

Isolation & molecular characterization of human parainfluenza virus in Chennai, India

C.P. Indumathi, P. Gunanasekaran, K. Kaveri, Kavita Arunagiri, S. Mohana, A. Khaleefathullah Sheriff, B.V. SureshBabu, P. Padmapriya, R. Senthilraja & Gracy Fathima

Department of Virology, King Institute of Preventive Medicine & Research, Chennai, India

Received April 25, 2013

Background & objectives: Human parainfluenza virus (HPIV) accounts for a significant proportion of lower respiratory tract infections in children as well as adults. This study was done to detect the presence of different subtypes of HPIV from patients having influenza like illness (ILI).

Methods: Throat and nasal swabs from 232 patients with ILI who were negative for influenza viruses were tested by multiplex reverse transcription polymerase chain reaction (mRT-PCR) for the detection of human parainfluenza virus. All samples were inoculated in rhesus monkey kidney (LLC-MK2) cell line.

Results: Of the 232 samples, 26 (11.2%) were positive by mRT-PCR and nine (34.6%) showed cytopathic effect with syncytium formation for HPIV and all were HPIV-3 serotype, other serotypes like 1,2,4 were negative. The HPIV-3 strains (*HN* gene) were sequenced and analysed. Two novel mutations were identified at amino acid residues 295 and 297.

Interpretation & conclusions: The mRT-PCR assay offers a rapid, sensitive and accurate diagnostic method for detection of HPIV which enables early detection and control. In our study there was a predominance of HPIV among 1-5 yr age group and the school going age group was less affected. Further studies need to be done to characterize HPIV isolated from different parts of the country.

Key words Human parainfluenza virus - mRT-PCR - isolation - ILI - prevalence

Human parainfluenza virus (HPIV) is known to cause acute respiratory infections (ARI) including lower respiratory tract infection, which is a leading cause of morbidity and mortality in infants and young children world-wide¹⁻³. HPIV belongs to the *Paramyxoviridae* family, subfamily *Paramyxovirinae*, and is classified into four serotypes (HPIV-1, HPIV-2, HPIV-3, and

HPIV-4). Serotype 4 can be further subdivided into two antigenic subtypes, HPIV-4A and HPIV-4B⁴. Infection with HPIV in immuno-compromised children is known to be associated with a range of diseases, from mild upper-respiratory symptoms to severe disease requiring mechanical ventilation and leading to death⁵. Of the four recognized serotypes, HPIV-3 is most

commonly associated with serious lower respiratory tract illness, followed by HPIV-1 and HPIV-2; HPIV-4 is rarely associated with serious illness⁶. HPIV is second only to respiratory syncytial virus (RSV) as a cause of hospitalizations for acute respiratory infection among children aged <5 yr; 2-17 per cent of such hospitalizations are due to HPIV infection⁶. It is important to know the mechanism resulting in genetic and antigenic diversity of HPIV for controlling the pathogen.

The use of classic diagnostic methods like viral isolation and serology is time consuming and takes several weeks till the results are available, and hence these methods are less useful for making therapeutic decisions⁷. Cell culture, often considered to be the gold standard, is delicate and sometimes too slow for it to be useful for diagnosis. Direct antigen detection methods are widely used for rapid diagnosis of HPIV infections⁸⁻¹⁰, but results can be variable^{5,11}. Multiplex reverse transcription polymerase chain reaction (mRT-PCR) assay can be a sensitive and specific tool for rapid diagnosis of HPIV infections^{5,12,13}. In this study, mRT-PCR was performed for the simultaneous detection of HPIV-1,2,3 and 4 in samples collected from patients with influenza like illness (ILI). The isolation positive samples were sequenced and analysed.

Material & Methods

Clinical samples: Throat and nasal swabs were collected during January 2011 to August 2012 from patients with ILI belonging to different age groups

attending outpatient departments (OPD) of tertiary care government hospitals in Chennai, Tamil Nadu, India [Institute of Child Health and Hospital (52 samples), Royapettah Government Hospital (20 samples), Saidapet Government Hospital (26 samples), Rajiv Gandhi Government Hospital (39 samples), Government Peripheral Hospital (42 samples), Kilpauk Medical College (29 samples) and Stanley Medical College (24 samples)].

Sample collection and processing: The study was conducted in the department of Virology, King Institute of Preventive Medicine and Research, Chennai. A total of 232 throat and nasal swabs were collected from infants, children, adolescents and adults. These samples were collected from patients with symptoms like fever, chills/rigors, nasal discharge, cough, sore throat, breathelessness and headache. Clinical samples were collected in 3 ml of cold viral transport medium (Hank's balanced salt solution) containing 0.5 per cent gelatin and transported in cold chain to the laboratory.

Nucleic acid extraction: QIAmp viral Qiagen RNA extraction kit was used for RNA extraction from clinical samples and Invitrogen superscript III platinum one step RT-PCR system for the detection of RNA. Oligonucleotide primers against hemagglutinin neuraminidase (HN) gene were used to detect HPIV-1, 2, 3¹⁴ and for HPIV-4, primers were directed against phosphoprotein gene¹⁵ (Table I).

Table I. Oligonucleotide primers for human parainfluenza virus

Primers	Sequence	Base pairs	Gene
HPIV-1 F ¹⁴	CCGGTAATTTCTCATACTATG	747-768	HN
HPIV-1R	CCTTGGAGCGGAGTTGTTAAG	694-714	HN
HPIV-2 F ¹⁴	AACAATCTGCTGCAGCATT	803-822	HN
HPIV-2 R	ATGTCAGACAATGGGCAAAT	620-639	HN
HPIV-3 F ¹⁴	CTCGAGGTTGTCAGGATATAG	632-652	HN
HPIV-3 R	CTTGGGAGTTGAACACAGTT	900-920	HN
HPIV-4A F ¹⁵	ATGATGGTGGAAACCAAGATT	240-259	P
HPIV-4 A R	AACCAGGAAACAGAGCTC	1103-1084	P
HPIV-4B F ¹⁵	CTGAACGGTTGCATTGAGGT	333-351	P
HPIV-4B R	AGGACTCATTCTTGATGCAA	1103-1084	P

Superscript numerals denote reference numbers

Optimized reagents and PCR cycling condition for HPIV: For primary PCR amplification, RNA was added to PCR mixture containing buffer, water, Taq polymerase, primers to a final volume of 50 μ l. Amplification was performed on ABI Thermal Cycler, USA, with cyclic conditions of 50°C for 30 min, 94°C for 15 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for one min and 72°C for 10 min for elongation¹⁴. PCR products were visualized in 1.5 per cent agarose gel electrophoresis and molecular weight marker of 100 bp used. Expected band sizes for HPIV-1,2,3 and 4 were 371, 507, 189 and 451 bp, respectively.

Virus and reagents: The human parainfluenza virus was propagated in rhesus monkey kidney cell line LLC-MK2 [National Institute of Virology (NIV), Pune] at 37°C with 5 per cent CO₂ in humidified conditions. The cells were maintained in Eagles minimal essential medium (Sigma, USA), supplemented with 10 per cent foetal bovine serum (FBS, Hi media, India) and 0.01 per cent antibiotic-antimycotic solution penicillin, streptomycin, kanamycin and fungizone, and trypsin-EDTA.

Viral isolation: All samples were inoculated into tissue culture T 25cm² flasks of LLC-MK2 cell line (NIV, Pune), allowed to adsorb for one hour and incubated at 37°C. Cell monolayers were observed for cytopathic effect (CPE) every 48 h. HPIV positive clinical isolates demonstrated focal rounding and destruction, occasional syncytia on initial isolation. The samples which showed CPE were confirmed by mRT-PCR.

Sequence analyzing: Three representative *HN* genes of HPIV were detected by mRT-PCR and isolation positive samples were sequenced and analysed. The HPIV-3 standard strain was downloaded along with strains from different countries from NCBI database (www.ncbi.nlm.nih.gov). Strain-gi/168481518/swine/USA/2009 was used as out group for construction of phylogenetic tree. All the sequences were analyzed by MEGA (version 5) program (mega.software.informer.com/5.0/) using maximum likelihood method with p distance¹⁶.

Results

Evaluation of multiplex reverse transcription PCR with clinical specimens: Oligonucleotide primers were used to amplify *HN* gene of HPIV (Table I). A total of 232 samples were tested for HPIV by mRT-PCR, of which 26 (11.2%) were positive for HPIV-3, other serotypes were negative. Positive samples showed amplicon size 189 bp for HPIV-3 (Fig. 1). Twenty mRT-PCR positives were among the paediatric age group (less than 12 yr) and six positives were in the group more than 12 yr.

Symptoms analyses for HPIV cases: In this study, 151 males were tested and 16 (10.5%) were positive. Among the 81 females tested, 10 (12.3%) were positive for HPIV-3 by mRT-PCR. Among all the signs and symptoms presented by the HPIV positive cases sore throat and, breathlessness were significant when compared with the negative cases ($P < 0.05$) (Table II).

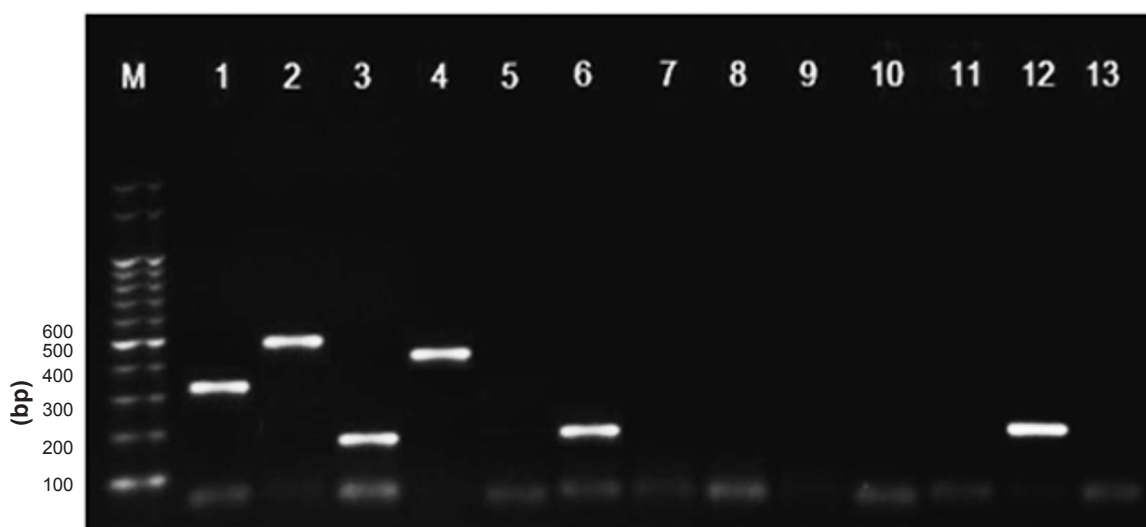


Fig. 1. Results of multiplex reverse-transcription (RT)-PCR with clinical samples and controls. Lane M, Marker; Lane 1, HPIV-1(Positive control); Lane 2, HPIV-2(PC); Lane 3, HPIV-3(PC); Lane 4, HPIV4(PC); Lane 5, Negative; Lane 6, HPIV3(clinical sample); Lanes 7 to 11, Negative; Lane 12, HPIV-3 (clinical sample); Lane 13, Negative control.

Table II. Clinical manifestations of human parainfluenza virus

Signs/symptoms	Samples positive for human parainfluenza virus (n=26)	Samples negative for human parainfluenza virus (n=206)
Fever	26	206
Chills/rigors	5 (19.2)	49 (23.7)
Nasal discharge	18 (69.2)	160 (77.6)
Cough	23 (88.4)	198 (96.1)
Sore throat	14 (53.8)	45 (21.8)
Breathlessness	12 (46.1)	63 (30.5)
Headache	6 (23)	70 (33.9)
Values in parentheses are percentages		

Comparison of multiplex reverse transcription PCR with tissue culture techniques: Viral isolation was attempted using LLC-MK2 cell lines. Of the 232 samples subjected to viral isolation in LLC-MK2, nine (34%) were positive. Negatives were discarded after three passages, and the samples that showed mild changes in the cell morphology were passaged further. If these samples produced clear CPE these were confirmed by PCR, if CPE was not seen after two more passages, these were checked by PCR and if negative, discarded. Clear CPE was observed in the ninth passage in seven samples and two samples showed CPE in 5th passage. These samples were reconfirmed by RT-PCR as HPIV-3.

Virus and sequence analysis: Among the 26 positives, three representative samples from different age groups and three geographical regions within Tamil Nadu were chosen and subjected to sequencing. The sequences of HPIV-3 strains were submitted to NCBI. Their accession numbers are (JQ901411-JQ901413).

Comparison of sequence with other strains

Sequences of HPIV strains from different countries were retrieved and compared with our strains using BLAST (blast.ncbi.nlm.nih.gov). (Table III). Phylogenetic analysis of our strains (Fig. 2) showed that these were highly similar to strains from Nagasaki, Fukuoka and Melbourne strains (97% similarity) and formed a same clade, The other strains that were significantly related were Washington (1973 and 1979), Oklahoma (2009), Bethesda (1997), Delhi (2005 and 2006), Riyadh (2008 and 2009), Texas (1980, 1982 and

1983), Logan (2009), Ontario (2001) and Guangdong (2007). The similarity confirmed the circulation of HPIV in Chennai and also its similarity to the strains in different countries.

Amino acid analysis: In our strains two mutations were identified at 295 and 297 amino acid residue. At 295 residue, glycine was replaced by serine, which can act as protein functional centres and hydrophobic amino acid. At position 297, histidine was replaced by tyrosine. Histidine is an essential amino acid with a positively charged imidazole functional group.

Discussion

It is known that among respiratory viruses namely RSV, HPIV and influenza viruses A and B, RSV has been documented to be the most common pathogen¹⁷ followed by human influenza virus and HPIV. Among the HPIVs, serotype 3 has been predominantly reported^{2,18} as also found in the present study.

In our study HPIV positivity was seen during the monsoon months of August-September and post monsoon months of November-January, with peak positivity in November. But in northern hemisphere HPIV infections were more frequent from January to April¹⁹. In temperate climates, HPIV-3,4 were detected in spring, summer and late fall and seasonal incidence varied for HPIV-1,2 in Chinese children²⁰.

A high proportion of males were found to be infected with respiratory viruses as compared to females in our study which was similar to another study in Delhi²¹. The patients' median age was 20 months for HPIV-4 infections and 7-11 months for HPIV-1, 2 and 3 infections, but the clinical manifestations did not differ significantly between HPIV-1, -2, -3, and -4 infections²⁰. It is known that influenza virus infections are more common in the paediatric age group, likewise in our study there was a predominance of HPIV among 1-5 age group. HPIV-3 was the prevalent serotype.

In this study the most common mode of presentation was fever, followed by sore throat and cough like any other ILI. Since samples were not collected from patients with SARI (severe acute respiratory illness) and long term follow up was not done, the rate of hospitalization among the ILI cases was not assessed in our study. Additional studies that include hospitalized controls are needed to clarify the clinical importance of HPIV infection in adults with community-acquired lower respiratory tract infection. One of the limitations of our study was that screening for RSV was not undertaken, as this could have been of

Table III. Strains of human parainfluenza virus reported from other countries and from the present study

GI and Accession No.	Strain/country	Year
gi332709, AAA46848	USA	1993
gi332701, AAA46844	USA	1993
gi351001320, AEQ39012	Oklahoma410/2009/USA	2011
gi1262233, AAB48689	JS/USA	1997
gi193888390, ACF28540	Del/88/06/India	2008
gi193888394, ACF28542	Del/139/05/India	2008
gi193888388, ACF28539	Del/322/06	2008
gi309252629, ADO60288	Riyadh149/2009/Soudi Arabia	2012
gi309252627, ADO60287	Riyadh11/2008/Soudi Arabia	2012
gi332715, AAA46851	USA	1993
gi332713, AAA46850	USA	1993
gi332711, AAA46849	USA	1993
gi167594039, ABZ85673	14702/Canada	2009
gi409057, AAA46855	Canada-Ontario	2001
gi163866868, ABY47606	ZHYMgz01 China	2007
gi37958139, AAP35240	Australia-Melbourne	2004
gi215794089, ACJ70090	China- Gansu Provence	2011
gi58430688, BAD89145	Fukuoka-Japan	2009
JQ901411(AFM78693)	Chennai, India (present study)	2011
JQ901412(AFM78694)	Chennai, India (present study)	2011
JQ901413(AFM78695)	Chennai, India (present study)	2011
gi3510305, BAA32574	GP/Japan-Nagasaki	2009
gi10937875, NP_067152	GP/Japan-Nagasaki	2009
gi356651382, AET35008	Wash/1957c243/USA	2011
gi15209379, CAC51081	MK9/UK	2001
gi332721, 46856	Canada-Ontario	1993
gi332718, AAA46853	USA-Ohio	1993
gi 332707, AAA46847	Australia	1993
gi168481518, ACA24945/swine	USA	2009

Source: BLAST: <http://www.blast.ncbi.nlm.nih.gov>

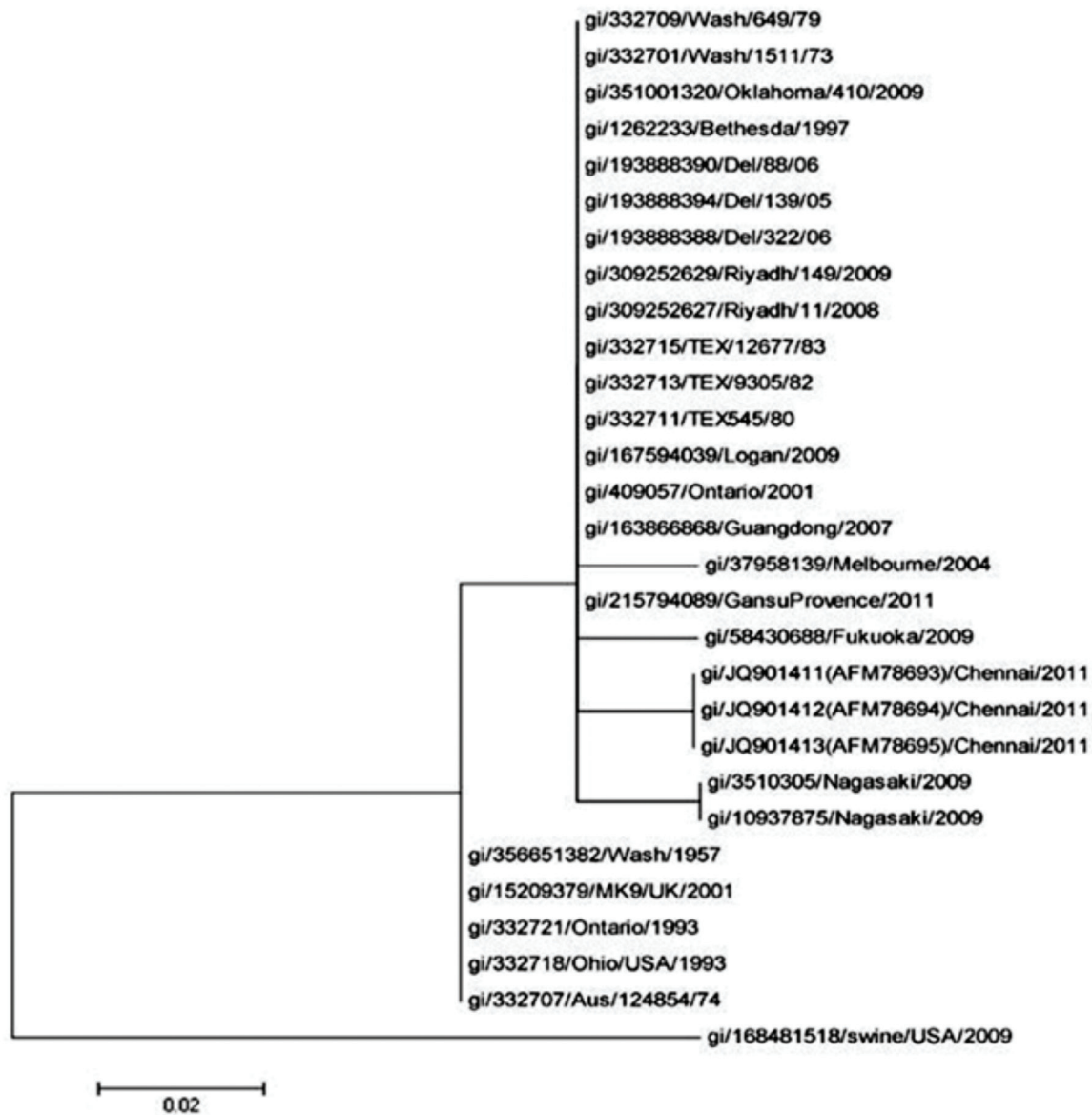


Fig. 2. Phylogenetic analysis of the deduced amino acid sequences of hemagglutinin neuraminidase gene of HPIV-3 to members of the family *Paramyxoviridae*. The tree was constructed by the maximum likelihood method with p distance.

great relevance in terms of the role of other respiratory pathogens in causing ILI.

In our study mRT-PCR assay was able to identify a greater number of positives in clinical specimens than cell culture as reported earlier^{4,11,15}. Of the 26 specimens that were mRT-PCR positive, only nine HPIV-3, strains could be isolated which was about one third of the total. Detection of HPIV in the remaining 17 specimens by mRT-PCR and not by isolation was due to the high sensitivity of mRT-PCR assay²². Hence compared to other methods, mRT-PCR seemed to be a better

method for detecting HPIV in suspected cases. There are similar studies indicating mRT-PCR to be a better diagnostic aid when compared with viral isolation and immunofluorescence tests¹⁵. The advantages are that mRT-PCR does not require cell line maintenance, as the isolation of HPIV requires multiple passages which is not only time consuming but also cost ineffective.

It was earlier identified that the point mutation at residues 278 and 281 coded for a single amino acid substitution in the HN protein²³. Another study also conferred the mutations at the residues in threonine

193 isoleucine and isoleucine 567 valine²⁴. The viral neuraminidase alters the host cells surface, modulating the number of available sialic acid receptors and thus determining the outcome of infection, fewer sialic acid receptors are available to interact with other viral HN molecules. Other studies have shown one point mutation in the *HN* gene corresponding to a single amino acid change in the HN glycoprotein, which converts aspartic acid 216 to an asparagine and proline 111 to a serine^{25,26}. We found two novel mutations at amino acid residues of 295 and 297.

In conclusion, mRT-PCR assay could be used for the accurate diagnosis and detection of HPIV. Further, expanded surveillance throughout the country will help in better epidemiological analysis, for implementation of better public health programmes in controlling virus induced respiratory infections.

Acknowledgment

Authors acknowledge the Director, Institute of Child Health and Hospital for Children and Deans, Royapettah Government Hospital, Rajiv Gandhi Government Hospital, Kilpauk and Stanley Medical College for permitting to collect samples from patients, and thank the Superintendent of Saidapet Government Hospital and Government peripheral Hospital for cooperation.

References

1. Yang HT, Jiang Q, Xu Z, Bai MQ, Hong-Li Si, Wang XJ, *et al*. Identification of natural human serotype 3 parainfluenza virus. *Viral J* 2011; 8 : 58.
2. Laurichesse H, Dedman D, Watson JM, Zambon M. Epidemiological features of parainfluenza virus infections: laboratory surveillance in England and Wales, 1975-1997. *Eur J Epidemiol* 1999; 15 : 475-84.
3. Ren L, Richard G, Wang Z, Xiang Z, Wang Y, Zhou H, *et al*. Prevalence of human respiratory viruses in adults with acute respiratory tract infections in Beijing, 2005-2007. *Clin Microbiol Infect* 2009; 15 : 1146-53.
4. Lau SKP, To WK, Tse PWT, Chan AKH, Patrick CYW. Human parainfluenza virus 4 outbreak and the role of diagnostic tests. *J Clin Microbiol* 2005; 43 : 4515-21.
5. Moscona A. Entry of parainfluenza virus into cells as a target for interrupting childhood respiratory disease. *J Clin Invest* 2005; 115 : 1688-98.
6. Fry AM, Curns AT, Harbour K, Hutwagner L, Holman RC, Anderson LJ. Seasonal trends of human parainfluenza viral infections: united states, 1990-2004. *J Clin Infect Dis* 2006; 43 : 1016-22.
7. Echevarria JE, Erdman DD, Swierkosz EM, Holloway BP, Anderson LJ. Simultaneous detection and identification of human parainfluenza viruses 1, 2 and 3 from clinical samples by multiplex PCR. *J Clin Microbiol* 1998; 36 : 1388-91.
8. Hierholzer JC, Johansson KH, Anderson LJ, Tsou CJ, Halonen PE. Comparison of monoclonal time-resolved fluorimmunoassay with monoclonal capture-biotinylated detector enzyme immunoassay for adenovirus antigen detection. *J Clin Microbiol* 1989; 25 : 1662-7.
9. Sarkkinen HK, Halonen PE, Salmi AA. Type specific detection of parainfluenza viruses by enzyme-immunoassay and radioimmunoassay in nasopharyngeal specimens of patients with acute respiratory disease. *J Gen Virol* 1981; 56 : 49-57.
10. Shen K, Zhaori G, Zwegberg-Wirgart B, Ying M, Grandien M, Wahren B, *et al*. Detection of respiratory viruses in nasopharyngeal secretions with immunofluorescence technique for multiplex screening an evaluation of the Chemicon assay. *Clin Diagnostic Virol* 1996; 6 : 147-54.
11. Lee JH, Chun JK, Kim DS, Park J, Choi JR, Kim HS. Identification of adenovirus, influenza virus, parainfluenza virus, respiratory syncytial virus by two kinds of Multiplex polymerase chain reaction (PCR) and a shell vial culture in pediatric patients with viral pneumonia. *Yonsei Med J* 2010; 51 : 761-7.
12. Karron RA, Froehlich JL, Bobo L, Belshe RB, Yolken RH. Rapid detection of parainfluenza virus type 3 RNA in respiratory specimens: use of reverse transcription-PCR-enzyme immunoassay. *J Clin Microbiol* 1994; 32 : 484-8.
13. Karron RA, Brien KLO, Froehlich JL, Brown VA. Molecular epidemiology of a parainfluenza type 3 virus outbreak on a pediatric ward. *J Infect Dis* 1993; 167 : 1441-5.
14. Bellau-Pujol S, Vabret A, Legrand L, Dina J, Gouarin S, Petitjean-Lecherbonnier J, *et al*. Development of the multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. *J Virol Methods* 2005; 126 : 53-63.
15. Aguilar JC, Perez-Brena MP, Garcia ML, Cruz N, Erdman DD, Echevarria JE. Detection and identification of human parainfluenza viruses 1, 2, 3 and 4 in clinical samples of pediatric patients by multiplex reverse transcription-PCR. *J Clin Microbiol* 2000; 38 : 1191-5.
16. Tamura K, Peterson D, Peterson P, Stecher G, Nei M, Kumar S. MEGA5 Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Mol Biol Evol* 2011; 28 : 2731-9.
17. Hall CB. Respiratory syncytial virus and parainfluenza virus. *N Engl J Med* 2001; 344 : 1917-28.
18. Broor S, Parveen S, Bharaj P, Prasad VS, Srinivasulu KN, Sumanth KM, *et al*. A prospective three-year cohort study of the epidemiology and virology of acute respiratory infections of children in rural India. *PLoS One* 2007; 2 : e491.
19. Maitreyi RS, Broor S, Kabra SK, Ghosh M, Dar L. Rapid detection of respiratory viruses by centrifugation enhanced cultures from children with acute lower respiratory tract infections. *J Clin Virol* 2000; 16 : 41-7.
20. Rena L, Gonzalez R, Xie Z, Xiong Z, Liu C, Xiang Z, *et al*. Human parainfluenza virus type 4 infection in Chinese children with lower respiratory tract infections: A comparison study. *J Clin Virol* 2011; 51 : 209-12.
21. Bharaj P, Sullender WM, Kabra SK, Mani K, Cherian J, Tyagi V. Respiratory viral infections detected by multiplex PCR among pediatric patients with lower respiratory tract

- infections seen at an urban hospital in Delhi from 2005 to 2007. *Virology* 2009; 6 : 89.
22. Weinberg GA, Erdman DD, Edwards KM, Hall CB, Walker FJ, Griffin MR, *et al*. Superiority of reverse-transcription polymerase chain reaction to conventional viral culture in the diagnosis of acute respiratory tract infections in children. *J Infect Dis* 2004; 189 : 706-10.
 23. Coelingh KLVW, Winter CC, Jorgenson BD, Murphy BR. Antigenic and structural properties of the HN glycoprotein of human parainfluenza virus type 3 sequence analysis of variant selected with monoclonal antibodies which inhibit infectivity, hemagglutination and neuraminidase activity. *J Virol* 1987; 61 : 1473-7.
 24. Murrell MT, Porotto M, Greengard O, Poltoratskaia N, Moscona A. A single amino acid alterations in the human parainfluenza virus-3 hemagglutinin neuraminidase glycoprotein confers resistance to the inhibitory effects of Zanamivir on receptor binding and neuraminidase activity. *J Virol* 2001; 75 : 6310-20.
 25. Huberman K, Richard , Peluso, Moscona A. Hemagglutinin neuraminidase of human parainfluenza virus 3; role of the neuraminidase in the viral life cycle. *J Virol* 1995; 214 : 294-300.
 26. Porotto M, Greengard O, Poltoratskaia N, Harga MA, Moscona A. Human parainfluenza virus type 3 HN-receptor interaction:effect of 4-Guanidino-Neu5Ac2en on a neuraminidase deficient variant. *J Virol* 2001; 75 : 7481-8.

Reprint requests: Dr K. Kaveri, Department of Virology, King Institute of Preventive Medicine & Research
Guindy, Chennai 600 032, Tamil Nadu, India
e-mail: kaveri_raj1967@yahoo.com