Diagnosis and Epidemiology of Echovirus 22 Infections

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To find out whether the appearance and pathogenicity of infections caused by echovirus 22 (EV22)—which has considerably different molecular characteristics from those of typical enteroviruses—exhibit extraordinary features, novel diagnostic approaches were applied. Staining of inoculated cell cultures with specific antibodies was followed by immunoperoxidase detection. Of fecal specimens from 140 children under 6 years of age, four were positive by the immunoperoxidase method. The nucleotide identity between these samples and additional EV22 isolates from Finland and the United States varied from 78% to 93% when studied by specific polymerase chain reaction, followed by sequence analysis of the amplicons. One-hundred ten single serum specimens collected from individuals in different age groups to find out the prevalence of EV22 antibodies were subjected to neutralization tests. Out of 21 neonates, 20 (95%) had EV22 antibodies, probably of maternal origin. Of 10 children aged 2–12 months, only two had neutralizing antibodies to EV22. The antibody levels increased rapidly by age, and among 30 adults tested, 29 (97%) were seropositive. A clear difference was observed with the occurrence of echovirus 30 (EV30) antibodies, which were less prevalent and appeared later in life. A review of previously published studies concerning the epidemiology of EV22 infections showed that gastroenteritis and respiratory infections are the most common symptoms observed in EV22 infections.

Echoviruses belong to the family of Picornaviridae, which is divided into five genera (aphthoviruses, cardioviruses, en-teroviruses, hepatoviruses, and rhinoviruses) and includes a number of important human and animal pathogens. Human enteroviruses are further subgrouped as polioviruses, type A coxsackieviruses (CAVs), type B coxsackieviruses (CBVs), echoviruses (EVs), and enteroviruses 68–71. EVs consist of ~30 serotypes and they have been associated with a wide range of clinical manifestations ranging from severe meningitis, encephalitis, and myocarditis to mild gastroenteritis and respiratory illnesses.

Recent studies on biological and molecular characteristics of echovirus 22 (EV22) suggest that this virus is genetically distant from other enteroviruses and that its protein processing appears to be unique among picornaviruses [1–4]. EV23 has been shown to share remarkable identity with EV22, and it also belongs to this previously unrecognized picornavirus group.

Because of the extensive differences in molecular and biological characteristics between EV22 and the typical enteroviruses, we were interested to know whether the epidemiology and disease associations of EV22 infections would have extraordinary features.

To improve diagnostic methods, we first used new approaches for identification of the virus, in addition to standard virus isolation. These included specific immunologic detection of infected cells after virus inoculation, as well as PCR followed by sequence analysis of the amplicons. Moreover, the prevalence of EV22 neutralizing antibodies in different age groups was determined.

Materials and Methods

Viruses and cells. EV11 (Gregory), EV22 (Harris), EV23 (Williamson), and EV30 (Bastianni) prototype strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Virus isolation, immunoperoxidase staining, and neutralization tests were carried out in the A549 (human lung carcinoma) cell line (ATCC).

Antibodies. EV22 antibodies used in immunoperoxidase staining were obtained by immunizing rabbits with purified virus, while EV11 antiserum was prepared by immunizing the animals with heat-treated virus in order to obtain an antiserum with wide reactivity against different enteroviruses. The latter antiserum recognizes all the prototypes of poliovirus serotypes, CBVs, CAV serotypes 18 and 21, and all the EV serotypes except 15, 22, and 23 (Waris et al., unpublished data).

Clinical specimens. The stool samples consisted of specimens sent to the Department of Virology at the University of Turku (Turku, Finland) for routine virus isolation and antigen detection during a 13-month period (from January 1991 to February 1992; 578 samples). A549 cells, susceptible to infection with the EV22 reference strain, were used for the study. Altogether, 140 specimens from children aged 2 days to 5.5
years were selected for this study. All specimens had been tested by rotavirus and adenovirus antigen detection and by virus isolation. Specimens from 25 patients were positive for rotavirus and five for adenovirus antigen. Viruses isolated were 2 adenoviruses, 4 CBV3, 1 CBV5, 2 EV30, 1 EV31, and 1 respiratory syncytial virus. The specimens had been stored at −70°C prior to testing.

Ten additional EV22 isolates for sequencing were obtained from Dr. Carl-Henrik von Bonsdorff (Department of Virology, Haartman Institute, University of Helsinki, Finland) and Dr. Mark Pallansch (Centers for Disease Control and Prevention, Atlanta). The five isolates from Finland had been recovered in Helsinki in 1991 (three samples), 1993, and 1995, while the isolates from the United States were recovered in Colorado (1973), Georgia (1979), Massachusetts (1982), Hawaii (1988), and Missouri (1992).

For the serological part of the study, 110 serum samples were obtained from individuals aged 1 day to 64 years. The samples from children were collected during a 1-month period during summer 1995 from the neonatal ward and from the children’s outpatient clinic at the Turku University Hospital. Twenty-one specimens were obtained from children aged 1–30 days, 19 were from those between 2 months and 2 years of age, and 20 were from those 2–5 years old. Ten samples each were collected from the age groups of 5–10 years and 10–15 years. Serum specimens from adults were obtained from Finnish Red Cross blood donors in Turku. Ten samples each were collected from the age groups of 18–25, 25–50, and >50 years. The sera were stored at −20°C until analyzed.

Virus isolation. For virus isolation, A549 cells were cultured in Hams F12 medium containing 10% fetal calf serum and antibiotics. Two hundred μL of specimen (suspended in PBS) was added to each tube, containing 1 mL of maintenance medium and ~0.5 × 10⁶ cells. The cultures were incubated in a 5% CO₂ atmosphere at 37°C, and the maintenance medium was changed weekly. Examinations for development of CPE were done three times a week during a 2-week period. Cultures exhibiting CPE were used for reinoculation, and confirmed positive isolates were used in further assays.

Immunoperoxidase staining. The method described by Ziegler et al. [5] was slightly modified. A549 cells were grown in 24-well cell-cluster dishes (Costar, Cambridge, MA). Isolates were added to duplicate wells, and EV22 standard strain (as a positive control) and a noninfected cell control were included. The plates were centrifuged at 35°C in a Technospin K centrifuge (Sorvall Instruments, DE) at 2,600 rpm for 45 minutes and then placed in a 5% CO₂ atmosphere at 37°C. After 48 hours’ incubation, the cells were washed twice with PBS-Tween (0.1% Tween-20) and fixed with methanol at 4°C for 15 minutes.

EV22 antibodies were diluted 1:800 in 5% milk powder (Valio, Finland) solution, while EV11 serum was used at a dilution of 1:100. Three hundred μL of each antiserum dilution was added to the wells, incubated at 37°C for 1 hour, and washed twice with 0.1% PBS-Tween, and then peroxidase-conjugated swine immunoglobulin (DAKO, Glostrup, Denmark), diluted 1:200 in 5% milk powder solution, was added. After incubation at 37°C for 1 hour, the wells were washed twice with PBS-Tween and rinsed once with PBS. Prior to use, 10 μL of H₂O₂ substrate was added to 10 mL of stain solution. After 30 minutes of incubation at room temperature, the results were scored by an inverted microscope at 40× magnification.

Reverse transcription and PCR. Virus RNA was isolated from the clinical samples with use of the Ultraspec RNA isolation system (Biotex Laboratories, Houston). The RNA pellet was dissolved in 30 μL of water and stored at −70°C until analyzed. The primers used for reverse transcription, PCR, and sequencing amplified a 237-nucleotide-long region from the 5’ untranslated region (5’UTR). The ev22+ (5’-CCCCACACAGCCATCCCT-3’) primer was complementary to the genomic RNA (2) at positions 312–328, while the ev22− (5’-TGCGGGTGACCTTCTTCGGG-3’) primer represents the complementary strand at positions 565–581. We also used primers common to typical enteroviruses, which amplify a 360-nucleotide-long sequence from the 5’UTR [6]. The 2+ (5’-CAAGCAGCCTCTGTTTTCGCCGG-3’) primer is of genomic polarity (positions 168–188), whereas the 4− (5’-GAAACACCGGACACCCAAAGTA-3’) primer is complementary to the sequence in positions 548–568 in the EV11 genome.

Synthesis of cDNA was performed in a reaction mixture containing 5 μL of specimen nucleic acid, 17.5 μL of water, 8 μL of 5× RT buffer (Promega Biotech, Madison, WI), 8 μL of dNTP mix (2.5 mM of dATP, dCTP, dGTP, and dTTP; Pharmacia, Uppsala, Sweden), 0.1 μL of ribonuclease inhibitor RNasin (40 U/μL; Promega), 1 μL of ev22− (73 pmol/μL) or 4− (58 pmol/μL) primer solution, and 0.1 μL of MMLV reverse transcriptase (200 U/μL; Promega). The reaction was carried out at 37°C for 1 hour.

For PCR, 5 μL of the cDNA reaction mixture was combined with 75 μL of water, 10 μL of 10× PCR buffer (Dynazyme, Finnzymes, Espoo, Finland), 8 μL of dNTP mix (2.5 mmol), 0.5 μL of Taq DNA polymerase (2 U/μL; Dynazyme) and 1 μL of ev22+ (73 pmol/μL) and ev22− (73 pmol/μL) or 2+ (62 pmol/μL) and 4-primer stocks. The mixture was amplified through 40 cycles of heating at 95°C for 2 minutes, primer annealing at 55°C for 2 minutes, and DNA synthesis at 70°C for 4 minutes. The PCR products were analyzed in 1% agarose gels containing ethidium bromide.

Sequence analysis. For sequencing, the PCR products were run in 1% agarose gels and the cDNA bands of expected size were excised. They were eluted with use of the QIAEX Gel extraction kit (Qiagen, Wilden, Germany) and suspended in 20 μL of TE. The nucleotide sequences of the cDNAs were determined by the dideoxynucleotide chain termination method in both directions with use of the Sequenase version 2.0 kit (Amersham, Cleveland). The purified amplicons were used directly as templates in the sequencing reactions; 9 μL of PCR-DNA was mixed with 1 μL of one of the primers used in the
PCR. To denature cDNA, the solution was incubated at 95°C for 5 minutes and quickly frozen in dry ice. The labeling mix (Sequenase) dilution used was 1:10.

The 35S-labeled reaction products were separated in 5% polyacrylamide gels in a 2010 Macrophor electrophoresis unit (LKB, Sweden). The results were visualized by autoradiography with Kodak film (Rochester, NY). Sequence data were entered into a VAX computer with use of the Genetics Computer Group (GCG) Seqed program [7], and the sequences were compared with previously published data by means of the Gap and PileUp programs (EMBL; GenBank).

Neutralization test. The test was carried out in 96-well cell-culture dishes (Costar). Paired serum specimens were tested at fourfold dilutions (1/4, 1/16, 1/64, 1/256, 1/1,024, and 1/4,096) in duplicate wells. EV22 dilution was added and the mixture was incubated at 37°C for 1 hour; then freshly trypsinized A549 cells were added (about 50,000 cells/well). The plates were incubated at 37°C in a 5% CO2 atmosphere, and CPEs were recorded after 4 days. Titers exceeding 1/10 were considered to be positive.

Results

Diagnostic procedures. For specific and rapid identification of EV22, an immunoperoxidase assay was developed. Rabbit antiserum produced by the use of purified EV22 as an antigen specifically detected EV22 and EV23 but not EV11 (figure 1). EV22 antiserum reactivity was also tested against three other enteroviruses (CAV16, CBV3, and poliovirus 1) and against viruses used in immunoperoxidase assays in routine diagnosis (adenovirus; herpes simplex viruses 1 and 2; influenza A and B viruses; parainfluenza viruses 1, 2, and 3; respiratory syncytial virus; and cytomegalovirus). EV22 antiserum did not show reactivity against any of these viruses. Broadly reactive rabbit antiserum produced against heat-treated EV11 (Waris et al., manuscript in preparation) did not show reactivity with EV22- or EV23-infected cells.

EV22 was detected in the stool samples of four of 140 patients by virus isolation followed by EV22-specific immunoperoxidase staining. One of the samples was positive with both EV22 and EV11 antiserum, suggesting coinfection. The EV22-positive patients were all under 2 years of age. Two of them had gastroenteritis diagnosed, one had acute respiratory infection, and one had pneumonia. All the positive specimens were from November and December 1991.

Samples from 21 patients were positive with use of the EV11 antiserum in immunoperoxidase staining, and 7 of them were also positive in rotavirus antigen detection; 4 specimens were enterovirus-positive by virus isolation. A predominant symptom in this group was diarrhea (seven patients). Other associated clinical symptoms were conjunctivitis in two and acute respiratory infection in another two children. Meningitis, tonsilitis, pneumonia, bronchitis, fever, and congenital malformation syndrome were diagnosed for other patients, and no diagnosis was available for five children.

EV22 was also detected in all four immunostain-positive samples by EV22-specific PCR. For PCR both EV22 and enterovirus primers were used; EV22 did not exhibit amplification with the enterovirus primers, and EV11 did not give a signal with the
from Helsinki and five EV22 isolates from different geographic locations in the United States were sequenced. The nucleotide sequence identity between the isolates and the EV22 reference strain varied from 78% to 93% (Figures 3 and 4). Two specimens from Turku, both isolated in 1991, and two specimens from Helsinki, isolated in 1993 and 1995, were completely identical with each other in this genome region. These two identical specimens isolated from Turku showed more homology with EV23 (91%) than with EV22 (85%).

However, when these two isolates were typed with neutralizing antiserum pools (WHO serum pools A–H), they were identified as EV22. An isolate from Colorado (1973) represented the same lineage as the prototype strain of EV22. Most of the EV22 isolates clustered in another lineage, including viruses from both Finland and the United States (from the period 1982–1991). An EV22 isolate from Georgia (1979) did not belong to any of these lineages but was more closely related to EV23 (84%) than to EV22 (78%) in this genome region.

EV22 and EV23 prototype strains and the sequences obtained from clinical specimens were also compared with previously published sequences of the representatives from other picornavirus groups: foot-and-mouth disease virus (FMDV, representing aphthoviruses), EV11 and poliovirus 1 (PV1) from the enterovirus genus, echoviruses, encephalomyocarditis virus (EMCV, 122 bases were sequenced from the 5′ UTR. In addition to our clinical material, five EV22-positive specimens
representative of cardioviruses), hepatitis A virus (HAV, a hepatovirus), and human rhinovirus 1B (HRV1B). In a dendrogram generated from these sequences, all the clinical isolates group in the same cluster with EV22 and EV23. In this genomic area, the nucleotide identity between EV22 and other compared picornaviruses varied from 34% with HAV to 55% with EMCV, while EV23 showed 60% identity with EMCV (figure 5).

**Epidemiology.** Twenty of 21 neonates (95%) had EV22 antibodies detected in the neutralization test (figure 6). These antibodies were evidently of maternal origin. Between 2 and 12 months of age, only two of 10 children had neutralizing antibodies. The titers of antibody to EV22 increased rapidly by age, and eight of nine children already had antibodies to EV22 at the age of 1–2 years. Among adults, 29 of 30 (97%) were seropositive. Seventy-two of the 79 individuals >1 year of age (91%) were EV22-seropositive.

For comparison, the prevalence of EV30 neutralizing antibodies in serum specimens was also determined. Among neonates, 8 of 21 (38%) had neutralizing antibodies against EV30, and from 2–12 months of age, 1 of 10 children was seropositive. Among children from 1 to 2 years old, no more than 1 of 9 had neutralizing antibodies to EV30. Of individuals >1 year old, 19 (24%) were seropositive, and among adults only 9 of 30 had neutralizing antibodies against EV30 (figure 6).

**Discussion**

EV22 and EV23 were first isolated in 1956, when Wigand and Sabin studied summer diarrhea in children [8]. Even in
their original characterization, growth properties different from those of typical enteroviruses were observed. More recently, sequence analysis has illuminated the molecular basis of the exceptional characteristics of EV22 and EV23. In molecular comparison of primary sequences, these two viruses differ from enteroviruses to the same extent as hepatitis A virus does in representing the hepatovirus genus of the picornavirus family [3]. Therefore, EV22 and EV23 are considered to be representatives of a distinct genus.

Because of the molecular differences from enteroviruses, EV22 and EV23 also require specific diagnostic techniques for their identification. Currently, these viruses are detected by virus isolation followed by neutralization typing with an antiserum panel including typical enterovirus as well as EV22 and EV23 antibodies. Since two cycles of cell culture are needed, the method is cumbersome and slow and cannot provide results during the acute phase of illness. Therefore, we accelerated the isolation procedure by immunoperoxidase staining [5] of the infected cells with EV22-specific antiserum, which makes early identification of the infected cells possible. As a method for routine diagnosis of EV22 infection, it is an easy and quick method and also sensitive, since a single stained cell indicates positivity.

Another technique used for identification of EV22 and EV23 was PCR, which has been shown in a number of reports to be a good alternative to enterovirus isolation [9–11]. The primers used in the enterovirus PCR allow detection of virtually all the serotypes but do not react with EV22 and EV23 because of the extensive sequence differences. We used primers from the conserved regions of the 5’UTR. All the samples that were positive by immunoperoxidase staining were also positive by PCR with the EV22 primers. In addition, EV23, but not EV11, was detected with these primers.

Among the four patients who were EV22-positive by immunoperoxidase staining and PCR, the clinical signs of diarrhea, pneumonia, and respiratory infection were typical for this virus. Somewhat surprisingly, a relatively high occurrence of diarrhea (seven of 21) was also noted among the enterovirus-positive patients.

The PCR primers were also used for sequence analysis of the amplicons. The 5’UTR is a highly conserved area in the genome of picornaviruses, and it seems to evolve more slowly than the other parts of the genome [2, 12]. When EV22 and EV23 prototype strains and the sequence obtained from the clinical isolates were compared with other picornaviruses in this region, they showed more relatedness with aphthoviruses (FMDV) and cardioviruses (EMCV) than with enteroviruses (EV11 and PV1). These results are in accordance with previously published data [2].

In our study three of the isolates exhibited more nucleotide identity with EV23 than with EV22 in the 5’UTR, but when typed with neutralizing antiserum pools, they were all identified as EV22. The explanation for this finding is that the present identification of the viruses is based on antigenic characteristics of the capsid proteins, and the serotypes do not directly correlate with the 5’UTR characteristics [12]. Furthermore, typing with polyclonal antisera does not have the sensitivity to distinguish between different strains of the same serotype, and if detailed information regarding epidemiology is required, sequence information for the virus can be successfully used.

A study of distribution of nonpolio enteroviruses in sewage and among clinical isolates was done in Finland in 1995, and according to this study EV22, together with CBV5, EV11, CAV9, CBV3, and EV30, was one of the six most common serotypes isolated [13]. This information suggests that EV22 infection might be more common than previously assumed. Our seroepidemiological data clearly show that EV22 infection is acquired early in childhood and is more common than EV30 infection, at least in Finland. EV30, frequently associated with aseptic meningitis [14, 15], was selected for this study to represent a typical member of the echoviruses.

All except one neonate (95%) had neutralizing antibodies to EV22, but only 38% of them were EV30-seropositive, suggesting low prevalence of antibodies. After 1 month of age, antibodies to both EV22 and EV30 rapidly disappeared, probably because the amount of maternal antibodies decreased. A rapid increase in titers of EV22 antibody was observed at ~1 year of age, and 91% of all individuals >1 year old had neutralizing antibodies against EV22, indicating that virtually everyone experiences the infection. EV30 seropositivity increased slowly, and there was no significant increase at any specific age.

In our study, clinical specimens were selected to match the situation in the general population as well as possible. Therefore, the samples from adults were obtained from blood donors. Since no such population of children was available, we used serum samples from the neonatal ward and from the children’s outpatient care unit. It is somewhat surprising that while EV22 infections seem to be common in early childhood on the basis of the seroepidemiological data, only four of 140 children excreted virus. This circumstance may be due to a short excretion
time, as in hepatitis A virus infections, or may reflect the epidemiological situation during the collection of the samples. Another explanation is that the age distribution of our patients (78 patients were <1 year old, 36 were 1–2 years old, and 26 were >2 years old) was not the most favorable.

EV22 infections have been reported from all around the world, and the data favor the idea that this virus infects mostly children. An extensive investigation of enterovirus infections, including those caused by EV22 and EV23, has been reported by Grist et al. [14], who summarized the data collected by the WHO Virus Unit during 1967–1974. These data show that 61% of the reported EV22 infections (altogether, 581 cases) involved children <1 year old and 97% involved children <15 years old. Among all EV infections, the figures were 17% and 79%, respectively.

In a retrospective study in Sweden, there were 109 EV22 isolations during the period from 1966 to 1990, and only two of the isolates were from adults. Of all the EV22-positive patients, 72% were <1 year of age [16]. A study carried out in 1970 in Japan showed that seroconversion for EV22 antibodies occurred in almost all children by shortly after 1 year of age [17].

In most of the studies the predominant symptom caused by EV22 has been diarrhea [14, 16–18], but respiratory infections are also common manifestations [14, 16, 19]. The first recorded cases were in three outbreaks of respiratory disease at a neonatal department in New York in 1964 and 1965 [19]. Altogether, 64 children fell ill, and 18 had a significant increase in titers of neutralizing antibody to EV22 or had the virus isolated from fecal specimens. Seven children developed pneumonia, and in 11 patients the infection was restricted to the upper airways.

In a prospective study in Sweden, the majority of EV22-infected children had diarrhea, which was nosocomial for 50%. Respiratory symptoms were evident in 14 patients, and the most common clinical finding was wheezing. EV22 was isolated from five patients who were somnolent and admitted on the suspicion of encephalitis. EV22 infection was also suspected to be associated with myocarditis in one patient, and for three children lymphadenopathy was the reason for sampling. These children were all febrile, and in two cases recent Epstein-Barr infection was demonstrated at the same time EV22 infection was diagnosed. EV22 was also isolated from two asymptomatic children [16].

In the WHO data, 29% of the 581 reported cases of EV22 infection involved patients with gastrointestinal symptoms, while 26% involved respiratory infection [14]. The incidence of gastroenteritis in all EV infections was 9%, and respiratory infection was detected in 13%. Diseases associated with the CNS were found in 12% of the patients infected with EV22, while the corresponding frequency among other EV infections was 57%.

Severe symptoms after EV22 involvement of the CNS have also been described. Acute flaccid paralysis was associated with EV22 in Jamaica in 1986. Six patients, aged 1–27 years, developed acute flaccid paralysis and were unable to walk [20]. EV22 was recovered from the stool samples of two patients, who also had a significant increase in titer of antibody to EV22. Koskineni et al. have also described a case in which EV22 was the probable cause of encephalitis in a previously healthy 5-month-old boy [21]. The virus was isolated from several stool samples, and seroconversion of both serum and CSF samples was later noted. EV22 has also been associated with other disease conditions, including myocarditis [22, 23] and hemolytic uremic syndrome [24] (table 1). Asymptomatic infections have also been reported [17, 25, 26].

In conclusion, the epidemiology of EV22 seems to differ from that of enteroviruses. In our study the seroepidemiology of EV22 seemed to differ from that of EV30; EV22 infection is more common than EV30 infection and is experienced in early childhood. According to the WHO data, supported by other reports, EV22 causes more gastrointestinal and respiratory but fewer CNS signs and symptoms than, for example, typical EVs. Most EV22 infections are still probably mild or asymptomatic. However, our knowledge about EV22 and EV23 infections may be rather incomplete since these viruses were only recently recognized to be biologically different from other enteroviruses.

Acknowledgments

The technical assistance of Marita Maaronen and Maija Rautiainen is greatly acknowledged by the authors. They thank Tapani Hovi for critical reading of the manuscript, Carl-Henrik von Bonsdorff and Mark Pallansch for the EV22 isolates, and Leena Kinnunen for help in sequence analysis.
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