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Rapid Detection and Identification of Human Parainfluenza Viruses 1, 2, 3 and 4 in Clinical Samples of Pediatric Patients by Multiplex Reverse Transcription-PCR.

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Abstract

We describe a multiplex reverse transcription polymerase chain reaction (RT-PCR) assay able to detect and differentiate all known human parainfluenza viruses. Serial dilution experiments with reference strains comparing cell culture isolation and multiplex RT-PCR showed sensitivities between 4 10^{-4} and 32 $TCDI_{50}$, depending on the virus. As few as ten plasmids containing HPIV PCR products could be detected in all cases. When 230 nasopharyngeal exudates of pediatric patients hospitalized for lower respiratory illness were tested, our multiplex RT-PCR assay detected 75 HPIVs (29 HPIV3, 28 HPIV1, 10 HPIV4 and 8 HPIV2), while only 44 of them (22 HPIV1, 15 HPIV3, 6HPIV2, 1HPIV4) grew in

cell culture and only 29 (17 HPIV1, 9 HPIV3, 2 HPIV2, 1 non typed) were detected by direct immunofluorescence (IF) staining with monoclonal antibodies. Our multiplex RT-PCR assay was more sensitive than either cell culture isolation or IF for the detection of HPIV infections. Also, HPIV4 was more frequent than HPIV2 in our series, suggesting that it has been underestimated as lower respiratory tract pathogen due to the insensitivity of classical detection techniques.

Introduction

Human parainfluenza viruses (HPIVs) are non-segmented RNA viruses that belong to the *Paramyxovirus* (HPIV1,-3) and *Rubulavirus* (HPIV2,-4) genera of the family *Paramyxoviridae* (22). HPIV 4 is further divided into two subtypes, A and B, based on antigenic differences (1). HPIV-1,-2 and -3 are important respiratory pathogens, a major cause of croup , bronchiolitis and pneumonia in infants and very young children (21, 30). They have been estimated to account for 40% of acute respiratory tract illnesses in children from which a virus is recoverable and for 20% of hospitalized pediatric respiratory illnesses (25). HPIV4, has been traditionally associated with mild upper respiratory tract infections in children and adults (5).

Etiological diagnosis of HPIV infections cannot be based exclusively on clinical signs and symptoms because other pathogens cause similar syndromes. Classic diagnostic methods, like viral isolation and serology, can result in delays of several weeks before test results are available and show variable diagnostic efficiencies depending on the virus (6). Rapid diagnosis is desirable both to assist therapeutic decisions and to prevent nosocomial infections (6, 20). Direct antigen detection on respiratory specimens provides rapid results but different methods like immunofluorescence (16, 24, 28, 31) or enzyme immunoassay (27) have reported variable sensitivities depending on the virus. Molecular

techniques based on reverse transcription polymerase chain reaction (RT-PCR) constitute another approach to rapid diagnosis with expected high sensitivity. RT-PCR assays have been applied for detection of HPIV1 and HPIV3 (10, 14, 16) in monospecific assays or in simultaneous amplification of HPIVs with other respiratory viruses (9, 11, 15, 23); multiplex RT-PCR assays permit detection of several viruses simultaneously, consuming less reagents, sample and time than single RT-PCR assays, wich can be an important consideration for high volume diagnostic laboratories.

In a previous report (8) we described a multiplex RT-PCR for detection of HPIV-1,-2, and -3. In the present work, this assay is evaluated with i) a more complete panel of clinical samples, ii) modified to simplify the protocol by using one step retrotranscription and first amplification and iii) using an internal control to prevent false negatives. This assay also includes primers for detection of HPIV4. The enlargement of the multiplex RT-PCR to detect HPIV4 was motivated by previous reports suggesting that HPIV4 can be underestimated as a cause of lower respiratory tract disease (19, 26).

Materials and Methods

Virus. Prototype strains of HPIV1 (C35), HPIV2 (Greer) , HPIV3 (C-243), HPIV4A (M-25) and HPIV4B (19.153) were obtained from the Center for Disease Control and Prevention (CDC) collections. Wild HPIV cell cultures isolates (two each HPIV1, 2 and 3) from multiple respiratory seasons were obtained from the Spanish National Center for Microbiology archives as well as three individual isolates of influenza A virus (2 H3 and 1 H1), three influenza B virus , two adenovirus, two mumps virus, two measles virus and three respiratory syncytial virus.

Clinical samples. Two hundred and thirty nasopharyngeal aspirates were collected from pediatric patients with lower tract respiratory illness recruited for a long-term prospective study of severe respiratory repeated infections. These patients were attended in the emergency room or required hospitalization at the Severo Ochoa Hospital in Leganés (Madrid, Spain). These samples corresponded to the periods of maximum HPIV activity detected by viral isolation and antigen detection on a larger series covering a complete year. One hundred and eighty four specimens covering from September 1997 to January 1998 were tested retrospectively and the remaining 46 covering from June 1998 to July 1998 prospectively (see study design below). Specimens were obtained with an aspirator device, placed in viral transport medium and processed within 24 h of collection. When they arrived in the laboratory they were diluted and homogenized with 5 ml of phosphate buffered solution (PBS) before testing. Three aliquots of 0.5 ml were stored at -70ºC.

Indirect immunofluorescence assay (IF). IF was performed directly on the respiratory secretions by standard methods using commercial reagents (Chemicon, Temecula, California, U.S.A) except for HPIV4A, 4B specific monoclonal antibodies (Mabs) that were obtained from CDC.

Virus isolation. Human laryngeal epidermoid carcinoma (HEp-2) cells, human lung mucoepidermoid carcinoma cells (NCI-H292) , Madin-Darby canine kidney (MDCK) and human embryonic lung fibroblast (Fp) cell cultures were used for primary viral isolation. Tubes with 80% confluent monolayers were inoculated with 0,3 ml of homogenized samples. HEp-2, Fp and MDCK cultures were enhanced to adsorb by centrifugation for 45 min. at 3000 rpm. HEp-2 and Fp were fed with 2 ml of 2% fetal calf serum in Basal Medium Eagle (BME). For MDCK, Minimal Essential Medium

(MEM) was supplemented with 3 μg/ml of trypsin. NCI-H292 was adsorbed for an hour without centrifugation and fed with MEM supplemented with 1,5 μg/ml of trypsin (4). Cell monolayers were observed for cytopathic effect (CPE) every 48 hours. When CPE was observed, or after ten days, the monolayer was scraped and tested for respiratory viruses by IF as described above. The IF negative cultures were subcultured and submitted again to blind IF after ten days.

Primers. Specific primers for HPIV1, 2 , 3 (8) and internal control (2) were previously published. For HPIV4 primer design, sequences of the phosphoprotein P gene were obtained from Genbank and aligned by using the Wisconsin Analysis Package version 8 (Genetics Computer Group ,Madison, WIS, USA). External primers PI4P+ (5'- CTGAACGGTTGCATTCAGGT - 3' [genome sense, bases 11-39]) and PI4P- (5'-AGGACTCATTCTTGATGCAA-3' [genome antisense, bases 433-452]) were chosen from conserved regions between HPIV4A and 4B subtypes. A second pair of internal primers PI4S+ (5'-AAAGAATTAGGTGCAACCAGTC-3' [genome sense, bases 158-179]) and PI4S- (5'-GCTGCTTATGGGATCAGACAC-3' [genome antisense, bases 382-402]) were selected for the nested reaction using the same criteria. Subtype A and B specific primers PI4SA+ (5'-ATGATGGTGGAACCAAGATT- 3' [genome sense, bases 226-245]) and PIASB+ (5-AACCAGGGAAACAGAGCTC- 3' [genome sense, bases 320-339]) were also selected within the first amplification fragment; PI4P+ in the 5' noncoding region and the rest of the primers in the P/V common region of the phosphoprotein P (19).

RNA extraction, RT and primary amplification. RNA was extracted from clinical samples and virus isolates as described previously (3). Briefly, 50 μl of each sample was treated with 200 μl of an extraction buffer (4 M guanidium thiocyanate, 0.5% N-lauryl sarcosine, 1 mM dithiothreitol, 25 mM

sodium citrate, 0,1 mg/ml of glycogen) including 100-molecules of a plasmid with an insert of the polymerase gene of the pseudorabies herpesvirus DNA as internal control template, followed by isopropanol and 70% ethanol precipitations. The pellet was resuspended in 10 µl of ribonuclease free water. Single step RT-amplification reaction was performed using the Promega Acces RT-PCR system kit (Promega Corporation, Madison, WI, USA) consisting of a PCR mixture containing 3 mM MgSO4, 500 mM of dNTPs, 0.5 µM HPIVs 1-4 and 0.2 µM internal control primary reaction primers, 10 µl of AMV/Tfl 5x buffer reaction, 5 units of AMV RT and 5 units of Tfl DNA Polymerase. PCR mixtures was overlaid with mineral oil and 5 µl of extracted RNA was added to a final volume of 50 µl. The tubes were centrifugated for a few seconds and placed in an Autocycler Plus Termocycler (Linus, Cultek S. L., Spain) programmed for 45 min of RT extension at 48ºC, 2 min of an intermediate AMV-RT inactivation at 94ºC and 30 cycles of 1 min of denaturation at 94ºC, 1 min of annealing at 50ºC, and 1 min of elongation at 72ºC. A final extension of 5 min was performed in the last cycle.

Nested amplification and product detection. One µl of the primary amplification product was added to 49 µl of a PCR mixture containing, 10 mM Tris-HCl (pH 8,3); 50 mM KCl; 3 mM MgCl2; 200 µM each dATP, dGTP, dCTP, and dTTP; 0.2 µM each nested HPIV 1-4 and internal control primers and 1,25 U of Taq polymerase (AmpliTaq, Perkin-Elmer Cetus). The thermal cycle program consisted of 30 cycles of 1 min of denaturation at 94ºC, 1 min of annealing at 58ºC, and 1 min of elongation at 72ºC and additional 2 min of denaturation preceded the first cycle and elongation was extended to 6 min in the last cycle. For HPIV4 subtyping, primers PIS4A+, PIS4B+ and PIS4- were used alone.

PCR products were sized by gel electrophoresis on 2% agarose containing 0,5 g/ml of ethidium bromide in Tris-Borate-EDTA (TBE) buffer and were visualized under UV light. Expected band sizes were 317bp for HPIV1, 203bp for HPIV2, 102bp for HPIV3 , 246 bp for HPIV4 and 140pb for the

internal control. Positive samples showed the specific HPIV band and the internal control band. When only internal control band appeared samples were considered negative. Samples showing no band were retested and those lacking any band after repetition were assumed to contain enzyme inhibitors. For HPIV4 subtyping, expected bands were 178 bp for HPIV4A and 84 bp for HPIV4B.

Standard precautions were taken throughout the procedure to avoid cross-contamination. Pipetting was performed with aerosol resistant tips. Reagent preparation, RNA extraction and sample addition to RT-primary amplification tubes and amplicon addition to nested tubes were performed in three different vertical flow cabinets. Two different thermal cyclers were used for primary and nested amplification and product detection was performed in a separate location. Negative and low tittered positive controls were included in every assay. All positive results were confirmed by retesting from a different specimen aliquot. In case of disagreement, a third RT-PCR was performed to decide the result.

PCR product cloning. Primary amplification products from prototype strains of HPIVs were purified using the GeneCleanII kit and ligated into pGEM-T plasmid vectors using the pGEM-T Plasmid Vector System (Promega ,Madison,WI, USA) following manufacturer directions. Plasmids were transformed into high efficiency competent cells (Epicurian coli XL1-Blue, Stratagene cloning systems, La Jolla, CA, USA) by electroporation. Transformants were selected in LB/ ampicillin / IPTG-X-Gal plates and the presence of the expected insert was confirmed with PCR. Plasmids were purified with the Wizard Plus SV Minipreps kit (Promega, Madison, WI, USA). The number of plasmid copies on final suspensions was estimated by UV spectroscopy at 260 nm OD.

Study design.

Prospective panel. Viral isolation, antigen detection by IF using all HPIV-specific Mabs and RT-PCR were performed on all fresh specimens arriving at the laboratory during the study period. RT-PCR positive specimens were confirmed from frozen aliquots.

Retrospective panel. Because HPIV4A and HPIV4B Mabs were not used for cell culture screening prior to the study, frozen aliquots from all historical specimens were recultured in NCI-H292 as described and rescreened for all HPIVs by IF. Multiplex RT-PCR was performed on frozen aliquots and all HPIV4 positives were subtyped using the primary amplification products as template.

Results.

Evaluation of multiplex RT-PCR sensitivity and specificity. DNA bands of expected size were obtained by RT-PCR with all HPIV reference strains and all HPIV wild-type isolates. No amplification was observed with the other respiratory viruses tested. A comparison of RT-PCR with virus culture in NCI-H292 cells using serial tenfold dilutions of the HPIV reference strains obtained sensitivities of 0.01 $TCID_{50}$ for HPIV1, 0.02 $TCID_{50}$ for HPIV2, 32 $TCID_{50}$ for HPIV3, 0.001 $TCID_{50}$ for HPIV4A and 0.0004 $TCID_{50}$ for HPIV4B. In dilution experiments using plasmids containing cloned HPIV cDNA, RT-PCR was able to detect as few as 10 molecules for all HPIVs.

Evaluation of multiplex RT-PCR with clinical specimens. Of the 230 clinical specimens available for testing by RT-PCR, 17 (7.4%) exhibited microbial contamination in cell culture, 9 (2.6%)

had scarce respiratory tract epithelial cells by IF, and 4 were suspected of enzymatic inhibition when tested by RT-PCR. These specimens were excluded from the analysis for the respected assays. HPIV was detected in 45 (21.1%) of the remaining 213 specimens by cell culture, 29 (13.2%) of 221 by IF and 75 (33.2%) of 226 by mRT-PCR. In all cases, the same virus was detected by RT-PCR and culture. Other respiratory viruses were identified in 50 specimens. Temporal variations in detection rates by cell culture and RT-PCR of the different HPIV types were observed, suggesting differing patterns of circulation of each HPIV (Figure 1).

HPIVs were recovered from 31 of the 173 cultured samples belonging to the retrospective panel; HPIV1 from 23 (12.5%), HPIV2 from 6 (3.5%), HPIV3 from 2 (1.1%) and HPIV 4 from 1 (0.6%). One specimen previously culture positive for HPIV1 was negative on rescreening. IF detected HPIVs in 20 (11.2%) of 177 samples; 17 (9.6%) HPIV1, 2 (1.1%) HPIV2 and 1 (0.6%) HPIV3 . RT-PCR yielded 49 (27.2%) positive samples of 180 distributed as follows: 28 (15,2%) HPIV1, 8 (4,4%) HPIV2 , 3 (1,6%) HPIV3 and 10 (5.5%) HPIV4. All 10 HPIV4 were typed as HPIV4A with the subtype primers as well as the only isolated HPIV4 with MAbs. One sample previously positive for RSV was found to be HPIV4 positive by RT-PCR. Dual infections were not observed by either culture or IF.

Only HPIV3 was detected within the prospective panel. Thirteen (32.5%) of the 40 clinical samples with cell culture result rended viral isolation, as well as 9 (20.5%) of 44 by IF and 26 (56.5%) of 46 samples by RT-PCR. HPIV3 could be amplified in three samples known to contain adenoviruses. As in the retrospective panel, no coinfection was observed by IF or culture.

In summary (see table 1), RT-PCR improved the diagnosis of all HPIVs. From the 75 positive results by PCR, 22 corresponded to HPIV culture negative samples and 34 to negative samples by IF. Cell culture results were not available due to contamination for 8 of these PCR positive samples and not enough cells were recovered for IF in three of them. Comparison between PCR and IF for the 10 HPIV4 was not possible because specific MAbs were not used at the time of the HPIV4 circulation. Consequently, only 45/67 (67,1%) of the RT-PCR positives could be detected by cell culture and only 29/62 (46,7%) by IF.

Discussion

Our data demonstrate that all four HPIVs can be detected by multiplex RT-PCR with greater sensitivity than viral isolation, the gold standard for HPIV detection. Previously reported mRT-PCR assays for HPIVs were not designed to detect HPIV4 (8, 11, 15, 23) or HPIV2 (15). Moreover, several design flaws were noted in these studies: i) no comparisons were made with other diagnostic techniques (15), ii) no culture negative specimens were included, and some HPIV culture positive specimens were negative by mRT-PCR (8), iii) mRT-PCR improved HPIV detection over cell culture isolation only for HPIV1, and iv) none of the clinical specimens tested were positive for HPIV1 or HPIV2 and the sensitivity of the assay for HPIV1 was low (250 $TCDI_{50}$) (11). In comparison, the lowest measured sensitivity of our mRT-PCR assay was 32 $TCDI_{50}$ for HPIV3, and we were able to detect as few as 10 plasmids containing cloned DNA of all HPIV types. More importantly, the sensitivity of our mRT-PCR assay was greater than IF or culture with clinical specimens for all HPIV types, particularly HPIV3 and HPIV4. Of the 28 specimens that were positive only by mRT-PCR, 10 were compromised by microbial contamination or coinfection with other faster growing viruses that could mask HPIVs

or contained too few epithelial cells for reliable IF detection. Detection of HPIVs in the remaining 18 specimens is likely due to the higher sensitivity of our mRT-PCR assay; false positives due to cross contamination could account for this difference, but it seems unlikely, since the temporal distribution of mRT-PCR and cell culture positives match, and all mRT-PCR positives were confirmed from a separate specimen aliquot. RT-PCR seems to be a better method for detecting co-infection, often missed by culture and IF, as observed before (7, 14).

Inclusion of an internal positive control template in all clinical specimens prevented reporting of false negative results in 13 cases (data not shown), illustrating the importance of establishing assay controls specific for each specimen. Four specimens repeatedly inhibited the internal control, probably due to the presence of enzyme inhibitors. The remaining nine specimens were positive on repetition, suggesting handling error as the cause of the first inhibition. Removal of supernatant during the RNA precipitation steps of the sample extraction procedure could be a critical point, since the RNA pellets can be lost. The inclusion of the internal template in the extraction buffer can control for this possibility as well (3). Consequently, enzyme inhibitors or mishandling could account for mRT-PCR negativity among cell culture positive samples observed in studies that did not use internal controls (8, 12). Alternatively, unexpected primer mismatches with different virus strains could also account for lack of reactivity. To address this possibility, our HPIV1, 2 and 3 specific primers were designed against multiple sequences of each virus and tested against temporally and geographically diverse isolates (8). However, only one reference strain for each HPIV4 subtype and 10 HPIV4a isolates from a single outbreak in Spain were available for this study. Additional isolates of both HPIV4 subtypes will be required to complete the evaluation of this method.

Of particular interest was the high number of HPIV4 identified in this study. HPIV4 appears to be the most difficult HPIV to grow in cell culture and is rarely isolated despite serologic studies showing that it is relatively ubiquitous (5). Monoclonal antibodies to HPIV4 have only recently become available commercially, which has also hindered identification of this virus (26). A recent study of hospitalized patients identified several cases with severe respiratory illnesses due to HPIV4 (19), suggesting that HPIV4 are not the banal respiratory pathogens once thought. Even though HPIV1 and HPIV3 were the most prevalent HPIVs identified in this study, as expected (13, 29), HPIV4 infections were more frequent than HPIV2, and were associated with severe clinical disease. In conclusion, our mRT-PCR assay provides both sensitive and specific identification of HPIVs in clinical specimens, especially HPIV4, whose clinical impact may have been underestimated due to the insensitivity of classical diagnostic techniques.

References.

1. **Canchola, J., A. J. Vargosko, H. W. Kim, R. H. Parrot, F. Chritsmas, B. Jeffries, and R. M. Chanock.** 1964. Amer. J. Hyg. **79:**357-364.

2. **Casas, I., A. Tenorio , J. M. Echevarría, P. E. Klapper, and G. M. Cleator.** 1997. Detection of enteroviral RNA and specific DNA of herpesviruses by multiplex genome amplification. J. Virol. Methods. **66:**39-50.

3. **Casas I., L. Powel, P. E. Klapper, and G. M. Cleator.** 1995. New method for the extraction of viral RNA and DNA from cerebrospinal fluid for use in the polymerase chain reaction assay. J. Virol. Methods. **53:**25-36.

4. **Castells, E., V. G. George, and J. C. Hierholzer.** 1990. NCI-H292 as an alternative cell line for the isolation and propagation of the human paramyxoviruses. Arch. Virol. **115:** 277-288.

5. **Collins, P. L., R. M. Chanock, and K. McIntosh.** 1996. Parainfluenza viruses, p. 1205-1241. In B. N. Fields, D. M. Knipe, and P. M. Howley (ed.) Virology, 3rd ed. Lippincott-Raven Publications, Philadelphia, Pa.

6. **Downham, M. A. P. S., J. McQuillin and, P. S. Gardner.** 1974. Diagnosis and clinical significance of parainfluenza virus infections in children. Arch. Dis. Child. **49:**8-15.

7. **Drews, A. L., R. L. Atmar, W. P. Glezen, B. D. Baxter, P. A. Piedra, and S. B. Greenberg.** 1997. Dual respiratory virus infections. Clin. Infect. Dis. **25:**1421-1429.

8. **Echevarría, J. E., D. D. Erdman, E. M. Swierkosz, B. P. Holloway, and L. J. Anderson.** 1998. Simultaneous detection and identification of human parainfluenza viruses 1, 2 and 3 from clinical samples by multiplex PCR. J. Clin. Microbiol. **36:**1388-1391.

9. **Eugene-Ruellan, G., F. Freymuth, C. Bahloul, H. Badrane, A. Vabret, and N. Tordo.** 1998. Detection of respiratory syncytial virus A and B and parainfluenzavirus 3 sequences in respiratory tracts of infants by a single PCR with primers targeted to the L-polymerase gene and differential hybridization. J. Clin. Microbiol. **36:**796-801.

10. **Fan, J., and K. J. Henrickson.** 1996. Rapid diagnosis of human parainfluenza type 1 infection by quantitative reverse transcription-pcr-enzyme hybridization assay. J. Clin. Microbiol. **34:**1914-1917.

11 **Fan, J., K. J. Henrickson,** and **L. L. Savatsky.** 1998. Rapid simultaneous diagnosis of infections with respiratory syncityal viruses A and B, influenza viruses A and B, and human parainfluenza virus

type 1, 2, and 3 by multiplex reverse transcription-polymerase chain reaction hybridization assay (Hexaplex). Clin. Infect. Dis. **26:**1397-1402.

12. **Freymuth, F., A. Vabret, F. Galateau-Salle, J. Ferey, G. Eugene, J. Petitjean, E. Gennetay, J. Brouard, M. Jokik, J-F. Duhamel, and B. Guillois.** 1997. Detection of respiratory syncytial virus, parainfluenzavirus 3, adenovirus and rhinovirus sequences in respiratory tract of infants by polymerase chain reaction and hybridization. Clin. Diagn. Virol. **8:**31-40.

13. **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.

14. **Gilbert, L. L., A. Dakhama, B.M. Bone, E. E. Thomas, Hegele R.G.** 1996. Diagnosis of viral respiratory tract infections in children by using a reverse transcription-PCR panel. J. Clin. Microbiol. **34:**140-143.

15. **Grondahl, B., W. Puppe, A. Hoppe, Y. Kuhne, J. A. Weigl, and H. J. Schmitt.** 1999. Rapid identification of nine microorganisms causing acute respiratory tract infections by single-tube multiplex reverse transcription-PCR: feasibility study. J. Clin. Microbiol. **37:**1-7.

16. **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.

17. Karron, R. A., J. L. Frohelich, L. Bobo, R. B. Belshe, and R. Yolken. 1994. Rapid detection of parainfluenza virus type 3 RNA in respiratory specimens: use of reverse transcription-PCR-enzyme immunoassay. J. Clin. Microbiol. **32:**484-488.

18. **Kondo, K., H. Bando, M. Tsurudome, M. Kawano, M. Nishio, and Y. Ito.** 1990. Sequence analysis of the phosphoprotein (P) genes of human parainfluenza type 4A and 4B viruses and RNA

editing at transcript of the P genes: The number of G residues added is imprecise. Virology. **178:**321-326.

19. Lindsquist S. W.**, A. Darnule, A. Istas, and Demmler G. J.** 1997. Parainfluenza virus type 4 infections in pediatric patients. Pediatr. Infect. Dis. J. **16:**34-38.

20. **Moisiuk, S. E., D. Robson, L. Klass, G. Kliewer, W. Wasyliuk, M. Davi, and P. Plourde.** 1998. Outbreak of parainfluenza virus type 3 in an intermediate care neonatal nursery. **17:** 49-53.

21. **Monto, A.S.** 1973. The Tecumseh study of respiratory illness. V. Patterns of infection with the parainfluenzaviruses. Am. J. Epidemiol. **97:**338-348.

22. **Murphy, F. A., C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jawis , G. P. Martelli, M. A. Mayo, and M.D. Summers (eds.).** 1995. Virus taxonomy. Sixth report of the international commitee on taxonomy of viruses. Arch. Suppl. Virol. **10:**1-586.

23. **Osiowy, C**. 1998. Direct detection of respiratory syncytial virus, parainfluenza virus, and adenovirus in clinical respiratory specimens by a multiplex reverse transcription-PCR assay. J. Clin. Microbiol. **36**:3149-3154.

24. **Ray, C. G., and L.L. Minnich.** 1987. Efficiency of inmunofluorescence for rapid detection of common respiratory viruses. J. Clin. Microbiol. **25:**355-357.

25. **Reed, G., P. H. Jewett, J. Thompson, S. Tollefson, and P. F. Wright.** 1997. Epidemiology and clinical impact of parainfluenza virus infectious in otherwise healthy infants and young children < 5 years old. J. Infect. Dis. **175:**807-813.

26. **Rubin, E. E., P. Quennec, and J. C. McDonald.** 1993. Infectious due to parainfluenza type 4 in children. Clin. Infect. Dis. **17:**998-1002.

27. **Sarkkinen, H. K., P. E. Halonen, and A.A. Salmi. 1981.** Type specific detection of parainfluenza viruses by enzyme-inmunoassay and radioinmunoassay in nasopharyngeal specimens of patients with acute respiratory disease. J. Gen. Virol. **56:**49-57.

28. **Stout, C., M. D. Murphy, S. Lawrence, and S. Julian.**1989. Evaluation of a monoclonal antibody pool for rapid diagnosis of respiratory viral infections. J. Clin. Microbiol. **27:**448-452.

29. **Tellez, A., P. Perez-Breña, M. V. Fernández-Patiño, P. León, P. Anda, and R. Nájera.** 1990. Acute respiratory disease in Spain: seven years of experience. Rev. Infect. Dis. **12:**745-753.

30. **Vainionpää, R., and T. Hyypiä.** 1994. Biology of parainfluenza viruses. Clin. Microbiol. Rev. **7:**265-75 .

31. **Wong, D. T., R.C. Welliver, K. R. Riddlesberger, M. S. Sun and, P.L. Ogra.** 1982. Rapid diagnosis of parainfluenzavirus infection in children. J. Clin. Microbiol. **16:**164-167.