Enterovirus surveillance reveals proposed new serotypes and provides new insight into enterovirus 5’-untranslated region evolution

Teemu Smura,1 Soile Blomqvist,1 Anja Paananen,1 Tytti Vuorinen,2 Zdenka Sobotová,3 Veronika Buboviča,4 Olga Ivanova,5 Tapani Hovi1 and Merja Roivainen1

Correspondence
Merja.Roivainen@ktl.fi

1Enterovirus Laboratory, Department of Viral Diseases and Immunology, National Public Health Institute (KTL), Mannerheimintie 166, FIN-00300 Helsinki, Finland
2Department of Virology, University of Turku, Kiinamyllynkatu 13, FIN-20520 Turku, Finland
3National Reference Centres, Public Health Office of the Slovak Republic, Trnavska 52, 82645 Bratislava, Slovak Republic
4State Agency ‘Public Health Agency’, Virology Laboratory, Klijanu Str. 7, LV-1012 Riga, Latvia
5M. P. Chumakov Institute of Poliomyelitis and Viral Encephalitides of Russian Academy of Medical Science, Kievskoe Shosse 27 km, 142782 Moscow Region, Russian Federation

Received 22 January 2007
Accepted 11 May 2007

Human enteroviruses are currently grouped into five species Human enterovirus A (HEV-A), HEV-B, HEV-C, HEV-D and Poliovirus. During surveillance for enteroviruses serologically non-typable enterovirus strains were found from acute flaccid paralysis patients and healthy individuals. In this study, we report isolates of recently described enterovirus types EV76 and EV90 of HEV-A species and characterize two new enterovirus type candidates, EV96 and EV97, to species HEV-C and HEV-B, respectively. Analysis of partial 3D regions of EV96 strains revealed sequence divergence consistent with several recombination events between EV96, other HEV-C viruses and polioviruses. Phylogenetic analysis of all available 5’-untranslated region sequences of human entero- and rhinovirus prototype strains and 10 simian enterovirus strains suggested interspecies recombination involving this region.

Enteroviruses (family Picornaviridae) are small RNA-viruses associated with several human diseases (Pallansch & Roos, 2001). The enterovirus genome is a single-stranded RNA molecule of approximately 7500 nt consisting of a single open reading frame flanked by non-coding 5’ and 3’ regions. The open reading frame is translated into a precursor polypeptide that is subsequently cleaved by viral proteases. The polypeptide is divided into three functional regions P1 to P3, of which the P1 region contains viral capsid proteins from VP4 to VP1. Within enterovirus species the sequence divergence is greatest in the capsid protein VP1-coding region of the virus genome, and the classifications based on sequence variation in this region correlate completely with the classification made by antigenic properties (Oberste et al., 1999, 2000). It has been suggested that enteroviruses should be classified in the same serotype if they have more than 75% nucleotide similarity in the VP1 capsid protein-coding sequence (85% amino acid similarity) and to different serotypes, if they have less than 70% nucleotide similarity (85% amino acid similarity) in this region (Oberste et al., 2004). Human enteroviruses are currently grouped into five species Human enterovirus A (HEV-A), HEV-B, HEV-C, HEV-D and Poliovirus by molecular and biological characteristics (Stanway et al., 2005). Molecular typing of serologically non-typable strains has led to the discovery of a large number of new enterovirus types (Oberste et al., 2001, 2004a, 2004b; Norder et al., 2003; Junttila et al., 2007; Smura et al., 2007). Most of the new serotypes are members of the HEV-B species.

During surveillance of enteroviruses, serologically non-typable enterovirus strains were found from acute flaccid paralysis (AFP) patients and healthy individuals. In this study, we report isolates of recently described enteroviruses and characterize two new enterovirus type candidates, EV96 and EV97, to species HEV-C and HEV-B, respectively. Analysis of partial 3D regions of EV96 strains revealed sequence divergence consistent with several recombination
events between EV96, other HEV-C viruses and polioviruses. Phylogenetic analysis of all available 5′-untranslated region (UTR) sequences of human entero- and rhinovirus prototype strains and 10 simian enterovirus strains suggested interspecies recombination involving this region.

Serologically non-typable virus isolates were found during enterovirus surveillance from Latvia, Kazakhstan, Slovakia and Finland. Viruses were isolated from faecal samples according to the WHO laboratory manual (WHO, 2001). The molecular characterization was performed as described in Smura et al. (2007). The initial characterization was made by partial sequencing of capsid protein VP1-coding region (Oberste et al., 2003). The isolates FIN03-2875, KAZ00-14550 and LVA02-10337 were plaque purified in RD cells (provided by the WHO Polio Labnet) and the isolate SVK03-24 was purified by the end-point titration method in the Ohio strain of HeLa cells (provided by Eurico Arruda, University of Virginia, Charlottesville, USA). The complete VP1 and partial 5′-UTR regions were sequenced using the primer-walking strategy. The partial 3D regions were sequenced as described by Pulli et al. (1995).

Nucleotide sequences were assembled and compared using programs ContigExpress and AlignX (Vector NTI advance 10.1; Invitrogen Corporation, 2005). The sequences were aligned using the CLUSTAL_X program (version 1.81) (Thompson et al., 1997). Phylogenetic trees were produced and visualized using the neighbour-joining method implemented in MEGA version 3.1 (Kumar et al., 2004). Bootstrap analysis was performed using 1000 replicates. Transition-transversion rate was estimated from the data and TN93 model of substitution (Tamura & Nei, 1993) was used to calculate distances. SimPlot 2.5 program was used for similarity plot and bootscanning analysis (Lole et al., 1999). For similarity plot analysis, a 200 nt window moved in 20 nt steps and Jukes–Cantor correction were used. Bootscanning analysis (Salminen et al., 1995) was run with the neighbour-joining algorithm and 100 pseudoreplicates.

Simian enterovirus sequences produced in previous work (Pöyry et al., 1999) were submitted to GenBank with accession numbers EF364414–EF364423. Supplementary Table S1 (available in JGV Online) shows the GenBank accession numbers of sequences used in this study.

We sequenced the VP1 and/or 5′-UTR and/or 3D regions of 10 serologically non-typable enterovirus isolates. The virus strains are shown in Table 1.

Two of the isolates (KAZ00-14550 and LVA02-10337) clustered in phylogenetic analysis with recently described HEV-A types EV76 and EV90 (Oberste et al., 2005), respectively. LVA02-10337 was isolated from a healthy child in Latvia. It clustered together with EV90 isolates (Fig. 1a) and had 77.2–85.8 % nucleotide and 91.5–97.3 % amino acid similarity with them in the VP1 region. KAZ00-14550 was isolated from an AFP patient in Kazakhstan. It clustered together with EV76 isolates and had 87.2–92.9 % nucleotide and 92.9–99.0 % amino acid similarities with them in the VP1 region.

The strain FIN03-2875 was isolated in Finland from a faecal sample of a healthy 3-year-old child. In phylogenetic analysis this isolate grouped together with HEV-B serotypes in the VP1 region (Supplementary Fig. S1 available in JGV Online), but had only 68.4 % nucleotide and 78.0 % amino acid similarity with the closest enterovirus prototype strain (echo 27), suggesting that this virus strain should be considered as a new enterovirus type. It was registered as a candidate new enterovirus with number EV97.

EV97 was also recently found from a sample in Bangladesh during virological surveillance in support of the global polio eradication initiative (Oberste et al., 2006). The FIN03-2875 strain had 88.7 % nucleotide and 97.6 % amino acid similarity with the Bangladeshi strain. Although the EV97 strain was isolated in Finland we cannot conclude the geographical origin of this virus, since the child from whom the virus was isolated had been recently adopted from India.

Table 1. Virus isolates characterized

<table>
<thead>
<tr>
<th>EV type</th>
<th>Virus isolate</th>
<th>Country*</th>
<th>Year of sample collection</th>
<th>Age of patient (years)</th>
<th>Clinical symptoms†</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV76</td>
<td>KAZ00-14550</td>
<td>Kazakhstan</td>
<td>2000</td>
<td>Not known</td>
<td>AFP</td>
</tr>
<tr>
<td>EV90</td>
<td>LVA02-10337</td>
<td>Latvia</td>
<td>2002</td>
<td>Child</td>
<td>Healthy</td>
</tr>
<tr>
<td>EV96</td>
<td>SVK03-24</td>
<td>Slovak Republic</td>
<td>2003</td>
<td>4</td>
<td>AFP</td>
</tr>
<tr>
<td>EV96</td>
<td>FIN04-7</td>
<td>Finland (Thailand)</td>
<td>2004</td>
<td>1</td>
<td>Healthy</td>
</tr>
<tr>
<td>EV96</td>
<td>FIN05-5</td>
<td>Finland (China)</td>
<td>2005</td>
<td>2</td>
<td>Healthy</td>
</tr>
<tr>
<td>EV96</td>
<td>FIN05-10</td>
<td>Finland (China)</td>
<td>2005</td>
<td>1</td>
<td>Healthy</td>
</tr>
<tr>
<td>EV96</td>
<td>FIN05-12</td>
<td>Finland (China)</td>
<td>2005</td>
<td>1</td>
<td>Healthy</td>
</tr>
<tr>
<td>EV96</td>
<td>FIN05-14</td>
<td>Finland (China)</td>
<td>2005</td>
<td>1</td>
<td>Healthy</td>
</tr>
<tr>
<td>EV96</td>
<td>FIN06-7</td>
<td>Finland (China)</td>
<td>2006</td>
<td>1</td>
<td>Healthy</td>
</tr>
<tr>
<td>EV97</td>
<td>FIN03-2875</td>
<td>Finland (India)</td>
<td>2003</td>
<td>3</td>
<td>Healthy</td>
</tr>
</tbody>
</table>

*The country where the virus was isolated. In cases where importation of the virus is suspected, the geographical origin of the virus is indicated in parentheses.
†AFP, Acute flaccid paralysis.
The strains SVK03-24, FIN04-7, FIN05-5, FIN05-10, FIN05-12, FIN05-14 and FIN06-7 were isolated from faecal samples of 1–4-year-old children in Finland and Slovakia. The strain SVK03-24 was isolated from an AFP patient who also had other symptoms including pain of the abdomen, anorexia, weakness, acute rhinopharyngitis and meningism. Also CVA24 and echo 1 viruses were isolated from the same patient. In phylogenetic analysis based on VP1 sequences, the serologically non-typable strains formed a monophyletic group within the HEV-C species (Fig. 1b). They had 75.3–98.7% nucleotide and 89.3–98.1% amino acid similarities with each other, and 66.3–68.1% nucleotide and 73.1–74.4% amino acid similarities with the closest enterovirus prototype CVA24 in the VP1-coding region, suggesting that these isolates belong to new enterovirus type, tentatively registered as EV96.

Arita et al. (2005) recently reported a non-typable HEV-C strain, CAM2083, from Cambodia. According to the VP1-coding sequence obtained from GenBank (accession no. AB207266) this strain also belongs to the proposed serotype EV96, since it has 78–81% nucleotide and

![Fig. 1. Phylogenetic trees constructed from complete VP1 sequences of HEV-A (a), HEV-C (b) and from partial 3D sequences of HEV-C (c). Strains sequenced in this study are indicated with arrows. Alignment with CLUSTAL_X version 1.8, phylogenetic tree construction with neighbour-joining method implemented in MEGA 3.1 program using Tamura–Nei (TN93) model of substitution, transition–transversion estimated from the data and visualization of the tree with MEGA 3.1.](image-url)
90.9–94.2 % amino acid similarities of the VP1-coding region with the EV96 isolates described in this paper. EV96 was also recently found from an AFP patient in Bangladesh (EV96-10488; AJ919472) (Oberste et al., 2006).

High prevalence of HEV-C viruses has been found in some of the tropical countries (Arita et al., 2005; Rakoto-Andrianarivelo et al., 2005), whereas in temperate regions the circulation of HEV-C viruses seems to be very restricted (Keshuriani et al., 2006; Witsø et al., 2006). The formation of subclusters in phylogenetic analysis and substantial divergence between EV96 isolates in both nucleotide and amino acid levels suggests independent circulation of different EV96 lineages. The finding of EV96 from both South Asia and Europe suggests that EV96 might be geographically widely distributed.

Intra- and intertypic recombination is a common phenomenon within enterovirus species HEV-A, HEV-B and HEV-C (Santti et al., 1999; Oprisan et al., 2002; Brown et al., 2003; Lindberg et al., 2003; Lukashev et al., 2003, 2005; Chevaliez et al., 2004; Oberste et al., 2004b, 2004c; Yoke-Fun & AbuBakar, 2006; Mirand et al., 2007; reviewed by Lukashev, 2005). We studied the possible recombination between EV96 and other enteroviruses by sequencing a part of the 3D protein-coding region of EV96 isolates as described by Pulli et al. (1995). The partial 3D sequences of EV96 isolates were not monophyletic (Fig. 1c). They had 75–100 % similarities with each other and 73–91 % similarities with other HEV-C and poliovirus serotypes in this region. The incongruity between phylogenetic trees based on VP1 and partial 3D regions suggests that EV96 can readily recombine with other serotypes of HEV-C species and polioviruses. The isolates SVK03-24, FIN04-7 and FIN06-7 grouped together with CVA1, CVA19 and CVA22, a group that has been previously suggested to be genetically distinct from other HEV-C serotypes (Brown et al., 2003). EV96 isolates FIN05-10, FIN05-12 and FIN05-14 grouped together with poliovirus 1 because of similarities in the 3D region, suggesting that EV96 may be able to recombine also with polioviruses. Circulating vaccine derived polioviruses (cVDPV) often contain a recombinant genome in which the capsid protein coding part of the genome is from Sabin strain poliovirus and the non-structural part is derived from an unknown HEV-C serotype (Arita et al., 2005; Brown et al., 2003; Guillot et al., 2000; Kew et al., 2002; Liu et al., 2003; Rousset et al., 2003; Shimizu et al., 2004; Yang et al., 2003; reviewed by Kew et al., 2005). It has been a concern that recombination between polioviruses and HEV-C viruses might increase the fitness (and hence transmissibility) of VDPVs, possibly via elimination of harmful mutations in the non-structural region (Agol, 2006). However, there is no experimental data available to confirm this hypothesis.

Most HEV-C serotypes are associated with mild respiratory symptoms. Although strains SVK03-24 and CAM2083 were isolated from AFP patients it is not known whether EV96 has a causative role in this disease, since the observed association may be only coincidental. Further studies are needed to elucidate any human disease associations of EV96. It is not known what the origin of EV96 is and how long it has been circulating in humans, since some enterovirus types may cause asymptomatic infections or only mild disease and thus may have been undetected due to a lack of efficient methods before molecular methods for enterovirus typing became widely accessible.

In addition to the VP1 regions, partial 5’-UTR regions of some isolates described in this study were sequenced. In the 5’-UTR, all human enteroviruses cluster into two major groups, of which polioviruses, HEV-C and HEV-D species are considered to constitute the enterovirus 5’-UTR cluster I and HEV-A and HEV-B constitute species cluster II (Hyypia et al., 1997). We constructed a phylogenetic tree on the basis of ~400 nt (positions 26–443 relative to the genome of PV1-Mahoney) in the 5’-UTR region of all known human enterovirus prototypes, human rhinovirus types HRV1B, HRV2, HRV14, HRV16, HRV39, HRV72 and HRV89 and simian enterovirus types SV4, SV6, SV19, SV26, SV28, SV35, SV43 and SV46 (Pöyry et al., 1999) (Fig. 2a).

Surprisingly, the newer HEV-A enterovirus serotypes EV90 (including LVA02-10337) and EV91 clustered together with HEV-C and HEV-D serotypes in this region, thus conflicting with the strict clustering of HEV-A serotypes to the 5’-UTR cluster II and suggesting interspecies recombination between the ancestor of EV90/EV91 and a member of the 5’-UTR cluster I (i.e. HEV-C, HEV-D or poliovirus strain). This is further supported by bootscanning analysis of complete enterovirus genomes (Fig. 2b), in which high bootstrap support of EV90 or EV91 clustering with a representative of 5’-UTR cluster I (EV94) is detected in the 5’-UTR, but in the other regions of the genome EV90/EV91 clusters with a representative of 5’-UTR cluster II (HEV-A serotype EV76). The separation of human enteroviruses to clusters I and II in the 5’-UTR was also evident in the similarity plot analysis using complete genomes of ‘newer’ HEV-A types and representatives of other human enterovirus species (Fig. 2c). There was a peak of similarity at the 3’ end of the 5’-UTR, corresponding to a highly conserved region in nucleotide alignment of EV76, EV90 and EV94 (nt 520–649 in the consensus sequence), which could act as a recombination site. Accordingly, EV90 5’-UTR sequence was clearly more similar to EV94 than to EV76 (sequence similarities of 88.0 and 75.7 %, respectively) upstream and more similar to EV76 than to EV94 (sequence similarities of 67.6 and 42.9 %, respectively) downstream of this conserved region. Formally, we cannot exclude the possibility of convergent evolution as an explanation for the grouping of EV90 and EV91 to the 5’-UTR cluster I, but given the substantial nucleotide divergence between EV90/EV91 and EV76/EV89 in this region, this seems unlikely.

In accordance with the original study (Pöyry et al., 1999), partial 5’-UTR sequences of the studied simian
Enteroviruses formed four groups, of which one clustered close to human enterovirus 5'-UTR cluster II. On the basis of VP1 sequences the human enterovirus types 76, 89, 90 and 91 form a distinct subgroup within the HEV-A species, which is closely related to simian enteroviruses A13, SV19/SV26/SV35 (a single serotype), SV43 and SV46 (Oberste et al., 2002, 2005; Fig. 1a). While the 5'-UTR sequences of EV90 and EV91 definitely clustered among group I strains, EV76 and EV89 formed a small cluster of their own between the bulk of group II strains and the simian viruses mentioned above.

Human rhinoviruses formed a separate cluster in the 5'-UTR that could be divided into two subclusters representing species HRV-A and HRV-B. However, we cannot conclude that all HRV-A and HRV-B serotypes fall into these clusters, since only seven rhinovirus sequences from this region were available.

In conclusion, we have proposed here new enterovirus serotypes EV96 and EV97 to HEV-C and HEV-B species, respectively. Partial 3D sequences of EV96 strains were not monophyletic, suggesting a potential to recombine with other HEV-C serotypes including poliovirus 1 and the previously reported subcluster including CVA1, CVA19 and CVA22. Furthermore, we observed that on the basis of the 5'-UTR sequences, the ‘newer’ HEV-A serotypes, including clinical isolates of EV76 and EV90 described here, do not cluster together with ‘conventional’ HEV-A serotypes, thus conflicting with the earlier exclusive grouping of HEV-A and HEV-B to cluster I and HEV-C and HEV-D to cluster II in this region.

Fig. 2. Phylogenetic tree constructed from all available partial 5'-UTR sequences of human entero- and rhinovirus prototype strains, 10 simian enterovirus strains and two porcine enterovirus B strains (a). Similarity plot of complete enterovirus genomes using a sliding window of 200 nt moving in 20 nt steps. EV90 sequence was compared to representatives of HEV-A (EV76 and EV91), HEV-B (CVB5), HEV-C (CVA24), poliovirus (PV2) and HEV-D (EV94) species (b). Bootscanning analysis of complete enterovirus genomes using a sliding window of 200 nt moving in 20 nt steps. EV90 was compared to the representatives of the 5'-UTR clusters I (EV94) and II (EV76 and CVB5) (c).
Acknowledgements

We thank Carita Savolainen-Kopra for helpful suggestions during manuscript preparation.

References


