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Classification of Respiratory Tract Picornavirus Isolates as Enteroviruses or Rhinoviruses by Using Reverse Transcription-Polymerase Chain Reaction

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The classification of picornaviruses isolated from respiratory secretions as human rhinoviruses (HRVs) or enteroviruses (EVs) by using reverse transcription-polymerase chain reaction was compared to that derived from acid lability testing. Of the 135 clinical isolates examined, 91 were found to be HRVs and 44 were EVs. There was 100% concordance between the two classification methods. Reverse transcription-polymerase chain reaction is an effective alternative to traditional methods for differentiating HRVs from EVs.

The family *Picornaviridae* is a large family of single-stranded RNA viruses that cause a variety of illnesses in humans. Human rhinoviruses (HRVs) and enteroviruses (EVs) are distinct genera of major clinical importance in humans; both may cause acute respiratory illness. Diagnosis of HRV and EV infection by immunoassays is considered to be impractical because of their antigenic diversity. Approaches to rapid diagnosis using broadly reactive genetic probes in nucleic acid hybridization assays have lacked sensitivity (8). Consequently, isolation of viruses in tissue culture remains the principal means for laboratory diagnosis of virus infection.

HRVs and EVs are distinguished by their different sensitivities to an acidic pH; HRVs are acid labile and EVs are acid stable (4). Olive et al. (8) recently used a reverse transcription-polymerase chain reaction (RT-PCR) assay to discriminate between these two genera. This assay takes advantage of the longer 5′ noncoding region of EVs by using degenerate oligonucleotide primers directed toward highly conserved regions of the 5′ noncoding and VP2 regions of the HRV and EV genomes. Using this assay, these investigators were able to appropriately classify laboratory strains of three serotypes of HRV and 26 serotypes of EV (8). Of the strains they examined, only echovirus type 22 was nonclassifiable. We compared a similar RT-PCR assay to standard acid lability tests for differentiating HRVs from EVs. Picornavirus isolates from persons with acute respiratory tract disease were tested at the first or second tissue culture passage level.

Clinical specimens were collected from 1 January through 31 December of 1991 from hospitalized patients, persons presenting to neighborhood and health maintenance organization clinics, volunteers in other studies, and symptomatic personnel at Baylor College of Medicine. Respiratory secretions were collected by nasal wash or aspiration, throat swab, or a combination of these methods. Specimens were inoculated onto WI-38, a human embryonic lung fibroblast cell line; Hep-2, a continuous human epithelial cell line; and LLC-MK2, a continuous rhesus monkey kidney cell line (Whittaker Bioproducts, Walkersville, Md.). Isolates were initially designated as picornaviruses on the basis of the appearance of typical cytopathic changes in the tissue culture. Tissue culture harvests were collected for further studies when 50 to 100% of the tissue culture monolayer showed cytopathic changes.

Acid lability tests were performed by a modification of previously described methods (3). One volume of 0.1 M citric acid buffer (pH 4) or one volume of 0.1 M phosphate buffer was added to an equal volume of tissue culture harvest fluid, and then the mixtures were incubated at 37°C for 1 h. Two volumes of 0.5 M phosphate buffer (pH 7.2) were then added to each sample. The harvest fluid incubated in citric acid buffer was then diluted 10-fold and inoculated into tissue culture tubes; the harvest fluid incubated in phosphate buffer was diluted 100-fold and 1,000-fold and also inoculated onto tissue cultures. Virus isolates with a ≥32-fold reduction in titer after incubation in the citric acid buffer were classified as HRVs, while the other isolates were classified as EVs. For some isolates, a repeat test at a higher or lower initial dilution of harvest fluid was required for interpretable results.

The RT-PCR assay was performed by a modification of the procedure described by Olive et al. (2, 8). Viral RNA was extracted from harvest fluids by previously described methods (2). The harvest fluids were extracted with an equal volume of phenol:water:chloroform (68:18:14) (Applied Biosystems, Foster City, Calif.). The interface was back-extracted with 3/4 volume of water, and the pooled aqueous phases were then extracted with chloroform and the nucleic acids were precipitated in 2.5 volumes of cold ethanol. Precipitated nucleic acids were suspended in 30 μl of RT reaction mix containing 10 mM Tris hydrochloride (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 3.3 μM degenerate primer OL68-1 (5′-GGTAAQTTCCACCAC CANCC-3′, where Q is C or T and N is A, C, G, or T), 667 μM deoxynucleoside triphosphates, 20 U of RNasin (Promega Corp, Madison, Wis.), and 5 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.). The RT mix was incubated for 1 h at 43°C and then placed on ice. Seventy microliters of PCR mix was added to the RT mix to yield a solution containing 10 mM Tris hydrochloride (pH 8.3), 50 mM potassium chloride, 2.0 mM magnesium chloride, 1 μM each of primers OL68-1 and

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OL24 (5′-CTACTTTGGGTGTCCG-3′), 200 μM deoxynucleoside triphosphates, and 5 U of *Taq* polymerase (Perkin Elmer-Cetus Corp., Norwalk, Conn.). The final mixture was overlaid with mineral oil, and the cDNA was amplified by using a PTC-100 thermal cycler (MJ Research, Inc., Cambridge, Mass.). After an initial 4-min heat denaturation at 94°C, 40 cycles of heat denaturation at 94°C for 1 min, primer annealing at 49°C for 1 min 30 s, and primer extension at 72°C for 1 min were followed by a final primer extension step at 72°C for 15 min. Amplified products were approximately 530 bp in length for HRVs and 650 bp in length for EVs. Ten microliters of the PCR products was analyzed by agarose gel electrophoresis using 1.5% SeaKem (FMC BioProducts, Rockland, Maine) agarose in 1× TBE buffer (89 mM Tris hydrochloride, 2.5 mM disodium EDTA, 89 mM boric acid) (9). Amplified products were visualized by UV light transillumination following staining with ethidium bromide. Negative reagent controls were run for each test and were always free of detectable amplification products.

Prior to testing of the clinical isolates, the RT-PCR assay was evaluated with stock laboratory strains. Poliovirus type 1 (LSC strain) and HRV types 1b, 13, and 15 were correctly identified as EV and HRVs, respectively. Using serial dilutions of an HRV type 1b virus pool of known titer, the level of detection of the RT-PCR assay was estimated to be approximately 10^3 50% tissue culture infective doses (data not shown). This is 1,000-fold less-sensitive detection than that reported by Olive et al. when they used this primer set on a poliovirus strain (8). Tissue culture cell lysates from uninfected monolayers (WI-38, HEp-2, and LLC-MK2) and from monolayers infected with other viruses and laboratory strains of other respiratory viruses [influenza virus A/Shanghai (H3N2), influenza virus A/Texas/36/91 (H1N1), influenza virus B/Panama/45/90, respiratory syncytial virus (strain 18537), and parainfluenza virus types 1-3] did not yield any amplified products.

On the basis of cytopathic changes in tissue culture monolayers, 140 specimens were initially designated as containing picornaviruses. Five of these could not be confirmed after further tissue culture passage and reisolation tests, leaving 135 isolates for study. Figure 1 shows the typical results of an RT-PCR assay. A total of 91 HRVs and 44 EVs were classified by RT-PCR and acid lability assays. The two assay systems agreed on the identification of all isolates.

This is the first report to evaluate the utility of the OL24 and OL68-1 primers to accurately classify a large number of clinical picornavirus isolates. Several investigators have reported on the utility of RT-PCR tests using other primer pairs for the identification of EVs in clinical specimens and tissue culture harvests and their classification as polioviruses or nonpolioviruses (1, 6, 10, 11). Olive et al. have shown that the EVs identified by the OL24 and OL68-1 primers also may be further classified into polioviruses and nonpolioviruses by using poliovirus-specific probes in hybridization studies on the amplified products (8).

Echovirus type 22 and related EV strains would not be classifiable by using the OL24 and OL68-1 primers because of the lack of homology of these viruses with other EVs and HRVs (5, 6). However, since we found complete agreement between the RT-PCR assay and our standard acid lability assay and each EV isolate generated a PCR amplification band of the expected size, it is likely that no echovirus type 22-like strains were grown from our respiratory samples.

The level of detection for our assay is probably suboptimal for using directly on clinical specimens, and no attempt was made to apply this assay directly to clinical specimens. Johnston et al. noted a similar lack of assay sensitivity when they used the OL68-1 primer in combination with a primer (OL26) homologous to a region approximately 100 nucleotides upstream of the region homologous to primer OL24 (6). However, the ability of the assay to correctly classify 100% of picornavirus isolates from our laboratory over a 1-year period suggests that it is a useful tool for epidemiologic studies of picornaviruses isolated from the respiratory tract. It may also have a role in the evaluation of EV isolates in diagnostic laboratories. The cost of reagents for an RT-PCR assay (ca. $5) is less than half of the reagent cost for an acid lability assay (ca. $12). RT-PCR has a potential disadvantage compared with acid lability assays, being subject to carryover contamination. Attention to basic quality control methods in the performance of this assay has prevented this from being a problem in our laboratory (7). The lower sensitivity of the OL68-1:OL24 primer pair may also help prevent this problem. Thus, the RT-PCR assay is a viable, cost-effective alternative to acid lability assays for the classification of picornavirus respiratory tract isolates as HRVs or EVs.

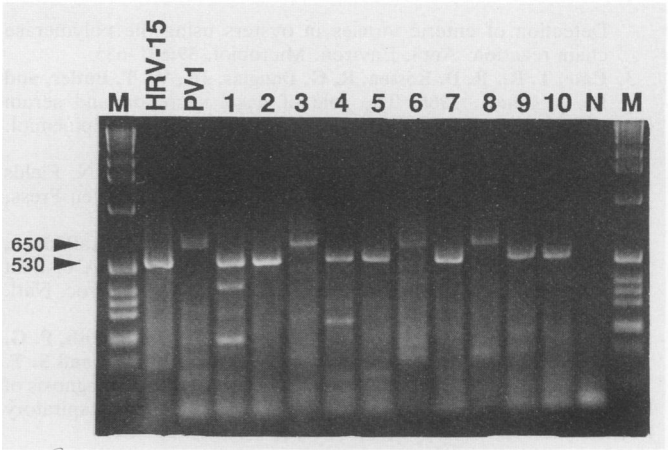

FIG. 1. Classification of picornavirus isolates by using RT-PCR. Lane M, DNA molecular size marker (1-kb ladder). Laboratory strains of rhinovirus type 15 (lane HRV-15) and poliovirus type 1 (lane PV1) were amplified, and their PCR products represent the appropriately sized products for rhinoviruses (530 bp) and enteroviruses (650 bp), respectively. Lanes 1 to 10, amplified products from 10 picornavirus isolates; isolates 1, 2, 4, 5, 7, 9, and 10 are rhinoviruses, and isolates 3, 6, and 8 are enteroviruses. Lane N, negative reagent control.

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REFERENCES

1. **Abraham, R., T. Chonmaitree, J. McCombs, B. Prabhakar, P. T. Lo Verde, and P. L. Ogra.** 1993. Rapid detection of poliovirus by reverse transcription and polymerase chain amplification: application for differentiation between poliovirus and nonpoliovirus enteroviruses. J. Clin. Microbiol. **31**:395–399.
2. **Atmar, R. L., T. G. Metcalf, F. H. Neill, and M. K. Estes.** 1993.

Detection of enteric viruses in oysters using the polymerase chain reaction. Appl. Environ. Microbiol. **59:**631–635.

3. **Cate, T. R., R. D. Rossen, R. G. Douglas, Jr., W. T. Butler, and R. B. Couch.** 1966. The role of nasal secretion and serum antibody in the rhinovirus common cold. Am. J. Epidemiol. **84:**352–363.
4. **Couch, R. B.** 1990. Rhinoviruses, p. 607–629. *In* B. N. Fields and D. M. Knipe (ed.), Fields virology, 2nd ed. Raven Press, New York.
5. **Hyypiä, T., C. Horsnell, M. Maaronen, M. Khan, N. Kalkkinen, P. Auvinen, L. Kannanen, and G. Stanway.** 1992. A distinct picornavirus group identified by sequence analysis. Proc. Natl. Acad. Sci. USA **89:**8847–8851.
6. **Johnston, S. L., G. Sanderson, P. K. Pattemore, S. Smith, P. G. Bardin, C. B. Bruce, P. R. Lambden, D. A. J. Tyrell, and S. T. Holgate.** 1993. Use of polymerase chain reaction for diagnosis of picornavirus infection in subjects with and without respiratory symptoms. J. Clin. Microbiol. **31:**111–117.
7. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339:**237–238.
8. **Olive, D. M., S. Al-Mufti, W. Al-Mulla, M. A. Khan, A. Pasca, G. Stanway, and W. Al-Nakib.** 1990. Detection and differentiation of picornaviruses in clinical samples following genomic amplification. J. Gen. Virol. **71:**2141–2147.
9. **Peacock, A. C., and C. W. Dingman.** 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. Biochemistry **7:**668–674.
10. **Rotbart, H. A.** 1990. Enzymatic RNA amplification of the enteroviruses. J. Clin. Microbiol. **28:**307–311.
11. **Zoll, G. J., W. J. Melchers, H. Kopecka, G. Jambroes, H. J. van der Poel, and J. M. Galama.** 1992. General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. J. Clin. Microbiol. **30:**160–165.