Identification of a New Strain of Fastidious Enterovirus 70 as the Causative Agent of an Outbreak of Hemorrhagic Conjunctivitis


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A 1994 outbreak of acute hemorrhagic conjunctivitis in Israel was caused by an enterovirus 70 strain that was distinct from previously reported strains. Characterization was by electron microscopy (eye washes), reverse transcription-PCR (RT-PCR; eyewash, specimens, eye swabs, and tears), and sequence analysis of RT-PCR-amplified fragments from the 5′ noncoding region and VP1.

Adenovirus strains 8, 19, and 37 have been associated with keratoconjunctivitis since 1977 (4–6, 13). Enterovirus 70 (EnV70) and the cosavirus A24 variant (CoxA24) also periodically cause outbreaks of acute hemorrhagic conjunctivitis (5, 7, 8, 11, 14). Alternative methods for identification of the infecting virus are isolation in tissue culture, which is laborious, time-consuming, and often unsuccessful; direct detection by transmission electron microscopy (EM); and amplification of viral nucleic acids with common primers. The last two methods allow for rapid diagnosis; however, EM can only identify the family of the virus involved, while use of nested and/or strain-specific primers and sequence analysis of the amplified viral nucleic acid allow for the identification of the virus type and strain.

An outbreak of acute hemorrhagic conjunctivitis (AHC) occurred in Israel and the Gaza District between September and November 1994. In addition to acute conjunctivitis with occasional reports of subconjunctival hemorrhages, patients had at least one or more of the following symptoms: itching, ocular discharge, keratitis, headache, nasal discharges, and sore throats. Eye swabs (n = 27) or eye wash specimens (n = 20) were collected from 47 patients (Table 1). The patients included newly born infants to adults 86 years of age. In some infants, symptoms appeared as early as 24 h after birth.

For identification of the virus involved in the outbreak, we have applied all three of the methods described above. Medium inoculated with eye swabs (2.5 ml) or eye wash samples (1 ml) from 47 clinical samples were initially cultured on HuKi cells (a human kidney epithelial cell line) and HEp-2 cells (a human larynx carcinoma epidermal cell line). Later, additional cell lines were used, as follows: MeWO cells (human melanoma cell line), BGM cells (Buffalo green monkey epithelial cell line), VeroE6 monkey kidney cells (primary cell line), MRC5 cells (human lung diploid cells), RD cells (human embryonal rhabdomyosarcoma cell line), and 293 cells (adenovirus type 5-transformed human embryonal kidney cell line). Culture tubes were maintained for 3 weeks with one to three passages. Only one isolate, isolate B2592 (eye swab), was obtained. Virus was not isolated from any other clinical sample, although some samples appeared to produce abortive infections (i.e., a weak cytopathic effect in early passages on MeWO cell monolayers within 48 h when it was passaged at a low titer).

Aliquots of clinical samples and/or tissue culture supernatants clarified by centrifugation at 4,000 × g for 10 min were prepared on grids by direct negative staining with uranyl acetate and were observed at ×40,000 magnification on a JEOL 100C electron microscope as described previously (1). Enterovirus-like particle morphology was found in 11 of 15 eyewash samples and in two of three supernatants from MeWO cell cultures with evidence of abortive infection. Eye swabs did not contain enough particles for analysis by EM.

Viral RNA, prepared by the guanidinium thiocyanate method (2) from 125 μl of eyewash sample, transport medium from eye swabs, tears, or tissue culture supernatant by using TRI-LS reagent (MRC, Cincinnati, Ohio) according to the manufacturer’s instructions, was amplified by reverse transcription-PCR (RT-PCR) with generic nested pairs of primers (12, 15) (Fig. 1). The nested primers recognize sequences of the 5′ noncoding region (5′-NC). These sequences are very highly conserved for all human enteroviruses. Outer primers AS1 and S1 amplify a 437-bp fragment. Nested primers AS2 and S2 amplify a 121-bp subfragment. The RT reaction mixture, consisting of 4 μl of 5× RT buffer, 0.3 μl of antisense primer (500 pmol/μl), 1.5 μl of avian myeloblastosis reverse transcriptase (8 U/μl; Promega, Madison, Wis.), RNA, and sterile water to a 20-μl final volume, was overlaid with 40 μl of mineral oil, incubated for 30 min at 42°C, heated to 96°C for 10 min, and then cooled to 4°C for at least 2 min. A total of 80 μl of PCR mixture (4 μl of 1 M KCl, 2 μl of 1 M Tris [pH 8.3], 0.3 μl of sense primer, 0.3 μl of AmpliTaq [Perkin-Elmer, Branchburg, N.J.] recombinant DNA polymerase [5 U/μl], and sterile water to complete the 80-μl final volume for each tube) was added and the tubes were subjected to 24 cycles of DNA amplification (annealing at 56°C for 45 s, elongation at 73°C for 45 s, and melting at 93°C for 1 min and 30 s). The reaction was terminated after a final 10 min of elongation at 73°C. The products were analyzed by electrophoresis on horizontal 3% agarose gels (2% low electroosmosis pronarose D1 [Hispanagar s.a., Burgos, Spain] and 1% NuSieve GTG agarose [FM, Rockland, Maine]) with ethidium bromide staining.

A positive enteroviral RT-PCR result with primer pair AS1-S1 was obtained for five of five eyewash samples containing enterovirus-like particles (EM positive), three of seven eye swabs, and one of one tear sample (Fig. 2A). Nested amplification with primer pair AS2-S2 (data not shown) confirmed these results.

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CoxA24 is distinguishable from EnV70 by RT-PCR with specific primer pairs derived from the VP1 region (7, 14; this report). Our specific primers were designed by aligning 16 published entero viral VP1 amino acid sequences (accession numbers are given at the end of this report) and choosing two areas of homology (boxed sequences in Fig. 3) bracketing an area of maximum sequence and length mismatch (173 bp for CoxA24 versus 114 bp for EnV70). Unique priming of CoxA24 or EnV70 templates is ensured by extensive nucleotide mismatches within these homologous amino acid sequences. The availability of more than one published EnV70 VP1 sequence allowed for the optimization of EnV70 primers by inclusion of dl (which complements all four nucleotides) at positions where the published sequences differed. RNAs from the four eyewash samples, one eye swab, two tissue culture supernatants, and one tear sample were amplified with EnV70 primers (AS2-S2), while RNAs from none of the samples were amplified by the CoxA24 primer set (AS3-S3) (Fig. 2B).

The 5′-NC region of the tissue culture isolate B2592 was identical to that of B2599, which failed to replicate; and the VP1 sequences of B2592, primary eyewash and tissue isolates, and virus from five of six other clinical samples had no sequence differences. Longer VP1 or even full-length viral sequences will be needed to correlate sequence differences with sequence differences. Longer VP1 or even full-length viral sequences will be needed to correlate sequence differences with the ability to grow in tissue culture. Microneutralization assay with Lim-Benyesh-Melnick antisera pools A-H (10) and J-P (9) identified isolate B2592 as EnV70. Post-convalescent-phase sera from those patients from whom they were obtained neutralized both the laboratory EnV70 prototype strain and isolate B2592, confirming that EnV70 was the cause of the outbreak.

Lastly, epidemiological information and phylogenetic relationships between the present and past outbreaks was obtained from direct nucleotide sequence analysis of RT-PCR-amplified fragments with primers AS1 and S1 for 5′-NC-amplified fragments and AS4 and S4 for VP1-amplified sequences. Samples...
from each area involved in the outbreak and each sample type (i.e., eye swabs, eye washes, tears, and tissue culture supernatant) were selected for analysis (Table 1). QIAquick columns-purified cDNA (Qiagen, Düsseldorf, Germany) was sequenced on an Applied Biosystems model 373 DNA Automatic Sequencing System (Biological Services, Weizmann Institute, Rehovot, Israel) by using PRISM Dye Deoxy Terminator Cycle Sequencing reactions (Applied Biosystems, Foster City, Calif.). Sequences were determined by comparative analysis of both complementary DNA strands with the SeqEd program (Applied Biosystems). The University of Wisconsin GCG gene analysis programs (3) were used to compare sequences and prepare phylogenetic trees by the unweighted pairwise grouping method (UPGMA) method.

All sequences from strains involved in the outbreak shared more than 98% homology in both their VP1-coding and 5′-NC regions. This high degree of homology (>98%) between viruses from all patient samples indicates a direct epidemiological link to a single source. The 5′-NC sequences from viruses from our clinical samples were most closely related (93 to 95% homology) to EnV70 (GenBank accession no. D00820) where, as they had less than 83% homology to CoxA24 (GenBank accession no. D00820), whereas the laboratory EnV70 prototype strain, and the published EnV70 sequence. A phylogenetic tree obtained from the published full-length EnV70 sequence (GenBank accession no. D00820) implies that this may not be the case and that observed differences occurred as a result of similar rates of randomly distributed mutations throughout the viral genome.

Nucleotide sequence accession numbers. The new nucleotide sequence data reported in this paper will appear in the GenBank and EMBL sequence data banks under accession numbers Z78125 (5′-NC; B2556), Z78126 (VP1; B2579), Z78127 (5′-NC; B2591), Z78128 (VP1; B2591), Z78129 (5′-NC; B2592), Z78130 (5′-NC; B2592; tissue culture isolate), Z78131 (VP1; B2592; tissue culture isolate), Z78132 (VP1; B2592), Z78133 (5′-NC; B2599), Z78134 (VP1; B2599), Z78135 (VP1; B2602), Z78136 (VP1; B2603), Z78137 (5′-NC; B3079), Z78138 (VP1; B3079), Z78139 (EnV70 5′-NC; J760/71 Israel laboratory passage), and Z78140 (EnV70 VP1; J760/71 Israel laboratory passage).

Other viral sequences referred to in this paper have the
following GenBank and EMBL accession numbers (designations in parentheses indicate the viral strain): coxsackie viruses type A, L28146 (CoxA02) D00627 (CoxA09), U05876 (CoxA16) D00538 (CoxA21), and D90457 (CoxA24); coxsackieviruses type B, M16560 (CoxB1), M33854 (CoxB3), D00149 (CoxB4), and X67706 (CoxB5); echoviruses, U16283 (EV06), X84981 (Echo 9), D10582 (EV11), X77708 (Echo 12), and X79047 (Echo 12wt); enteroviruses, D00820 (EnV70CG, EnV70H, J670/71), D17597 (EnV70C, G10/72), D17597 (EnV70D, HP185/81), D17599 (EnV70E, HP85/78), D17600 (EnV70F, I72/72), D17601 (EnV70G, J648/71), D17602 (EnV70H, J670/71), D17603 (EnV70I, M51/76), D17604 (EnV70J, M67/72), D17605 (EnV70K, R20/71), D17606 (EnV70L, R6/71), D17607 (EnV70M, SEC32/71), D17608 (EnV70N, T260/74), D17609 (EnV70O, T62/73), D17610 (EnV70P, TW266/81), D17611 (EnV70Q, V1250/81), and polioviruses, J02281 (poliovirus)

FIG. 3. Amino acid alignment of the 5' end of enteroviral VP1 genes. Published VP1 nucleotide sequences of enteroviruses (see accession numbers in the text) were translated, and gaps were introduced to obtain optimal amino acid alignment. Two areas of high similarity (in boxes) flank an area of dissimilarity at the 5' end of VP1. Only this portion of VP1 is shown. Specific primers for CoxA24 and EnV70 were designed from the sequences within the boxes. Echo, echovirus, Polio, poliovirus.

FIG. 4. Phylogenetic comparison of the 5'-NC regions of clinical samples with other enteroviruses. The phylogenetic tree was generated for outbreak sequences and published enteroviral sequences located between primers AS1 and S1 by the UPGMA method by using the University of Wisconsin GCG gene analysis programs (3). The virus serotype and accession number are listed for each entry. w.t., wild type; Polio, poliovirus.

FIG. 5. Phylogenetic comparison of enteroviral VP1 sequences. Phylogenetic relationship (determined by the UPGMA method by using the University of Wisconsin GCG gene analysis programs [3]) for the 5' end of VP1 between primers AS4 and S4 from direct sequencing of RT-PCR-amplified clinical samples, the equivalent sequences of 18 previously published EnV70 isolates (original references reviewed elsewhere [14]), and computer-generated RT-PCR amplification of the equivalent 5' end of VP1 for non-EnV70 enteroviruses (i.e., the sequences between the similarity boxes in Fig. 3). The virus serotype and accession number are listed for each entry. w.t., wild type; Polio, poliovirus.
type 1, Mahoney), V01150 (poliovirus type 1, Sabin 1), M12197 (poliovirus type 2), and X00925 (poliovirus type 3).

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