CHAPTER 4

The editorial style of Water Research was followed in this chapter

4 DETECTION OF ENTEROVIRUSES IN TREATED DRINKING WATER

4.1 Abstract

This study deals with the routine monitoring of drinking water for the presence of enteroviruses, over a period of one year. A rapid and simple method was employed for the simultaneous detection and typing of enteroviruses in large-volume water samples. This included an integrated cell culture/nested PCR approach, followed by restriction enzyme analysis. The drinking water supplies were derived from acceptable quality surface water sources using treatment processes, which conform to international specifications for the production of safe drinking water. Enteroviruses (predominantly coxsackie B viruses) were detected in 11% - 16% of the drinking water samples. This study confirmed that acceptable water quality indicators do not necessarily reflect the virus content of drinking water.

4.2 Introduction

Routine surveillance of drinking water quality is essential for the protection of public health. The WHO Scientific Group has concluded that the presence of even a few enteric viruses in a large volume of drinking water poses a threat to public health (WHO, 1979). Early detection of viruses in drinking water samples will enable effective management of public water supplies and the implementation of appropriate preventive control measures.

Numerous studies have documented the presence of enteroviruses in raw and treated water (Keswick *et al.*, 1984; Gilgen *et al.*, 1995; Reynolds *et al.*, 1997; Abbaszadegan *et al.*, 1999; Grabow *et al.*, 2000). Enteroviruses are stable in the environment and are resistant to chlorine and UV disinfection (Payment, 1985; Bartigelli *et al.*, 1993). Enteroviruses are the most common agents of acute myocarditis and aseptic meningitis (Muir and Van Loon, 1997). Neonates are at particular risk of infection as well as other sensitive populations such as the eldery and immunocompromised. There is evidence that enteroviruses contribute to chronic diseases, including insulin dependant diabetes mellitus (Roivainen *et al.*, 1998) and dilated cardiomyopathy (Kandolf, 1999).

The standard method for detecting viruses by inoculation in cell culture require 6 – 10 days, thereby defeating the very purpose of analysis with respect to preventive measures. The PCR method can be used to enzymatically amplify nucleic acid sequences that are present in low copy numbers in water samples. The speed, specificity, low cost and ease of this procedure have led to its use in the detection of enteroviruses in water samples (Gilgen *et al.*, 1995; Reynolds *et al.*, 1997; Abbaszadegan *et al.*, 1999; Grabow *et al.*, 2000). However, the detection of the viral genome does not provide any information about the infectivity of the viruses. Numerous studies have described the use of a combined cell culture/PCR technique for the detection of infectious enteroviruses (Egger *et al* 1995; Reynolds *et al.*, 1997; Grabow *et al.*, 2000). Kuan (1997) has described the use of restriction enzyme (RE) analysis for the rapid identification of PCR positive samples. Restriction enzyme analysis yields valuable information on viruses circulating in the community and reduces the requirement for complete nucleotide sequencing.

This study deals with the routine monitoring of drinking water for the presence of enteroviruses, over a period of one year. The drinking water supplies were derived from acceptable quality surface water using treatment processes which conform to international specifications for the production of safe drinking water.

4.3 Materials and Methods

4.3.1 Virus Stocks

Enterovirus controls included: poliovirus 1 - 3, coxsackievirus B1 – B6, coxsackievirus A9 and echovirus 1. Viruses were clinical isolates, obtained from the National Institute of Virology (NIV), Johannesburg, South Africa. These different serotypes of enteroviruses were recovered in buffalo green monkey kidney cells (BGM cell line, passage 80 - 95). After demonstrating a complete cytopathic effect, the cultures were frozen and thawed three times. Debris were removed by centrifugation at 600 x g (Eppendorf Centrifuge 5415D) for 10 min at room temperature. The virus suspensions were stored at –70°C.

4.3.2 Drinking Water Supplies

A total of 172 samples of drinking water were collected weekly over a one year period. The supplies were derived from acceptable quality surface water sources using treatment processes, which conform to international specifications for the production of safe drinking water (WHO,

1996; WHO, 1997). Glass wool filters were used for the on-site and in-line recovery of viruses from 100 to 1 000 litre volumes of water (Vilaginès *et al.*, 1997).

4.3.3 Virus Recovery

The enteroviruses were concentrated by absorption-elution on glass wool (Saint Gobian, Isover-Orgel, France) (Grabow *et al.*, 1993; Vilaginès *et al.*, 1997). After filtration, viruses were eluted with 100 ml of a glycin-beef-extract buffer (0.05 M glycin; 0.5 % beef extract, pH 9). The eluting solution was left in contact with the glass wool for 5 min before being passed through the filter under pressure, where after the pH was adjusted to pH 7. A second concentration step was performed, using the polyethylene glycol (PEG 6000, Merck, Darmstadt) method (Minor, 1985). The resulting pellet was resuspended in 15 ml phosphate buffered saline (PBS) (Sigma Chemical Co.Louis, USA). The concentrates were treated with a penicillin/streptomycin fungizone mix (Bio Whittaker) for 30 min at room temperature before inoculation onto cell monolayers. The primary liver carcinoma cell line (PLC/PRF/5) and the buffalo green monkey kidney cell line (BGM) were used for the propagation of enteroviruses (Grabow *et al.*, 1999). Cells were grown to confluent monolayers in 25 cm^2 plastic flasks (Corning, USA). One millilitre of the final sample concentrate was inoculated onto each of two flasks and incubated at 37°C with 0.5% CO_2. Cells were passaged after 7 days and harvested after 14 days. Cells were investigated on a regular basis for any cytopathogenic effect. A total of 1.5 ml of suspended cells was centrifuged at 300 x g (Eppendorf centrifuge 5402D) for 5 min. The pellet was dissolved in the appropriate volume of buffer RLT (Rneasy Viral RNA extraction kit, Qiagen, Germany). The sample was homogenized by using a QIAshredder column (Qiagen, Germany). The lysate was pipetted directly onto a QIAshredder column and centrifuged for 2 min at 13 000 x g (Eppendorf Centrifuge 5415D).

4.3.4 RNA Extraction

Enteroviral RNA was extracted by means of a RNEasy Viral RNA extraction kit (RNEasy, Qiagen, Germany). The extraction was performed according to the manufacturer's instructions. A DNase step was included in the extraction to remove contaminating DNA from RNA preparations.

4.3.5 RT-PCR Amplification

A Promega Access RT-PCR System (Promega, USA) was used for the reverse transcription (RT) and PCR amplification of enteroviral RNA. A 50 µl reaction volume, containing the following was prepared: AMV/Tfl reaction buffer (5x), dNTP mix (final concentration of 0.2 mM) PCR primers EP1 and EP4 (50 pmol) (Table 4-1), 1.5 mM $MgSO_4$ and 5U of AMV Reverse Transcriptase and Tfl DNA polymerase. The reaction was incubated at 49°C for 45 min and then subjected to 30 amplification cycles consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C (Hybaid OmniGene Thermocycler). The final annealing step was performed for 7 min at 72°C.

4.3.6 Nested Amplification

A second PCR run was undertaken as described by Kuan (1997). Briefly 1 µl of the amplified product was added to 49 µl of the PCR mixture. The PCR mixture contained the following: 10 x PCR buffer (10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1% Triton X-100), $MgCl_2$ (final concentration of 1.5 mM), dNTP mix (final concentration of 0.2 mM), primers E1 and E2 (50 pmol each) (Table 4-1) and 1.5 U of Taq DNA polymerase. Cycling was carried out 35 times with denaturation at 94°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 1 min (Hybaid OmniGene thermocycler). The amplified products were separated using 2% agarose (Seakem LE Agarose, Bioproducts, USA) gel electrophoresis (Midicell Primo gel apparatus, Holbrook, New York).

4.3.7 Sabin Specific Triplex RT-PCR

A Sabin specific RT-PCR was described by Yang *et al.* (1991) to distinguish between poliovirus 1 to 3 (Sabin strains). Three sets of primers that were specific for Sabin strains were combined in a triplex RT-PCR (Table 4-1). Water samples that were positive for poliovirus were confirmed as vaccine strains with the Sabin specific RT-PCR. A 50 µl reaction volume containing the following was prepared: 5 x AMV/Tfl reaction buffer, dNTP mix (final concentration of 0.2 mM), PCR primers S1-1, S1-2, S2-1, S2-2, S3-1a, S3-2 (25 pmol of each) (Table 4-1), 1.5 mM $MgSO_4$ and 5 U of AMV reverse transcriptase and Tfl DNA polymerase (Promega, Madison, USA). The amplification was performed in 30 cycles in a Mini thermocycler (MJ Research, USA). The conditions were as follows: reverse transcription for 45 min at 42°C, denaturation for 30 sec at 95°C annealing for 45 sec at 56°C and extension for 1 min at 72°C.

4.3.8 Quantification of the Nested PCR

Sensitivities were determined by using enteroviral RNA extracted from virus culture supernatant of known titre. Viral RNA was extracted from 10-fold dilutions of poliovirus 1 infected culture supernatant as described previously (4.3.4). It was amplified using the single RT-PCR and the nested PCR amplification procedures. PCR products synthesized by single PCR and nested PCR were analysed by 2% agarose (Seakem LE Agarose, Bioproducts, USA) gel electrophoresis using a Midicell Primo gel apparatus (Holbrook, New York).

4.3.9 Restriction Enzyme Analysis

Aliquots of 10 µl of n-PCR products were incubated with 5U RE in a 30 µl reaction volume with the buffer recommended by the manufacturers (Promega, Madison, USA) (Kuan, 1997). Restriction enzymes used were: StyI, BglI and XmnI (Promega, Madison, USA). Samples were incubated at 37°C for 3 h and were analyzed with 7% polyacrylamide (BioRad, Hercules, CA) gel electrophoresis using a Hoefer electrophoresis unit (San Francisco, USA).

4.3.10 Quality Control of the Amplification Method

Standard precautions were applied in all the manipulations to reduce the possibility of sample contamination by amplified DNA molecules. Separate laboratories were used for reagents, treatment of samples, and manipulation of amplified fragments. Negative controls for cell culture, RNA extraction, RT-PCR and n-PCR were included in each assay.

4.4 Results and Discussion

Enterovirus reference strains were successfully amplified with both sets of primers, resulting in DNA bands of the expected sizes (Table 4-1). A high level of sensitivity was obtained with the n-PCR approach. The RT-PCR detected 10 plaque forming units (PFU) of PV1 in 140 µl volume of sample (Figure 4-1). The n-PCR increased the sensitivity to 10^{-2} PFU. Since it has been estimated that the ratio of virus particles to infectious units is between 100 and 1 000 for the enteroviruses (Rotbart, 1990, Severini *et al.*, 1993, Melnick, 1996), we concluded that the n-PCR allowed the detection of between 1 and 10 copies of enteroviral RNA per 140 µl volume of sample. These levels of sensitivity are in agreement with findings reported by Kämmerer *et al.* (1994) and Kuan (1997).

Figure 4-1: Gel electrophoresis of RT-PCR products from enteroviral RNA in suspensions which contained in tenfold dilutions 10^5 (lane 1) to 10 (lane 5) PFU of poliovirus 1 per ml. M: 100 bp molecular marker (Promega, Madison, USA).

A total of 172 samples were analysed over a one year period. Treated water samples were collected at 2 different treatment units designated A and B. Eleven percent (n=88) of treated water samples from treatment unit A were positive and 16% (n=84) of treated water samples from treatment unit B were positive for enteroviral RNA. All the drinking water supplies had heterotrophic plate counts of less than 100 cfu.ml^{-1}, total and faecal coliform counts of 0 cfu.100 ml^{-1}, and negative results in qualitative presence-absence tests for somatic and F-RNA coliphages on 500 ml samples. These results support earlier findings on shortcomings of conventional indicators for assessment of the virological quality of drinking water (Payment *et al.*, 1997; Grabow *et al.*, 2000). No cytopathogenic effect (CPE) was observed in the cell cultures. This shows that many enteroviruses in water supplies are still capable of infecting susceptible host cells and of replicating their RNA, but they have lost the ability to produce a CPE in the cell cultures and test conditions used. Similar findings have been reported by Seidel *et al.* (2000).

PCR products obtained from treated water samples were analysed with restriction enzyme analysis (RE). The n-PCR RE analysis enabled us to differentiate between various serotypes of prototypical enteroviruses. Most of the samples exhibited restriction enzyme patterns identical to those described by Kuan (1997) (Figure 4-2). Amongst the enteroviruses detected were polioviruses (12%) and coxsackie B viruses (88%) (Table 4-2). Coxsackie viruses B5 and B3 were the most abundant of the coxsackie B viruses. This is in agreement with results obtained by Payment *et al.* (1985a) and Hejkal *et al.* (1982). Polioviruses were confirmed to be poliovirus Sabin 1 using the Sabin-specific triplex RT-PCR.

Figure 4-2: Restriction enzyme digestion of the 297 bp product of enteroviruses detected in the treated drinking water. M: Molecular marker. Lane 1 – 9: StyI, BglI, XmnI digested PCR product.

4.5 Conclusions

The drinking water supplies analyzed in this study meet specifications for raw source, treatment, disinfection and quality in terms of indicator organisms (WHO, 1996; WHO, 1997). Microbiological data on the water supplies showed standard plate counts of less than 100 cfu.1 ml^{-1}, the absence of coliform bacteria per 100 ml samples, and negative results in presence-absence tests for somatic and F-RNA coliphages on 500 ml samples.

The results reported here confirm the presence of viable enteroviruses in these treated drinking water supplies. The viruses detected were evidently damaged to the extent that they failed to produce a CPE in the cell cultures used and under the experimental conditions applied. However, the ability of these viruses to infect susceptible host cells and to replicate their RNA, confirms that they are viable and infectious and therefore constitute a health risk (Reynolds *et al.*, 1997; Grabow *et al.*, 2000; Seidel *et al.*, 2000). The extent of this health risk is due to be investigated using the latest risk assessment models (Haas *et al.*, 1999). Other non-cytopathogenic viruses, including adeno, astro, and hepatitis A viruses have likewise been detected in these drinking water supplies (Grabow *et al.*, 2000; Taylor *et al.*, 2000). The detection of viruses in treated drinking water supplies has been recorded in many studies (Keswick *et al.*, 1984; Payment *et al.*, 1985b; Reynolds *et al.*, 1997; Seidel *et al.*, 2000).

Reasons for the survival of these viruses have not been investigated. Occlusion in protective material may play a role (Payment *et al.*, 1985a). The results showed that previously used methods for the virological analysis of drinking water, based on CPE in cell cultures (WHO, 1996; WHO, 1997; SABS, 1999; Grabow *et al.*, 2000), would fail to detect these viruses.

The detection of viruses in these treated drinking water supplies is in agreement with epidemiological data which associate substantial levels of enteric infections with drinking water supplies which meet all specifications for treatment, disinfection and counts of indicator organisms (Rose *et al.*, 1986; Bosch *et al.*, 1991; Payment *et al.*, 1997).

4.6 References

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Table 4-1: Primers used for the detection of enteroviral RNA in drinking water samples.

	Primer	Sequence	Product	Reference
RT-PCR	EP1 (64 – 83)	5'CGGTACCTTTGTGCGCCTGT3'		
	EP4 (459 – 478)	5'TTAGGATTAGCCGCATTCAG3'	414	Gow *et al.*, 1991
Nested PCR	E1 (166 – 182)	5'AAGCACTTCTGTTTCCC3'		
	E2 (447 – 463)	5'ATTCAGGGGCCGGAGGA3'	297	Kuan, 1997
Sabin specific RT-PCR	S1-1 (2584 - 2601)	5'TCCACTGGCTTCAGTGTT3'		
	S1-2 (2505 – 2523)	5'AGGTCAGATGCTTGAAAGC3'	97	Yang *et al.*, 1991
	S2-1 (2580 – 2595)	5'CGGCTTGTGTCCAGGC3'		
	S2-2 (2525 – 2544)	5'CCGTTGAAGGGATTACTAAA3'	71	Yang *et al.*, 1991
	S3-1a	5'AGTATCAGGTAAGCTATCC3'		
	S3-2 (2537 – 2553)	5'AGGGCGCCCTAACTTTG3'	54	Yang *et al.*, 1991

Table 4-2: Enteroviruses detected in treated drinking water.

	Water distribution site A		Water distributions site B	
Date	**n-PCR**	**Volume**	**n-PCR**	**Volume (B)**
1999-04-06	-	260	-	212
	-	260	-	58
1999-04-19	-	216	+ (PV1)	251
	-	216	-	155
1999-05-03	-	216	-	149
	-	216	+ CBV5	20
1999-05-17	-	260	-	310
	-	260	-	370
1999-05-24	-	216	-	72
	-	216	-	9.5
1999-05-31	-	260	-	142
	-	260	-	56-
1999-06-14	-	216	-	47
	-	216	-	43
1999-06-21	-	260	-	119
	-	260	-	119
1999-07-05	-	216	-	81
	-	216	-	25
1999-07-19	-	260	ND	ND
	-	260	ND	ND
1999-07-26	-	216	-	199
	-	216	-	29
1999-08-02	-	216	-	147
	-	216	-	86
1999-08-10	+ (PV1)	220	-	185
		220	+ (CBV5)	246
1999-08-16	-	160	-	144
	-	192	-	100
1999-08-23	-	360	-	260
	-	360	-	360
1999-08-30	-	120	-	347
	-	146	-	244
1999-09-06	-	200	-	244
	-	186		
1999-09-13	+ (CBV5)	260	+ (CBV5)	126
	-	260	-	373
1999-09-20	-	200	-	220
	-	126	-	200
1999-09-27	-	180	-	103

Table 4-2: Enteroviruses detected in treated drinking water. (continued)

	Water distribution site A		Water distributions site B	
Date	**n-PCR**	**Volume**	**n-PCR**	**Volume (B)**
1999-09-27	-	196	-	80
1999-10-04	-	246	-	280
	-	284	-	166
1999-10-11	+ polio 1	180	-	112
	-	166	+ (CBV5)	110
1999-10-18	+ CBV3	200	-	180
	-	200	-	140
1999-10-25	-	345		
	-	240		
1999-11-01	-	126	-	200
	-	200	+ (CBV3)	100
1999-11-08	-	186	-	251
	-	210	-	150
1999-11-15	-	145	-	100
	-	145	-	100
1999-11-22	-	210	-	220
	-	220	-	146
1999-11-29	-	134	-	134
	-	141	-	136
1999-12-06	-	404	-	135
1999-12-13	-	154	-	154
	-	150		
1999-12-20	-	144	-	80
	-	144	-	120
2000-01-10	ND	ND	-	142
	ND	ND	-	137
2000-01-17	-	148	-	137
	-	156	-	132
	-	146	-	143
2000-01-24	-	200	+ (CBV3)	180
	+ (CBV3)	200	+ (CBV3)	180
	+ (CBV3)	200		
2000-01-31	-	200	-	198
	-	200	-	85
2000-02-07	-	200	-	58
	-	180	-	204
2000-02-14	-	200	-	144
	-	220	-	205
2000-02-21	-	180	+ (CBV2)	149
	-	200	+ (CBV2)	261

Table 4-2: (continued) **Enteroviruses detected in treated drinking water.**

	Water distribution site A		Water distribution site A	
Date	**n-PCR**	**Volume**	**n-PCR**	**Volume (B)**
2000-02-28	+ (CBV3)	200	-	126
	-	190	+(CBV3)	243
2000-03-06	-	120	-	220
	-	120	-	220
2000-03-13	-	144	-	176
	-	140	+ (CBV3)	163
2000-03-20	+ (CBV5)	200	-	143
	-	200	-	181
2000-03-27	-	200	-	201
	-	200	-	200

CHAPTER 5

The editorial style of Water Research was followed in this chapter

5 ASSESSMENT OF THE RISK OF INFECTION ASSOCIATED WITH COXSACKIE B VIRUSES IN DRINKING WATER

5.1 Abstract

The risks of infection constituted by enteroviruses detected in the drinking water supplies analysed in this study were assessed. Coxsasckie B viruses (CBV) were used as model in these assessments. A high proportion of coxsackie B virus infections is asymptomatic. However, clinical manifestations may range from mild, undifferentiated febrile illness or upper respiratory tract infection to a severe, systemic and sometimes fatal disease of sensitive populations. Dose-response studies suggested that an exponential model best describes infectivity of CBV. The analysis of 172 samples of treated drinking water supplies described in this study (Chapter 4) revealed the presence of CBVs in 11% (water treatment unit A) and 16% (water treatment unit B) of the samples. This incidence of CBV was used as basis for risk assessment. The results indicated that the drinking water supplies concerned constitute a risk of CBV infection of 3.91 x 10^{-3} (unit A) and 7.4 x 10^{-3} (unit B) per year. The estimated risks of infection are about an order of magnitude higher than the yearly acceptable risk of one infection per 10 000 consumers proposed for drinking water supplies.

5.2 Introduction

The potential for human disease contracted from exposure to waterborne microorganisms is a growing public health concern. Drinking water has been recognised as a potential vector for the transmission of communicable diseases (Craun, 1991, Grabow, 1996, Payment, 1997). Although concentrations of pathogenic microorganisms may be quite low, drinking water reaches every member of a population, so that even a low risk of infection may affect a significant number of consumers. Although the mortality of many waterborne diseases is relatively low, the socio-economic impact of non-fatal infections is phenomenal (Payment, 1993).

The detection, quantification and characterization of pathogens in drinking water is essential for quantitative risk assessment and is now considered useful, if not yet essential, for monitoring the

quality and microbiological safety of source and treated waters (Haas *et al.*, 1999). These data are essential for the formulation of acceptable risks. The United States Environmental Protection Agency (US EPA) has recommended the following acceptable risk for drinking water: Not more than one infection per 10 000 consumers per year (Macler and Regli, 1993).

Coxsackie B virus infection may result in serious or even fatal disease such as acute myocarditis and aseptic meningitis (Muir and Van Loon, 1997). Neonates are at particular risk of infection as well as the eldery and immunocompromised. Hospitalization rates of 50 – 364 infants per 100 000 live births per year with a mortality of 3.9 per 100 000 live births per year have been reported (Kaplan *et al.*, 1988). There is evidence that enteroviruses contribute to common chronic diseases, including insulin dependant diabetes mellitus (Roivainen *et al.*, 1998) and dilated cardiomyopathy (Kandolf *et al.*, 1999).

Several studies have documented the presence of enteroviruses in raw and treated water (Keswick *et al.*, 1984; Gilgen *et al.*, 1995; Pallin *et al.*, 1997; Reynolds *et al.*, 1997; Abbaszadegan *et al.*, 1999; Grabow *et al.*, 2000). Enteroviruses in drinking water may pose a potential health risk to consumers.

The objective of this study was to assess the risks of infection constituted by CBVs, detected in the drinking water supplies analysed in this study. The quality of the raw surface water source, treatment by coagulation, sedimentation, filtration, and disinfection by chlorine, complied with international specifications for drinking water supplies (WHO, 1996; WHO, 1997). The drinking water supplies were monitored for a one year period for the presence of enteroviruses (Vivier *et al.*, 2000). Enteroviruses (predominantly coxsackie B viruses) were detected in 11% (water treatment unit A) and 16% (water treatment unit B) of the drinking water samples by an integrated cell culture/n-PCR approach (Chapter 4).

The risk assessment approach followed in this study involves four basic steps: (1) hazard identification; (2) dose-response assessment; (3) exposure assessment; and (4) risk characterization (NAS, 1983; Haas *et al.*, 1999). These steps were used to assess the risk of contracting a coxsackie B virus infection from the consumption of drinking water.

5.3 Hazard Identification

Coxsackie B viruses belong to the enterovirus group and are members of the family Picornaviridae (Melnick, 1996). There are six serotypes of these small nonenveloped, single-stranded RNA viruses (CBV1 – 6) (Melnick, 1996). In temperate climates, infections are

predominantly spread during the late summer-early fall, especially in young children (Cromwell and Landau, 1997). Coxsackieviruses are ubiquitous agents and can spread rapidly within the community causing epidemics (Moore, 1982; Pallansch, 1997). Although a high proportion of these virus infections are subclinical, presentation may range from mild, undifferentiated febrile illness or upper respiratory tract infection to a severe, systemic and sometimes fatal disease of neonates (Baboonian *et al.*, 1997). Reports have associated CBV infection with various diseases and syndromes. These associations are based on studies of outbreaks of enteroviral infections (Pallansch, 1997). Some are based on one or several clinical case discriptions associated with large numbers of people with similar symptoms who showed evidence of infection with the same serotype of CBV (Pallansch *et al.*, 1997). CBV infection has been ascribed in these studies as the cause of aseptic meningitis, encephalits, pleurodynia, myocarditis, and pericarditis (Pallansch, 1997). The most common outcome of CBV infection is asymptomatic infection, an undifferentiated febrile illness or mild upper respiratory symptoms (Pallansch, 1997).

Coxsackieviruses are transmitted by the faecal-oral route, thus exposure to contaminated food, water or fomites can result in acquisition of an infection (Melnick, 1996). Coxsackieviruses can be shed in the faeces as long as three months after acquiring an infection (Hirschman and Hammer, 1974). Numerous common source outbreaks of coxsackievirus have been documented including two documented waterborne outbreaks (Hawley *et al.*, 1973; Denis *et al.*, 1974). Coxsackieviruses have been found in raw sewage, recreational waters and drinking water (Lucena *et al.*, 1985; Payment *et al.*, 1985; Krikelis *et al.*, 1986; Dahling *et al.*, 1989). Coxsackieviruses are reported to be more resistant to chloramines and ultraviolet light disinfection than polioviruses (Payment *at al.*, 1985; Bartigelli *et al.*, 1993) and are persistent in the environment (Lo *et al.*, 1976). Lo *et al.* (1976) found that coxsackievirus type B5 was more stable than echovirus type 6 and poliovirus type 1 at any temperature.

5.4 Exposure Assessment

5.4.1 Drinking Water Supplies

Eleven (unit A) and sixteen (unit B) percent of drinking water samples (n = 172), monitored over a one year period, were positive for coxsackie B viruses (Vivier *et al.*, 2000). The supplies were derived from acceptable quality surface water sources using treatment processes which conform to international specifications for the production of safe drinking water (WHO, 1996; WHO, 1997). The water treatment units supply water to approximately 10 million consumers in South Africa (Grabow *et al.*, 2000).

5.4.2 Exposure Analysis

The exposure analysis was based on: (1) concentration of CBV in the treated drinking water, (2) recovery of the detection procedure, (3) the viability of the organisms and (3) the average volume of treated water consumed per individual. The daily exposure (N) was determined using the following equation (Teunis *et al.*, 1997; Haas *et al.*, 1999):

$$N = C \times {}^{1}/_{R} \times I \times 10^{-DR} \times Vc \qquad (5.1)$$

C = concentration of pathogenic micro-organisms (CBV) in treated drinking water

R = Recovery of the detection method

I = Fraction of the detected pathogens that is capable of infection (viability)

DR = removal or inactivation efficiency of the treatment process (DR = 0 when concentrations in drinking water are used)

Vc = Daily individual consumption of unboiled drinking water

5.4.3 Concentration

The polymerase chain reaction gave qualitative and not quantitative values. In order to determine the concentration of coxackieviruses in treated drinking water quantitatively, we assumed a random distribution of organisms within and between samples, which is described by a Poisson distribution (Havelaar, personal communication). The Poisson parameter λ was determined for water treatment units A and B (Table 5-1). Due to the uncertainty associated with the sampling, a random number of possible samples was simulated by means of bootstrapping (@RISK, version4, Pallisade Corporation, 2000). This simulates another possible test result in any volume of the original sample, using the concentrations estimated in Table 5-1. The distribution of 1 000 bootstrap replications of concentrations is given in Figure 5-1 and Figure 5-2. The concentration data for units A and B have been fitted with a normal and log logistic distribution, using the Chi-square test. The mean concentration of coxsackie B viruses was 4.67×10^{-4} L^{-1} for unit A and 8.90×10^{-4} L^{-1} for unit B (Table 5-2).

5.4.4 Recovery of the Detection Method

The efficiency (recovery) of a detection method is the fraction of the pathogenic microorganisms detected by the method used. On the basis of published data (Vilaginès *et al.*, 1997) and unpublished findings in our laboratory the recovery of the glass wool adsorption-elution method used in this study was conservatively estimated as 40%.

5.4.5 Viability

Test samples were inoculated onto cell cultures prior to RT-PCR screening to amplify viral nucleic acid. This amplification of viral nucleic acid gives an indication of the viability and infectivity of viruses because in vivo amplification of nucleic acid requires infection of host cells and activation of the replication cycle (Egger *et al.*, 1995; Reynolds *et al.*, 1997; Grabow *et al.*, 2000; Seidel *et al.*, 2000). Only viral capsids are known to attach to glass wool and there is no evidence that naked nucleic acid sequences are recovered by the glass wool adsorption-elution procedure (Grabow *et al.*, 2000). Furthermore, naked RNA is rapidly degraded and would not survive in the environment (Pallin *et al.*, 1997). The viruses detected in this study are therefore considered viable and infectious.

5.4.6 Consumption

As a default number, 2 L per person per day was used in this study to estimate drinking water exposure. This value (2L/person) was used in studies conducted in the USA (Haas, 1993; Macler and Regli, 1993). A survey conducted in the greater Cape Town area (analysed by sex, age, population group, income and season) found differences in water consumption between population groups (Bourne *et al.*, 1987). The average water consumption for the one population group was 2.19 L per day and for the other population group 1.26 L per day (Bourne *et al.*, 1987). The assumption of 2 L per day represents the higher consumption rate of the population groups and will therefore not underestimate risks (Genthe and Rodda, 1999).

5.5 Hazard Characterization

The exponential risk assessment model was used (Haas, 1983) to estimate the daily risk of infection related to the daily ingested dose of coxsakie B viruses:

$$Pi/day = 1 - \exp(-rN) \qquad (5.2)$$

where, Pi = probability of becoming infected

N = number of organisms

r = dose response parameter

The naïve estimate for the annual risk was simply estimated as:

$$P_{\aleph} = 1-(1-Pi/day)^{\aleph} \qquad (5.3)$$

where, P_{χ} = probability (risk) of one or more infections over period χ

χ = number of days of exposure

P_i/day = daily risk

5.5.1 Estimated Risk of Infection, Morbidity and Mortality

The dose response parameter r (7.75×10^{-3}) used in this study was estimated based on dose response experiments with coxsackievirus (type B4 and A21 strains pooled) (Suptel, 1963; Crabtree *et al.*, 1995; Haas *et al.*, 1999). The probability of becoming ill from that infection as well as the probability of mortality was determined. The probability of clinical illness was calculated by multiplying P_i (the probability of infection) by the morbidity rate of 0.75 reported for coxsackievirus (Cherry, 1981). The probability of death from an infection was calculated by multiplying P_i x morbidity rate (0.75) x case/fatality rate. The mortality rate used in this risk assessment was 0.0059 (Gerba and Haas, 1988). Point estimates for the daily risk and yearly risk of infection as well as the risks for morbidity and mortality for both treatment units are presented in Table 5-5. The daily risks of infection from CBV posed by drinking water from units A and B are 1.81×10^{-5} and 3.45×10^{-5} respectively. These daily risks of infection are two logs higher than the proposed acceptable daily risk of 2.7×10^{-7} per person (Haas *et al.*, 1999). The yearly risk estimates from treatment units A and B were 6.59×10^{-3} and 1.25×10^{-2} respectively. This is considerably higher than the mean yearly risk of 10^{-4} recommended by the EPA for treated drinking water (Macler and Regli, 1993).

5.5.2 Uncertainty Analysis

Point estimates of risk do not reveal the degree of uncertainty in the risk estimate. There are two kinds of uncertainty associated with risk. The first is the uncertainty associated with the sample

data and the second is the uncertainty associated with the model parameters (Haas *et al.*, 1999; Teunis *et al.*, 1999).

Uncertainty in the data and model parameters are quantified by a probability distribution, which yields the probability of the whole range of data values. If the probability distribution is identified, then a large number of samples or outcomes can be generated from the distribution. This method is known as the Monte Carlo simulation (Haas *et al.*, 1999). In this technique a set of random samples for water consumption, virus concentration and the dose response parameter is obtained. Individual calculations using the sets of random samples are combined to reveal an estimated distribution of risk (Teunis *et al.*, 1999; Haas *et al.*, 1999).

Using the distributions of various parameters in Table 5-4, the variation in risk of infection was estimated by sampling from the distributions for each of the factors (concentration and drinking water consumption). The Latin hypercube sampling method was employed in a simulation run of 1 000 model iterations. Summary statistics on 1 000 iterations of the Monte Carlo model are shown in Table 5-6. Figure 5-3 and Figure 5-4 show the distributions for single (daily) exposure to coxsackie viruses from distribution units A and B. The risk of infection from units A and B followed a lognorm2 and gamma distribution respectively. The mean individual daily risk for treatment units A and B were 1.02×10^{-5} and 1.95×10^{-5} respectively (Table 5-6).

The naïve estimate for the annual risk for units A and B was 6.59×10^{-3} and 1.25×10^{-2} respectively. This assumes that for a given individual, the daily risk is constant throughout the year. A more realistic simulation is to estimate 365 independent values. The yearly risk was calculated as a product of all estimated daily risks over a one year period (Havelaar, personal communication). The yearly risk of CBV infection from unit A was 3.91×10^{-3} and from unit B 7.4×10^{-3}.

5.6 Sensitivity Analysis

The purpose of the sensitivity analysis is to demonstrate the model's response to variation in certain input parameters. The effect on the model output can be determined by varying one or more parameter values over a range of likely values. A sensitivity analysis was conducted to determine the influence of variation in recovery, consumption and dose-response values on the model output. Figure 5-7 shows the effect of the variation of recovery efficiency (R), ranging from 20% - 80%, on the estimate of probability of infection per year. The influence is

exponential and the effect of a R value less than 50% has an important influence on the model output (Figure 5-7).

Figure 5-8 shows that drinking water consumption (C) has a marked effect on the model output value. Drinking water consumption values ranged from 0.5 – 2.0 L per day. The probability of infection per year increased significantly at higher values of C. The effect of the dose-response parameter (r) on the output value (Pinf/year) is presented in Figure 5-9. The probability of infection per year increased linear with the dose-response parameter (r).

5.7 Discussion

The risk of infection associated with exposure to coxsackie B viruses in two drinking water supplies has been outlined in this chapter. The estimated risks of infection of 3.91×10^{-3} (unit A) and 7.4×10^{-3} (unit B) (determined with the probabilistic model) were an order of magnitude higher than the acceptable risk of one infection per 10 000 consumers per year proposed by the US EPA (Macler and Regli, 1993). Risks of infection constituted by enteroviruses, which exceed this acceptable risk, have also been reported for other drinking water supplies. Crabtree *et al.* (1995) calculated that coxsackieviruses in drinking water supplies in the USA and Canada constituted a risk of infection of 2.55×10^{-4} (CBV concentration of 5×10^{-5} PFU/L) and 7.30×10^{-1} (CBV concentration of 0.31 PFU/L) per year. Previously reported data on the concentrations of coxsackieviruses in drinking water (Hejkal *et al.*, 1982; Payment *et al.*, 1985) were used in their assessment. The results of risk assessment carried out on a drinking water supply in South Africa indicated that the risk of enterovirus infection was as high as 4×10^{-1} (one infection per 40 consumers) per year (Genthe *et al.*, 1999).

Both deterministic and probabilistic models were used for the estimation of the risk of infection in this study. The purpose of the probabilistic model was to quantify the uncertainty associated with the risk of infection by coxsackie B viruses. Variability in recovery efficiency, viability and dose-response was not accounted for, because of lack of data, but can be included to refine the risk assessment model, if the data become available. Lack of information on the dose-response parameters is a major shortcoming. A deterministic dose response parameter, which does not reflect variability has been used. To obtain data that could be used for the purpose of predicting the probability of infection with low numbers of viruses, large numbers of individuals would have to be exposed to a highly virulent microorganism. Even if such experiments could be done, there would still be a great deal of uncertainty when extrapolating dose-response curves to

low exposure levels. Furthermore, it is uncertain that a pool of volunteers would reliably reflect the distribution of susceptibilities characterising the population as a whole (Gerba *et al.*, 1988).

The numbers of viruses, including entero and CBV, detected in the drinking water supplies analysed in this study (Chapter 4), exceed limits recommended for drinking water supplies (WHO, 1996; WHO, 1997; SABS, 1999; Grabow et al., 2000). Calculations carried out in this study indicated that the risk of infection constituted by the CBVs in these drinking water supplies exceeds acceptable risks. These findings warrant further investigation. This would include further details on the variables which affect the accuracy of risk assessment. More accurate data on the risk of infection constituted by the viruses in the drinking water supplies concerned would facilitate decisions about upgrading the treatment and disinfection processes to obtain levels of viruses within acceptable risks of infection.

Sensitivity analysis indicated that variation in the input parameters (recovery, drinking water consumption and dose-response) have a significant influence on the model output value. Dose-response data for CBV-infection used are based on experiments carried out on healthy, normal individuals (Gerba *et al.*, 1996). However, a substantial component of consumer populations may be more susceptible to infection as well as at risk to develop clinical illness (Gerba *et al.*, 1996). These highly susceptible individuals would include the very young, the eldery, undernourished individuals, pregnant women and immuno-compromised people notably organ transplant-, cancer- and AIDS patients (Gerba *et al.*, 1996). In many parts of the world this highly susceptible component of populations would appear to increase (Gerba *et al.*, 1996). This would include South Africa, where an exceptionally high incidence of HIV infection in many communities (Editorial, 1999) may increase dose-response figures. An increase in the dose-response parameter would increase the risk of infection constituted by CBVs in drinking water supplies.

Revision of some other parameters may decrease the risk of infection to meaningful extent. For instance, the efficiency of recovery (EOR) of the glass wool adsorption-elution method used for the recovery of viruses from drinking water supplies has conservatively been estimated as 40%. If the EOR were 80% (Vilaginès *et al.*, 1997), the risk of infection constituted by the CBVs in the drinking water supplies concerned would be substantially lower. Likewise, all the CBVs detected in this study were considered infectious. If this was incorrect, and some of the viruses were not capable of infecting humans, the risk of infection constituted by the CBVs in the drinking water supplies concerned would be reduced. The volume of unboiled water consumed daily would affect the risk of infection. Consumption of less than 2.0 litres per day used in the risk

assessment, would reduce the risk of infection and consumption of more than the 2.0 litres per day would increase the risk of infection.

In view of available information, the figures used for the above variables tended to be rather conservative, which implies that the actual risk of infection constituted by the water supplies may actually be lower than indicated. Therefore more detailed experiments need to be conducted on recovery efficiency of the glass wool adsorption-elution procedure and drinking water consumption in South Africa to determine these parameters more accurately.

Risk assessment can be expanded to give an indication of economic impact, which is determined by integrated measures of health, such as Disability Adjusted Life Years (DALY) (Havelaar *et al.* 1999). The loss in healthy life years in a population is measured in DALY's and weighed with a factor between 0 and 1 for the severity of the disability. This assessment requires more data and is outside the scope of this investigation.

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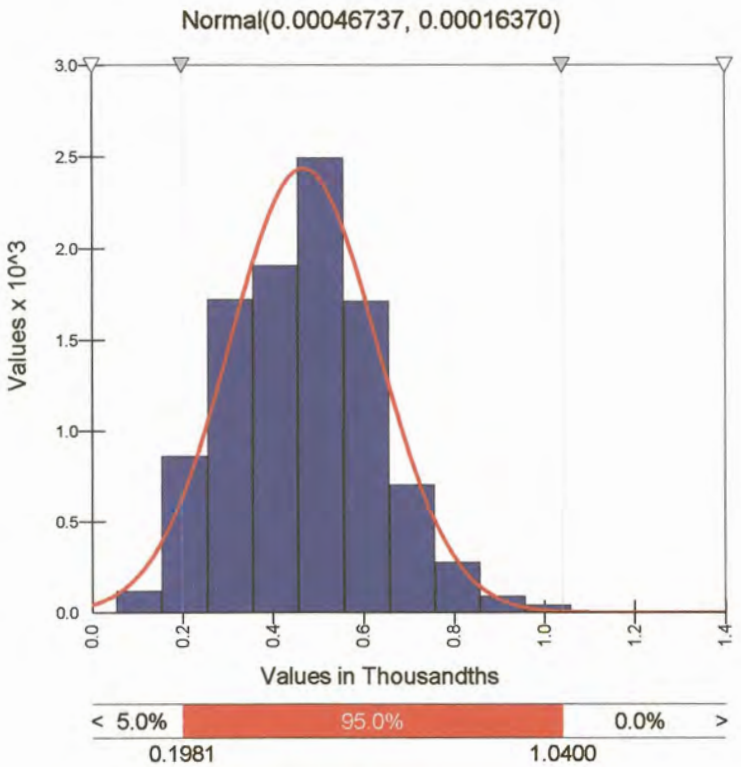

Figure 5-1: Probability distribution on bootstrap values for enterovirus concentrations in water samples from unit A.

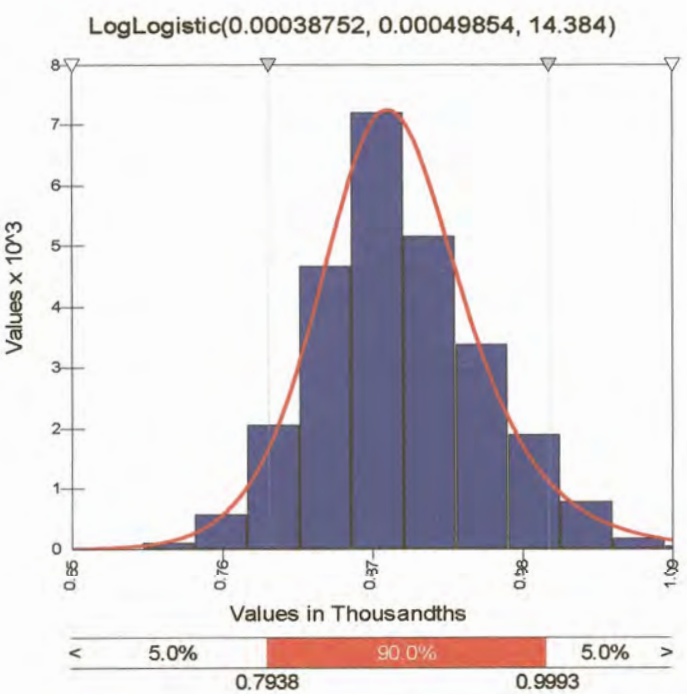

Figure 5-2: Probability distribution on bootstrapped values for enterovirus concentrations in water samples from unit B.

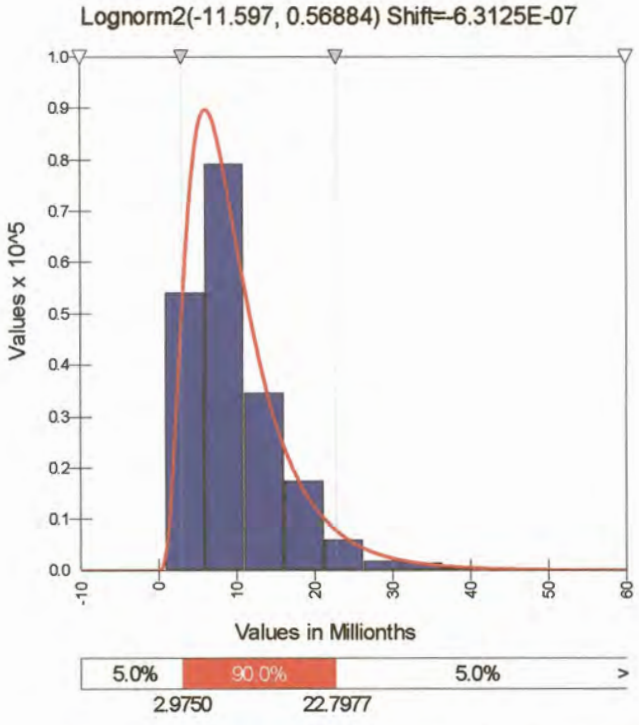

Figure 5-3: Distribution of Probability of infection/day from water treatment unit A.

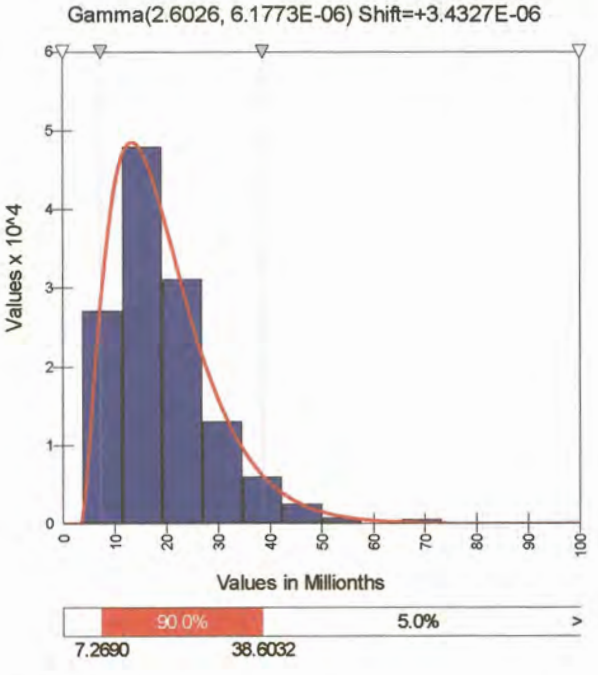

Figure 5-4: Distribution of Probability of infection/day from water treatment unit B.

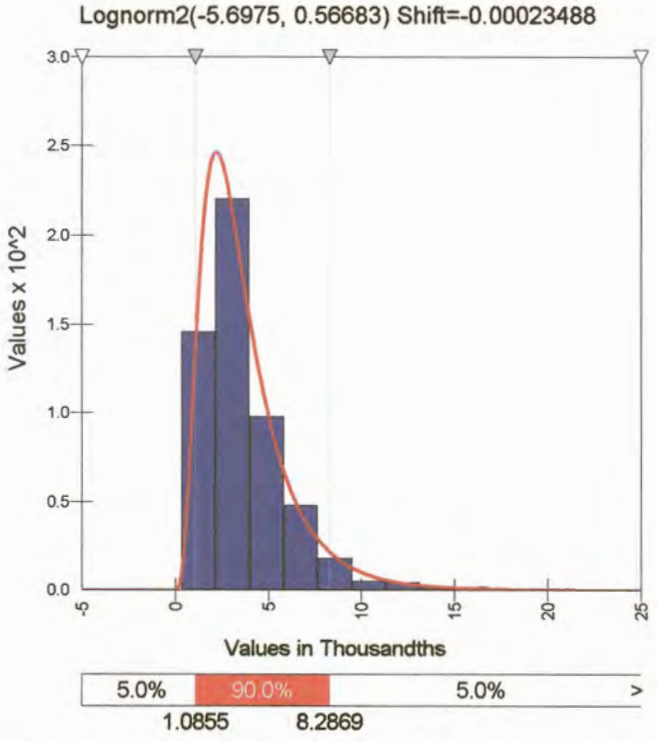

Figure 5-5: Distribution of Probability of infection/year from water treatment unit A.

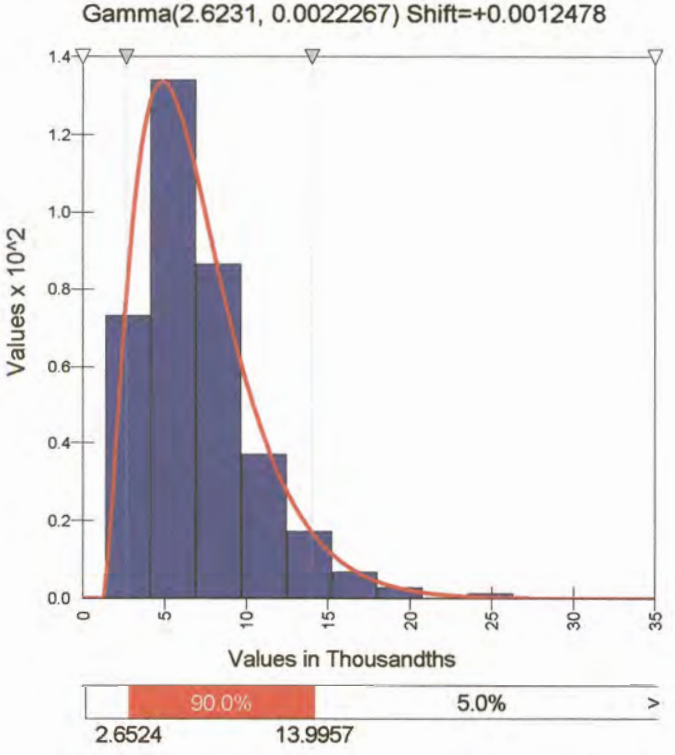

Figure 5-6: Distribution of Probability of infection/year from water treatment unit B.

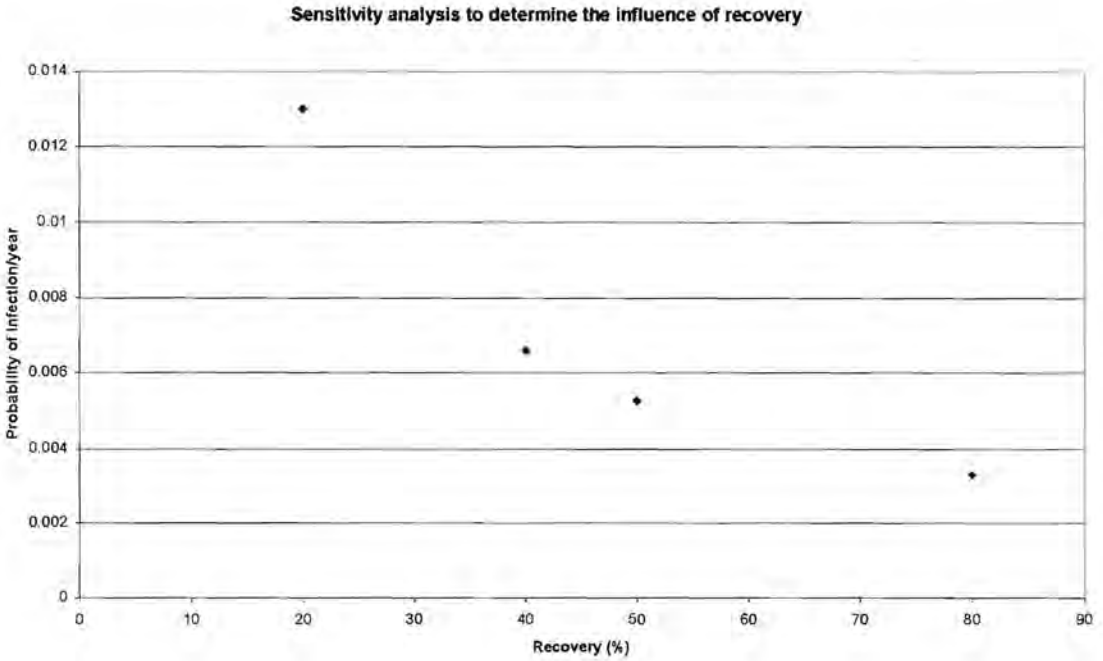

Figure 5-7: **Sensitivity analysis to determine the influence of recovery efficiency on the annual risk of infection (deterministic model).**

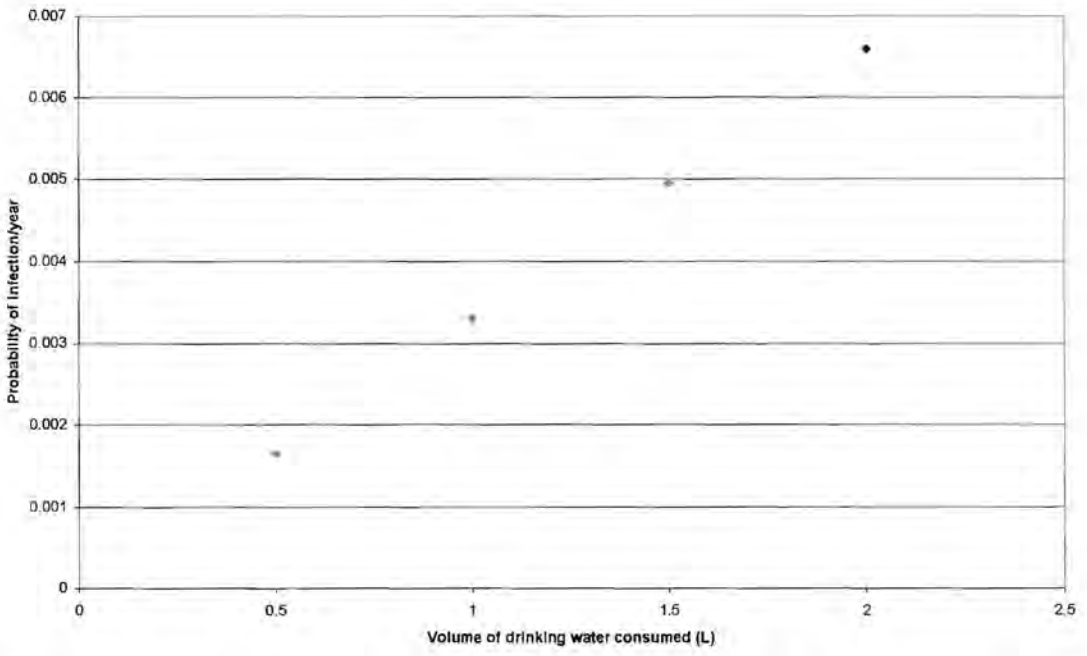

Figure 5-8: **Sensitivity analysis to determine the influence of consumption on the annual risk of infection (deterministic model).**

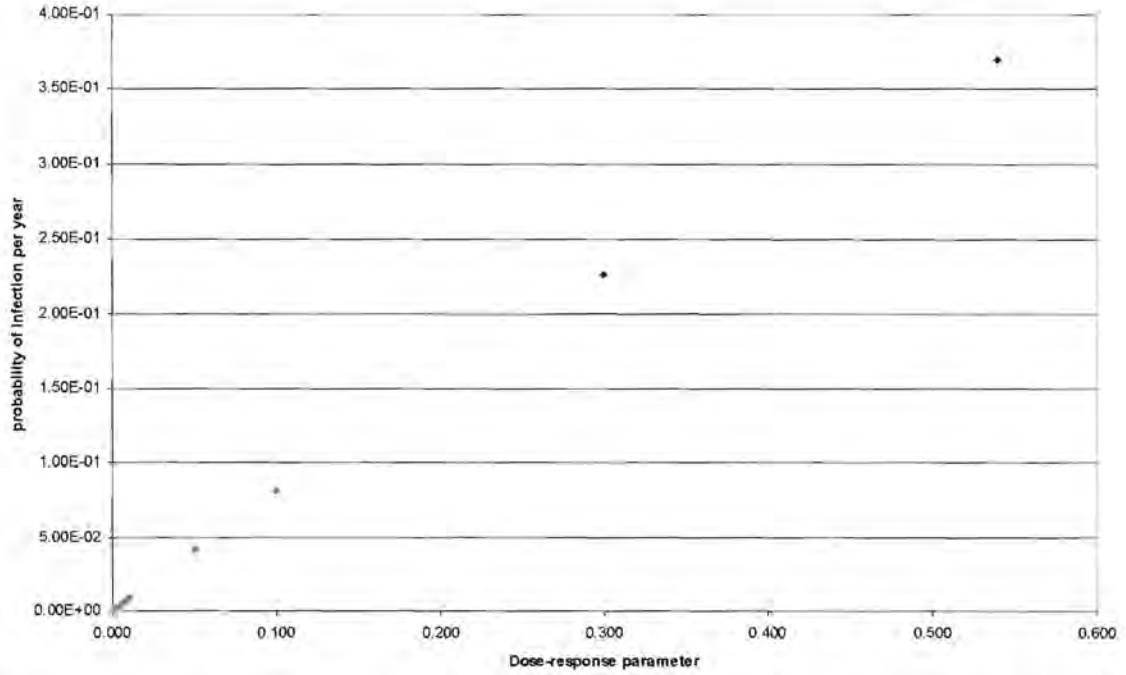

Figure 5-9: Sensitivity analysis to determine the influence of the dose response parameter (r) on the annual risk of infection (deterministic model).

Table 5-1: Determination of the poisson parameter and concentration for water from treatment units A and B.

	Equation	A	B
Poisson parameter (λ)	$\lambda = -\ln[P(0)]$	0.0953	1.422
Mean Volume (V)		204.3523	162.5723
Concentration (C)	$C = \lambda / V$	0.0005	0.0009

Table 5-2: Summary of Monte Carlo trials. Probability distribution of bootstrap values for enterovirus concentrations in water treatment units A and B.

Statistic	Enterovirus Concentration (unit A)	Enterovirus Concentration (unit B)
Mean	4.67×10^{-4}	8.9×10^{-4}
Median	4.67×10^{-4}	8.86×10^{-4}
Standard Deviation	1.63×10^{-4}	6.398×10^{-5}
Lower 95% Confidence Limit	2.00×10^{-4}	7.937×10^{-4}
Upper 95% Confidence Limit	1.00×10^{-3}	9.993×10^{-4}

Table 5-3: Model parameters used in the deterministic model to estimate risk associated with water consumption from treatment units A and B.

Model Parameters	Mean Value (A)	Mean Value (B)	Dimension
Concentration in drinking water (C)	4.67×10^{-4}	8.90×10^{-4}	Viruses/L
Recovery (R)	4.00×10^{-1}	4.00×10^{-1}	
Infectivity (I)	1	1	
Decimal Reduction by Treatment (DR)	NA	NA	
Volume Consumed (Vc)	2	2	L/day
Dose Response Parameter (CBV4)	7.75×10^{-3}	7.75×10^{-3}	

Table 5-4: Model parameters used in the probabilistic model to estimate risk associated with water consumption from treatment units A and B.

Model Parameters	Median Value		Probability distribution	
	Unit A	Unit B	Unit A	Unit B
Concentration in drinking water (C)	4.67×10^{-4}	8.86×10^{-4}	Bootstrap	Bootstrap
Recovery (R)	0.4	0.4	Fixed	Fixed
Infectivity (I)	1	1	Fixed	Fixed
Decimal Reduction by Treatment (DR)	NA	NA	NA	NA
Volume Consumed (Vc)*	1.13	1.13	Lognorm2 (0, 0,5)	Lognorm2 (0, 0.5)
Dose Response Parameter (CBV4)	7.75×10^{-3}	7.75×10^{-3}	Fixed	Fixed

* (Havelaar, personal communication)

Table 5-5: Risks associated with Coxsackieviruses in Drinking Water (Deterministic Model)

	Treatment unit A		Treatment unit B	
	Day	Year	Day	Year
Probability of infection	1.81×10^{-5}	6.59×10^{-3}	3.45×10^{-5}	1.25×10^{-2}
Probability of illness	1.36×10^{-5}	4.94×10^{-3}	3.59×10^{-5}	3.38×10^{-3}
Probability of death	8.02×10^{-8}	2.91×10^{-5}	1.53×10^{-7}	5.53×10^{-5}

Table 5-6: Summary of Monte Carlo Trials. Daily and yearly risks of Coxsackie B virus infection (Probabilistic Model).

	Treatment unit A		Treatment unit B	
Statistic	**Individual daily risk**	**Individual yearly risk**	**Individual daily risk**	**Individual yearly risk**
Mean	1.02×10^{-5}	3.7×10^{-3}	1.95×10^{-5}	7.01×10^{-3}
Median	8.56×10^{-6}	3.12×10^{-3}	1.75×10^{-5}	6.37×10^{-3}
Standard Deviation	6.68×10^{-6}	2.42×10^{-3}	9.96×10^{-6}	3.61×10^{-3}
Lower 95% Confidence Limit	2.28×10^{-5}	8.3×10^{-3}	3.86×10^{-6}	13.99×10^{-3}
Upper 95% Confidence Limit	2.975×10^{-6}	1.08×10^{-3}	7.26×10^{-6}	2.65×10^{-3}

CHAPTER 6

The editorial style of Clinical Virology was followed in this chapter

6 DETECTION AND TYPING OF ENTEROVIRUSES IN CLINICAL SPECIMENS

6.1 Abstract

Enterovirus (EV) infection, the most common cause of aseptic meningitis can be rapidly diagnosed with an EV-specific reverse transcriptase polymerase chain reaction (RT-PCR) method. This assay was compared with virus isolation in cell culture. A panel of 129 stool specimens, 71 CSF specimens and 19 specimens, which included blister fluid, throat swabs and brain tissue, were assayed for enteroviral RNA. Enteroviruses were detected in 32% of culture negative specimens, mostly in children less than 14 years of age (78%). Nonpolio enterovirus serotypes detected were CBV1 (8%), CBV2 (6%), CBV3 (22%), CBV5 (11%), ECV7 (8%), ECV9 (6%), ECV11 (6%) and untypeable enteroviruses (19%). A positive EV-PCR result may affect clinical decision making and can promote rapid discharge of patients from hospital.

6.2 Introduction

The enteroviruses comprise a large genus belonging to the picornaviridae. Sixty-six immunologically distinct enterovirus serotypes are known to cause infections in humans (Melnick, 1996). The enterovirus group includes polioviruses (PV), the cause of paralytic poliomyelitis, which results in significant disability in many parts of the world (Izurieta *et al.*, 1997). Worldwide eradication of PV seems within reach, following a World Health Organization (WHO) effort to eradicate the virus by active immunization (Hull *et al.*, 1994). Although polio may be a "submerging" infectious disease, there are other members of the family that are emerging (Pollard and Dobson, 2000). Approximately 80% - 92% of aseptic meningitis cases for which an etiologic agent is identified are caused by enteroviruses (Ramers *et al.*, 2000). Some serotypes may lead to serious clinical syndromes, including encephalitis, acute paralysis, sepsis-like syndrome in infancy, myocarditis and chronic infection in immunocompromised persons (Pollard and Dobson, 2000). There is evidence that enteroviruses contribute to common chronic diseases, including dilated cardiomyopathy (Baboonian *et al.*, 1997; Kandolf *et al.*, 1999), insulin-dependant diabetes mellitus (Roivainen *et al.*, 1998), and chronic fatigue syndrome (Gow *et al.*, 1991).

Clinical presentation of enterovirus meningitis is difficult to distinguish from meningitis due to bacteria, fungi or other viruses, resulting in unnecessary hospitalization and inappropriate treatment. Therefore specific and rapid diagnosis of enterovirus meningitis appears to be of paramount importance for patient management (Chonmaitree *et al.*, 1989). Attempts to isolate enteroviruses from cerebrospinal fluid (CSF) are laborious, time-consuming and are frequently unsuccessful (Chonmaitree *et al.*, 1988). Results of viral cultures can take 5 to 8 days and are frequently not available before discharge of the patient from hospital.

Enteroviruses are now increasingly being detected by PCR rather than by cell culture (Hyypia *et al.*, 1989; DeLeon *et al.*, 1990; Rotbart, 1990, Zoll *et al.*, 1992, Muir *et al.*, 1993). PCR-based assays could shorten hospital stays and reduce antibiotic therapy, thus decreasing overall costs (Sawayer *et al.*, 1994; Yearly *et al.*, 1996; Ahmed *et al.*, 1997). The main disadvantage of PCR based diagnosis is the loss of epidemiological information. An alternative means of enterovirus typing, employing PCR in conjunction with molecular techniques such as nucleotide sequencing (Oberste *et al.*, 1999), restriction enzyme analysis (Kuan, 1997) or nucleic acid hybridisation (Chapman *et al.*, 1990) may overcome some of the problems associated with serotyping and would provide additional information regarding the epidemiology and biological properties of enteroviruses.

The objective of this study was to evaluate RT-PCR followed by RE analysis as an alternative method to cell culture for the detection and serotyping of enteroviruses in clinical specimens.

6.3 Materials and Methods

6.3.1 Virus Stocks

Enterovirus controls included: poliovirus 1 - 3, coxsackievirus B1 – B6, coxsackievirus A9, CAV19, CAV22 and echovirus 1. Viruses were clinical isolates, obtained from the National Institute of Virology (NIV), Johannesburg, South Africa. These different serotypes of enteroviruses were recovered in buffalo green monkey kidney cells (BGM cell line, BioWhittaker, passage 80 - 95). After demonstrating a complete cytopathic effect, the cultures were frozen and thawed three times. Debris were removed by centrifugation at 600 x g (Eppendorf Centrifuge 5415D) for 10 min at room temperature. The virus suspensions were stored at –70°C.

6.3.2 RNA Extraction

6.3.2.1 Cerebrospinal Fluid Samples

Trizol (Gibci BRL), QiaAmp Viral RNA extraction kit (QiaAmp, Hilden, Germany), Dyna Bead RNA extraction (mRNA direct kit, Dynal, Norway) and heat inactivation (96°C for 10 min) procedures were compared for the extraction of enteroviral RNA in cerebrospinal fluid samples. Nine hundred microlitres of an enterovirus negative cerebrospinal fluid sample were seeded with 100 µl of a 10^5 PFU.ml^{-1} stock of poliovirus 1. One hundred and forty microlitres of the seeded sample was used for each assay.

6.3.2.2 Muscle Biopsies

Newborne mice were inoculated intraperitoneally with CAV19 and CAV22 suspensions. Mice were killed 7 days after inoculation. Heart and brain specimens were collected aseptically and stored at –70°C for nucleic acid extraction. Total RNA from the brain and heart muscle specimens were isolated with the Trizol (Gibco, BRL) and Rneasy (Qiagen, Hilden, Germany) techniques respectively.

6.3.3 Clinical Specimens

Clinical specimens were obtained from the specimen collection at the Department of Medical Virology, University of Pretoria, South Africa. Four hundred and eighty four assorted specimens (stools, cerebrospinal fluid, throat swabs, blister fluid and heart biopsy tissue) from patients with a suspected enterovirus infection were send to the laboratory from January 1997 to December 1998 for virus isolation. Archival specimens (stored at –20°C) were subsequently tested using a reverse transcriptase polymerase chain reaction (RT-PCR) method.

RNA was extracted from CSF specimens, throat swabs, blister fluid and heart muscle biopsy with the Trizol reagent (Gibco, BRL). The extraction was performed according to the manufacturer's instructions. The QiaAmp viral RNA extraction kit (QiaAmp, Hilden, Germay) was used to extract RNA from 10% stool suspensions. Stools were treated with an equal volume of 1.1.2-trichloro-trifluoro-ethane (Signma, St Louis, USA), prior to RNA extraction.

6.3.4 RT-PCR Amplification

A Promega Access RT-PCR System (Promega, USA) was used for the reverse transcription (RT) and PCR amplification of enteroviral RNA. A 50 µl reaction volume, containing the following was prepared: AMV/Tfl reaction buffer (5x), dNTP mix (final concentration of 0.2 mM) PCR primers EP1 and EP4 (50 pmol) (Table 6-1), 1.5 mM $MgSO_4$ and 5U of AMV Reverse Transcriptase and Tfl DNA polymerase. The reaction was incubated at 49°C for 45 min and then subjected to 30 amplification cycles consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C (Hybaid OmniGene Thermocycler). The final annealing step was performed for 7 min at 72°C.

6.3.5 Nested Amplification

A second PCR run was undertaken as described by Kuan (1997). Briefly 1 µl of the amplified product was added to 49 µl of the PCR mixture. The PCR mixture contained the following: 10 x PCR buffer (10 mM Tris-HCl, pH 9; 50 mM KCl; 0.1% Triton X-100), $MgCl_2$ (final concentration of 1.5 mM), dNTP mix (final concentration of 0.2 mM), primers E1 and E2 (50 pmol each) (Table 6-1) and 1.5 U of Taq DNA polymerase. Cycling was carried out 35 times with denaturation at 94°C for 1 min, annealing at 45°C for 1 min and extension at 72°C for 1 min (Hybaid OmniGene thermocycler). The amplified products were separated using 2% agarose (Seakem LE Agarose, Bioproducts, USA) gel electrophoresis (Midicell Primo gel apparatus, Holbrook, New York).

6.3.6 Sabin Specific Triplex RT-PCR

A Sabin specific RT-PCR was described by Yang *et al.* (1991) to distinguish between poliovirus 1 to 3 (Sabin strains). Three sets of primers that were specific for Sabin strains were combined in a triplex RT-PCR (Table 6-1). Clinical specimens that were positive for poliovirus were confirmed as vaccine strains with the Sabin specific RT-PCR. A 50 µl reaction volume containing the following was prepared: 5 x AMV/Tfl reaction buffer, dNTP mix (final concentration of 0.2 mM), PCR primers S1-1, S1-2, S2-1, S2-2, S3-1a, S3-2 (25 pmol of each) (Table 6-1), 1.5 mM $MgSO_4$ and 5 U of AMV reverse transcriptase and Tfl DNA polymerase (Promega, Madison, USA). The amplification was performed in 30 cycles in a Mini thermocycler (MJ Research, USA). The conditions were as follows: reverse transcription for 45 min at 42°C, denaturation for 30 sec at 95°C annealing for 45 sec at 56°C and extension for 1 min at 72°C.

6.3.7 Restriction Enzyme Analysis

Aliquots of 10 µl of n-PCR products were incubated with 5U of restriction enzyme (RE) in a 30 µl reaction volume with the buffer recommended by the manufacturers (Promega, Madison, USA) (Kuan, 1997). Restriction enzymes used were: StyI, BglI and XmnI (Promega, Madison, USA). Samples were incubated at 37°C for 3 h and were analyzed with 7% polyacrylamide (BioRad, Hercules, CA) gel electrophoresis using a Hoefer electrophoresis unit (San Francisco, USA).

6.3.8 Quantification of the Nested PCR

Sensitivities were determined by using enteroviral RNA extracted from virus culture supernatant of known titer. Viral RNA was extracted from 10-fold dilutions of poliovirus 1 infected culture supernatant as described previously (6.3.3). It was amplified using the single RT-PCR and the nested PCR amplification procedures. PCR products synthesized by single PCR and nested PCR were analysed by 2% agarose (Seakem LE Agarose, Bioproducts, USA) gel electrophoresis using a Midicell Primo gel apparatus (Holbrook, New York).

6.3.9 Quality Control

Precautions to prevent carryover contamination were the following: Separate laboratories were used for reagents, treatment of samples, and manipulation of amplified fragments. Dedicated reagents, disposable sterile tubes and filtered pipette tips were used. Negative and positive controls were included in each experiment and processed together with patient specimens.

6.4 Results

6.4.1 RNA Extraction Methods

Nested PCR results of the CSF specimens were negative after heat-denaturation and RNA extraction with the Dyna Bead RNA extraction kit. The specimens showed specific amplified bands when tested after RNA extraction with the Qiagen extraction kit and the Trizol reagent. Enteroviral RNA extraction from the brain specimen was successful with both the RNEasy and Trizol extraction methods. The Trizol method however gave higher RNA yields than the RNEasy extraction kit with the heart specimens.

6.4.2 Specificity and Sensitivity of PCR

The specificity of the assay was assessed by testing a limited series of enteroviral and non-enteroviral reference strains. The sets of primers evaluated gave successful amplification for all enteroviruses, resulting in DNA bands of the expected sizes. Cell cultures infected with CAV19 and CAV22 were negative, but positive results were obtained when RNA was extracted from the original sample (homogenized mouse tissue).

A high level of sensitivity was obtained with the n-PCR. PCR products obtained from the nested PCR were detected in 0.01 PFU/140 µl sample volume of PV1. This corresponds to between 1 and 10 copies of enteroviral RNA per 140 µl volume of sample (Rotbart, 1990; Severini *et al.*, 1993; Melnick, 1996). The n-PCR proved to be 3 orders of magnitude more sensitive than the RT-PCR.

6.4.3 Direct Detection of Uncultured Specimens from Patients with EV Infection by PCR-RE Analysis

Routine enteroviral culture yielded 1.2% positive results out of 484 specimens tested (Table 6-2). One hundred and twenty-nine culture negative stool specimens were submitted for PCR of which 39 (30.2%) were positive (Table 6-2). Twenty percent of the PCR positive specimens were typed as vaccine strain polioviruses (Table 6-2). Twenty eight percent (n=71) of culture negative CSF specimens were positive for enteroviral RNA. Enterovirus serotypes detected by n-PCR - restriction enzyme analysis in clinical specimens are presented in Figure 6-1.

Nonpolio enterovirus serotypes detected were CBV1 (8%), CBV2 (6%), CBV3 (22%), CBV5 (11%), ECV7 (8%), ECV9 (6%), ECV11 (6%) and untypeable enteroviruses (19%). Untypeable enteroviruses were viruses that gave RE profiles different to those described by Kuan (1997). Three of the untypeable enteroviruses, detected in July 1997 gave the same RE profiles, suggesting that they were of the same serotype. Sequencing analysis of the VP1 region was not performed on these samples due to insufficient material. Enteroviruses were detected from clinical specimens during all seasons. The proportion of enterovirus positive specimens peaked in March and October. Most of the cases occurred amongst children less than 14 years of age (78%) and some cases involved those under 1 year of age (35%). A five year old child who presented with an ECV11 infection was HIV positive. Clinical information on PCR-positive specimens yielded clinical pictures compatible with enteroviral disease (Table 6-3). Most of the patients presented with aseptic meningitis (37%).

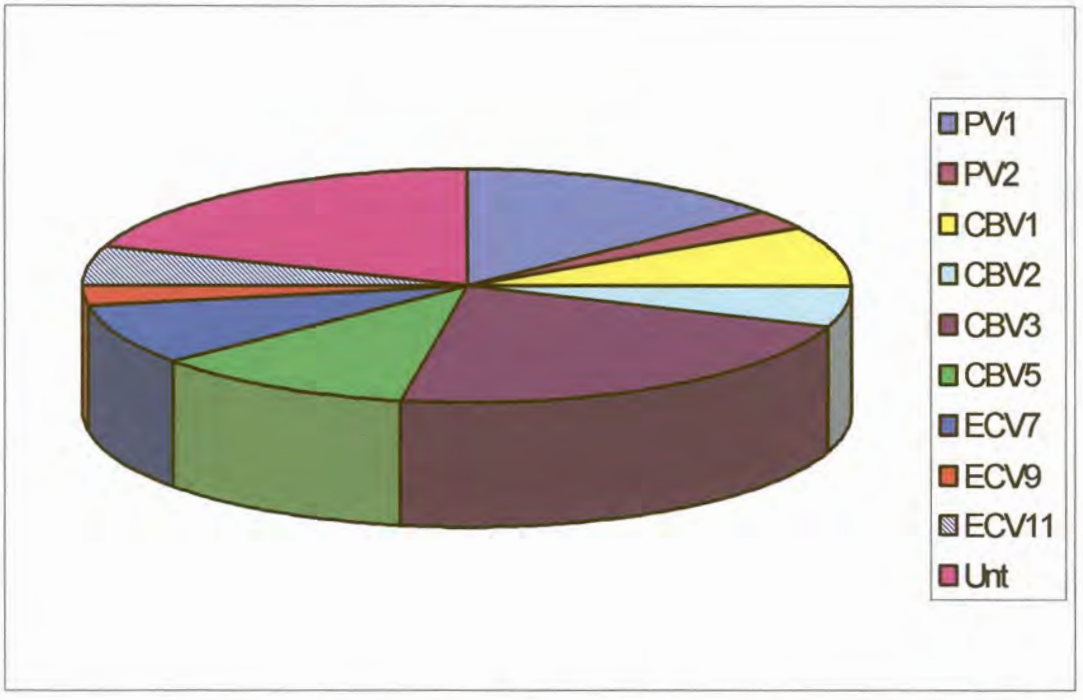

Figure 6-1: Enterovirus serotypes detected in clinical specimens by n-PCR followed by RE analysis.

6.5 Discussion

PCR was compared with virus isolation to detect enteroviruses in specimens from patients with suspected enteroviral infection. This study confirms that RT-PCR is more sensitive than cell culture isolation. Thirty two percent of the virus-culture negative samples were RT-PCR positive, which is in accordance with earlier findings (Sawayer *et al.*, 1994). The high degree of sensitivity and specificity of n-PCR make this method a valuable tool for the general detection of cytopathogenic as well as non-cytopathogenic enteroviruses in clinical specimens. The sensitivity of the detection of enteroviral RNA in CSF was improved by employing the Trizol RNA extraction method instead of heat inactivation (the method previously used in our diagnostic laboratory).

The age distribution amongst reported enterovirus-positive individuals, showing a predominance amongst the youngest age groups, is consistent with earlier findings (Melnick, 1996). Young children are known to be the principal reservoir of human enteroviruses. In the present study NPEV infections peaked in the months of March and October. The increased summer/fall occurrence of non-polio enteroviral illnesses is consistent with previous reports on the seasonal distribution of these viruses in temperate climates (Melnick, 1996; Nairn and Clement, 1999).

Coxsackie B viruses were the predominant viruses detected during the study period. These results contrast with results obtained by Nairn and Clement (1999). They found that echoviruses represented 77% of isolates and coxsackie A and B types accounted for 23% in Glasgow. However isolation data from Belgium showed that Coxsackie B virus isolates accounted for a larger proportion of NPEV isolates than in Glasgow (Druyts-Voets, 1997). In the current report, different serotypes were not particularly associated with specific symptoms. Coxsackie B viruses are important agents of acute heart disease (Kandolf, 1998), but in this study symptoms of cardiac involvement were evident in only two cases.

The detection of enterovirus in the faeces of a patient does not completely confirm an enterovirus diagnosis, because the excretion of enterovirus may persist for several weeks after an enterovirus infection in some patients. This is emphasized by the fact that most enteroviral isolates from stool specimens and nasopharyngeal aspirates of young children are vaccine strain poliovirus. Reporting an enterovirus in this setting without specifying poliovirus vs. nonpolio enterovirus can lead the physician to wrongly discontinue antibiotic treatment in the belief that an enteroviral aetiology has been established. The CNS is a nonpermissive site of enteroviral infection, implying true invasive infection when an enterovirus is detected and a high likelihood of association with a current illness. Therefore the detection of an enterovirus from the spinal fluid has special diagnostic significance and may play a major role in the proper management of a patient.

A positive EV-PCR result affect clinical decision making and may reduce unnecessary hospitalisation, expensive diagnostic procedures and antibiotic therapy (Ramers *et al.*, 2000). PCR may establish an enteroviral aetiology when other methods have been unsuccessful, as was indicated in this study. The assay should be utilised early in the evaluation process to have a maximum impact on patient management.

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Table 6-1: Primers used for the detection of enteroviral RNA in clinical specimens.

PCR	Primer	Sequence	Product	Reference
RT-PCR	EP1 (64 – 83)	5'CGGTACCTTTGTGCGCCTGT3'		
	EP4 (459 – 478)	5'TTAGGATTAGCCGCATTCAG3'	414	Gow *et al.*, 1991
Nested PCR	E1 (166 – 182)	5'AAGCACTTCTGTTTCCC3'		
	E2 (447 – 463)	5'ATTCAGGGGCCGGAGGA3'	297	Kuan, 1997
Sabin specific RT-PCR	S1-1 (2584 - 2601)	5'TCCACTGGCTTCAGTGTT3'		
	S1-2 (2505 – 2523)	5'AGGTCAGATGCTTGAAAGC3'	97	Yang *et al.*, 1991
	S2-1 (2580 – 2595)	5'CGGCTTGTGTCCAGGC3'		
	S2-2 (2525 – 2544)	5'CCGTTGAAGGGATTACTAAA3'	71	Yang *et al.*, 1991
	S3-1a	5'AGTATCAGGTAAGCTATCC3'		
	S3-2 (2537 – 2553)	5'AGGGCGCCCTAACTTTG3'	54	Yang *et al.*, 1991

Table 6-2: Enteroviruses detected in clinical specimens during the period January 1997 – December 1998.

Patient no	Age	Specimen	Virus isolated	RFLP results	Clinical diagnosis
1	2 months	Nasopharyngeal aspirate	PV1 (sabin)	PV1	Pneumonia
2	11 years	Stool	CBV	CBV	Spastic paralysis
3	unknown	Stool	Unt	ND	Unknown
4	unknown	Stool	PV1	PV1	Convulsions, encephalitis
5	10 years	CSF	CBV1	CBV1	Epilepsy
6	unknown	CSF	CBV5	CBV5	Meningitis
7	17 years	Stool	ND	EV9	Unknown
8	8 months	stool	ND	PV3	Diarrhoea
9	11 months	stool	ND	CBV5	diarrhoea
10	1 year	stool	ND	CBV1	Diarrhoea, pyrexia
11	unknown	SSV	ND	CBV3	Meningitis
12	unknown	SSV	ND	EV7	Meningitis
13	Unknown	stool	ND	PV1	Enteritis
14	1 month	stool	ND	PV1	Unknown
15	10 months	stool	ND	CBV2	meningitis
16	8 months	stool	ND	PV1	Unknown
17	3 years	stool	ND	EV7/CBV1	Guillian Bare
18	12 years	stool	ND	EV7/CBV1	Guillian Bare
19	unknown	stool	ND	Unt	Encephalitis
20	unknown	stool	ND	Unt	Unknown
21	1.5 years	stool	ND	EV4	Unknown
22	10 months	stool	ND	unt	Diarrhoea
23	5 years (HIV+)	stool	ND	EV11	Unknown
24	unknown	stool	ND	CBV3	Diarrhoea
25	2 years	stool	ND	EV11	Diarrhoea
26	3 years	stool	ND	EV11	diarhoea
27	9 months	stool	ND	EV4/CAV2/CAV3	Chronic diarrhoea
28	4 years	stool	ND	CBV2	Unknown
29	10 months	SSV	ND	CBV3	Comateus
30	unknown	SSV	ND	CBV3	Coma, diabetes
31	unknown	SSV	ND	CBV3	Meningitis
32	30 years	CSF	ND	CBV5	Meningitis
33	11 year	CSF	ND	unt	Abnormal gait
34	75 years	CSF	ND	CBV5	Guillian Bare
35	5 years	CSF	ND	unt	Encephalopathy
36	unknown	CSF	ND	CBV2	Meningitis
37	unknown	CSF	ND	CBV1	Meningitis
38	unknown	CSF	ND	unt	Unknown
39	unknown	Blister fluid	ND	Unt	Miocarditis
30	3 years	ND	ND	CBV5	Meningitis, diarrhoea

Table 6-2: Enteroviruses detected in clinical specimens during the period January 1997 – December 1998. (continued)

Patient no	Age	Specimen	Virus isolated	RFLP results	Clinical diagnosis
31	31 years	Heart biopsy	ND	CBV3	unknown
32	unknown	CSF	ND	ND	meningitis
33	32 years	CSF	ND	ND	Post encephalitis
34	unknown	CSF	ND	ND	Encephalitis
35	2 months	CSF	ND	ND	Meningitis
36	33 years	CSF	ND	CBV3	Meningitis
37	3 years	CSF	ND	CBV3	Meningitis
38	2 years	CSF	ND	ND	meningitis

ND: Not done

Unt: untypeable

Table 6-3: Clinical information on patients with a PCR-positive result.

Clinical picture	Number of patients
Meningitis	14
Encephalitis	4
Coma	2
Neonatal sepsis	2
Abnormal Gait	1
Guillian Bare syndrome	3
Encephalopathy	1
Miocarditis	2
Unknown	9

CHAPTER 7

7 GENERAL DISCUSSION

In view of the major health impact of enteroviruses, sensitive and reliable techniques for the detection and identification of this large group of closely related viruses are essential (Chapter 2). Traditionally enteroviruses have been detected in cell culture and their serotypic identity has been established by neutralization of infectivity with serotype-specific antisera (Melnick, 1996). This procedure is limited by the difficulty in culturing some enteroviruses, the availability of antisera for serotyping and the cost and technical complexity of serotyping procedures (Melnick, 1996). The use of molecular techniques is becoming a more rapid and sensitive method to identify enteroviruses from clinical specimens and environmental samples. The objective of this study was to optimise and assess molecular techniques for the rapid and sensitive detection and typing of enteroviruses in both clinical specimens and environmental samples.

The molecular techniques, optimised and assessed in this study, proved suitable for the rapid and sensitive detection and typing of enteroviruses in clinical specimens and environmental samples. The polio-nonpolio RT-multiplex PCR proved useful for the rapid and sensitive detection of poliovirus (PV) and for its distinction from nonpolio enteroviruses (NPEV) in river water samples. In addition to the sensitivity and specificity of the PV detection protocol, the PVs detected can be subjected to strain specific typing using the Sabin-specific triplex RT-PCR. Nonpolio enteroviruses detected in the river water samples were successfully typed by restriction enzyme (RE) analysis. By employing three restriction enzymes (Sty1, Bgl1 and Xmn1), a digestive pattern was obtained that was easily interpretable. A drawback of RE analysis is that single point mutations within the recognition sequence may give rise to unclassifiable results. Sequencing within the VP1 region of the enterovirus genome proved useful for the typing of enteroviruses that were not typeable by RE analysis. This technique proved effective to determine whether viruses isolated from water sources are related (Chapter 3). Sequencing is however laborious and expensive and thus not suitable for the routine monitoring of water samples.

The integrated cell culture (ICC) nested PCR approach, followed by restriction enzyme analysis proved 4 logs more sensitive than the multiplex RT-PCR for the detection of enteroviruses in drinking water samples and clinical specimens (Chapters 4 and 6). The high degree of sensitivity and specificity of n-PCR followed by RE analysis make this method a valuable tool for the

general detection and typing of low numbers of cytopathogenic as well as non-cytopathogenic enteroviruses. Results indicated that molecular techniques proved suitable to replace conventional detection and typing methods. Conventional methods include cell culture neutralization tests and inoculation of newborne mice.

Amongst the enteroviruses detected in treated drinking water were vaccine strain polioviruses (12%) and coxsackie B viruses (88%). The detection of these viruses in the treated drinking water supplies is in agreement with epidemiology data which associate substantial levels of enteric infections with drinking water supplies which meet specifications for treatment, disinfection and counts of indicator organisms (Payment *et al.*, 1997). The ability of these viruses to infect susceptible host cells and to replicate their RNA, confirms that they are viable and infectious and therefore constitute a health risk (Reynolds *et al.*, 1997; Grabow *et al.*, 2000; Seidel *et al.*, 2000). The incidence of CBVs in the treated drinking water supplies was used as basis for risk assessment using models described by Haas *et al.* (1999). The results indicated that the drinking water supplies concerned constitute an annual risk of CBV infection of 3.91×10^{-3} (treatment unit A) and 7.4×10^{-3} (treatment unit B). The estimated risks of infection are an order of magnitude higher than the yearly acceptable risk of one infection per 10 000 consumers proposed for drinking water supplies by the United States Environmental Protection Agency (Macler and Regli, 1993).

The high incidence of coxsackie B viruses in both patients and water environments from the same region would seem to reflect the value of analysing wastewater discharges to obtain an indication of viruses circulating in communities. One benefit in this regard is illustrated by the detection of vaccine strains of polioviruses in wastewater effluents, which reflects the success of poliomyelitis immunisation in the communities concerned. Likewise, wastewater monitoring may prove a useful tool for screening the potential presence of wild type strains of polioviruses in communities (Grabow *et al.*, 1999). The ability to differentiate between wild and vaccine derived PV and NPEV in the environment is of fundamental importance to international endeavours regarding the eradication of poliomyelitis (Grabow *et al.*, 1999; Yoshida *et al.*, 2000). Although tremendous progress has been made, polio eradication is not yet assured. Outbreaks of poliomyelitis are currently in progress in Cape Verdi, Haiti and the Dominican Republic (Reports i.e. electronic ProMed mail, December 2000). The outbreak in the Dominican Republic and Haiti has raised serious concerns because the Western Hemisphere has been free of wild poliovirus circulation since 1991. The virus identified was an unusual derivative of the Sabin type 1 oral poliovirus vaccine (Reports i.e. electronic ProMed mail, December 2000).

Details on enteroviruses circulating in communities, as determined by wastewater monitoring, have other important benefits. The incidence of enterovirus infections and their public health impact would appear to increase worldwide. This is illustrated by reports on regular outbreaks of enterovirus infections, in many parts of the world. Enterovirus 71 seems to be the cause of meningitis and hand-foot-and-mouth disease in many of these outbreaks (AbuBakar *et al.*, 1999; Pollard and Dobson, 2000). However, other enteroviruses, notably coxsackievirus A16, may also be involved (Reports i.e. electronic ProMed mail, October 2000). Details on the circulation of these viruses in communities, and on the possible role of water in their transmission, is of major importance in the development of strategies aimed at controlling these infections which have far-reaching public health implications.

An outbreak of aseptic meningitis is currently (November to December 2000) in progress in the eastern parts of Johannesburg and surrounding areas. Initial findings suggested that coxsackie B2 and B3 viruses might be involved. Even though this is the time of the year when the incidence of enterovirus infections is typically high, initial reports indicated that the incidence is higher than would be expected in terms of normal seasonal fluctuation. Details on the viruses concerned are urgently required, because fears have been expressed about the potential importation of enterovirus strains responsible for devastating outbreaks in parts of the world such as Malaysia, Singapore, Taiwan and Hong Kong. Questions have been raised by health authorities concerning the possible role of water in the transmission of the viruses concerned. Answers to these questions require accurate typing of enteroviruses in patients as well as water sources and supplies which would cast light on the possible role of water in the epidemiology of the infections. The techniques developed in this study proved suitable for the sensitive detection and accurate typing of enteroviruses in both patient specimens and water samples.

Future research should include refinement of the risk assessment model. More detailed experiments need to be conducted on recovery efficiency of the glass wool adsorption-elution procedure and drinking water consumption in South Africa. Risk assessment can be expanded to give an indication of economic impact, which is determined by integrated measures of health, such as Disability Adjusted Life Years (DALY). The application of molecular detection and typing techniques on the routine monitoring of water supplies should also be considered a goal of research.

7.1 References

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