Full Length Research Paper

Diagnostic significance of real time PCR for sensitive detection of respiratory syncytial virus and human metapneumovirus in a tertiary care hospital in India

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Abstract

A total of 225 NPAs (103 for RSV and 122 for hMPV) were analyzed by real time RT-PCR, conventional PCR and virus culture. Real time RT-PCR detected RSV in 75 and hMPV in 29 samples as compared to 35 and 16 detected by conventional RT-PCR. By culture, only 27 and 16 NPAs revealed RSV and hMPV respectively. Hence real time RT-PCR detected 53% and 64% additional RSV infection as compared to conventional RT-PCR and culture and 45% additional hMPV infection when compared to both. Positive correlation between viral loads and disease severity for RSV but not for hMPV was observed. Low birth weight with both RSV and hMPV whereas bronchiolitis with RSV and prematurity with hMPV infection showed significant association. Eight cases of co-infection were detected by real time RT-PCR; conventional RT-PCR detected one and culture none. Real time RT-PCR should be the method of choice for rapid and sensitive detection of RSV and hMPV alongwith culture.

Keywords: Real time RT-PCR, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), quantitation, co-infection.

INTRODUCTION

Acute viral respiratory tract infection is the leading cause of pediatric hospitalizations worldwide (Murray and Lopez 1997, WHO 1999, Rao 2003). Around 156 million new cases of clinical pneumonia in children <5 years of age occur each year globally of which 151 million are in developing countries (Rudon et al., 2008). Of these, 30% (43 million) occur in India alone. Of the pneumonia related deaths in year 2000, India had the highest mortality rate (408,000 deaths) in children below 5 years of age (WHO 2007).

Respiratory viruses account for 50–90% of acute lower respiratory tract infections (ALRI) in young children with respiratory syncytial virus (RSV) being most

commonly identified (Fan et al., 1998; Simoes EAF 1999, Broor and Bharaj, 2007). In 2001, human metapneumovirus (hMPV) was identified as an important respiratory pathogen and was soon reported worldwide, including from New Delhi, India (Banerjee et al., 2007, Bharaj et al., 2009). Respiratory infections caused by viruses usually present with clinical features that are nearly indistinguishable (Debbia et al., 2001). In young children, these viral illnesses may cause fever and result in empiric antibiotic use. Timely detection of these viruses may reduce the length of hospitalization and treatment costs in both pediatric and adult populations (Woo et al., 1997).

A variety of diagnostic methods are available for detection of respiratory viruses ranging from rapid antigen detection assays to virus culture (Landery and Ferguson, 2000; Falsey, 2002). The use of real-time RT-PCR for the diagnosis of respiratory viral infections has

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been advocated over conventional methods due to its advantages including sensitivity, specificity, and speed (Weinberg et al 2004). We sought to determine the sensitivity and specificity of three different assays for RSV and hMPV detection, virus culture, conventional PCR and real time RT-PCR. In lieu of association of several risk factors with both RSV and hMPV, we extended our analysis to determine the impact of these factors for severe disease for both the viruses.

MATERIALS AND METHODS

Clinical definition

Acute respiratory tract infections (ARI) were classified into upper respiratory tract infection (URI) if the infection was from nasopharynx to epiglottitis, acute lower respiratory tract infection (ALRI) on basis of tachypnea for which cut offs were per age of child age 2–11 months: ≥50/minute; age 1–5 years: ≥40/minute. Classification was further done to severe ALRI if chest indrawing was present along with symptoms of ALRI and very severe ALRI as presence of central cyanosis or severe respiratory distress or inability to drink along with symptoms of severe ALRI (WHO Handbook 2000).

Patients

Two hundred and twenty five nasopharyngeal aspirates (NPAs) were collected from children < 5 years of age who presented to All India Institute of Medical Sciences (AIIMS) Hospital, New Delhi, India at the outpatient department (OPD) or were admitted to the pediatric ward with ARI as part of an earlier study between April 2005 to March 2007. The demographic profile of children, clinical symptoms and risk factors were recorded in a predesigned proforma. Clinical features (fever >38 °C, difficulty in breathing, runny nose, cough) and risk factors (prematurity, history of ARI in family, presence of smokers in the household and low birth weight <2.5kg) along with clinical diagnoses of bronchiolitis or pneumonia were recorded. The Ethical Committee of AIIMS approved the study. Informed consent was obtained from the parents of the children.

Of 225 NPAs, 103 NPAs were tested for RSV and 122 NPAs for hMPV separately by real time RT-PCR, conventional RT-PCR and virus culture. The goal of the study was to compare the sensitivity and specificity of real time RT-PCR with conventional RT-PCR and virus culture. Furthermore we wanted to determine the correlation between viral load and severity of disease in these samples and define the role of risk factors for these viruses.

Specimen collection and processing

Sample collection and processing was done as described before (Parveen et al., 2006). Briefly, NPAs were collected via suction using hand vacuum pump through an infant feeding tube into 2-3 ml cold viral transport media, transported to the Virology Laboratory on ice within 4-6 hours and processed for virus identification.

Mucous present in the NPAs was broken down using glass beads and the samples were then split into three parts for viral culture, conventional RT-PCR and real time RT-PCR respectively. The PCR aliquots were not processed further and were stored at -80ºC until PCR was done. The virus isolation aliquots were spun down and clarified supernatants were used for virus inoculation. The pellet was saved for indirect immunofluorescence.

Virus inoculation in Tissue Cultures and indirect immunofluorescence

Cell culture and virus culture was done in HEp-2 and LLCMK-2 cells for RSV and hMPV respectively. Briefly, cell lines grown in 24 well culture plates were inoculated with 200µl of the NPA diluted 1:100 in viral growth medium (VGM) as described before (Parveen et al., 2006; Banerjee et al., 2007).

The virus was adsorbed for 1 hour at 37°C, the inoculum was discarded and the infected cells were refed with VGM specific for each virus. For RSV, infected cells were harvested at 5-7 days post infection (p.i.) and for hMPV 12-17 days p.i. when cytopathic effect (CPE) of either syncytia for RSV or refringent cells for hMPV were observed.

For virus identification in cell culture, indirect immunofluorescence was done on cell spots coated on Teflon slides (Cel-Line/Erie Scientific Co USA). Spots were air dried, fixed and subjected to indirect immunofluorescence using monoclonal antibodies (MAb) (Chemicon. Inc, USA) specific for each virus. Tissue culture grown standard viruses (RSV A2 and 18537 and hMPV CAN97-83) were used as a positive control for every set.

Conventional RT-PCR

RSV and hMPV were detected by conventional PCR using self designed N gene specific primers (Banerjee et al., 2007; Bharaj et al., 2009) from RNA extracted from NPAs by MagNA Pure compact RNA Extraction System using MagNA Pure Compact RNA Isolation Kit (Roche Diagnostics, Switzerland) as per the manufacturer's instructions. cDNA synthesis was done using random primers and AMV-RT (both from Promega, Madison,

USA) in a 25µl reaction and subjected to N gene PCR for both RSV and hMPV separately. For validation, G gene PCR for RSV and F gene for hMPV were done using primers and method as described before (Parveen et al., 2006; Banerjee et al., 2011).

Real Time RT-PCR

For virus detection by real time RT-PCR, published primers were used to generate PCR products for RSV, hMPV and GAPDH (Guedin et al., 2003; Maertzdorf et al., 2004; O Shea and Cane 2004) that were individually cloned into TOPO TA vector, (Invitrogen Inc, USA). For generation of *in-vitro* RNA transcripts, the plasmid DNA was linearized by *SpeI* and RNA transcripts were generated using Riboprobe in-vitro transcription system (Promega, Madison, USA) as per manufacturer's instructions. Following DNAse treatment, RNA concentration and purity were estimated by spectrophotometry and copy numbers were expressed as copies/ml. Serial 10 fold dilutions were made to generate standard curves in each assay.

An aliquot of RNA extracted from NPAs with a MagNA Pure compact RNA Extraction System was subjected to real time RT-PCR. Published probes for real time RT-PCR for RSV, hMPV and human GAPDH were used in this study (Guedin et al., 2003; O Shea and Cane, 2004, Maertzdorf et al., 2004). RSV probe was modified by removing the hairpin loop of the beacon structure. Real time RT-PCR reactions were performed in triplicate using SuperScript™ III Platinum® One-Step Quantitative RT-PCR System kit (Invitrogen, USA) in a 25µl reaction for 40 cycles. The standard curve was plotted between the threshold cycle (C_t) value and the log of the copy number of the standards. The threshold cycles of clinical samples were interpolated on the C_t values of standards and the viral loads were expressed as virus copies per milliliter (copies/ml).

hMPV sequencing

Sequencing of F gene of hMPV was done using primers and method as described before (Banerjee et al., 2007). Briefly, RNA was extracted from NPAs using RNeasy kit (Qiagen, GmBH, Germany), cDNA synthesized using AMV-RT and random primers and F gene PCR was done using primers which amplified 407 base region (607-965bp) of standard strain of hMPV. This PCR product was sequenced using Big Dye terminator DNA sequencing kit v3.1 (ABI, USA) and phylogenetic tree was constructed using MEGA v3.0 software using Kimura-2 parameter and neighbor joining algorithm (bootstrap 1000 replicates).

Statistical analysis

Statistical analysis was done using STATA9 (STATA Corp, Texas, USA) at Department of Biostatistics of AIIMS. A p value of <0.05 was calculated using Mann-Whitney U test and considered statistically significant.

RESULTS

Real time RT-PCR is highly sensitive method of detection for RSV and hMPV

RSV was tested in 103 NPAs by all three assays. Of these, 75 samples were positive for RSV by real time RT-PCR whereas conventional RT-PCR assay and virus culture detected RSV in only 35 and 27 samples respectively. Real time RT-PCR detected 48 additional samples as compared to culture that has been taken as the gold standard and 40 additional samples when compared to conventional RT-PCR. The sensitivity of conventional RT-PCR and real time RT-PCR was 100% when compared to culture. The specificity was 89.5% for conventional RT-PCR whereas for real time RT-PCR it was 36.8%. The positive likelihood ratios for conventional RT-PCR and real time RT-PCR were 25 and 1.6 respectively. There was no sample that was detected by culture for RSV and not detected by conventional RT-PCR and/or real time RT-PCR respectively. Additionally, G gene PCR for RSV confirmed all the samples detected by conventional RT-PCR.

The average age of 75 children in whom RSV was detected by real time RT-PCR was 15 months±12.8 months (median 11 months, range 2-61 months) as compared to those in whom RSV was not detected , the average age was 18.9 months± 17.3 months (median 12 months, range 2-62 months). Hence children infected with RSV were younger than the RSV negative group (p≥0.05, unpaired t test). A higher male to female ratio was observed in both RSV positive and RSV negative groups.

Of the 122 NPAs tested for hMPV, real time RT-PCR detected the virus in 29 samples. Of these 29 samples, hMPV was detected in 16 both by conventional RT-PCR and culture. Real time RT-PCR detected 13 additional samples as compared to culture and conventional RT-PCR. The sensitivity of conventional RT-PCR and real time RT-PCR was 100% when compared to culture. The specificity was 100% for conventional RT-PCR whereas for real time RT-PCR it was 86.8%. The positive likelihood ratio for real time RT-PCR was 7.5. Like RSV, there was no sample that was detected by culture and not detected by conventional RT-PCR and/or real time RT-PCR respectively for hMPV. F gene PCR for hMPV confirmed all the samples detected by conventional N

Table 1. Viral load detection of RSV and hMPV by real time RT-PCR and its comparison to viral load in samples detected by either conventional RT-PCR or culture

Assay (# of samples)	Viral load (copies/ml)	Range
Positive for RSV by culture (n=27)	$7.9X10^6 \pm 2.2 \pm 10^{7a}$	$8X10^3 \text{-} 1.2X10^8$
Positive for RSV by conventional RT-PCR but negative by culture (n=8)	$3.7X10^3 \pm 2.5X10^{3b}$	$1.4X10^3 \text{-} 8.5X10^3$
Positive for RSV by real time RT-PCR only (n=40)	$1.4X10^2 \pm 2.1X10^{2c}$	$1.0X10^1 \text{-} 8.7X10^2$
Positive for hMPV both by culture and conventional RT-PCR (n=16)	$2.4X10^6 \pm 4.9 \pm 10^{6d}$	$1.2X10^3 \text{-} 2X10^7$
Positive for hMPV by real time RT-PCR only (n=13)	4.0X102±4.5X102e	1.1X101-1.2X102

gene RT-PCR.

The average age of 29 children with hMPV infection was 15.5±12.4 months (median 12 months, range 2-57 months) as compared to those without hMPV infection that had an average age of 17.1±15.8 months (median 12 months, range 2-72 months). The difference in the average ages between the two groups was not statistically significant (p≥0.05, unpaired t test). Similar to RSV, a higher male to female ratio was observed in both hMPV positive and hMPV negative groups.

Correlation between viral loads and clinical severity of disease

The mean viral load of RSV samples positive by real time RT-PCR was $2.8X10^6 \pm 1.4X10^7$ copies/ml (range $1.1X10^1$-$1.2X10^8$ copies/ml) and for hMPV was $1.3X10^6 \pm 3.8X10^6$ copies/ml (range $1.1X10^1$-$2.1X10^7$ copies/ml). The viral loads of RSV and hMPV detected by real time RT-PCR in samples are shown in Table 1. It was observed that a majority of samples for both RSV and hMPV that were detected by real time RT-PCR only had very low viral loads that were not detected by either conventional RT-PCR or culture (p≤0.05, Mann-Whitney U test).

For RSV, viral loads in patients with ALRI (n=51) was $7X10^5 \pm 3.7X10^6$ copies/ml (mean±SD) as compared to those with severe ALRI (n=23) of $7.7X10^6 \pm 2.4X10^7$ copies/ml (p≤0.05, Mann-Whitney U test). Hence significantly higher viral loads appeared to correlate with disease severity for RSV. For hMPV, the viral loads between URI (n=5) ($1.2X10^6 \pm 1.6X10^6$ copies/ml), ALRI (n=14) ($1.9X10^6 \pm 5.5X10^6$ copies/ml), and severe ALRIs (n=9) ($7.9x10^5 \pm 9.8x10^5$ copies/ml) did not seem to show an association with disease severity.

Clinical features and risk factors associated with RSV and hMPV infection

Amongst various clinical symptoms that were analyzed

for their association with RSV, bronchiolitis appeared to be associated with RSV infection (41/75) as compared to the RSV negative patients (5/28, p ≤ 0.01). For hMPV, we did not observe any of the clinical symptoms analyzed in this study to be significantly associated with its infection. Among risk factors, low birth weight for RSV and both low birth weight and prematurity for hMPV showed positive correlation with virus infection (Table 2).

An important feature of real time RT-PCR is its ability to detect mixed infections. Fifty samples (randomly selected twenty-five samples each from 103 and 122 NPA groups) were separately tested for the co-presence of RSV and hMPV by all the three detection methods. Culture failed to identify co infection by these viruses in any sample whereas conventional RT-PCR detected it in one sample only. Real time RT-PCR on the other hand was able to detect eight co infections. Hence, the ability to detect co infections in samples appeared to be significantly enhanced by real time PCR.

In our attempt to correlate hMPV viral load to its subtypes, we sequenced 16 hMPV isolates by F gene. Electrophenograms revealed that 8 of these samples were hMPV subtype A2b, 7 were B1, and one was B2 by F gene sequencing (Banerjee et al., 2007). The average viral load of hMPV in A2b subtype samples was $1.4X10^6 \pm 1.3X10^6$ copies/ml (range $1.2X10^3$-$3.8X10^6$ copies/ml), in B1 was $3.8X10^6 \pm 7.5X10^6$ copies/ml (range $9.6X10^4$-$2X10^7$ copies/ml) and B2 was $2.1X10^6$ copies/ml respectively. It appeared that hMPV viral loads in samples were not associated with its subtypes.

DISCUSSION

The present study aims to evaluate the use of real time RT-PCR for quantification of the RSV and hMPV RNA load in respiratory secretions as compared to conventional RT-PCR and culture for their ability to detect RSV and hMPV in children with ARI. Real time RT-PCR showed an increase in the diagnostic yield of RSV by 64% and 53% over culture and conventional RT-PCR.

Table 2. Comparison of clinical symptoms and risk factors associated with RSV and hMPV infection as detected by real time RT-PCR

Variables	RSV positive (n=75)	RSV negative (n=28)	p value	OR (95% CI)		hMPV positive (n=29)	hMPV negative (n=93)	p value	OR (95% CI)
Clinical symptoms									
Cough	75	26	0.07	-		27	85	0.77	1.27 (0.23, 12.98)
Difficulty in breathing	39	17	0.43	0.7 (0.25, 1.84)		13	58	0.09	0.5 (0.2, 1.23)
Runny nose	32	9	0.33	1.57 (0.58, 4.47)		12	37	0.87	1.06 (0.41, 2.7)
Fever	67	23	0.33	1.82 (0.42, 7.03)		21	76	0.27	0.6 (0.2, 1.8)
Pneumonia						17[*]	71	0.06	0.4 (0.16, 1.18)
Bronchiolitis	41	5	**0.01**	5.54 (1.7, 20.3)		7[*]	22	0.96	1.02 (0.32, 2.93)
URI	-	-	-	-		5	0	-	-
Risk factors									
ARI in family	29	11	0.95	0.97 (0.36, 2.65)		12	35	0.71	1.16 (0.45, 2.95)
Prematurity	10	4	0.99	0.92 (0.23, 4.42)		6	7	**0.04**	3.2 (0.79, 12.25)
Smokers in family	27	11	0.75	0.87 (0.33, 2.37)		11	33	0.81	1.11 (0.42, 2.83)
Low birth weight	48	12	**0.05**	2.37 (0.89, 6.33)		17	20	**0.001**	4.95 (1.85, 13.32)

For hMPV, the yield increased over 45% for both assays. The significantly higher number of samples identified for both the viruses detected by real time PCR could be attributed to a number of reasons. The detection limit of real time RT-PCR in our hands ranged from 10^1-10^8 copies/ml. We observed that majority of the samples detected positive for virus by real time PCR only had copy number less than 10^3 copies/ml. Real time PCR is a highly sensitive technique and has been shown to detect low copy numbers (Gueudin et al., 2003; Mentel et al., 2003; Hu et al., 2003; Scheltinga et al., 2005) nonetheless the interpretation of the viral loads should be observed with great deal of caution. The recovery of virus infected cells from NPAs is a technique dependent process and may vary significantly which are probably reflected in the average viral load titers cited here. Moreover, for some calculations the number of samples was not big enough for analysis. Even though the high diagnostic yield of real time PCR as compared to culture is well elucidated (Templeton et al., 2004; Kuypers et al., 2006; Carr et al., 2008) probably including a different gene detection assay for real time RT-PCR for the detection of these viruses would have clarified if the low positives were truly positive.

Low detection rates in culture as compared to real time RT-PCR could be explained based on the explanations described previously on difficulty of RSV due to its thermolability and for hMPV by its requirement of several blind passages before any CPE can be observed (Falsey et al., 2002; Matsuzaki et al., 2010). It is believed that hMPV detection by viral culture is possible in only one third of the cases in which NP samples are inoculated particularly after primary inoculation (Matsuzaki et al., 2010). Thus, the low sensitivity of culture for RSV and hMPV necessitates the need for adequate evaluation by real-time RT-PCR as a means of quantitative detection.

As RSV pathogenesis is multifactorial and varied (Collins and Graham 2007), the relationships between viral loads and disease severity need to be evaluated. Association of viral load with disease severity has been controversial. The diagnostic value of determination of viral loads in respiratory tract infections is still unclear as respiratory specimens are difficult to standardize in terms of amount of virus or virus infected cells within specimens and non standardized dilutions of samples. Nonetheless, there are studies that have shown positive correlation of viral load with disease severity for both RSV and hMPV (Buckingham et al., 2000; Bosis et al., 2008; Houben et al., 2010); alongside those that prove otherwise (Wright et al., 2002). The results in the current study show a trend of positive correlation between RSV viral load and disease severity and none for hMPV. However, this study included children presented to the OPD or admitted to the ward that may have presented severe symptoms; hence, the relation between viral load and disease severity may not reflect an accurate presentation.

In multivariate analysis, clinical symptoms, and risk factors and their relation to RSV infection were assessed. Bronchiolitis was found to be frequently associated in children with RSV infection and has been shown to be an independent risk factor for the same (Choi et al., 2006;

Thomazelli et al., 2007). Numerous risk factors have been shown to be associated with RSV infection including age, environmental factors, passive smoking, type of fuel used in the home, breastfeeding and underlying medical conditions like prematurity and low birth weight (Law et al., 1998; Leader and Kohlhase 2003; Sampalis et al., 2008; Fiqueras-Aloyet al., 2004, 2008). However, the number of patients in the latter group was less; hence these results should be interpreted with caution. We also found that only low birth weight was significantly associated with RSV infection. For hMPV, there are relatively few studies that have analyzed association between the virus and risk factors. Moreover, majority of them have reported no significant difference between them (Noyola et al., 2005; Vicenete et al., 2006; Garci-Garcia et al., 2006). Contrastingly, we observed that both gestation (less than 35 weeks) and birth weight were (less than 2500g) were associated with the increased identification of hMPV among the patients from whom the samples were obtained. The differences in the observation could be differences in population selection or the fact that children in this study probably includes sicker patients based on hospital visit so prematurity and low birth weight were probable reasons of consultation (parents are more worried/babies are followed closer/babies are sicker) and not a risk factor for virus infection. Thus, clinical presentation and risk factors associated at the time of admission may contribute to difference in appearance of disease severity and its association to risk factors and hence should be carefully evaluated.

An important aspect of this study was the increase in detection of RSV and hMPV co infections by real time RT-PCR. The impact of a co infection on the clinical course of illness remains controversial. Hence it is quite essential to determine the causative agents of the disease. In patients in whom multiple viruses are detected real time RT-PCR may help discriminate between the virus actually causing the acute respiratory disease and those simultaneously detected but without a causative relationship to the actual clinical symptoms. Real time RT-PCR in the present study detected 8 co-infections where as conventional RT-PCR was able to detect only one and culture detected none. A significantly increased risk of more severe disease or of admission to a pediatric intensive care unit has been reported for dual infections as compared to single respiratory virus infection by some (Aberle et al., 2005; Richard et al., 2008)

Bosis et al. (2008) investigated association between hMPV viral loads with its subtypes. We extended our analysis to see if there was an association between hMPV viral loads and its subtypes in the present scenario. Similar to observations by Bosis et al, we did not observe any correlation between hMPV viral load and its subtypes by F gene sequencing. However, the number of samples in the present study demands further investi-

gation by including samples from a larger and more variable population.

In conclusion, our study gave an insight on high infection rates by RSV and hMPV in a selected group of children presented to the OPD. This highlights the necessity for vaccine development and faster laboratory detection in order to reduce antibiotic utilization. The real time RT-PCR assay described in this study has been used in the testing of vaccine surveillance samples at the clinical virology laboratory of All India Institute of Medical Sciences, New Deilhi, India.

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