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Short Communication

Molecular characterization of an Enterovirus 71 causing neurological disease in Germany

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Enterovirus 71 (EV71) is mainly known as a cause of hand-foot-and-mouth disease (HFMD) but sometimes associated with neurological disease, even as fatal brainstem encephalitis. In Europe, EV71 infections are extremely rare, in contrast to the worldwide situation. This is the first report of molecular characterization of an EV71 strain isolated in Europe that had caused neurological disease. The german strain is closest related to sublineage B2 strains isolated in the United States, which where mainly associated with neurological disease. Phylogenetic analysis also showed that the strain must have been imported to Germany several years ago, and continues to circulate since then. *Journal of NeuroVirology* (2003) **9,** 126–128.

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Enterovirus 71 (EV71) is the most recently described serotype of the genus *Enterovirus* (family Picornaviridae). Infections with this virus induce a variety of clinical manifestations and the disease spectrum ranges from febrile disease, hand-foot-and-mouth disease (HFMD), and herpangina to major complications involving the central nervous system (CNS), including aseptic meningitis, poliomyelitis-like paralysis, brainstem encephalitis, and even death (Landry *et al*, 1995).

Recently, several large and severe EV71 outbreaks have occurred periodically throughout the world, mainly in the Asia-Pacific region (Chan *et al*, 2000; Ho *et al*, 1999; McMinn *et al*, 2001), where in a small proportion of cases rapid clinical deterioration and death occurred due to severe neurological disease as described for the outbreaks in Malaysia in 1997 (Chan *et al*, 2000) and in Taiwan in 1998 (Ho *et al*, 1999).

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The association of severe neurological disease, including death, with recent large outbreaks emphasizes the need to understand the pathogenesis and epidemiology of EV71. Several studies of EV71 evolution on isolated strains (McMinn *et al*, 2001; Brown *et al*, 1999) demonstrated the development of three independent genetic lineages (A, B, and C) and several sublineages within these three genogroups (B1–B4, C1, and C2), elucidating close genetic relations between strains isolated at the same time points and geographic regions (McMinn *et al*, 2001; Brown *et al*, 1999).

In Europe, there were two large epidemics of EV71 in Bulgaria and Hungary in the years 1975 and 1978, with 91 fatal cases. Since then, EV71 has been isolated only very rarely. So far there have been no reports on the molecular characterization of EV71 strains from Germany or other European countries that could provide information about the epidemiology of EV71 infections in Europe.

In August 1998, two cases of meningitis occurred in a village in southern Germany. The patients showed on admission at the local hospital the typical neck stiffness accompainied by nausea, fever, vomiting, and diarrhea. Furthermore, one patient (patient 1) had a disturbed conciousness (drowsiness and somnolence) during the initial 2 days of disease. Lumbar puncture revealed the presence of pleocytosis (patient 1: 800 cells/μl and patient 2: 130 cells/μl;

mainly neutrophils). Other values were within normal range. No electroencephalograph (EEG) or magnetic resonance imaging (MR) was performed. The patients course over the subsequent few days showed gradual improvement. One week after admission, the patients had recovered fully and were discharged. We obtained throat and cerebrospinal fluid (CSF) samples of the two 3-year-old girls, who had been in contact with one another, but had otherwise no contact with a person of a foreign country, on day 3 of admission. The samples were tested for presence of bacterial and viral pathogens. Throat and CSF samples of both girls were strongly positive for enterovirus RNA by reverse transcriptase–polymerase chain reaction (RT-PCR) following the method of Chapman *et al* (1990). Additionally, virus isolation was performed using primary green monkey cells (PMK). The enteroviral cytopathic effect (CPE) was confirmed by immunofluorescence antibody staining (IFA) and the virus was typed by an EV71-specific antiserum in the neutralization test. We decided to sequence the complete VP1 gene, encoding the most abundant surface protein, and to perform a phylogenetic analysis by comparing our data to VP1 sequences of strains isolated worldwide so far, to gain insight into the genetic relationships and thus the molecular epidemiology of the isolated strain.

Viral RNA was isolated from CSF samples using the High Pure Viral RNA kit (ROCHE Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. RT-PCR was performed using primers 5′-GGG GAC AGA GTG GCA GAT GTG-3′ (forward primer) and 5′-CCC GAG CGT AGT GAT TGC C-3′ (reverse primer) under standard conditions. Alignment of the complete VP1 gene sequence (891 nucleotides) of the strain that was isolated in Germany (accession number AY079098) with strains isolated in the United States, Australia, Singapore, Malaysia, Canada, Bulgaria, and Hungary (sequences from GenBank) was performed with the Clustal W algorithm (Thompson *et al*, 1994). Phylogenetic trees were constructed by neighbor-joining using the Saitou and Nei sequence distance method (Saitou *et al*, 1987). Previously published VP1 sequences were used to reconstruct the three EV71 genogroups (A, B1–4, C1/2) identified by Brown *et al* (1999) and McMinn *et al* (2001).

Sequence alignment showed that the German strain is genetically closest related to the strain 2219-IA-87 (AF009539) and to several other neurotropic strains that all belong to sublineage B2 (figure), and that all are closely related with each other, although isolated in widely separated regions of the United States (Iowa, Connecticut, Pennsylvania, Mississippi, Alaska, and Washington). The nucleotide identity to B2 strains was 94%, compared to 89% to 93% for B1, B3, and B4 strains, and 83% to 84% to C1 or C2 strains. All the strains within the B2 sublineage, closest related to the German strain,

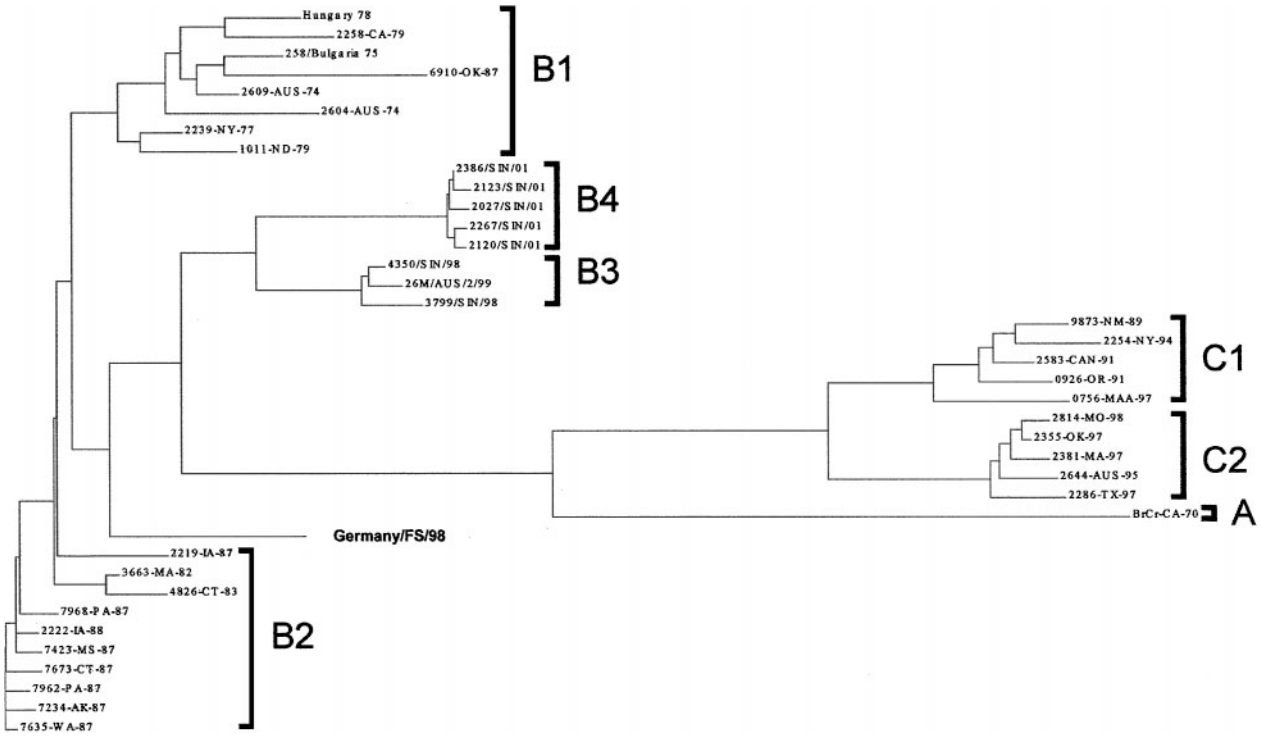

Figure 1 Phylogenetic tree showing the genetic relationship between the strain isolated in Germany and strains isolated worldwide based on the complete VP1 gene sequence. Most strain names indicate a unique number/country or U.S. state of isolation/year of isolation. Dendrogram shows genogroups A, B1–B4, and C1, C2. The strain isolated in this study is highlighted.

and for which information about the outcome of disease were available produced neurological disease (meningitis: 2219-IA-87 and 7635-WA-87; paralysis: 7423-MS-87, 7962-PA-87, and 7234-AK-87), except strain 2222-IA-88 (fever). Interestingly, all the above mentioned strains within cluster B2 were isolated between 1982 and 1988 in the United States, most of them during the nationwide outbreak of EV71 in 1987 (Brown *et al*, 1999).

No strains belonging to genetic sublineage B2 have been isolated in the United States since 1988 (Brown *et al*, 1999). The fact that the strain isolated in Germany is closely related to sublineage B2 strains isolated between 1982 and 1988, and also that it produced neurological disease, makes it very possible that it had been introduced to Germany from the United States during the 1980s and has continued to circulate at least in some part of Germany since then. The fact that the German strain is not a typical representative of the B2 sublineage but seems to be to some extent distinct (Figure 1) strengthens this hypothesis, because it was isolated at least 10 years after the isolation of other B2 strains, and the calculated evolutionary rate for B lineage strains lies between $1.6 \approx 10^{\approx 2}$ at the third position and $4.2 \approx 10^{\approx 3}$ for all codon positions, per nucleotide and year (Brown *et al*, 1999).

During 1998, we identified a total of 74 positive enterovirus cases from patients with neurological disease (54 patients with meningitis, 18 with encephalitis, and 2 patients with paresis) by means of RT-PCR and virus isolation in cell culture. From these and hundreds more cases identified until the present, however, only the two cases described herein were caused by EV71. Additional surveillance is required to ascertain whether other strains possibly circulating in Germany or other European countries belong to the same genetic cluster as the strain described herein, which would suggest that EV71 was introduced just once into Germany, or that EV71 was introduced several times.

This is the first report of molecular characterization of an EV71 from Europe, in particular Germany. The strain isolated had caused at least two identified cases of neurological disease. Although the cases of meningitis did not cause irreversible damage of the CNS, EV71 outbreaks in Europe should not be taken lightly. The symptoms produced by single neurogenic strains can differ from person to person, from aseptic meningitis to even death as seen during the Taiwan outbreak in 1998 (Ho *et al*, 1999). Increased surveillance, together with improved specific laboratory diagnosis of EV71 infection, will enable public health authorities in Germany and other European countries to rapidly recognize an outbreak of EV71 disease and thereby implement measures to limit further virus transmission.

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