

Rapid Detection of Parainfluenza Virus Type 3 RNA in Respiratory Specimens: Use of Reverse Transcription-PCR-Enzyme Immunoassay

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Parainfluenza virus type 3 (PIV-3), an important lower respiratory tract pathogen in young children and immunocompromised individuals, may be underdiagnosed because of the insensitivity of available culturing systems and delay in identification of virus in cell culture. We developed a reverse transcription-PCR-enzyme immunoassay (RT-PCR-EIA) for PIV-3, using primers specific for a highly conserved region of the hemagglutinin-neuraminidase gene. Testing of nasal washes spiked with PIV-3 or other respiratory viruses showed that this assay detected seven strains of PIV-3 but not other respiratory viruses. Of 103 respiratory tract samples obtained from children experimentally infected with a live PIV-3 vaccine or naturally infected with wild-type PIV-3, 51 were positive by culture and 48 were positive by RT-PCR-EIA. Eleven of the culture-positive samples were negative by RT-PCR-EIA; however, none of these grew virus upon reinoculation into cell culture, indicating that virus was lost or was present at a very low titer. Eight of the culture-negative samples were positive by RT-PCR-EIA: two were obtained from a subject who was culture negative but had a serologic response to PIV-3, four were obtained 7 to 9 days after the first positive culture, and two were obtained 1 day prior to the first positive culture. Thus, this RT-PCR-EIA for PIV-3 is sensitive and specific and can detect viral RNA in samples from which virus cannot be cultivated. This assay could be used for diagnosis late in the course of PIV-3 infection and for accurate detection of disease outbreaks.

Parainfluenza virus type 3 (PIV-3) causes croup and pneumonia in infants, young children, and immunocompromised individuals (2, 6, 7, 11, 12) and upper respiratory tract illness in older children and adults (8). It has been estimated that in the United States, PIV-3 infection leads to hospitalization of approximately 1 in 400 children less than 2 years of age and is second only to respiratory syncytial virus (RSV) as a cause of lower respiratory tract (LRT) illness in this population (17). PIV-3 also causes nosocomial respiratory illness in newborn nurseries, infant and toddler wards, and bone marrow transplant units (9, 13, 15, 16, 19, 23, 24).

Despite the importance of PIV-3 as a respiratory pathogen, severe LRT illness caused by PIV-3 may be underdiagnosed because sensitive and specific rapid diagnostic tests for PIV-3 are not readily available (23). Isolation of PIV-3 in cell culture, which remains the standard for diagnosis of infection, may require several weeks; moreover, cultures from the nasopharynx may be negative in patients with LRT disease (23). Development of a sensitive, specific, and relatively rapid test for PIV-3 might therefore eliminate the need for invasive diagnostic procedures in patients with serious LRT illness (23), guide the choice of antimicrobial therapy for such patients, and allow hospital personnel to implement adequate infection control measures to prevent the nosocomial transmission of PIV-3.

Amplification of viral nucleic acids by PCR has been used

for the rapid detection of paramyxoviruses (18, 22, 27) and influenza virus (3) and might be successfully employed to develop a rapid diagnostic assay for PIV-3. We have previously described a reverse transcription (RT)-PCR-sequencing assay for a hypervariable region of the fusion protein gene of PIV-3 which we used to investigate the epidemiology of a PIV-3 outbreak on a pediatric ward (13). We have also used enzyme immunoassay (EIA) techniques to develop assays for the rapid quantitative detection of amplified nucleic acids in clinical specimens (PCR-EIA) (5, 25). We now describe the development of an RT-PCR-EIA for a highly conserved region of the hemagglutinin-neuraminidase (HN) gene of PIV-3. We applied this new assay to sequential respiratory tract samples obtained from children naturally infected with wild-type PIV-3 or infected with a live attenuated PIV-3 vaccine during clinical trials.

MATERIALS AND METHODS

Virus stocks. PIV-3 strains Texas/536/80, Texas/9305/82, Texas/12477/83, Washington/47885/57, Washington/1511/73, Washington/641/79, and JS were provided by Brian Murphy (National Institutes of Health, Bethesda, Md.). The JS strain of PIV-3 has been attenuated by cold adaptation as a vaccine candidate (see below); the wild-type strain is referred to as JS, and the cold-adapted strain used in vaccine trials is referred to as cp (for cold passaged)-18JS (1). Influenza virus strains A/Kawasaki/9/86, A/Los Angeles/2/87, and B/Ann Arbor/1/86 were also provided by Brian Murphy. PIV-1 (C35 strain), PIV-2 (Greer strain), and RSV (Long strain) were obtained from the American Type Culture Collection (Rock-

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ville, Md.). A mumps virus isolate was provided by Michael Foreman (Johns Hopkins Hospital, Baltimore, Md.), and measles virus vaccine (Edmonston-Zagreb strain) was provided by Neal Halsey (Johns Hopkins School of Hygiene and Public Health, Baltimore, Md.). To determine the sensitivity of the RT-PCR-EIA, the PIV-3 JS strain was serially diluted in aliquots of undiluted pooled culture-negative nasal washes obtained from pediatric subjects participating in respiratory virus vaccine studies.

Volunteer specimens. Specimens from 15 children enrolled in live-virus vaccine trials at the Center for Vaccine Development, St. Louis University, St. Louis, Mo., and at the Center for Immunization Research, Johns Hopkins University, Baltimore, Md., were tested for the presence of PIV-3 RNA. These specimens were stored frozen for up to 2 years prior to use in this assay. Nine of these children had been enrolled in a trial of the live cp-18 JS strain PIV-3 vaccine (1). In this study, nasopharyngeal swab specimens were obtained daily from each subject from day 0 (the day of inoculation) through day 11; a subset of these specimens was available for testing by RT-PCR-EIA. Two of the children shed wild-type PIV-2 during the study. Six children had been enrolled in trials of other respiratory virus vaccines, but wild-type PIV-3 grew from the nasal washes obtained from each child (by previously described methods [21]). All of the specimens from these six children were available for testing by RT-PCR-EIA. In all instances, informed, witnessed, written consent had been obtained from the parents prior to participation in vaccine trials.

Quantitative data on viral shedding were generated for specimens from cp-18 JS strain PIV-3 vaccine study participants which were tested by RT-PCR-EIA. In both of the aforementioned studies, nasal wash or swab specimens were immediately inoculated in cell culture (Rhesus monkey kidney cells [Whittaker MA Bioproducts, Walkersville, Md.] or LLC-MK2 [American Type Culture Collection, Rockville, Md.]) for primary isolation. Viral titrations were performed by using L-132 cells (American Type Culture Collection) on frozen specimens as previously described (1). For purposes of calculation, specimens that were culture negative were assigned a titer of $10^{0.5}$ PFU/ml. Specimens that grew PIV-3 in the initial culture but which were culture negative upon titration were assigned a titer of $10^{0.75}$ PFU/ml so that they could be distinguished from true culture-negative specimens. Phenotypic analysis to distinguish wild-type PIV-3 from vaccine strain PIV-3 was performed in the study of the live attenuated PIV-3 vaccine (1).

RT-PCR-EIA. For RT-PCR, specimens were diluted 1:10 in distilled water, extracted, and amplified by previously described methods (13). Briefly, samples were extracted with guanidinium thiocyanate, phenol, and chloroform, ethanol precipitated, dried, resuspended in diethyl pyrocarbonate-treated water, and reverse transcribed into cDNA using random hexamers. For PCR amplification, primers for highly conserved sequences of the HN gene (4) (5'-CTCGAGGT TGTCAGGATATAG-3', bp 706 to 726 [mRNA sense] and 5'-CTTTGGGAGTTGAACACAGTT-3', bp 893 to 914 [genome sense]) were added to 100 μ l of a reaction mixture which contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 0.01% gelatin, 0.2 mM deoxynucleoside triphosphates, and 2.5 U of *Taq* DNA polymerase. Each sample was overlaid with mineral oil and was subjected to 30 cycles of amplification in a DNA thermal cycler (Perkins-Elmer Cetus, Norwalk, Conn.), also as previously described (13). For some specimens, the amplified product was detected by

polyacrylamide gel electrophoresis (PAGE) and silver staining.

For the EIA, a biotinylated RNA probe was prepared by using nested HN primers 5'-TTAATACGACTCACTAT AGGGCAAGTATTACAGATA-3' (mRNA sense; T7 promoter sequences are underlined) and 5'-ATCTGTATTTAG GAGTGCT-3' (genome sense) to reamplify the PCR product made from the Texas/9305/82 PIV-3 strain by using the outer HN primers. A biotinylated RNA probe was made from this amplified product by RT with biotin-11 UTP (Enzo, New York, N.Y.). The probe was purified as previously described (5, 26) and stored at -70°C . The nonisotopic solution hybridization reaction and EIA for detection of amplified sequences were also performed as previously described (5, 26).

Positive and negative control samples were included in each assay. The positive PCR control was an RT preparation of PIV-3 stock that had been PCR positive in previous assays. The positive EIA control was a titration of PCR product from a PIV-3 stock preparation that was amplified in quantity and stored frozen at -70°C . In studies of tissue culture-grown virus, supernatant from uninfected Rhesus monkey kidney cells was included in each RT-PCR-EIA as a negative control. In studies using nasal wash or nasal swab specimens, an aliquot of the pooled culture-negative nasal washes described above was included in each RT-PCR-EIA as a negative control. All samples were run in duplicate. A sample was considered to be positive if it generated a fluorescence activity three standard deviations greater than the mean activity generated by the negative control samples included in the assay. Positive samples were further quantified by subtracting the mean fluorescence of the negative controls from the mean fluorescence of the sample. All data are expressed in fluorescence units, defined as mean fluorescence of the sample minus mean fluorescence of the negative control.

Statistical analysis. The correlation between viral titer and specific activity in PCR-EIA was calculated by the method of Spearman, as described by Snedecor and Cochran (20).

RESULTS

Amplification of viral RNA from stock viruses. The specificity of our assay for PIV-3 was initially assessed by RT-PCR-EIA of seven strains of PIV-3 and of high-titered stocks of PIV-1, PIV-2, measles virus, mumps virus, RSV, and influenza A (H1N1 and H3N2 subtypes) and B viruses. The results of the PCR portion of this assay are shown by the silver-stained polyacrylamide gel in Fig. 1A, and the results of the RT-PCR-EIA are shown by the graph depicting virus-specific fluorescence in Fig. 1B. An appropriately sized band (208 bp) is visible on the gel for all seven PIV-3 strains and none of other viruses; by RT-PCR-EIA, all seven PIV-3 strains showed an activity of ≥ 300 fluorescence units, whereas the other paramyxoviruses or influenza viruses showed an activity of ≤ 45 fluorescence units.

The sensitivity of the RT-PCR-EIA compared with cell culture for tissue culture-grown virus was determined by amplification of serial dilutions of the PIV-3 JS strain. The results of this experiment are shown in Fig. 2. The limit of detection of PIV-3 by the RT-PCR-EIA was approximately $1.53 (10^{0.18})$ PFU/ml. The linear regression correlation coefficient (r) was 0.93 ($P < 0.001$).

Amplification of viral RNA in patient samples. A total of 103 samples were tested by RT-PCR-EIA for the presence of PIV-3 RNA. The results of these assays are shown in Table

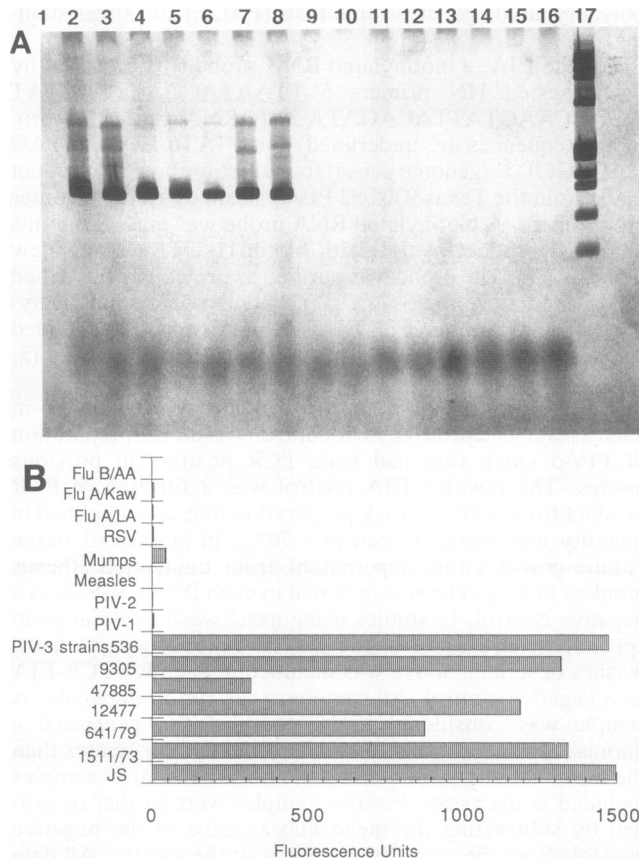


FIG. 1. (A) PAGE and silver staining of PCR products from PIV-3 Texas/9305/82 (lane 2), Texas/536/80 (lane 3), Washington/641/79 (lane 4), Texas/12477/83 (lane 5), Washington/47885/57 (lane 6), Washington/1511/73 (lane 7), and JS (lane 8) and from PIV-1 (lane 9), PIV-2 (lane 10), measles virus (lane 11), mumps virus (lane 12), RSV (lane 13), influenza virus A/Los Angeles/2/87 (lane 14), influenza virus A/Kawasaki/9/86 (lane 15), and influenza virus B/Ann Arbor (lane 16). Lane 17, gel markers. (B) Fluorescence by RT-PCR-EIA of the PIV-3 strains and the other respiratory viruses listed for panel A. Fluorescence units are corrected by subtracting the mean fluorescence of negative controls, as described in the text.

1. Forty samples were positive by viral culture and had PIV-3 RNA detected by PCR-EIA. There were 11 culture-positive, RT-PCR-EIA-negative samples and 8 culture-negative, RT-PCR-EIA-positive samples. Of the 11 culture-positive RT-PCR-EIA-negative specimens, none grew PIV-3 upon reinoculation in cell culture (Table 1). The RT-PCR-EIA also appeared to be specific for PIV-3: all but one of the day 0 specimens were RT-PCR-EIA negative (see below), and all of the specimens which were culture positive for PIV-2 were RT-PCR-EIA negative.

As mentioned above, the day 0 specimen from one study subject (no. 17) was culture negative but RT-PCR-EIA positive. Specimens from this child, who received the live attenuated cp-18 JS strain PIV-3 vaccine, were culture negative for PIV-3 but RT-PCR-EIA positive on days 0 and 1 (438 and 57 fluorescence units, respectively). The child also had an eightfold rise in serum hemagglutinating antibody to PIV-3 in paired serum specimens obtained on days 0 and 28 of the study. The child's sister, who was a placebo recipient, shed wild-type PIV-3 on day 6 of the study. These data suggest that subject 17 was naturally infected with

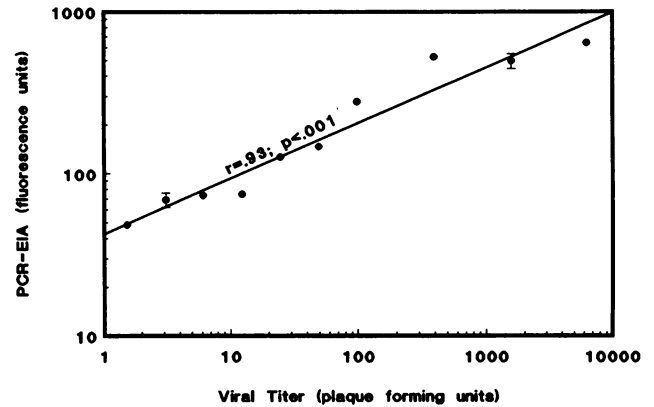


FIG. 2. Comparison of viral titer and RT-PCR-EIA for serial dilutions of the JS strain of PIV-3 by linear regression analysis.

wild-type PIV-3 prior to the onset of the vaccine trial and that these RT-PCR-EIA-positive, culture-negative specimens represent true positives.

Correlation between viral titer and RT-PCR-EIA specific activity. The Spearman rank correlation coefficient (r_s , corrected for ties) for viral titer and specific activity was 0.684 and was highly significant ($P = 0.0001$).

DISCUSSION

This study documents the sensitivity and specificity of our RT-PCR-EIA for the detection of PIV-3 in cell culture-grown virus stocks and, more importantly, in respiratory tract samples obtained from children infected with PIV-3. Our assay, which amplified a highly conserved region of the HN gene (4), was able to detect seven strains of PIV-3 isolated over 26 years but did not detect other types of PIV (either in stock specimens or in respiratory tract samples), paramyxoviruses other than PIV, or influenza viruses.

TABLE 1. Comparison of cell culture and RT-PCR-EIA for detection of PIV-3 in patient specimens

Specimen group	Culture result	RT-PCR-EIA		Total no.
		No. positive	No. negative	
Children receiving PIV-3 vaccine	Positive	19	5	24
	Negative	4	17	21
Total		23	22	45
Other children participating in vaccine studies	Positive	21	6	27
	Negative	4	27	31
Total		25	33	58
All specimens	Positive	40	11	51 ^a
	Negative	8	44	52
Total		48	55	103

^a Eleven samples were culture positive but RT-PCR-EIA negative. None of these specimens grew PIV-3 upon reinoculation in cell culture. Thus, 19 specimens from children receiving PIV-3 vaccine and 21 specimens from other children participating in vaccine studies (a total of 40 specimens) were culture positive upon reinoculation.

As measured by serial titration of a single strain of PIV-3, the RT-PCR-EIA was at least as sensitive as cell culture, the limits of detection being approximately 1 PFU/ml. This finding was confirmed in the experiments using volunteer specimens, in which 40 specimens were culture positive and RT-PCR-EIA positive, 8 specimens were RT-PCR-EIA positive but culture negative, and 11 specimens were culture positive but RT-PCR-EIA negative (although none of these last specimens were culture positive after a single cycle of freeze-thawing, suggesting that virus was present at a very low titer). The sensitivity of this assay might be enhanced 10-fold by using undiluted nasal wash specimens. We diluted specimens for this study because of the limited volumes available and concern about the presence of PCR inhibitors in patient samples (25). However, our titration experiments, in which we serially diluted virus into undiluted pooled nasal washes, suggest that our PCR is not inhibited by nasal secretions.

If these preliminary observations are confirmed in subsequent studies, the PIV-3 RT-PCR-EIA might be a particularly useful diagnostic tool for immunocompromised patients with PIV-3 LRT disease. Rapid diagnosis of PIV-3 infection in these patients might lessen the use of broad-spectrum antibiotics, or, in certain patients, promote the timely use of ribavirin, which has been reported anecdotally to ameliorate PIV-3 disease (10, 14). Rapid diagnosis would also promote the use of appropriate infection control measures (patient cohorting and isolation procedures) to control nosocomial transmission of PIV-3 in these settings. Because nasopharyngeal viral cultures are often not obtained from immunocompromised patients at the time of the upper respiratory tract illness and may be negative (or require weeks in culture to become positive) at the time of LRT disease (23), an RT-PCR-EIA for PIV-3 which can detect viral RNA in the nasopharynx at the time of LRT disease might, in selected patients, also eliminate the need for invasive diagnostic procedures. Alternatively, RT-PCR-EIA of LRT specimens from those patients who require bronchoscopy or open lung biopsy would permit more rapid diagnosis of PIV-3 infection than typically occurs with standard culture techniques (2 days with the RT-PCR-EIA versus a median of 9 days with standard techniques [23]). As nucleic acid amplification assays have been described for other respiratory viruses (3, 18), this assay might ultimately be used as part of a panel to detect the presence of respiratory viral RNA in high-risk individuals. Thus, the use of this RT-PCR-EIA for rapid diagnosis of PIV-3 infection in hospital settings could benefit patients with PIV-3 infection and their contacts.

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