

Molecular identification of new picornaviruses and characterization of a proposed enterovirus 73 serotype

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Enteroviruses (EV) have traditionally been identified by using serotype-specific antisera in a virus-neutralization test. Three EV strains isolated in California, USA, in 1955, 1964 and 1978, and a 1995 Oman isolate, were found to be antigenically related to one another; however, the strains were not neutralized by standard EV typing antisera, suggesting that they may represent a new EV serotype. The isolates were characterized genetically by RT-PCR coupled with amplicon sequencing and comparison to a database of enterovirus nucleotide sequences. The strains were 75.3 to 87.2% identical to one another in complete VP1 nucleotide sequence, but no more than 68% identical in sequence to the prototype strain of any EV serotype. Their complete capsid sequences were closely related to one another, but only distantly related to those of any EV prototype strain. The California and Oman isolates were most closely related to members of EV cluster B, suggesting that they are unclassified members (i.e. a new serotype) of cluster B. The complete genome sequence was determined for one isolate, CA55-1988, and the predicted polyprotein sequence was 86.5 to 89.2% identical to those of other cluster B EV and 56.7 to 61.9% identical to the polyprotein sequences of EV belonging to other clusters. Isolation of this new EV serotype from samples obtained on two continents and over a period of 40 years suggests continued circulation over a wide geographical area. In keeping with standard picornavirus nomenclature, we propose that this new serotype be named 'enterovirus 73' (EV73).

Introduction

Viruses in the family *Picornaviridae* were initially classified on the basis of physical characteristics and antigenic relationships measured by neutralization test (Committee on Enteroviruses, 1962; Melnick, 1996; Minor *et al.*, 1995). Over 200 serotypes have been identified and nine genera have been defined (King *et al.*, 2000; Mayo & Pringle, 1998): *Enterovirus* (89 serotypes), *Rhinovirus* (103 serotypes), *Cardiovirus* (3 serotypes), *Aphthovirus* (8 serotypes), *Hepatovirus* (2 serotypes), *Parechovirus* (2 serotypes), *Teschovirus* (10 serotypes), *Kobuvirus* (1 serotype) and *Erbovirus* (1 serotype). Of the 89 *Enterovirus*

serotypes, 64 are known to infect humans (Melnick, 1996). Most of the human enterovirus serotypes were discovered and described between 1948 and 1963 as a result of the application of cell culture and suckling mouse inoculation to investigations of cases of infantile paralysis (paralytic poliomyelitis) and other central nervous system diseases (Committee on Enteroviruses, 1962; Panel for Picornaviruses, 1963).

The accepted approach to classification requires the investigator to generate antisera against each potentially new serotype and to perform reciprocal cross-neutralization testing, using a complete panel of prototype strains and antisera (Committee on Enteroviruses, 1962). The effect of this labour-intensive approach on the pace of enterovirus discovery was recognized as early as 1962, when a leader in the field remarked, 'new human enteroviruses are still being discovered. The rate of making new discoveries has slowed, probably only because the labours involved in establishing "new" serotypes

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are now so very great!' (Wenner, 1962). Since that time, only nine new human enterovirus serotypes have been identified (Melnick *et al.*, 1974; Panel for Picornaviruses, 1963; Rosen & Kern, 1965; Rosen *et al.*, 1973; Schieble *et al.*, 1967) and none have been identified in the last 25 years.

Recognizing the technical difficulties and limitations inherent in the classic approach to enterovirus identification, we have developed rapid molecular techniques for enterovirus typing (Oberste *et al.*, 2000, 1999*a, b*). In the present study, we describe a comparison of immunological and molecular methods for the characterization and classification of a group of related human enteroviruses isolated over a period of 40 years and propose the classification of these isolates as members of a new human enterovirus serotype.

Methods

■ **Virus isolation and antigenic characterization.** The viruses analysed in this study are described in Table 1. CA55-1988, CA64-4454 and CA78-1480 were isolated in the Viral and Rickettsial Diseases Laboratory (VRDL), California Department of Health Services, Berkeley, CA, USA. The VRDL serves as a reference laboratory for the State of California, performing primary isolation and identification of viruses, as well as identification of viral cultures referred from other laboratories. CA55-1988 was isolated in primary rhesus monkey kidney cells (RMK). CA64-4454 was isolated in RMK and in human foetal diploid kidney fibroblast cells (HFDK). CA78-1480 was isolated in RMK, HFDK and diploid human foetal lung cells (IMR-90). None of the strains were lethal in intracerebrally inoculated newborn mice. Typing by neutralization test was attempted using the Lim and Benyesh-Melnick (LBM) or in-house immune serum pools and standard methods (Grandien *et al.*, 1989; Lim & Benyesh-Melnick, 1960; Schmidt *et al.*, 1961).

OMA95-6498 and OMA95-6499 were isolated in the National Poliovirus Laboratory, Department of Laboratory Services, Ministry of Health, Muscat, Oman, during the course of poliovirus surveillance activities in support of the Global Poliomyelitis Eradication Initiative. Specimens were inoculated into RD and HEp2(C) cells, observed for the development of enterovirus-like cytopathic effect, and tentatively identified as untypeable non-polio enteroviruses on the basis of the absence of neutralization using antisera against the three poliovirus

serotypes and with pooled antisera specific for 27 non-polio enteroviruses (World Health Organization, 1997).

Immune sera were prepared against CA55-1988 and CA78-1480 in hamsters and against CA64-4454 in monkeys. Antisera were standardized with all other enterovirus serotypes, as previously described (Schnurr *et al.*, 1996), and used to assess the antigenic relationships of CA55-1988, CA64-4454, CA78-1480 and OMA95-6498 by neutralization, using standard methods (Grandien *et al.*, 1989).

■ **Physical characterization.** The California isolates were tested for stability to acid by incubation of the virus in pH 3.0 buffer for 1 h at 4 °C and inoculation of cell cultures after adjustment to pH 7 (Gwaltney *et al.*, 1989). The isolates were purified by three terminal dilutions and further characterized by determination of size and morphology using electron microscopy, stabilization to heat by divalent cations, determination of genome type (DNA or RNA) and measurement of ether stability. The Oman isolates were not physically characterized.

■ **Molecular characterization of viruses.** Viral RNA extraction, RT-PCR, nucleotide sequencing and sequence analysis were performed as described previously (Oberste *et al.*, 2000, 1999*a*). For initial molecular characterization, viral RNA from each isolate was amplified by RT-PCR, using VP1 primers 012-011, 040-011, 187-222, 188-222 and 189-222, as described previously (Oberste *et al.*, 2000, 1999*a*). The partial VP1 sequences were compared with a database of all complete enterovirus VP1 sequences (Oberste *et al.*, 1999*b*) and other picornavirus VP1 sequences that are available in the GenBank database, as described previously (Oberste *et al.*, 2000, 1999*a*), to determine whether the isolates were genetically related to any known picornavirus serotype. Sequences spanning the 3' portion of the 5'NTR to the 5' end of VP2 were determined as described previously (Oberste *et al.*, 1998). Complete capsid sequences were determined using the primer-walking method to amplify and sequence the region between the 5'-NTR-VP4-VP2 and VP1 sequences. The complete genome sequence of CA55-1988 was also determined by primer walking.

Results

Physical and antigenic properties

As part of routine enterovirus typing procedures, neutralization of the California isolates was attempted using standard pools of enterovirus typing antisera (Lim & Benyesh-Melnick,

Table 1. Virus strains, date and place of isolation, patient information and clinical disease association

Strain	Year	Specimen	Age	Sex	Clinical symptoms
CA55-1988	1955	Stool	NA	F	NA
CA64-4454	1964	Stool	1 yr	M	Croup*
CA78-1480	1978	Stool	4 yr	M	Fever/convulsions
OMA95-6498	1995	Stool	2 yr	M	Asymptomatic contact of AFP† case
OMA95-6499	1995	Stool	2 yr	M	Asymptomatic contact of AFP case

* Parainfluenzavirus type 1 was isolated from throat swab.

† AFP, acute flaccid paralysis.

NA, Information not available.

Table 2. Antigenic relationships assayed by neutralization test

Titres are shown as reciprocal of 50% endpoint, determined by the method of Reed & Muench (1938). Numbers in parentheses are the virus dose (50% cell culture infectious dose), calculated from a back-titration. NT, Not tested.

Virus	Antiserum		
	CA55-1988	CA64-4454	CA78-1480
EV prototypes (48 strains)*	NT	< 8	NT
CA55-1988	1024 (320)	≥ 2048 (3200)	32 (1000)
CA64-4454	< 8 (320)	4096 (100)	8 (100)
CA78-1480	256 (32)	2048 (32)	2048 (32)
OMA95-6498	512 (320)	1024 (320)	4096 (320)
OMA95-6499	1024 (32)	2048 (32)	≥ 4096 (32)

* Strains tested included the recognized prototype strains of poliovirus types 1–3, coxsackieviruses A7, A9, A16 and B1–B6; echoviruses 1–9, 12–29 and 31–33; and EV69–71, as well as non-prototype reference strains of echovirus types 11 and 30.

Table 3. VP1 nucleotide sequence relationships (percent identity) of candidate EV73 strains to other picornaviruses

	CA55-1988	CA64-4454	CA78-1480	Oman95*
EV prototypes (64 strains)	47.0–67.0	45.9–67.8	46.0–67.4	45.4–66.7
EV Cluster A	47.0–50.5	45.9–50.2	46.0–50.4	45.4–51.2
EV Cluster B	59.1–67.0	58.5–67.8	58.7–67.4	58.8–66.7
EV Cluster C	49.3–54.7	49.8–53.9	49.8–55.5	50.1–55.1
EV Cluster D	48.1–48.9	49.5–51.2	48.1–49.2	47.2–49.4
Rhinoviruses†	47.6–51.5	47.5–50.8	48.5–51.6	48.0–50.7
Other picornaviruses	≤ 41.1	≤ 41.7	≤ 43.1	≤ 42.0

* 'Oman95' includes both OMA95-6498 and OMA95-6499, whose VP1 sequences were identical to one another. Because the VP1 sequences were identical, OMA95-6499 was excluded from further analyses.

† Human rhinovirus VP1 sequences available from the GenBank database: types 1B, 2, 3, 14, 16 and 89.

1960; Schmidt *et al.*, 1961). None of the three isolates were neutralized by any of the pools (data not shown). At the time the viruses were isolated, it was standard practice to produce antisera against untypeable isolates to determine whether the isolate might be antigenically related to a known serotype. Monkey anti-CA64-4454 antisera failed to neutralize any of the enterovirus prototype strains (Table 2). Typing of the Oman isolates was attempted using standard pools of enterovirus typing antisera distributed by the World Health Organization to laboratories in the Global Poliovirus Laboratory Network (World Health Organization, 1997), and neither isolate was neutralized by any of the pools (data not shown). Anti-CA64-4454 antiserum neutralized CA55-1988, CA64-4454, CA78-1480, OMA95-6498 and OMA95-6499 to high titre (≥ 1:1024). Anti-CA55-1988 antiserum neutralized CA55-1988, CA78-1480, OMA95-6498 and OMA95-

6499, but did not neutralize CA64-4454 (titre < 1:8). Anti-CA78-1480 antiserum neutralized CA78-1480, OMA95-6498 and OMA95-6499, but only partially neutralized CA55-1988 (titre 1:32) and did not neutralize CA64-4454 (titre 1:8). The non-reciprocal antigenic relationship between CA64-4454 and the other California isolates suggests that CA64-4454 may be a 'prime' strain of CA55-1988 and CA78-1480.

Infectivity of CA64-4454 and CA78-1480 was not reduced by treatment with ether, by incubation at pH 3.0 for 1 h, or by incubation at 56 °C in the presence of 1 M MgCl₂ (data not shown). CA55-1988, OMA95-6498 and OMA95-6499 were not tested for ether sensitivity, stability to acid or stabilization by MgCl₂. For CA78-1480, typical picornavirus particles (round, non-structured virus particles 28–29 nm diameter) were observed by electron microscopy (data not

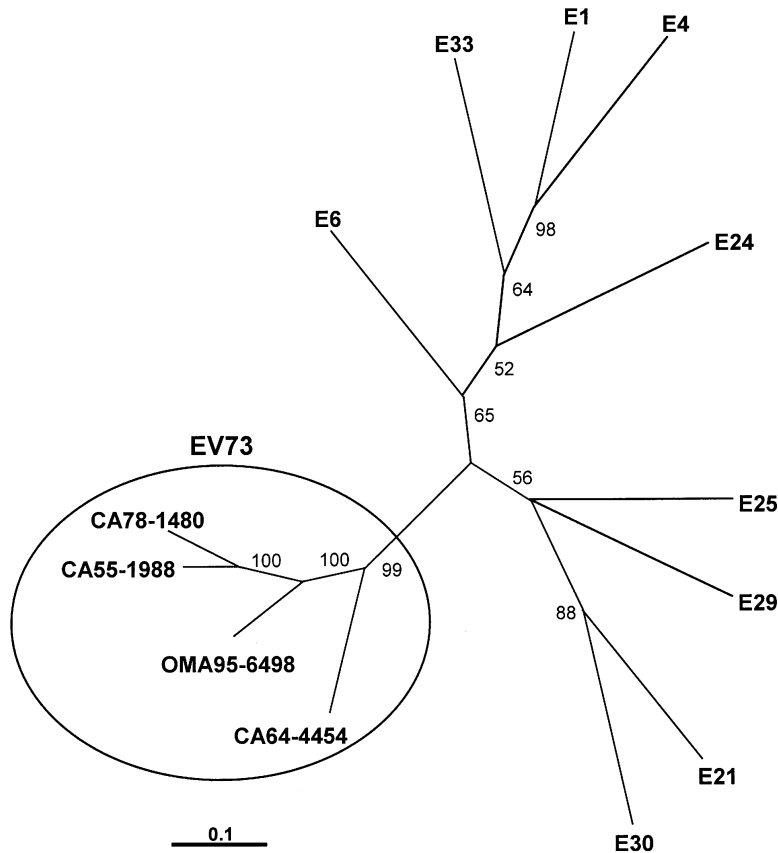


Fig. 1. Phylogenetic tree generated from complete VP1 sequences by the quartet puzzling method with a maximum likelihood distance matrix. Branch lengths are proportional to genetic distance, indicated by the scale bar. For clarity, CA55-1988, CA64-4454, CA78-1480, OMA95-6498, and only the nine closest heterologous prototype strains are shown.

shown). Electron microscopy was not performed for CA55-1988, CA64-4454, OMA95-6498 or OMA95-6499.

Molecular serotyping and genetic relationships

The complete VP1 sequence (867 nucleotides) of each of the isolates was determined and compared with those of the human enterovirus prototype strains and of other picornaviruses. In all cases, the nearest taxa were viruses in the *Enterovirus* genus, and the highest pair-wise nucleotide sequence identity score was between 67 and 68% (Table 3). The highest scoring serotypes were members of enterovirus cluster B, which contains the coxsackie B viruses, the echoviruses, coxsackievirus A9 and EV69. Scores for comparisons with available human rhinovirus sequences were between 47 and 52%, similar to the scores for comparisons with cluster A enteroviruses (Table 3). Scores for comparisons with picornaviruses of all other genera were below 44%. For the complete VP1 sequence comparisons, a highest identity score below 70% indicates that the query sequence is heterologous in serotype to all sequences in the database (Oberste *et al.*, 2000, 1999*a, b*), suggesting that the California and Oman isolates are unclassified members (a new serotype) of cluster B. The complete VP1 sequence of OMA95-6499 was identical to that of OMA95-6498, and therefore OMA95-6499 was not further characterized genetically.

Phylogenetic relationships were inferred from complete VP1 coding sequences by the quartet puzzling method, using maximum likelihood distances. CA55-1988, CA64-4454, CA78-1480 and OMA95-6498 were monophyletic with respect to the prototype strains of all known enterovirus serotypes, with a 99% support value (Fig. 1 and data not shown). The nearest sibling taxa were E25, E29 and E6, in general agreement with the nucleotide identity scores. Within the new group, the phylogenetic relationships mirrored the antigenic relationships, as CA55-1988 and CA78-1480 clustered together (100% support) and CA64-4454 was more distant.

The nucleotide sequence for the region encoding the complete capsid protein was determined for CA55-1988, CA64-4454, CA78-1480 and OMA95-6498. Overall, the capsid-coding sequences were 75.9 to 87.0% identical to one another and the amino acid sequences of the predicted capsid polyproteins were 90.3 to 98.1% identical to one another (Table 4). Of the 854 predicted amino acids in the capsid polyprotein, 758 residues were invariant among all four isolates. Only five of 96 variant sites occurred in regions predicted to participate in conserved β -barrel structures (data not shown), and four of the differences in these sites were conservative changes (A-S, F-Y, I-V and K-R). VP1 was the most divergent capsid protein, varying by up to 12.5% (CA64-4454 versus CA78-1480). Nucleotide identity scores

Table 4. Nucleotide and amino acid sequence relationships (percent identity) among complete capsid sequences of CA55-1988, CA64-4454, CA78-1480 and OMA95-6498

Numbers above the diagonal are percent nucleotide sequence identity and numbers below the diagonal are percent amino acid sequence identity.

	CA55-1988	CA64-4454	CA78-1480	OMA95-6498
CA55-1988	100	76.0	87.0	81.0
CA64-4454	90.3	100	76.8	75.9
CA78-1480	98.1	90.4	100	80.5
OMA95-6498	96.1	91.4	96.0	100

Table 5. Comparison of coding and noncoding sequences of CA55-1988 with those of representative enteroviruses

	Cluster A	Cluster B	Cluster C	Cluster D
5'NTR (nt 1-744)*	84.1-84.4†	79.9-91.0	68.2-71.3	73.4
Complete polyprotein	56.7-58.1	86.5-89.2	60.7-61.9	60.4
P1 (nt 745-3306)	46.0-48.9	71.4-77.7	54.5-55.6	47.8
P2 (nt 3307-5040)	65.2-65.7	93.8-96.4	57.6-60.8	63.7
P3 (nt 5041-7311)	61.0-62.7	96.0-98.7	68.3-68.8	72.0
3'NTR (nt 7312-7411)	43.0	73.0-99.0	41.0-49.0	44.0

* Nucleotide position relative to the complete genome sequence of CA55-1988.

† Numbers are percent nucleotide identity for 5' and 3' non-translated regions (NTR) and percent amino acid identity for protein coding regions.

for comparisons of the complete capsid sequences to available enterovirus prototype sequences were similar to those for comparisons of VP1 alone (data not shown).

Complete sequence of CA55-1988

The complete nucleotide sequence of CA55-1988 was 7411 residues long, exclusive of the poly(A) tract (Table 5). The genome organization was typical of the human enteroviruses, with a predicted 744 nucleotide non-translated region at the 5' end and a 100 nucleotide non-translated region at the 3' end of the genome. Eight AUG codons occur in the first 750 nucleotides of the genome, but comparison with other cluster B enteroviruses suggested that translation probably begins at nucleotide 745 (eighth AUG) and continues to a termination codon at residues 7309 to 7311, resulting in a primary translation product of 2188 amino acids. The complete polyprotein sequence was compared, by genome region (P1, P2 and P3), with each of the complete enterovirus sequences available in the GenBank database (21 different serotypes). Overall, the predicted polyprotein sequence of CA55-1988 was 86.5 to 89.2% identical to those of other cluster B

enteroviruses (13 different serotypes) and 56.7 to 61.9% identical to the polyprotein sequences of enteroviruses belonging to other clusters (Table 5). The CA55-1988 P1 (capsid) region was the most divergent, differing from other cluster B capsid sequences by 22.3 to 28.6%. By contrast, the P2 and P3 (nonstructural) regions were much more highly conserved, with a maximum divergence from other cluster B viruses of 6.2% in P2 and 4.0% in P3.

Discussion

In the past, antigenic tests, including neutralization, haemagglutination and complement fixation, have been used to dissect the relationships among enterovirus isolates (Duncan, 1968; Wenner *et al.*, 1967), to suggest modifications to the taxonomy of enteroviruses (Harris *et al.*, 1973) and to establish new serotypes (Committee on the Enteroviruses, 1957; Melnick *et al.*, 1974; Panel for Picornaviruses, 1963). Molecular sequence data and comparative genomics have recently been applied to the classification of newly discovered picornaviruses (Marvil *et al.*, 1999; Niklasson *et al.*, 1999; Yamashita *et al.*, 1998) and to the reclassification of existing picornaviruses

(Doherty *et al.*, 1999; Hyypiä *et al.*, 1992). Our previous studies have shown that human enterovirus VP1 sequences correlate with antigenically defined serotypes (Oberste *et al.*, 1999*b*), and we have successfully applied this observation to the typing of clinical isolates, including those that could not be typed by traditional antigenic methods (Oberste *et al.*, 2000, 1999*a*). Comparisons of VP1 sequences supported the suggestion, based upon antigenic relatedness (Melnick, 1996), that CA15 and CA18 should be reclassified as variants of CA11 and CA13, respectively (Oberste *et al.*, 1999*b*). A number of other studies have also demonstrated the utility of using picornavirus capsid region sequences, and VP1 sequences in particular, as a molecular surrogate for antigenic type (Brocchi *et al.*, 1997; Callens & De Clercq, 1997; Hyypiä *et al.*, 1997; Santti *et al.*, 1999). In the present study, sequences encoding the VP1 protein, as well as the complete capsid of the Oman and California isolates, correlated with the antigenic relationships of the viruses to one another. Sequence comparisons also fully supported the antigenic data that indicated CA55-1988 and related strains are distinct from the known human enterovirus serotypes.

Presumably, enteroviral structural diversity, and hence the number of possible serotypes, is constrained by the ability of different primary capsid sequences to form a functional viral particle capable of interacting with and infecting a susceptible host cell. However, the large number of enterovirus serotypes suggests that the viable conformation space may be very large, so that many more serotypes may remain to be discovered, either as historical, previously untyped enteroviruses, or as newly emerging serotypes. In either case, the molecular methods described here will be invaluable in rapidly identifying and characterizing these agents. For example, national enterovirus surveillance figures indicate that 3% of all enterovirus isolates for the years 1970 to 1983 (Strikas *et al.*, 1986), and 3·8% of isolates for 1993 to 1996 (Centers for Disease Control and Prevention, 1997), were reported by state public health laboratories as 'untyped enterovirus'. In our own collection, more than 12% of isolates from the period 1962 to 1997 were originally reported as untypeable (Centers for Disease Control and Prevention, 1999). This higher figure is partially explained by the fact that the Centers for Disease Control and Prevention is a reference centre and may be expected to receive those isolates that were most difficult to type in the primary clinical or public health laboratory. Our recent pilot study suggests that VP1 sequencing and database comparison will easily identify the vast majority of untypeable isolates as variants of known serotypes (Oberste *et al.*, 2000). In that study, we identified one potentially new serotype, represented by four isolates from three states, isolated between 1985 and 1987, and those isolates could be analysed by methods similar to those outlined in the present study. Broadly reactive VP1-specific primers are available to rapidly screen untypeable isolate collections for potentially novel serotypes (Oberste *et al.*, 2000, 1999*a*).

Practical criteria must be established before molecular sequence information can be applied routinely to picornavirus identification. A partial or complete VP1 nucleotide sequence identity of at least 75% (minimum 85% amino acid sequence identity) between a clinical enterovirus isolate and serotype prototype strain may be used to establish the serotype of the isolate (Oberste *et al.*, 2000, 1999*a, b*). These criteria also appear to apply to comparisons among isolates of foot-and-mouth-disease virus (Vosloo *et al.*, 1992), but a study directly comparable to the enterovirus studies has not yet been performed. A best-match nucleotide sequence identity of between 70 and 75% or a second-highest score of greater than 70% may provide a tentative identification, pending confirmation by other means, such as neutralization with monospecific antisera (Oberste *et al.*, 2000) or more extensive sequencing. A best-match nucleotide sequence identity below 70% (less than 85% amino acid sequence identity) may indicate that the isolate represents an unknown serotype. Sequencing of the complete capsid-coding region may be useful in confirming this result, but complete capsid sequences are available for less than half of the known enterovirus serotypes, limiting the utility of complete capsid sequence comparisons. More extensive characterization, possibly including complete genome sequences, may be required for viruses that appear to represent previously unknown genera (Hyypiä *et al.*, 1992; Marvil *et al.*, 1999; Niklasson *et al.*, 1999; Yamashita *et al.*, 1998). Due to the high frequency of recombination among picornaviruses (King, 1988; Kopecka *et al.*, 1995; Santti *et al.*, 1999), sequence information from non-capsid regions is of little value in characterizing new serotypes within known genera.

On the basis of the antigenic and molecular comparisons presented here, we propose that CA55-1988, CA64-4454, CA78-1480, OMA95-6498 and OMA95-6499 be recognized as isolates of a new human enterovirus serotype, 'enterovirus 73' (EV73), subject to approval by the appropriate taxonomic authority, and that CA55-1988 be designated as the prototype strain. RT-PCR coupled with amplicon sequencing is a simple and rapid method for the typing and classification of picornaviruses and may lead to the identification of many new picornavirus serotypes. We propose this method as a general approach for the molecular classification of newly discovered picornaviruses and for the reclassification of known serotypes. In light of the increased use of molecular sequence data in virus classification (Calisher *et al.*, 1995; Mayo & Pringle, 1998; Van Regenmortel *et al.*, 1997) and the correlation of picornavirus capsid sequence data with classic antigenic typing, we further recommend that molecular data be given increased weight in the classification of picornaviruses.

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