CHAPTER 1

1 INTRODUCTION

The enteroviruses comprise a large genus belonging to the family Picornaviridae (Melnick, 1996). Sixty-six immunologically distinct serotypes are known to cause infections in humans. Enteroviruses were initially classified by their pathogenic properties in animal and cell culture, resulting in four major groups: polioviruses (PV, 3 serotypes), coxsackie A viruses (CAV, 23 serotypes), coxsackie B viruses (CBV, 6 serotypes) and echoviruses (ECV, 30 serotypes). Recently isolated enteroviruses have been named with a system of consecutive numbers: EV68 – EV71 (King *et al.*, 2000).

Enteroviruses have a world-wide distribution. They are transmitted by the faecal-oral route, thus exposure to contaminated water, food or vomits can result in acquisition of infection (Fogarty *et al.*, 1995). Infection has been associated with a wide range of diseases including aseptic meningitis, myocarditis, respiratory illness and insulin dependant diabetes mellitus (Melnick, 1996). Neonates are at particular risk of infection as well as other sensitive populations, such as the elderly and immunocompromised (Melnick, 1996).

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. Although serotyping may be of limited importance for the management of individual patients with an enterovirus infection, it is essential for understanding the epidemiology of enterovirus infections, for monitoring the emergence of new types or possible changes in virulence and for facilitating early, rapid detection of circulating types during the epidemic season (Van Loon *et al.*, 1999). To date enteroviruses have generally been isolated by cell culture propagation, and typed by neutralization tests using specific antisera. Inoculation of newborne mice remains the most common procedure to distinguish between coxsackie A and B viruses. These techniques have major shortcomings. They are slow, labor intensive, lack specificity and sensitivity, and type specific neutralization antibodies are not readily available.

Difficulties with the detection and typing of enteroviruses may be solved by molecular techniques. These techniques are based on the polymerase chain reaction (PCR) and are specific enough to readily distinguish between vaccine and wild type strains of polioviruses, which in the past was a complicated exercise. Indications are that these techniques may substantially facilitate the detection of enteroviruses in patient specimens as well as in water environments (Zoll *et al.*,

1992; Abbaszadegan *et al.*, 1993; Kopecka *et al.*, 1993; Muir *et al.*, 1993; Abbaszadegan *et al.*, 1999). Primer pairs, specific for the conserved 5' non-translated region of the enterovirus genome, may be used as a screening procedure for a spectrum of enteroviruses which would serve a valuable purpose as general viral indicator. The detection of enteroviruses may be followed by more specific molecular techniques for typing of the viruses (Egger *et al.*, 1995). New developments in molecular techniques concerned include reverse transcription followed by restriction enzyme analysis (RE) (Kuan, 1997), antigen capture-PCR (Shen *et al.*, 1997) and sequencing (Oberste *et al.*, 1999) which may prove useful for differentiating between genotypes of enteroviruses.

The basic objective of this study was to use advanced molecular techniques to develop a rapid, simple and inexpensive assay for the detection and serotyping of enteroviruses in water sources and clinical specimens and to apply these techniques in research on the diagnosis and incidence of enterovirus infection. This included the investigation of the role of enteroviruses in conditions such as aseptic meningitis, myocarditis and upper respiratory diseases. The technology will be utilized in monitoring the eradication of poliomyelitis by the typing of viruses in cases of acute flaccid paralysis and viruses isolated from water.

The objectives of this study were therefore:

- To select primers and probes for the typing of enteroviruses.
- To optimise the polymerase chain reaction (PCR) and gene probe hybridization techniques for typing of enteroviruses.
- To apply the new molecular techniques for typing of enteroviruses isolated from patients and the environment.
- To assess the extent to which the new techniques can be used to type non-cytopathogenic enteroviruses detected by PCR.
- To investigate the replacement of presently used procedures for typing coxsackieviruses based on the inoculation of newborne mice and cell culture neutralization tests, by molecular techniques.
- To assess the risk of infection constituted by enteroviruses detected in drinking water supplies.

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CHAPTER 2

2 LITERATURE REVIEW

2.1 From Ancient Egypt to the 20th Century

Polio has probably caused death for most of human history. The oldest identifiable reference to paralytic poliomyelitis is an Egyptian stele (stone engraving) over 3 000 years old (Figure 2-1). Sporadic cases of paralytic poliomyelitis have occurred for at least as long as human history has been recorded. From ancient times into the late 1800's, polioviruses were widely distributed in most of the world's populations. Polioviruses (PV) survived in an endemic fashion by continuously infecting susceptible infants. Paralytic poliomyelitis was mentioned in the medical literature in the mid 1700's, predominantly in infants or young children (Melnick, 1996). In the mid 1800's outbreaks of paralytic polio were detected in urban, industrialised parts of Europe and North America (Melnick, 1996). Epidemics followed that grew more severe, frequent and widespread. Cases were observed in adolescents and young adults. Large epidemics spread across the United States and Europe in the first half of the 20th century (Melnick, 1996).

In the United States, in the summer of 1916, over 27 000 people were paralysed, with 6 000 deaths (Melnick, 1983). The 1916 epidemic caused widespread panic and thousands of people fled from the cities to nearby mountain resorts. Increased public awareness and fear, together with the ongoing developments that had taken place in medical science, led to the intensified study of the disease and its control by vaccination.

For 30 years, poliovirus was considered a strictly neurotropic disease (Melnick *et al.*, 1946). Neither the spread of the virus in nature nor the pathogenesis of the disease was clearly understood. In the late 1930's, investigators rediscovered a finding, originally made in 1912 in Sweden: the virus could be isolated not only from the spinal cord but also from the faeces of both patients and healthy carriers. In the 1940's, poliomyelitis was regarded as an enteric infection (Melnick *et al.*, 1946).

Figure 2-1: Ancient Egyptian stone engraving portraying polio (picornaviruses at www-micro.msb.le.ac.uk)

This finding initiated the following question: If polio was an enteric infection, was it waterborne? During 1940-1945, investigators studied the occurrence of poliovirus in the sewage of New York City, Chicago and other cities (Melnick *et al.*, 1947). Although this investigation could not prove waterborne transmission of polio, it indicated that when paralytic cases were prevalent in the community, large numbers of polioviruses were present in the sewage. It remained infectious for several weeks in flowing sewage (Melnick *et al.*, 1947).

The development of simpler testing methods became a necessity with more scientists entering the field. In 1936, Sabin and Olitsky reported the successful growth of poliovirus in tissue fragments, cultivated in glass vessels (Sabin *et al.*, 1936). Another 13 years elapsed before the landmark report by Enders *et al.* (1949). By using different strains of poliovirus, Enders and colleagues proved that poliovirus could multiply in a variety of tissue cultures, particularly in the cells that grew from tissue fragments (Enders *et al.*, 1949).

The discovery of the large group of viruses to which the poliovirus belongs came about in 1948. Dalldorf and Sickles (1948) inoculated newborn suckling mice with faecal suspensions from two suspected polio patients from the town of Coxsackie, New York. These pathogens induced paralysis in the newborn mice, in contrast to poliovirus, which usually causes disease only in

primates (Dalldorf and Sickles, 1948). The viruses were called "coxsackie" viruses referring to the first geographical site of isolation (Dalldorf and Sickles, 1948).

Coxsackie A viruses (CAV's) induced flaccid paralysis, which was different from the spastic paralysis observed after inoculation of mice with other virus strains (Godman *et al.*, 1952). Today these strains are known as coxsackie B viruses (CBV's). Further studies revealed that in experimental animals, CAV's affect skeletal and heart muscle, while CBV's replicate in a wide range of tissue (Godman *et al.*, 1952). These included the central nervous system, liver, exocrine pancreas, brown fat and striated muscle (Godman *et al.*, 1952). Twenty-three CAV serotypes and six CBV serotypes (1-6) were recognised (Melnick, 1983). These viruses were not easy to study because there were many serotypes and a number of new strains (Melnick, 1983). Furthermore, funds to study these viruses decreased once a vaccine had been developed for the polioviruses (Melnick, 1983).

The introduction of cell culture techniques enabled the isolation of viruses, which did not replicate in experimental animals. These agents were first called "orphan viruses" - i.e. viruses in search of a parent disease. A more explicit name was later agreed upon: enteric cytopathogenic human orphan viruses, which soon became echoviruses (ECV) (Melnick, 1955).

As studies of polioviruses, coxsackieviruses and echoviruses continued, it became clear that these viruses shared more properties than a human enteric habitat. Recognising that they form a distinct genus they were named enteroviruses - the name by which they are known today (Minor, 1991).

2.2 Classification and General Characteristics

The name picornavirus was introduced in 1963 to designate a larger grouping that included both the enteroviruses and the rhinoviruses (International Enterovirus Study Group, 1963). The name **Picorna** is an acronym, which was derived from, **P**oliovirus, **I**nsensitivity to ether, **C**oxsackievirus, **O**rphan virus, **R**hinovirus, **R**ibo-**N**ucleic **A**cid.

The picornavirus species definition is as follows: "A picornavirus species is a polythetic class of phylogenetically related serotypes or strains which would normally be expected to share: (i) a limited range of hosts and cellular receptors, (ii) a significant degree of compatibility in proteolytic processing, replication, encapsidation and genetic recombination, and (iii) essentially identical genome maps" (King *et al.*, 2000). The most recent revision of virus taxonomy has erected 9 genera within the family: Enterovirus, Rhinovirus, Hepatovirus, Cardiovirus,

Aphtovirus, Erbovirus, Kobuvirus, Teschovirus and Parechovirus (Figure 2-2) (King *et al.*, 1999; Pringle, 1999, King *et al.*, 2000). Previous sequencing studies have shown that ECV22 and ECV23 are distinct from all other enteroviruses (EV) and it has been suggested that ECV22 and ECV23 be reclassified as parechovirus (a separate genus within the picornaviridae) (Stanway *et al.*, 1994; Oberste *et al.*, 1998, Pringle, 1999; King *et al.*, 2000).

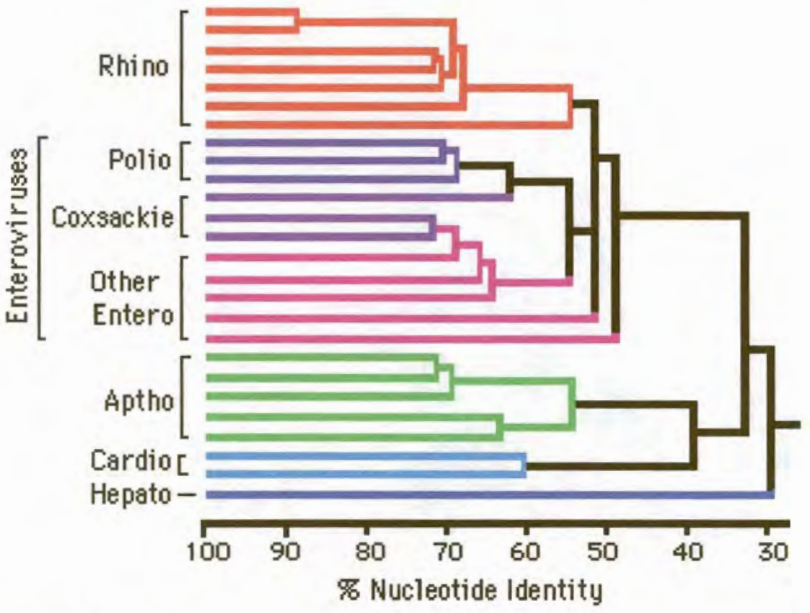

Figure 2-2: Schematic illustration of Picornavirus species (picornaviruses at www-micro.msb.le.ac.uk).

2.3 Viral Morphology

Picornavirus virions are icosahedral, 22 – 30 nm in diameter, and have no envelope (Melnick, 1983). The viruses lack essential lipids and carbohydrates (Melnick, 1983). The enterovirus genome is a single-stranded RNA molecule, approximately 7 500 nucleotides long and of positive polarity (Figure 2-3) (Rueckert, 1996). A 750- nucleotide 5' untranslated region (5'UTR) is followed by a long open reading frame coding for a 2 100 amino acid polyprotein (Melnick, 1983). This is followed by a short 3'untranslated region (3'UTR) and a poly (A) tail (Melnick, 1983).

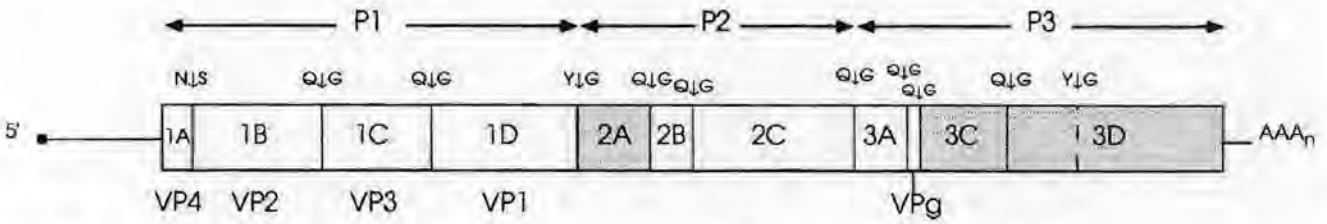

Figure 2-3: Genomic structure of a generalized picornavirus (Muir, 1998)

2.3.1 5'UTR

The 5'UTRs of picornaviruses form 8 - 12% of the total virus genetic information (Toyoda *et al.*, 1984). Toyoda *et al.* (1984), indicated that the 5'UTR could be divided into conserved (nucleotides 1 to 650) and hypervariable (nucleotides 651 to 750) regions according to the variation seen among the three Sabin strains. There are a number of short nucleotide stretches, particularly between nucleotides 440 and 600 that showed almost perfect conservation among all enterovirus groups (Stanway *et al.*, 1984). In all picornaviruses the 5'UTR has a different nucleotide composition from the rest of the genome, being lower in A and higher in C residues (Stanway *et al.*, 1984). The increased G+C content may reflect the fact that secondary structure is important in RNA stability and plays an important role in the function of the 5' UTR (Stanway *et al.*, 1984). Several important functions have been mapped to the 5'UTR (Andino *et al.*, 1993). The first 100 nucleotides of PV play a role in viral replication (Andino *et al.*, 1993). Enteroviruses use internal initiation of translation rather than the ribosome-scanning model proposed for cellular mRNAs (Pelletier *et al.*, 1988). The internal initiation has been shown to require a large portion (nucleotides 130 to about 600) of the PV 5'UTR (Bienkowska-Szewczyk and Ehrenfeld, 1988), but the exact ribosome binding sequence is not known. Point mutations in the 5'UTR affected virulence (Cann *et al.*, 1984; Kawamura *et al.*, 1989; Moss *et al.*, 1989), temperature sensitivity and plaque morphology (Ramsingh *et al.*, 1995).

2.3.2 Open Reading Frame

The open reading frame following the 5'UTR is translated into a polyprotein which is co- and posttranslationally cleaved to give four structural proteins (VP4, VP2, VP3 and VP1), which form the viral capsid (Figure 2-4) and seven non-structural proteins (Kitamura *et al.*, 1981). VP1 to VP3 are partially exposed on the virion surface, while VP4 is completely internalised in infectious virions (Kitamura *et al.*, 1981). Each of the capsid proteins is composed of conserved elements that form the β-barrel structures (Kitamura *et al.*, 1981). Many of the loops are exposed

on the virion surface and studies of monoclonal antibody resistant mutants have shown that a number of the loops contribute to specific antigenic neutralization sites (Kitamura *et al.*, 1981). Three or four neutralization determinants have been identified for each PV serotype by using monoclonal antibody neutralization escape mutants, and they have been mapped to VP1, VP2, and/or VP3 (Minor, 1992). Neutralization determinants have been identified on VP1 for CBV3 (Haarman *et al.*, 1994) and CBV4 (Reimann *et al.*, 1991). Determination of attenuation of virulence (Ramsingh *et al.*, 1995), virion thermostability (Minor, 1992), altered host range (Couderc *et al.*, 1993), persistent infection (Calvez *et al.*, 1993; Pavio *et al.*, 1996), and plaque morphology (Ramsingh *et al.*, 1995) have been mapped to the capsid-encoding region.

Some functions of the non-structural proteins and their precursor forms are known. Protein 2A is one of the viral proteinases that cleaves the polyprotein in trans between VP1 and 2A and frees the capsid protein precursor from the rest of the polyprotein (Toyoda *et al.*, 1986). Proteinase 2A participates in host cell shutoff by indirectly inducing cleavage of the cellular p220 protein, which is an important factor in cap-dependant initiation of translation (Sonenberg, 1990). The specific functions of 2B and 2C are not known, although protein 2C and its precursor form 2BC have been found in the replication complex of PV (Bienz *et al.*, 1992). Protein 2C has a helicase activity (Bienz *et al.*, 1992). Protein 3AB is a precursor of 3B, the small polypeptide covalently linked to the 5'UTR of picornavirus RNA molecules (Flanegan *et al.*, 1977). Protein 3C is the second viral proteinase which does most of the polyprotein processing (Hanecak *et al.*, 1982), while 3D is the RNA dependant RNA polymerase (Van Dyke and Flanegan, 1980).

2.3.3 3'UTR

The coding region is followed by a 70 – 100 nucleotide 3'UTR (Iizuka *et al.*, 1987). This region is important in the initiation of the minus-strand RNA synthesis, but no specific sequences have been identified for polymerase binding (Iizuka *et al.*, 1987). Several secondary and tertiary structures (pseudoknots) have been proposed for this region (Iizuka *et al.*, 1987). A genomic poly(A) tail with an average length of 75 nucleotides follows the 3'UTR of enteroviruses (Armstrong *et al.*, 1972).

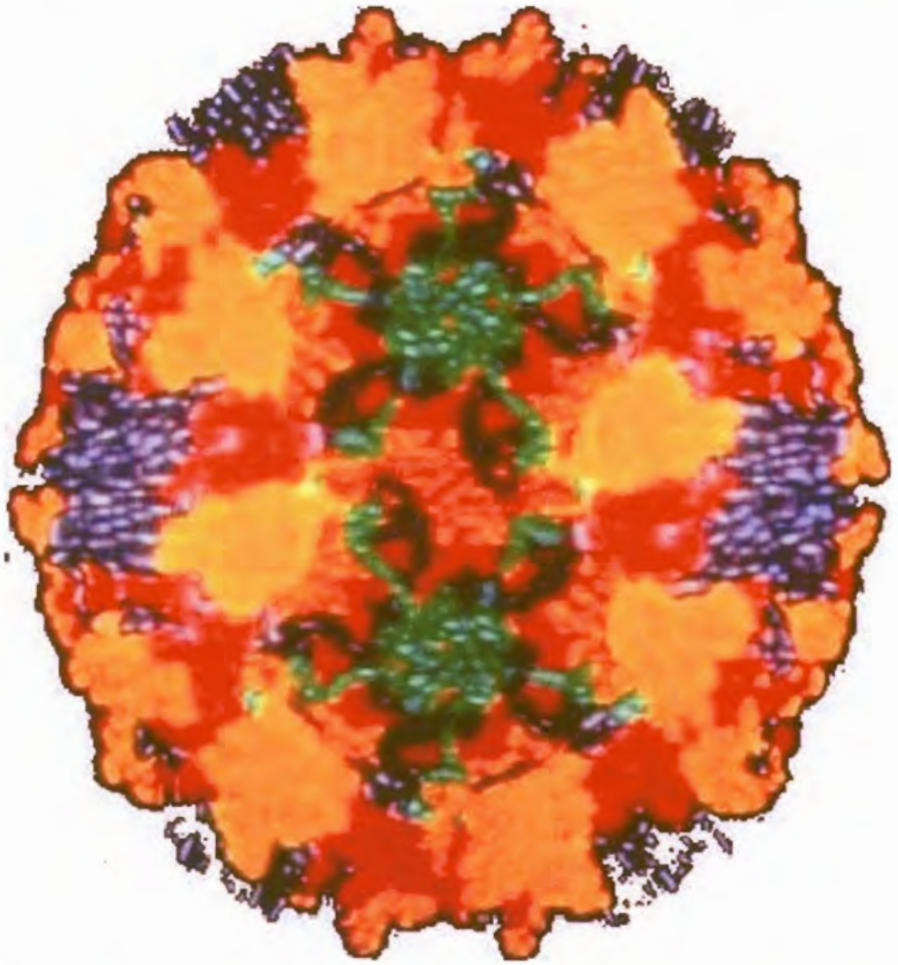

Figure 2-4: Capsid structure of a picornavirus (picornaviruses at www-micro.msb.le.ac.uk).

2.4 Properties of Enteroviruses

Enteroviruses and the antigens associated with them are resistant to all known antibiotics and chemotherapeutic agents (Melnick, 1996). Alcohol, phenol, quaternary ammonium compounds and similar laboratory disinfectants are not effective (Melnick, 1996). They are insensitive to ether, deoxycholate and various detergents that destroy other viruses (Melnick, 1996). Treatment with 0.3% formaldehyde, 0.1 N HCl, or free residual chlorine at a level of 0.3-0.5 ppm causes rapid inactivation, but the presence of extraneous organic matter protects the virus from such inactivation (Melnick, 1996).

Liu *et al.* (1971) compared the effect of chlorine disinfection on twenty human enteric viruses. Except for poliovirus type 2, coxsackievirus A5 and B5 were the most resistant to chlorine treatment. Jensen *et al.* (1980) studied the effect of chlorine at various pH levels on the inactivation of enteroviruses. Inactivation of coxsackievirus types B3 and B5 by chlorine (pH 6)

was approximately half that of inactivation of poliovirus (Jensen *et al.*, 1980). When 0.1 M NaCl was added, the inactivation rate of poliovirus increased three-fold while coxsackievirus types B3 and B5 were not affected (Jensen *et al.*, 1980). Coxsackievirus B5 was 1.6 - 10 times more resistant to inactivation by chlorine than the four other enteroviruses studied (Jensen *et al.*, 1980). Barttigelli *et al.* (1993) compared the effects of ultraviolet (UV) light disinfection on coxsackievirus B5 and four other viruses, including the bacteriophage MS-2. Coxsackievirus B5 was as resistant to UV irradiation as rotavirus, which has been considered the enteric virus that is the most resistant to this form of treatment (Wolfe, 1990). Payment (1999) determined the efficacy of residual chlorine disinfectant in drinking water to inactivate waterborne pathogens in distribution systems. His results suggested that the maintenance of a free residual concentration in a distribution system does not provide a significant inactivation of pathogens (Payment, 1999). Due to the high resistance of coxsackieviruses to inactivation by chlorine and UV, it may be necessary to test treated water for these viruses (Payment, 1999).

Enteroviruses are resistant to low pH (i.e. pH 3.0) (Melnick, 1983). As a result they pass through the gastric acidity of the stomach and replicate in the mucosal cells of the small intestine (Melnick, 1983). Enteroviruses are thermolabile and exposure of these viruses to a temperature of 50°C destroys them rapidly (Melnick, 1983). However, Lo *et al.* (1976) found that coxsackievirus B5 was the most stable virus (compared with echovirus type 6 and poliovirus type 1) at any temperature. Magnesium chloride inhibited the inactivation of enteroviruses at all temperatures tested (Melnick *et al.*, 1961). This property has led to the wide-spread use of $MgCl_2$ as a stabiliser of oral poliovirus vaccine (Melnick *et al.*, 1961).

2.5 The Need for Typing of Enteroviruses

Although serotyping may be of limited importance for the management of individual patients with an enterovirus infection, it is essential for understanding the epidemiology of enterovirus infections, for monitoring the emergence of new types or possible changes in virulence and for facilitating early, rapid detection of circulating types during the epidemic season (Van Loon *et al.*, 1999).

2.5.1 Correct Identification of Polioviruses

Drawing inspiration from the successful smallpox initiative, the World Health Assembly (WHA) resolved to eradicate polio from the world by the year 2000 (World Health Assembly, 1988). The following reasons make poliovirus a candidate for eradication: poliovirus causes acute, non-

persistent infections, humans are the only reservoir, virus survival in the environment is finite and immunization with vaccine interrupts virus transmission. The World Health Organization (WHO) is leading a global partnership of international organisations and governments to implement the proven strategies for polio eradication in all remaining polio-endemic countries (Hull *et al.*, 1994). The strategies recommended by the WHO for polio eradication are as follows: maintaining high routine immunization coverage, conducting nation wide mass immunization campaigns, building effective, laboratory-based surveillance for acute flaccid paralysis (AFP) and conducting localized immunization campaigns directed at the final reservoirs of virus transmission. Since most poliovirus infections are subclinical, eradication means not just that there will be no cases of clinical poliomyelitis caused by naturally occurring (wild) polioviruses. Eradication means in addition, that wild poliovirus transmission will cease and that no polioviruses can be found despite intensive efforts to do so.

Although tremendous progress has been made, polio eradication is not yet assured. One obstacle is inadequate development of surveillance systems. AFP surveillance is at an early stage of development in southern Africa. The shift from simple reporting of physician-diagnosed polio to the detailed investigation of all cases of suspected polio (AFP), as required for the national certification of polio-free status, was established in 1994 (Biellik *et al.*, 1997).

Developing a surveillance system is a long-term commitment that must be maintained until global eradication is certified after the year 2000. AFP surveillance poses difficulties in countries with inadequate health infrastructure. War and civil disorder poses a second obstacle to eradication. Immunization campaigns are difficult in the face of armed conflict. The major obstacle is inadequate political and financial support for the eradication initiative. Although all countries were committed to the eradication goal through the World Health Assembly Resolution and the World Summit for Children, not all countries in which polio is endemic have initiated polio eradication activities (Hull *et al.*, 1997).

Poliomyelitis remains endemic in most of the countries in the African region (Figure 2-5). This is illustrated by an outbreak of poliomyelitis with more than 600 reported cases during March and April 1999 in Angola (Reports i.e. electronic ProMed mail, 30 April 1999). Some of these countries serve as reservoirs for wild poliovirus and may periodically export viruses to areas that had previously been considered free of indigenous wild poliovirus. The reported annual incidence of poliomyelitis cases has declined by 70%, from 5126 cases in 1980 to 1597 in 1995 (Hull *et al.*, 1997). In 1995, 897 cases (representing 59% of reported cases) were notified by countries in "difficult circumstances" such as Angola, Ethiopia, Nigeria, and Zaire.

Polio outbreaks were reported in Namibia in 1993 - 1995 (Biellik *et al.*, 1994; Van Niekerk *et al.*, 1994; Biellik *et al.*, 1997). The outbreak in Namibia was attributed to the importation of wild poliovirus from neighbouring Angola, where polio remains endemic (Izurieta *et al.*, 1997). The 1994 - 1995 epidemic was focal in nature and appears to have been associated with a new importation of wild poliovirus from Kuando Kubango province in Angola, which is located directly accross the border (Izurieta *et al.*, 1997). More than half the confirmed cases were associated directly or indirectly with persons of Angolan origin (Izurieta *et al.*, 1997). Kuando Kubango province has been under rebel control for >10 years (Biellik *et al.*, 1997). Since then, the province has been isolated from Luanda and other government controlled areas and vaccination services were suspended (Biellik *et al.*, 1997). The wild type 1 poliovirus isolated during the 1994 - 1995 Namibian outbreak differed from PV isolates in Luanda in 1994. This indicated that a separate strain was circulating in south-eastern Angola (Izurieta *et al.*, 1997).

A large epidemic of poliomyelitis, due to poliovirus type 1, was reported from Zaire (Lambert *et al.*, 1995). Zaire shares a long frontier with Angola. Isolates from Zaire have been sequenced at the National Institute of Virology in South Africa (Lambert *et al.*, 1995). The isolates exhibited 97% sequence homology with isolates from cases in Luanda during May 1994, suggesting close epidemiological linkage between the cases (Lambert *et al.*, 1995).

Despite the encouraging progress toward polio eradication, especially in southern and eastern Africa, the risk of a poliomyelitis outbreak, such as the outbreaks that occurred in Namibia in 1993, and 1994 - 1995, remains high. Polio is a disease that does not respect national borders or continental boundaries, and in that sense is a problem of global concern requiring large scale international co-operation. The ability to identify wild and vaccine-derived PV in clinical and environmental samples is necessary to: (i) monitor the effectiveness of polio vaccination strategies (Bottiger *et al.*, 1992; Miyamura *et al.*, 1992; Tambini *et al.*, 1993), (ii) identify and monitor PV outbreaks (Hovi *et al.*, 1986; Conyn-van Spaendonck *et al.*, 1996), (iii) diagnose cases of vaccine associated paralytic poliomyelitis and (iv) exclude other enteroviruses as a cause of poliomyelitis like disease (Asaad and Cockburn, 1972; Chumakov *et al.*, 1979).

2.5.2 Studying the Relationship between Enterovirus (Sub)type and Disease Manifestation

Although the disease spectra of the different serotypes overlap, certain disease syndromes are caused by one or a few serotypes eg. poliovirus that is the main cause of paralytic poliomyelitis. Associations between enterovirus serotype and disease syndrome have been observed (Grist *et al.*, 1978; Moore, 1982; Strikas *et al.*, 1986), and the use of sensitive molecular detection methods

has proved of value in further delineating these associations. It was found that most cases of enteroviral meningitis are caused by CBV and ECV serotypes (Berlin *et al.*, 1993) whereas cases of viral myocarditis are associated with CBV (Kämmerer *et al.*, 1994; Khan *et al.*, 1994; Nicholson *et al.*, 1995).

According to Cherry (1992), 50% of infections due to coxsackievirus type A and 80% due to type B are symptomatic. Enterovirus data compiled by the WHO between 1975 - 1983 indicated a greater frequency of symptomatic cases: 83.2% for type A and 78% for type B (Grist and Reid, 1988). Table 2-1 showed the percentage of various diseases caused by different serotypes of enteroviruses (total %) reported for 1975 - 1983 (Grist and Reid, 1988). During this period 58 956 enterovirus infections were reported to the WHO and 35% of these were coxsackievirus infections (9.8% type A and 25.3% type B) (Grist and Reid, 1988). Diseases associated with the coxsackieviruses reported during the time period were cardiovascular disease, respiratory illness, gastroenteritis and central nervous system disorder (Grist and Reid, 1988).

Table 2-1: Enterovirus infections by enteroviral serotype (Grist and Reid, 1988)

Disease	Total %	% CVA	% CVB	% Echovirus	% Poliovirus
Respiratory illness	14.0	9.0	34.9	56.0	0.1
Central Nervous System Disorder	38.4	7.2	19.2	73.5	0.06
Cardiovascular Disorder	1.6	6.2	64.8	28.9	0.1
Gastroenteritis	16.4	8.5	25.0	66.4	0.03
Skin Disorder	4.4	48.5	13.9	37.5	0.1
Other	21.8	8.6	29.8	61.5	0.1

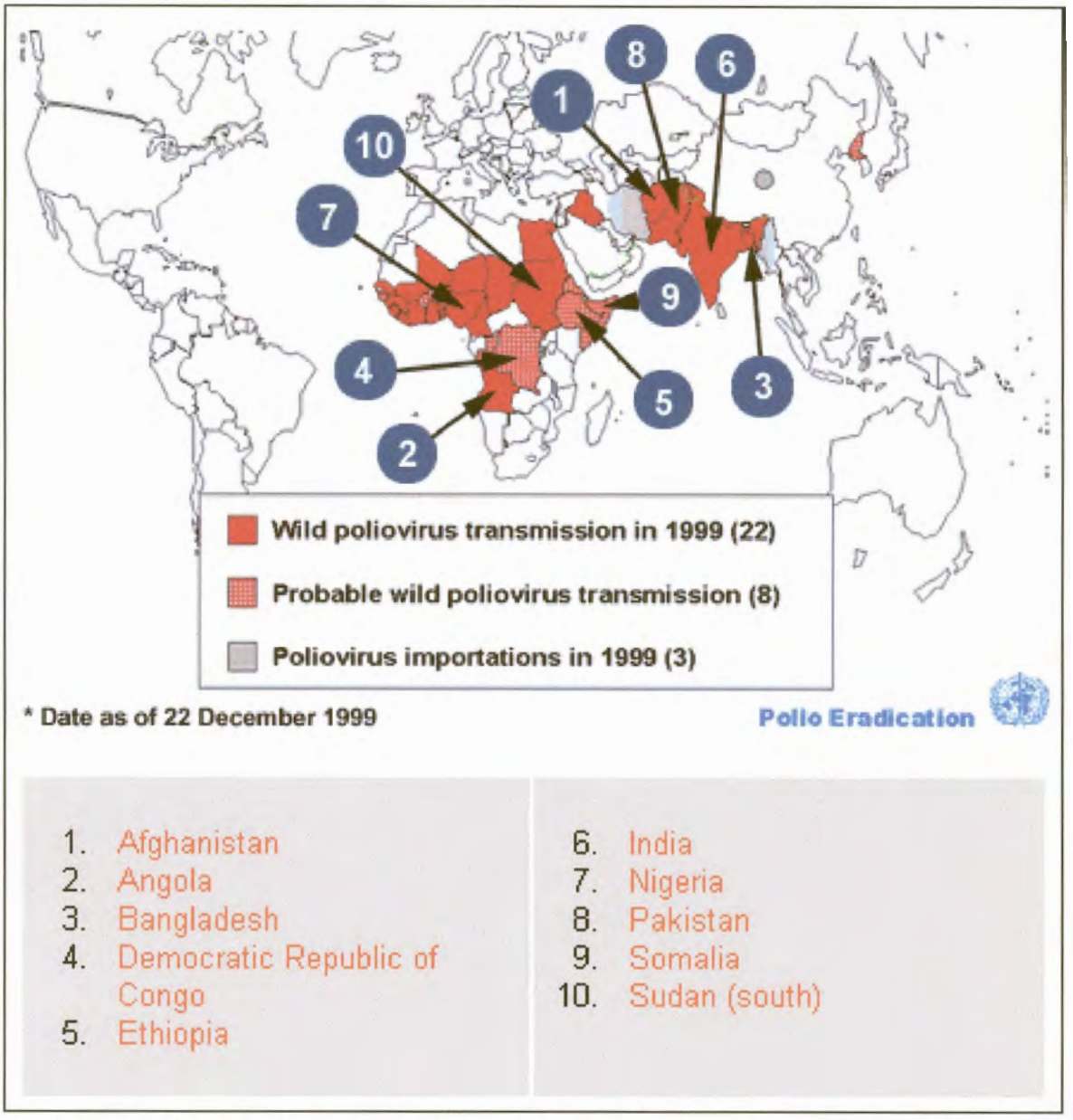

Figure 2-5: Polio endemic countries (picornaviruses at www-micro.msb.le.ac.uk).

2.5.2.1 Enterovirus Infection of the Central Nervous System

Infection of the central nervous system (CNS) occurs either by direct hematogenous spread or by axonal transport from peripheral nerves, which occurs more frequently if nerve cells or muscles are injured (Ren and Racaniello, 1992). Damage to CNS tissue results directly from viral replication and attendant cell damage and destruction, or indirectly from immune-mediated mechanisms (Muir and Van Loon, 1997). The mechanism of cell death is not known; direct virus-induced mechanisms may involve viral inhibition of cellular macromolecular synthesis, toxicity of viral polypeptides, or virus induced apoptosis (Muir and Van Loon, 1997).

2.5.2.1.1 Paralytic Poliomyelits

The cardinal feature of paralytic poliomyelitis (PPM) is acute flaccid paralysis (AFP) (Figure 2-6), resulting from lower neuron damage (Muir and Van Loon, 1997). PPM is by definition caused by PV. Other enterovirus serotypes have been associated with a similar paralytic illness, most notably CAV7 (Grist, 1962) and enterovirus type 70 (Barucha *et al.*, 1972) and 71 (Chumakov *et al.*, 1979; Takimoto *et al.*, 1998). In situations where elimination of wild poliovirus is nearing completion, cases associated with nonpolio enteroviruses may constitute a greater proportion of AFP cases.

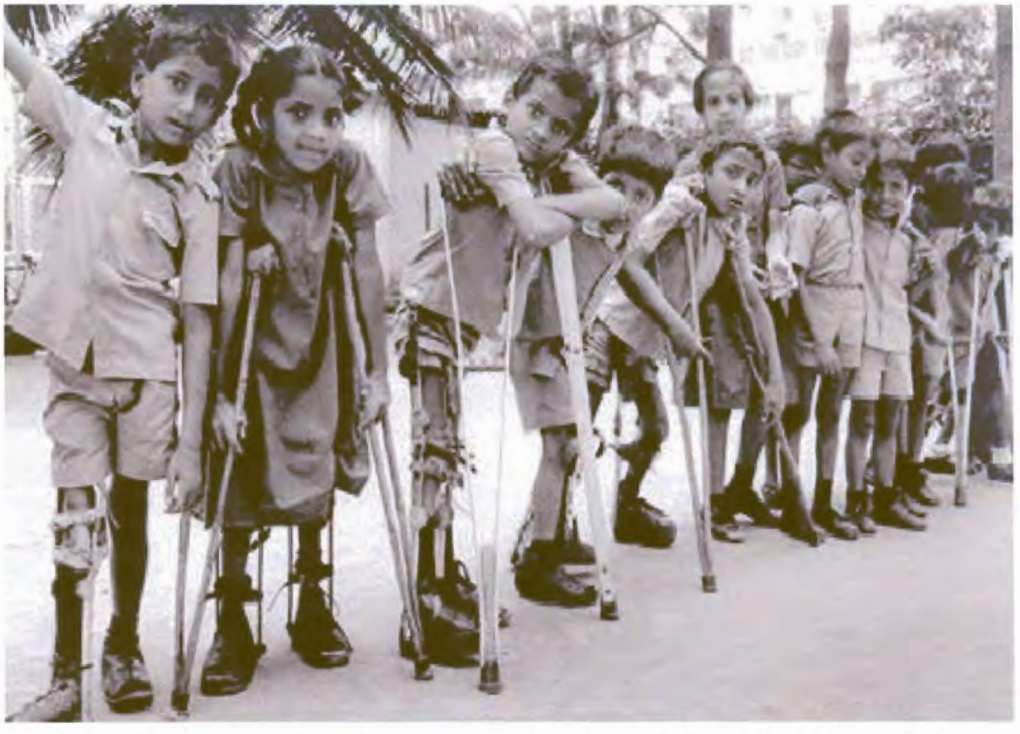

Figure 2-6: Young polio victims in a hospital in India (picornaviruses at www-micro.msb.le.ac.uk).

2.5.2.1.2 Aseptic Meningitis

Enteroviruses are the most commonly identified agents of aseptic meningitis (AM), particularly during late summer and early fall (Muir and Van Loon, 1997). Most serotypes, including PV have been associated with AM, although some serotypes are more frequently implicated than others, particularly ECV types 6, 9, 11 and 30 and the CBV (Rotbart, 1995). The significant contribution of enterovirus and CBV infection to aseptic meningitis in infants (less than two years of age) has been shown by Berlin *et al.* (1993). They attempted to establish the aetiology for hospitalized children over a five year period. There was laboratory evidence of enterovirus

infection in 62% of these cases and nearly half of laboratory diagnosed cases had evidence of CBV infection (Berlin *et al.*, 1993). Among the different CBV serotypes, types 2, 4 and 5 appear to be the most common associated with undifferentiated fever and aseptic meningitis in young infants (Berlin *et al.*, 1993). Dagan and colleagues (1988) found that the infants infected with CBV (and echovirus serotypes 11 and 30) were more likely to have meningitis than infants infected with other serotypes.

2.5.2.1.3 Encephalitis

Encephalitis due to enteroviruses is well known but thought to be an unusual complication (Rotbart, 1995). Unlike aseptic meningitis, encephalitis due to enteroviruses causes acute disease and long term sequellae (Rotbart, 1995). Enteroviruses are demonstrable by brain biopsy in 13% of patients suspected of having herpes simplex encephalitis, the most commonly identified cause of focal encephalitis (Whitley *et al.*, 1989); several of those patients had CBV serotypes. Modlin *et al.* (1991) reported four cases of focal enterovirus encephalitis and reviewed an additional three from the literature. A variety of focal neurologic findings were seen as well as abnormalities by imaging studies; one of the seven patients had CBV2 documented infection but most were coxsackievirus A serotypes (Modlin *et al.*, 1991). Agammaglobulinemic individuals infected with the enteroviruses may develop chronic meningitis or meningoencephalits lasting years, often with fatal outcome (McKinney *et al.*, 1987). The echovirus serotypes have been associated with this form of chronic meningoencephalitis but cases of CBV infection have been reported and are clinically indistinguishable from those due to other enteroviruses (McKinney *et al.*, 1987).

2.5.2.2 Cardiac Disease

Coxsackievirus type B is the most common viral etiological agent associated with heart disease (Bowles *et al.*, 1986) and is associated with > 50% of all cases of viral myocarditis (See and Tilles, 1991). In some investigations, 39% of patients infected with coxsasckievirus B5 experienced heart problems (Melnick, 1982) and coxsackievirus antigens are often found in damaged heart tissues.

Viral myocarditis in children is a significant cause of morbidity and mortality (Baboonian *et al.*, 1997). Clinical symptoms associated with CBB in children may be localized to the heart or involve multiple organs. Hilton *et al.* (1993) used in situ hybridization and PCR to investigate the presence of coxsackie virus RNA in childhood myocarditis using formalin fixed tissue. Two out of ten cases studied were positive (Hilton *et al.*, 1993). Viral RNA was predominantly associated

with areas showing inflammatory cell infiltrates and myofibre necrosis, suggesting a lytic action of the virus (Hilton *et al.*, 1993). Work by Towbin (1996) strengthened the belief that enteroviruses are one of the agents involved in myocardial disease. In a detailed study of 135 pediatric patients with myocarditis, Towbin found 23 specimens to be PCR positive for enteroviruses from the myocardium (Towbin, 1996).

Several series of autopsy, explant or endomyocardial biopsy specimens have been described in which molecular techniques were successfully applied in search of enteroviral genomes (Table 2-2) (McManus and Kandolf, 1991; Martino *et al.*, 1994). Bowles and colleagues (1986) were one of the first groups to report the detection of CBV RNA in myocardial tissue of 53% of patients with myocarditis. The detection system used did not discriminate between enteroviruses as a group from the CBV (Bowles *et al.*, 1986). Nucleotide sequencing of PCR products later confirmed the high rate of positivity and established that the detected sequences were similar to CBV3 (Khan *et al.*, 1994). Regarding pathogenic mechanisms of the development of myocardial enterovirus infection, major insights have been obtained from *in situ* hybridization studies of human autopsy hearts (Kandolf, 1998).

Table 2-2: Evidence for the association of enteroviruses with acute idiopathic myocarditis

Reference	Method used	Percentage positive	Control
Bowles *et al.*, 1986	Slot blot (enterovirus specific)	50 % (4/8)	0 % (0/4)
Jin *et al.*, 1990	RT-PCR (enterovirus specific)	7 % (2/28)	0 % (0/9)
Tracy *et al.*, 1990	In situ hybridization (enterovirus specific)	18 % (3/17)	ND
Koide *et al.*, 1992	RT-PCR (enterovirus specific)	33 % (3/9)	0 % (0/9)
Giacca *et al.*, 1994	RT nested PCR (enterovirus specific)	33 % (1/3)	3.6 % (1/28)
Satoh *et al.*, 1994	RT nested PCR (enterovirus specific)	33 % (12/36)	0 % (0/10)
Kandolf *et al.*, 1998	In situ hybridization	Not applicable	Not applicable

2.5.2.3 Respiratory Illness

Enteroviruses are responsible for approximately 15% of upper respiratory infections for which an aetiology is identified (Chonmaittree and Mann, 1995). Enterovirus serotypes were identified in respiratory infections, approximately equally divided among the major subgroups (Grist *et al.*, 1978). Coxsackievirus type A is associated with respiratory illnesses, particularly types A21 and A24 (Grist *et al.*, 1978). In some cases, coxsackievirus type A7 resulted in fatal pneumonia

(Melnick, 1982). According to Cherry (1992) type A21 is the only enterovirus that can be labelled as a "common cold virus". Bloom and colleagues, (1962) studied the association between type A21 and respiratory illness in military personnel. Twenty six percent of patients showed an association between upper respiratory infection and the virus (Bloom *et al.*, 1962). Outbreaks of coxsackievirus type B - associated respiratory illness among children have been documented (Cherry, 1992). In adults bronchopneumonia has been associated with coxsackievirus B3 and echovirus 9 (Cherry, 1992; Chonmaittree and Mann 1995).

2.5.2.4 Insulin-Dependant Diabetes Mellitus

Several findings support a link between enteroviruses and insulin-dependant diabetes mellitus (IDDM) (Barrett-Connor, 1985; Fohlman and Friman, 1993). First, coxsackievirus B3 and B4 have been isolated from the pancreas of patients with recent-onset IDDM (Yoon *et al.*, 1979; Nigro *et al.*, 1986). Secondly, genetically susceptible mice developed IDDM after infection with strains of coxsackievirus B4 (Yoon *et al.*, 1987). Thirdly, the seasonal variability of IDDM follows a pattern similar to that of enteroviruses: the peak incidence of enterovirus infections occurs in fall (Morens *et al.*, 1991), while that of IDDM appears to occur in fall and early winter (Gamble and Taylor, 1969). Forthly, a number of epidemiological studies have found an association between IDDM and antibodies to enteroviruses (Helfand *et al.*, 1995).

Approximately 50% of children with insulin-dependant diabetes mellitus have antibodies to coxsackievirus (Barrett-Connor, 1985). Toniolo *et al.* (1988) have reported that 33.3% of all cases of IDDM are preceded by coxsackievirus type B infections. Several studies have been conducted that investigated the association between coxsackievirus infections and the development of IDDM. Mertens *et al.* (1983) studied 166 IDDM patients who had experienced a coxsackievirus infection prior to the onset of IDDM. Eighty percent of these patients had neutralizing antibodies to coxsackievirus type B (Mertens *et al.*, 1983). In a prospective multicentre study on pediatric events in siblings of index cases with insulin dependent diabetes mellitus, 31 children developed clinical diabetes during the observation period (Mertens *et al.*, 1983). Fifty-one of these children seroconverted for islet cell antibodies or insulin autoantibodies (Mertens *et al.*, 1983). By using non-serotype specific EIA and RIA, it has been shown that enterovirus infections in both groups were associated with increases of islet cell antibody and/or insulin autoantibody titres (Mertens *et al.*, 1983). Serum specimens sequentially collected from twelve children were tested for serotype specific neutralizing antibodies (Roivainen *et al.*, 1998). Plaque neutralization assays were carried out for coxsackievirus A9,

coxsackievirus B types 1 to 6 and echovirus types 1 and 11 (Roivainen *et al.*, 1998). An unequivocal monotypic increase in neutralizing antibodies was observed on seven occasions in six children (Roivainen *et al.*, 1998). The following results were obtained: one coxsackievirus A9, one coxsackievirus B1, two coxsackievirus B2, two coxsackievirus B3 and one coxsackievirus B5 (Roivainen *et al.*, 1998). These results suggested that the association of enterovirus infections with insulin-dependant diabetes mellitus is not restricted to serotype 4 of coxsackie B viruses as suspected previously, but that several different serotypes play a role in the pathogenisis of the disease (Roivainen *et al.*, 1998).

2.5.3 Epidemiological Monitoring

Enteroviruses have a worldwide distribution (Melnick, 1996). Within a given geographic locality, some serotypes may be endemic, with little change in the range of serotypes present from year to year (Melnick, 1996). In temperate climates there is an increased circulation in summer and early fall (Moore, 1982). In contrast, some serotypes may be introduced periodically, causing epidemics, with few isolations reported in intervening years (Moore, 1982). Transmission may be directly from person to person via faecal-oral spread or contact with pharyngeal secretions (Melnick, 1996).

Transmission of enteroviruses may be by air, contact with vomits and by water (Melnick, 1996). Enterovirus infections are known to occur in communities with susceptible individuals such as newborne wards and children's day care centres (Melnick, 1996). Unlike influenza viruses, enteroviruses exceptionally cause pandemics (Melnick, 1996), but tend to be geographically restricted in transmission (Rico-Hesse *et al.*, 1987). Since most enterovirus infections are subclinical or cause minor non-specific symptoms, data based on isolates from hospitalized patients does not reflect overall circulation of different serotypes in human populations. The latter is becoming more interesting with accumulating evidence of the role of coxsackie B and other enterovirus infections in the pathogenesis of insulin-dependant diabetes (Hyöty *et al.*, 1995).

Epidemiological surveillance plays an important role in the control of infectious diseases. Information on the serotypes of enteroviruses detected in clinical and environmental samples has formed an important part of surveillance programs. Such information may help in understanding changing patterns of enterovirus infection and disease association, and may facilitate the rapid diagnosis of enterovirus infections. This may reduce unnecessary hospitalisation and other medical expenditure and allow immune globulin batches with high

titers of frequently circulating serotypes to be reserved for intravenous therapy of neonates or immunodeficient patients (Muir, 1998).

One thousand one hundred and sixty-one non-polio enterovirus strains, isolated during regular screening of Finnish sewage specimens were analysed for serotype distribution seasonally over a 20 year period (Hovi *et al.*, 1996). The findings were compared with similar data based on 1681 clinical isolates (Hovi *et al.*, 1996). Coxsackievirus B4 (CBV4), CBV5, echovirus 11 (ECV11), ECV6, CBV2 and CBV3 were the most common serotypes in sewage, whilst CBV5, ECV11, coxsackievirus A9, ECV22, CBV3 and ECV30 were the most common clinical isolates (Hovi *et al.*, 1996). Seasonal variation in enterovirus occurance in both sources showed an expected peak in autumn with a decrease in spring (Hovi *et al.*, 1996). The occurrence of enteroviruses correlated with monthly recordings of mean relative humidity (Hovi *et al.*, 1996). A further observation concerning clinical specimens in Finland was the relative excess of serotypes, such as echovirus 22 and coxsackievirus A9, and paucity of others, for instance, echovirus 4 and 9, when compared to published data from other countries (Grist *et al.*, 1978; Strikas *et al.*, 1986). This was consistent with the idea of geographically restricted circulation of enteroviruses (Hovi *et al.*, 1996).

In the United States, enterovirus surveillance data are collected and analysed by the Centres for Disease Control and Prevention. The data have been reported since the beginning of the program in 1961 (Moore, 1982). During the period 1961 - 1995 there were four major epidemics of CBV5 in the United States, in 1961, 1967, 1972 and 1983 (Moore, 1982). By contrast, endemic viruses are isolated nearly every year, with minor differences (Moore, 1982). This is the pattern seen with CBV2 and CBV4, although larger outbreaks do occur occasionally, as with CBV3 in 1980 (Moore, 1982).

Molecular epidemiologic studies, using sequencing have been reported for CBV1 and CBV5 (Drebot *et al.*, 1994; Zoll *et al.*, 1994; Kopecka *et al.*, 1995). The study of the CBV5 isolates examined the pattern of genetic changes over three separate outbreaks in the United States. The nucleotide sequences from multiple isolates from the epidemics showed that each of the epidemics was caused by a single genotype (Pallansch, 1997). The genotype of CBV5 observed in the 1967 epidemic showed more similarity to the virus observed in the 1983 epidemic than to viruses isolated during the intervening years (Pallansch, 1997).

Nairn *et al.* (1999) conducted a study in which the number and range of enteroviruses isolated in the Regional Virus Laboratory, Glasgow during 1977 - 1997 were determined. During this

period, 3 039 enterovirus isolations were reported (Nairn *et al.*, 1999). The echoviruses represented 67% of isolations with echovirus 4 (due to an outbreak in 1990), echovirus 30 and echovirus 11 being the most frequently isolated types (Nairn *et al.*, 1999). The pattern of prevalence of non-polio enteroviruses had changed from the previous 20 year period with echovirus types isolated more frequent (77% vs. 55.4%) and coxsackieviruses isolated less often (23% vs. 44.6%) (Nairn *et al.*, 1999). The 5' untranslated region (UTR, bases 63 - 475) and the VP4/VP2 region (bases 581 – 1 199) of selected echovirus 30 and coxsackie B3 isolates were sequenced (Nairn *et al.*, 1999). Sequences represented endemic and epidemic types respectively and were shown to be closely related within their type, but different from the published sequences (Nairn *et al.*, 1999). The current echovirus 30 strains differed from the 1966 isolates by 16% - 20% in both the 5' UTR and VP4/VP2 region (Nairn *et al.*, 1999). The coxsackie B3 isolates, which caused a small outbreak in 1997 (after a 5 year absence) differed by 27% from previous isolated strains (Nairn *et al.*, 1999).

Large outbreaks of aseptic meningitis due to echovirus have been observed in Japan (type 9 in 1997 and type 30 in 1998) (Akasu, 1999; Yoshida *et al.*, 1999), Germany (type 30 in 1996) (Vieth et al., 1999), France (type 30 in 1997) (Chambon *et al.*, 1999), Switzerland (type 30 in 1996) (Schumacher *et al.*, 1999) and Israel (type 4 in 1997) (Handsher *et al.*, 1999). In the latter, it appeared that the causative agent was a new varient of echovirus type 4 that had not previously circulated among the population (Handsher *et al.*, 1999).

In June 1997, 21 children from a single community in Germany were hospitalised with aseptic meningitis (Reintjes *et al.*, 1999). Reintjes *et al.* (1999) conducted an epidemiological investigation to determine the extent of the outbreak and risk factors for illness. Echovirus 30 caused substantial morbidity during this community outbreak (Reintjes *et al.*, 1999). Household contacts, day-care centres and playgrounds were prominent risk factors for transmission (Reintjes *et al.*, 1999).

An outbreak of enterovirus 71 (EV71) infection occurred in Taiwan in 1998 (Wang *et al.*, 1999). The clinical spectrums and laboratory findings for 97 patients with virus culture-proven to be EV71 infections were analysed (Wang *et al.*, 1999). Eighty seven percent of patients were younger than 5 years (Wang *et al.*, 1999). Hand-foot-and-mouth syndrome occurred in 79% of the children and central nervous system involvement in 35%, including nine fatal cases (Wang *et al.*, 1999). The predominant neurological presentations were myoclonus (68%), vomiting (53%), and ataxia (35%) (Wang *et al.*, 1999). Brain stem encephalitis was the cardinal feature of EV71 CNS involvement during this outbreak (Wang *et al.*, 1999).

Little is known about population immunity to EV71 and the circumstances that predispose to the development of an epidemic. Before the Taiwan epidemic, 50% of adults had antibody to EV71 (Pollard and Dobson, 2000). Examination of the EV71 isolates from the 1997 Malaysian outbreak showed that the EV71 5'UTR was distinct from those of previous isolates, but there were no differences between severe and trivial cases (AbuBakar *et al.*, 1999), suggesting that viral mutation might have triggered the outbreak. Molecular epidemiology of EV71 in North America has demonstrated that the virus is rapidly evolving and is genetically diverse; a finding that suggests new epidemics are inevitable (Brown *et al.*, 1999).

2.5.4 Identification of New Enterovirus Types of Variants

The ability to recognize previously unknown enterovirus types (Barucha *et al.*, 1972; Schmidt *et al.*, 1974) provides a basis for understanding changing patterns in the epidemiology and clinical manifestations of enterovirus infections. This has been of particular importance when the new type may cause poliomyelitis-like illness (Kono *et al.*, 1977; Chumakov *et al.*, 1979). Molecular detection methods are increasingly accepted as a means of identifying and characterizing previously unknown, noncultivable microorganisms and establishing their etiologic relationship to disease (Fredericks and Relman, 1996). This approach may identify additional enterovirus types. One study identified novel enterovirus sequences, possibly representing previously unrecognised enterovirus types, in patients with chronic fatigue syndrome (Clements *et al.*, 1995; Galbraith *et al.*, 1995).

2.6 Conventional Methods for Diagnosis and Typing of Enteroviruses

Conventional diagnostic methods for human picornavirus infections include culturing of the virus in susceptible cell lines, followed by neutralization typing (Melnick and Wimberley, 1985). Serological detection of enterovirus-specific antibodies provides indirect evidence of the etiology of the disease. The isolation methods currently used for enterovirus identification are cumbersome, laborious and insensitive (Santti *et al.*, 1999).

2.6.1 Virus Isolation

Virus isolation has been regarded as the "gold standard" for enterovirus identification (Morens *et al.*, 1991). These procedures are however poorly standardized and virus isolation data may vary considerably between laboratories (Morens *et al.*, 1991). Sensitivity is highly dependant on the type and quality of the specimen, the timing of specimen collection and storage before arrival in

the virus laboratory (Morens *et al.*, 1991). The choice of cell types used for enterovirus isolation is also important, because no single cell line currently in use supports the growth of all known enterovirus serotypes (Rotbart, 1995). To ensure maximal isolation efficiency, combinations of cell lines are generally used (Grabow *et al.*, 1999). In monolayers of cultured cells, the growth of enteroviruses is generally associated with a characteristic cytopathogenic effect (CPE).

The usual host range of human enteroviruses, regarding both cell cultures and laboratory animals is summarised in Table 2-3. Most of the common cytopathogenic enterovirus serotypes can be grown in primary and passage cultures of monkey kidney cells (Melnick, 1996). However, if recovery of all possible types is being attempted from clinical specimens, at least one human tissue culture system (WI-38 or HEK cells) should be included (Cooney *et al.*, 1972). The Buffalo green monkey (BGM) line of African green monkey kidney cells has been reported as being more sensitive than primary rhesus or green monkey kidney cells for titration of certain enterovirus types and for recovery of plaque forming enteric viruses from sewage and water (Menegus and Hollick, 1982).

Coxsackie A virus serotypes are difficult to grow in cell culture (Schmidt *et al.*, 1975). Most have been propagated in Rhabdosarcoma (RD) cells (Schmidt *et al.*, 1975), but isolation from clinical material is often unsuccessful. The use of suckling mice for isolation of CAV has been more successful, but because of practical difficulties this technique is rarely used. As a consequence, CAV infections are probably underdiagnosed and underrepresented in epidemiological surveys.

Table 2-3: Usual host range of human enteroviruses: Animal and tissue culture spectrum (Ginsberg *et al.*, 1990).

Virus	Antigenic Types	Cytopathogenic effect (CPE)		Illness and Pathology	
		Monkey kidney tissue culture	Human tissue culture	Suckling mouse	Monkey
Polioviruses	1-3	+	+	-	+
Coxsackieviruses group A	1-24	+/-	+/-	+	-
Coxsackieviruses group B	1-6	+	+	+	-
Echoviruses	1-34	+	+/-	-	-
Enteroviruses	68-71	+	+	-	-

Other factors affecting the efficiency of isolation are the physiological condition of the cells and whether blind passages are performed (Lipson *et al.*, 1988). Blind passages are necessary in some cases before a cytopathic effect in cell culture or paralysis in suckling mice becomes apparent

(Lipson *et al.*, 1988). This may be due to low initial titer of virus or the requirement for adaptation to growth in the isolation system (Lipson *et al.*, 1988). Under optimal conditions, a positive enterovirus isolation can be reported within a few days, but a presumptive diagnosis may require more than 14 days when dealing with difficult isolates and even longer for samples containing mixtures of viruses (Melnick, 1996).

2.6.2 Enterovirus Serotyping

For identification of serotype, neutralization of the isolated virus is performed using specifically formulated pools of antibodies (Melnick and Wimberley, 1985). Enterovirus isolates are incubated with each antisera pool and then re-inoculated onto susceptible cells (Melnick and Wimberley, 1985). After incubation for several days the incubation pattern is recorded. From this, the enterovirus serotype can be inferred, since pools are designed so that neutralization patterns are distinct for each individual serotype (Melnick and Wimberley, 1985). Finally, the suspected enterovirus type can be confirmed by neutralization with the type-specific single antiserum (Melnick and Wimberley, 1985).

The Lim Benyesh-Melnick (LBM) pool scheme consists of eight pools (designated A to H) containing antisera to 42 different enterovirus types (Lim and Benyesh-Melnick, 1960; Melnick *et al.*, 1973). Antibodies against 19 additional CAV serotypes in seven additional pools (designated J to P) can be used if identification with pools A to H cannot be achieved (Melnick *et al.*, 1977).

There are several drawbacks to the use of intersecting pools for typing of enteroviruses. First the method is time-consuming, labour intensive and costly. Secondly, the supply of antisera is limited and WHO has advocated a conservative approach to the use of pools, which should not be used to type every clinical isolate (Melnick and Benyesh-Melnick, 1985). Thirdly the problem with untypeable enteroviruses is frequently encountered (Morens *et al.*, 1991). There are several reasons for this: (i) untypeable isolates may contain mixtures of enteroviruses (Van Loon *et al.*, 1999), (ii) not all enterovirus serotypes can be identified with intersecting pools (CAV3, 11, 15, 17, and 24 and enterovirus types 68 - 71 cannot be typed using LBM pools), (iii) enteroviruses sometimes form aggregates that cannot be neutralized (Kapsenberg *et al.*, 1980), (iv) the isolates may be prime strains. These are antigenic variants of recognised serotypes which are neutralized poorly or not at all by antiserum to the homologous prototype strain (Melnick, 1996), (v) failure to type isolates may represent a new or previously unrecognised enterovirus type (Melnick, 1996).

2.7 Enterovirus Detection by the Polymerase Chain Reaction

The technique of enzymatic amplification and the widespread use of heat-stable DNA polymerases have simplified the task of detecting enteroviruses. By using repeated cycles of PCR, a 10^6-fold amplification of target DNA can be completed within a few hours. The decreased time and cost and the increased sensitivity of PCR facilitate the detection of low numbers of target DNA and RNA found in clinical and environmental samples (Hyypia *et al.*, 1989; DeLeon *et al.*, 1990; Rotbart, 1990; Zoll *et al.*, 1992; Abbaszadegan *et al.*, 1993; Kopecka *et al.*, 1993; Muir *et al.*, 1993; Abbaszadegan *et al.*, 1999).

PCR has become an important diagnostic tool in both clinical and environmental applications to detect enteroviruses (Hyypia *et al.*, 1989; DeLeon *et al.*, 1990; Rotbart, 1990; Zoll *et al.*, 1992; Abbaszadegan *et al.*, 1993; Kopecka *et al.*, 1993; Muir *et al.*, 1993; Abbaszadegan *et al.*, 1999). The design of the enteroviral genome and studies designed to locate probe sequences useful for the detection of enteroviruses suggested that the relatively well-conserved 5'-nontranslated region of the genome is the most logical target sequence for the detection of the greatest number of human enteroviruses.

PCR is not without problems. For environmental water samples, direct PCR must be able to detect viruses after concentration from large volumes (100 - 1000 l) of water (APHA, 1989). Concentrated compounds, such as humic substances and metals can interfere with enzymes used in PCR (Santos and Gouvea, 1994). Substances interfering with reverse transcription or with PCR amplification have been described (Santos and Gouvea, 1994), especially when stool or sewage is the sample to be tested. In addition, the viability or infectivity of viruses in a water sample is an important issue and cannot be determined by direct PCR alone (Egger *et al.*, 1995; Reynolds *et al.*, 1997; Grabow *et al.*, 2000). Although PCR allows the detection of viruses which fail to grow in available cell culture systems, amplification of non-cytopathogenic viruses can be misleading since their presence may not constitute a significant health risk (Grabow *et al.*, 2000). Furthermore, PCR is limited by small reaction volumes (Ma *et al.*, 1995). Typical PCR samples are 10 - 50 µl whereas typical water sample concentrates are 20 - 30 ml. Therefore, it would be impractical to evaluate the entire sample using direct PCR, but this can be done easily using cell culture. The use of a combined cell culture/PCR technique utilises the major advantages of each separate methodology while overcoming the disadvantages (Egger *et al.*, 1995; Reynolds *et al.*, 1997; Grabow *et al.*, 2000). Samples are first assayed on cells for 24 – 48 h followed by PCR on cell harvests prior to CPE (Egger *et al.*, 1995; Reynolds *et al.*, 1997). The combined cell culture

and molecular techniques increased the PCR equivalent volume, reduced the effects of toxicity in cell culture and reduced inhibitory effects on the PCR. This combined techniques maximise the detection of infectious viruses with the speed and specificity of direct PCR (Egger *et al.*, 1995; Reynolds *et al.*, 1997; Grabow *et al.*, 2000).

If the amount of foreign nontarget DNA is high, carrying out a second, nested or semi-nested PCR by changing both or one of the primers enhance the specificity of the assay (Kuan, 1997). Nested PCR (n-PCR) is a more sensitive and time-saving technique (Severini *et al.*, 1993) than those employing PCR only or a combination of PCR and hybridization (Kämmerer *et al.*, 1994; Kuan, 1997). Andreoletti *et al.* (1996) described a rapid method of enteroviral RNA detection in clinical samples using PCR and a microwell capture hybridization assay. The sensitivity and specificity of this procedure was compared with an improved semi-nested PCR assay (Andreoletti *et al.*, 1996). PCR-microwell hybridization was shown to be as sensitive as semi-nested PCR and capable of detecting 0.01 plaque forming units (PFU) per 100 µl of biological sample with a result obtained within 8 h (Andreoletti *et al.*, 1996). A multi-centric evaluation of a commercial kit by Gantzer *et al.* (1999) showed that the Amplicor kit (Roche) is well suited for the detection of enterovirus genomes in treated waste water. The results are comparable to results obtained with semi-nested PCR (Gantzer *et al.*, 1999). However, the Amplicor kit technique is simpler and has the advantage of providing a standardized technique useful for comparative studies (Gantzer *et al.*, 1999).

Muir *et al.* (1999) conducted a multicenter evaluation of commercial and in-house PCR methods for the detection of enteroviruses. Three coded panels of test and control RNA samples, artificial clinical specimens and representative enterovirus serotypes were used to assess amplification methods, RNA extraction methods and reactivities with different serotypes (Muir *et al.*, 1999). Despite several differences between PCR methods, good agreement, with some variation in sensitivity was observed (Muir *et al.*, 1999). Most PCR methods were able to detect enterovirus RNA from at least 1 $TCID_{50}$ of enteroviruses in cerebrospinal fluid, stool, or throat swab specimens (Muir *et al.*, 1999). The PCR methods were able to detect a wide range of serotypes, although serotypic identification was not possible (Muir *et al.*, 1999). Some laboratories experienced false-positive results due to PCR contamination, which appeared to result from cross-contamination of specimens during RNA extraction (Muir *et al.*, 1999). Provided that this problem is overcome, these PCR methods will be sensitive and rapid alternatives to cell culture for diagnosis of enteroviral infection. Mechanisms for quality

assessment will be required to ensure that test sensitivity, specificity and standardization are maintained (Muir *et al.*, 1999, Van Loon *et al.*, 1999).

2.8 Molecular Basis of Molecular Typing for Enteroviruses

Development of a molecular typing system for enteroviruses requires an understanding of the structure and function of the enterovirus genome and knowledge of the variability of genome sequences among enteroviruses (Muir, 1998). There is a high degree of phylogenetic similarity among enterovirus serotypes in most genomic regions (Muir, 1998). The regions showing the greatest divergence among serotypes also show greater variability within serotypes (Muir, 1998). The genomic regions that should be targeted for sequence analysis will be determined by the objectives of molecular typing.

2.8.1 Enterovirus Genome Structure

The enterovirus genome is a single-stranded RNA molecule, approximately 7 500 nucleotides long and of positive polarity (Figure 2-3) (Muir, 1998). An approximately 750-nucleotide 5' UTR is followed by a long open reading frame coding for an approximately 2 100-amino-acid polyprotein. This is followed by a short 3'UTR and a poly(A) tail (Muir, 1998). The enterovirus genome structure is detailed in section 2.3.

2.8.2 Genetic Relationships among Human Enteroviruses

Complete genome sequences for a number of human enterovirus serotypes have been published (Nomota *et al.*, 1982; Hughes *et al.*, 1986; Chang *et al.*, 1989; Hughes *et al.*, 1989; Hyypia *et al.*, 1992; Supanaranond *et al.*, 1992; Gratsch and Righthand, 1994; Poyry *et al.*, 1994; Brown and Palansch, 1995; Dahllund *et al.*, 1995; Kraus *et al.*, 1995). While sequence comparisons partially support the classical subgrouping of enterovirus serotypes into PV, CAV, CBV and ECV, the genetic relationships do not correlate with this devision (Muir, 1998). Nucleotide sequence comparison of several genomic regions indicate that while serotypes within the PV and CBV groups are genetically related, CAV and ECV are genetically diverse subgroups (Muir, 1998).

The UTRs are highly conserved parts of the enterovirus genome (Stanway *et al.*, 1984; Toyoda *et al.*, 1984). The conserved regions within the 5'UTR have been exploited as primer and probe recognition sequences for broadly reactive diagnostic assays (Muir, 1998). Recent studies have indicated that human enterovirus genomes can be phylogenetically divided into two distinct groups in the 5' noncoding region (nt 1 to 750); PVs, CAV21, CAV24 and enterovirus 70 belong

to group I, while all sequenced Evs, CBVs, CAV9, CAV16 and enterovirus 71 belong to group II (Poyry *et al.*, 1994; Poyry *et al.*, 1996; Hyypia *et al.*, 1997) (Figure 2-7).

Each of the two clusters based on sequence similarity in the 5'UTR can be further subdivided into two separate clusters based on nucleotide and amino acid sequence similarity in the coding region (Muir, 1998). When the partial sequence of VP2 protein is compared, CAV2, CAV3, CAV5, CAV7, CAV8, CAV10, CAV14, CAV16, and EV 71 form one cluster (A); all CBV serotypes, CAV9, ECV11, ECV12 and EV69 form another cluster (B); all PV serotypes, CAV1, CAV11, CAV13, CAV17, CAV18, CAV20, CAV21, and CAV24 form the third cluster (C); and EV 68 and EV 70 form the fourth cluster (D) (Pulli *et al.*, 1995) (Figure 2-8). The clustering of enteroviruses is the same regardless of which protein is used in the comparison, provided that complete protein-coding sequences are used in the analysis (Poyry *et al.*, 1996).

Sequence variation is the highest in VP1, which codes for the major antigenic sites and the most type-specific neutralization determinants (Oberste *et al.*, 1999). Sequencing studies (Oberste *et al.*, 1999) of VP1 confirmed the presence of sequence domains that are conserved among all members of the enterovirus genus, as well as intervening domains that vary in sequences between strains of different serotypes and in some cases within a serotype. Phylogenetic trees constructed from complete VP1 sequences produced the same four clusters as published trees based on VP2 sequences (Figure 2-8) (Oberste *et al.*, 1998a). In contrast to the VP2 trees, the VP1 tree strains were monophyletic (Oberste *et al.*, 1999). In pairwise comparisons of complete VP1 sequences, enteroviruses of the same serotype were distinguished from those of heterologue serotypes and the limits of intraserotypic divergence appeared to be 25% nucleotide sequence difference or 12% amino acid sequence difference (Oberste *et al.*, 1999). Molecular assays directed to specific sequences in VP1 have been applied to serotyping, genotyping and group identification of polioviruses (De *et al.*, 1995; De *et al.*, 1997; Kilpatrick *et al.*, 1998). Based on the success of poliovirus molecular diagnostics targeted at VP1 and the shortcomings of current molecular methods for identifying non-poliovirus enteroviruses (Muir, 1998), Oberste *et al.* (1999) suggested that future molecular development efforts should be targeted at the genomic region encoding VP1.

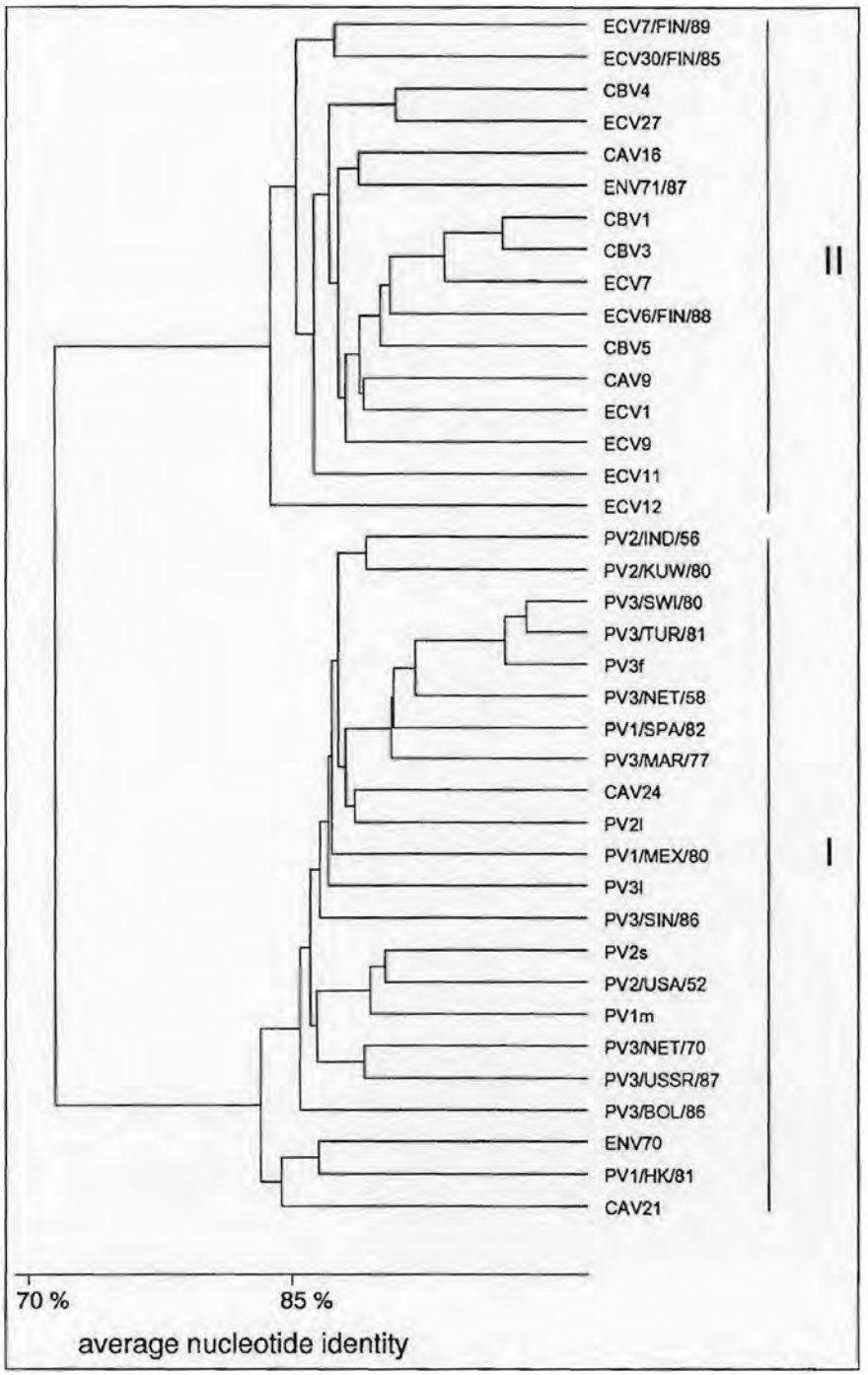

Figure 2-7: Dendrogram based on the nucleotide identity of the 5'UTR of the enterovirus genome (Muir, 1998).

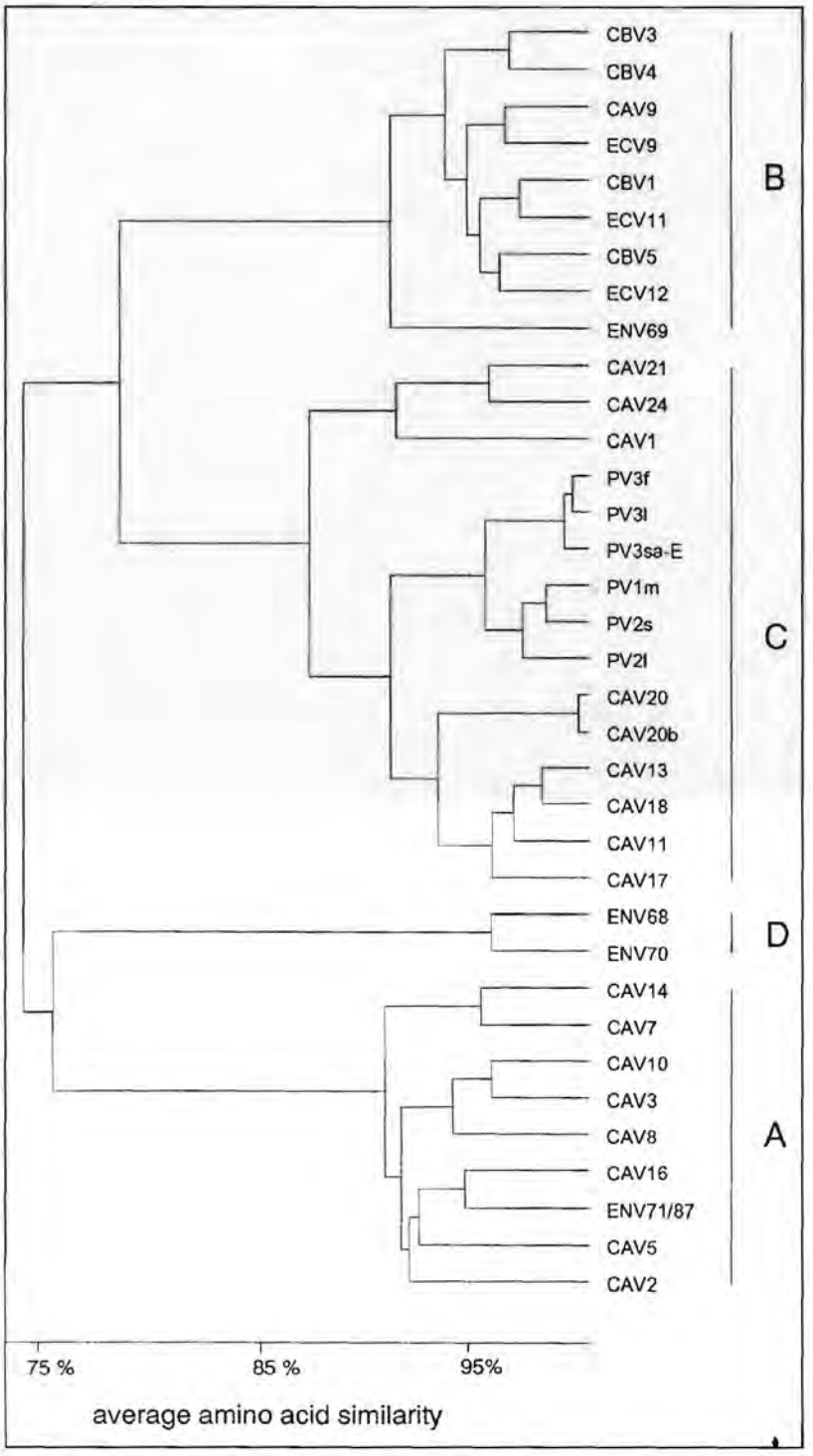

Figure 2-8: Genetic relationship among human enteroviruses. Dendrogram based on amino acid similarity of a 70-amino-acid sequence in the junction of VP4 and VP2 (Muir, 1998).

The non-structural proteins show considerably less variation than the structural proteins (Muir, 1998). While variation in the capsid proteins may be advantageous, enabling the virus to escape

from host immune responses, variation in the non-structural proteins, might be deleterious (Muir, 1998). In general, amino acid similarity in non-structural proteins is high, usually up to 90% within a cluster, being the highest in the 3D protein, which is the RNA polymerase (Muir, 1998).

Clustering of the 3'UTR is the same as that seen in the coding region, with a minimum nucleotide identity of 76% within a cluster (Poyry *et al.*, 1996). The most prominent variation between the clusters is its length: PV-like viruses have the shortest 3'UTR (69 – 72 nucleotides), while the CBV-like viruses have the longest (102 – 106 nucleotides) and CAV 16-like viruses and EV 70 have an intermediate length 3'UTR (82 – 86 nucleotides) (Poyry *et al.*, 1996). A similar secondary structure can be predicted for all members in each cluster, although there are minor differences within each cluster (Poyry *et al.*, 1996). It seems that the need to maintain some identical nucleotides as well as the secondary structure limits the variation in the 3'UTR (Poyry *et al.*, 1996).

2.8.3 Genotypic Classification of Enteroviruses

Human enteroviruses can be classified into two major phylogenetic groups based on the 5'UTR sequence similarity and into four major groups based on similarity in the coding region or the 3'UTR (2.8.2). A PCR assay combined with amplicon sequencing, which allows enteroviruses to be assigned to one of the four clusters has been described (Arola *et al.*, 1996). It has been suggested that this should form the basis of an alternative genetic classification of enteroviruses (Pulli *et al.*, 1995; Arola *et al.*, 1996; Hyypia *et al.*, 1997). It is notable that polioviruses cannot be discriminated from CAV's in the C cluster by this means and other genomic regions are required for this purpose (Oberste *et al.*, 1999). Oberste *et al.* (1999) determined the homologue partial VP2 sequences for the 12 prototype strains for which the VP2 sequence was unavailable and for eight well-characterized antigenic variants. Phylogenetic analysis of all prototype strains produced four major groups, consistent with published enterovirus phylogenies (Arola *et al.*, 1996). Many antigenic variants failed to cluster with their respective prototype strains, suggesting that this portion of VP2 may be inappropriate for consistent molecular inference of serotype and for detailed study of enterovirus evolution (Oberste *et al.*, 1999).

Studies on the three serotypes of poliovirus have shown that a partial VP1 sequence correlates well with PV serotype (Kilpatrick *et al.*, 1998). To test whether the VP1 sequence might be applied to the classification of nonpolio enteroviruses and to the analysis of phylogenetic relationships among the human enteroviruses, Oberste *et al.* (1999) determined the complete

nucleotide sequences of 47 human enterovirus prototypes and 10 well-characterised antigenic variants. These data, together with previously available sequences, comprise a database of complete VP1 sequences for all known human enterovirus serotypes and 12 natural antigenic variants (Oberste *et al.*, 1998a). Phylogenetic trees constructed from complete VP1 sequences produced the same four major clusters as published trees based on partial VP2 sequences (Oberste *et al.*, 1998). VP1 sequences correlated better with the serotype than do the sequences of either the 5'UTR or the VP4-VP2 junction (Oberste *et al.*, 1999). Oberste *et al.* (1999) showed that there is a 100% correlation between the nucleotide sequence of the 3' half of VP1 and antigenic typing by the standard neutralization test for clinical isolates of various serotypes. They have developed a molecular typing system based on reverse transcription-PCR and nucleotide sequencing of the 3' half of the genomic region encoding VP1 (Oberste *et al.*, 1999). This method reduced the time required to type an enterovirus isolate and can be used to type isolates that are difficult or impossible to type with standard immunological reagents (Oberste *et al.*, 1999). The technique is useful for the rapid determination of whether viruses isolated during an outbreak are epidemiological related (Oberste *et al.*, 1999).

In the 5' UTR the clustering of enterovirus serotypes is different and does not provide a useful tool for typing, but can be used in some epidemiological studies (Nicholson *et al.*, 1995; Muir *et al.*, 1996; Zhang *et al.*, 1997; Santti *et al.*, 1999). Some researchers have attempted to identify enterovirus serotypes on this basis, for example, by analyzing PCR products from the 5' UTR generated with enterovirus group-reactive primers by high-stringency hybridization (Chapman *et al.*, 1990), PCR-restriction fragment length polymorphism analysis (RFLP) (Kämmerer *et al.*, 1994; Nicholson *et al.*, 1994; Kuan, 1997), PCR-single strand conformation polymorphism (SSCP) analysis (Fujioka *et al.*, 1995) or nucleotide sequence determination (Nicholson *et al.*, 1995; Muir *et al.*, 1996; Zhang *et al.*, 1997). A drawback of RFLP analysis is that single point mutations within the recognition sequence may give rise to unclassifiable results (Kuan, 1997). The use of SSCP for the analysis of enterovirus genotypes by using PCR products from the 5' UTR has been described (Fujioka *et al.*, 1995). Different electrophoretic profiles were reported for 14 enterovirus serotypes investigated (Fujioka *et al.*, 1995). However this method is susceptible to minor sequence variation (Muir, 1998). Furtermore, SSCP analysis requires additional equipment and is time-consuming (Muir, 1998). PCR-RFLP or PCR-SSCP could allow the rapid identification of all PCR-positive samples with an identical pattern ("the outbreak pattern"), thus yielding valuable epidemiological information and reducing the requirement for complete nucleotide sequence analysis (Muir, 1998).

Holland *et al.* (1998) developed and standardized a computerized method for the typing and characterization of enteroviruses with radiolabeled viral protein fingerprints. Each serotype has a discrete protein pattern and the most important pattern differences for determining each type are in the region of the viral capsid proteins, VP1, VP2 and VP3 (Holland *et al.*, 1998). Their results showed that most of the strains currently isolated from the community have protein patterns different from those of their older prototype strains (Holland *et al.*, 1998). The method was specific and 97% accurate, but the radiolabeled proteins generate radioactive waste and the method requires the use of specialized instrumentation for data acquisition and analysis (Oberste *et al.*, 1999). In addition, the database of protein patterns available for comparison contains representatives of fewer than one-third of the 66 known human EV serotypes (Oberste *et al.*, 1999).

Antigen capture (AC) PCR is a technique that combines PCR with immune affinity concentration and purification of viruses (Shen *et al.*, 1997). Shen *et al.* (1997) reported the use of AC-PCR to detect and type human enteroviruses. The system combines advantages of culture/neutralization with those of PCR based assays by providing a rapid method of detecting and serotyping enteroviruses. In addition, the immune capture step is a procedure reported to purify virus (Jansen *et al.*, 1990).

Molecular methods for intratypic differentiation of PV currently used by the WHO polio laboratory network include RNA probe hybridisation, strain specific PCR, and PCR-RFLP analysis (Muir, 1998). In a study comparing the reliability of these and currently used serological methods in five WHO laboratories with a coded panel of wild and vaccine strain viruses, all the methods gave correct results in 91.9 to 97.8% of the tests (Van der Avoort *et al.*, 1995). However, only the enzyme immunoassay was able to detect and confirm the presence of both Sabin-like and wild non-Sabin-like viruses in samples containing mixtures of vaccine and wild PV. It was recommended that at least two methods of intratypic differentiation based on fundamentally different principles (e.g. antigenic differences or sequence differences) be used (Van der Avoort *et al.*, 1995). The use of molecular biological methods for both detection and intratypic differentiation of PV in clinical and environmental samples may lead to further improvement in the streamlining, standardization and sensitivity of test procedures (Muir, 1998). This would require an assay that detects all PV types and strains, but not other enteroviruses, to identify PV containing samples for subsequent intratypic differentiation. A number of methods have been described. Using primers located in the 5'UTR designed to selectively amplify PV, Abraham *et al.* (1993) reported a sensitivity of 100% on testing 81 PV isolates and a specificity of

96% on testing 50 NPEV isolates. Egger *et al.* (1995) reported a sensitivity of 100% on testing 81 PV isolates when two primer pairs located in the P2-P3 region were used and a specificity of 98% on testing 45 NPEV isolates. Using primers in the VP1-2A region, Chezzi (1996) reported 100% sensitivity and specificity for testing 125 PV and 38 NPEV isolates.

2.9 Risk Assessment

Quantitative risk assessment provides a comprehensive tool to integrate relevant information from different sources into a single coherent analysis (Gerba, 2000). It bridges a wide range of disciplines and provides a direct link between water management decisions and public health, thereby providing a basis for decision making (Gerba, 2000).

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 that drinking water treatment processes should be designed to ensure that human populations are not subjected to risk of infection higher than 1 infection per 10 000 consumers for a yearly exposure (Gerba, 2000).

Although no accepted formal framework for microbial risk assessment exists, it generally follows the steps used in other health-based risk assessments – hazard identification, exposure assessment, dose-response and risk characterization (Gerba, 2000). The differences are in the specific assumptions, models and extrapolation methods used (Haas *et al.*, 1999). The results of such assessments can be used to inform risk managers of the probability and extent of environmental impacts resulting from exposure to different levels of contaminants (Gerba, 2000). This information can be used to weigh the cost and benefits of control options and to develop standards or treatment options (Gerba, 2000).

2.10 Summary

The enteroviruses comprise a large genus belonging to the piconaviridae. Sixty-six immunologically distinct serotypes are known to cause infections in humans (Melnick, 1996). The enterovirus group includes the 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 *et al.*, 2000). As many as 90% of cases of culture-positive aseptic meningitis are caused by enteroviruses, and 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 *et al.*, 2000). There is evidence that enteroviruses cause or 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). These are not emerging infectious diseases but are of emerging importance, because of the recent availability of pleconaril, an orally active broad-spectrum antipicornaviral agent, which offers the possibility of antiviral therapy (Pollard *et al.*, 2000).

Diagnosis of enterovirus infections cannot be made on clinical grounds alone, due to the diversity of disease manifestations (Chonmaitree and Mann, 1995). Diagnosis is important to distinguish between enterovirus-induced disease and other causes of clinically similar disease, to identify PV outbreaks, and to assess the prognosis (Chonmaitree and Mann, 1989). Traditionally enteroviruses have been detected by isolation in cell culture, and their serotypic identity has been established by neutralization of infectivity with serotype-specific antisera (Lim and Benyesh-Melnick, 1960). This prodedure is limited by the difficulty in culturing some enteroviruses, the availability of antisera for serotyping and the cost and technical complexity of serotyping procedures. Enteroviruses are now increasingly being detected by PCR rather than by cell culture isolation (Hyypia *et al.*, 1989; DeLeon *et al.*, 1990; Rotbart, 1990; Pillai *et al.*, 1991, Zoll *et al.*, 1992, Abbaszadegan *et al.*, 1993; Kopecka *et al.*, 1993; Muir *et al.*, 1993). Classical typing methods will therefore no longer be possible in most instances. An alternative means of enterovirus typing, employing PCR in conjunction with molecular genetic 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. Molecular typing methods would provide additional information regarding the epidemiology and biological properties of enteroviruses.

Molecular typing methods for viruses should aim to provide clinically and biologically useful information about viruses, particularly with regard to virulence, viral epidemiology, and virus serotype identification (Muir, 1998). The extent to which molecular classification systems for viruses have aimed toward, or achieved these goals varies, and in some cases further

development and refinement of the methods are required. For enteroviruses, to these general aims must be added the requirement to differentiate wild PV and NPEV, at least until global eradication of wild poliovirus is verified. Even if wild PV eradication is achieved by the year 2000, a period of surveillance will be required to verify this. The ability to differentiate between wild and vaccine derived PV and NPEV is therefore likely to be required until at least 2010 (Muir, 1998). In view of this, molecular detection of PV should be considered a goal of research and development of molecular typing, which should be achievable by methods discussed.

The genomic regions that should be targeted for sequence analysis will be determined by the objective of molecular typing. Molecular methods allow the rapid and specific detection of human EVs, by targeting the 5'UTR (Rotbart, 1995). However, variability of the 5'UTR sequence within a serotype has prevented the use of this region for identification of clinical isolates to the serotype level (Drebot *et al.*, 1994). Phylogenetic studies targeting the VP4-VP2 junction suggested that this region may be more suitable than the 5'UTR for development of serotype specific diagnostics (Poyry *et al.*, 1996), but this region appeared to correlate only partially with serotype (Oberste *et al.*, 1998). Sequences of the 3' half of the VP1 region has proved to be an excellent genetic correlate for the enterovirus serotype (Oberste *et al.*, 1999). Oberste and colleagues (1999) showed that there is a 100% correlation between the nucleotide sequences of the 3' half of VP1 and antigenic typing by the standard neutralization test. Phylogenetic typing is a realistic intermediate target for enterovirus molecular typing and methods such as those described by Arola *et al.* (1996) and Oberste *et al.* (1999) should be persued further.

The objective of this study was to develop molecular techniques for the typing of enteroviruses and to apply these techniques in research on the impact of enterovirus infections. This includes investigation of the role of enteroviruses in conditions such as aseptic meningitis, myocarditis and upper respiratory disease. The technology will be applied in monitoring the eradication of poliomyelitis by the typing of viruses in cases of acute flaccid paralysis and viruses isolated from water.

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CHAPTER 3

The editorial style of Water Research was followed in this chapter

3 EVALUATION OF MOLECULAR TYPING METHODS

3.1 Abstract

The objective of this study was to assess the application and efficiency of molecular techniques for the detection and serotyping of enteroviruses from environmental water samples. Samples of water were collected at regular intervals up- and downstream of an informal settlement. Techniques for the detection of enteroviruses included a multiplex RT-PCR and Sabin specific triplex PCR. A specific 297-base pair (bp) fragment was amplified and subjected to restriction enzyme (RE) analysis to differentiate between various serotypes of prototypical enteroviruses. Enteroviruses that gave inconclusive restriction patterns were typed by partial sequencing of the VP1 region. Results indicated a high incidence of enteroviruses, predominantly coxsackie B viruses. The results on polioviruses and other enteroviruses, contributed valuable information on enteroviruses circulating in the community. The molecular approach described here proved suitable for the rapid, sensitive, specific and cost effective, simultaneous detection and typing of enteroviruses in water.

3.2 Introduction

Non-polio enteroviruses are responsible for 10 million to 30 million illnesses each year in the United States (Strikas *et al.*, 1986) and the population most affected is under 10 years of age (Melnick, 1996). Enteroviruses are the most common cause of aseptic meningitis and other illnesses ranging from minor respiratory type infections to paralysis and carditis (Cherry., 1992).

Presumptive laboratory confirmation of an enterovirus infection is by the distinctive cytopathic effect produced on a selection of host cells. Although cell culture remains the "gold standard" for enterovirus infection diagnosis, the use of reverse transcription-PCR (RT-PCR) is becoming a more rapid and sensitive method to identify an enterovirus from clinical specimens and environmental samples (Rotbart, 1990; Abbaszadegan *et al.*, 1999). Neither cell culture nor PCR can provide an enterovirus type identification when used as diagnostic tests.

Virus neutralization with the Lim and Benyesh-Melnick (LBM) antiserum pools, followed by confirmation of the serotype with monospecific antiserum is the classic method of typing enteroviruses in the laboratory (Lim and Benyesh-Melnick, 1960). The antisera of these pools were raised against enteroviral prototype strains and problems have been noted when the pools were used to type new variants because of pronounced intratypic antigenic variation (Melnick and Wimberley, 1985). The World Health Organization (WHO) has recommended conserving the use of the limited stock of LBM reference pools. These recommendations and the expense of virus neutralization have resulted in many laboratories abandoning serotyping in favour of making an enterovirus (EV) identification only by cell culture (Centers for Disease Control and Prevention, 1997). This approach has resulted in less effective epidemiological surveillance and will consequently limit future knowledge of the etiology of enteroviral disease.

Alternative typing methods such as PCR with restriction fragment length polymorphism analysis (RFLP) (Kuan, 1997), PCR-single stranded conformation polymorphism analysis (Fujioka *et al.*, 1995) and antigen capture PCR (Shen *et al.*, 1997) have been described. Some laboratories have used nucleotide sequencing of the 5' nontranslated region (NTR) and the VP4-VP2 junction as diagnostic and epidemiologic tools, with some success (Drebot *et al.*, 1994; Arola *et al.*, 1996). However, sequences in these regions do not always correlate with serotype (Kopecka *et al.*, 1995; Arola *et al.*, 1996; Oberste *et al.*, 1998). Oberste *et al.* (1999) demonstrated that the VP1 sequence correlated better with the serotype than does the sequence of either the 5' NTR or the VP4-VP2 junction.

The technique of viral protein fingerprinting has recently been used for the typing and characterization of clinical EV isolates (Holland *et al.*, 1998). The method was specific and 97% accurate, but the radiolabeled proteins generate radioactive waste and the method requires the use of specialized instrumentation for data acquisition and analysis (Oberste *et al.*, 1999). In addition, the database of protein patterns available for comparison contains representatives of less than one third of the 66 known human enterovirus serotypes (Oberste *et al.*, 1999).

The primary objective of this research was to use advanced molecular techniques to develop a rapid, simple and inexpensive assay for the detection and serotyping of enteroviruses in water sources. The approach we used included laboratory studies in which we developed and optimized PCR-based methods and determined the specificity of the PCR primers used for the detection of enteroviruses. This was followed by a field evaluation of the methods, performed with stream water polluted by an informal settlement.

3.3 Materials and Methods

3.3.1 Viruses and Cells

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, 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.

3.3.2 Environmental Samples

Water samples were collected at regular intervals over a 9 month period from a stream polluted by an informal settlement. The water was sampled 3 km upstream and 1 km downstream from the settlement. Enteroviruses were isolated from unconcentrated samples by conventional cell culture propagation using the buffalo green monkey (BGM) kidney and PLC/PRF/5 human liver cell lines and primary vervet kidney cells (Grabow *et al.*, 1999). Enteroviruses were typed after demonstrating a complete cytopathogenic effect (CPE) by neutralization serotyping (Lim and Benyesh-Melnick, 1960; Grabow *et al.*, 1996) and molecular detection and typing techniques (Kämmerer *et al.*, 1994; Egger *et al.*, 1995; Kuan, 1997; Oberste *et al.*, 1999).

3.3.3 RNA Extraction

A total of 1.5 ml of infected cell culture supernatant was centrifuged at 100 000 x g in a Beckman TL-100 Ultra Centrifuge at 4°C for 1 h. The pellet was resuspended in 140 µl guanidium thiocyanite homogenization buffer (4 M Guanidium Thiocyanite, 0.1 M Tris-HCl, 1% β Mercaptoethanol). Cell cultures were homogenised using a biopolymer shredding system (Qiashredder™, Hilden, Germany) prior to RNA extraction. The RNA was extracted from infected tissue culture fluid using a commercial viral RNA extraction kit (Qiagen, Hilden, Germany). The extraction was performed according to the manufacturer's instructions. The nucleic acids were digested with RQ1 RNAse free DNAse (Promega, Madison, USA) for 15 min at 37°C. The enzyme was then inactivated at 80°C for 15 min (Minicycler, MJ Research USA).

3.3.4 Polio-Nonpolio RT-Multiplex PCR

A RT-multiplex PCR was described (Egger *et al.*, 1995) for the rapid detection of poliovirus (PV) and for its distinction from nonpolio enteroviruses (NPEV). The primers that are specific for either EV or PV are combined in a multiplex RT-PCR and give rise to amplicons of different sizes (Table 3-1). RT and PCR were performed with a Promega Access RT-PCR system (Promega, Madison, USA). Optimized final concentrations in a total volume of 50 µl were 2.0 mM $MgSO_4$, dNTP mix (final concentration of 0.2 mM), 50 pmol of primers E1, E2, PO1, PO2, PO3 and PO4 (Table 3-1), and 5 U of AMV reverse transcriptase and Tfl polymerase. After 35 cycles (1 min at 94°C, 1.5 min at 48°C and 1 min at 72°C), 15 µl of the PCR mixture was subjected to agarose gel electrophoresis (2%) (Seakem LE agarose, Bioproducts, USA) (Egger *et al.*, 1995).

3.3.5 Quantification of the Polio-Nonpolio RT-Multiplex PCR

Poliovirus 1 stocks were prepared and their titers determined using standard plaque assays with a 2% agarose (Seakem ME, FMC Bioproducts, Rockland, USA) overlay (Cromeans *et al.*, 1987). Cells used for virus titration were BGM cells (Bio Whittaker, passage 80 – 95). Viral RNA was extracted from 10-fold dilutions of poliovirus type 1 infected culture supernatant as described previously (3.3.3). It was then amplified using the polio- nonpolio RT-multiplex PCR (Egger *et al.*, 1995).

3.3.6 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 3-1). Water samples that were positive for poliovirus were confirmed as vaccine strains with the Sabin specific RT-PCR. The Sabin specific PCR was performed as described by Chezzi (1997) with a few modifications. 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 3-1), 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.

3.3.7 Restriction Enzyme Analysis

Aliquots of 10 µl of the multiplex RT-PCR products were incubated with 10 U of RE (Promega, Madison, USA) in a 30 µl reaction volume with the buffer recommended by the manufacturers (Table 3-2). Samples were incubated at 37°C for 3 h and were analyzed by (7%) polyacrylamide (BioRad, Hercules, CA) gelelectrophoresis (Hoefer electrophoresis unit, San Fransisco, USA).

3.3.8 Sequencing

Samples that gave inconclusive restriction enzyme patterns were typed by means of sequencing of the 3' end of the VP1 region of the enterovirus genome (Oberste *et al.*, 1999). cDNA synthesis and PCR were performed with a Promega Access RT-PCR system (Promega, Madison, USA). Five microlitres of the RNA was added to the PCR mixture (total volume of 50 µl) containing the following: 5 x AMV/Tfl reaction buffer, dNTP mix (0.2 mM), PCR primers 011 and 012/040 (50 pmol) (Table 3-1), 1.5 mM $MgSO_4$ and 5 U of AMV reverse transcriptase and Tfl DNA polymerase. The final mixture was overlaid with sterile mineral oil and was amplified in a DNA thermal cycler (MiniCycler, MJ Research, USA). After an initial step of reverse transcription at 42°C for 45 min, 30 cycles of denaturation (1 min at 95°C), annealing (1.5 min at 46°C) and extension (1.5 min at 72°C) were performed. This was followed by a final extension period of 10 min at 72°C. Ten microliters of the final products were analyzed on 2% agarose gels (Seakem LE agarose, Bioproducts, USA) containing 1 $\mu g.ml^{-1}$ of ethidium bromide (Sigma, St Louis, USA) in a Midicell® Primo gell-apparatus (Holbrook, New York).

Nucleotide sequencing of the RT-PCR products was carried out using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) with the 011 and 012/040 primers and modified T7 DNA polymerase (Sequenase version 2.0 PCR product sequencing kit, Amersham Life Science, USA). The sequencing reactions were run at 60 watt on an 8% polyacrylamide (BioRad, Hercules, CA)-6 M urea (Merck, Darmstadt, Germany) gel in 1 x TBE buffer, pH 8.3 (Amresco, Solon, Ohio) in a sequencing tank (Hybaid, Middlesex). Gels were vacuum dried (Slab gel drier SE 1160, Hoefer, San Fransisco) and exposed to X-ray film for 12 h at room temperature. The sequences were compared with the sequences in the EV VP1 sequence database by sequential pairwise allignment of the query sequence by using Blast (Altschul *et al.*, 1997). Sequences of the amplified products were analysed using the CLUSTAL program of the PC/GENE software, version 6.8 (IntelliGenetics, Mountain View, CA).

3.4 Results and Discussion

The polio-nonpolio RT multiplex PCR proved useful for the rapid, specific and sensitive detection of PV and for its distinction from NPEVs. Poliovirus 1 – 3, coxsackievirus B1 - B6, coxsackievirus A9 and echovirus type 1 were amplified with the polio- nonpolio RT-multiplex PCR. The patterns of the amplicons of different sizes obtained with the poliovirus and nonpolio enterovirus strains were summarized in Table 3-3. Poliovirus strains showed the poliovirus specific bands of 193 and 565 bp (Figure 3-1). Poliovirus 3 showed only the 193 poliovirus specific band. All nonpolio enteroviruses and polioviruses were positive with the enterovirus specific primers that generate a 297 bp product. All negative controls were negative.

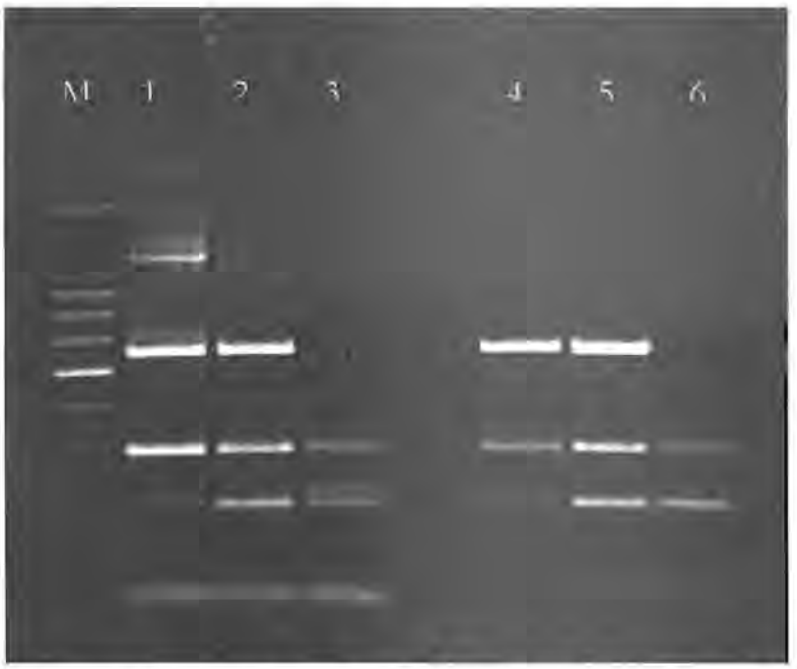

Figure 3-1: Band patterns observed with PCR of the Sabin strains type 1 – 3. M: 100 bp molecular marker (Promega, Madison, USA). Lane 1 – 3: PV 1 – 3 (100 pmol primer). Lane 4 – 6: PV1 – 3 (50 pmol primer).

The RT multiplex PCR detected 10^2 plaque forming units (PFU) of PV1 in 140 µl volume of sample. Since it has been estimated that the ratio of virus particles to infectious units is between 100 and 1 000 for the enteroviruses, it was concluded that the RT- multiplex PCR allowed the detection of 10^4 to 10^5 copies of entroviral RNA per 140 µl volume of sample (Rotbart, 1990; Severini *et al.*, 1993; Melnick, 1996).

In addition to the sensitivity, specificity and speed of the PV detection protocol, there is the advantage that in parallel to the demonstration of PV and its distinction from NPEV, the poliovirus detected can be subjected to strain specific typing. The Sabin specific strains (PV1–3)

were successfully amplified with the Sabin specific triplex RT-PCR, resulting in DNA bands of the expected sizes (Table 3-1). None of the other enterovirus prototype strains amplified with the Sabin specific primers.

Non-polio enteroviruses (samples that only exhibited the 297 bp product) were successfully typed with RE analysis. By employing three REs (StyI, BglI and XmnI), a digestive pattern was obtained that was easily visible and not too complicated to interpret. The thirteen prototype enterovirus strains were successfully amplified with the E1 - E2 primer pair, resulting in DNA bands of the expected sizes (297 bp). RE patterns were as predicted for all serotypes (Figure 3-2) and are summarized in Table 3-4 (Kämmerer *et al.*, 1994; Kuan, 1997).

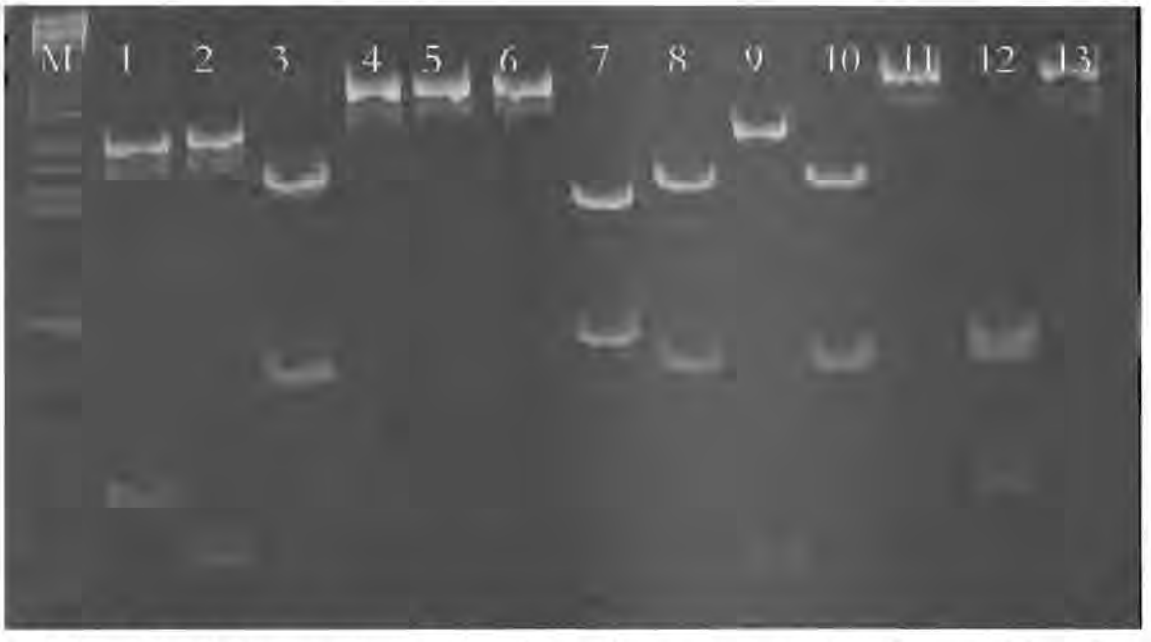

Figure 3-2: Restriction enzyme digestion of prototype enteroviruses. M: pGem marker. Lane 1-2: PV1 (Sty1, BamH1). Lane 3-4: PV2 (Sty1, Xmn1). Lane 5-7: PV3 (Sty1, Bgl1 and Mlu1). Lane 8-9: CBV1 (Sty 1, Xmn1). Lane 10-11: CBV2 (Sty 1, Bgl1). Lane 12-13: CBV3 (Sty1, Bgl1).

Molecular Typing of Environmental Samples

A total of 374 enteroviruses were isolated from stream water collected in and below the settlement during March 1994 to December 1994 (Grabow *et al.*, 1996). The isolates consisted of polioviruses (17% PV1, 3% PV2, 3% PV3), coxsackie B viruses (3% CBV1, 26% CBV2, 12% CBV3, 14% CBV4, 6% CBV6), echoviruses (9% EV7, 2% EV29) and untypeable enteroviruses (5%) in the inflow samples (Figure 3-4).

The following isolates were detected in the outflow samples: polioviruses (14% PV1, 5% PV2, 3% PV3), coxsackie B viruses (12% CBV1, 21% CBV2, 9% CBV3, 9% CBV4, 2% CBV5, 4% CBV6), echoviruses (9% EV7, 1% EV29, 10% EV15) and untypeable enteroviruses (10%) (Figure 3-4). There was an increase in the coxsackie B virus serotypes (CBV2, CBV3 and CBV4) during September to December 1994 in both inflow and outflow samples. One hundred and thirty seven isolated enteroviruses were typed with the polio- nonpolio RT-Multiplex PCR to distinguish between polio and nonpolio enteroviruses. One hundred and twenty two (89%) samples showed the 297 bp band, specific for polio- and nonpolio enteroviruse. Thirty-four (28%) of the samples showed the PV specific bands of 193 and 565 base pairs. Twenty-two of these samples showed an additional 1 000 bp product, resulting from a read-through between primers PO1 and PO4. These results were in agreement with the neutralization results (Table 3-6). Polioviruses detected by the polio- nonpolio multiplex PCR were typed with a sabin specific triplex RT-PCR (Figure 3-3). All the polioviruses detected were vaccine strains, with PV1 most prevalent. No wild type polioviruses were detected in these samples.

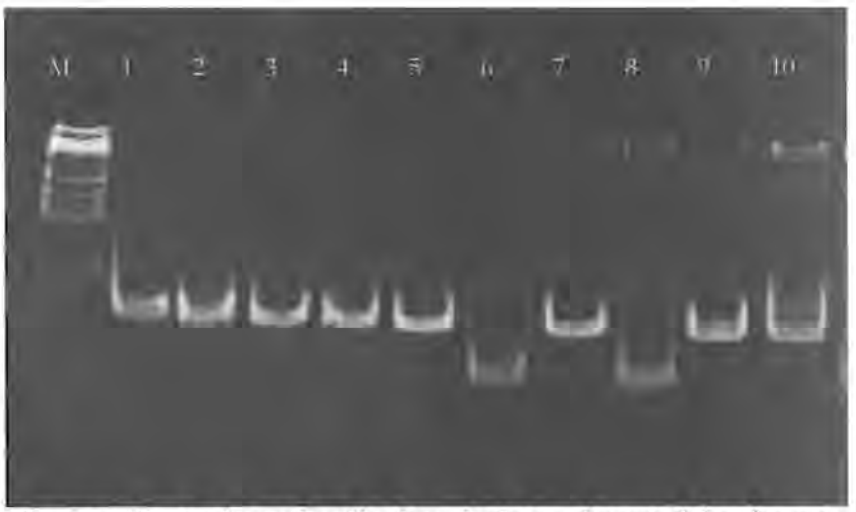

Figure 3-3: Strain specific typing of poliovirus isolates detected in the stream water. M: Marker V (Boehringer). Lanes 1-5, 7, 9 - 10: PV1 (Sabin strain). Lanes 6, 8: PV2 (Sabin strain).

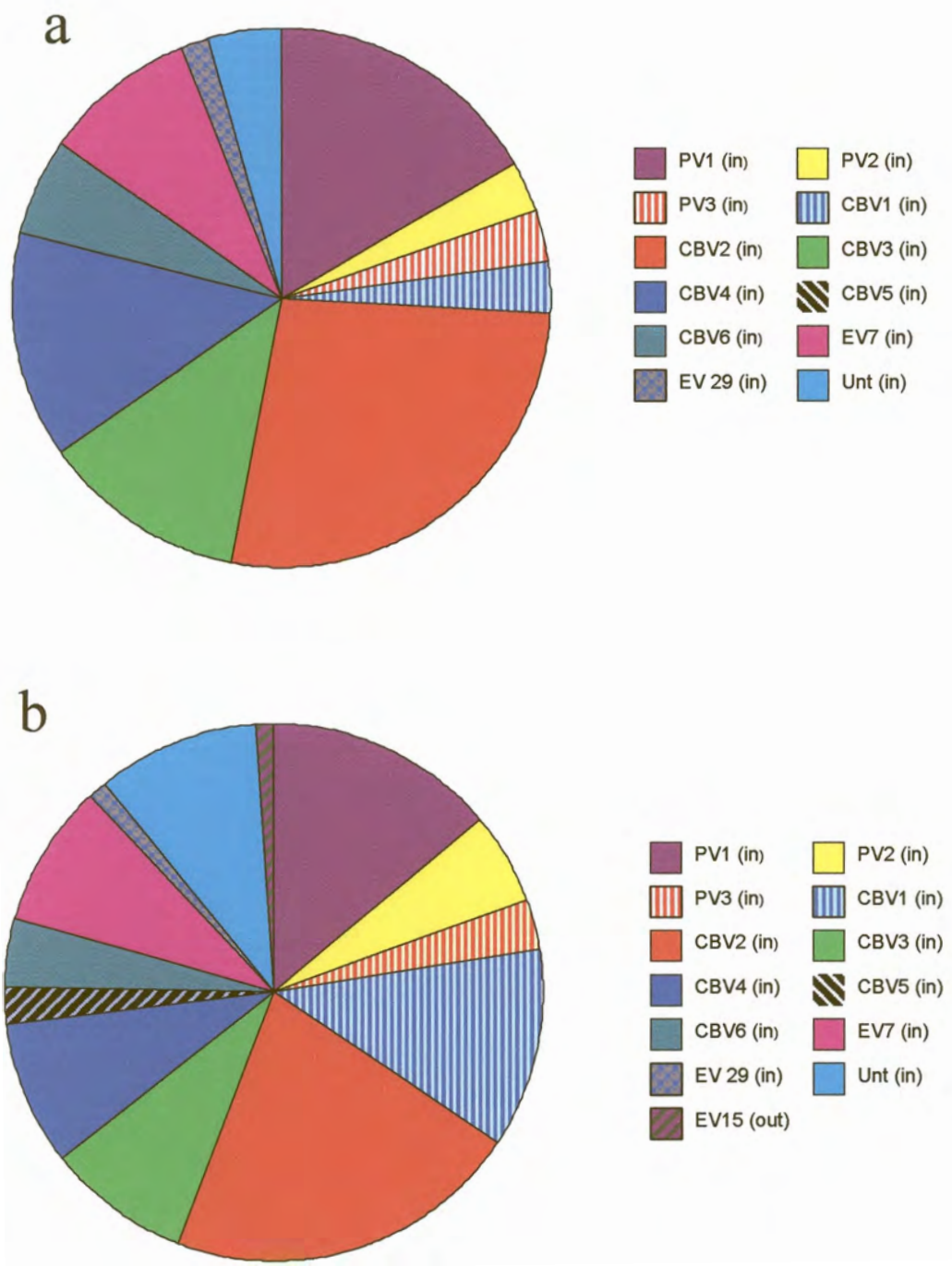

Figure 3-4 (a and b): Enterovirus serotypes detected upstream (a) and downstream (b) from an informal settlement.

Nonpolio enteroviruses were typed by means of restriction enzyme analysis of the 297 bp product (Figure 3-5). In most of the cases the restriction patterns corresponded to those described by Kämmerer *et al.* (1994) and Kuan (1997). Restriction enzyme results from 84% of the CBV2, 94% of the CBV3, 100% CBV1, 75% CBV5, 100% CBV6 and 100% EV7 corresponded with the neutralization results (Table 3-6). Coxsackie B2 viruses isolated in the outflow samples from the end of October 1994 to the end of December 1994 exhibited restriction patterns different from those of the corresponding prototype strains. It is possible that these strains carry mutations or recombination sequences in the BglI restriction site. The CBV4, EV 15 and EV 29 serotypes yielded restriction patterns that were different from those described by Kämmerer *et al.* (1994) and Kuan (1997). The coxsackie B4 serotypes exhibited the same recognition site as the coxsackie B2 serotypes for StyI. It is therefore necessary to evaluate additional REs to distinguish between the CBV4 and CBV2 serotypes.

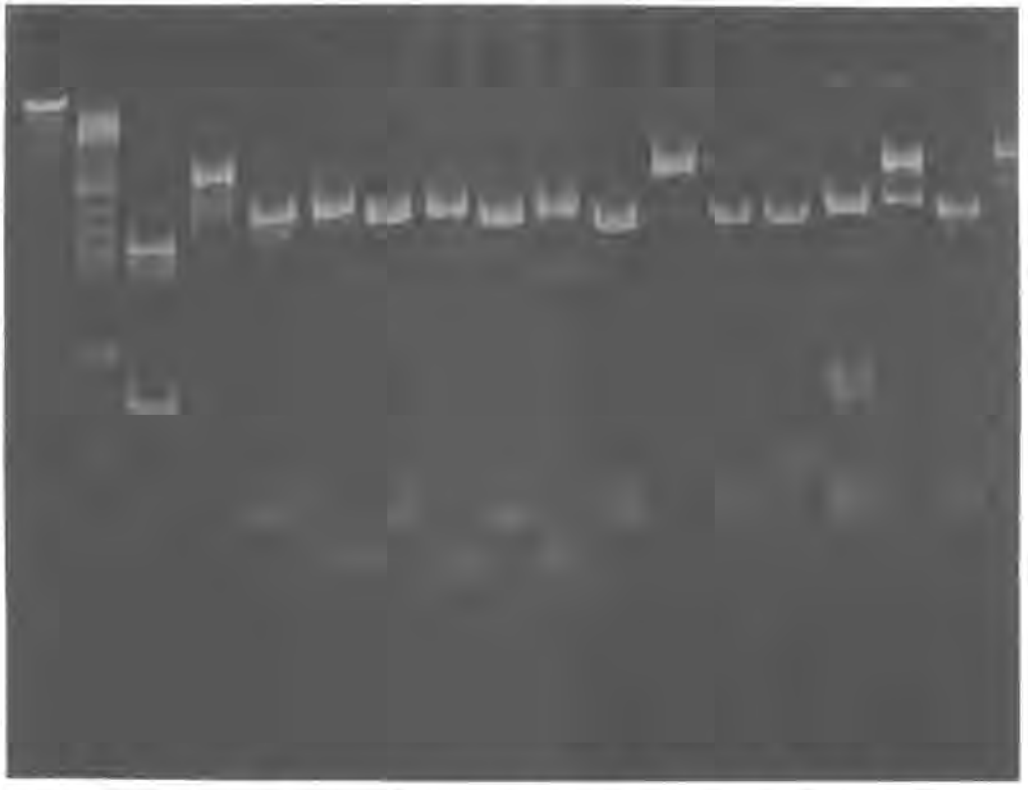

Figure 3-5: Restriction enzyme digestion of the 297 bp product of enteroviruses detected downstream from the informal settlement. Lane 1, 2: pGem marker; Lanes 3, 5, 7, 9, 11, 13, 15, 17: Styl digested products; Lanes 4, 6, 8, 10, 12, 14, 16: Bgll digested products.

Enteroviral RNA was amplified with the sequencing primers 011 and 012/040 in cases where the neutralization and restriction enzyme results differed. The CBV3, CBV2, CBV1, CBV5 and EV7 serotypes (typed by neutralization) were successfully amplified with the 011/012 primer pair resulting in a 440 bp product. Partial VP1 sequences of 11 environmental EV isolates, when compared to the VP1 sequences of prototype human EV strains, correlated with the serotype determined by the conventional neutralization tests (Table 3-5). The nucleotide sequences of the environmental isolates were 83 – 90% identical to the sequences of their respective prototype strains and 69 – 72% identical to the sequences of the highest scoring heterologous prototype strains. Enteroviruses of the same serotype were distinguishable from those of heterologous serotypes and the limits of intraserotypic divergence indicated a 25% nucleotide sequence difference.

None of the CBV4 serotypes amplified with the primer pairs 011/012 or 011/040. This is in agreement with results obtained by Oberste *et al.* (1999). In these cases the 297 bp product in the 5' UTR was sequenced with primer pairs E1 and E2. Results indicated that variability of the 5' UTR within serotype has prevented the use of this region for identification to the serotype level.

3.5 Conclusions

- The polio-nonpolio RT multiplex PCR proved useful for the rapid and sensitive detection of PV and for its distinction from NPEVs in water samples.

- In addition to the sensitivity, specificity and speed of the PV detection protocol, there is the advantage that in parallel to the demonstration of PV and its distinction from NPEVs, the poliovirus detected can be subjected to strain specific typing with the Sabin specific triplex RT-PCR.

- The observations on polioviruses indicated successful vaccination campaigns in the settlement.

- Non-polio enteroviruses were successfully typed with restriction enzyme analysis. PCR-RFLP allowed the rapid identification of all PCR-positive samples with an identical pattern, thus yielding valuable information on viruses circulating in the environment and reducing the requirement for complete nucleotide sequence analysis.

- A drawback of RFLP analysis is that single point mutations within the recognition sequence may give rise to unclassifiable results.

- The sequences of the 3'end of the VP1 region proved to be an excellent genetic correlate for the EV serotype. The technique is useful for the rapid determination of whether viruses isolated are epidemiologically related.

- In the 5'UTR the clustering of enterovirus serotypes is different from the corresponding prototype strains and does not provide a useful tool for typing, but can be used in some epidemiological studies.

- The polio-nonpolio RT multiplex PCR, followed by restriction enzyme analysis or the sabin specific RT-PCR may provide one of the most efficient methods currently available for rapid detection and typing of enteroviruses in water samples.

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Table 3-1: Sequences of the oligonucleotides used for the detection and typing of enteroviruses.

	Primer region and map position	Sequence	Primer	Product length	Specificity	Reference
Polio nonpolio multiplex PCR	163 - 178	5'AAGCACTTCTG TTTCC3'	E1		EV	Kämmerer *et al.*, 1994; Egger *et al.*, 1995; Kuan, 1997.
	443 - 460	5'CATTCAGGGGC CGGAGGA3'	E2	297	EV	Kämmerer *et al.*, 1994; Egger *et al.*, 1995; Kuan, 1997.
	4460 - 4478	5'CAGTTCAAGAG CAAACACC3'	P1		PV	Egger *et al.*, 1995
	4634 - 4653	5'TCGTCCATATC ACCACTCC3'	P2	193	PV	Egger *et al.*, 1995
	4922 - 4941	5'GAAATGTGTAA GAACTGTCA3'	P3		PV	Egger *et al.*, 1995
	5467 5487	5'GTAACAATGTT TCTTTTAGCC3'	P4	565	PV	Egger *et al.*, 1995
Sabin specific PCR	2584 - 2601	5'TCCACTGGCTTC AGTGTT3'	S1-1	97	Poliovirus 1 (Sabin strain)	Yang *et al.*, 1991
	2505 - 2523	5'AGGTCAGATGC TTGAAAGC3'	S1-2		Poliovirus 1 (Sabin strain)	Yang *et al.*, 1991
	2580 - 2595	5'CGGCTTGTGTC CAGGC3'	S2-1	71	Poliovirus 2 (Sabin strain)	Yang *et al.*, 1991
	2525 - 2544	5'CCGTTGAAGGG ATTACTAAA3'	S2-2		Poliovirus 2 (Sabin strain)	Yang *et al.*, 1991
		5'AGTATCAGGTA AGCTATCC3'	S3 – 1a	54	Poliovirus 3 (Sabin strain)	Yang *et al.*, 1991
	2537 - 2553	5'AGGGCGCCCTA ACTTTG3'	S3 - 2		Poliovirus 3 (Sabin strain)	Yang *et al.*, 1991
Sequencing VP1	2875 – 2894	5'ATGTAYGTICCI CCIGGIGG3'	012	450	EV	Oberste *et al.*, 1999
	2905 – 2924	5'ATGTAYRTICCI MCIGGIGC3'	040		EV	Oberste *et al.*, 1999
	3311 – 3292	5'GCICCIGAYTGIT GICCRAA3'	011	450	EV	Oberste *et al.*, 1999

Table 3-2: Restriction enzymes used for the genotyping of enteroviruses

Restriction Enzyme	Recognition Site	Assay Temperature (^{O}C)
Sty 1	C▲C(A/T)(A/T)GG	37
Bgl 1	GCCNNNN▲NGGC	37
Xmn 1	GAANN▲NNTTC	37
Bam H1	G▲GATCC	37
Mlu 1	A▲CGCGT	37
Nhe 1	G▲CTAGC	37

Key: N = A, G, C or T

▲ = recognition site for restriction enzyme

Table 3-3: Specificity of the polio- nonpolio RT-multiplex PCR

Serotypes	Primers E1 and E2 (297 bp product)	Primers PO1 and PO2 (193 bp product)	Primers PO3 and PO4 (565 bp product)
PV1	+	+	+
PV2	+	+	+
PV3	+	+	-
CB1	+	-	-
CB2	+	-	-
CB3	+	-	-
CB4	+	-	-
CB5	+	-	-
CB6	+	-	-
CA9	+	-	-
EV1	+	-	-
Rotavirus	-	-	-
Adenovirus	-	-	-
Astrovirus	-	-	-
Hepatitis A virus	-	-	-
Uninfected cell culture supernatant (BGM)	-	-	-

Table 3-4: Products obtained after RE analysis of the 297 bp product

Virus	Enzyme	Products
PV1	Sty 1	226 + 71
	Bam H1	239 + 85
PV2	Sty 1	195 + 100
	Xmn 1	297
PV3	Sty 1	297
	Bgl 1	297
	Mlu 1	192 + 105
CBV1	Sty 1	195 + 100
	Xmn 1	236 + 61
CBV2	Sty 1	212 + 73 + 10
	Bgl 1	217 + 80
CBV3	Sty 1	212 + 73 + 10
	Bgl 1	297
CBV4	Sty 1	297
	Bgl 1	217 + 80
	Nhe 1	297
CBV5	Sty 1	112 + 102 + 83
	Bgl 1	217 + 80
CBV6	Sty 1	212 + 73 + 10
	Bgl 1	196 + 80 + 21
CAV9	Sty 1	297
	Bgl 1	217 + 80
	Nhe 1	268 + 29

Table 3-5: Correspondence between typing by sequence comparison and by neutralization.

Strain	Neutralization type	Highest scoring prototype		Second-highest-scoring prototype (s)	
		Type	% nt sequence identity	Type	% nt sequence identity
94-12-15 (M40)	CB2	CBV2	86	CBV4	73
94-10-13 (M44)	CB2	CBV2	89	CBV4	72
94-11-03 (M31)	CB2	CBV2	89	CBV4	72
94-09-29 (M47)	CB2	CBV2	85.13	CBV4	72.28
94-11-24 (M61)	CB2	CBV2	90.36	CBV4	72.69
94-09-06 (VK)	CB3	CBV3	84	CBV4	69
94-09-06 (BGM)	CB3	CBV3	84	CBV4	69
94-06-21 (BGM)	EV7	EV7	86	CBV2	70
94-03-10 (M59)	EV7	EV7	86	CBV2	70
94-07-05 (M56)	CB1	CBV1	83.2	ECV6	69.8
94-07-12 (M53)	CB5	CBV5	89.3	CBV1	70.8
94-08-02	CBV4	EV11	96	EV30	93
94-09-13	CBV2	CBV2	90	EV9	90

Table 3-6: Enterovirus serotypes detected in a stream polluted by an informal settlement. Molecular detection methods compared with neutralization typing.

Date:	Cell culture	Neutralization Results	Polio- nonpolio PCR	Sabin Specific PCR	RE Analysis
1994-05-03	BGM	PV (Sabin 1)	PV	PV1	PV1
1994-05-26	BGM	PV (Sabin 1)	PV	PV1	PV1
1994-06-14	BGM	PV(Sabin 1)	PV	PV1	PV1
1994-06-21	PLC	PV (Sabin 3)	PV	PV3	PV3
1994-06-21	BGM	CBV6	NPEV	-	CBV6
1994-06-23	PLC	PV (Sabin 1)	PV	PV1	PV1
1994-06-23	BGM	CBV1	NPEV	-	CBV1
1994-06-23	PLC	PV (Sabin 1)	PV	PV1	PV1
1994-06-28	BGM	CBV3	NPEV	-	CBV3
1994-06-28	PLC	PV (Sabin 1)	PV	PV1	PV1
1994-06-28	BGM	CBV3	NPEV	-	CBV3
1994-06-28	VK	Untyped	Negative	-	-
1994-07-05	BGM	CBV1	NPEV	-	CBV1
1994-07-07	PLC	PV (Sabin 1)	PV	PV1	PV1
1994-07-07	VK	CBV6	NPEV	-	CBV6
1994-07-07	BGM	EV7	NPEV	-	EV7
1994-07-12	PLC	PV (Sabin 1)	PV	PV1	PV1
1994-07-12	PLC	Untyped	NPEV	-	EV6
1994-07-14	VK	EV7	NPEV	-	EV7
1994-07-14	BGM	EV29	Negative	-	Unt
1994-07-14	BGM	CBV6	NPEV	-	CBV6
1994-07-14	PLC	-	Negative	-	-
1994-07-19	VK	PV (Sabin 1)	PV	PV1	PV1
1994-07-19	VK	CBV3	NPEV	-	CBV3
1994-07-19	PLC	Echo 7	Negative	-	-
1994-07-21	BGM	CBV1	NPEV	-	CBV1
1994-07-21	VK	CBV6	NPEV	-	CBV6
1994-07-26	BGM	PV (Sabin 1)	NPEV	-	-
1994-07-26	BGM	PV (Sabin 1)	PV	PV1	PV1
1994-07-28	BGM	CBV2	NPEV	-	CBV2
1994-08-02	PLC	CBV4	NPEV	-	CBV2
1994-08-02	BGM	PV (Sabin 1)	PV	PV1	PV1
1994-08-02	PLC	PV (Sabin 3)	PV	PV3	PV3
1994-08-04	VK	EV7	NPEV	-	EV7
1994-08-04	BGM	EV7	NPEV	-	EV7
1994-08-09	BGM	EV7	NPEV	-	EV7
1994-08-09	PLC	PV (Sabin 1)	PV	PV1	PV1

Table 3-6: Enterovirus serotypes detected in a stream polluted by an informal settlement. Molecular detection methods compared with neutralization typing. (continued)

Date:	Cell culture	Neutralization Results	Polio- nonpolio PCR	Sabin Specific PCR	RE Analysis
1994-08-16	VK	CBV2	NPEV	-	CBV2
1994-08-25	PLC	PV (Sabin 2)	PV	PV2	PV2
1994-08-25	BGM	PV (Sabin 2)	PV	PV2	PV2
1994-09-06	VK	CBV3	Negative	-	-
1994-09-06	PLC	CBV4	NPEV	-	CBV2
1994-09-06	BGM	CBV4	NPEV	-	CBV2
1994-09-13	VK	CBV4	Negative	-	-
1994-09-13	BGM	CBV4	NPEV	-	CBV2
1994-09-13	VK	CBV4	NPEV	-	CBV2
1994-09-13	PLC	CBV4	NPEV	-	CBV2
1994-09-15	VK	CVB2	NPEV	-	CBV2
1994-09-15	BGM	CBV2	NPEV	-	CBV2
1994-09-15	BGM	CBV2	NPEV	-	CBV2
1994-09-15	PLC	CBV2	Negative	-	-
1994-09-20	VK	CBV2	NPEV	-	CBV2
1994-09-20	BGM	CBV2	NPEV	-	CBV2
1994-09-22	BGM	CBV4	NPEV	-	CBV2
1994-09-22	PLC	CBV2	NPEV	-	CBV2
1994-09-22	VK	CBV2	NPEV	-	CBV2
1994-09-27	PLC	CBV2	NPEV	-	CBV2
1994-09-29	PLC	CBV2	Negative	-	-
1994-09-29	BGM	CBV3	NPEV	-	CBV3
1994-09-29	PLC	CBV2	Negative	-	-
1994-10-03	VK	CBV2	NPEV	-	CBV2
1994-10-06	BGM	CBV2	NPEV	-	CBV2
1994-10-13	VK	CBV2	NPEV	-	CBV2
1994-10-13	PLC	CBV4	NPEV	-	CBV2
1994-10-13	BGM	CBV3	NPEV	-	CBV3
1994-10-18	VK	CBV4	NPEV	-	CBV2
1994-10-18	PLC	CBV4	Negative	-	-
1994-10-20	VK	CBV2	NPEV	-	CBV2
1994-10-20	BGM	CBV4	Negative	-	-
1994-10-25	VK	CBV4	NPEV	-	CBV2
1994-10-25	BGM	CBV3	NPEV	-	CBV3
1994-10-25	PLC	CBV3	Negative	-	CBV3
1994-10-27	PLC	CBV3	NPEV	-	CBV3
1994-10-27	PLC	CBV3	NPEV	-	CBV3
1994-10-27	VK	CBV3	NPEV	-	CBV3
1994-11-01	PLC	CBV2	NPEV	-	CBV2
1994-11-01	VK	CBV2	NPEV	-	CBV2

Table 3-6: Enterovirus serotypes detected in a stream polluted by an informal settlement. Molecular detection methods compared with neutralization typing. (continued)

Date:	Cell culture	Neutralization Results	Polio- nonpolio PCR	Sabin Specific PCR	RE Analysis
1994-11-03	BGM	PV2	PV	PV2	PV2
1994-11-03	VK	CBV2	NPEV	-	CBV2
1994-11-15	PLC	CBV2	NPEV	-	CBV2
1994-12-06	PLC	CBV2	NPEV	-	CBV2
1994-12-06	BGM	CBV2	NPEV	-	CBV2
1994-12-08	PLC	CBV2	NPEV	-	CBV2
1994-12-13	PLC	CBV2	NPEV	-	CBV2
1994-12-13	BGM	PV2	PV	PV2	PV2
1994-12-13	BGM	CBV4	NPEV	-	CBV2
1994-12-15	PLC	CBV2	NPEV	-	CBV2
Downstream					
Date:	**Cell culture**	**Neutralization Results**	**Polio- nonpolio PCR**	**Sabin Specific PCR**	**Restriction Enzyme Analysis**
1994-03-10	VK	CBV6	NPEV	-	CBV6
1994-03-22	VK	CBV2	NPEV	-	CBV2
1994-05-03	VK	PV1	PV	PV1	PV1
1994-05-24	BGM	PV1	PV	PV1	PV1
1994-05-26	BGM	PV1	PV	PV1	PV1
1994-05-26	PLC	PV1	PV	PV1	PV1
1994-06-02	PLC	PV1	PV	PV1	PV1
1994-06-09	PLC	PV1	PV	PV1	PV1
1994-06-14	BGM	CBV6	NPEV	-	CBV6
1994-06-16	BGM	Unt	NPEV	-	-
1994-06-21	PLC	CBV5	NPEV	-	CBV5
1994-06-21	BGM	CBV2	NPEV	-	CBV2
1994-06-21	PLC	PV1	PV	PV1	PV1
1994-06-23	PLC	PV1	PV	PV1	PV1
1994-06-28	PLC	PV1	PV	PV1	PV1
1994-06-28	BGM	EV7	NPEV	-	EV7
1994-06-28	VK	EV27	NPEV	-	Unt
1994-07-05	BGM	CBV1	NPEV	-	CBV1
1994-07-05	PLC	PV1	PV	PV1	PV1
1994-07-05	VK	EV7	NPEV	-	EV7
1994-07-05	VK	EV15	NPEV	-	Unt
1994-07-07	BGM	PV2	PV	PV2	PV2
1994-07-07	PLC	PV1	PV	PV1	PV1
1994-07-12	BGM	PV1	Negative	-	-
1994-07-12	PLC	PV3	PV	PV3	PV3
1994-07-12	VK	PV1	PV	PV1	PV1
1994-07-12	PLC	CBV5	NPEV	-	CBV5

Table 3-6: Enterovirus serotypes detected in a stream polluted by an informal settlement. Molecular detection methods compared with neutralization typing. (continued)

Date:	Cell culture	Neutralization Results	Polio- nonpolio PCR	Sabin Specific PCR	RE Analysis
1994-07-14	PLC	PV3	PV	PV3	PV3
1994-07-14	PLC	CBV3	Negative	-	-
1994-07-21	BGM	CBV1	NPEV	-	CBV1
1994-07-21	BGM	CBV6	NPEV	-	CBV6
1994-07-26	PLC	CBV1	NPEV	-	CBV1
1994-07-26	BGM	PV	NPEV	-	CBV3
1994-08-02	PLC	CBV1	Negative	-	-
1994-08-02	BGM	PV1	PV	PV1	PV1
1994-08-04	PLC	PV3	PV	PV3	PV3
1994-08-04	VK	CBV2	NPEV	-	CBV2
1994-08-04	PLC	CBV1	NPEV	-	CBV1
1994-08-04	VK	EV7	NPEV	-	EV7
1994-08-09	PLC	PV1	PV	PV1	PV1
1994-08-11	BGM	CBV1	NPEV		CBV1
1994-08-11	VK	CBV6	NPEV		CBV6
1994-08-23	BGM	CBV2	NPEV		CBV2
1994-09-06	BGM	CBV4	NPEV		CBV2
1994-09-06	BGM	CBV3	NPEV		CBV3
1994-09-13	PLC	CBV2	NPEV		CBV2
1994-09-13	BGM	CBV2	NPEV	-	CBV3
1994-09-13	PLC	CBV1	NPEV	-	CBV1
1994-09-13	VK	CBV2	NPEV	-	CBV2
1994-09-15	BGM	CBV4	Negative	-	-
1994-09-08	BGM	CBV3	NPEV	-	CBV3
1994-09-20	BGM	CBV2	NPEV	-	CBV2
1994-09-20	PLC	CBV2	NPEV	-	CBV2
1994-09-29	PLC	CBV2	NPEV	-	CBV2
1994-10-04	BGM	CBV2	NPEV	-	CBV2
1994-10-13	PLC	CBV3	NPEV	-	CBV3
1994-10-13	BGM	CBV5	NPEV	-	CBV5
1994-10-13	VK	CBV4	NPEV	-	CBV2
1994-10-18	BGM	CBV4	NPEV	-	CBV2
1994-10-18	VK	CBV4	NPEV	-	CBV2
1994-10-18	PLC	CBV4	NPEV	-	CBV2
1994-10-20	BGM	CBV4	NPEV	-	CBV2
1994-10-20	PLC	CBV2	NPEV	-	CBV2
1994-10-25	PLC	CBV2	NPEV	-	CBV2
1994-10-27	BGM	CBV2	NPEV	-	CBV3*
1994-11-03	PLC	CBV2	NPEV	-	CBV3*
1994-11-24	BGM	CBV2	NPEV	-	CBV3

Table 3-6: Enterovirus serotypes detected in a stream polluted by an informal settlement. Molecular detection methods compared with neutralization typing. (continued)

Date:	Cell culture	Neutralization Results	Polio- nonpolio PCR	Sabin Specific PCR	RE Analysis
1994-11-29	BGM	CBV2	Negative	-	-
1994-11-24	VK	CBV3	PV	PV1	PV1
1994-11-24	VK	CBV3	NPEV	-	CBV3
1994-12-01	PLC	CBV3	Negative	-	-
1994-12-06	PLC	CBV2	NPEV		CBV3
1994-12-13	BGM	CBV2	NPEV		CBV3
1994-12-13	PLC	EV7	NPEV	-	EV7
1994-12-15	PLC	CBV2	NPEV		CBV3
1994-12-15	VK	CBV2	NPEV		CBV3