Enteroviruses 76, 89, 90 and 91 represent a novel group within the species *Human enterovirus A*

M. Steven Oberste, Kaija Maher, Suzanne M. Michele, Gaël Belliot, Moyez Uddin and Mark A. Pallansch

Molecular methods have enabled the rapid identification of new enterovirus (EV) serotypes that would have been untypable using existing neutralizing antisera. Nineteen strains of four new EV types termed EV76 (11 isolates), EV89 (two isolates), EV90 (four isolates) and EV91 (two isolates), isolated from clinical specimens from patients in France (one isolate) and Bangladesh (18 isolates), are described. Nucleotide sequences encoding the VP1 capsid protein (882–888 nt) are less than 65 % identical to the homologous sequences of the recognized human EV serotypes, but within each group the sequences are more than 78 % identical. The deduced amino acid sequences of the complete capsid (P1) region are more than 94 % identical within type but less than 76 % identical to those of the recognized serotypes. For both VP1 and P1, the 19 isolates are monophyletic by type with respect to all other EV serotypes. Using the proposed molecular typing scheme, these data support their identification as four new types within the species *Human enterovirus A* (HEV-A). In almost all cases, the VP1 sequences were more similar to those of some simian EVs than to the human EVs. Partial 3D sequences of all 19 isolates also clustered within HEV-A; they were monophyletic as a group, but not by type, suggesting that recombination has occurred among viruses of these four types. Partial 3D sequences were more closely related to those of simian EVs than to human viruses in HEV-A. These results suggest that the four new types may represent a new subgroup within HEV-A, in addition to the existing human and simian subgroups.

**INTRODUCTION**

The genus *Enterovirus* (family *Picornaviridae*) is comprised of more than 80 serotypes, most of which are known human pathogens (Pallansch & Roos, 2001). Most enterovirus infections are asymptomatic or result in only mild illness, such as non-specific febrile illness or mild upper respiratory symptoms (common cold). However, enteroviruses can also cause a wide variety of clinical illnesses including acute haemorrhagic conjunctivitis, aseptic meningitis, undifferentiated rash, acute flaccid paralysis, myocarditis and neonatal sepsis-like disease (Pallansch & Roos, 2001). The human enteroviruses are presently classified into five species, *Poliovirus* and *Human enterovirus* (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 addition to the human enteroviruses, four serotypes of simian enteroviruses, A13, SV19/SV26/SV35, SV43 and SV46 are provisionally classified as members of HEV-A (Oberste et al., 2002).

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., 1999a, 2000, 2003). 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). Four additional new types, EV74, EV75, EV77 and EV78, have also been identified by similar methods (Norder et al., 2003; Oberste et al., 2004c). The identification of these new types suggested that there may be many other additional enterovirus serotypes awaiting identification.

In this study, 19 enterovirus isolates are characterized as members of four new types within HEV-A. Isolates within
each of these four groups are significantly different from all known enterovirus serotypes, as determined on the basis of sequences from multiple genome regions. Sequence comparisons identified all four groups as members of the species HEV-A, but analysis of partial 3D sequences showed that they also form a distinct subgroup within HEV-A. We propose that these isolates be classified as members of four new human enterovirus types in the species HEV-A.

**METHODS**

**Virus isolation.** The 19 virus isolates characterized in this study are listed in Table 1. Strain FRA91-10369 was obtained from a French military recruit with gastroenteritis, during a 1991 outbreak of astrovirus-associated gastroenteritis among military recruits in Caen, France, by inoculation of stool specimen into Caco-2 cell culture (Belliot et al., 1997). Astrovirus was also detected in this patient’s stool specimen by using RT-PCR. The Bangladeshi strains were isolated from stool specimens obtained from patients presenting with acute flaccid paralysis (AFP), during AFP surveillance activities in support of global polio eradication, by inoculation of stool specimen by using RT-PCR. The Bangladeshi strains were isolated from stool specimens obtained from patients presenting with acute flaccid paralysis (AFP), during AFP surveillance activities in support of global polio eradication, by inoculation of stool specimen by using RT-PCR. The Bangladeshi strains were isolated from stool specimens obtained from patients presenting with acute flaccid paralysis (AFP), during AFP surveillance activities in support of global polio eradication, by inoculation of stool specimen by using RT-PCR.

**Molecular characterization of isolates.** Viral RNA extraction, RT-PCR and nucleotide sequencing for molecular serotyping were performed as described previously (Oberste et al., 1999a, 2000, 2003). Isolates were initially characterized by partial VP1 sequence (Oberste et al., 2003). The partial VP1 sequences were compared to one another and to those of known enterovirus serotypes to determine whether related isolates were available. To confirm the relationships observed with the partial VP1 sequences, complete VP1 sequences were amplified and determined using primers 486–488 and 487–489 as described (Oberste et al., 2004d). The complete VP1 sequences were compared with the VP1 sequence database as described above. Isolates were further characterized by sequencing of the complete capsid (P1) region. RT-PCR primers were designed to anneal to sites encoding amino acid motifs that are highly conserved among members of the species HEV-A (Oberste et al., 2004d). Specific, non-degenerate primers were designed from preliminary sequences to close gaps between the original PCR products. The complete genome sequences of each of the proposed new prototype strains were also determined using a similar strategy, with additional primer-walking to close large gaps as necessary. For the non-prototype strains, a portion of the 3D region was amplified and sequenced using primers 782 (5’-TAGCCTTCTCCCGCTTCG-3’, EV76 nt 6672–6690) and 783 (5’-GAYGCACTGRTCTTCTGIGT-3’, EV76 nt 7205–7186). For comparison with isolates that are members of previously recognized HEV-A serotypes, the partial 3D sequences of 16 contemporary Bangladeshi HEV-A isolates were determined using primers 233 (5’-TTGAYTTACWCGNWATAGTG-3’, PV1 nt 6681–6702) and 130 (5’-WGRRTCTTGTCCCATC-3’, PV1 nt 7209–7191) (Oberste et al., 2004e). For all sequence determinations, the PCR products were purified for sequencing by using a High-Pure PCR product purification kit (Roche Molecular Biochemicals), and both strands were sequenced by automated methods, using fluorescent dideoxy-chain terminators (Applied Biosystems).

**Sequence analysis.** The nucleotide and deduced amino acid sequences of the isolates were compared to one another and to those of other enteroviruses by using the programs Gap and Distances (Wisconsin Package, version 10.3). Nucleotide sequences were aligned using the PILEUP program (Wisconsin Package) and adjusted manually to conform to the optimized alignment of deduced amino acid sequences. Phylogenetic relationships were inferred from the aligned sequences. The nucleotide and deduced amino acid sequences of the isolates were compared to one another and to those of other enteroviruses by using the programs Gap and Distances (Wisconsin Package, version 10.3). Nucleotide sequences were aligned using the PILEUP program (Wisconsin Package) and adjusted manually to conform to the optimized alignment of deduced amino acid sequences. Phylogenetic relationships were inferred from the aligned sequences.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Strain</th>
<th>Country</th>
<th>Locale*</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV76</td>
<td>FRA91-10369</td>
<td>France</td>
<td>Caen</td>
<td>1991</td>
</tr>
<tr>
<td>EV76</td>
<td>BAN99-10370</td>
<td>Bangladesh</td>
<td>Jhenaidah, Khulna</td>
<td>1999</td>
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<td>BAN99-10371</td>
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<td>Paña, Rajshahi</td>
<td>1999</td>
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<td>Bangladesh</td>
<td>Narayanganj, Dhaka</td>
<td>1999</td>
</tr>
<tr>
<td>EV76</td>
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<td>Patuakhali, Barisal</td>
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<td>Bangladesh</td>
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<td>Jhalakati, Barisal</td>
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<td>Brahmanbaria, Chittagong</td>
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<tr>
<td>EV76</td>
<td>BAN00-10382</td>
<td>Bangladesh</td>
<td>Comilla, Chittagong</td>
<td>2000</td>
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<tr>
<td>EV89</td>
<td>BAN00-10359</td>
<td>Bangladesh</td>
<td>Bholia, Barisal</td>
<td>2000</td>
</tr>
<tr>
<td>EV89</td>
<td>BAN00-10360</td>
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<td>Rajbari, Dhaka</td>
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<tr>
<td>EV90</td>
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<td>1999</td>
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<tr>
<td>EV90</td>
<td>BAN00-10402</td>
<td>Bangladesh</td>
<td>Dinajpur, Rajshahi</td>
<td>2000</td>
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<tr>
<td>EV90</td>
<td>BAN02-10404</td>
<td>Bangladesh</td>
<td>Khulna, Khulna</td>
<td>2002</td>
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<td>EV90</td>
<td>BAN02-10405</td>
<td>Bangladesh</td>
<td>Cox’s Bazar, Chittagong</td>
<td>2002</td>
</tr>
<tr>
<td>EV91</td>
<td>BAN00-10406</td>
<td>Bangladesh</td>
<td>Barisal, Barisal</td>
<td>2000</td>
</tr>
<tr>
<td>EV91</td>
<td>BAN00-10407</td>
<td>Bangladesh</td>
<td>Mymensingham, Dhaka</td>
<td>1999</td>
</tr>
</tbody>
</table>

*City for France; city and administrative division for Bangladesh.
nucleic acid sequences by the neighbour-joining method implemented in MEGA, version 2.1 (Kumar et al., 2001), using the Kimura two-parameter substitution model (Kimura, 1980) and a transition-transversion ratio of 10. Regions containing alignment gaps were omitted from the analysis. Support for specific tree topologies was estimated by bootstrap analysis with 1000 pseudo-replicate datasets.

**Nucleotide sequence accession numbers.** The sequences described here have been deposited in the GenBank sequence database, accession numbers AY697458 to AY697507; the accession numbers are listed in Table S1 (JGV Online) by strain and region sequenced. Other enterovirus sequences used in comparisons are listed in Table S2 (JGV Online).

### RESULTS

**VP1 sequences**

The 19 enterovirus isolates were initially characterized by RT-PCR amplification and sequencing of a portion of the VP1 coding region, using an established molecular serotyping protocol (Oberste et al., 2003). For all of the isolates, the partial VP1 sequence was less than 70% identical to those of the recognized enterovirus prototype strains, suggesting that these isolates may represent one or more new types (data not shown). As putative new types are identified, their partial VP1 sequences are added to the database of enterovirus prototype strain VP1 sequences as a secondary search set to allow the subsequent identification of related strains. Using this strategy, four groups of genetically related isolates were identified (Table 1 and data not shown).

To confirm the typing identifications that were based on the partial VP1 sequences, complete VP1 sequences were determined for each of the isolates. The sequences were compared with each other and with the complete VP1 sequences of all known enterovirus serotypes. Like the partial VP1 sequences, the complete VP1 nucleotide sequences of all 19 isolates are less than 70% identical to those of the established enterovirus serotypes, and they are most closely related to those of members of HEV-A (54–9–69·4% nucleotide sequence identity) (Table 2); members of other HEV-A serotypes are 54–5–73·2% identical to one another (Oberste et al., 1999b, 2004d). We have previously shown that strains that are at least 75% identical in VP1 sequence belong to the same serotype, whereas those that are less than 70% identical to one another belong to different serotypes (Oberste et al., 1999a, b, 2000, 2001, 2003). In almost all cases, the VP1 sequence of each of the 19 isolates is most closely related to that of one of the simian enteroviruses in HEV-A, A13, SV19/SV26/SV35 (a single serotype), SV43 or SV46 (Fig. 1a). Comparison of the complete VP1 sequences with one another confirmed that they formed four distinct groups (provisional new types). Within each of the four new types, the VP1 sequences are at least 78·1% identical to one another (93·2% amino acid identity), but members of the different types differ from one another by at least 29·7% (Table 2). These new types have been provisionally named enteroviruses 76, 89, 90 and 91. EV77 and EV78, both members of HEV-B, have been recently described (Bailly et al., 2004; Norder et al., 2003); enteroviruses numbered from 79 to 88 are also members of HEV-B and will be described elsewhere (M. S. Oberste, unpublished data).

The pairwise comparison results were confirmed by phylogenetic reconstruction. Members of each of the four new types are monophyletic in VP1 with respect to all other serotypes (Fig. 1a). The VP1 sequences of the 19 isolates are also monophyletic as a group, with respect to all other serotypes in HEV-A, but with low bootstrap support (48%). The EV76-EV89-EV90-EV91 cluster appears to be most closely related to the simian enterovirus cluster (Fig. 1a). The 11 EV76 capsid sequences cluster into three major phylogenetic groups of one, three and seven isolates (Fig. 1a). The Bangladeshi isolates are distributed between two groups, while FRA91-10369 forms an independent branch. The sequences in a group are at least 90% identical to one another, but members of different groups differ from one another by 19–22% (Table 2).

**P1 sequences**

As in VP1, all of the complete capsid sequences are monophyletic, both by type and as a group (Fig. 1b), and the capsid sequence clustering is more firmly supported by bootstrap analysis (97%). Complete capsid sequences are not yet available for the simian viruses in HEV-A. The 11 EV76 capsid sequences remain clustered in the same three subgroups, with 100% bootstrap support for each subgroup. The deduced capsid protein sequences are highly conserved within a type (94–100% identity), whereas capsid sequence identity between the four types ranges

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**Table 2.** Comparison of the VP1 sequences of EV76, EV89, EV90 and EV91 isolates with one another and with those of other enteroviruses (nucleotide identity %)

<table>
<thead>
<tr>
<th></th>
<th>EV76</th>
<th>EV89</th>
<th>EV90</th>
<th>EV91</th>
<th>HEV-A*</th>
<th>HEV-B</th>
<th>HEV-C</th>
<th>HEV-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV76</td>
<td>78·1–99·2</td>
<td>68·5–70·3</td>
<td>64·4–67·7</td>
<td>66·6–69·5</td>
<td>55·5–66·1</td>
<td>44·9–52·5</td>
<td>44·4–52·3</td>
<td>51·3–54·9</td>
</tr>
<tr>
<td>EV89</td>
<td>97·6</td>
<td>65·6–66·2</td>
<td>68·8–69·4</td>
<td>58·2–67·9</td>
<td>45·3–51·6</td>
<td>48·1–51·3</td>
<td>52·0–53·0</td>
<td></td>
</tr>
<tr>
<td>EV90</td>
<td>89·8–98·2</td>
<td>65·0–67·1</td>
<td>57·3–66·0</td>
<td>44·5–52·6</td>
<td>45·3–51·9</td>
<td>50·9–52·9</td>
<td></td>
<td></td>
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<tr>
<td>EV91</td>
<td>97·6</td>
<td>57·9–67·4</td>
<td>45·2–49·5</td>
<td>46·3–50·2</td>
<td>51·8–52·1</td>
<td></td>
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</tbody>
</table>

*Includes simian viruses in HEV-A.
from 79 to 82% (Table 3). However, they are no more than 76% identical to those of any other enterovirus serotype. By comparison, other members of HEV-A are 66–85% identical to one another in deduced capsid protein sequence (Oberste et al., 2004d). The capsid protein sequences of the EV76 candidates are 94–100% identical to one another, and at least 99% identical within each subgroup (Table 3 and data not shown). Sites at which the amino acid sequences vary among the EV76 isolates are distributed throughout the three major capsid proteins, 16 in VP2, 13 in VP3 and 20 in VP1; the VP4 sequences are identical in all isolates (data not shown). Eight of 16 variable sites in VP2 are in the ‘puff’ region and 10 of 13 variable sites in VP3 are in the ‘knob’ region. In enteroviruses of known 3D structure, the puff and knob are predominant surface protrusions and contribute to neutralizing epitopes on the capsid surface (Muckelbauer et al., 1995). In VP1, most of the variable sites are near the ends of the mature protein: eight in the first 27 residues at the amino terminus and six in the last 26 aa at the carboxyl terminus. The capsid sequences of the EV89, EV90 and EV91 isolates are very highly conserved within type (99.4, 98.2–99.2 and 99.0% identity, respectively) (Table 3).

Non-capsid coding sequences

The P2 and P3 sequences of the four prototype strains (EV76, FRA91-10369; EV89, BAN00-10359; EV90, BAN00-10399; and EV91, BAN00-10406) are typical of those of the established HEV-A serotypes in overall length and in the predicted proteolytic processing sites. Like the VP1 and P1 sequences, the P2 and P3 sequences are monophyletic as a group, forming a cluster that is within HEV-A, but distinct from all other members of the species (Fig. 2).

To determine whether the distinct non-capsid sequence clustering was possibly an artefact due to temporal and

Table 3. Comparison of the deduced capsid protein sequences of EV76, EV89, EV90 and EV91 isolates with one another and with those of other enteroviruses (amino acid identity %)

<table>
<thead>
<tr>
<th></th>
<th>EV76</th>
<th>EV89</th>
<th>EV90</th>
<th>EV91</th>
<th>HEV-A*</th>
<th>HEV-B</th>
<th>HEV-C</th>
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<tr>
<td>EV76</td>
<td>94.3–100</td>
<td>81.7–82.2</td>
<td>78.8–79.7</td>
<td>79.5–80.9</td>
<td>68.3–75.7</td>
<td>47.0–50.5</td>
<td>45.9–48.0</td>
<td>51.6–52.1</td>
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<tr>
<td>EV89</td>
<td>99.4</td>
<td>80.3–81.0</td>
<td>80.9–81.6</td>
<td>68.3–73.2</td>
<td>47.2–50.4</td>
<td>45.9–48.4</td>
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<tr>
<td>EV90</td>
<td>98.2–99.2</td>
<td>80.7–81.4</td>
<td>69.1–73.9</td>
<td>46.6–50.8</td>
<td>46.5–49.5</td>
<td>53.2–54.0</td>
<td></td>
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<tr>
<td>EV91</td>
<td>99.0</td>
<td>96.6–72.9</td>
<td>46.3–49.4</td>
<td>45.9–49.0</td>
<td>52.8–53.4</td>
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</table>

*Includes simian viruses in HEV-A.
geographical differences in the isolation of EV76, EV89, EV90 and EV91, versus other HEV-A serotypes, 16 isolates of nine HEV-A types were chosen for comparison from among the typed non-polio enterovirus isolates identified in Bangladesh during the period August 1999 to January 2003 (unpublished data). Partial 3D sequences were determined and compared with those of the EV76, EV89, EV90 and EV91 isolates. Like the P2 and P3 sequences, the EV76, EV89, EV90 and EV91 partial 3D sequences are monophyletic as a group (Fig. 3). The 3D sequences of the isolates of ‘conventional’ HEV-A serotypes clustered with those of the recognized prototype strains isolated in other countries 30–50 years earlier, and they were distinct from the isolates of the new types that were isolated in the same area during the same period. For both the conventional and new serotypes, isolates of a given type did not necessarily cluster together, suggesting that RNA recombination is likely to occur frequently among HEV-A clinical isolates, as it does in HEV-B and HEV-C (Lindberg et al., 2003; Liu et al., 2000, 2003; Lukashev et al., 2003, 2004; Oberste et al., 2004a, e; Oprisan et al., 2002).

**DISCUSSION**

The most recently described enterovirus types, EV73-75 and EV77-78, have been defined on the basis of pairwise sequence comparisons and phylogenetic clustering, using sequences encoding the VP1 capsid protein or those encoding the complete capsid polyprotein (Norder et al., 2003; Oberste et al., 2001, 2004c). In the current typing scheme, viruses of the same type share at least 75% VP1 nucleotide sequence identity (>85% VP1 amino acid identity), and viruses of different serotypes are less than 70% identical in VP1 (<85% amino acid identity) (Oberste et al., 1999a). When complete capsid sequences are considered, homotypic isolates share at least 90% identity in deduced complete capsid amino acid sequence (Brown & Pallansch, 1995; Chua et al., 2001; Oberste et al., 2001, 2004c; Paananen et al., 2003; Zimmermann et al., 1996), whereas isolates of different serotypes share ≤87% capsid identity (Brown et al., 2003; Oberste et al., 2001, 2004a, b, d). In most cases, VP1 sequence is sufficient to identify the serotype, but isolates may occasionally have a highest match value between 70 and 75% (Oberste et al., 2000). Thus, the use of complete capsid sequence for serotype identification may provide additional resolution and permit identification of isolates that are in or near the ambiguous range of 70–75% VP1 identity. Regardless,
there is complete correspondence between serotype defined by classic antigenic methods and the molecular definition of type and it is likely that these two definitions are functionally equivalent for species of human enteroviruses.

Analysis of enterovirus prototype strains has suggested that recombination is a frequent event in enterovirus evolution and that recombination only occurs between viruses of the same species (Andersson et al., 2002; Brown et al., 2003; Oberste et al., 2004a, d; Santi et al., 1999). Similar results have been obtained by analysis of more recent clinical isolates (Lindberg et al., 2003; Lukashev et al., 2003, 2004; Oberste et al., 2004e). Phylogenetic analysis of partial 3D sequences suggests that EV76, EV89, EV90 and EV91 have recombined with one another but not with viruses of other HEV-A serotypes (Fig. 3). Similarly, the analysis of 3D sequences shows that the Bangladesh enteroviruses of other types are also recombinant with respect to each other and to the HEV-A prototype strains, as the recent clinical isolates do not necessarily cluster by type in the 3D tree. The biological factors underlying the apparent restriction of recombination within a species remain unknown.

Four serotypes of simian enteroviruses, A13, SV19/SV26/SV35, SV43 and SV46 are provisionally classified as members of HEV-A (Oberste et al., 2002). Other than A13, all of these viruses were originally isolated from Asian primates of the genus Macaca, most commonly Macaca mulatta (rhesus macaque) and Macaca fascicularis (cynomolgus macaque) (Heberling & Cheever, 1965; Hoffert et al., 1958; Hull et al., 1958). While complete capsid sequences are not yet available for A13, SV19, SV43 or SV46, the VP1 sequence phylogeny suggests that EV76, EV89, EV90 and EV91 may be most closely related to the simian enterovirus serotypes in HEV-A (Fig. 1a). In the partial 3D phylogeny, EV76 and EV89-91 form a distinct group within HEV-A, separate from both the human and simian prototype strains of other serotypes, suggesting that these viruses may occupy a unique ecological niche (Fig. 3). Rhesus macaques are indigenous to Bangladesh and elsewhere in South Asia. Isolation of enteroviruses from wild primates species in South Asia may yield additional clues to the genetic relationships between the human and simian viruses in HEV-A.

EV76, EV89, EV90 and EV91 represent the first new HEV-A types identified since the isolation of EV71 in 1972 (Kennett et al., 1974; Schmidt et al., 1974). Whereas EV71 is closely related to the previously identified members of HEV-A, particularly to coxsackievirus A16 (Brown & Pallansch, 1995), the four newest members of the species are clearly distinct from other HEV-A viruses throughout their genomes. It will be interesting to see whether additional HEV-A types will be discovered in the future and whether these new types more closely resemble the ‘conventional’ HEV-A viruses or the members of the EV76-EV89-EV90-EV91 group.

ACKNOWLEDGEMENTS

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