Enterovirus 94, a proposed new serotype in human enterovirus species D

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The genus Enterovirus (family Picornaviridae) contains five species with strains isolated from humans: Human enterovirus A (HEV-A), HEV-B, HEV-C, HEV-D and Poliovirus. In this study, a proposed new serotype of HEV-D was characterized. Four virus strains were isolated from sewage in Egypt and one strain from acute flaccid paralysis cases in the Democratic Republic of the Congo. The complete genome of one environmental isolate, the complete coding sequence of one clinical isolate and complete VP1 regions from the other isolates were sequenced. These isolates had 66.6–69.4 % nucleotide similarity and 74.7–76.6 % amino acid sequence similarity in the VP1 region with the closest enterovirus serotype, enterovirus 70 (EV70), suggesting that the isolates form a new enterovirus type, tentatively designated enterovirus 94 (EV94). Phylogenetic analyses including sequences of the 5‘UTR, VP1 and 3D regions demonstrated that EV94 isolates formed a monophyletic group within the species HEV-D. No evidence of recombination was found between EV94 and the other HEV-D serotypes, EV68 and EV70. Further biological characterization showed that EV94 was acid stable and had a wide cell tropism in vitro. Attempts to prevent replication with protective antibodies to known enterovirus receptors (poliovirus receptor, vitronectin x₁β₃ receptor and decay accelerating factor) were not successful. Seroprevalence studies in the Finnish population revealed a high prevalence of this virus over the past two decades.

INTRODUCTION

The genus Enterovirus (family Picornaviridae) contains a large group of human pathogens. Although the majority of enterovirus infections are subclinical, enterovirus infection can lead to a variety of acute and chronic diseases including mild upper respiratory illness, febrile rash, aseptic meningitis, encephalitis, acute haemorrhagic conjunctivitis, pleurodynia, acute flaccid paralysis (AFP), diabetes, myocarditis and neonatal sepsis-like disease (Pallansch & Roos, 2001). The primary site of enterovirus infection is the mucosal tissue of the respiratory or gastrointestinal tract. After spreading through the lymphatic system and circulation, the virus can infect secondary target tissues. The secondary replication sites largely define the clinical manifestations of a given enterovirus strain. In the intestinal mucosa, virus replication can continue for several weeks, during which time progeny virus is shed into faeces.

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 5’ UTR contains an internal ribosome-binding site, which is essential for translation initiation (Pelletier & Sonenberg, 1988; Molla et al., 1992; Chen & Sarnow, 1995). The 3’ UTR forms highly conserved secondary and tertiary structures that are thought to be important in replication initiation (Pilipenko et al., 1992, 1996; Mirmomeni et al., 1997). The open reading frame is translated into a single, large polypeptide, which is subsequently cleaved by viral proteases (reviewed by Palmenberg, 1990). The polypeptide is divided into three domains, P1 to P3, consisting of three to four proteins each. The P1 region contains viral capsid proteins VP1 to VP4, whilst P2 and P3 contain the non-structural proteins.

Enteroviruses were originally classified by their antigenic and pathogenic properties in humans and mice. As phylogenetic methods to classify enteroviruses became available, it became apparent that pathogenic properties were not sufficient to classify the evolutionarily related viruses into correct groups. Using molecular properties, human enteroviruses have been classified into five species, Human enterovirus A (HEV-A), HEV-B, HEV-C, HEV-D and Poliovirus (Stanway et al., 2005). Polioviruses are genetically...
related to HEV-C and will probably be reclassified into this species (Brown et al., 2003).

Within an enterovirus species, sequence divergence is greatest in the capsid protein (VP1)-coding region of the virus genome, and classifications based on sequence variation in this region correlate completely with the classification made using antigenic properties (Oberste et al., 1999, 2000). It has been suggested that enteroviruses should be classified in the same serotype if they have >75% nucleotide similarity in the VP1-coding sequence (>85% amino acid sequence similarity) and into different serotypes if they have <70% nucleotide similarity (<85% amino acid similarity). Molecular typing of serologically non-typable strains has led to the discovery of a large number of new enterovirus types (Oberste et al., 2001, 2004c, 2005; Norder et al., 2003).

Detection of circulating wild-type poliovirus strains has become more and more important as the World Health Organization (WHO)-coordinated global poliomyelitis eradication initiative is approaching its goal (Hovi, 2006). Poliomyelitis typically presents itself as a rapidly progressing, usually unilateral AFP, often with fever and residual paralysis at day 60 after onset. Along with standard clinical case-driven surveillance of AFP patients, environmental surveillance of sewage waters has been adopted as a supplementary method for the detection of poliovirus circulation in some countries, including Egypt. Samples are collected from water bodies contaminated with human feces, concentrated and analysed using standard methods (El-Bassioni et al., 2003; Hovi et al., 2005). During environmental surveillance, non-polio enteroviruses are also found regularly.

In this study, we have reported the isolation and characterization of several strains of a proposed new serotype strain of HEV-D from an AFP case in the Democratic Republic of the Congo and from waste water in Egypt. Only two other serotypes have been found previously in the HEV-D species, EV68 and EV70. The new serotype was found to have an unusually wide tissue tropism and host species range in vitro. Seroprevalence studies in the Finnish population revealed a high prevalence of this virus over past two decades.

**METHODS**

**Cell lines.** A human rhabdomyosarcoma (RD) cell line and recombinant mouse L cells expressing human poliovirus receptor (L20B) (Pipkin et al., 1993) were provided by the WHO Polio Labnet. The Ohio strain of HeLa cells was kindly provided by Eurico Arruda (University of Virginia, Charlottesville, USA). The green monkey kidney cell line GMK and Vero have been maintained in our laboratory since the 1960s. Human colorectal adenocarcinoma (CaCo-2), human lung carcinoma (A549), human larynx epidermoid carcinoma (Hep-2C), mouse embryo fibroblast (3T3), hamster kidney (BHK21), rabbit kidney (RK13), canine kidney (MDCK) and bovine kidney (MDBK) cell lines were purchased from ATCC. Human neuroblastoma (SK-N-SH) cells were kindly provided by Antti Vaheri (University of Helsinki, Finland). Human hepatocellular carcinoma (HUH-7) cells were kindly provided by Darius Moradpour (University Hospital Freiberg, Germany). Chinese hamster ovary (CHO) cells expressing human Coxackie- and adenovirus receptor (HCAR) were kindly provided by Jeffrey Bergelson (Children’s Hospital of Philadelphia, USA). Mouse fibroblast M4 (LPREG−/−, LDLR−/−) cells (Reithmayer et al., 2002) and intracellular adhesion molecule (ICAM-1)-expressing M4 cells were kindly provided by Dieter Blaas (Medical University of Vienna, Austria).

**Isolation of viruses.** Collection of a 1 l sewage sample and concentration using a two-phase separation method were performed as described previously (El-Bassioni et al., 2003; Hovi et al., 2005). Virus isolation was carried out in RD and L20B cell lines as described by Hovi et al. (2005). Virus strains producing cytopathic effect (CPE) in both cell lines were subjected to neutralization with polyclonal antisera specific for poliovirus types PV1, PV2 and PV3 (National Institute of Public Health and the Environment, Bilthoven, The Netherlands) according to the protocol recommended by WHO. Virus strains escaping neutralization with PV-specific antisera were passaged once in L20B cells before molecular typing. The isolation and sequencing of viruses from stool samples has been described elsewhere (Junttila et al., 2007).

**Partial VP1 RT-PCR and sequencing.** Viral RNA was extracted from 100 μl infected cell cultures with an RNeasy Total RNA kit (Qiagen) according to the manufacturer’s instructions. RT-PCR using primers 292 and 222 (nt 2612–2627 and 2969–2951 relative to the genome of PV1-Mahoney) was carried out as described by Oberste et al. (2003). PCR amplicons were purified with the QIAquick gel extraction kit (Qiagen) and used as templates in cycle sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit, version 1.1; Applied Biosystems) in an automated sequencer (model 310; Applied Biosystems). Electropherograms were analysed with Vector NTI Advance 10.1 (Invitrogen) and the partial VP1 sequences obtained were compared with sequences available in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Virus isolates showing <70% nucleotide sequence identity with known enterovirus serotypes were subjected to further analysis.

**Plaque purification of environmental virus strains.** Viruses were purified using a plaque assay in monolayer cultures of RD cells and passaged once in RD cells at 36 °C, freeze-thawed three times and clarified by centrifugation at 250 g for 10 min. All subsequent experiments were performed using these plaque-purified virus preparations.

**Full-length genome sequencing.** Total RNA was extracted from cells infected with virus strains using Trizol reagent (Gibco-BRL Life Technologies) according to the manufacturer’s instructions. A SuperScript First-strand Synthesis System for RT-PCR kit (Gibco-BRL Life Technologies) was used for cDNA synthesis and a SuperScript One-step RT-PCR kit was used for PCRs. PCR products were purified using a QIAEXII agarose gel extraction kit (Qiagen). Complete or partial genomes of the virus isolates were sequenced using a primer-walking strategy.

**Phylogenetic analysis.** Nucleotide and amino acid sequences were assembled and compared using the programs CONTIGEXPRESS and ALIGNX (Vector NTI Advance 10.1; Invitrogen). The sequences were aligned using CLUSTAL X (version 1.81) (Thompson et al., 1997). Phylogenetic trees were produced using the maximum-likelihood method implemented in TREE-PUZZLE version 5.2 (Schmidt et al., 2002) and the neighbour-joining method implemented in MEGA version 3.1 (Kumar et al., 2004). Bootstrap analysis was performed using 1000 replicates. The transition–transversion rate was estimated from the data and the TN93 model of substitution (Tamura & Nei, 1993) was used to calculate distances. Phylogenetic trees were visualized using MEGA version 3.1. The SIMPLOR 2.5 program was used for
similarity plot and bootscanning analyses (Lole et al., 1999). For similarity plot analysis, a 200 nt window was moved in 20 nt steps and Jukes–Cantor correction was used. Bootscanning analysis (Salminen et al., 1995) was run with the neighbour-joining algorithm and 100 pseudoreplicates.

**GenBank accession numbers.** The following sequences were obtained from GenBank: HEV-D serotype EV68 strains EV68-Fermom (AY426331), EV68-TX03 (AY426390), EV68-MD99 (AY426499), EV68-TX99 (AY426498), EV68-MN98 (AY426497), EV68-TX02-2 (AY426496), EV68-TX02-1 (AY426495), EV68-W100 (AY426494), EV68-MO00 (AY426493), EV68-MD02-2 (AY426492), EV68-MD02-1 (AY426491), EV68-NY93 (AY426490), EV68-MN89 (AY426489), EV68-CA62-3 (AY426488), EV68-CA62-2 (AY426487) and EV68-CA62-1 (AY426486); HRV87-Corn (AY355268); serotype EV70 strains EV70-J670/71 (NC_001430), EV70-ENG/71 (D17595), EV70-FB/73 (D17596), EV70-G10/72 (D17597), EV70-HP185/81 (D17598), EV70-HP85/78 (D17599), EV70-I72 (D17600), EV70-M51/76 (D17603), EV70-M8/72 (D17604), EV70-R20/71 (D17605), EV70-R6/71 (D17606), EV70-SEC32/71 (D17607), EV70-TE60/74 (D17608), EV70-TE62/73 (D17609), EV70-TE266/81 (D17610) and EV70-V1250/81 (D17611); HEV-A serotypes CVA8 strain Donovan (AY421766), CVA8 strain G-10 (U05876), CVA8 strain Nancy (M16572) and CVA9 strain Griggs (D00627); HEV-C serotypes CVA11 strain Belgium-1 (AF498636) and CVA20 strain IH35 (AF499642), poliovirus 2 strain Lansing (M12197) and porcine enterovirus 8 (PEV8) strain V13 (AF068613).

**Viruses replication in different cell lines.** Confluent cell cultures in 24-well plates were infected with EV94-E210 at an apparently high m.o.i. After 30 min of adsorption at room temperature and a 30 min internalization period at 36°C in 5% CO₂, the remaining inoculated virus was removed and cells were washed twice with Hank’s balanced salts solution supplemented with 20 mM HEPES (pH 7.4), and growth medium (Eagle’s minimum essential medium supplemented with 20 mM HEPES, 20 mM MgCl₂, 1% FBS and penicillin/streptomycin solution) was added to all cultures. Incubation was continued at 36°C in a 5% CO₂ atmosphere.

For virus titration, cell cultures were harvested at different intervals and freeze-thawed three times. The total infectivity of each sample was subsequently determined by endpoint titration in microwell cultures of the corresponding cell line or in RD cells for detection of possible replication in non-human cell lines. CPE was determined on day 6 after infection by microscopy and TCID₅₀ titles were calculated using the Kärber formula (Lennette, 1969).

**Human sera.** The study group for the serum neutralization assay consisted of 181 women at the end of the first trimester of pregnancy. The sera were derived from the Finnish Maternity Cohort and had been collected in 1983 (n = 86) and 2002 (n = 95).

**Neutralization assay.** Aliquots of sera were inactivated at 56°C for 20 min and stored at 4°C. Serial 4-fold dilutions of sera were mixed with an equal volume of virus (100 TCID₅₀). The mixtures were neutralized at 36°C for 1 h and then overnight at 4°C. The mixtures were transferred into 96-well cell culture plates with RD cells (~3 x 10⁵ cells ml⁻¹) and incubated at 36°C in 5% CO₂ for 6 days. The highest dilution that completely inhibited viral CPE was taken as the end-point titre of the serum.

**CPE protection assay.** Monoclonal antibodies to the second and third short consensus repeats of decay accelerating factor (DAF) were kindly provided by Douglas Lublin (Washington University School of Medicine, St Louis, USA). Polyclonal rabbit antisera to the vitronectin x₂β₃ receptor and to the poliovirus receptor (PVR) were produced in house (Ylipaasto et al., 2004). The antibodies were added to confluent 96-well cell culture plate monolayers in volumes of 30 μl and incubated for 2 h at 36°C in 5% CO₂, after which pre-titrated virus (~10⁰ TCID₅₀ per well) was administered. The mixture was incubated at 36°C in 5% CO₂ for 20 h and the level of protection was examined by light microscopy.

**Assay for acid sensitivity.** The acid sensitivities of environmental EV94 strains were tested using a standard protocol (Couch, 1992). Human rhinovirus 2 (HRV2) and PV1 Sabin strain were included as acid-sensitive and acid-insensitive control viruses, respectively. Briefly, an equal volume of buffer A [0.1 M citric acid buffer (pH 4.0) or 0.1 M phosphate buffer (pH 7.0)] was added to a virus-infected cell supernatant. The mixtures were incubated for 1 h at 36°C and then neutralized using 0.5 M phosphate buffer (pH 7.2). The infectivities of the acid-treated and untreated viruses were assayed in RD cells in 96-well microplates at 36°C in 5% CO₂. CPE was examined daily and end-point titres were calculated using the Kärber formula on day 7.

**Immunofluorescence assay of virus-infected cells.** Infected cells were fixed with cold methanol for 15 min at −20°C, washed three times in PBS without calcium or magnesium (PBS⁻) and incubated for 1 h at 36°C in 5% CO₂ with enterovirus-specific polyclonal rabbit antiserum (KTL-482) produced against the peptide EAIPALTAVETGHTSQVC, which was designed according to the VP1 region of the enterovirus genome (Härkönen et al., 2002). Unbound antibody was removed by washing three times with PBS⁻ and once with PBS supplemented with 0.1% BSA. The conjugate for virus antiserum (anti-rabbit FITC, cat. no. 711-095-152, Jackson ImmunoResearch Laboratories) was incubated for 30 min at 36°C. After staining, slides were washed three times with PBS⁻ and once with water. Slides were analysed under a confocal microscope (Leica TGS NT).

**RESULTS**

**Identification of a new serotype of HEV-D**

Sewage specimens collected in Egypt during environmental surveillance for wild-type polioviruses were examined for cytopathic viruses in both RD and L20B cells. Virus strains that produced CPE in L20B cells and escaped neutralization with poliovirus-specific antisera were characterized using molecular methods in Helsinki. Several non-serotypable enterovirus strains were isolated. Initial characterization was performed by partially sequencing the VP1-coding region. Four strains clustered close to EV70 and were subjected to a more detailed study. Meanwhile, three non-serotypable virus strains isolated from faecal specimens of AFP patients in the Democratic Republic of the Congo were analysed in more detail. One of these isolates turned out to be EV68, whilst the other two grouped closer to the above-mentioned Egyptian isolates.

The complete VP1 sequence of one of the virus strains (E210) was submitted to the Picornavirus Study Group (University of Essex, Colchester, UK) for comparison with those of previously proposed new enterovirus serotypes, and consequently was registered as a new candidate enterovirus serotype, EV94. According to this proposal, from here on, the virus strains characterized in this study are referred to as strains of EV94.

Possible cross-reactions of EV94 with EV68 and EV70 were studied using a neutralization assay with virus-specific anti-
rabbit antisera raised against EV68 and EV70. The antiserum against EV68 was not able to protect RD cells from EV70 or EV94 infection. Likewise, the antiserum against EV70 was not able to protect GMK cells from EV68 infection or RD cells from EV94 infection, indicating that EV94 differed antigenically from these two previously determined HEV-D serotypes.

**Further genetic characterization of EV94**

The genome of EV94-E210 sequenced in this study was 7364 nt. The non-coding 5' end was 714 nt, followed by an open reading frame encoding a 2190 aa polypeptide. The length of the non-coding 3' region was 80 nt. The overall base composition of the genome was 30.1 % A, 22.2 % G, 21.5 % C and 26.2 % U.

<table>
<thead>
<tr>
<th>EV94-19-04</th>
<th>EV70-J670/71</th>
<th>EV68-Fermon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete genome</td>
<td>ND</td>
<td>75.8</td>
</tr>
<tr>
<td>5' UTR</td>
<td>ND</td>
<td>83.5</td>
</tr>
<tr>
<td>VP4</td>
<td>85.0</td>
<td>79.7</td>
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<td>76.3</td>
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<tr>
<td>3D</td>
<td>89.9</td>
<td>82.7</td>
</tr>
<tr>
<td>3' UTR</td>
<td>ND</td>
<td>80.7</td>
</tr>
</tbody>
</table>

ND, Not determined.

Nucleotide similarities in the complete VP1 region ranged from 96.3 to 98.3 % among the four environmental EV94 isolates and from 85.7 to 86.3 % among clinical isolate 19-04 and the environmental isolates, confirming clustering of all six strains in the same serotype. The environmental isolate EV94-E210 and the clinical isolate EV94-19-04 had 81.5–89.9 % nucleotide similarity and 92.9–100 % amino acid similarity in the coding region (Tables 1 and 2).

A similarity plot analysis with prototype sequences of other enterovirus serotypes suggested that EV94 was most closely related to the HEV-D species in all genome regions except in 5' UTR (Fig. 1). In the 5' region, HEV-D and HEV-C serotypes were about equally close to EV94. As expected, the sequence similarities were lowest in the capsid-coding region and highest in the non-structural protein-coding regions. The complete genome nucleotide similarities between EV94-E210 and the prototype strains of EV70 (EV70-J670/71) and EV68 (EV68-Fermon) were 75.8 and 73.7 %, respectively. In the VP1 region, the nucleotide

**Table 1. Nucleotide sequence similarities (%) between EV94-E210 and strains EV94-19-04, EV70-J670/71 and EV68-Fermon**

**Table 2. Amino acid sequence similarities (%) between EV94-E210 and strains EV94-19-04, EV70-J670/71 and EV68-Fermon**

<table>
<thead>
<tr>
<th>EV94-19-04</th>
<th>EV70-J670/71</th>
<th>EV68-Fermon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyprotein</td>
<td>97.0</td>
<td>86.9</td>
</tr>
<tr>
<td>VP4</td>
<td>97.1</td>
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<td>98.8</td>
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<td>3C</td>
<td>96.7</td>
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</tr>
<tr>
<td>3D</td>
<td>97.8</td>
<td>95.2</td>
</tr>
</tbody>
</table>

ND, Not determined.

![Fig. 1. Similarity plot of complete enterovirus genomes using a sliding window of 200 nt moving in 20 nt steps. The sequence of EV94-E210 was compared with representatives of HEV-A (CVA16), HEV-B (CVB3), HEV-C (CVA20), poliovirus (PV2) and HEV-D (EV68 and EV70) species.](image-url)
identities were 67.7 and 67.4 %, respectively. The nucleotide similarities between the EV94 isolates and the EV68 and EV70 isolates obtained from GenBank ranged from 66.1 to 69.6 % and from 66.6 to 69.4 %, respectively, in the complete VP1 regions (for EV68 vs EV94) or in the overlapping 912 nt in the VP1 region (for EV70 vs EV94).

We used the complete genome sequence of EV68 prototype strain Fermon (GenBank accession no. AY426531) for tentative estimation of the EV94 polypeptide cleavage sites. The amino acid sequences of individual estimated EV94 proteins had 71.4–90.6 % similarity with those of EV68-Fermon and 73.6–95.7 % similarity with EV70-J670/71 proteins (Table 2). The VP1 amino acid sequences of the EV68 and EV70 strains obtained from GenBank had 69.1–72.0 % and 74.7–76.6 % sequence similarity with EV94-E210 in the overlapping regions.

**Phylogenetic analysis**

Phylogenetic trees were generated from HEV-D 5′ UTR, VP1 and 3D sequences (Fig. 2). On the basis of the VP1 and 3D regions, the EV94 isolates were closely related and formed a monophyletic group within the HEV-D species. In the 5′ UTR region, EV94 grouped together with other HEV-D strains and HEV-C serotypes. The phylogenetic trees of the VP1 and 3D regions were essentially congruent, providing no evidence of recombination within HEV-D species. In order to verify the apparent lack of recombination between HEV-D serotypes, we analysed the nucleotide sequences of EV94-E210, EV94-19-04, EV70-J670/71 and EV68-Fermon using similarity plot and bootscanning methods (data not shown). No evidence of recombination was found.

**Acid sensitivity**

Sensitivity to acid treatment and optimum growth temperature infer the natural target tissue and route of transmission of the virus. Rhinoviruses and EV68, which multiply mainly in the respiratory tract epithelia, are sensitive to low pH and have a lower optimal growth temperature than most enteroviruses, which are enteric pathogens. In order to assess the possible transmission route of EV94, we tested the acid sensitivity of environmental strain EV94-E210 using a standard assay (Couch, 1992). Acid treatment did not affect the infectivities of the environmental EV94 isolates (Table 3), whereas the titre of the acid-sensitive control, HRV2, was reduced 10 000-fold. The titre of the acid-insensitive control, PV1-Sabin, was not affected by acid treatment.

**Infectivity and virus replication**

We infected a variety of human continuous cell lines with EV94-E210. Virus replication was studied by light microscopy and by measuring the infectivity of samples collected at different time points. The virus was able to induce CPE and clear progeny virus production was detected in all of the human cell lines studied (Fig. 3a).

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**Fig. 2.** Phylogenetic trees constructed from complete 5′ UTR (a), VP1 (b) and 3D (c) nucleotide sequences. Phylogenetic trees were constructed with TREE-PUZZLE 5.2 using the Tamura–Nei model of substitution; the transition–transversion rate was estimated from the data. Six representatives of both human enterovirus 5′ UTR clusters were used to construct the tree in (a). The viruses sequenced in this study and representatives of each human enterovirus species were used to construct the trees in (b) and (c). PEV8 was used as an outgroup to root the trees in (b) and (c).
Most human enteroviruses have strict host specificity and can infect only human or other primate cells. To determine the host species specificity of EV94 in vitro, we studied infection and replication of EV94-E210 in a variety of mammalian cell lines, as described above. EV94 was able to induce CPE in green monkey kidney cells (GMK and Vero), recombinant mouse L cells expressing human PVR (L20B) and hamster kidney BHK21 cells (Fig. 3b). Virus replication without a strong CPE was found in rabbit RK13 cells. No signs of replication or altered morphology were found in mouse fibroblastic 3T3 cell line. In bovine kidney (MDBK) and canine kidney (MDCK) cells, the virus infection altered cell morphology and induced cell death, but no viable virus progeny formation was detected in the replication assay using RD cells. However, an enterovirus-specific antibody produced a positive signal in an immunofluorescence assay in MDBK cells, suggesting that EV94 is able to internalize into these cells and that there is at least limited gene expression (Fig. 3c). No viral proteins were detected in MDCK cells (not shown).

**Table 3. Stability of environmental EV94 isolates under acidic conditions**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>pH 4</th>
<th>pH 7</th>
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<tbody>
<tr>
<td>EV94-E210</td>
<td>7.1</td>
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</tr>
<tr>
<td>EV94-E430</td>
<td>6.6</td>
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</tr>
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<td>EV94-E435</td>
<td>7.1</td>
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<tr>
<td>EV94-E438</td>
<td>6.6</td>
<td>6.6</td>
</tr>
<tr>
<td>HRV2</td>
<td>&lt;1.6</td>
<td>5.6</td>
</tr>
<tr>
<td>PV1-Sabin</td>
<td>7.1</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Receptor usage

The ability of viruses to use cell-surface molecules as receptors is considered to be an important determinant of tissue tropism, host range and pathogenesis. The role of DAF (CD55), vitronectin receptor (αvβ3) and PVR (CD155) in cell attachment and virus internalization was studied using CPE protection assays (Nobis et al., 1985). None of the blocking antibodies was able to prevent infection in RD cells.

We used CHO cell lines expressing either the α2 subunit of α2β1 integrin or HCAR, and M4 cells expressing (ICAM-1) to assess the role of α2β1, HCAR and ICAM-1, respectively, in EV94 infection. Equal amounts of progeny formation were found in both α2- and HCAR-expressing CHO cells. No progeny virus production was found in M4 or M4–ICAM cells, but mild CPE was detected in M4–ICAM cells.

Neutralization assays with human sera

As EV94 is a previously unknown enterovirus, we assessed the prevalence of antibodies against EV94 in Finland using a serum neutralization assay. Serum samples of 181 pregnant women, collected in 1983 (n = 86) and 2002 (n = 95), were studied. Neutralizing antibodies against EV94 were found in 80 % of subjects in the year 1983 and 78.9 % in the year 2002 (Fig. 4).
DISSCUSSION

Members of an enterovirus species are considered to share >70 % amino acid similarity in regions coding for the capsid (P1) and non-structural proteins 2C and 3CD, to share a limited number of host-cell receptors and natural host range, to have a genomic G+C content that varies by no more than 2.5 mol% and to share a significant degree of compatibility in proteolytic processing, replication, encapsidation and genetic recombination (Stanway et al., 2005). Enterovirus strains should be classified in the same serotype if they have >75 % nucleotide similarity in the VP1-coding sequence (>85 % amino acid sequence similarity) and into different serotypes if they have <70 % nucleotide similarity (<85 % amino acid similarity) (Oberste et al., 2000, 2001, 2003).

The sequence similarities and phylogenetic analyses suggested that the virus isolates described in this study belonged to the HEV-D species and formed a new type, preliminarily designated EV94. We suggest that E210 should be considered as the prototype strain of this new enterovirus type. The natural host range and the cellular receptor for this virus are not known, but the similarities between EV70 and EV94 in cellular host range in vitro suggest that these serotypes might share some biological characteristics. The genomic G+C content of EV94 and EV68 varied by 2.6 mol%, thus only just failing to meet the species demarcation criterion. We did not study proteolytic processing or encapsidation of EV94, but given the overall genomic similarity of EV94, EV70 and EV68, it is unlikely that there would be any major differences in these properties among HEV-D serotypes.

Strain EV94-19-04 was isolated from a patient with AFP. Moreover, the environmental strain EV94-E210 was able to induce cytoplastic infection in neuroblastic SK-N-SH cells, suggesting that this new enterovirus serotype might also be neurovirulent in vitro. However, based on this data, we cannot conclude that EV94 is necessarily the causative agent of these AFP cases, as the observed association may be coincidental.

The EV94 isolates originating from Egypt and the Democratic Republic of the Congo had substantial divergence in nucleotide sequences but only a few amino acid substitutions were detected. The nucleotide substitutions were distributed evenly throughout the genome. This suggests that these are geographically divergent, independently circulating strains that probably share antigenic and other biological properties but have accumulated mostly neutral mutations over time. In phylogenetic analysis, the EV94 isolates were monophyletic with other HEV-D serotypes in all genome regions except the 5′ UTR. In this region, all enteroviruses cluster into two major groups of which HEV-A and HEV-B species constitute the enterovirus 5′ UTR cluster II and HEV-C and HEV-D species cluster I (Hyypiä et al., 1997). The phylogenetic trees constructed on the basis of amino acid sequences were essentially similar to nucleotide alignment-based trees (data not shown).

Intra- and intertypic recombination is common 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., 2004a; reviewed by Lukashev, 2005). However, no evidence of recombination between HEV-D serotypes has been found in this study or by others (Oberste et al., 2004b). Although bootscanning analysis showed some possible recombination sites, the similarity plot analysis suggested that EV68 and EV70 are almost equally distant from EV94, and any evidence of recombination may be a product of convergent evolution between lineages rather than a definite recombination event. Moreover, the phylogenetic trees of VP1 and 3D were essentially congruent. Apparently, any possible recombination event must have taken place a relatively long time ago. Our seroprevalence studies suggested that EV94 is a common virus; hence, co-infection of this virus with other HEV-D strains is possible and the apparent lack of recombination may be due to different tissue tropisms, as suggested previously by Oberste et al. (2004b). More complete genome sequences of field isolates of HEV-D serotypes are needed to elucidate the role of recombination within this species.

Most enteroviruses use the faecal–oral route of transmission. Interestingly, the other serotypes of HEV-D, EV68 and EV70, differ from most enteroviruses by infecting their primary target tissue directly. EV68 is associated with respiratory disease and EV70 is a causative agent of acute haemorrhagic conjunctivitis. In order to access primary replication sites in the alimentary channel mucosa, the virus has to get through the acidic environment of the stomach. It has been shown previously that EV68 (Blomqvist et al., 2002) and some strains of EV70 (Oberste et al., 2004b) are acid sensitive, and EV68 has a lower optimum growing temperature than most other enteroviruses. In this study, we showed that EV94-E210 is acid stable and thus is likely also to be able to access the small intestine in vivo. Moreover,
EV94 was able to replicate in a cell line of alimentary channel origin, the colon epithelial CaCo-2 cell line, and the strains described in this study were originally isolated from sewage and faecal specimens, suggesting that this virus might use the faecal–oral route of transmission.

Most enteroviruses have a restricted cell tropism in vitro. To characterize the in vitro cell tropism of EV94, we studied its replication in various human and other mammalian cell lines. EV94 was cytopathic in a wide variety of human cell lines including muscle, glandular, lung epithelial, epithelioid and neuroblastic cells. Moreover, clear virus progeny production was observed in all of the human cell lines studied. EV94 was also able to replicate in some non-human cell lines including primate (GMK and Vero), hamster (BHK21), rabbit (RK13), mouse (L20B) and bovine (MDBK) cell lines, suggesting that EV94 resembles EV70 in having a wider host range in vitro than most other enteroviruses (Yoshii et al., 1977). The ability of the virus to infect and replicate in mouse L cells expressing human PVR presents an important practical issue. L20B cells are used in poliovirus diagnostics and thus EV94 may cause false-positive results if the serotype is not confirmed by other means. Continuous cell lines differ from cells in vivo in many respects; for example, virus receptor expression levels may be different in cultured cells and the corresponding cells in vivo. Therefore, further studies are needed to determine the tissue tropism and possible pathogenesis of EV94 in vivo.

Both virus and host determinants affect the tissue tropism of a virus. These determinants include receptor specificity, host-cell factors participating in translation and replication initiation (reviewed by Whitton et al., 2005) and the local cytokine (alpha/beta interferon) milieu at the site of infection (Ida-Hosonuma et al., 2005; Yoshikawa et al., 2006). The ability of EV94 to infect L cells expressing human PVR presents an important practical issue. L20B cells are used in poliovirus diagnostics and thus EV94 may cause false-positive results if the serotype is not confirmed by other means. Continuous cell lines differ from cells in vivo in many respects; for example, virus receptor expression levels may be different in cultured cells and the corresponding cells in vivo. Therefore, further studies are needed to determine the tissue tropism and possible pathogenesis of EV94 in vivo.

In conclusion, our results suggest that EV94 is a new serotype of the HEV-D species and has an unusually wide tissue tropism and host species range in vitro. EV94 is probably able to use the faecal–oral route of transmission, is potentially neuropathogenic and has an unexpectedly high prevalence in the Finnish population. Further studies are needed to elucidate the pathogenic and epidemiological properties of EV94.

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REFERENCES


echovirus 11 strains circulating in Europe during an epidemic of multisystem hemorrhagic disease of infants indicates that evolution generally occurs by recombination. *Virology* 325, 56–70.


