Pergamon

Wat. Sci. Tech. Vol. 31, No. 5-6, pp. 345–350, 1995.

0273–1223/95 $9·50 + 0·00

0273-1223(95)00290-1

DETECTION OF ENTERIC ADENOVIRUSES IN SOUTH AFRICAN WATERS USING GENE PROBES

B. Genthe*, M. Gericke**, B. Bateman**, N. Mjoli*** and R. Kfir**

* *Division of Water Technology, CSIR, P.O. Box 320, Stellenbosch, 7599, South Africa*
** *Division of Water Technology, CSIR, P.O. Box 395, Pretoria, 0001, South Africa*
*** *Department of Microbiology, University of Durban-Westville, South Africa*

ABSTRACT

Gene probes developed locally for both enteric Adenoviruses 40 and 41 were used to determine whether these viruses were present in both raw and treated waters. Approximately sixty water samples were concentrated by ultrafiltration and analysed directly for the presence of enteric adenoviruses. Three pretreatment techniques, namely sephadex columns, cellulose fibre and Geneclean™ were tested for the removal of inhibitory substances from concentrated water samples. The effect of chlorine treatment on viral detection using gene probe hybridization was also examined by exposing adenoviruses to chlorine concentrations of up to 20mg/l for 1 hour. Enteric adenoviruses were detected in up to 59% of both raw and treated waters analysed. Cellulose fibre and Geneclean™ were found to successfully remove inhibitory substances from concentrated raw waters. Viral DNA was detected after exposure to a range of chlorine concentrations indicating that the viruses detected in the treated waters may have been inactivated virus particles.

KEYWORDS

Enteric Adenoviruses types 40 and 41; gene probes; nucleic acid hybridization; sephadex; cellulose-fibre; Geneclean™; inhibitory substances; South Africa.

INTRODUCTION

Adenoviruses, particularly types 40 and 41 (subgroup F) have been recognized as a major cause of gastroenteritis. Subgroup F enteric adenoviruses (EAds) are apparently second only to rotavirus as a viral cause of gastroenteritis in hospitalized infants and young children (Kidd *et al.*, 1983). Very little information is available about the occurrence of Adenoviruses in water.

Studies have shown that Adenoviruses in sewage were far more abundant than any other enteric viruses (Cabelli, 1982). Large amounts of virus particles, in excess of 10^{11} particles per gram of stool are reported to be excreted at the peak of acute infection (Buitenwerf *et al.*, 1985). One can therefore expect the EAds 40 and 41 to be present in water in large numbers, if contamination with untreated sewage has occurred.

Conventional detection methods for enteric Adenoviruses are both complicated and time consuming. These viruses are also known as "fastidious Adenoviruses" because in contrast to most other Adenoviruses, they do not readily replicate with a cytopathogenic effect in conventional cell culture systems using the primary cells and cell lines routinely used to detect the Adenoviruses types 1-39 (Tiemessen and Kidd, 1988; Witt and Bousquet, 1987). Due to difficulties in cultivation in cell-lines, alternative methods for their detection are necessary. With the advent of nucleic acid hybridization techniques, gene probes for the enteric Adenoviruses were developed and are reported to provide a rapid, sensitive and accurate detection method for these viruses (Kidd *et al.*, 1985; Hurst *et al.*, 1988). Gene probes may be used to detect viral nucleic acid either directly or indirectly (e.g. *in situ* hybridization). Indirect methods, although highly sensitive, lengthen the detection time and require the use of cell culture which further complicates the methods, due to EAds being "fastidious" viruses. Therefore, the reported speed and sensitivity of the direct method provides an attractive alternative for the detection of EAds in environmental water samples.

In South Africa, although there is an abundance of information on the presence of enteric viruses in water, very little information is available on the prevalence of Adenoviruses. In this study the suitability of the use of a direct gene probe method for the detection of EAds 40 and 41 was investigated.

METHODS AND MATERIALS

Water concentration procedure

Pressure ultrafiltration was used to concentrate 10 litre water samples as previously described by Nupen *et al.* (1980).

Approximately 60 water samples were concentrated and analysed directly for the presence of the enteric Adenoviruses using the gene probe method. Both raw (n=21) and treated waters (n=38) were analyzed.

Viral stocks

Adenovirus 40 (ATCC VR-931, originating from an infantile gastroenteritis faeces) and 41 (ATCC VR-930, originating from the faeces of a 14 month old child with gastroenteritis) were obtained from the American Type Culture Collection. Frozen viral stocks were stored at -70°C. Freon extraction of viral stocks was performed to ensure single particles for experimental work, using equal volumes of virus suspension and freon.

Preparation of concentrated environmental sample for direct testing

Environmental water samples concentrated by ultrafiltration were extracted with an equal volume of phenol and chloroform, precipitated with 2 volumes of 95% ethanol and resuspended in tris-EDTA solution before being boiled in 20% formamide.

Gene probe/nucleic acid hybridization

Adenovirus 40 and 41 specific probes as cloned fragments of EAds 40 and 41 were kindly donated by C Tiemessen (described in Kidd *et al.*, 1985). These probes are DNA probes made up of relatively large fractions of the EAds 40 and 41 genomes (5.3% or 1800 base pairs and 18.7% or 6500 base pairs, respectively) in the *Escherichia coli* plasmid, pBR322.

Plasmids with the Adenovirus DNA inserts were extracted using alkaline lysis treatment. The purified plasmid DNA containing the Adenovirus inserts were then digested using the restriction enzyme Pst 1 at 37°C for 90 minutes to linearize the DNA before labelling by the random priming method using the non-radioactive digoxigenin (DIG) system (Boehringer Mannheim).

Hybridization procedures

After samples had been blotted onto nylon membranes the membranes were prehybridized at 50°C for 2-4 hours in hybridization buffer (5X SSC, 2% blocking reagent, 0.1% w/v N-lauroyl sarcosine, 0.02% w/v SDS). Denatured labelled probe was then added to the hybridization buffer and hybridized for 16-18 hours at 50°C. The DIG labelled DNA was visualized with anti-DIG alkaline phosphatase conjugate. The bound alkaline phosphatase conjugate was reacted with lumigen-PPD, a chemiluminescent substrate for alkaline phosphatase. The blots were then exposed to X-ray film for 1-2 hours at room temperature and the film developed. Positive results are described as either 1+, 2+, or 3+ according to the strength of the signal detected with 3+ meaning the strongest reaction and 1+ the weakest.

Specificity of probes

Negative controls using nucleic acid extracts of rotavirus (SA11) and positive controls using EAds 40 and 41 DNA were included in each test.

Pretreatment of concentrated environmental water samples

Three pre-treatment techniques namely, sephadex columns, cellulose fibre and Geneclean™ were examined for the removal of inhibitory substances from the concentrated water samples. Approximately 45 environmental raw water samples were spiked with 100 ml EAds 40 and 41 stock cultures. The pretreatment methods were then compared with the conventional phenol-chloroform method.

Sephadex columns were made in a 3ml syringe by using Sephadex G-75 (Pharmacia) equilibrated with 5mM Tris buffer. Concentrated environmental samples (100ml sample size) were run through the column and collected after centrifugation at 700rpm. The purified DNA was then tested using the gene probe method for EAds as described above.

Purification of environmental water samples was carried out using glassmilk supplied by the manufacturers of Geneclean™ (Bio 101 Inc.). The concentrated environmental water samples (100ml) were mixed with NaI and glassmilk, left to stand for 5 minutes, followed by 3 washes with the wash solution. Tris-EDTA was added and incubated at 55°C for 3 minutes to elude the DNA. The supernatant containing the purified DNA was then tested using the gene probe method for EAds as described above.

The concentrated environmental water sample (100ml) was mixed with 200ml extraction buffer (0.2M glycine, 0.1M Na_2HPO_4, 0.6M NaCl, 1% SDS, pH 9.5) and vortexed for 30 seconds. An extraction with an equal volume phenol was followed with an extraction using phenol-chloroform-isoamylalcohol (24:24:1). The aqueous phase was mixed with 95% ethanol to bring to a final concentration of 15% ethanol. CF-11 cellulose (30 mg) was added and the mixture vortexed for 10 seconds. The samples were then put on a rotating mixer for 90 minutes at 4°C. The sample was centrifuged for 1 minute, followed by 3 washes in STE buffer (0.1M NaCl, 1mM EDTA, 0.05M Tris pH7) containing 15% ethanol (v/v). The DNA was eluded from the cellulose with a fourth wash in STE without ethanol. The solution was centrifuged for 5 minutes and the DNA precipitated with 0.3M NaAc (pH 5.4) and ethanol (Wilde *et al.*, 1990). The purified DNA was then tested using the gene probe method for EAds as described above.

Chlorination of enteric Adenoviruses

To determine the implications and significance of detecting EAds in treated drinking water samples, water samples were seeded with 100ml of purified virus stock cultures, treated with various concentrations of chlorine ranging from 0,25 mg/l to 20mg/l for 15 and 60 minutes before the chlorine was neutralised with sodium thiosulphate. Treated EAds were tested directly after exposure to chlorine using the gene probe method.

RESULTS

Results using the gene probes for the detection of both enteric Adenoviruses 40 and 41 showed that the viruses occur in high numbers of South African waters, with 58-59% percent of both raw and treated waters positive for Adenovirus 40 and 47% positive for Adenovirus 41 (Figure 1.). The same percentage of treated waters as raw waters were found to be positive for both Adenovirus 40 and 41, but the positive scores in treated waters were generally higher than in raw waters. On one occasion the raw water tested negative where the corresponding treated water tested positive.

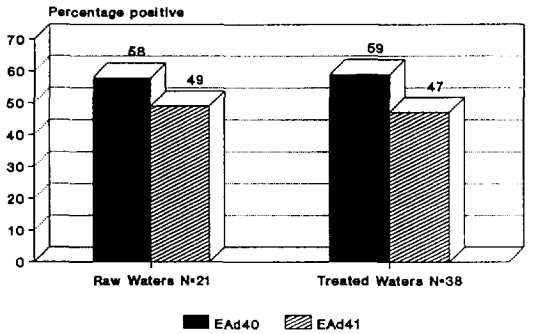

Figure 1. Percentage of water samples positive for EAds 40 and 41 using gene probes.

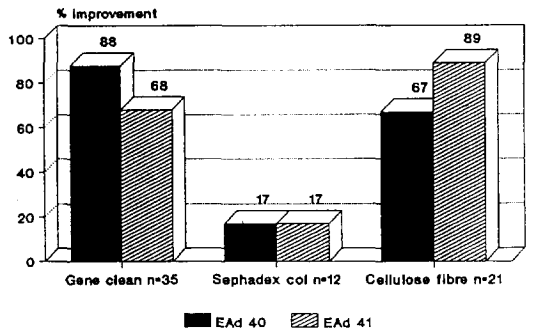

Figure 2. Comparison of pretreatment methods for the removal of inhibitory substances.

Both the Geneclean™ and the cellulose fibre method were efficient in the removal of inhibitory substances from concentrated water samples prior to the detection of both EAd 40 and 41 (Figure 2). The Geneclean™ method was found to be the most successful in removing inhibitory substances for the detection of EAd 40 (88% improvement), whereas the cellulose fibre method was the most suitable for EAd 41 (88% improvement). Sephadex columns were not successful in removing inhibitory substances from raw water samples in comparison to the above-mentioned methods, with only a 17% improvement for both EAd 40 and 41.

Results of virus detection after chlorination with various concentrations of chlorine are presented in Table 1. Treatment of water samples seeded with EAds 40 and 41 and treated with various concentrations of chlorine for both 15 minutes and 1 hour did not reduce the signal produced when directly testing for the presence of viral DNA using the gene probe method. The same signal intensity was obtained whether the virus was exposed to no chlorine or to 20mg/l chlorine (Table 1).

Table 1. Detection of EAds 40 & 41 using gene probes after exposure to chlorine*

Chlorine mg/ℓ	0	0,25	0,5	0,75	1,0	1,25	1,5	1,75
Gene probe	+	+	+	+	+	+	+	+
Chlorine mg/ℓ	2,0	2,5	5,0	7,5	10	15	20	
Gene probe	+	+	+	+	+	+	+	

* The same results were obtained for 15 minutes and 1 hour contact time with chlorine

DISCUSSION

Enteric Adenoviruses were detected in a high percentage of both raw and treated drinking waters. Concentrated water samples were tested directly using the gene probe method. As higher levels of EAds were found in treated water than in raw water the removal of inhibitory substances from raw water was examined. Inhibitory substances were successfully removed by pretreatment using either the cellulose fibre or the Geneclean™ method. It is therefore recommended to use either of these methods prior to the application of the gene probe method when testing environmental water samples.

Because of the relatively high percentage of drinking waters positive for EAd 40 and 41, further investigations were conducted exposing the viruses to chlorine concentrations ranging from 0-20mg/l with an exposure time of up to 1 hour. The viral DNA could still be detected even after exposure to such high doses of chlorine. Viability tests, using cell culture techniques were thus far not able to establish whether these viruses were still infectious. It is known that the enteric Adenoviruses are able to survive longer than other enteroviruses under certain stress conditions. Enriquez and Gerba (1994) found that the enteric Adenoviruses survive longer than poliovirus in sewage, tap water and seawater. The enteric Adenoviruses are double stranded DNA viruses enclosed in an icosahedral capsomere, which may contribute to their apparent resistance. As far as could be established, no information is available on the survival pattern of EAds 40 and 41 during chlorination. Published studies conducted on other waterborne viruses, examining survival patterns after chlorination have never shown viruses to be capable of surviving after exposure to such high concentrations of chlorine. The results of this study imply that the viruses are either extremely resistant to chlorine, or the viruses were inactivated, but the viral DNA remained detectable without the viruses being infectious. Moore and Margolin (1994) found similar results after exposure of poliovirus to chlorine, chlorine dioxide, ozone and ultraviolet radiation. They found that the viral nucleic acid was not degraded after disinfection and could still be detected using gene probes. Since EAds 40 and 41 are DNA viruses, the likelihood that their nucleic acids will not be degraded during chlorination is much higher than that of the nucleic acids of polioviruses, which are composed of RNA and are known to be more sensitive to degradation in comparison to DNA.

The results of this study demonstrate that gene probes used for the direct detection of EAds 40 and 41 are highly sensitive and can be used for the detection of EAds in environmental water samples. However the

viability of the enteric Adenoviruses detected in chlorinated water using this method is questionable and is being further addressed.

REFERENCES

Buitenwerf, J., Louwerens, J.J. and de Jong, J.C. (1985). A simple and rapid method for typing Adenoviruses 40 and 41 without cultivation. *J. Virol. Methods*, **10,** 38-44.

Cabelli, V.J. (1982). The potential for water related, pollution associated infectious disease as seen from bacterial monitoring data. *Intergovernmental oceanographic commission report*, **8.11,** 157-177.

Enriquez, C.E. and Gerba, CP. (1994). Inactivation of the enteric Adenoviruses 40 and 41 and poliovirus 1 in seawater, tap water and wastewater. Presented at the *94th ASM General Meeting,* May 1994, Los Vegas, USA.

Hurst, C.J., McClellan, K.A. and Benton, W.H. (1988). Comparison of cytopathogenicity, immunofluorescence and *in situ* hybridization as methods for the detection of Adenoviruses. *Wat. Res.* **22,** (12), 1547-1552.

Kidd, A.H., Rosenblatt, A., Besselaar, T.G., Erasmus, M.J., Tiemessen, C.T., Berkowitz, F.E. and Schoub, B.D. (1983). Characterization of rotaviruses and subgroup F Adenoviruses from acute summer gastroenteritis in South Africa. *National Institute for Virology and Baragwanath Hospital, Johannesburg, South Africa:* Document.

Kidd, A.H., Harley, E.H. and Erasmus, M.J. (1985). Specific detection and typing of adenovirus types 40 and 41 in stool specimens by dot-blot hybridization. *J Clin Microbiol,* **22,** 934-939.

Moore, N.J. and Margolin, A.B. (1994). The comparison of a digoxigenin DNA probe assay to cell culture for the detection of poliovirus in water samples treated with chlorine, chlorine dioxide, ozone, or ultraviolet radiation. Presented at the *94th ASM General Meeting,* 4 May 1994, Los Vegas, USA.

Nupen, E.M., Basson, N.C. and Grabow, W.O.K. (1980). Efficiency of ultrafiltration for the isolation of enteric viruses and coliphages from large volumes of water in studies on wastewater reclamation. *Prog. Water Technol.* **12** (2), 851-863.

Tiemessen, C.T. and Kidd, A.H. (1988). Helper function of adenovirus 2 for adenovirus 41 antigen synthesis in semi-permissive and non-permissive cells. *Arch. Virol.* **103,** 207-218.

Wilde, J., Eiden, J. and Yolken, R. (1990). Removal of inhibitory substances from human faecal specimens for the detection of group A rotaviruses by reverse transcriptase and polymerase chain reactions. *J. Clin. Microbiol.* **28** (6), 1300-1307.

Witt, D.J. and Bousquet, E.B. (1988). Comparison of enteric Adenovirus infection in various human cell lines. *J. Virol Methods*, **20,** 295-308.