Molecular typing of enteroviruses associated with viral meningitis in Cyprus, 2000–2002

Jan Richter, Dana Koptides, Christina Tryfonos
and Christina Christodoulou

Correspondence

Jan Richter
richter@cing.ac.cy

Department of Molecular Virology, Cyprus Institute of Neurology and Genetics, PO Box 23462,
1683 Nicosia, Cyprus

Human enteroviruses are responsible for a wide spectrum of clinical diseases affecting many different organ systems. Although infection is usually asymptomatic, infections of the central nervous system manifested as meningitis or encephalitis can pose a serious public health problem, especially during outbreaks. In this study, samples from 218 patients diagnosed with enteroviral meningitis between January 2000 and December 2002 were analysed in order to assess the epidemiology of human enteroviruses as a cause of viral meningitis in Cyprus. A new typing strategy, based on partial sequencing of the 5' non-coding region (5'NCR), prediction of type, and selection of type-specific primers for sensitive VP1 PCR amplification, was developed. As clustering in the 5'NCR was concordant with clustering in the VP1 region, quick and reliable typing by VP1 sequencing was achieved without virus isolation in cell culture. The most frequent enterovirus serotypes identified were Human echovirus 30 (55.5%), Human echovirus 13 (15.1%), Human echovirus 6 (13.8%) and Human echovirus 9 (8.3%). Human coxsackieviruses B2, B1 and B5, Human echovirus 4, Human enterovirus 71 and Human coxsackievirus A6 represented rather rare serotypes. This is the first molecular epidemiological study of enterovirus meningitis in Cyprus. Serotype distribution corresponded basically with observations in other European countries, suggesting the spread of enteroviruses by tourism.

Received 30 November 2005

Accepted 28 March 2006

INTRODUCTION

Non-polio human enteroviruses (HEVs) belonging to the *Picornaviridae* family represent the most common cause of aseptic meningitis, which, conversely, is the most commonly identified illness in association with enterovirus infections (Rotbart, 1995). HEVs are ubiquitous faecal-orally transmitted small RNA viruses with a seasonal peak of infection in summer and autumn. Outbreaks of disease caused by a single serotype strain are frequently reported and represent a major public health problem (Ozkaya *et al.*, 2003; Avellon *et al.*, 2003; Thoelen *et al.*, 2003).

The typing of enteroviruses is based conventionally on virus isolation in cell culture, followed by neutralization with intersecting pools of type-specific antisera and confirmation with monospecific antisera to identify the serotype. Due to the large number of antigenically distinct serotypes, serotyping procedures are time-consuming, labour-intensive and costly. Moreover, the limited supply of reference sera and their inability to detect new antigenic variants or emerging

serotypes are major drawbacks of neutralization typing (el Sagey *et al.*, 1998).

Because VP1 is the major surface-accessible protein in the picorna virion that exhibits relevant serotype-specific neutralization epitopes, the sequence of the VP1-encoding gene has been shown to correlate well with the classical serotype classification (Oberste *et al.*, 1999, 2003). However, assays utilizing highly degenerated primers to amplify a portion of the VP1 gene in all HEV serotypes usually require cell culture prior to extraction due to a lack of sensitivity (Oberste *et al.*, 1999, 2003; Caro *et al.*, 2001).

Since sample material is often used up completely for RNA and DNA extractions, for diagnostic purposes the typing of HEVs utilizing cell culture is often not possible. For this reason a typing strategy based on the sequencing of a large part of the 5' untranslated region and amplification of a part of the VP1 region was developed.

In this study, samples from 218 patients with viral meningitis, who were diagnosed enterovirus positive between January 2000 and December 2002, have been analysed.

Data obtained by sequencing a 399 bp fragment in the 5'NCR of the enterovirus genome, together with information about currently circulating HEV strains, were used to

Abbreviations: CSF, cerebrospinal fluid; CV, human coxsackievirus; HEV, human enterovirus; 5'NCR, 5' non-coding region.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are AY898964–AY899181.

design serotype-specific primers amplifying a portion of the VP1 gene directly from clinical specimens in order to unambiguously identify the serotype.

METHODS

Patients and clinical samples. Between February 2000 and December 2002 cerebrospinal fluid (CSF) and/or stool samples from 298 patients with suspected viral meningitis from all parts of Cyprus were sent to our laboratory for analysis. The median patient age was 6 years, ranging from one month to 77 years, with a male:female ratio of 1.5 (Fig. 1). For routine laboratory diagnosis, an in-house nested RT-PCR assay targeting a sequence in the highly conserved 5'NCR was employed. One hundred and sixty-eight of 228 CSF samples (74%) and 50 of 70 stool samples (71%) were found to be enterovirus positive. Subsequently, these 218 positive samples were subjected to molecular typing for epidemiological surveillance.

Extraction and 5'NCR nested RT-PCR. Stool samples were treated as previously described (World Health Organization, 2004). Viral RNA was extracted from 140 µl of clinical specimen (CSF or stool) by the QIAamp Viral RNA Mini kit (Qiagen), according to the manufacturer's instructions. For reverse transcription, 10 pmol primer HEV 7 (see Table 1) and 8 µl 2.5 mM dNTP mix were added to 5 µl RNA. The mixture was heated to 70 °C for 5 min and subsequently snap-cooled on ice for 1 min. To each sample were added 5 µl of a reaction mixture containing 1 × Taq buffer, 20 U ribonuclease inhibitor and 50 U M-MuLV reverse transcriptase (New England BioLabs). cDNA synthesis was performed for 30 min at 42 °C. cDNA product (2.5 µl) was added to 22.5 µl of a PCR mixture containing 1 × RT buffer, 5 nmol dNTPs, 5 pmol primers HEV 2 and HEV 7, and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems). Amplification involved 30 cycles of denaturation at 95 °C for 30 s, annealing at 42 °C for 45 s and elongation at 72 °C for 1 min. For nested PCR, 2.5 µl of first PCR product was used in an equivalent assay with primers HEV 5 and HEV 8. The 399 bp products were visualized in a 1.5% agarose gel containing 1 µg ethidium bromide ml⁻¹.

Sequencing. Amplicons were purified using the Montage DNA Gel Extraction kit (Millipore) and sequenced on a CEQ 8000 sequencer

(Beckman Coulter) in the forward and reverse directions using the respective PCR primers and the CEQ Dye Terminator Cycle Sequencing with Quick Start kit (Beckman Coulter).

Sensitivity. The sensitivity and specificity of the 5'NCR nested RT-PCR assay was verified using the 2004 Quality Control for Molecular Diagnostics (QCMD) HEV proficiency panel. The panel consisted of 12 lyophilized samples, of which nine were HEV positive, one was Human rhinovirus 16 (RV16) positive and two were negative. The positive samples contained various dilutions of tissue-culture-grown heat-inactivated viruses in a virus transport medium or a PBS/fetal calf serum matrix, the lowest concentration being 0.03 tissue-culture infective dose 50 (TCID₅₀) ml⁻¹. All HEV-positive samples were successfully amplified, while the rhinovirus and negative samples did not yield a product.

Amplification of VP1-encoding sequences. Primers in the VP1 region of relevant enterovirus serotypes were developed based on prototype strain sequences available from GenBank. The selected serotype-specific primers were tested for heterologous priming with BLAST and are listed in Table 1. A VP1 one-tube two-step RT-PCR was carried out as follows: 10 pmol antisense primer and 4 µl 2.5 mM dNTP mix were added to 2.5 µl viral RNA template. The mixture was heated to 70 °C for 5 min and subsequently snap-cooled on ice for 1 min. To each sample were added 2.5 µl of a reaction mixture containing 1 × Taq buffer, 20 U ribonuclease inhibitor and 25 U M-MuLV reverse transcriptase (New England BioLabs). cDNA synthesis was performed for 30 min at 42 °C. After completing cDNA synthesis, to each tube were added 40 µl of a PCR mixture containing 1 × Taq buffer, 10 pmol of the respective sense primer and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems). Amplification involved 40 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 45 s and elongation at 72 °C for 1 min. The PCR products were visualized in a 1.5% agarose gel containing 1 µg ethidium bromide ml⁻¹. Purification and sequencing of the VP1 amplicons were carried out as described above.

Phylogenetic analysis. Multiple sequence alignments were built with CLUSTALW. Phylogenetic analyses were conducted using MEGA version 2.1 (Kumar *et al.*, 2001). Genetic distances were calculated using the Kimura two-parameter model with a transition:transversion ratio of 2. The phylogenetic tree was constructed using the neighbour-joining method. Statistical significance of phylogenies was estimated by bootstrap analysis with 500 pseudoreplicates. For reasons of clarity, interior branches representing distinct clusters were collapsed into elongated triangles, whose thickness was proportional to the number of taxa condensed and whose width was proportional to the maximum distance between the taxa.

RESULTS AND DISCUSSION

Two hundred and eighteen CSF and/or stool samples collected over a period of 3 years from patients diagnosed with enteroviral meningitis were analysed for epidemiological surveillance. A nested RT-PCR was employed to amplify a 399 bp fragment from the highly conserved 5'NCR. A phylogenetic tree was constructed using the neighbour-joining method to group the samples for further characterization (see Fig. 2a). Representative prototype sequences were included in the alignment. The 5'NCR tree revealed 12 clusters. Since it had been reported previously that clustering in the 5'NCR tree is concordant with clustering in the VP1 phylogenetic tree (Thoelen *et al.*, 2003, 2004), it was assumed that samples belonging to one cluster represented the same enterovirus type. Consensus sequences for each

Fig. 1. Age distribution of patients diagnosed with enteroviral meningitis in Cyprus between January 2000 and December 2002.

Table 1. Oligonucleotide primers used for 5'NCR nested RT-PCR and VP1 RT-PCR

The positions of the primers in the 5'NCR are relative to the genome of Poliovirus 1 Mahoney. The positions of the primers in the VP1 region are relative to the genomes of Human echovirus 30 strain Bastianni (AF162711), Human echovirus 13 strain Del Carmen (AY302539), Human echovirus 9 isolate DM (AF524867), Human echovirus 6 lytic strain (U16283), Human echovirus 4 (AY302557), HEV71 polyprotein gene (U22522), CVA6 strain Gdula (AY421764), CVB1 (M16560), CVB2 strain Ohio (AF081485) and CVB5 (X67706), respectively.

Primer	Polarity	Region	Position	Sequence (5'–3')
HEV 2	Sense	5'NC	65–84	CGGTACCTTTGTGCGCCTGT
HEV 5	Sense	5'NC	162–182	CAAGCACCTCTGTTTCCCCGG
HEV 7	Antisense	5'NC	575–594	ATTGTCACCATAAGCAGCCA
HEV 8	Antisense	5'NC	540–559	AAACACGGACACCCAAAGTA
E30 VPA	Sense	VP1	2547–2567	GACAGCAGTGGAGACAGGGCA
E30 VPB	Antisense	VP1	2904–2924	GCCAGGCAAAGTCTTCATAAC
E13 VPA	Sense	VP1	2528–2547	GCTGAGACAGGGCACACATC
E13 VPB	Antisense	VP1	2895–2915	GCCATGCGTAATCGTCCACAC
E9 VPA	Sense	VP1	2487–2506	GGGTTGCTGACACAATACGC
E9 VPB	Antisense	VP1	2941–2960	GCATTCCCTTCAGTCCAAAA
E6 VPA	Sense	VP1	2472–2493	GTCAATTGTTCTGTAGCGGAC
E6 VPB	Antisense	VP1	2926–2947	GTTGGTGGAAAGTTTGCCAGTT
E4 VPA	Sense	VP1	2547–2566	TGGAGACTGGCCATACCTCG
E4 VPB	Antisense	VP1	2958–2977	CGCATTCCCTTCAGTCCAGA
EV71 VPA	Sense	VP1	2597–2616	TGGGGCATCGTCAAATACTA
EV71 VPB	Antisense	VP1	3013–3034	GCGGGTGACATGAATGGAACGTG
CVA6 VPA	Sense	VP1	2666–2688	GTGGAACAGTTTACTCCCCTGCG
CVA6 VPB	Antisense	VP1	3029–3054	GTAGGGTAACCATCATAAAACCACTG
CVB1 VPA	Sense	VP1	2665–2688	GCTTGTGTGTATTACGCCACCTAC
CVB1 VPB	Antisense	VP1	3107–3130	CTTGGCCTGCCTCATTTACGTGCC
CVB2 VPA	Sense	VP1	2634–2657	CCGAATCCAGCGTAGAGAACTTCC
CVB2 VPB	Antisense	VP1	2922–2947	GCATATATTGTGCCCATATTGTTAAG
CVB5 VPA	Sense	VP1	2505–2527	AGCGGTCCAGTCAACTCAGAGTC
CVB5 VPB	Antisense	VP1	3024–3045	TAGCCCACCCATCATAGAACAT

cluster were determined and subjected to a BLAST search against GenBank.

Based on these results, primers amplifying a part of the VP1 region of potential enterovirus serotypes were designed using prototype enterovirus strain sequences available from GenBank. One-step RT-PCR was then performed in the VP1 region to verify the serotype of each cluster, starting with the highest match of the 5'NCR sequencing result. For six clusters, the serotype could already be determined in this manner after one RT-PCR [Human echovirus 4, Human echovirus 9, Human echovirus 6 strain A, Human echovirus 30 strain A, HEV71 and Human coxsackievirus (CV)A6]; three further clusters yielded a positive result after testing them for the second-highest identity score (CVB1, CVB2 and CVB5). However, the differences between the highest and second-highest matches were in all three cases only minor, i.e. 1 or 2 nucleotides, thus making a clear decision difficult. For the remaining three clusters, however, a positive result was not obtained at this stage. Since two of these clusters contained a significantly large number of samples, it was decided to probe them for the predominant serotypes

found in Europe during 2000 and 2001. Human echovirus 30 was one of the enteroviruses isolated most frequently, causing outbreaks of aseptic meningitis in France, Iceland, Kosovo and the Netherlands in 2000, and in England and Wales, Scotland, Ireland and Germany in 2001 (Noah & Reid, 2002; Communicable Disease Surveillance Centre, 2001; Bernit *et al.*, 2004). Human echovirus 13, previously regarded a rare enterovirus serotype, caused outbreaks in England and Wales, Scotland, Ireland, Germany, France and the Netherlands in 2000 (Chomel *et al.*, 2003; Noah & Reid, 2002). In an epidemiological study of an outbreak of aseptic meningitis in the summer of 2000 in Belgium, human echoviruses 30, 13 and 6 were identified most frequently during the epidemic (Thoelen *et al.*, 2003).

Using primers in the VP1 region for human echoviruses 30 and 13, two more clusters could be identified (Human echovirus 13 and Human echovirus 30 strain B). The last remaining cluster was then tried for the next highest matches of the 5'NCR BLAST result. All VP1 amplicons generated were sequenced and BLAST analysis was carried out. Each cluster was assigned the serotype that yielded the

1038

Journal of Medical Microbiology 55

Table 2. Enterovirus types identified from 218 samples in Cyprus between 2000 and 2002

Enterovirus serotype	Number of samples in:			Total	Percentage of total
	2000	2001	2002		
Human echovirus 30	43	78	0	121	55.5
Human echovirus 13	33	0	0	33	15.1
Human echovirus 6	2	15	13	30	13.8
Human echovirus 9	0	0	18	18	8.3
CVB2	7	0	0	7	3.2
Human echovirus 4	0	0	3	3	1.4
HEV71	0	0	2	2	0.9
CVB1	2	0	0	2	0.9
CVA6	0	0	1	1	0.5
CVB5	1	0	0	1	0.5
Total	88	93	37	218	—

monophyletically as they did in the VP1 tree. However, samples forming a distinct subcluster in the 5'NCR tree were found to be of the same serotype by VP1 typing.

Table 2 summarizes the distribution of serotypes identified in Cyprus from 2000 to 2002. Ten different enterovirus serotypes were identified, with human echoviruses 30, 13, 6 and 9 being the most prevalent enteroviruses encountered. CVB2, B1 and B5, Human echovirus 4, HEV71 and CVA6 represented rather rare serotypes isolated in this study. In Fig. 3 is depicted the temporal distribution of the four most prevalent serotypes mentioned above. In spring 2000, Human echovirus 30 was identified most frequently as the cause of aseptic meningitis, but its incidence declined rapidly in summer as it was superseded by Human echovirus 13, which dominated until the end of November 2000. In 2001, another outbreak of Human echovirus 30 was observed, which lasted from April to September with some sporadic cases of Human echovirus 6 in May and June.

Human echovirus 6 then became the predominant enterovirus serotype from October 2001 to February 2002, when it was superseded by Human echovirus 9, which was isolated until July 2002. From Fig. 3 it is notable that the different virus strains caused temporally separated outbreaks that succeeded each other, having a mean duration of about 5 months.

Recently, many RT-PCR assays have been developed for the molecular typing of enteroviruses (Ishiko *et al.*, 2002; Caro *et al.*, 2001; Santti *et al.*, 1999a; Oberste *et al.*, 2003). In the past, different regions of the enteroviral genome have been evaluated for the correlation of genotype with serotype, and it has been shown that only the VP1 region fulfils the criterion of absolute correlation (Oberste *et al.*, 1999; Casas *et al.*, 2001; Caro *et al.*, 2001). The VP1 protein is immunodominant among the enterovirus capsid proteins (Mateu, 1995; Minor, 1990). In particular, the BC loop, which covers amino acids 82–93 of the 292 amino acid VP1

Fig. 3. Temporal distribution of the four most prevalent serotypes isolated in Cyprus between 2000 and 2002.

protein, has been shown to be important for the reactivity of type-specific antibodies (Norder *et al.*, 2001; Reimann *et al.*, 1991). In this study, data obtained by sequencing a 399 bp fragment in the 5'NCR, together with information about currently circulating HEV strains, were used to design serotype-specific primers for the VP1 region of the most probable serotypes. The partial VP1 sequences amplified encompassed entirely the hypervariable BC loop, making an unambiguous identification of the serotype possible. In most instances, knowledge of the 5'NCR clustering was very useful to guide the choice of primers for the VP1 RT-PCR. With additional information about currently circulating HEV strains it was also possible to identify the few remaining clusters. A major benefit of this approach is that it can be carried out directly on clinical specimens without the requirement for virus propagation in cell culture. In addition, the sample material is often used up for RNA and DNA extractions for diagnostic purposes, leaving this approach as the only opportunity for possible typing. Furthermore, results can be obtained in as little as 2 days.

Our results are in agreement with the findings of Thoelen *et al.* (2003), who observed that clustering in the 5'NCR is concordant with clustering in the VP1 region, but seem to contradict studies by Oberste *et al.* (2004a, b), who suggest that there is only minimal correlation between the 5'NCR and serotype due to recombination between different enteroviruses. Recombination, intratypic as well as intertypic, has been observed previously, and is thought to play an important role in enterovirus evolution (Santti *et al.*, 1999b; Oprisan *et al.*, 2002). However, the predictive power of the 5'NCR sequencing depends largely on the existence in GenBank of recent 5'NCR sequence data for currently circulating strains. The close relationship of the Cypriot isolates to previously identified European strains whose sequences have already been published may account for the success of this approach.

In the summer of 1996, an outbreak of viral meningitis occurred in Cyprus (Ramsay *et al.*, 1996; Communicable Disease Surveillance Centre, 1996a, b). More than 400 cases were admitted to hospital, the majority of whom were infants and young children. Most cases were mild and were discharged from hospital within a few days. No deaths or serious complications were observed. To our laboratory were sent 217 samples from the Limassol district, 88 from the Nicosia district, 84 from the Larnaca district and 16 from the Paphos district for analysis. Cell culture and RT-PCR confirmed the presence of enteroviruses in 292 samples (72%). Sequencing of a small fragment (135 bp) of the 5'NCR of 38 samples suggested CVB5 as the causative agent (unpublished data). Several stool samples were sent to the Public Health Laboratory Service (PHLS), Virus Reference Division, Colindale, UK, which isolated CVB5 from 14 of 21 specimens. CVB5 was also the most common cause of viral meningitis in England and Wales in 1996 (Communicable Disease Surveillance Centre, 1996c).

In this study, samples from 218 patients diagnosed with enteroviral meningitis between January 2000 and December 2002 were analysed. The most frequent enterovirus serotypes identified were human echoviruses 30 (55.5%), 13 (15.1%), 6 (13.8%) and 9 (8.3%) (see Table 2). Human echovirus 30 is one of the most commonly isolated enteroviruses, causing outbreaks of aseptic meningitis around the world every few years, and outbreaks in Europe in 2000 and 2001. Human echovirus 13, rarely reported previously, emerged in Cyprus as the second most predominant serotype in 2000. Many reports about Human echovirus 13 outbreaks in 2000 and 2001 across Europe and the USA have confirmed the emergence of this enterovirus as a human pathogen (Mullins *et al.*, 2004; Kirschke *et al.*, 2002; Avellon *et al.*, 2003; Noah & Reid, 2002; Chomel *et al.*, 2003). In general, the pattern of the most frequently isolated enterovirus serotypes in Cyprus seems to follow those in European countries. The large number of tourists arriving in Cyprus each year (~2 400 000), mainly from European countries, compared to the size of the population (~730 000), is probably a major factor in the epidemiology of enteroviral infections in Cyprus.

In conclusion, this study is the first molecular epidemiological analysis of enterovirus meningitis cases in Cyprus. A serotype distribution that basically corresponded with observations in other European countries during the same period was observed. Our approach showed that sequencing of a large part of the 5'NCR is very helpful in guiding the choice of primers for the VP1 RT-PCR, thus allowing a reliable typing of enteroviruses from clinical samples. This approach is especially useful in cases in which sample volume is limited or insufficient for cell culture, or the virus is uncultivable.

REFERENCES

- Avellon, A., Casas, I., Trallero, G., Perez, C., Tenorio, A. & Palacios, G. (2003). Molecular analysis of echovirus 13 isolates and aseptic meningitis, Spain. *Emerg Infect Dis* **9**, 934–941.
- Bernit, E., de Lamballerie, X., Zandotti, C., Bearger, P., Veit, V., Schleinitz, N., de Micco, P., Harle, J. R. & Charrel, R. N. (2004). Prospective investigation of a large outbreak of meningitis due to echovirus 30 during summer 2000 in Marseilles, France. *Medicine (Baltimore)* **83**, 245–253.
- Caro, V., Guillot, S., Delpeyroux, F. & Crainic, R. (2001). Molecular strategy for 'serotyping' of human enteroviruses. *J Gen Virol* **82**, 79–91.
- Casas, I., Palacios, G. F., Trallero, G., Cisterna, D., Freire, M. C. & Tenorio, A. (2001). Molecular characterization of human enteroviruses in clinical samples: comparison between VP2, VP1, and RNA polymerase regions using RT nested PCR assays and direct sequencing of products. *J Med Virol* **65**, 138–148.
- Chomel, J. J., Antona, D., Thouvenot, D. & Lina, B. (2003). Three echovirus serotypes responsible for outbreak of aseptic meningitis in Rhone-Alpes region, France. *Eur J Clin Microbiol Infect Dis* **22**, 191–193.
- Communicable Disease Surveillance Centre (1996a). Outbreak of viral meningitis in Cyprus – update. *Commun Dis Rep CDR Wkly* **6**, 293.

- Communicable Disease Surveillance Centre (1996b).** Viral meningitis in Cyprus, summer 1996. *Commun Dis Rep CDR Wkly* 6, 275.
- Communicable Disease Surveillance Centre (1996c).** Viral meningitis in England and Wales. *Commun Dis Rep CDR Wkly* 6, 275.
- Communicable Disease Surveillance Centre (2001).** Viral meningitis in England and Wales associated with an increase of echovirus type 30. *Commun Dis Rep CDR Wkly* 11, 275.
- el Sageyer, M. M., Szendroi, A., Hutter, E. & 12 other authors (1998).** Characterisation of an echovirus type 11' (prime) epidemic strain causing haemorrhagic syndrome in newborn babies in Hungary. *Acta Virol* 42, 157–166.
- Ishiko, H., Shimada, 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.
- Kirschke, D. L., Jones, T. F., Buckingham, S. C., Craig, A. S. & Schaffner, W. (2002).** Outbreak of aseptic meningitis associated with echovirus 13. *Pediatr Infect Dis J* 21, 1034–1038.
- Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001).** MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* 17, 1244–1245.
- Mateu, M. G. (1995).** Antibody recognition of picornaviruses and escape from neutralization: a structural view. *Virus Res* 38, 1–24.
- Minor, P. D. (1990).** Antigenic structure of picornaviruses. *Curr Top Microbiol Immunol* 161, 121–154.
- Mullins, J. A., Khetsuriani, N., Nix, W. A. & 10 other authors (2004).** Emergence of echovirus type 13 as a prominent enterovirus. *Clin Infect Dis* 38, 70–77.
- Noah, N. & Reid, F. (2002).** Recent increases in incidence of echoviruses 13 and 30 around Europe. *Eurosurveillance Weekly* 6 (Issue 7), 14 February 2002. <http://www.eurosurveillance.org/ew/2002/020214.asp#4>
- Norder, H., Bjerregaard, L. & Magnius, L. O. (2001).** Homotypic echoviruses share aminoterminal VP1 sequence homology applicable for typing. *J Med Virol* 63, 35–44.
- Oberste, M. S., Maher, K., Kilpatrick, D. R., Flemister, M. R., Brown, B. A. & Pallansch, M. A. (1999).** Typing of human enteroviruses by partial sequencing of VP1. *J Clin Microbiol* 37, 1288–1293.
- Oberste, M. S., Nix, W. A., Maher, K. & Pallansch, M. A. (2003).** Improved molecular identification of enteroviruses by RT-PCR and amplicon sequencing. *J Clin Virol* 26, 375–377.
- Oberste, M. S., Maher, K. & Pallansch, M. A. (2004a).** Evidence for frequent recombination within species human enterovirus B based on complete genomic sequences of all thirty-seven serotypes. *J Virol* 78, 855–867.
- Oberste, M. S., Penaranda, S. & Pallansch, M. A. (2004b).** RNA recombination plays a major role in genomic change during circulation of coxsackie B viruses. *J Virol* 78, 2948–2955.
- Oprisan, G., Combiescu, M., Guillot, S., Caro, V., Combiescu, A., Delpeyroux, F. & Crainic, R. (2002).** Natural genetic recombination between co-circulating heterotypic enteroviruses. *J Gen Virol* 83, 2193–2200.
- Ozkaya, E., Hizel, K., Uysal, G., Akman, S., Terzioğlu, S. & Kuyucu, N. (2003).** An outbreak of aseptic meningitis due to echovirus type 30 in two cities of Turkey. *Eur J Epidemiol* 18, 823–826.
- Ramsay, M. E., Brown, D. W., Appleton, H., Megson, B., Polynikis, A., Papantonou, L. & Eleftheriou, A. (1996).** Viral meningitis in Cyprus and England: Summer 1996. *Euro Surveill* 1, 28.
- Reimann, B. Y., Zell, R. & Kandolf, R. (1991).** Mapping of a neutralizing antigenic site of Cocksackievirus B4 by construction of an antigen chimera. *J Virol* 65, 3475–3480.
- Rotbart, H. A. (1995).** Meningitis and encephalitis. In *Human Enterovirus Infections*, pp. 271–289. Edited by H. A. Rotbart. Washington, DC: American Society for Microbiology Press.
- Santti, J., Vainionpää, R. & Hyypia, T. (1999a).** Molecular detection and typing of human picornaviruses. *Virus Res* 62, 177–183.
- Santti, J., Hyypia, T., Kinnunen, L. & Salminen, M. (1999b).** Evidence of recombination among enteroviruses. *J Virol* 73, 8741–8749.
- Thoelen, I., Lemey, P., Van, D. D. I., Beuselinck, K., Lindberg, A. M. & Van Ranst, M. (2003).** Molecular typing and epidemiology of enteroviruses identified from an outbreak of aseptic meningitis in Belgium during the summer of 2000. *J Med Virol* 70, 420–429.
- Thoelen, I., Moes, E., Lemey, P., Mostmans, S., Wollants, E., Lindberg, A. M., Vandamme, A. M. & Van Ranst, M. (2004).** Analysis of the serotype and genotype correlation of VP1 and the 5' noncoding region in an epidemiological survey of the human enterovirus B species. *J Clin Microbiol* 42, 963–971.
- World Health Organization (2004).** *Polio Laboratory Manual*. Geneva, Switzerland: World Health Organization.