Journal of Medical Microbiology (2005), **54**, 63–69 DOI 10.1099/jmm.0.45695-0

Enteroviruses in Tunisia: virological surveillance over 12 years (1992–2003)

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Received 13 April 2004
Accepted 14 September 2004

This report is an overview of enterovirus epidemiology in Tunisia during a 12-year period from 1992 to 2003. A total of 4700 clinical samples were collected as part of the national poliovirus surveillance programme and the routine diagnostic programme for aseptic meningitis. Enterovirus detection was performed by isolation on cell culture according to World Health Organization recommended protocols. Serotype identification was performed by seroneutralization of the cytopathic effect using pools of specific antisera and sequencing in the VP1 region of the genome. Poliovirus isolates were assessed for their wild or vaccine-related origin by standard World Health Organization recommended methods (PCR, probe hybridization and ELISA). The results confirm the interruption of wild poliovirus circulation since 1995. A total of 236 non-polio enterovirus (NPEV) strains were isolated; seroneutralization allowed typing of 93 % (219 out of 236) of them. The antisera used allowed the identification of the most common enterovirus serotypes. The remaining 17 isolates were sequenced; 16 of them belonged to enterovirus serotypes that were not targeted by the antisera pools used. A total of 29 different serotypes of NPEV were detected in the country during the study period. Echoviruses of serotypes 6, 11 and 30 were the most frequently isolated, almost every year; other serotypes had a cyclic occurrence and others were detected during a limited period with very few isolates. The NPEV isolation rate varied from year to year but was steadily under 10 %, suggesting a relatively low prevalence of these viruses in comparison to that in other developing countries. A seasonal variation was also noted; the high transmission period starts in March and peaks in September–November. This study is the first report of the epidemiology of NPEV in Tunisia. These viruses are associated with various diseases and epidemiological data may help to clarify their impact on human health.

INTRODUCTION

Human enteroviruses (HEV) are small non-enveloped RNA viruses belonging to the family *Picornaviridae* (Chapman *et al.*, 1990). Initially, on the basis of their pathogenesis in humans and experimental animals, 64 immunologically distinct serotypes of HEV were recognized, including polioviruses (PV, serotypes 1–3), coxsackieviruses A and B (CAV, serotypes 1–22 and 24; CBV, serotypes 1–6), echoviruses (ECV, serotypes 1–7, 9, 11–27 and 29–33) and the other enteroviruses (EV, serotypes 68–71). In 1999, a new classification of HEV, based on nucleotide and deduced amino acid sequences, was proposed. The genus was subgrouped into five species: polioviruses, HEV-A (11 CAV and EV71), HEV-B (CBV, ECV, CAV9 and EV69), HEV-C (11 CAV) and HEV-D (EV68, EV70 and EV73) (Ishiko *et al.*, 2002; Rakoto-Andrianarivelo *et al.*, 2002; Oberste *et al.*, 2000). Since 1988, wild PV have been targeted by a global eradication programme based on mass vaccination of the population and a complete virological investigation of all acute flaccid paralysis (AFP) cases (World Health Organization, 1988). Poliomyelitis became very rare: at the close of 2002, wild PV remained endemic in only six countries throughout the world and less than 2000 cases were recorded during that year. However, non-polio enteroviruses (NPEV) remain widespread globally. They cause a wide variety of clinical diseases, especially in children, most of them being mild or asymptomatic, but serious illnesses may occur, such as viral meningitis, paralysis, pericarditis and myocarditis. Other illnesses with chronic course, such as type I diabetes mellitus, have also been associated with enterovirus infections (Hovi *et al.*, 1996).

In Tunisia, a triple dose of oral polio vaccine (OPV) was introduced in the national immunization programme in 1979 and given to all neonates before the age of 12 months. The vaccine coverage rate increased during the 1980s and has

Abbreviations: AFP, acute flaccid paralysis; CPE, cytopathic effect; CSF, cerebrospinal fluid; ITD, intratypic differentiation; OPV, oral polio vaccine.

been maintained at over 90 % since 1988 (Triki *et al.*, 1999). At the end of 1991, a national programme for polio eradication was initiated. This programme is based on two main components: (i) a reinforced vaccination of infants mainly through National Immunization Days conducted from 1995 to 1997 during which all infants aged 1–5 years received annually two doses of OPV, regardless of their prior vaccination history; (ii) an attentive virological investigation of all detected polio-suspected cases (AFP cases). The Laboratory of Clinical Virology in the Pasteur Institute of Tunis was designated to serve as the national reference laboratory and, accordingly, all faecal samples collected from suspected polio cases and their healthy contacts were forwarded for virological investigation (World Health Organization, 1994). The laboratory also conducts other viral diagnostic activities, including enterovirus detection in aseptic meningitis cases. This paper reports the results of this enterovirus surveillance in Tunisia from 1992 to 2003 and gives an overview of the detected serotypes and their patterns of occurrence in polio-suspected paralytic cases, healthy contacts and aseptic meningitis cases.

METHODS

Studied population and sampling. A total of 4700 samples, collected during the 12-year period between January 1992 and December 2003, were investigated (Table 1): 4278 stool samples and 422 from cerebrospinal fluid (CSF). Stool samples were obtained from 525 polio-suspected paralytic cases ($n = 1086$, most of the cases had two stools collected at a 24–48 h interval), 3122 of their healthy contacts ($n = 3122$, one sample from each individual) and 70 patients with aseptic meningitis. A CSF sample was collected from each of the 422 patients with aseptic meningitis.

EV detection in clinical samples: virus isolation on cell culture. All samples were inoculated onto the WHO-recommended cell lines for HEV detection (World Health Organization, 2000): RD and HEp-2cincinatti for all the samples, and L20B for samples obtained since 1997. L20B cells are known to be highly sensitive to PV isolation. When complete cytopathic effect (CPE) was obtained, the infected cells were harvested and kept frozen (−20 °C) until typing.

Identification of viral isolates

(i) Serotype identification by seroneutralization of the CPE on cell culture. Microneutralization tests with pools of antisera specific for the most common HEV serotypes were used, according to standard protocols (World Health Organization, 2000), for all virus isolates obtained: type-specific PV antisera, enterovirus A–G pools (National Institute of Public Health and the Environment, Bilthoven, The Netherlands) and in-house-produced monospecific immune antisera specific for the CBV serotypes.

(ii) Serotype identification by partial sequencing in the VP1 genomic region. This technique was used for all isolates that could not be typed by seroneutralization of the CPE. Virus RNA extraction, reverse transcription and PCR amplification were conducted using the 'in-house' protocol previously described (Rezig *et al.*, 2004), and the primers published by Oberste *et al.* (2000). The serotype was determined, as recommended by Oberste *et al.* (1999a, 2000), by comparing the obtained 356 bp sequence to the sequences of the same region existing in the GenBank database. In this scheme, a VP1 sequence identity of at least 75 % to any HEV prototype strain and a second-highest identity score less than 70 % indicates that the isolate is of the

Table 1. Studied population and frequency of enterovirus isolation

	Number of cases												Total studied cases (stools, CSF samples)	PV-positive cases (stool samples)	NPEV-positive cases (stool samples)
	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003			
Paralytic cases	25	36	31	45	56	59	41	38	50	52	47	45	525 (1086, 0)	24 (42)	63 (71)
Healthy contacts	679	268	204	380	265	283	245	160	137	168	166	167	3122 (3122, 0)	83 (83)	153 (153)
Aseptic meningitis cases	7	16	7	11	11	37	7	14	110	64	51	87	422 (70, 422)	0 (0)	20 (20)
Total	711	320	242	436	332	379	293	212	297	284	264	299	4069 (4278, 422)	107 (125)	236 (244)

homologous serotype. A high score between 70 and 75 % or a second-highest score of more than 70 % indicates a tentative identification that must be confirmed by other means, whereas a high score of less than 70 % indicates that the sequence of the isolate does not match any sequence in the database.

Intratypic differentiation (ITD) of polioviruses. All isolated PV were tested for their wild or vaccine origin systematically by two distinct methods according to WHO-recommended protocols (World Health Organization, 2000): one antigenic and one genetic. The antigenic method is the ELISA test using cross-absorbed Sabin-specific or wild-specific rabbit antisera; the genetic method is the hybridization assay using recombinant riboprobes specific for vaccine-related isolates or the PCR test using Sabin-specific primers.

RESULTS AND DISCUSSION

This report gives an overview of PV and NPEV circulation in Tunisia over a 12-year period, based on data collected as part of PV surveillance and a routine diagnostic programme for aseptic meningitis.

Poliovirus isolation

A total of 125 samples were PV-positives. They were collected from 107 individuals: 24 AFP cases and 83 of their healthy contacts. No PV were isolated from aseptic meningitis cases (Table 1). Serotyping identified a total of 115 PV isolates: 47 of serotype 1, 22 of serotype 2 and 46 of serotype 3. For 10 AFP cases, PV was isolated from the two samples collected, thus we considered only one isolate per case. Six individuals had a mixture of two PV serotypes: PV1 plus PV2 in four individuals; PV2 plus PV3 in two individuals. One case had a mixture of the three PV serotypes: PV1 plus PV2 plus PV3. Among all PV isolates, 86 were obtained from 1992 to 1997 and 29 from 1998 to 2003. The PV isolation rate in stool samples was lower during the second period: 1·7 % (29 out of 1677 stools tested) versus 3·3 % (86 out of 2601 stools) with a statistically significant difference ($P = 0{\cdot}0018$). All isolates had concordant ITD results (vaccine or wild origin) between ELISA and probe hybridization or PCR. The number of PV-positive isolates per year and their ITD results are given in Fig. 1. All PV type 2 were found to be vaccine-related. Wild-type PV1 was isolated in 1994 from a healthy contact of an AFP case. Wild-type PV3 was detected in four AFP cases and 13 healthy contacts in 1992 and in two healthy contacts in 1994. From 1995 to 2003, all isolated PV strains were vaccine-related. In a previous study (Triki *et al.*, 1999), we reported the results of PV surveillance in Tunisia up to 1996 and the genetic characteristics of the wild PV isolated during the 1980s and the early 1990s. All wild PV isolates from serotype 3 belonged to the same genotype, which seems specific to the country; wild isolates from serotype 1 belonged to two different genotypes. The present study confirms that the wild viruses from 1994 are the latest wild strains isolated in the country. These findings are certainly due to the effectiveness of the extensive supplemental immunization activities conducted in the country up to 1997, particularly the annual National Immunization Days organized from 1995 to 1997. It is likely that these enlarged mass vaccination activities resulted in the interruption of the circulation of endemic PV strains and induced high levels of poliovirus immunity in the population, which constituted an effective barrier against the importation of wild strains from other regions of the world. Most of the PV isolated during the study period were vaccine-derived. OPV is, in fact, the main vaccine used in the country as part of the national immunization programme; the inactivated vaccine is used for a very low proportion of neonates vaccinated in the private sector. Vaccine strains are excreted by vaccinees and may be transmitted to susceptible individuals in the community. The higher rate of PV isolation during the period 1992–1997 likely results from the extensive use of OPV up to 1997. During the past 6 years, OPV has been used only for routine

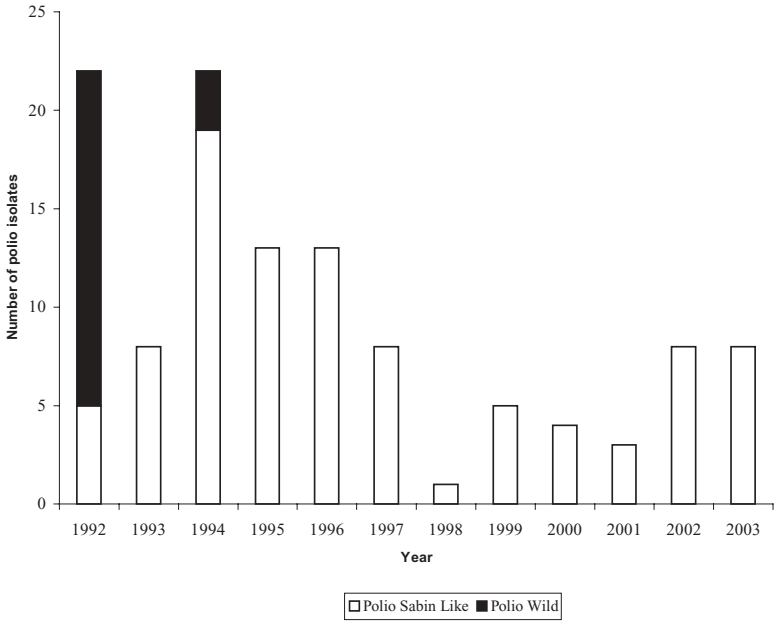

Fig. 1. Intratypic differentiation of isolated polioviruses.

vaccination of neonates with much more restricted supplemental vaccination activities. Given the risk of wild PV importation, attentive PV surveillance is to be maintained in all countries of the world, including those where endemic wild PV transmission has been interrupted, until global poliomyelitis eradication is achieved.

NPEV isolation

A total of 244 samples were positive for NPEV. They were collected from 236 individuals: 63 AFP cases, 153 healthy contacts and 20 aseptic meningitis cases (Table 1). Seroneutralization of CPE on cell culture was performed by the enterovirus-specific pools which allow the identification of the 27 most common enterovirus serotypes (ECV1–7, 9, 11–14, 20–22, 25, 27, 29, 30, 33, CBV1–6 and CAV9). The method enabled the identification of 93 % ($n = 219$) of the NPEV isolates. Twenty-three different serotypes were identified: ECV1, ECV2, ECV3, ECV4, ECV6, ECV7, ECV9, ECV11, ECV12, ECV13, ECV14, ECV20, ECV21, ECV22, ECV25, ECV29, ECV30, ECV33, CBV1, CBV2, CBV3, CBV4 and CBV5. Most echoviruses were isolated on RD cells, and coxsackieviruses on HEp-2c. For the remaining 17 isolates the serotype was assessed by partial sequencing of the VP1 region of the genome. Sixteen isolates belonged to six supplemental serotypes, ECV15, ECV19, CAV18, CAV20, CAV21 and CAV24, which are not covered by the RIVM A–G antisera pools used in seroneutralization. The VP1 sequence of the remaining isolate suggested serotype ECV1, which is normally identifiable by seroneutralization. The lack of neutralization with the antisera pools may be due to evolution of this strain so that it is no longer neutralized with the antiserum produced against the reference strain of the same serotype. Two isolates of CBV detected in 1995 remained untypable. They were neutralized by CBV group specific antisera included in enterovirus A–G pools but could not be neutralized by monospecific immune antisera specific for the CBV serotypes. Moreover, their amplification by PCR failed. With worldwide PV surveillance and the recent development of new molecular tools for enterovirus detection and identification, more interest is being given to NPEV. Standard methods for enterovirus detection and identification are based on virus isolation on cell culture followed by serotyping the isolated viruses by serum neutralizing assays using pools of serotype-specific antisera (Muir *et al.*, 1998). This procedure is time-consuming and labour-intensive and the availability of specific antisera gradually becomes restricted. Several techniques for rapid detection of the enterovirus genome in clinical samples, most of them based on PCR amplification in the 5′ non-coding (5′NC) of the genome, have been developed; however, these methods do not allow serotype identification and genetic characterization of the detected viruses beyond the genus level (Chapman *et al.*, 1990; Rotbart *et al.*, 1994; Lina *et al.*, 1996; Kuan, 1997; Pozo *et al.*, 1998; Van Loon *et al.*, 1999). Thus when information on the serotype is needed, virus isolation on cell culture remains the most appropriate technique. To overcome the problems related to the specific antisera, more recent work attempts to develop new methods for typing enteroviruses by PCR amplification and partial sequencing in the VP1 region of the genome (Oberste *et al.*, 1999a, b; Norder *et al.*, 2001; Caro *et al.*, 2001). Different parts of this region were targeted by the authors and all proved to contain serotype-specific information. In this study, we used one of these methods to identify the isolates that could not be serotyped by the enterovirus-specific pools available in the laboratory.

NPEV isolation rate

The NPEV isolation rate varied from year to year, ranging from 3·0 to 10·8 % per year with a mean rate of 5·3 %. These rates under 10 % during all of the 12-year study period reflect a relatively low circulation of NPEV in the country. The annual NPEV isolation rate counts among the WHO-recommended indicators that may be used to monitor the sensitivity of national PV surveillance programmes. In fact, except L20B cells, the two other cell lines used for PV surveillance (HEp-2c and RD) are susceptible to all PV and NPEV. In a given country/region, the PV isolation rates may decrease as a result of the effectiveness of the eradication programme; however, this may also be due to a loss in the sensitivity of laboratory methods or to inadequate conditions for sample collection and transportation. One of the indicators that may be used to differentiate between the two situations is the NPEV isolation rate, given that these viruses have a worldwide distribution and are not targeted by any control programme. Previous studies suggested that, in most developing countries, the annual NPEV rate is at a minimum of 10 % of stool samples assessed. However, it was well recognized that in some countries or regions, the NPEV rate may be less than 10 % (Sanders *et al.*, 1997), depending on the socio-economic level and climatic factors.

The NPEV isolation rate varied throughout the year: a low period of transmission was observed during winter and a high period of transmission from March to November with a peak in autumn (September–November). This period of the year is, in fact, marked by a high degree of humidity associated with elevated temperatures, two climatic factors which have been reported as facilitating enterovirus transmission (Druyts-Voets, 1997). It is also known that NPEV isolation varied with season in temperate climates with the occurrence of NPEV infections during the summer and autumn, in comparison with tropical climates where these viruses are isolated throughout the year (Hovi *et al.*, 1996; Nairn & Clements, 1999).

NPEV isolation periodicity

During the 12-year period studied, a total of 29 different serotypes of NPEV were detected in Tunisia. The periodicity of their detection by year is shown in Table 2. ECV11, ECV6 and ECV30 were the most frequently isolated, almost every year (Table 2). These three serotypes were also reported to be among the most frequently isolated in other countries (Strikas *et al.*, 1986; Druyts-Voets, 1997; Khalfan *et al.*, 1998;

Table 2. NPEV serotype isolation and their distribution by year

Serotype	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	Total
Endemic NPEV serotypes													
ECV11	1	2	1			1	4	1		2	1	1	14
ECV6	3	5			1	4	1		1	2	1		18
ECV30			1	1	2		2	1		3	1	3	14
NPEV serotypes isolated with recurrence at variable intervals													
ECV20				4	4	4			3		1		16
ECV13		2					3	1	1		2		9
ECV1			1	1	1			1					4
ECV9				1	1		1			1			4
ECV25					1	13			1			3	18
ECV3				2		7	1			1			11
ECV33				7	1							1	9
CBV3	31					1		1				3	37
ECV7	12							1				1	14
CAV18	3			3		2							8
ECV12		2				2	2						6
CBV5		2						1	7				10
ECV21		1		1									2
ECV4		1			1								2
CAV24			3	3	1				1				8
CBV2			1			4							5
CBV1				7					2				9
NPEV serotypes isolated episodically													
CAV21				2									2
ECV29										1	3		4
CBV4		3											3
ECV2			1										1
ECV15				1									1
CAV20				1									1
ECV19						1							1
ECV14						1							1
ECV22												2	2
CBV				2									2

Trallero *et al.*, 2000; Meqdam *et al.*, 2002); they seem to be endemic with constant circulation among non-immune persons. Other serotypes showed a tendency to recur at variable intervals (ECV20, ECV13, ECV1, ECV9, ECV25, ECV3, CBV3, ECV7, ECV12 and CBV5) (Table 2). This cyclic occurrence was also reported in previous studies suggesting that some serotypes circulate during a period of time in a country/region undergoing high specific immunization of the population. This period is then followed by an interruption of virus circulation until a build-up of susceptibles occurs, resulting in the reintroduction of the virus and its transmission. Finally, some serotypes (CBV4, CAV20, ECV2, ECV14, ECV15) were isolated only during a limited period of 1–2 years (Table 2) with very few isolates and did not reappear during the study period. As these were detected only during a limited period, this pattern suggests that they were not endemic and were occasionally introduced in the country.

The pattern of prevalence of NPEV also varied with time throughout the study period. Up to 1997, 5–13 different serotypes were isolated each year with a total of 28 different serotypes detected during the 6-year period, and the proportion of NPEV positive individuals was 6·7 % (161 out of 2420 individuals). The NPEV prevalence and the serotype diversity decreased during the 6 subsequent years, from 1998 to 2003: only 19 different serotypes were detected during the whole period, with a maximum of seven different serotypes detected each year and a proportion of NPEV positives of 4·6 % (75 out of 1649 individuals). The difference observed between the two periods was statistically significant ($P = 0{\cdot}004$). Thus one may question if, beside their proven effect in reducing and even interrupting wild PV circulation, nationwide PV vaccination campaigns may have a similar impact on NPEV circulation; such campaigns have been conducted in Tunisia up to 1997.

Table 3. NPEV serotype detection according to the clinical status of the infected individuals

Virus serotype	Aseptic meningitis	Paralytic cases	Asymptomatic individuals (contact of paralytic cases)	Total
ECV6	2	6	10	18
ECV11	2	5	7	14
ECV30	3	5	6	14
ECV13	1	3	5	9
CBV1	2	1	6	9
CBV5	7	3		10
ECV1	1		3	4
ECV9	1		3	4
ECV20		3	13	16
ECV3		3	8	11
ECV25		4	14	18
ECV7		1	13	14
ECV12		2	4	6
ECV29		2	2	4
ECV21		1	1	2
ECV4		1	1	2
CBV3		6	31	37
CAV24		2	6	8
CAV18		3	5	8
ECV22		1	1	2
ECV19		1		1
CAV20		1		1
ECV33			9	9
CBV2			5	5
CBV4			3	3
CAV21			2	2
ECV2			1	1
ECV14			1	1
ECV15			1	1
CBV			2	2
				236

Table 3 shows serotype detection according to the clinical status of the infected individuals. Some serotypes, including the most frequent ones (echoviruses of serotypes 11, 6 and 30), were detected in meningitis, paralytic cases and healthy contacts. Some serotypes were detected in paralytic cases and healthy individuals; others were isolated only from healthy people. Most of the serotypes were isolated from asymptomatic individuals. This highlights the important capacity of enteroviruses to have silent circulation in the healthy population. Echoviruses of serotypes 1, 6, 9, 11, 13 and 30 and CBV of serotypes 1 and 5 were isolated from aseptic meningitis cases. Previous studies also reported the association of most of these serotypes, particularly ECV11, ECV6, ECV1, ECV9 and ECV13, with meningitis (CDC, 2002; Chomel *et al.*, 2003). In contrast to aseptic meningitis cases where the viral isolates were obtained from CSF, the viruses isolated from paralytic cases were obtained from stool samples, and thus their implication as the aetiological agent of the paralytic disease is not so evident. Their presence in the stool sample may also result from an asymptomatic carriage of the virus, the paralytic disease being due to other viral or non-viral aetiology.

This study is a first report of NPEV epidemiology in Tunisia. Although enterovirus detection was conducted only as part of poliovirus surveillance and of a routine diagnostic programme for aseptic meningitis, circulation of multiple enterovirus serotypes was detected and probably associated with a significant disease burden. A better knowledge of the transmission and the implication of these viruses in other diseases such as myocarditis, diabetes mellitus and outbreaks of haemorrhagic conjunctivitis seems to justify future studies on NPEV epidemiology.

ACKNOWLEDGEMENTS

This work was supported by the Tunisian State Secretariat for Scientific Research and Technology (SERST, grant UR 03/02) and Pasteur

Institutes Network (FSP 2001-168 Reseau de surveillance des resistances aux anti-infectieux") The authors are thankful to Ahlem Djebbi for critical comments on the article.

REFERENCES

Caro, V., Guillot, S., Delpeyroux, F. & Crainic, R. (2001). Molecular strategy for 'serotyping' of human enteroviruses. *J Gen Virol* **82**, 79–91.

CDC (2002). Enterovirus type 13 – United States, 2001. *Morb Mortal Wkly Rep* **50**, 777–780.

Chapman, N., Tracy, S. & Guantt, C. (1990). Molecular detection and identification of enteroviruses using enzymatic amplification and nucleic hybridisation. *J Clin Microbiol* **28**, 843–850.

Chomel, J. J., Antona, D., Thouvenot, D. & Lina, B. (2003). Three echovirus serotypes responsible for outbreak of aseptic meningitis in Rhône-Alpes region, France. *Eur J Clin Microbiol Infect Dis* **22**, 191–193.

Druyts-Voets, E. (1997). Epidemiological features of entero non-poliovirus isolations in Belgium 1980-94. *Epidemiol Infect* **119**, 71–77.

Hovi, T., Stenvik, M. & Rosenlew, M. (1996). Relative abundance of enterovirus serotypes in sewage differs from that in patients: clinical and epidemiological implications. *Epidemiol Infect* **116**, 91–97.

Ishiko, H., Shimamda, Y., Yonaha, M., Hashimoto, O., Hayashi, A., Sakae, K. & Takeda, N. (2002). Molecular diagnosis of human enteroviruses by phylogeny-based classification by use of the VP4 sequence. *J Infect Dis* **185**, 744–754.

Khalfan, S., Aymard, M., Lina, B., Thouvenot, D., Shuffenecker, I., Foray, S., Fernandes, E. & Baig, B. (1998). Epidemics of aseptic meningitis due to enteroviruses following national immunization days in Bahrain. *Ann Trop Paediatr* **18**, 101–109.

Kuan, M. M. (1997). Detection and rapid differentiation of human enteroviruses following genomic amplification. *J Clin Microbiol* **35**, 2598–2601.

Lina, B., Pozzetto, B., Andreoletti, L. & 22 other authors (1996). Multicenter evaluation of a commercially available PCR assay for diagnosing enterovirus infection in a panel of cerebrospinal fluid specimens. *J Clin Microbiol* **34**, 3002–3006.

Meqdam, M., Khalousi, M. & Al-Shurman, A. (2002). Enteroviral meningitis in northern Jordan: prevalence and association with clinical findings. *J Med Virol* **66**, 224–228.

Muir, P., Kämmerer, U., Korn, K., Mulders, M., Pöyry, T., Weissbrich, B., Kandolf, R. & Cleator, G. (1998). Molecular typing of enteroviruses: current status and future requirements. *Clin Microbiol Rev* **11**, 202–227.

Nairn, C. & Clements, G. B. (1999). A study of enterovirus isolations in Glasgow from 1977 to 1997. *J Med Virol* **58**, 304–312.

Norder, H., Bjerregaard, L. & Magnius, L. O. (2001). Homotypic echoviruses share aminoterminal VP1 sequence applicable for typing. *J Med Virol* **63**, 35–44.

Oberste, M. S., Maher, K., Kilpatrick, D. R. & Pallansch, M. A. (1999a). Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. *J Virol* **73**, 1941–1948.

Oberste, M. S., Maher, K., Kilpatrick, D. R., Flemister, M. R., Brown, B. A. & Pallansch, M. A. (1999b). Typing of human enteroviruses by partial sequencing of VP1. *J Clin Microbiol* **37**, 1288–1293.

Oberste, M. S., Maher, K. & Flemister, M. R. (2000). Comparison of classic and molecular approaches for the identification of untypeable enteroviruses. *J Clin Microbiol* **38**, 1170–1174.

Pozo, F., Casas, I., Tenorio, A., Trallero, G. & Echevarria, J. M. (1998). Evaluation of a commercially available reverse transcription-PCR assay for diagnosis of enteroviral infection in archival and prospectively collected cerebrospinal fluid specimens. *J Clin Microbiol* **36**, 1741–1745.

Rakoto-Andrianarivelo, M., Rousset, D., Razafindrastsimandresy, R. & Delpeyroux, F. (2002). Nouvelle méthode de typage moléculaire des *Entérovirus* humains: caractérisation des souches malgaches ''non sérotypables''. *Arch Inst Pasteur Madag* **68**, 55–58.

Rezig, D., Ben Yahia, A., Ben Abdallah, H., Bahri, O. & Triki, H. (2004). Molecular characterization of coxsackievirus B5 isolates. *J Med Virol* **72**, 268–274.

Rotbart, H. A., Sawyer, M. H., Fast, S., Lewinski, C., Murphy, N., Keyser, E. F., Spadoro, J., Kao, S. Y. & Loeffelholz, M. (1994). Diagnosis of enteroviral meningitis by using PCR with colorimetric microwell detection assay. *J Clin Microbiol* **32**, 2590–2592.

Sanders, R., Maher, C., Aylward, R. B., Bilous, J., Schnur, A., Sato, Y., Omi, S. & Tangermann, H. (1997). Development and coordination of the polio laboratory network in the western pacific region of the world health organization. *J Infect Dis* **175**, S117–S121.

Strikas, R. A., Anderson, L. J. & Parker, R. A. (1986). Temporal and geographic patterns of isolates of nonpolio enterovirus in the United States, 1970-1983. *J Infect Dis* **153**, 346–351.

Trallero, G., Casas, I., Tenorio, A., Echevarria, J. E., Castellanos, A., Lozano, A. & Brena, P. (2000). Enteroviruses in Spain: virological and epidemiological studies over 10 years (1988–97). *Epidemiol Infect* **124**, 497–506.

Triki, H., Bahri, O., Guillot, S. & 7 other authors (1999). Molecular epidemiology of poliovirus infection in Tunisia. *J Med Microbiol* **48**, 569–576.

Van Loon, A. M., Cleator, G. C. & Ras, A. (1999). External quality assessment of enterovirus detection and typing. *Bull W H O* **77**, 217–223.

World Health Organization (1988). Global eradication of poliomyelitis by the year 2000. *Bull W H O* **63**, 161–168.

World Health Organization (1994). Poliomyelitis eradication, Tunisia. *Bull W H O* **69**, 341–348.

World Health Organization (2000). *Manual for the Virological Investigation of Poliomyelitis*. Geneva, Switzerland: World Health Organization.