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Investigation into a school enterovirus outbreak using PCR detection and serotype identification based on the 5′ non-coding region

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SUMMARY

A summer camp was followed by an outbreak of illness involving around 90 children. Investigations included individual questionnaires, inspection of the camp facilities, and laboratory analysis of water and clinical samples. Contamination of drinking and swimming water was demonstrated. An enterovirus was detected by polymerase chain reaction (PCR) and/or culture in 4/4 cerebrospinal fluid samples, 9/15 (60 %) stool samples from symptomatic children and 2/9 (22 %) stool samples from asymptomatic children. The virus was identified as an echovirus 3 by sequencing and phylogenetic analysis of a short 5′ non-coding region (NCR) PCR product. Viruses from the outbreak clustered closely and an echovirus 3 from a temporally associated non-outbreak case could be readily distinguished. Despite the lack of a standardized approach, direct molecular detection and identification of enteroviruses is an efficient epidemiological tool. Here the 5′-NCR was successfully used for both detection and 'serotyping', and the close genetic relatedness of isolates was proven.

INTRODUCTION

The Grade 5 class of a primary school in the Cape Peninsula, South Africa, attended an educational camp for 3 days in February 2001. The camp site was in the Villiersdorp district, a farming area ~100 km from Cape Town. Camp activities included lectures, nature walks, swimming and other water sports.

Some children were unwell at the camp, complaining of headache and sore eyes and one child had a bout of vomiting. Initially these symptoms were attributed to factors such as daytime temperatures of 38 °C, change of environment and lack of sleep. However, many children were ill on the first day of their return home and absenteeism peaked early in the following week. About 25 children had severe symptoms, with four being admitted to local hospitals for meningitis. In all, approximately 90 children were affected by the camp outbreak.

The local health authority was requested to investigate the outbreak by the school principal, and the Division of Medical Virology, University of Cape Town, was independently alerted to the problem by a teacher.

Our investigations led to the conclusion that the cause of the outbreak was an enterovirus. Enteroviruses form a large genus of the family Picornaviridae. These viruses have a worldwide distribution. Sixty-six immunologically distinct serotypes are known to cause disease in humans. Classically, these serotypes are subgrouped as polioviruses (1–3), coxsackieviruses A (1–22, 24), coxsackieviruses B (1–6) and echoviruses (1–7, 9, 11–27, 29–33). More

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recent additions to the genus have simply been assigned enterovirus serotypes (6–71). The classical subgroupings are only partially supported by sequence comparisons and the latter approach has given rise to new, genetically defined subgroups [1]. Be that as it may, individual enteroviruses are currently still identified according to their classical serotype names.

Enteroviruses are associated with an array of syndromes involving the central nervous system (meningitis, meningoencephalitis, paralytic poliomyelitis), skeletal and cardiac muscle (Bornholm's disease, myocarditis), skin and mucosae (hand, foot and mouth disease, herpangina, haemorrhagic conjunctivitis) and still others. Certain serotypes are commonly responsible for particular syndromes.

Spread of enteroviruses occurs via the faecal–oral and respiratory routes, and via hands and fomites, with the dominant mode of spread depending on the circumstances. Spread via faecal–oral contact appears to require compromised levels of hygiene so that it is more common among very young children and unsupervised older children. School sports teams and activity groups (such as this school camp group) may be at increased risk for enterovirus spread due to a combination of crowding, poor hygiene and other factors [2]. Outbreaks of enterovirus disease have occasionally been linked to contaminated food, but foodborne transmission is seemingly rare [3]. Viruses are excreted from the throat during the first week of infection and in faeces for 1–5 weeks. These viruses are hardy and can potentially survive in the environment for some time. They are regularly found in sewage. They can be found in ground and surface water and in recreational swimming water in the absence of obvious faecal contamination, and even in the presence of recommended chlorine levels [4].

SUBJECTS AND METHODS

The 130 school pupils involved in the outbreak were 10–11 years old, resident in the suburbs and an informal settlement near to their school.

Epidemiological investigations

A questionnaire was issued to all Grade 5 children for completion with the help of their parents. The questionnaire covered the nature of symptoms and their time of onset, consumption of meals and participation in water sports. In total, 116 sheets were returned. An interview was held with the Grade 5 teachers, including the three teachers who attended the camp, and the school secretary, who had been in communication with parents and children. The clinicians caring for the four hospitalized children were contacted regarding their clinical picture.

An inspection of the camp facilities was made, a detailed menu was obtained and water samples were collected from various sites.

Principals of schools that attended the camp in the weeks immediately before and after the group in question were contacted by telephone.

Microbiological investigations

Cerebrospinal fluid (CSF) samples of the four children with meningitis were forwarded to our laboratory for viral culture and enterovirus PCR, after routine bacterial cultures proved negative. Ten days after returning from the camp, stool specimens were collected from 25 other children, 15 of whom were symptomatic and 10 asymptomatic. A detailed symptom list was obtained for this symptomatic subgroup. The stool specimens underwent viral culture and enterovirus PCR.

One additional CSF sample included for analysis in this study was from an 11-year-old child presenting with aseptic meningitis at the same time as the school camp outbreak. However, this child was attending a different school in the Cape Peninsula and was unconnected to the camp outbreak. The sample was submitted for viral culture by the child's paediatrician who was also caring for one of the children with meningitis from the outbreak.

Bacterial culture

The camp water samples were submitted to the Scientific Services Department of the City of Cape Town. Cultures for bacterial indicator organisms were carried out to evaluate the water quality and specifically to look for evidence of faecal contamination.

Membrane filters were incubated on m-Endo agar at 37 °C for 24 h to obtain the total coliform count, and on m-TEC agar for 2 h at 37 °C followed by 44·5 °C for 22 h to obtain the faecal coliform count. An *in-situ* urease test was used to identify *Escherichia coli*.

Viral culture

The CSF samples were inoculated directly into primary African green monkey kidney (PMK) cell cultures. Stool samples (~1 g) were diluted 1/10 in 'intake

medium' (0·2 M sucrose in PBS) and clarified by centrifugation at 10 000 rpm (~9000 *g*) for 10 min. Supernatant fluids were then inoculated into PMK cell culture tubes. The cell culture tubes were incubated at 37 °C in a roller drum. Cell cultures were examined three times per week by light microscopy for viral cytopathic effect (CPE). If CPE was observed, cell culture supernatant fluid was passaged into fresh cell culture tubes. On completion of 10 days' culture, or sooner if CPE was evident, the cell culture coverslips were stained with haematoxylin and eosin to allow more detailed morphological examination by light microscopy. Typical CPE of the enterovirus group shows rounding up of cells, large ground glass eosinophilic intra-cytoplasmic inclusions and 'twisted pancake' nuclei.

An attempt was made to serotype the enteroviral isolates by neutralization. In this laboratory neutralization assays are performed in PMK cell cultures using pooled monovalent antisera to polioviruses 1–3 or coxsackieviruses B1–B6 (Diagnostici Ismunit, Pomezia, Italy). Briefly, a viral isolate is passaged to increase the viral titre to a level where ~25% CPE appears after overnight culture. Culture supernatant (virus) and antiserum are co-incubated for 1 h at 37 °C before re-inoculation into fresh cultures. Failure of development of CPE after 5 days indicates neutralization by that serum pool. An isolate can then be broadly classified as a poliovirus, a coxsackievirus B or a non-polio, non-coxsackievirus B enterovirus. Identification of individual members of the group can then be pursued by neutralization assays using individual antisera, following a similar method.

RNA extraction

RNA was extracted from 200 μl CSF or stool samples using a QIAamp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. The RNA was eluted in 50 μl elution buffer and stored at −20 °C until required.

Reverse transcription and PCR

Ten microlitres of RNA was denatured in the presence of 1 μM hexanucleotide mix and 200 μM deoxynucleotide (dNTP) mix (Roche Molecular Biochemicals, Mannheim, Germany) at 65 °C for 5 min, cooled on ice for 5 min before the addition of 50 U RNase inhibitor, 200 U Superscript II reverse transcriptase (RT), RT buffer and 0·1 M dithiothreitol (Gibco-BRL, Paisley, UK). The mixture was incubated at room temperature for 10 min followed by incubation at 42 °C for 60 min. The RT was inactivated at 70 °C for 15 min.

Diagnostic PCR for enteroviruses was based on previously published general primers reported to detect at least 60 of the enterovirus serotypes [5]. These primers ('primers 2 and 3') target the highly conserved 5′ non-coding region (NCR) of the enteroviral genome and yield a 155-bp product. However, in order to increase the sensitivity of the PCR assay, an additional outer primer ('primer 1') was designed for use in a semi-nested reaction step.

The cDNA (10 μl) was amplified using primer 1 [5′-TGG(tc)(cg)(cg)(at)GGCTGCGT) (359–374 nt)] and primer 3 (5′-ATTGTCACCATAAGCAGCCA) (580–599 nt) at 1 μM in a standard PCR reaction mixture comprising 200 μM dNTP, 1·25 U High Fidelity PCR System *Taq* DNA polymerase and PCR buffer (Roche Molecular Biochemicals) for 40 cycles with the following cycling parameters: 94 °C for 20 s, 50 °C for 30 s and 72 °C for 40 s. A final extension step of 7 min at 72 °C was also performed. Five microlitres of the outer PCR reaction was added to an inner PCR reaction containing the same components as above but using primer 2 (5′-TCCTCCGGCC CCTGAATGCG) (445–464 nt) with primer 3. The cycling conditions were the same as above, however, the annealing temperature was increased to 55 °C.

The PCR products (155 bp) were separated in a 2% agarose gel and visualized with ethidium bromide staining under UV illumination. Recommended steps to avoid amplicon contamination were followed and included spatial partitioning of the PCR mastermix preparation, specimen preparation and product detection areas, and the use of dedicated equipment in these areas [6]. Each amplification experiment included a positive control (a laboratory enterovirus isolate) and negative control (distilled water).

The specificity of the PCR products was confirmed by their subsequent sequencing.

Cloning and sequencing

The nested PCR products (155 bp) were cloned into a pGEM-T vector (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. DNA was extracted from the appropriate clones (containing the correct size insert), using the High Pure Plasmid Isolation kit (Roche Molecular Biochemicals). Each strand was sequenced using the M13 forward and reverse sequencing primers in an ALFexpress

DNA Automated Sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden).

Phylogenetic analysis

The manually edited DNA sequences obtained in this study were aligned with known enteroviral sequences obtained from the GenBank database using the CLUSTAL_X program [7]. These latter sequences were also manually edited to obtain a sequence of 155 bp corresponding to the PCR fragment amplified.

The TREECON for Windows program [8] was used to construct a phylogenetic tree using the neighbour-joining method [9], and a human rhinovirus sequence was used as the outgroup. The reliability of the phylogenetic analysis was assessed using 1000 bootstrap samplings.

One isolate provisionally identified as poliovirus type 1 was referred to the National Institute for Communicable Diseases, South Africa, for further molecular analysis using poliovirus reagents provided by the Centres for Disease Control (Atlanta, GA, USA).

RESULTS

Epidemiological investigations

Boys and girls had been accommodated separately, in six dormitories, with ~20 children per dormitory. Roughly equal numbers of boys and girls had been ill and there was no apparent association of illness with a particular dormitory.

Ablution facilities included flush toilets, hand basins and showers, which were all found to be in a clean condition. The sewage was on a septic tank and soak-away system. Drinking water was drawn from a mountain stream 3 km away and filtered but not chlorinated (Fig. 1). No association with illness was apparent for any item on the menu. No unpasteurized dairy products were consumed.

The swimming pool was a concrete pool fed by overflow from the domestic water supply, in turn with a run-off into the local river (Fig. 1). The swimming-pool water was not chlorinated. Due to the lack of rain in the preceding months, there was little replacement of the swimming- pool water. Canoeing took place on a farm dam which was filled by pump from the local river.

Villiersdorp is a deciduous fruit-farming area but there was no evidence that the children had been exposed to any agricultural poison at the camp.

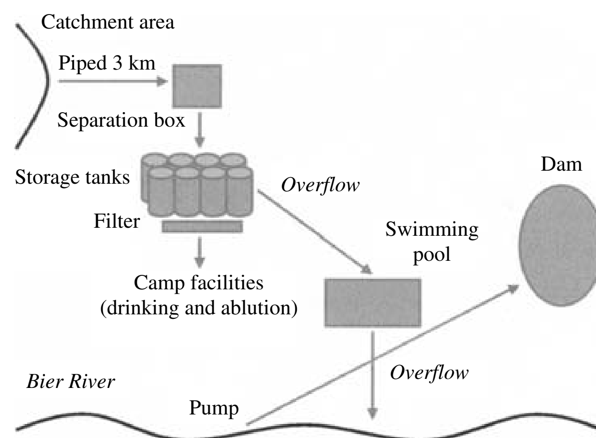

Fig. 1. The camp reticulation system: domestic and recreational water supply.

Four children had symptoms which led to clinical diagnosis of meningitis, and were hospitalized for investigation and observation. Two of these children were twin boys. CSF chemistry, cell counts and negative bacterial cultures indicated aseptic meningitis in all four children.

From the 116 completed questionnaires, 82 other children recorded at least one symptom, most commonly headache (41/116) and/or abdominal discomfort (33/116). A detailed description of symptoms was obtained for 13 children from whom stool specimens were collected. These were considered representative of the group of approximately 25 more severely affected children. In this subgroup the most common symptoms were fever, headache, sore throat, abdominal pain or cramps, loss of appetite, nausea and vomiting. A few children reported one or more of: rash, sore muscles, weakness, cough, sinusitis, laryngitis, mouth ulcers, diarrhoea, photophobia and lymphadenopathy.

It was established that school groups that had attended the camp before and after the group under study did not experience any adverse health events. An anecdotal report that local school children in Villiersdorp were affected by an illness similar to the study group could not be verified. An increased number of diagnoses of viral meningitis were being made by paediatricians in the Cape Peninsula at the time of this outbreak and one other school outbreak of viral meningitis was reported to the Department of Virology. In addition, enterovirus activity, including cases of meningo-encephalitis, had been noted by the National Institute for Virology in Gauteng Province since the previous November (2000), spreading to Kwazulu-Natal Province over the

Table 1. *Culture of indicator organisms from camp water samples*

Sample location	Total coliforms (per 100 ml)	Faecal coliforms (per 100 ml)	*Escherichia coli* (per 100 ml)
Separation box	**10**	1	1
Kitchen	**34**	1	1
Swimming pool	**Overgrown**	<1	<1
Dam	>100	33	32

Results in bold type are above the acceptable limits for domestic or recreational water as laid down by the 1999 recommendations of the South African Bureau of Standards, the 1991 draft guidelines of the Department of National Health and Population Development (South Africa) and the 1996 South African National Water Quality Guidelines.

Table 2. *Enterovirus detection in clinical samples from 29 children*

		Stools	
	CSF: children with meningitis ($n=4$)	Symptomatic children ($n=15$)	Asymptomatic children ($n=10$)
Culture positive	2 (50%)	2 (13%)	0 (0%)
PCR positive	3 (75%)	7 (47%)	2 (20%)
Culture or PCR positive	4 (100%)	9 (60%)	2 (20%)

Christmas holiday season, and with subsequent reports of enteroviral illness from the Eastern Cape Province early in 2001.

Microbiological investigations

Water indicator organisms

Results are summarized in Table 1. Results for the separation box and the kitchen pertain to the domestic water supply, which includes the drinking water. Results for the swimming pool pertain to children who swam ('full contact water sport'), whereas results for the dam pertain to those who engaged in canoeing.

Viral studies

Viral culture yielded an enterovirus from 2/4 CSF samples, from 2/15 stool samples of symptomatic children and from 0/10 stool samples of asymptomatic children. Enterovirus PCR was positive in 3/4 CSF samples, in 7/15 stool samples of symptomatic children and in 2/10 stool samples of asymptomatic children (Table 2).

The enterovirus implicated in this outbreak proved difficult to grow in cell culture, with slow growth and very low titres achieved. This hindered attempts to serotype the virus by neutralization and this mode of identification was abandoned in favour of a molecular approach. PCR and sequencing of enteroviruses directly from samples has been shown to be a successful route to their identification when culture is not possible [10].

Despite the short 155-bp sequence of 5′-NCR used to construct the phylogenetic tree (Fig. 2), the distribution of the human members of the enterovirus family was very similar to that of a previous phylogenetic analysis of the entire 5′-NCR [11]. This prior analysis found that the human enteroviruses formed two clusters, with cluster 1 comprising the polioviruses, coxsackieviruses A21 and 24 and enterovirus 70, and with cluster 2 comprising the coxsackieviruses B, the echoviruses, coxsackieviruses A9 and 16 and enterovirus 71.

Viruses 235, 247, 251 and 252 were recovered from CSF, but virus 252 was derived from a paediatric case of meningitis temporally associated with, but not a part of, the camp outbreak. Viruses 278, 279, 280, 286 and 287 were recovered from stool samples of symptomatic children and virus 291 was obtained from the stool sample of an asymptomatic child.

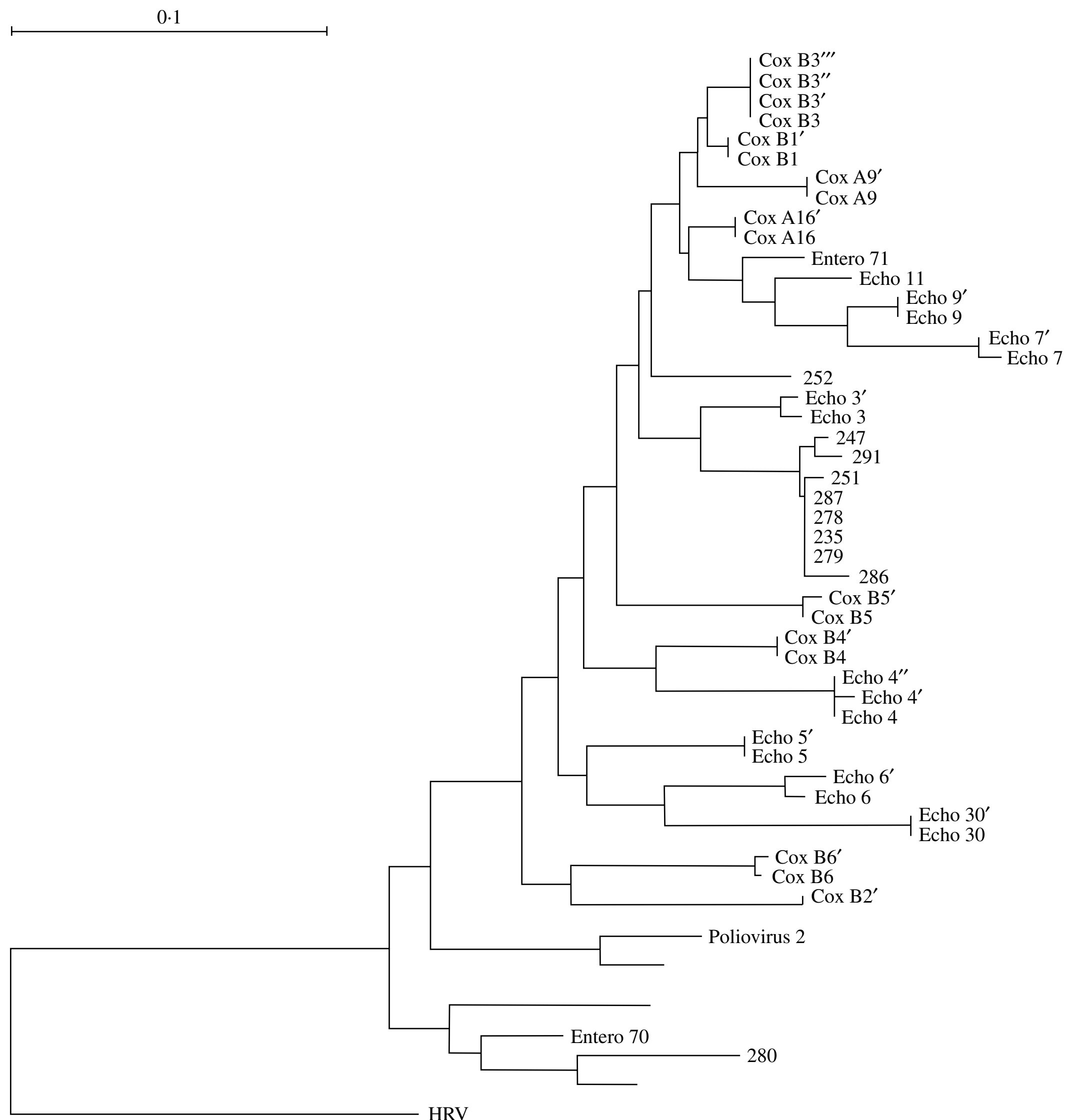

Fig. 2. Phylogenetic tree of enteroviruses rooted to human rhinovirus (HRV), showing the school camp outbreak viruses grouped with echovirus 3. Viruses 247, 251 and 235 were from children with meningitis, 287, 278, 279, 286 and 280 were from other symptomatic children and 291 was from an asymptomatic child. Virus 252 was from a child with meningitis who presented concurrently but independently.

One virus, detectable only by PCR in the stool of a symptomatic child, grouped with poliovirus type 1 (sample 280), and was initially presumed to be a Sabin strain. However, further phylogenetic analysis of the 155-bp 5′-NCR region of poliovirus type 1 isolates including the Sabin strain did not show that 280 was related to the vaccine strain (data not shown). In addition, no amplification was obtained using the Centres for Disease Control primers supplied for wild-type and Sabin polio strains (testing undertaken at the National Institute for Communicable Diseases, Sandringham, Johannesburg), although this result may have been compromised by the minimal amount of remaining RNA sample. Unfortunately further investigation was not possible due to insufficient sample.

DISCUSSION

In temperate climates, enteroviruses are prevalent typically in the summer and this was an important clue to the aetiology of this outbreak. Many of the same enteroviruses associated with aseptic meningitis are known to cause non-specific febrile illness in children. The specific syndrome of aseptic meningitis can

have accompanying respiratory or gastrointestinal symptoms, myalgia, conjunctivitis or rash. It is, therefore, plausible that the spectrum of symptoms seen in the 90 children could have been caused by a single enterovirus. In this outbreak we were able to demonstrate enterovirus infection in the four children with clinically apparent meningitis and in 60% of a subset of children with other significant symptomatology. Also, $\sim$20% of the asymptomatic children were infected. Although it varies for different members of the group, it is estimated that, overall, 50% of enterovirus infections are asymptomatic [2].

The concurrent onset of symptoms in a large number of children initially suggested a common source outbreak. However, the affected children were not clustered in a particular dormitory and no link with a particular meal was discernible from the questionnaire. The suspicion of unhygienic swimming-pool water was confirmed by the total coliform count, although faecal contamination was not specifically demonstrated. Enteroviruses are suspected, but not proven, to spread via recreational waters, probably by swallowing contaminated water. A study in the 1970s showed that children who swam at public beaches along the Wisconsin lakes were at higher risk for enterovirus illnesses compared to children who did not swim at all or who only swam in private swimming pools [12]. In the course of an investigation of a coxsackievirus outbreak at a boys' summer camp on Lake Champlain, coxsackievirus B5 was isolated from a sample of the lake water [13]. In addition to contamination of the swimming water, the presence of faecal coliforms in the drinking water was a concern. Although we considered testing recreational and drinking-water samples for the presence of enteroviruses, our laboratory had no prior experience in the field of water testing and lacked the apparatus to concentrate viruses from large volumes of water (a concentration step appearing to be a pre-requisite for reliable viral culture or even PCR detection in such samples). Regardless of whether or not contaminated drinking water played a role in this outbreak, this matter was addressed with the camp management.

Countering the hypothesis of a common source outbreak is the fact that the incubation period for non-polio enteroviruses is fairly short, with that of a number of echoviruses being in the range of 1–3 days [2]. It is possible that one or more children departed for the camp in the early stages of an enterovirus infection. Subsequently, the close contact between the children during the outgoing and return bus journeys and the sharing of dormitories and ablution facilities could all have contributed to rapid child-to-child spread of the virus, by both the respiratory and faecal–oral routes.

Many of the 71 human enteroviruses have been found to cause meningitis, but worldwide echoviruses 3, 4, 6, 9, 11, 16 and 30, coxsackievirus A9, coxsackievirus B1-5, enterovirus 71 and polioviruses 1–3 have most often been responsible [2]. Between 1981 and 1989, echovirus 4, echovirus 9 and coxsackievirus A9 were responsible for most viral meningitis in Cape Town [14]. Since then, economic constraints on our health services have prevented routine viral culturing for suspected viral meningitis and secondarily prevented monitoring of enteroviral epidemiology.

Through molecular techniques we showed that the enterovirus in this outbreak was an echovirus 3 in the majority, if not all, of the children. Our phylogenetic analysis of a short region of the 5′-NCR is validated by its similarity to an earlier definitive study of enterovirus phylogenetics using the 5′-NCR, capsid coding and 3′-NCR regions [11]. Successful phylogenetic analysis of the 5′-NCR diagnostic PCR product avoided the need for PCR and sequencing of a second, more variable region, such as VP1, which has been advanced as the optimal region for the purpose of enterovirus identification [15, 16].

The viruses recovered from children in the outbreak clustered very closely, suggesting their origins from a single or very limited number of introductions into this group of children. An isolate obtained coincidentally in the same week from a child in the Cape Peninsula, who presented with meningitis at the same time as our camp outbreak, but who was unconnected to this outbreak, also proved to be an echovirus 3 but was distinctly divergent from the outbreak cluster. A clearly unrelated enterovirus was found in one symptomatic child, but its identity could not be established.

Viral culture and PCR appeared to detect virus in our clinical samples in a *semi*-independent manner. Of the 38 samples tested by both modalities, enterovirus was detected in 11 by PCR only and in three by culture only, but in only one sample by both PCR and culture. We tested all 38 samples with PCR for the single copy human β-actin gene [17] in case the presence of PCR inhibitors was playing a role in these results. However, no samples could be successfully amplified with this non-nested PCR, probably reflecting minimal quantities of cellular RNA in the samples (data not shown). We are uncertain why this virus grew poorly in culture since echoviruses are

ordinarily readily cultivable in PMK cells [18]. The National Institute for Communicable Diseases experienced similar difficulty in the isolation of enteroviruses from samples received during the national outbreak preceding and concurrent with the camp outbreak in the Cape Peninsula. One explanation for the unusual behaviour of this echovirus 3 in cell culture could be that the virus represents a variant strain, which might also explain the high attack rate in our camp outbreak and the concurrent national outbreak.

This study once again demonstrated the usefulness of molecular techniques in clinical and epidemiological investigation of enteroviral disease, although a standardized approach to molecular typing of enteroviruses is still awaited [1].

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