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# Molecular evolution and epidemiology of echovirus 6 in Finland

Teemu Smura [a,*], Laura Kakkola [b], Soile Blomqvist [a], Päivi Klemola [a], Alun Parsons [b], Hannimari Kallio-Kokko [c], Carita Savolainen-Kopra [a], Denis E. Kainov [b], Merja Roivainen [a]

[a] National Institute for Health and Welfare (THL), P.O. Box 30, FI-00271 Helsinki, Finland
[b] Institute for Molecular Medicine Finland, FIMM, FI-00290 Helsinki, Finland
[c] Institute of Clinical Medicine, FI-00290 Helsinki, Finland

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ABSTRACT

Echovirus 6 (E-6) (family *Picornaviridae*, genus *Enterovirus*) is one of the most commonly detected enteroviruses worldwide. The aim of this study was to determine molecular evolutionary and epidemiologic patterns of E-6. A complete genome of one E-6 strain and the partial VP1 coding regions of 169 strains were sequenced and analyzed along with sequences retrieved from the GenBank. The complete genome sequence analysis suggested complex recombination history for the Finnish E-6 strain. In VP1 region, the phylogenetic analysis suggested three major clusters that were further divided to several subclusters. The evolution of VP1 coding region was dominated by negative selection suggesting that the phylogeny of E-6 VP1 gene is predominantly a result of synonymous substitutions (i.e. neutral genetic drift). The partial VP1 sequence analysis suggested wide geographical distribution for some E-6 lineages. In Finland, multiple different E-6 lineages have circulated at the same time.

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## 1. Introduction

Enteroviruses (genus *Enterovirus*, family *Picornaviridae*) are small non-enveloped positive stranded RNA viruses. Seven of the species in genus *Enterovirus*, *Human enterovirus A* to *D* (HEV-A, -B, -C and -D) and *Human rhinovirus A* to *C* (HRV-A, HRV-B and HRV-C), cause infections in humans. Enterovirus genome consists of a single open reading frame (ORF) that is flanked by 5′ and 3′ untranslated (or non-coding) regions (5′UTR and 3′UTR). The ORF is translated to a single polypeptide that is cleaved to P1, P2 and P3 peptides by autocatalytic mechanism. P1 is further cleaved to four capsid proteins VP1 to VP4, whereas P2 and P3 are cleaved to so-called non-structural proteins 2A to 2C and 3A to 3D, respectively. Each of the enterovirus species are further classified to serotypes (or types) by their sequence similarities. According to current typing system, the virus strains that share >75% nucleotide and >85% amino acid similarities in VP1 coding region are classified into same type.

Echovirus 6 (E-6), a member of HEV-B species, is among the most commonly detected enteroviruses worldwide (Belguith et al., 2007; Centers for Disease Control and Prevention (CDC), 2010; Khetsuriani et al., 2006; Trallero et al., 2010; Tryfonos et al., 2011; Blomqvist et al., 2008; Antona et al., 2007; Sedmak et al., 2003; Richter et al., 2011; Baek et al., 2011; Fares et al., 2011; Khetsuriani et al., 2010). It has been associated with aseptic meningitis outbreaks (Tryfonos et al., 2011; Cabrerizo et al., 2008; Chomel et al., 2003; Gharbi et al., 2006; Joo et al., 2005; Kim et al., 2012a; Lee et al., 2010; Mao et al., 2010; Richter et al., 2006; Thoelen et al., 2003; Papa et al., 2009; Ashwell et al., 1996; Dos Santos et al., 2006), and occasionally with encephalitis, exanthema and gastrointestinal illness cases (Lee et al., 2010; Santos et al., 2008; Abe et al., 2000). In neonates E-6 may induce severe diseases such as pneumonitis, hepatitis, type 1 diabetes and may even be fatal (Yen et al., 2003; Ventura et al., 2001; Blokziji and Koskiniemi, 1989; Boyd et al., 1987; Otonkoski et al., 2000). E-6 has also frequently been found from upper respiratory tract samples (Trallero et al., 2010; Jacques et al., 2008) and co-circulation of entero/rhinoviruses with influenza viruses has been detected during influenza pandemics and epidemics (Chang et al., 2012; Navarro-Mari et al., 2012; Kim et al., 2012b; Yang et al., 2012).

Although E-6 has been among the five most commonly detected enterovirus types in Finland (Blomqvist et al., 2008), detailed

*Abbreviations:* E-, echovirus; EV, enterovirus; CSF, cerebrospinal fluid; GTR, general time reversible model of substitution; HEV, Human enterovirus; HKY, Hasegawa-Kishino-Yano model of substitution; HRV, Human rhinovirus; MCMC, Monte Carlo Markov Chain; ML, maximum likelihood method; MRCA, most recent common ancestor; NJ, Neighbour-joining method; NPA, nasopharyngeal aspirate; ORF, open reading frame; TN93, Tamura-Nei model of substitution; UTR, untranslated region.

* Corresponding author. Tel.: +358 29 524 8891.
*E-mail address:* Teemu.Smura@thl.fi (T. Smura).

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molecular epidemiological and evolutionary analysis of Finnish or other Northern European E-6 strains has not been conducted previously. Here we studied the molecular epidemiology of E-6 in Finland by sequencing partial VP1 coding region of 169 strains isolated during enterovirus surveillance. Complete genome was sequenced for one strain. These E-6 strains were analyzed along with E-6 sequences available in GenBank to infer the evolutionary patterns of this enterovirus type.

## 2. Material and methods

### 2.1. Viruses

Finnish E-6 strains were isolated from sewage samples collected during environmental surveillance for polioviruses using a two-phase concentration method, as described previously (Hovi et al., 2005). Human rhabdomyosarcoma (RD), human colorectal adenocarcinoma (CaCo-2), human cervical carcinoma (HeLa), human lung carcinoma A549 (ATCC CCL 185) and green monkey kidney (GMK) cell lines were used for virus isolation. In addition, clinical E-6 isolates sent to the national enterovirus reference laboratory (National Institute for Health and Welfare, THL) from other laboratories were included in the study (Blomqvist et al., 2008). The strains isolated are listed in Supplementary Table 1.

### 2.2. Partial VP1 RT-PCR and sequencing

Viral RNA was extracted from infected cell cultures with RNeasy Total RNA kit (Qiagen, Hilden, Germany) or E.Z.N.A. Total RNA Kit Omega (Bio-Tek Inc., Doraville, GA, USA) according to the manufacturer's instructions. RT-PCR was carried out as described by Oberste et al., (2003b) using primers 292 and 222. PCR amplicons were purified with the QIAquick gel extraction kit (Qiagen). Sequencing reactions with BigDye Terminator cycle sequencing ready reaction kit v3.1 (Life Technologies, Carlsbad, CA, USA) and sequencing with ABI3730 Automatic DNA Sequencer (Life Technologies) were performed by Institute for Molecular Medicine Finland (FIMM) Sequencing Laboratory. The electropherograms were analysed using Geneious Pro 5.6 software (Biomatters Ltd, Auckland, New Zealand, http://www.geneious.com).

### 2.3. Isolation and complete genome sequencing of strain E-6-FIN-09-NPA

Nasopharyngeal aspirate (NPA) sample was collected at the Helsinki University Central Hospital, Finland, in 2009 from a hospitalized patient suffering from influenza-like illness. Influenza virus A(H1N1)pdm09 infection was confirmed by RT-PCR assay at Helsinki University Hospital Laboratory Services (HUSLAB), Helsinki, Finland (Ronkko et al., 2011). Viruses from the NPA sample were propagated in A549 cells as described previously (Denisova et al., 2012). Viruses were collected from the culture medium by centrifugation at 150,000*g* for 4 h at 4 °C. RNA was isolated using RNeasy Plus Mini Kit (Qiagen). Extracted RNA was screened by visual inspection in a 0.75% agarose gel. It appeared that the RNA had a size of 7500 nt. Knowing that, during the 2009 influenza pandemic season, the most common co-infections were influenza A and respiratory syncytial virus B (genome size approximately 15000 nt), and influenza A and enterovirus/rhinovirus (genome size approx. 8000 nt) (Peci et al., 2012), it was concluded that the isolated RNA could belong to *Enterovirus*-genus (comprising enteroviruses and rhinoviruses).

DNase-treated (NucleoSpin TriPrep, Macherey-Nagel, Düren, Germany) RNA was reverse transcribed to cDNA using SuperScript II reverse transcriptase (Life Technologies) and random hexamers (New England BioLabs, Ipswich, MA, USA). Illumina-compatible Nextera technology (Epicentre Biotechnologies, Madison, WI, USA) was used for RNA-sequence library preparation. In this technology, DNA fragmentation and tagging was performed by *in vitro* cut-and-paste transposition with a few modifications. Briefly, HMW Buffer from Nextera DNA Sample Prep Kit, 100 ng ds-cDNA and Nextera Enzyme Mix diluted 1:10 were used. The tagged cDNA was purified with RNeasy MinElute Cleanup Kit (Qiagen).

In order to add the Illumina–specific bridge PCR (bPCR)-compatible sites, limited-cycle PCR (9 cycles) was performed. In short, for a bar coded library, 50X Nextera Adaptor 2 was replaced with a bar coded Illumina-compatible Adaptor 2 from the Nextera Bar Codes kit (Illumina-compatible) in PCR setup. Agencourt AMPure XP system (Beckman Coulter, Indianapolis, IN, USA) was used for purification of the PCR-products and the quality of the library was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The library was used as an input material for bPCR and cluster generation. Nexter Read Primers 1 and 2 as well as Nextera Index Read Primer from the Nextera DNA Sample Prep Kit were used for paired-end sequencing and index read sequencing, respectively. The sequencing was performed on Illumina HiSeq2000 platform using standard Illumina protocol.

The resulting reads were aligned against human genome to filter human sequences out, re-aligned against several enterovirus/rhinovirusreference genomes and assembled against Echo6/Henan/116/2008 (HM185055) using pipeline described in (Sulonen et al., 2011; McKenna et al., 2010). The variants were called with samtools mpileup and indel including dindel and pindel (Albers et al., 2011). Sequences were aligned with blastn/blastp and SNPs were identified.

### 2.4. Dataset collection

Three parallel datasets were constructed from the sequences. The first dataset contained the complete genome of E-6 strain FIN09-NPA characterized here, the complete E-6 genomes derived from the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) as well as the complete genome sequences of other human enteroviruses that showed substantial (over 85%) similarity with E-6-FIN09-NPA in BLAST search (search 18.4.2012) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The second dataset contained all complete E-6 VP1 sequences found from the GenBank (search 18.4.2012), and the third dataset contained partial VP1 sequences characterized here and the overlapping sequences of the complete VP1 dataset.

### 2.5. Sequence analysis

The sequences were aligned using ClustalW algorithm (for proteins) implemented in MEGA version 5.05 (Tamura et al., 2011) followed by manual correction.

Phylogenetic trees were constructed using neighbor-joining (NJ) and maximum-likelihood (ML) methods implemented in MEGA version 5.05 (Tamura et al., 2011) and Bayesian Monte Carlo Markov Chain (MCMC) method implemented in BEAST version 1.7.4 (Drummond et al., 2012). For NJ and ML trees, bootstrap resampling with 1000 replicates was conducted. Various substitution models including Tamura-Nei (TN93) (Tamura and Nei, 1993), general time reversible (GTR) (Tavaré, 1986) and maximum composite likelihood models (Tamura et al., 2004) were utilized.

The SimPlot 3.5.1 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 was used. For the bootscanning analysis (Salminen et al., 1995) a 500-nt window moved in 20-nt steps and NJ-algorithm run with 100 pseudoreplicates were used. In addition, GARD method (Kosakovsky Pond et al.,

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