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Detection of Hepatitis A Virus in Sewage Sludge by Antigen Capture Polymerase Chain Reaction

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Antigen capture polymerase chain reaction (PCR) was tested as a sensitive and rapid method for detecting hepatitis A virus (HAV) in raw sewage sludge. The antigen capture PCR was performed both with and without solid-phase virus-catching monoclonal antibodies. Similar results proved that both methods were equally sensitive. Sewage sludge samples from different regions in Germany were examined for evidence of HAV contamination by antigen capture PCR. This method of detection was compared with that used in a previous study of these sewage sludge samples, in which the HAV was detected through indirect immunofluorescence after cell culture inoculation. The results obtained by antigen capture PCR matched those obtained in the earlier cell culture investigations, when HAV was detected in raw as well as digested sewage sludge samples. The advantage of the PCR method, however, lies in the fact that it needs only two days while the cell culture propagation of HAV takes about 8 to 10 weeks.

Hepatitis A virus (HAV) is a member of the family *Picornaviridae* and was previously classified as an enterovirus on the basis of its biophysical and biochemical characteristics (7). Significant differences between the HAV and existing members of the genus *Enterovirus*, however, have been observed with respect to heat and acid stability (4, 13), replication, genome organization, and behavior in cell culture (14). HAV generally does not effect any cytopathogenic changes in cell culture during primary isolation but does, after prolonged incubation, lead to persistent infection (3, 15). It was therefore suggested that HAV ought to be classified under the new genus *Hepatovirus* (10) within the family *Picornaviridae*.

HAV is a principal cause of acute hepatitis worldwide and may lead to severe illness or even death. It is transmitted by the fecal-oral route and results in widespread endemic, asymptomatic infection in young children. Clinical disease occurs, and even a mortality rate of 1 to 2% is estimated among nonimmune adolescent and adult populations in industrialized countries. Although the incidence of hepatitis A infection is declining in Europe, the risk of the infection is still high in the Mediterranean region because of food and water contamination. Inadequate sewage treatment also likely plays a role in the transmission of hepatitis A. Despite the low risk of infection in Germany, HAV is still present in sewage sludge. In a study of sewage sludge samples from different regions in Germany, infectious HAV was isolated from both untreated and treated sludge (5). These samples were collected during the various stages of sewage treatment.

Detection of traces of HAV in the environment and in food could prevent outbreaks of hepatitis. A sensitive and rapid method of detection is essential when sporadic outbreaks of HAV infection occur in order to determine the source of the infection. For many enteroviruses, cell culture infection is a useful method of virus detection as cytopathic effects are clearly apparent. HAV, however, does not usually show any cytopathic effects in cell culture and, besides this, its replication is very slow. A minimum of 8 weeks must elapse before positive results of primary HAV isolation can be obtained by indirect immunofluorescence of infected cell cultures. The sensitivity of solid-phase immunoassay is inadequate for detecting traces of HAV in environmental samples. Therefore, we have looked for a sensitive and rapid method based on the detection of viral genome fragments. Nucleic acid hybridization and polymerase chain reaction (PCR) show considerable promise as rapid methods for detecting virus in the environment. Hybridization with genomic viral RNA has been reported to detect 10^4 particles of HAV (9). PCR, however, provides a 10^5 to 10^6 enrichment of a specific nucleic acid sequence and can detect a single copy of DNA (12). The detection of HAV RNA in sewage sludge, on the other hand, may be inhibited by various inorganic and organic substances.

We therefore used and modified the antigen capture PCR (AC-PCR) method as reported by Jansen et al. (8) to demonstrate the specificity and sensitivity of this method for detecting HAV in sewage sludge. In a previous study, 8 of 154 investigated sludge samples were shown to be HAV positive after the prepared sewage sludge was added to confluent monolayers of FRhK-4 cells (fetal rhesus monkey kidney-derived cells) and the mixture was incubated at 37°C for at least 8 weeks (5). By indirect immunofluorescence, the cells were periodically tested for HAV infection, while the supernatant was examined by antigen detection radioimmunoassay (Ag-RIA). These eight sludge samples with HAV-positive cell cultures were then investigated for HAV contamination by AC-PCR.

MATERIALS AND METHODS

Sewage sludge. To determine sensitivity, cell culture supernatant containing $10^{5.2}$ 50% tissue culture infective doses ($TCID_{50}$) per ml of HAV was mixed with untreated raw sewage sludge. A dilution series of this material in sewage sludge was then used for comparing HAV detection by

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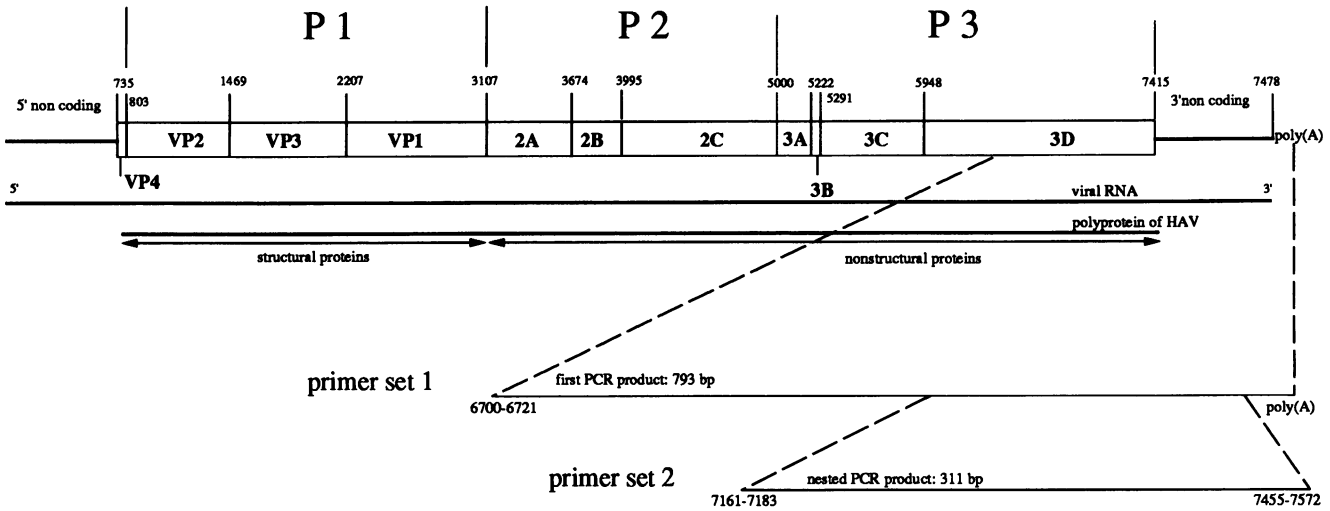

FIG. 1. HAV genome organization and PCR products obtained in this study. The enlarged region shows the predicted sizes of the products of the first and second (nested) PCR amplifications within the 3D and 3'-end region of the HAV genome. The positions of the PCR primers are shown (1).

Ag-RIA and by AC-PCR with or without monoclonal antibody (MAb).

Eight sewage sludge samples from different regions of Germany were used for comparing HAV detection by AC-PCR, Ag-RIA, and cell culture infection. These samples, from various stages of municipal sewage treatment, were proven to be positive for HAV in cell culture (5). They had been processed by the beef extraction method (6). Briefly, 10 ml of a 30% beef extract solution was added to 100 ml of sewage sludge. The pH was adjusted to pH 9.0, and the suspension was sonicated on ice. After centrifugation for 15 min at 4,000 × *g*, the supernatant was adjusted to pH 7.2, 10% freon was added, and the mixture was agitated for 15 min at 4°C. In order to neutralize cytopathic enteroviruses, the supernatant was mixed with a serum pool containing antibodies against all enteroviruses except HAV (Rijksinstitut voor Volksgezondheit, Bilthoven, The Netherlands).

AC-PCR. The AC-PCR method of Jansen et al. (8) was slightly modified. PCR tubes (Perkin-Elmer Cetus) were coated with MAb 7e7 (11) and blocked with 1% bovine serum albumin (BSA) solution. After three washes with phosphate-buffered saline (PBS) containing 0.05% Tween 80, 80 μl of HAV-contaminated material was loaded into the antibody-coated tubes and incubated overnight at 4°C for effective binding of the antigen. The samples were washed five times with a buffer containing 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, and 75 mM KCl to eliminate inhibitory substances for PCR. Denaturation of the virus was then carried out for 5 min at 95°C in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–1.5 mM $MgCl_2$–0.25 mM each dATP, dCTP, dGTP, and dTTP–100 nM sense primer 1–100 nM antisense primer 1 (Fig. 1). The now-accessible HAV RNA was transcribed into cDNA after 5 U of avian myeloblastosis virus reverse transcriptase (Promega), 40 U of RNasin (Promega), and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus) had been added for 20 min at 43°C. The HAV cDNA was amplified by 30 automated cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. This procedure is based on single-tube reverse transcription and PCR, as described elsewhere (2). Negative controls were performed with antibody-coated tubes inoculated with PBS free of HAV.

To increase the sensitivity of HAV detection, a nested PCR was done with 5 μl of the first PCR amplification product by means of an inner primer set 2 (Fig. 1), followed by 30 automated cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C.

Primer sets 1 and 2 were kindly provided by S. M. Feinstone (Food and Drug Administration, Bethesda, Md.) and correspond to the 3' end of the HAV genome HM175 (1) (Fig. 1).

The optimal concentration of the MAb 7e7 was assayed with HAV from the supernatant of cell cultures. This HAV pool had a $TCID_{50}$ of $10^{4.2}$/ml. The protein concentration of MAb 7e7 stock was 2.4 μg/ml. PCR tubes coated with various concentrations of MAb 7e7 and saturated with BSA were prepared prior to loading of 80-μl volumes from the known HAV pool. The AC-PCR was performed as described above.

AC-PCR was also performed without antibodies and BSA saturation. Dilutions of HAV in raw sewage sludge were put into uncoated PCR tubes as well as MAb 7e7-coated tubes. Following incubation, washing, and denaturation, cDNA transcription and amplification were performed as described above. A nested PCR followed the first round of amplification to increase the sensitivity of this test.

Since AC-PCR for HAV detection was also performed without the selective use of the HAV MAb 7e7, the cross-reactivity of other enteroviruses was determined. PCR tubes, both coated with MAb 7e7 and left without any antibody, were loaded with a pool of different enteroviruses (polioviruses 1 through 3 and coxsackieviruses B3 through B5), and AC-PCR was performed with primer sets 1 and 2, containing one primer complementary to the poly(A) tail which is common to all enteroviruses.

Agarose gel electrophoresis and hybridization analysis. An aliquot of each amplified DNA product was tested and its size was determined by agarose gel electrophoresis through 2% Nusieve 3:1 (FMC Bioproducts) in Tris acetate-EDTA

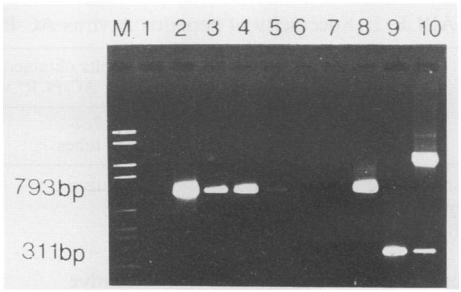

FIG. 2. Agarose gel-ethidium bromide analysis of AC-PCR products obtained with various concentrations of MAb 7e7. The captured HAV was from a cell culture supernatant pool with a $TCID_{50}$ of $10^{4.2}$/ml. Lanes: M, DNA marker VI (Dig labeled; Boehringer Mannheim); 1, negative control consisting of MAb 7e7-coated tubes inoculated with PBS free of HAV; 2 through 6, titration of MAb 7e7 (concentrations of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}, respectively); 7, no MAb 7e7 but BSA saturation; 8, uncoated PCR tube (no MAb 7e7 and no BSA saturation); 9, nested PCR product obtained with an uncoated PCR tube; 10, positive control with a primer set corresponding to a different genome region of HAV (VP1).

buffer, and then it was visualized by staining with ethidium bromide.

A Southern hybridization was performed with PCR products to verify the binding capacity of HAV onto uncoated PCR tubes. After transfer of the DNA onto a nylon membrane (positively charged; Qiagen), hybridization was performed with a digoxigenin (Dig)-labeled cDNA probe specific to the 3′ region of HAV. Labeling of the probe and detection after hybridization were performed with a Dig DNA labeling and detection kit according to the protocol of the manufacturer (Boehringer Mannheim).

RESULTS

Determination of optimal concentration of MAb 7e7 for AC-PCR. Dilutions of MAb 7e7, 10^{-2} to 10^{-6}, were tested to determine the optimal concentration for AC-PCR by using a HAV pool with a known $TCID_{50}$ of $10^{4.2}$/ml. The electrophoretic separation of the PCR amplification products is shown in Fig. 2. Primer set 1 (Fig. 1) was used for the AC-PCR, during which a DNA fragment with a predicted size of 793 bp was amplified. An optimal yield of the AC-PCR product was obtained with a dilution of MAb 7e7 of 10^{-2} and following BSA saturation (Fig. 2, lane 2). If, however, HAV was loaded onto uncoated PCR tubes, a PCR product with the expected size of 793 bp was also obtained (Fig. 2, lane 8). The intensity of this DNA band was approximately equal to that of the band derived from the AC-PCR in which MAb 7e7 was used at a concentration of 10^{-3} (Fig. 2, lane 3).

To verify whether this product actually represented the HAV genome, a nested PCR was performed with the inner primer set 2 (Fig. 1). This assay created a DNA product with the expected size of 311 bp (Fig. 2, lane 9). In addition, Southern hybridization, with a Dig-labeled HAV cDNA probe corresponding to the 3′ region, gave a positive result for the PCR products obtained by AC-PCR with or without MAb 7e7 and BSA saturation (Fig. 3, lanes 2 to 9). In contrast, only a faint signal was received when an uncoated PCR tube blocked with BSA was used (Fig. 3, lane 7). Figure 2, lane 10, shows PCR products derived from the VP1 region of the HAV genome, which did not give any nonspecific signal with the HAV probe used (Fig. 3, lane 10).

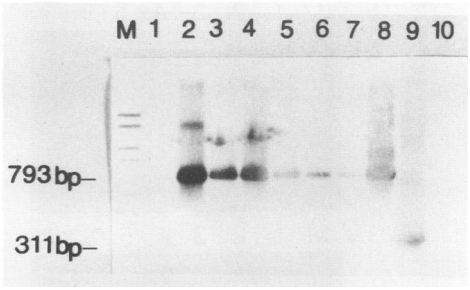

FIG. 3. Southern hybridization of agarose gel shown in Fig. 2. The hybridization was performed with a Dig-labeled cDNA probe from the 3′ region of a HAV strain from our laboratory. Lanes are as described in the legend to Fig. 2.

Sensitivity of AC-PCR for environmental samples. The sensitivity of AC-PCR for testing environmental samples was determined with HAV-seeded sewage sludge. To determine possible inhibitory effects of the sewage sludge, dilutions were made from HAV-positive cell culture supernatant ($10^{5.2}$ $TCID_{50}$/ml) in raw sewage sludge as well as in cell culture medium. The PCR-amplified products were separated by agarose gel electrophoresis as shown in Fig. 4. The same primer sets as shown in Fig. 1, primer sets 1 and 2, were used.

We detected $10^{2.2}$ $TCID_{50}$/ml after the first round of PCR amplification and $10^{-2.2}$ $TCID_{50}$/ml after the nested PCR had been done. These results were obtained with MAb 7e7 as the capturing agent and HAV diluted in sewage sludge. The

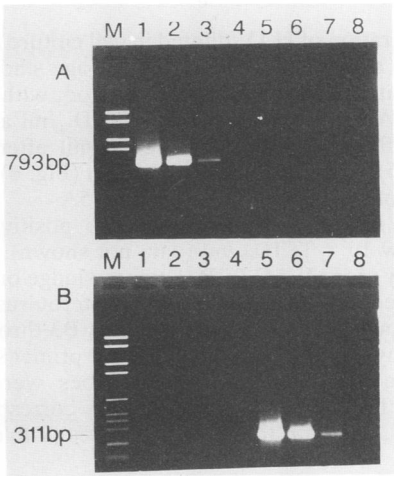

FIG. 4. Agarose gel-ethidium bromide analysis of first (A) and second (nested) (B) AC-PCR amplification products obtained with HAV-positive cell culture supernatant diluted in raw sewage sludge and loaded on MAb 7e7 (dilution, 1:100)-coated PCR tubes. (A) Lanes 1 through 8 show results obtained for a titration of HAV ($TCID_{50}$ = $10^{5.2}$/ml) in raw sewage sludge (concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}, respectively). Amplification of the predicted 793-bp product was detected through a concentration of 10^{-3}. (B) Lanes 5 through 8 show results obtained with a 5-μl aliquot of the first PCR product, shown in panel A, lanes 5 through 8 (titration of HAV in sewage sludge; concentrations of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}, respectively). For both panels, lane M contains DNA marker VI (Boehringer Mannheim). Only the negative samples from the first PCR were tested by nested PCR in order to reduce the possibility of contamination. The predicted 311-base product was detected through a concentration of 10^{-7}.

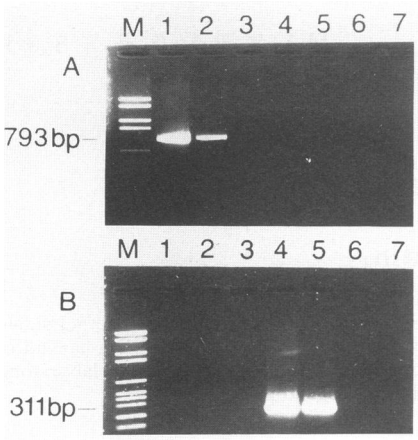

FIG. 5. Agarose gel-ethidium bromide analysis of first (A) and second (nested) (B) AC-PCR amplification products obtained with uncoated PCR tubes loaded with HAV-seeded sewage sludge. (A) Lanes 1 through 6 show results obtained for a titration of HAV ($TCID_{50}$ = $10^{5.2}$/ml) in raw sewage sludge (concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}, respectively); lane 7 is a negative control, consisting of PBS free of HAV. Amplification of the predicted 793-bp product was detected through a concentration of 10^{-2}. (B) Lanes 4 through 6 show results obtained with a 5-μl aliquot of the first PCR product, shown in panel A, lanes 4 through 6 (titration of HAV in sewage sludge; concentrations of 10^{-4}, 10^{-5}, and 10^{-6}, respectively); lane 7 is a negative control. For both panels, lane M contains DNA marker VI (Dig labeled; Boehringer Mannheim). Only the negative samples from the first PCR were tested by nested PCR in order to reduce the possibility of contamination. The predicted 311-base product was detected through a concentration of 10^{-5}.

endpoint titration of HAV diluted in cell culture medium was identical to that of HAV diluted in sewage sludge.

The sensitivity of the detection method, without antibodies and BSA saturation, was $10^{3.2}$ $TCID_{50}$/ml after the first PCR amplification and $10^{-1.2}$ $TCID_{50}$/ml after the nested PCR (Fig. 5). A Southern hybridization (Fig. 6) verified the specificity of the results shown in Fig. 5A.

Ag-RIA, in comparison, showed no positive results at levels below $10^{5.2}$ $TCID_{50}$/ml (data not shown).

Specificity of HAV AC-PCR. Sewage sludge or cell culture medium seeded with a selection of enteroviruses (polioviruses 1 through 3 and coxsackieviruses B3 through B5) did not show any amplified product with the primer sets used for HAV, even when uncoated PCR tubes were used and despite the fact that one of the primers corresponds to the poly(A) tail, common to all enteroviruses (Table 1).

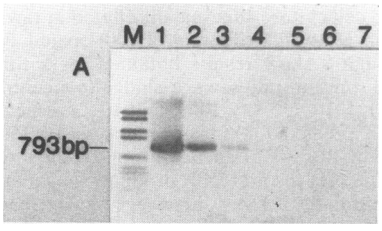

FIG. 6. Southern hybridization of agarose gel shown in Fig. 5A. The hybridization was done with a Dig-labeled DNA probe from the 3′ region of a HAV strain from our laboratory. The lanes are as described in the legend to Fig. 5A.

TABLE 1. Specificity of hepatitis A virus AC-PCR

Sample tested	Results obtained for HAV AC-PCR[a] with:	
	MAb 7e7-coated tubes	Uncoated tubes
Sewage sludge containing a pool of enteroviruses[b] (dilutions from 10^{-1} to 10^{-6})	Negative	Negative
Cell culture medium containing a pool of enteroviruses[b]	Negative	Negative
Sewage sludge containing a pool of enteroviruses[b] plus HAV	Positive	Positive

[a] The primer set used for the HAV AC-PCR, shown in Fig. 1, contained one primer corresponding to the poly(A) tail, common to all enteroviruses as well as HAV.

[b] The enterovirus pool consisted of poliovirus types 1, 2, and 3 and coxsackieviruses B3, B4, and B5. The cytopathogenic titers per ml of cell culture were 10^9 for poliovirus type 1, 10^7 each for poliovirus types 2 and 3, 10^5 each for coxsackieviruses B3 and B5, and 10^4 for coxsackievirus B4.

Detection of HAV in sewage sludge samples. Figure 7 shows agarose gel separation of the PCR amplification products derived from eight sewage sludge samples from different regions in Germany. These samples were treated with beef extract before AC-PCR was performed.

The first round of PCR, using primer set 1, gave amplified products with the expected size of 793 bp (Fig. 7A, lanes 1 to 3 and 6). Primer set 2, used for nested amplification, gave PCR products with the expected size of 311 bp (Fig. 7B, lanes 4, 5, and 7). Only negative samples from the first PCR were tested by nested PCR. The negative controls, virus-free sewage sludge and PBS, did not show any signal on the agarose gel (Fig. 7, lanes 10 and 11).

Of 154 samples tested (raw and digested sludge), 8 demonstrated infectious HAV in cell culture (5). While all eight were HAV negative in the Ag-RIA, seven of these were

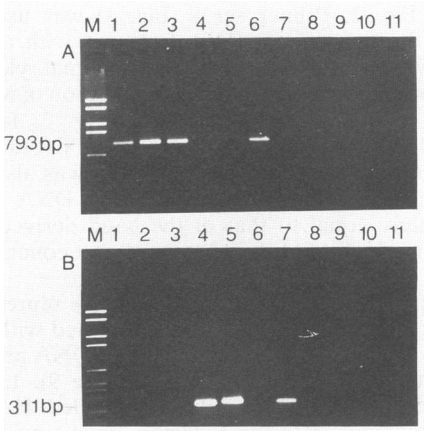

FIG. 7. Agarose gel-ethidium bromide analysis of first (A) and second (nested) (B) AC-PCR amplification products obtained with sewage sludge collected from different regions in Germany. Lanes: M, DNA marker VI (Boehringer Mannheim); 1, 2, 8, and 9, digested sludge samples; 3 through 7, undigested sludge samples; 9, sludge sample in lane 8 loaded onto an uncoated PCR tube; 10, uninfected sewage sludge; 11, negative control, consisting of PBS without HAV.

TABLE 2. Comparison of various HAV detection methods used to investigate sewage sludge samples collected from different regions in Germany

Sludge sample[a]	Result obtained with HAV detection method			
	RIA[b]	Cell culture infection[b]	AC-PCR	Nested PCR
A1r	−	+	+	NT[c]
D1r	−	+	−	+
F1d	−	+	−	−
K1d	−	+	−	+
K2d	−	+	+	NT
L1r	−	+	+	NT
M1r	−	+	+	NT
N1r	−	+	−	+

[a] r, raw sludge; d, digested sludge.
[b] Results were obtained in a previous study with the same samples (6).
[c] NT, not tested.

HAV positive after AC-PCR was performed (Table 2). Three raw sludge samples proved positive only after the nested PCR (Fig. 7B, lanes 4, 5, and 7) was done. Two raw (Fig. 7A, lanes 3 and 6) and two digested (Fig. 7A, lanes 1 and 2) sludge samples turned positive upon amplification during the first PCR. One of the eight samples remained negative after nested PCR in which a MAb 7e7-coated tube, as well as an uncoated PCR tube, was used for antigen capture (Fig. 7B, lanes 8 and 9).

DISCUSSION

The fact that HAV is a very stable virus in the environment was underlined by our results. Traces of HAV were detectable even in digested sewage sludge. The risk of transmission of HAV via contaminated food makes a sensitive and rapid method of detection helpful.

While PCR has been shown to be a very sensitive tool for detecting even minute amounts of nucleic acid from any source, we compared indirect immunofluorescence after cell culture inoculation, Ag-RIA, and AC-PCR for the analysis of HAV-contaminated environmental samples. PCR may be more valuable than cell culture for detecting HAV in sludge because it is more rapid, not all wild-type strains of HAV will propagate in cell culture, and virus is probably lost during the extraction process. Intrinsic to the AC-PCR technique are specific purification of HAV from environmental samples and separation from other viruses, via capture by MAb, and enrichment of the virus through binding to a solid phase. Besides this, each step of the procedure (antigen capturing, virus denaturation to release RNA, reverse transcription into cDNA, and DNA amplification) can be performed in the same reaction tube (2, 8), thus avoiding the loss of material due to extraction. Our results were identical when avian myeloblastosis virus reverse transcriptase and *Taq* polymerase were added together for AC-PCR and when the two enzymes were added sequentially. Collectively, our results underscore the fact that the AC-PCR is a specific, sensitive, rapid, and easy-to-handle method.

The sensitivity of AC-PCR for detecting HAV in raw sewage sludge was equivalent to that achieved with a HAV-containing cell culture supernatant. Chloroform extraction of HAV-seeded sewage sludge did not enhance the sensitivity of the detection method (data not shown). Therefore, we concluded that inorganic and organic substances in sewage sludge did not seem to influence the sensitivity of AC-PCR.

Further experiments showed that AC-PCR could be successfully used for detection of HAV if uncoated PCR tubes were used, although sensitivity was slightly lower. The ability of HAV to attach to a polypropylene surface presumably explains these positive results.

Cross-reactions between primers for the 3′ end of HAV and different enteroviruses such as polioviruses 1 through 3 and coxsackieviruses B3 through B5 were not found.

In our investigations of HAV seeded in sewage sludge, nested PCR was more sensitive than cell culture infection. HAV RNA was still detectable in sewage sludge even when the $TCID_{50}$ of the HAV pool used was already negative. This point raises the question of whether the detection level obtained with the nested PCR actually reflects the level of the infectious virus, consisting of total HAV RNA coated with P1 proteins. Since the AC-PCR was based on capturing the antigen by using a MAb, and as reverse transcription reaction showed that the presence of viral RNA led to amplified DNA, it can be assumed that the captured particles represented infectious viruses.

Eight sewage sludge samples used in an earlier study (5) were available as a natural source of virus-contaminated environmental samples for our investigations. In that study, 154 sludge samples were assayed for HAV by cell culture inoculation and Ag-RIA. Raw as well as digested sludge samples were then processed by the beef extraction method. This was carried out to enable virus recovery and to eliminate solid-associated cytotoxic agents. Our recent experiments demonstrated that this extraction step is not necessary if AC-PCR is the chosen method, because of the sensitivity shown with the HAV-seeded raw sewage sludge compared with the sensitivity with HAV diluted in cell culture medium. In each of the eight samples, the presence of HAV could be demonstrated by immunofluorescence 2 months after inoculation of the cell cultures. HAV determination by Ag-RIA yielded not a single positive result. Solid-phase immunoassay is, in general, not sensitive enough for such environmental samples. Seven of the eight sludge samples also proved positive when the faster AC-PCR was used, although three of them became positive only after a nested PCR. One of the sludge samples, positive by cell culture techniques, remained negative. To rule out the possibility that the MAb 7e7 is unable to bind the virus out of this sludge sample, AC-PCR was also performed with uncoated PCR tubes. This experiment, however, failed to yield a positive result. Inhibitory effects of the sewage sludge for the amplification could be excluded because of the identical results obtained with HAV diluted in sewage sludge and HAV diluted in cell culture medium. The negative PCR result is due either to the protracted length of storage or to the possibility that the sample contained a HAV strain which did not react with our primers. Less stringent conditions for annealing of the primer or a primer set corresponding to a different HAV region might lead to positive results, but because of the limited amount of this sample, further analysis was not possible.

On the basis of our results, AC-PCR is a highly sensitive method for detecting HAV in environmental sources, and it could replace the time-consuming cell culture technique.

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REFERENCES

1. **Cohen, J. I., J. R. Ticehurst, R. H. Purcell, A. Buckler-White, and B. M. Baroudy.** 1987. Complete nucleotide sequence of wild-type hepatitis A virus: comparison of different strains of hepatitis A virus and other picornaviruses. J. Virol. **61**:50–59.
2. **Eldadah, Z. A., D. A. Asher, M. S. Godec, K. L. Pomeroy, L. G. Goldfarb, S. M. Feinstone, H. Levitan, C. J. Gibbs, Jr., and D. C. Gajdusek.** 1991. Detection of flaviviruses by reverse-transcriptase polymerase chain reaction. J. Med. Virol. **33**:260–267.
3. **Flehmig, B.** 1981. Hepatitis A virus in cell culture. II. Growth characteristics of hepatitis A virus in Frhk-4/R cells. Med. Microbiol. Immunol. **170**:73–81.
4. **Flehmig, B., A. Billing, A. Vallbracht, and K. Botzenhart.** 1985. Inactivation of hepatitis A virus by heat and formaldehyde. Water Sci. Technol. **17**:43–45.
5. **Flehmig, B., and J. Weckerle.** 1988. Isolierung von infektiösem Hepatitis A Virus aus Klärschlamm. Z. Gesamte Hyg. Grenzgeb. **34**:43–45.
6. **Goyal, S. M., and C. P. Gerba.** 1982. Methods for concentration of enteroviruses from water, p. 91–109. *In* C. P. Gerba and S. M. Goyal (ed.), Methods in environmental virology, vol. 7. Marcel Dekker, Inc., New York.
7. **Gust, I. D., A. G. Coulepis, S. M. Feinstone, S. A. Locarnini, Y. Moritsugu, R. Najera, and G. Siegl.** 1983. Taxonomic classification of hepatitis A virus. Intervirology **20**:1–7.
8. **Jansen, R. W., G. Siegl, and S. M. Lemon.** 1990. Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. Proc. Natl. Acad. Sci. USA **87**:2867–2871.
9. **Jiang, X., M. K. Estes, T. G. Metcalf, and J. L. Melnick.** 1986. Detection of hepatitis A virus in seeded estuarine samples by hybridization with cDNA probes. Appl. Environ. Microbiol. **52**:711–717.
10. **Minor, P. D.** 1991. Picornaviridae, in classification and nomenclature of viruses. Arch. Virol. (Suppl. 2):320–326. (Fifth report of the international committee on taxonomy of viruses.)
11. **Pfisterer, M., U. Heinricy, and B. Flehmig.** 1987. Development and characterization of a monoclonal antibody against hepatitis A virus, abstr. 46, p. 17A. Int. Symp. Viral Hepatitis, London, England. 1987.
12. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer directed enzymatic amplification of DNA with thermostable DNA polymerase. Science **239**:487–491.
13. **Scholz, E., U. Heinricy, and B. Flehmig.** 1989. Acid stability of hepatitis A virus. J. Gen. Virol. **70**:2481–2485.
14. **Ticehurst, J., J. I. Cohen, S. M. Feinstone, R. H. Purcell, R. W. Jansen, and S. M. Lemon.** 1989. Replication of hepatitis A virus: new ideas from studies with cloned cDNA, p. 27–50. *In* B. L. Semler and E. Ehrenfeld (ed.), Molecular aspects of picornavirus infection and detection. American Society for Microbiology, Washington, D.C.
15. **Vallbracht, A., L. Hoffmann, K. G. Wurster, and B. Flehmig.** 1984. Persistent infection of human fibroblasts by hepatitis A virus. J. Gen. Virol. **65**:609–615.