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# Characterization of echovirus 25 (ECV 25) in the VP1/2A gene junction region

Brief Report

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**Summary.** Genetic relationships among echovirus type 25 (ECV 25) isolates associated with aseptic meningitis in Germany 1997/98 and a 40-year-old ECV 25 prototype strain were investigated using RT-PCR and direct sequencing the VP1/2A gene junction region. Sequences were compared to each other and to nonpolio enterovirus representatives (phylogenetic analysis). The analysis indicated that the sequences of recent isolates have drifted over time distinctly away from the prototype strain sequence. Genetic drift may change biological features of isolates possibly leading to new antigenic variants.

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Echoviruses are small, single-stranded RNA viruses. They are still the largest subgroup of the enterovirus genus of *Picornaviridae* family with lately 28 serotypes [3, 5]. Clinical manifestations are ranging from mild nonspecific illness with fever or gastroenteritis to severe meningitis, encephalitis and myocarditis [4]. Echovirus 25 (ECV 25) causes a widespread maculopapular rash, respiratory tract illness, diarrhea or meningitis/encephalitis.

We investigated cases of severe neonatal disease caused by ECV 25 occurring in Germany in 1997/98. We have developed an RT-nested PCR assay in a variable genome region to detect ECV 25 genomes. Nucleotide sequencing of PCR amplicons was used to identify ECV 25 isolates and to analyse their genomic variation by a phylogenetic tree.

Virus was isolated from stool or cerebrospinal fluid (CSF) by conventional cell culture methods as previously described [1]. ECV 25 isolates investigated in this study were supplied by three different medical centers as infected cell cultures.

In total, five clinical isolates recovered in Germany and an ECV 25 prototype strain were characterised. The ECV 25 prototype strain was isolated in USA

in 1957. The present isolates were as follows: 792/Frankfurt,M./97, 1362/Potsdam/98, 1368/Potsdam/98, 1369/Potsdam/98, and 2529/Stuttgart/98.

Cultures with an enterovirus cytopathic effect (CPE) were typed by microneutralization assay using equine antiserum pools supplied by the European Reference Center for Poliomyelitis, RIVM, Bilthoven, The Netherlands [6, 9].

The procedure for extraction of total RNA from supernatant of infected cell cultures was accomplished using QIAamp Viral RNA Kit according to manufacturers instruction (QIAGEN GmbH, Hilden, Germany). RNA was extracted from 140 μl supernatant of infected cell cultures.

Alignment studies of available echovirus sequences (GenBank) revealed some relatively conserved sequence segments at the VP1/2A gene junctions of these viruses. These small areas of nucleotide conservation were tested for use as primers for RT-PCR in the VP1/2A gene junction region of ECV 25. Nucleotide sequences of suitable primers and their location in the ECV 25 genome are shown in Table 1.

cDNA synthesis was performed with the EC1 primer and MMLV reverse transcriptase at 42 °C for 1 h. The first-round PCR amplified a segment of the VP1/2A genome region with a size of 444 bp and was performed with primer pair EC1 and EC2. Nested PCR amplification with primer pair EC3 and EC4 resulted in a 362 bp product. The amplifications were carried out in 35 cycles consisting of 30 sec at 94 °C, 30 sec at 42 °C, and 45 sec at 72 °C.

We sequenced the nested PCR products directly, using a dye terminator cycle sequencing kit (Perkin Elmer) and ABI 373A DNA sequencer (Applied Biosystems). The primers used were the same as in the PCR.

All sequence data estimated in this work are available in GenBank database. The accession numbers are as follows:

AF107280 (792/Frankfurt,M./97), AF107281 (1362/Potsdam/98), AF107282 (1368/Potsdam/98), AF107283 (1369/Potsdam/98), AF107284 (2529/Stuttgart/98), and AF107285 (ECV25 prototype).

For analysis, sequence data were truncated at genome positions 3095–3414. The truncated 320 bp segments covering the C-terminal region of capsid protein VP1 gene (240 nt) and the N-terminal region of 2A proteinase gene (80 nt) were shown as a comparative alignment in Fig. 1. The alignment of corresponding deduced amino acids is given in Fig. 2.

**Table 1.** Primers used in RT-PCR and sequencing

| Primer | Sequence | Polarity | Location[a] (5′-3′) |
|---|---|---|---|
| EC1 | 5′ CCGTGGGCTGTGGTGGTGCT 3′ | antisense | 3480–3461 |
| EC2 | 5′ TAGCTTTTATGATGGATGGTC 3′ | sense | 3037–3057 |
| EC3 | 5′ TCTCTGTTGTAGTCCTCCCA 3′ | antisense | 3450–3431 |
| EC4 | 5′ AACACTTTAAACAACATGGG 3′ | sense | 3089–3108 |

[a]Position numbers refer to ECV 11 (GenBank accession number X80059)

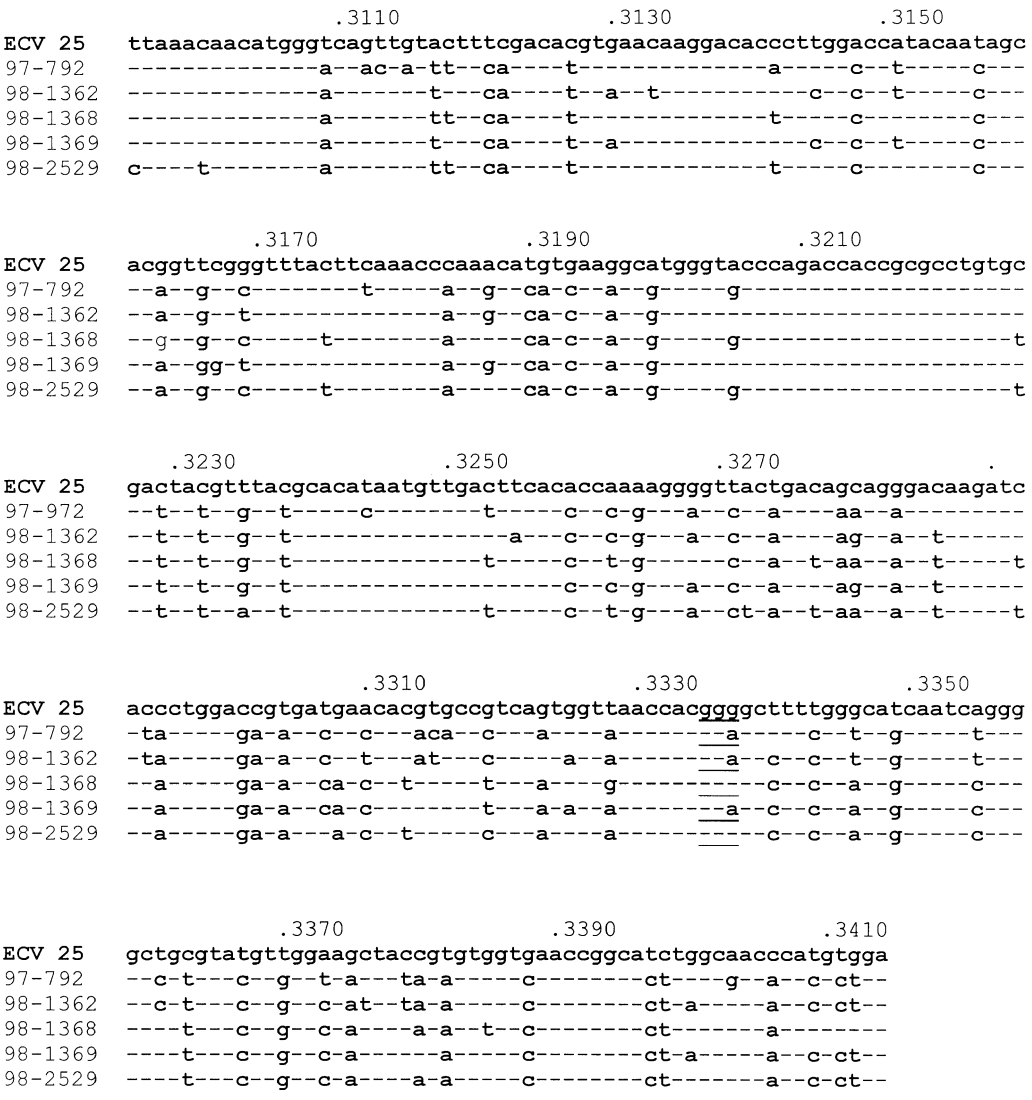


**Fig. 1.** C-terminal part of the VP1 (pos. 3095–3334, 240 nt) and N-terminal part (pos. 3335–3414, 80 nt) of 2A genome region. Position numbering according to ECV 11 (GenBank accession X80059). The first codon of the 2A gene in position 3335 is underlined. Dashes indicate presence of identic nucleotides in both ECV 25 prototype strain and isolates

Nucleotide and amino acid differences obtained by pairwise comparison between sequences of ECV 25 prototype strain, recent isolates and ECV 11 (GenBank, accession X80059) as an arbitrarily chosen representative of nonpolio enteroviruses are shown in Table 2.

Nucleotide differences of 3–12% found between isolates were sufficient to discriminate individual isolates. Deduced amino acid sequences of the isolates showed only 1–6% differences because of up to 84% silent mutations. Distinctly more nucleotide sequence differences (21–23%) were found between isolates and the ECV 25 prototype strain. The corresponding amino acid sequences showed a

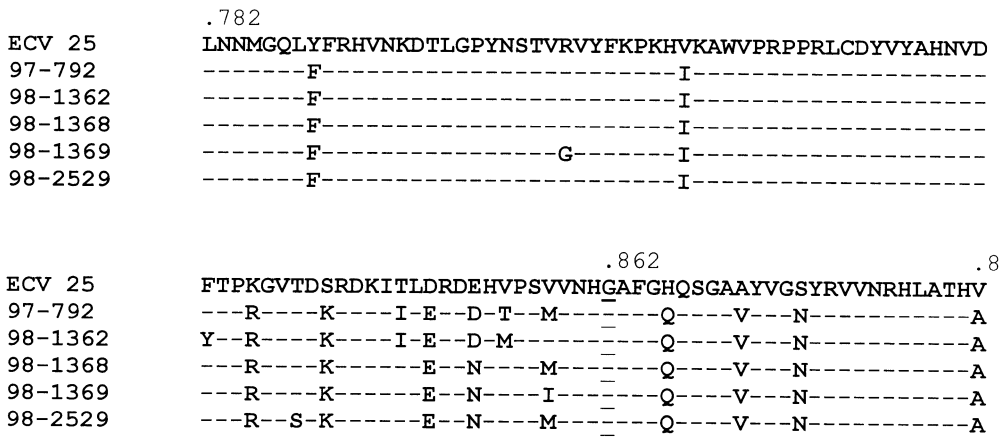


**Fig. 2.** Amino acid sequence similarities in a part of the VP1/2A genome region (Pos. 782–887, 106 aa). Position numbering according to ECV 11 (GenBank accession X80059). The first amino acid of the 2A gene in position 862 is underlined. A dash indicates the presence of the same amino acid in both isolate and ECV 25 prototype strain

diversity range from 10–12% in account of silent mutations up to 75%. Similar levels of intratypic divergence (up to 25% nucleotide sequence difference or 12% amino acid sequence difference) were observed lately by Oberste et al. [7], too.

However, sequence comparisons revealed clearly greater interserotypic differences, averageing 37% for nucleotide as well as amino acid levels, between the serotype ECV 11 and both ECV 25 isolates and the ECV 25 prototype strain.

For phylogenetic analysis, the deduced amino acid sequence data were aligned using the CLUSTAL W68 K program [8]. The alignment was visually verified and then submitted to phylogenetic analysis. The analysis was performed using the programs PROTDIST and NEIGHBOR included in the PHYLIP (Phylogeny Inference Package) Version 3.57c [2]. To check the reliability of the branches

**Table 2.** Nucleotide (nt) and deduced amino acid (aa) differences obtained by pairwise comparison of a 320 nt (106 aa) segment (pos. 3095–3414) of the VP1/2A junction region in the ECV 25 genome

| Isolates | ECV 11 | ECV 25[a] | 792/97 | 1362/98 | 1368/98 | 1369/98 | 2529/98 |
|---|---|---|---|---|---|---|---|
| | | | | nucleotide differences [nt] | | | |
| ECV 11 | – | 115 | 113 | 113 | 125 | 120 | 125 |
| ECV 25[a] | 39 | – | 75 | 71 | 66 | 67 | 70 |
| 792/97 | 38 | 13 | – | 26 | 34 | 30 | 33 |
| 1362/98 | 39 | 13 | 3 | – | 37 | 14 | 36 |
| 1368/98 | 38 | 11 | 3 | 5 | – | 25 | 11 |
| 1369/98 | 39 | 12 | 5 | 6 | 2 | – | 26 |
| 2529/98 | 39 | 12 | 4 | 6 | 1 | 3 | – |
| | | | | amino acid differences [aa] | | | |

[a]ECV 25 prototype strain

ECV 11 strain was included for comparison

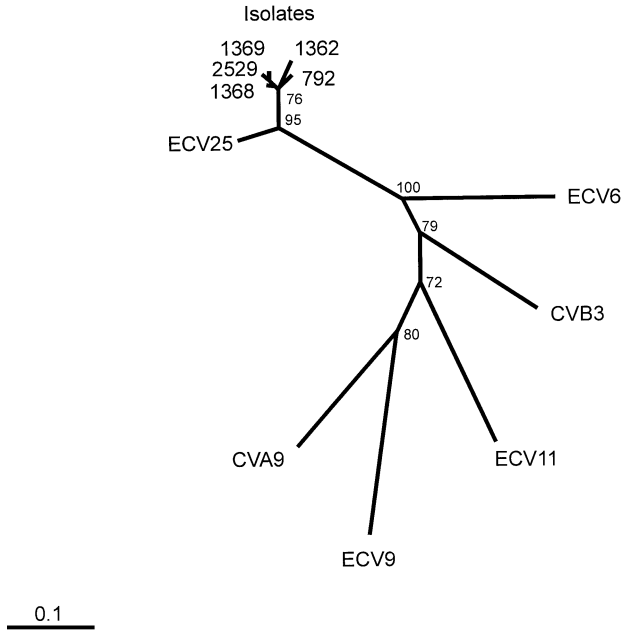


**Fig. 3.** Genetic relationships among ECV 25 prototype strain, five actual isolates and five other nonpolio enteroviruses (ECV 6, ECV 9, ECV 11, CVB3, and CVA9) for comparison. The tree based on amino acid similarity of a 106-amino-acid-sequence in the junction of VP1 and 2A genes and is an unrooted tree. Statistical reliability for branching points obtained by bootstrapping (100 replicates) are given in percent by numbers at the branches. The line in the left-hand bottom represents a scale for the tree in terms of proportion of amino acids substituted per site

defined by the tree, we used the programs SEQBOOT (with 100 replicates) and CONSENSE also included in the PHYLIP package.

Genetic relationships among recent isolates themselves, the ECV 25 prototype strain, and five other representatives of nonpolio enterovirus serotypes clustering phylogenetically within one of the four major human enterovirus groups [7] were shown as such a phylogenetic tree in Fig. 3. The sequences of ECV 6, ECV 9, ECV 11, coxsackievirus B3 (CVB3), and coxsackievirus A9 (CVA9) were obtained from GenBank under accession numbers U16283, X92886, X80059, M33854, and D00627.

As can be seen in the phylogenetic tree, the five isolates could be grouped into an ECV 25 cluster distinctly distant from the prototype strain but far away from the other enterovirus serotypes. In view of the present results it will be interesting to investigate whether alterations at antigenic sites may have also occurred during the recent evolution of ECV 25.

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