Molecular identification and characterization of two proposed new enterovirus serotypes, EV74 and EV75

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Sequencing of the gene that encodes the capsid protein VP1 has been used as a surrogate for antigenic typing in order to distinguish enterovirus serotypes; three new serotypes were identified recently by this method. In this study, 14 enterovirus isolates from six countries were characterized as members of two new types within the species *Human enterovirus B*, based on sequencing of the complete capsid-encoding (P1) region. Isolates within each of these two types differed significantly from one another and from all other known enterovirus serotypes on the basis of sequences that encode either VP1 alone or the entire P1 region. Members of each type were $\geqslant$77·2 % identical to one another (89·5 % amino acid identity) in VP1, but members of the two different types differed from one another and from other enteroviruses by $\geqslant$31 % in nucleotide sequence (25 % amino acid sequence difference), indicating that the two groups represent separate new candidate enterovirus types. The complete P1 sequences differed from those of all other enterovirus serotypes by $\geqslant$31 % (26 % amino acid sequence difference), but were highly conserved within a serotype ($<$8 % amino acid sequence difference). Phylogenetic analyses demonstrated that isolates of the same serotype were monophyletic in both VP1 and the capsid as a whole, as shown previously for other enterovirus serotypes. This paper proposes that these 14 isolates should be classified as members of two new human enterovirus types, enteroviruses 74 and 75 (EV74 and EV75).

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INTRODUCTION

The genus *Enterovirus* (family *Picornaviridae*) comprises more than 80 serotypes; most are known human pathogens (Pallansch & Roos, 2001), but the genus also contains

viruses that infect cattle, swine and non-human primates. Most enterovirus infections are asymptomatic or result in only mild symptoms, e.g. non-specific febrile illness or mild upper respiratory symptoms (common cold). In addition, enteroviruses can cause a wide variety of other clinical illnesses, including acute haemorrhagic conjunctivitis, aseptic meningitis, undifferentiated rashes, acute flaccid paralysis (AFP), myocarditis and neonatal sepsis-like disease (Pallansch & Roos, 2001). The human enteroviruses are classified into five species: *Poliovirus* and *Human enterovirus A–D* (HEV-A–D) (King *et al.*, 2000), but recent data suggest that the polioviruses should be reclassified as members of HEV-C (Brown *et al.*, 2003).

In previous studies, we used analysis of partial or complete VP1 nucleotide sequences as a surrogate for antigenic typing to distinguish enterovirus serotypes (Oberste *et al.*, 1999b, 2000). This method was used to identify a new enterovirus serotype, EV73, from among enterovirus isolates that were deemed untypable by classical identification methods (Oberste *et al.*, 2001). Isolation of additional strains of EV73 from Bangladesh, India, Korea and Oman (Oberste *et al.*, 2001; Norder *et al.*, 2002; M. S. Oberste, unpublished data) confirmed its worldwide circulation. Three additional new proposed types, EV76 (M. S. Oberste, unpublished data), EV77 and EV78 (Norder *et al.*, 2003; Bailly *et al.*, 2004), were also identified by similar methods. The discovery of these new types suggested that numerous additional enterovirus types await identification.

In this study, 14 enterovirus isolates were characterized as members of two new types. Isolates within each of these two types were significantly different from one another and from all other known enterovirus serotypes, based on sequences that encode either the VP1 capsid protein or the entire capsid region. We propose that these isolates should be classified as members of two new human enterovirus types, enteroviruses 74 and 75 (EV74 and EV75).

METHODS

Virus isolation. The 14 virus isolates that were characterized in this study are listed in Table 1. Two California (USA/CA) strains, USA/CA75-10213 and USA/CA84-10214, were isolated in the Viral and Rickettsial Diseases Laboratory, California Department of Health Services, Berkeley, CA, USA, by inoculation of human fetal diploid kidney and primary monkey kidney cells. The Bangladesh (BAN), China (CHN), Iraq (IRQ) and Oman (OMA) strains were isolated from stool specimens of patients who presented with AFP during AFP surveillance activities in support of global polio eradication. Strains BAN00-10217, BAN00-10367, BAN00-10368 and IRQ00-10218 were isolated in the Enterovirus Laboratory, CDC, Atlanta, GA, USA, by inoculation of stool specimens into RD cells (a human rhabdomyosarcoma cell line; CDC, Atlanta, GA, USA). CHN97-10215 was isolated in the Poliovirus Laboratory, Queen Mary Hospital, Hong Kong Special Administrative Region, China, by inoculation of RD cells. OMA98-10366 was isolated in the National Poliovirus Laboratory, Muscat, Oman, by inoculation of RD cells. All isolates were amplified by one additional passage in RD cells prior to molecular characterization. The French strain (FRA99-130) was isolated in the Centre National de Référence des Entérovirus, Lyon, France, by inoculation of a bronchoalveolar fluid specimen into MRC-5 cells (a human lung fibroblast cell line). The strain from Ethiopia (ETH74-1341) was isolated from an Ethiopian child (who was adopted in Sweden) during routine health screening upon entry into Sweden. ETH74-1341 was isolated in the Department of Virology, Swedish Institute for Disease Control, Solna, Sweden, by inoculation of a stool specimen into human diploid lung fibroblast cells.

Table 1. Virus isolates characterized

Proposed prototype strains are indicated in bold type. Abbreviations: BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; NP, nasopharyngeal; URI, upper respiratory tract infection; NK, not known.

Candidate serotype/strain	Year	Country	Specimen	Clinical symptoms
EV74				
USA/CA75-10213	1975	California, USA	Stool	Fever, seizures
USA/CA84-10214	1984	California, USA	NP wash	NK
CHN97-10215	1997	China	Stool	AFP
FRA99-130	1999	France	BAL	Pneumopathy
BAN00-10217	2000	Bangladesh	Stool	AFP
IRQ00-10218	2000	Iraq	Stool	AFP
EV75				
USA/OK85-10362	1985	Oklahoma, USA	CSF	Unspecified CNS disease
ETH74-1341	1974	Ethiopia	Stool	None*
USA/VA86-10363	1986	Virginia, USA	Throat swab	Acute URI
USA/CT87-10364	1987	Connecticut, USA	NK	Neonatal jaundice
USA/CT87-10365	1987	Connecticut, USA	NK	'Failure to thrive'
OMA98-10366	1998	Oman	Stool	AFP
BAN00-10367	2000	Bangladesh	Stool	AFP
BAN00-10368	2000	Bangladesh	Stool	AFP

*Isolated during routine health screening from a 6-year-old adoptee upon entry into Sweden.

Molecular characterization of isolates. The isolates from Bangladesh, China, Iraq and the United States were characterized as described for the initial analysis of EV73 (Oberste *et al.*, 2001). Briefly, viral RNA extraction, RT-PCR and nucleotide sequencing for molecular serotyping were performed as described previously (Oberste *et al.*, 1999b, 2000, 2003b). Isolates were characterized initially by amplification and determination of partial VP1 sequence. The partial VP1 sequences were compared with a database of complete enterovirus VP1 sequences of all serotypes (Oberste *et al.*, 1999b, c, 2000, 2003b) to determine whether the isolates were related genetically to any known enterovirus serotype. Sequences that were $<70\,\%$ identical to the sequences of known enterovirus serotypes were flagged to be potential new types. The partial VP1 sequences were also compared with sequences of new type candidates that had been identified previously, to determine whether related isolates had already been characterized. To confirm the relationships of partial VP1 sequences, complete VP1 sequences were amplified and determined (Oberste *et al.*, 1999a). The French and Ethiopian isolates were characterized by a similar strategy that used degenerate primers to amplify and sequence the VP1-encoding region, as described previously (Norder *et al.*, 2001, 2002).

Isolates were further characterized by sequencing of the complete capsid (P1) region. RT-PCR primers were designed to anneal to sites that encode amino acid motifs that are highly conserved among members of the species *Human enterovirus B* (HEV-B) (Oberste *et al.*, 2004). Specific, non-degenerate primers were designed from preliminary sequences to close gaps between the original PCR products. Complete capsid sequences were determined by the 'primer-walking' method. PCR products were purified for sequencing by the use of a High Pure PCR product purification kit (Roche). Both strands were sequenced by automated methods, by the use of fluorescent dideoxy chain terminators (Applied Biosystems). The complete genome sequences of the candidate prototype strains, USA/CA75-10213 and USA/OK85-10362, were also determined by the strategy of degenerate primer RT-PCR and sequencing by 'primer-walking'.

Sequence analysis. Nucleotide and deduced amino acid sequences of candidate EV74 and EV75 isolates were compared with one another and with those of all HEV-B serotypes by using the programs GAP and DISTANCES (Wisconsin Package, version 10.2; Accelrys). Nucleotide sequences were aligned by using the PILEUP program (Wisconsin Package) and were adjusted manually to conform to the optimized alignment of deduced amino acid sequences. Phylogenetic relationships were inferred from the aligned nucleic acid sequences by the neighbour-joining method, implemented in the programs DNADIST and NEIGHBOR (PHYLIP version 3.57; University of Washington, Seattle, WA, USA), by using the Kimura two-parameter substitution model (Kimura, 1980) and a transition–transversion ratio of 10 (Oberste *et al.*, 2004). Support for specific tree topologies was estimated by bootstrap analysis with 1000 pseudoreplicate datasets. Branch lengths in consensus trees were calculated by the maximum-likelihood quartet-puzzling method, using the nucleotide substitution model of Tamura & Nei (1993), as implemented in Tree-Puzzle 5.0 (Strimmer & von Haeseler, 1996).

Nucleotide sequence accession numbers. The sequences that are described here have been deposited in GenBank under accession nos AY556057–AY556070. The data have also been furnished to the Picornavirus Study Group of the International Committee on Taxonomy of Viruses (ICTV) in support of a proposal to establish the new types EV74 and EV75. The Picornavirus Study Group has agreed to act as registrar for new enterovirus types (G. Stanway, personal communication). For registration (and reservation of the next available number), the complete VP1 sequence must be submitted to the Study Group and must be $<70\,\%$ identical to the VP1 sequences of all serotypes and proposed types that have been registered previously. The contact for registration is Dr Glyn Stanway (stanwg@essex.ac.uk), chair of the Picornavirus Study Group.

RESULTS

VP1 sequences

The 14 clinical isolates were characterized initially by RT-PCR amplification and sequencing of a portion of the gene encoding VP1. The initial characterization of isolates USA/OK85-10362, USA/VA86-10363, USA/CT87-10364, USA/CT87-10365 and FRA99-130 (W553-130/99 in the original reference) was described briefly as part of two previous, larger studies (Oberste *et al.*, 2000; Norder *et al.*, 2003). The partial VP1 sequence was $<70\,\%$ identical to that of each of the enterovirus prototype strains for all of the clinical isolates, suggesting that they may represent one or more new types. To confirm this result, complete VP1 sequences of all 14 isolates were determined and compared with each other and with the complete VP1 sequences of all enterovirus serotypes. The complete VP1 sequences of six isolates were 858 nt in length (encoding a 286 aa protein) and those of the other eight isolates were 873 nt long (encoding a 291 aa protein). The complete VP1 sequences of all 14 isolates were related most closely to those of members of HEV-B (59·5–68·5 % nucleotide sequence identity) and were $<57\,\%$ identical to those of members of other enterovirus species (Table 2). We showed previously that strains that are 75 % identical in VP1 sequence belong to the same serotype, whereas those that are $<70\,\%$ identical to one another belong to different serotypes (Oberste *et al.*, 1999b, c, 2000, 2001). Comparison of the VP1 sequences from the clinical isolates with one another showed that they formed two distinct groups. Members of each group were $\geqslant 77{\cdot}2\,\%$ identical to one another (89·5 % amino acid identity) (Table 3), but members of the two different groups differed from one another by $\geqslant 34\,\%$ in nucleotide sequence (31 % amino acid sequence difference) (data not shown). These data indicate that the two groups represent separate new candidate enterovirus types, which we have provisionally named enteroviruses 74 and 75 (EV74 and EV75). This result was confirmed by phylogenetic reconstruction using the clinical isolate VP1 sequences and those of all established members of HEV-B (Fig. 1). The EV74 and EV75 isolates were each monophyletic with respect to other serotypes (Fig. 1a), but bootstrap support was generally low (Figs 1b and c). The Asian EV74 strains (CHN97-10215, IRQ00-10218 and BAN00-10217) clustered together, whereas the two Californian isolates (CA75-10213 and CA84-10214) segregated into two separate branches (Fig. 1b); FRA99-130 clustered with CA75-10213. The phylogenetic relationships mirrored the pairwise sequence identities (Table 3). The two EV75 strains from Bangladesh clustered closely together, as expected for two strains that were isolated in the same location and year, as did the two USA/CT87 strains, which were isolated from two infants who were in the same hospital at the same time (Fig. 1c).

Table 2. Comparison of VP1 nucleotide sequences of clinical isolates to those of human enterovirus prototype strains

Proposed prototype strains are indicated in bold type. Values are nucleotide identity (%). USA/CT87-10365 is essentially identical to USA/CT87-10364 and was excluded from this comparison.

Candidate serotype/strain	HEV-A	HEV-B	HEV-C	HEV-D
EV74				
USA/CA75-10213	46·6–51·0	60·0–67·4	51·0–55·7	50·4–51·8
USA/CA87-10214	47·8–52·1	60·1–68·5	52·3–56·3	50·4–53·3
CHN97-10215	47·0–51·6	59·8–68·3	51·1–55·3	50·5–51·2
W553-130/99	46·7–52·5	60·2–67·4	52·6–55·9	50·1–50·6
BAN00-10217	47·4–51·0	59·5–68·5	51·9–56·2	50·3–51·0
IRQ00-10218	47·0–51·6	59·9–67·7	51·7–55·6	50·2–50·4
EV75				
USA/OK85-10362	45·9–50·7	60·0–67·4	49·4–55·6	51·4–51·8
ETH74-1341	45·1–49·7	58·4–67·5	50·7–53·5	50·4–50·7
USA/VA86-10363	45·5–50·1	58·9–67·5	49·4–54·7	49·4–50·9
USA/CT87-10364	46·6–51·0	60·0–67·4	51·0–55·7	50·4–51·8
OMA98-10366	45·2–49·3	60·7–67·7	50·4–54·9	51·8–53·3
BAN00-10367	45·4–50·5	59·6–66·5	50·0–53·3	50·2–51·2
BAN00-10368	44·3–50·9	60·0–66·9	49·5–53·9	50·5–52·9

Capsid (P1) sequences

The complete capsid-encoding nucleotide sequences were the same length for isolates of the same candidate type (2562 nt, encoding an 854 aa protein, for the EV74 isolates; and 2577 nt, encoding an 859 aa protein, for the EV75 isolates). The EV74 and EV75 capsid sequences were ⩾31 % different from those of all other enterovirus serotypes (26 % amino acid sequence difference). The capsid gene sequences of the EV74 candidates were 79·5–95·1 % identical to one another (92·4–98·7 % amino acid identity) (Table 4). Sites at which the amino acid sequences varied in the EV74 isolates were distributed throughout the capsid sequence: seven in VP4, 19 in VP2, 19 in VP3 and 37 in VP1 (data not shown). Nine of the 19 variable sites in VP2 were in the 'puff' region. In VP1, most of the variable sites were near the ends of the mature protein: seven in the first 20 aa at the amino-terminus and eight in the last 20 aa at the carboxyl-terminus. The complete capsid-encoding sequences of the EV75 candidates were 82·3–96·9 % identical to one another;

Table 3. VP1 nucleotide and deduced amino acid sequence identities among candidate EV74 and EV75 isolates

Nucleotide sequence identities (%) are above the diagonal and amino acid sequence identities (%) are below the diagonal. VP1 sequences of EV74 isolates were <66 % identical to those of EV75 isolates (<69 % amino acid identity).

Serotype/isolate	1	2	3	4	5	6	7	8	9	10	11	12	13
EV74													
1. CA75-10213		79·8	81·8	90·8	82·4	82·3							
2. CA87-10214	93·7		79·9	77·2	80·2	79·3							
3. CHN97-10215	94·8	93·4		80·7	95·9	91·0							
4. FRA99-130	93·0	89·5	90·2		80·8	80·8							
5. BAN00-10217	95·5	94·1	98·6	90·9		91·3							
6. IRQ00-10218	94·4	93·4	97·2	90·6	97·2								
EV75													
7. OK85-10219								86·1	83·7	84·9	85·9	87·9	87·5
8. ETH74-1341							97·9		82·6	85·9	85·0	84·8	84·9
9. VA86-10220							98·6	97·3		84·1	82·7	82·7	81·7
10. CT87-10221							98·6	97·3	97·9		84·4	86·0	84·8
11. OMA98-10223							98·6	98·6	97·9	97·9		85·5	85·0
12. BAN00-10224							99·0	97·9	98·3	98·3	98·6		96·9
13. BAN00-10225							98·3	97·6	97·6	97·6	97·9	99·3	

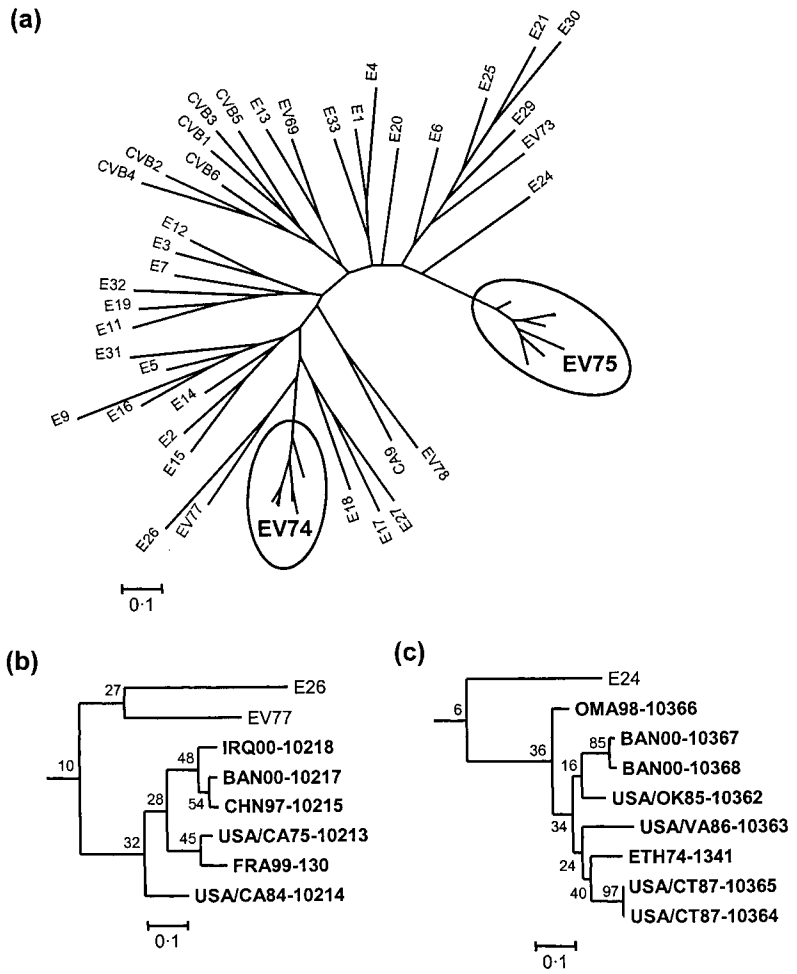

Fig. 1. Phylogenetic relationships among isolates of EV74 and EV75, based on complete VP1 nucleotide sequences (858 and 873 nt, respectively) and comparison with prototype strains of other serotypes in HEV-B. Strains of EV74 and EV75 are indicated in bold type. (a) Tree showing EV74, EV75 and the prototype strains of all other members of HEV-B. (b) Subtree showing details of the EV74 branch. Numbers at nodes are percentages of 1000 bootstrap replicates supporting that node. (c) Subtree showing details of the EV75 branch. Numbers at nodes are percentages of 1000 bootstrap replicates supporting that node.

Table 4. P1 nucleotide and deduced amino acid sequence identities among candidate EV74 and EV75 isolates

Nucleotide sequence identities (%) are above the diagonal and amino acid sequence identities (%) are below the diagonal. P1 sequences of EV74 isolates were <69% identical to those of EV75 isolates (<74% amino acid identity).

Serotype/isolate	1	2	3	4	5	6	7	8	9	10	11	12	13
EV74													
1. CA75-10213		80·5	81·8	91·8	81·8	82·0							
2. CA87-10214	95·6		80·0	79·5	80·3	79·8							
3. CHN97-10215	96·5	94·7		81·2	95·1	91·4							
4. FRA99-130	95·6	92·4	93·7		80·8	81·0							
5. BAN00-10217	97·1	95·3	98·7	94·0		90·7							
6. IRQ00-10218	96·6	95·0	98·4	94·0	98·7								
EV75													
7. OK85-10219								85·9	83·7	84·6	84·6	87·0	87·3
8. ETH74-1341							98·7		82·7	86·7	84·6	83·9	84·4
9. VA86-10220							99·0	98·1		82·8	82·4	82·5	82·3
10. CT87-10221							99·2	98·5	98·6		83·6	83·4	83·7
11. OMA98-10223							99·0	98·7	98·4	98·8		84·6	84·8
12. BAN00-10224							99·2	98·4	98·6	98·8	98·7		96·9
13. BAN00-10225							99·3	98·6	99·0	99·0	98·8	99·4	

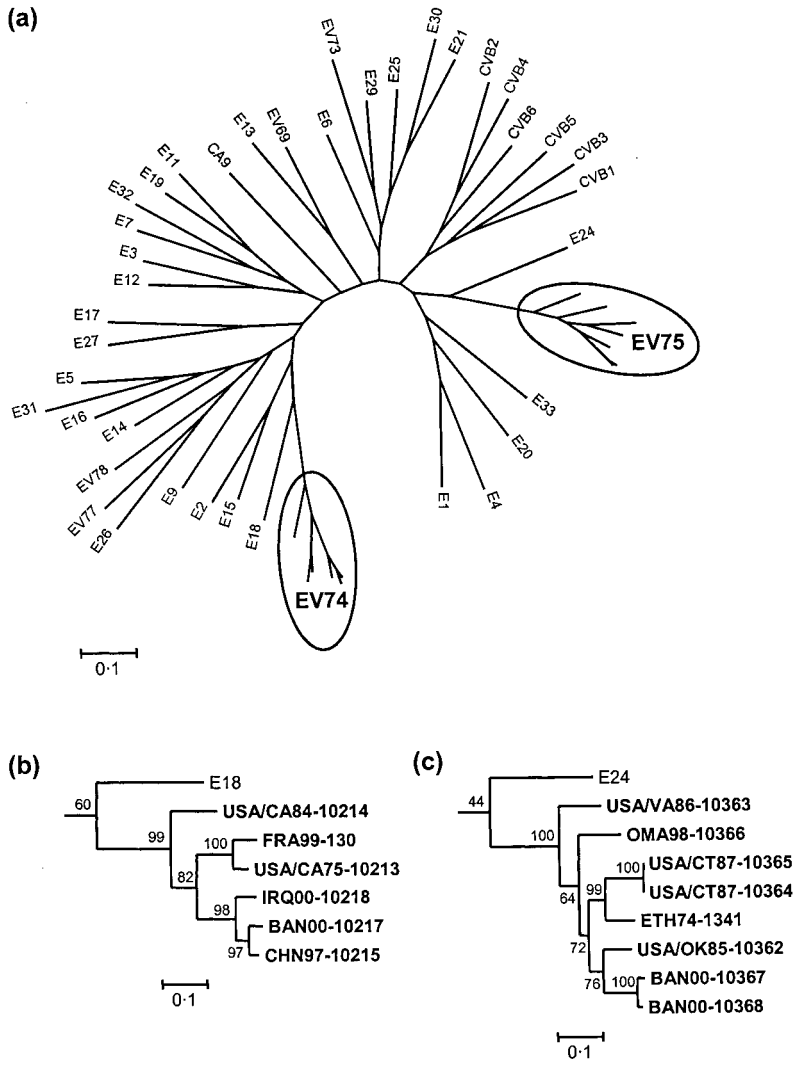

Fig. 2. Phylogenetic relationships among isolates of EV74 and EV75, based on complete capsid nucleotide sequences (2562 and 2577 nt, respectively) and comparison with prototype strains of other serotypes in HEV-B. Strains of EV74 and EV75 are indicated in bold type. (a) Tree showing EV74, EV75 and the prototype strains of all other members of HEV-B. (b) Subtree showing details of the EV74 branch. Numbers at nodes are percentages of 1000 bootstrap replicates supporting that node. (c) Subtree showing details of the EV75 branch. Numbers at nodes are percentages of 1000 bootstrap replicates supporting that node.

however, the amino acid sequences were very highly conserved (98·4–99·4 % identity) (Table 4). The EV75 capsid sequences only contained about one-third as many variable sites as the EV74 sequences: three in VP4, eight in VP2, four in VP3 and 15 in VP1. Overall, 828 of 859 (96·4 %) sites were invariant in the EV75 capsid, compared to 772 of 854 (90·4 %) in EV74. As in VP1, the EV74 and EV75 complete capsid sequences were each monophyletic with respect to all other enterovirus serotypes (Fig. 2), but with 100 % bootstrap support (Figs 2b and c).

Other genome regions

To further characterize the proposed new types, one isolate of each type (the proposed prototype strain) was sequenced in its entirety. The topology and sequences of the USA/CA75-10213 and USA/OK85-10362 non-capsid regions were consistent with membership of HEV-B, but there were no other type-specific distinguishing features (data not shown). These data are in agreement with analysis of other HEV-B serotypes that was reported previously (Oberste *et al.*, 2004).

DISCUSSION

The EV74 strains that are described here were isolated over a 25 year period from five countries on three continents (North America, Europe and Asia); EV75 was isolated over a 26 year period from four countries (in North America, Asia and Africa). Although we have described a small number of isolates, their wide temporal and geographical distribution suggests that EV74 and EV75 may be relatively common enterovirus types. EV73, the first new enterovirus serotype to be established by molecular criteria, is also distributed widely, both temporally and geographically (Oberste *et al.*, 2001; Norder *et al.*, 2002). Taken together, these data suggest that 'new' serotypes may be relatively common in collections of uncharacterized enterovirus isolates; this is in agreement with our previous proposal (Oberste *et al.*, 2001).

The French EV74 isolate and the United States EV74 and EV75 isolates were obtained from patients that were diagnosed with a variety of illnesses, including respiratory tract infections, neonatal disease and unspecified central

nervous system disease (Table 1). Additional isolates of both EV74 and EV75 were obtained from stool specimens of children who presented with AFP during poliovirus surveillance activities in southern and western Asia. Non-polio enteroviruses are often isolated from the stools of children with AFP (Gear, 1984; Grist & Bell, 1984; Melnick, 1984; Chaves *et al.*, 2001; Kapoor *et al.*, 2001; Oberste *et al.*, 2001; Santos *et al.*, 2002; Grimwood *et al.*, 2003), but isolation of a ubiquitous infectious agent from a non-sterile site is not sufficient to imply causality. On the other hand, it is likely that at least some non-polio AFP cases are attributable to infection with non-polio enteroviruses, as they have occasionally been isolated directly from neural tissues of paralysed patients (Chumakov *et al.*, 1979). Molecular identification of new enterovirus serotypes provides a tool to assist in the epidemiological investigation of AFP cases that are associated with non-polio enterovirus infection.

Whilst the non-translated regions and non-structural proteins may influence enterovirus replication and translation, the serotype-specific properties of an enterovirus are encoded in the viral capsid. Enteroviruses have been identified by the antigenic properties of the capsid for over 50 years (Bodian *et al.*, 1949) and, more recently, by the partial sequences of their capsid-encoding genes (Oberste *et al.*, 1999c, 2003b). The antigenic properties of the capsid are also critical for host immunity, as the host immune response is serotype-specific and the humoral response to capsid antigens is both necessary and sufficient for protection against most systemic enterovirus disease. Specific sites on the capsid surface also control receptor specificity and virus binding to the host cell. Receptor-binding specificity can contribute to cell and tissue tropism, which affect the distribution of virus replication sites within the infected host. Once the virus enters a host cell, the presence or absence of specific host-cell proteins may further restrict replication in a given tissue or cell type.

In previous studies, we and others have shown that complete or partial VP1 sequence correlates completely with antigenic typing by neutralization assay and that it may serve as a molecular surrogate for traditional serotyping methods (Oberste *et al.*, 1999b, c, 2000, 2001; Caro *et al.*, 2001; Casas *et al.*, 2001; Norder *et al.*, 2001). We have also recommended specific criteria for the interpretation of VP1 sequence data when a VP1 sequence is compared to reference sequences for all human enterovirus serotypes: (i) a partial or complete VP1 nucleotide sequence identity of $\geqslant$75 % (85 % amino acid sequence identity) between a clinical enterovirus isolate and serotype prototype strain may be used to establish the serotype of the isolate, on the provision that the second highest score is $<$70 %; (ii) a best-match nucleotide sequence identity of $<$70 % may indicate that the isolate represents an unknown (that is, new) serotype; and (iii) a sequence identity between 70 and 75 % indicates that further characterization is required before the isolate can be identified firmly (Oberste *et al.*, 2000, 2001, 2003b).

We recognize that the term 'serotype' implies identification by antigenic means (usually by neutralization in the case of the human enteroviruses). The term 'genotype' might be more acceptable to describe an identification that has been made by molecular methods, but this term has been used widely to discriminate among lineages of several enterovirus serotypes that have been defined genetically, including the polioviruses and coxsackieviruses A9 and B4 (Rico-Hesse *et al.*, 1987; Mulders *et al.*, 2000; Santti *et al.*, 2000). The term 'genogroup' has also been used in this way (Brown *et al.*, 1999; Oberste *et al.*, 2003a). We suggest that the term 'serotype' should be maintained in the description of isolates that have been identified by using the VP1 sequence as a surrogate marker for antigenic type, until a consensus can be reached among enterovirologists on a more appropriate term. We also recommend that molecular typing, by complete sequencing of VP1 and application of these criteria, should be recognized as a substitute for the extensive cross-neutralization studies that have traditionally been required for the establishment of new enterovirus serotypes. Finally, we recommend that a complete capsid (P1) sequence should be determined for the strain that is proposed as the prototype of a new enterovirus serotype and, preferably, also for other isolates that are included in the original description of the serotype. Complete genome sequences, if made available, will facilitate additional studies (e.g. on the evolution of related viruses within a species). These additional data will allow for further validation by the virology community and contribute to refinement of molecular-typing criteria.

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