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Rapid Diagnosis of Human Parainfluenza Virus Type 1 Infection by Quantitative Reverse Transcription-PCR-Enzyme Hybridization Assay

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The detection and quantitation of human parainfluenza virus type 1 (HPIV-1) RNA in nasal wash specimens from 49 children with lower respiratory infections were performed by a reverse transcription-PCR-enzyme hybridization assay (RT-PCR-EHA). The HPIV-1 RT-PCR-EHA was then used to test 40 samples from asymptomatic children. Primers and probes were designed from regions within the HPIV-1 hemagglutinin-neuraminidase gene which are highly conserved among all known genotypes. HPIV-1 was detected in all nine children who were culture positive. Other common respiratory viruses (HPIV-2, -3, and -4, mumps virus, respiratory syncytial virus, measles virus, and influenza virus) were not detected by the HPIV-1 assay. Forty symptomatic children were negative by culture, and four of these were positive by RT-PCR-EHA. All of the samples from asymptomatic children were negative by culture and RT-PCR-EHA. RT-PCR-EHA was 100% sensitive (95% confidence interval, 0.66 to 1.00) and 95% specific (95% confidence interval, 0.88 to 0.99) compared with culture. The four false-positive results (relative to the results of culture) were in children with lower respiratory infections compatible with HPIV-1 infection and suggest that RT-PCR-EHA may be more sensitive than culture. Our data indicate that HPIV-1 may be underdiagnosed by routine culturing methods. RT-PCR-EHA has been demonstrated to be an easy, rapid, sensitive, and specific test for diagnosing HPIV-1 infection and provides a methodology for the rapid detection of closely related respiratory viruses.

Human parainfluenza virus type 1 (HPIV-1) is a major lower respiratory tract pathogen that causes epidemics in infants and children in the United States as well as other parts of the world (1, 7, 8, 21, 23). At least 50 percent of croup cases in the United States can be linked to this virus (5, 9, 11). HPIV-1 is also responsible for a significant proportion of cases of bronchiolitis, tracheobronchitis, and pneumonia in infants, young children, immunocompromised patients, and elderly people. HPIV-1 infections have been estimated to cause the hospitalization of 35,000 children during each biennial epidemic in the United States (9, 11, 22), with an estimated cost of $93 million for emergency room visits and hospitalization (14).

Current methods used in the diagnosis of HPIV-1 infection include virus isolation in tissue culture, shell vial assay, antigen detection, and serology. These same methods are used for the diagnosis of HPIV-2, HPIV-3, and other common respiratory viruses. In the United States, the most common method of detecting HPIV-1 is viral culture with tissue culture confirmation by using direct or indirect immunofluorescence (15). The major disadvantages of viral culture are the length of time required to grow HPIV-1 (usually 3 to 5 days, but it can be as long as 14 days) and the poor sensitivity (approximately 50%) (15). HPIV-1 and all common respiratory viruses may be underdiagnosed because sensitive, specific, and rapid diagnostic tests are not readily available. The development of an improved method for the diagnosis of HPIV-1 infection may facilitate early diagnosis, allow elimination of antimicrobial therapy, shorten hospital stays, decrease costs, limit unneeded diagnostic procedures, improve hospital infection control measures, expand epidemiologic studies, and provide methodologies useful for the diagnosis of other respiratory viruses.

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The development of molecular techniques such as gene amplification (e.g., PCR) has allowed the detection of small numbers of viral genomes in clinical specimens. We have developed a modification and application of a reverse transcription (RT)–PCR–enzyme-linked assay that involves liquid hybridization and have named this assay the RT-PCR enzyme hybridization assay (EHA; RT-PCR-EHA) for the direct rapid detection, identification, and quantification of HPIV-1 virus RNA in nasal wash specimens.

MATERIALS AND METHODS

Virus stocks. HPIV-1 (HA-2, strain C39; American Type Culture Collection, Rockville, Md.), HPIV-2 (LRS-76 clinical isolate, 1991, Milwaukee), HPIV-3 (LRS-75 clinical isolate, 1991, Milwaukee), HPIV-4 (strain M-25; American Type Culture Collection), respiratory syncytial virus (RSV; 9320; American Type Culture Collection), influenza virus A (LRS-147 clinical isolate, 1991, Milwaukee), adenovirus (gift from Geoff Kitchingman), measles virus (Edmonston strain), and mumps virus (Jones strain; American Type Culture Collection) were used and prepared in our laboratory as described previously (16).

Nasal wash specimens. Nasal secretions were obtained from children 5 years of age or younger who presented to the Emergency Room of Children's Hospital of Wisconsin with signs and symptoms of lower respiratory infection during the fall of 1991 (10). Nasal wash specimens were obtained as described by Hall and Douglas (10). The average volume of the specimens was 1 to 2 ml. The specimens were immediately emptied into transport tubes containing 2 ml of minimum essential medium supplemented with 0.5% bovine serum albumin, gentamicin (5 μg/ml), penicillin (500 units/ml), and amphotericin B (2.5 μg/ml). Transport tubes were kept at room temperature for 0.5 to 3 h in the emergency room before being refrigerated at 4°C. Specimens were centrifuged at 2,000 × *g* for 15 min. The supernatants were divided into 0.5- to 1-ml aliquots and were refrigerated at 4°C until culture or were frozen at −80°C until RNA extraction.

Viral culture. Primary cynomolgus monkey kidney (CMK) cells (Viromed Laboratories, Minnetonka, Minn.) were obtained as a suspension culture and were plated one time only. LLC-MK_2 cells (American Type Culture Collection) are continuously passaged in our laboratory. The supernatants were immediately inoculated onto confluent CMK cells and LLC-MK_2 tissue culture monolayers in 96-well microtiter plates (12 wells per specimen). After adsorption for 30 to 45 min, 200 μl of fresh serum-free acetylated trypsin and antibiotics were added. The cultures were then incubated at 34°C and were examined on days 1 to 3, 4 to 5, 7 to 8, and 14 for cytopathic effects and hemadsorption by using 0.5% guinea pig erythrocytes and immunofluorescence. The identities of the infecting viral

agents were determined with a commercial indirect immunofluorescence kit (Baxter, West Sacramento, Calif.). This kit detects adenovirus, RSV, influenza viruses A and B, and parainfluenza virus types 1, 2, and 3. All assays were performed as recommended by the manufacturer (15). The supernatants of nasal wash specimens from 49 symptomatic children were also cultured in HEL, AGMK, HEp-2, MDCK, and HFS tissue culture lines at the City of Milwaukee Virology Laboratory.

Construction of quantitation standard. To construct a quantitation standard, HPIV-1 cDNA was synthesized from HPIV-1 genomic RNA by RT. The cDNA was amplified with the primer pair HN1B (ACT CTG GAC TCA AGA ATG AGA AAT) and HN2A (CAT ATT TGA CAA ATA GGC AGG CAT) to yield a 2,070-bp hemagglutinin-neuraminidase (HN) gene product. The PCR product and plasmid PCR II (Invitrogen, San Diego, Calif.) were ligated under standard conditions. Transformation of INVaF′-competent cells (Invitrogen) with the ligated plasmid was carried out according to the supplier's protocol. A clone was obtained and was designated PCR II2-1. It contained the 2,070-bp HPIV-1 HN gene insert. The clone was confirmed first by *Bam*HI, *Xba*I, and *Bam*HI-*Xba*I digestions and then by sequencing with the sequences provided with the PCR product sequencing kit (United States Biochemicals, Cleveland, Ohio). PCR II2-1 DNA was transcribed to RNA with SP6 RNA polymerase (Promega, Madison, Wis.). The RNA was examined on a denatured agarose gel, quantitated on a spectrophotometer to obtain the calculated copy number, and frozen at −70°C. A known copy number of the transcript was introduced into lysis buffer, isolated by the same procedure used to isolate virus genomic RNA, and then used as a quantitation standard.

Viral genomic RNA isolation. Viral genomic RNA was extracted from frozen nasal wash specimens by previously described methods (4). Briefly, samples were treated with guanidinium-isothiocyanate (4 M) in sodium citrate (25 mM) buffer (pH 7.0) with 0.5% sarcoryl and 0.1 M β_2-mercaptoethanol; 0.1 volume of 2 M sodium acetate was added together with 1 volume of water-saturated phenol and 0.2 volume of chloroform-isoamyl alcohol (49:1). After centrifugation, RNA was extracted in the aqueous phase, and the phenol-chloroform extraction was repeated once more. The RNA was then precipitated with isopropanol at −20°C for 1 h. After centrifugation, the pellet was washed twice with 70% ethanol and was dissolved in 50 μl of diethyl pyrocarbonate-treated water containing 20 U of RNase inhibitor.

cDNA synthesis. cDNA was synthesized from random hexamers by incubation at 42°C for 60 min and 99°C for 5 min and was soaked at 5°C for 5 min with Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer Cetus, Norwalk, Conn.) and 1 mM (each) deoxynucleoside triphosphates. Fifteen nanograms of viral RNA from virus stocks or 3 μl of viral RNA extracted from nasal wash samples was used for a single test (6).

Amplification of cDNA by PCR. For PCR amplification, primers from highly conserved sequences of the HPIV-1 HN gene (17–19), PF 526 (ATT, TCT, GGA, GAT, GTC, CCG, TAG, GAG, AAC, base pairs 526 to 552; sense strand) and PR 678 (CAC, ATC, CTT, GAG, TGA, TTA, AGT, TTG, ATG, A; base pairs 678 to 705; antisense strand), were used for amplification. Primer PR 678 was biotinylated at the 5′ end (Operon, Alameda, Calif.). The PCR mixture contained 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, 1 mM (each) primers, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). After denaturation at 95°C for 2 min, aliquots were then amplified by two cycles at 95°C for 1 min, 55°C for 45 s, and 72°C for 45 s and 28 cycles of 95°C for 1 min, 60°C for 45 s, and 72°C for 45 s and were then held at 72°C for 7 min. After a final extension at 72°C for 7 min, the PCR products were denatured by heating at 95°C for 5 min and were then kept on ice. Positive and negative controls which included transcript RNAs from plasmids PCR II2-1 and PCR II were added to each assay.

The PCR products were analyzed by electrophoresis on a 2% agarose gel in TBE (Tris-borate-EDTA) buffer at 80 V for 1 h and 15 min and were stained with ethidium bromide.

Detection and quantitation of PCR product by RT-PCR-EHA. To detect and quantitate the PCR product, 96-well microplates were coated with streptavidin (Pierce, Rockford, Ill.). Previously published methodologies were followed (20), and 0.5 pmol of horseradish peroxidase-labeled HPVI-1 HN probe 640 (TAC, CTT, CAT, TAT, CAA, TTG, GTA, AGT, CAA, TAT, ATG; base pairs 640 to 672; sense strand) was added to each well. A capture and hybridization reaction was then carried out, and 200 μl of substrate solution (trimethylbenzidine; Life Technologies, Grand Island, N.Y.) was added to each well (19). After 15 min the reaction was stopped with 1 N H_2SO_4, and the optical density of each well was measured at 450 nm on a spectrophotometer (EL-3; Biotek, Winooski, Vt.). The positive cutoff value was calculated from the mean absorbance obtained for a group of seronegative samples plus 3 standard deviations (minimum of 1.5 times the absorbance for the negative controls). The virus copy number in clinical samples was determined by using the absorbance values obtained from the serial dilution of the quantitative standard described above.

RESULTS

Specificity and sensitivity of HPIV-1 RT-PCR-EHA. The specificity of the RT-PCR-EHA for HPIV-1 was initially assessed by testing closely related and common human respiratory viruses. Nine different strains of HPIV-1 and one strain each of HPIV-2, HPIV-3, HPIV-4, measles virus, mumps virus, RSV, influenza virus type A, and adenovirus were tested by RT-PCR-EHA. The results of the RT-PCR portion of this assay are shown in the ethidium bromide-stained agarose gel in Fig. 1. A band of the appropriate size (180 bp) was found for the HPIV-1 strains only. The results of the complete RT-PCR-EHA agreed with this result (Table 1). Only HPIV-1 clinical isolates gave positive A_{450} values. The HPIV-1 RNA quantitation standard was used to illustrate the sensitivity of RT-PCR-EHA as an amplification and detection system. Various amounts of RNA transcript equivalent to 5, 10, 50, 500, 1,000, 5,000, and 10,000 copies were amplified and detected by RT-PCR and RT-PCR-EHA. The relative sensitivities of RT-PCR by agarose gel electrophoresis (Fig. 2) were compared with those of RT-PCR-EHA (calculated). RT-PCR-EHA detected as few as 600 copies per ml of nasal wash and was more sensitive than RT-PCR (1,000 copies per ml, which is equal to 5 copies per 18 μl of loaded sample).

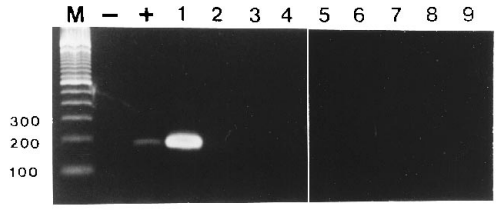

FIG. 1. Specificity of RT-PCR for detection of HPIV-1. HPIV-1 (lane 1), HPIV-2 (lane 2, HPIV-3 (lane 3), HPIV-4 (lane 4), RSV (lane 5), influenza virus A (lane 6), adenovirus (lane 7), measles virus (lane 8), and mumps virus (lane 9) were reverse transcribed and amplified. PCR products were analyzed on a 2% agarose gel and were stained with ethidium bromide. RNA transcripts from PCR II2-1 plasmid with the HPIV-1 HN gene insert were used as the positive control, and RNA transcripts from plasmid without the insert were used as the negative control. Lane M, molecular mass markers (indicated on the left in base pairs).

Reproducibility of HPIV-1 RT-PCR-EHA. To test the reproducibility of the RT-PCR-EHA, duplicate aliquots of two HPIV-1-negative samples (samples 1 and 2) and two HPIV-1-positive samples were subjected to RT, amplification, and EHA. First, the intra-assay variability of the EHA was tested.

TABLE 1. Optical densities for different respiratory viruses tested by HPIV-1 RT-PCR-EHA

Virus	A_{450}
Common respiratory viruses	
Negative control	0.25
HPIV-1	1.66
HPIV-2	0.28
HPIV-3	0.25
HPIV-4	0.27
RSV	0.31
Influenza virus	0.27
Adenovirus	0.26
Measles virus	0.28
Mumps virus	0.25
Representative clinical HPIV-1 isolates	
Negative control	0.08
CI-93	0.48
CI-20	0.42
CI-21	0.42
CI-23	1.44
CI-105	0.47
CI-67	0.44

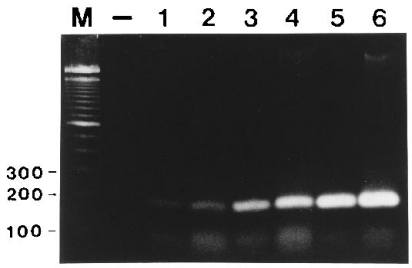

FIG. 2. Sensitivity of RT-PCR for detection of HPIV-1. The HPIV-1 RNA positive control transcripts were reverse transcribed and amplified. PCR products were analyzed on a 2% agarose gel and were stained with ethidium bromide. Lanes 1 to 6, 5, 10, 50, 100, 500, and 1,000 copies, respectively. RNA transcripts from the PCR II plasmid without the insert were used as a negative control (lane −). Lane M, molecular mass markers (indicated on the left in base pairs).

The mean of the optical density reading from sample 1 was 0.0390 (standard error of the mean [SEM], 0.00075), that from sample 2 was 0.0725 (SEM, 0.00150), that from sample 3 was 0.4287 (SEM, 0.00850), and that from sample 4 was 0.5275 (SEM, 0.01300). Multiple replicates of the same PCR sample yielded absorbances that varied <10% between wells.

Detection and quantitation of HPIV-1 RNA from nasal wash specimens by RT-PCR-EHA. Nasal wash specimens from 49 children with signs or symptoms of lower respiratory infection and 40 specimens from asymptomatic children were tested for HPIV-1 RNA by RT-PCR-EHA. Thirteen of 89 nasal wash specimens showed a positive band on agarose gel electrophoresis (Fig. 3) and in the EHA demonstrated significant absorbance above the background (Table 1). HPIV-1 was detected in samples from all 9 children who were culture positive (copy numbers from 5.3×10^4 to 6.5×10^6/ml; sensitivity, 100%) and in 4 of 80 samples from children who were negative by culture (specificity, 95%). The four false-positive samples were from children with lower respiratory infection compatible with HPIV-1 infection. Furthermore, the samples were collected during an HPIV-1 epidemic and suggest that RT-PCR-EHA may be more sensitive then culture. Except for the nine HPIV-1-positive nasal wash specimens mentioned previously, no other infectious agent was isolated.

DISCUSSION

The epidemiology and clinical diagnosis of HPIV-1 are major public health concerns (1, 5, 7–9, 11, 14, 21–23). A rapid definitive diagnosis may improve proper clinical management of many patients, especially hospitalized premature infants, immunodeficient patients, patients with chronic heart or lung disease, and elderly people. A definitive diagnosis of HPIV-1 is provided by viral culture followed by indirect immunofluorescence confirmation or HPIV-1 antibody detection in acute- or convalescent-phase serum (15). These methods require at least several days (range, 2 to 14 days) (15). The only other rapid test for the detection of HPIV-1 is direct antigen detection with fluorescent monoclonal antibodies. However, this test has a poor sensitivity, reported to be between 43 and 52% (13). RT-PCR-EHA amplification and quantitation of viral RNA provides a rapid technique for the detection and identification of HPIV-1 within 8 h. The sensitivity and specificity of HPIV-1 detection were increased by using PCR primers designed from highly conserved sequences of the HPIV-1 HN gene (18), an elevated annealing temperature after the second cycle, and oligonucleotide probe hybridization. Nine different samples containing HPIV-1 clinical isolates showed a positive RT-PCR-EHA result, but all other samples containing the human viruses tested showed a negative result. There are at least three distinct genotypes (genotypes A, C, and D) among HPIV-1 strains (18), and the primers and probe which were used in this assay detected HPIV-1 strains of all three genotypes.

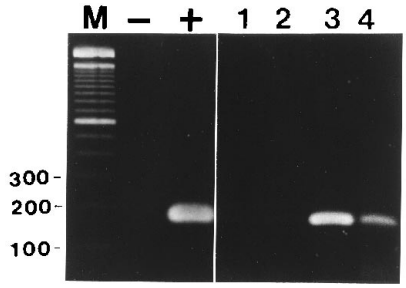

FIG. 3. Detection of HPIV-1 from clinical samples by RT-PCR. The HPIV-1 RNAs extracted from nasal wash samples were reverse transcribed and amplified. PCR products were analyzed on a 2% agarose gel and were stained with ethidium bromide. The samples in lanes 1 and 2 show negative results; the results for the samples in lanes 3 and 4 are representative of the results for the 13 positive samples. RNA transcripts from plasmid PCR II2-1 with the HPIV-1 HN gene insert were used as positive controls (lane +) and RNA, transcripts from plasmids without the inserts were used as negative controls (lane −). Lane M, molecular mass markers (indicated on the left in base pairs).

The RT-PCR-EHA was equivalent (or superior) to the standard virus isolation and the indirect immunofluorescence protocols in terms of test specificity (95%; 95% confidence interval [CI], 0.88 to 0.99) and sensitivity (100%; CI, 0.66 to 1.00). Positive predictive values were either 100% (CI, 0.75 to 1.000) or 69% (CI, 0.30 to 0.91), depending on whether the four RT-PCR-EHA-positive specimens from symptomatic children during an HPIV-1 epidemic are considered false positives. The negative predictive value was 100% (CI, 0.95 to 1.00). All samples classified by standard virus culture as positive were positive by RT-PCR-EHA (14). Many factors lead to the decreased ability to recover respiratory viruses from clinical samples. Temperature, humidity, pH, and proteolytic enzymes have all been implicated (2, 12, 13). However, these factors are expected to have little effect on the ability to detect virus by RT-PCR-EHA (3).

The HPIV-1 RT-PCR-EHA was able to detect as few as 600 copies of HPIV-1 RNA per ml. The RT-PCR-EHA yielded linear detection of from 5 to 10,000 copies. In the current assay, RNA was extracted from a sample containing 90 to 280 μl of nasal wash, and the equivalent of only 16 μl of nasal wash was analyzed per amplication. Therefore, the HPIV-1 RT-PCR-EHA is much more rapid, uses less clinical specimen, and is probably more sensitive than culture. It also has an excellent negative predictive value. This test should allow for a fuller understanding of the true epidemiology of HPIV-1 and should be useful for diagnosing HPIV-1 infections in immunocompromised patients, hospitalized children, and elderly people. Furthermore, it serves as a model for future assays for closely related respiratory viruses. A multiplex RT-PCR-EHA for HPIV-1, -2, and -3 has recently been shown to be equivalent to the HPIV-1 assay (unpublished data) for the detection of each virus individually or during a dual infection.

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REFERENCES

1. **Assaad, F., and W. C. Cockburn.** 1974. A seven-year study of WHO virus laboratory reports on respiratory viruses. Bull. W. H. O. **51:**437–45.

2. **Blanding, J. G., M. G. Hoshiko, and H. R. Stutman.** 1989. Routine viral culture for pediatric respiratory specimens submitted for direct immunofluorescence testing. J. Clin. Microbiol. **27:**1438–1440.
3. **Cherian, T., L. Bobo, M. C. Steinhoff, R. A. Karron, and R. H. Yolken.** 1994. Use of PCR-enzyme immunoassay for identification of influenza A virus matrix RNA in clinical samples negative for cultivable virus. J. Clin. Microbiol. **32:**623–628.
4. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenolchloroform extraction. Anal. Biochem. **162:**156.
5. **Denny, F. W., T. F. Murphy, W. A. Clyde, Jr., A. M. Collier, and F. W. Henderson.** 1983. Croup: an 11-year study in a pediatric practice. Pediatrics **71:**871–876.
6. **Fan, J., H. Z. Bass, and J. L. Fahey.** 1993. Elevated IFN-γ and decreased IL-2 gene expression are associated with HIV infection. J. Immunol. **151:** 5031–5040.
7. **Glezen, W. P., and F. W. Denny.** 1973. Epidemiology of acute lower respiratory disease in children. N. Engl. J. Med. **288:**498–505.
8. **Glezen, W. P., A. L. Frank, L. H. Taber, and J. A. Kasel.** 1984. Parainfluenza virus type 3: seasonality and risk of infection and reinfection in young children. J. Infect. Dis. **150:**851–857.
9. **Glezen, W. P., F. A. Loda, W. A. Clyde, Jr., et al.** 1971. Epidemiologic patterns of acute lower respiratory disease of children in a pediatric group practice. J. Pediatr. **78:**397–406.
10. **Hall, C. B., and R. G. Douglas, Jr.** 1975. Clinically useful method for the isolation of respiratory syncytial virus. J. Infect. Dis. **131:**1–5.
11. **Hall, C. B., J. M. Geiman, B. B. Breese, and R. G. Douglas.** 1977. Parainfluenza viral infection in children: correlation of shedding with clinical manifestations. J. Pediatr. **91:**194–198.
12. **Henrickson, K. J.** 1994. Lower respiratory viral infections in immunocompetent children, p. 59–96. *In* S. C. Aronoff. Advances in pediatric infectious diseases—1994. Mosby-Year Book, Inc., Chicago.
13. **Henrickson, K. J.** 1995. Human parainfluenza viruses, p. 481–494. *In* E. H. Lennette (ed.), Diagnostic procedures for viral rickettsial, and chlamydia infections—1995. American Public Health Association, Washington, D.C.
14. **Henrickson, K. J., S. M. Kuhn, and L. L. Savatski.** 1994. Epidemiology and cost of infection with human parainfluenza virus types 1 and 2 in young children. Clin. Infect. Dis. **18:**770–779.
15. **Henrickson, K. J., S. M. Kuhn, L. L. Savatski, and J. Sedmak.** 1994. Recovery of human parainfluenza virus types one and two. J. Virol. Methods **46:**189–206.
16. **Henrickson, K. J., and A. Portner.** 1990. Antibody response in children to antigenic sites or human PIV-3 HN: correlation with known epitopes mapped by monoclonal antibodies. Vaccine **8:**75–80.
17. **Henrickson, K. J., and L. L. Savatski.** 1992. Genetic variation and evolution of human parainfluenza virus type 1 hemagglutinin-neuraminidase: analysis of 12 clinical isolates. J. Infect. Dis. **166:**995–1005.
18. **Henrickson, K. J., and L. Savatski.** 1996. Two distinct human parainfluenza virus type 1 genotypes detected during the 1991 Milwaukee epidemic. J. Clin. Microbiol. **34:**695–700.
19. **Hetherington, S. V., A. S. Watson, K. A. Scroggs, and P. Portner.** 1995. Human parainfluenza virus type 1 evolution combines cocirculation of strains and development of geographically restricted lineages. J. Infect. Dis. **169:**248–252.
20. **Holodniy, M., D. A. Katzenstein, D. M. Israelski, and T. C. Merigan.** 1994. Reduction in plasma human immunodeficiency virus ribonucleic acid after dideoxynucleoside therapy as determined by the polymerase chain reaction. J. Clin. Invest. **88:**1755–1759.
21. **Murphy, B. R., G. A. Prince, P. L. Collins, et al.** 1988. Current approaches to the development of vaccines effective against parainfluenza and respiratory syncytial viruses. Virus Res. **11:**1–15.
22. **Skolnik, N. S.** 1989. Treatment of croup: a critical review. Am. J. Dis. Child. **143:**1045–1049.
23. **Welliver, R., D. T. Wong, T. S. Choi, and P. L. Ogra.** 1982. Natural history of parainfluenza virus infection in childhood. J. Pediatr. **101:**180–187.