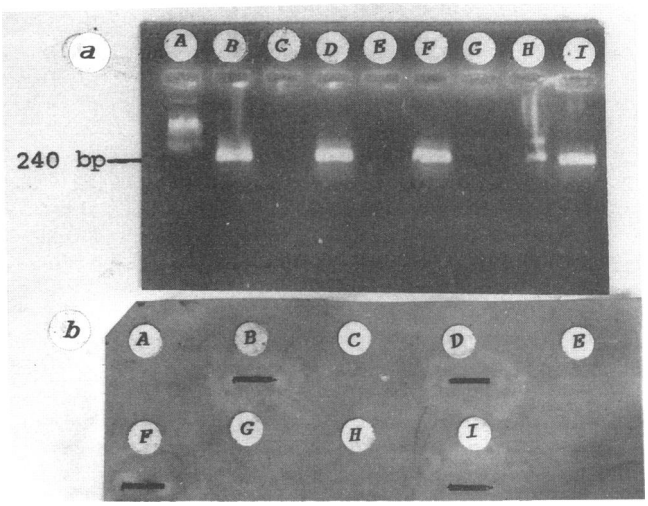

FIG. 3. Analysis of HEV PCR products obtained from raw sewage samples adjusted to pHs 5.0 and 3.5. (a) Gel electrophoresis of amplified HEV sequences showing the 240-bp band. Lanes: A, molecular weight standards (*Hae*III-digested bacteriophage ϕX174 DNA); B and C, Nesapakkam influent samples adjusted to pHs 5.0 and 3.5, respectively; D and E, Kodungaiyur influent samples adjusted to pHs 5.0 and 3.5, respectively; F and G, Koyambedu influent samples adjusted to pHs 5.0 and 3.5, respectively; H, negative control (no RNA); I, positive control (vector insert containing ET-1.1 DNA). (b) Slot blot analysis of HEV PCR products of panel a. hybridized with a nonradiolabelled probe.

20-μl sample via a slot blot filtration manifold (Bio-Rad). The membrane was then baked for 2 h at 80°C under a vacuum and prehybridized at 65°C for 30 min in 5× SSC containing 1% blocking reagent. The probe was then added to the 5× SSC–1% blocking reagent, and hybridization was done at 65°C overnight. Following hybridization, the membrane was washed twice in 2× SSC–0.1% SDS for 30 min at room temperature and twice in 0.1 × SSC–0.1% SDS for 30 min at 65°C.

Hybridization detection. Digoxigenin-labelled probes were detected by using an antidigoxigenin antibody-alkaline phosphatase conjugate (Boehringer Mannheim) and the substrates 5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) and nitroblue tetrazolium.

RESULTS

Recovery of enterovirus. Quantitative enumeration of HEV in sewage samples could not be conducted because of the absence of a suitable cell culture for isolation. Therefore the procedure developed during the course of this investigation was evaluated for the ability to concentrate poliovirus 1 and determine the percentage of its recovery from seeded sewage samples. The concentration method presented in Fig. 1 gave an average of 90% recovery of poliovirus 1 at concentration step 2. Control experiments showed no recovery of enterovirus from heat-treated sewage samples and ensured that indigenous enteroviruses were inactivated. Experiments were performed in triplicate.

Specificity of HEV primers. cDNA synthesis was followed by PCR amplification of nucleic acid extracted from cells infected with sewage samples. The nucleic acid was negative by PCR amplification with HEV primers and positive with enterovirus primers (Fig. 2). This confirmed that HEV primers did not cross-react with enteroviral RNA isolated from environmental samples. The specificity of the HEV PCR was further tested on poliovirus types 1 and 2, coxsackievirus B5, and rotavirus, none of which reacted in the PCR. The specificity of the primer set used in the PCR was confirmed with a positive control (ET 1.1). By using the sample preparation method given in Fig. 1, we obtained 240-bp ethidium bromide-stained amplified DNA fragments on an agarose gel from sewage samples containing HEV.

The PCR results were considered reliable because (i) negative control samples remained PCR negative during the whole observation period, (ii) we obtained identical results with sewage samples tested in duplicate, and (iii) we confirmed the results by slot blot hybridization with a nonradiolabelled probe (ET-1.1).

Influence of pH on recovery of HEV from sewage samples. The effectiveness of the membrane filter-based U-APB method for concentration of viruses in sewage samples adjusted to pHs 3.5 and 5.0 was tested for recovery of HEV and further detection by PCR with appropriate primers after RT. The results in Fig. 3 demonstrate that at pH 3.5 no HEV was recovered but at pH 5.0 a distinct 240-bp PCR product was observed in all three raw sewage samples. It seemed logical to select pH 5 for further studies of HEV concentration. PCR results for HEV and enteroviruses (PFU) obtained with three sets of both influent and effluent sewage samples collected from three treatment plants are presented in Table 1, and one set of results obtained by gel electrophoresis of the HEV PCR product is shown in Fig. 4.

Nesapakkam treatment plant. A raw sewage sample from the Nesapakkam plant which showed an average of 506 PFU/liter was reduced on treatment to an average of 100 PFU/liter in the final effluent, for an average percentage of reduction of 81%. The raw sewage samples contained HEV during three sampling periods, and only one of three samples of the effluent contained HEV.

Kodungaiyur treatment plant. The average enteroviral titer of raw sewage from the Kodungaiyur plant was 900 PFU/

TABLE 1. Detection of viruses in sewages samples collected from sewage treatment plants[a]

Treatment plant	Enterovirus detection an denumeration (PFU/liter of sewage)					
	Influent sampling period:			Effluent sampling period:		
	1	2	3	1	2	3
Nesapakkam	+ (560)	+ (520)	+ (440)	+ (200)	− (60)	− (40)
Kodungaiyur	+ (840)	+ (920)	+ (940)	+ (240)	+ (360)	+ (320)
Koyambedu	+ (580)	+ (620)	+ (540)	− (60)	− (80)	− (40)

[a] Samples were judged for the HEV 240-bp PCR product in ethidium bromide-stained gels, and the results were confirmed with hybridization studies. Enumeration of enteroviruses was done by in vitro cell culture.

liter, which was reduced on treatment to 307 PFU/liter in the final effluent. Thus, an average percentage of removal of 67% was achieved. Both influent and final effluent samples collected at the treatment plant during the three sampling periods contained HEV.

Koyambedu treatment plant. The average enterovirus concentration in a raw sewage sample from the Koyambedu plant was 580 PFU/liter. This was reduced to 60 PFU/liter in the final effluent. Thus, the average percentage of virus removal at this treatment plant was 89% (Table 1). HEV was present in all three of the influent samples, but there was no detectable HEV in the effluent samples during this period.

DISCUSSION

Detection of HEV in sewage confirms the epidemiological importance of monitoring and reveals a public health hazard since sewage samples can cross-contaminate public water supplies, especially at places where drinking water inlets are sited in close proximity to sewage treatment plant outflows. In the past, a practical problem posed for HEV recovery from environmental samples was the absence of suitable cell culture techniques to prove a cytopathic effect. In the present study, direct concentration of HEV through a membrane filter-based adsorption technique, filter elution, reconcentration, and RT-PCR amplification demonstrated a breakthrough in the detection of HEV in environmental samples. Moreover, we found that use of digoxigenin-labelled probes in a hybridization assay gave particularly rapid results and no nonspecific background color, demonstrating good assay specificity. The PCR technique showed a high degree of sensitivity for detection of HEV in sewage samples and a high degree of specificity.

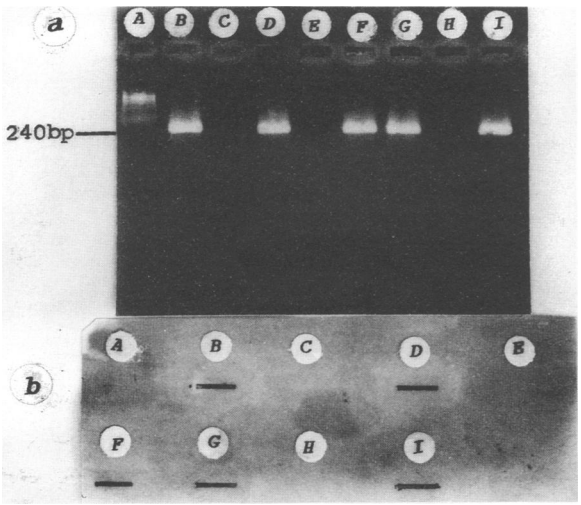

FIG. 4. Analysis of HEV PCR products obtained from influent and effluent sewage samples collected from treatment plants. (a) Gel electrophoresis of amplified HEV sequences showing the 240-bp band. Lanes: A, molecular weight standards (*Hae*III-digested phage ϕX174 DNA); B and C, Nesapakkam influent and effluent samples, respectively; D and E, Kodungaiyur influent and effluent samples, respectively; F and G, Koyambedu influent and effluent samples, respectively; H, negative control (no RNA); I, positive control (vector insert containing ET-1.1 DNA). (b) Slot blot analysis of HEV PCR products of influent and effluent sewage samples given in panel a.

The present study indicates that HEV is sensitive to pH 3.5, presumably because the difference in electrostatic charge on the virus affects adsorption to the membrane filter. This conclusion was reached because virus nucleic acid was recovered when pH 5.0 was used in the concentration procedure but not when pH 3.5 was used. Virus reduction in the different sewage treatment plants varied considerably. All of the samples positive for HEV were likewise positive for enteroviruses. Some of the sewage effluent samples that were free of HEV still showed a significant residual concentration of enteroviruses. This reveals that sewage treatment practices are less than satisfactory from a public health point of view and suggests that enterovirus may be present in the absence of detectable HEV.

This is the first report on the isolation and detection of HEV directly from environmental samples. Earlier studies on HEV detection were restricted to clinical samples, and no commercial kits for HEV detection are available. Most of the laboratory tests for detection of HEV rely on exclusion of hepatitis A, B, C, and D infections along with exclusion of cytomegalovirus and Epstein-Barr virus, all of which can be excluded by serologic testing (2, 9, 12, 13). Ranjit Ray et al. (10) have demonstrated that PCR can be used to detect HEV in stool samples from patients.

non-A, non-B hepatitis epidemics in developing countries are attributed mainly to contamination of drinking water, poor sanitation facilities, and poor personal hygiene. Since environmental samples contain many different groups of viruses, simultaneous amplification and detection of sequences of several different viruses in the same sample by using different primer sets may be possible (7). It is intriguing to consider the possibilities of this approach for simultaneous detection and identification of hepatitis A virus and HEV in sewage samples.

The present study revealed that wastewaters from sewage treatment plants can be a constant and important source of viral hepatitis caused by enterically transmitted HEV. The membrane filter-based U-APB–RT–PCR virus concentration and detection method reported in this study is relatively rapid. It consists of concentration by adsorption and elution of viruses with membrane filters, extraction of nucleic acid, cDNA synthesis, and amplification of DNA. The method takes 1 day plus overnight to confirm the presence of HEV in sewage samples and will be useful for monitoring of wastewater reclamation and reuse. The high rate of HEV prevalence in environmental samples has important implications for epidemiologists who try to determine the exact relationship between the presence of viruses in water and human disease.

ACKNOWLEDGMENTS

We thank G. R. Reyes, D. W. Bradley, and R. Ray for the primers and plasmid DNA (ET-1.1) containing the cloned polymerase gene. We also thank G. P. Talwar for continued encouragement and support.

REFERENCES

1. **American Public Health Association.** 1989. Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association, Washington, D.C.
2. **Bradley, D. W.** 1990. Enterically transmitted non-A, non-B hepatitis. Br. Med. Bull. **46:**442–461.
3. **Jothikumar, N., D. Aparna, and P. Khanna.** 1990. A simple elution and reconcentration technique for viruses concentrated on membrane filters from drinking water. Water Res. **24:**367–372.
4. **Jothikumar, N., D. Aparna, and P. Khanna.** 1991. Evaluation of urea-arginine phosphate buffer (UAPB) for reconcentration of viruses of field samples. Int. J. Environ. Stud. **39:**231–236.
5. **Jothikumar, N., P. Khanna, S. Kamatchiammal, and R. P. Murgan.** Rapid detection of waterborne virus using polymerase chain reaction and gene probe. Intervirology, in press.
6. **Khuroo, M. S.** 1991. Hepatitis E: enterically transmitted non-A, non-B hepatitis. Indian J. Gastroenterol. **10:**96–100.
7. **Li, H. O., U. A. Gyllensten, X. Cui, R. K. Saiki, H. A. Erlich, and N. Arnhim.** 1988. Amplification and analysis of DNA sequences in single sperm and diploid cells. Nature (London) **335:**414–417.
8. **Purcell, R. H., and J. R. Ticehurst.** 1988. Viral hepatitis and liver disease, p. 13–137. Alan R. Liss, Inc., New York.
9. **Ramalingaswami, V., and R. H. Purcell.** 1988. Waterborne non-A, non-B hepatitis. Lancet **ii:**571–573.
10. **Ray, R., R. Aggarwal, P. N. Salunke, N. N. Mehrotra, G. P. Talwar, and S. R. Naik.** 1991. Hepatitis E virus genome in stools of hepatitis patients during large epidemic in north India. Lancet **ii:**438–442.
11. **Reyes, G. R., M. A. Purdy, and J. P. Kim.** 1990. Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science **297:**1335–1339.
12. **Viswanthan, R.** 1957. Infectious hepatitis in Delhi (1955–56): a critical study: epidemiology. Indian J. Med. Res. **45**(Suppl.)**:**1–30.
13. **Zuckermann, A. J.** 1990. Hepatitis E virus. Br. Med. J. **300:** 1475–1476.