

One Step RT- PCR for Detection of Respiratory Syncytial Virus in Children with Lower Respiratory Tract Infection.

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ABSTRACT

To assess the diagnostic utility of Reverse Transcriptase Polymerase chain reaction (RT-PCR) in detecting the respiratory syncytial virus in lower respiratory tract infection (LRTI). Cross sectional diagnostic accuracy study in tertiary care hospital. Total 130 cases with LRTI between 2 months to 5 years of age were included in the study. Flocked nasal swabs samples were processed for detection of RSV virus by both rapid antigen detection method and RT-PCR. RSV was detected in 14 patients by rapid method and by RT-PCR in 16 patients. Out of the 14 positive cases by rapid antigen method, 13 subjects were also positive by RT-PCR but one was negative. There were 3 more subjects, positive only by RT-PCR. The sensitivity and specificity of RT-PCR was 92.85 percent and 97.41 percent respectively. RSV was demonstrated in much higher percentage of bronchiolitis, especially below 6 months of age compared to pneumonia. RT-PCR assay is a highly sensitive method for detection of RSV. If an appropriate turnaround time can be offered, molecular detection should be used for RSV diagnosis.

INTRODUCTION

Acute lower respiratory tract infection (ALRTI) is one of the major causes of mortality and morbidity in young children worldwide ^[1]. Viruses account for 50–90% of ALRTI in young children ^[2] with *respiratory syncytial virus* (RSV), *parainfluenza viruses* (PIV), *influenza viruses A and B* and *human metapneumoviruses* (hMPV) being most commonly identified ^[3,4,5]. RSV is the single most important viral cause of lower respiratory tract infection (LRTI) during infancy and early childhood worldwide ^[6]. The RSV infection rate in young children approaches 70 percent in the first year of life, with peak incidence occurring between ages 2 - 4 months ^[7].

The virus accounts for approximately 50 percent of all pneumonia and up to 90 percent of the reported cases of bronchiolitis in infancy ^[6]. It may also predispose children to subsequent development of asthma, the most common chronic illness of childhood ^[8].

LRTI is frequently diagnosed on clinical grounds with minimal effort to identify accurately the etiological agent. This is especially so in the case of viral infections. This limitations shell reflect in the restricted utility of precise epidemiological data imposing suboptimal intervention measures as and when such measures become available. RSV bronchiolitis targets young children, predominantly those less than two years of age.

Laboratory diagnosis of RSV infection is beset with difficulties due to lability of the virus, and a requirement for cell-culture facilities and appropriate diagnostic reagents. However RSV diagnosis is important for the proper management of the afflicted children and to anticipate future complications. Present study is an attempt to understand the one step RT-PCR to identify RSV etiology and its diagnostic utility by comparing this test with an approved rapid antigen detection test.

MATERIALS AND METHODS

This is a cross-sectional study and was conducted in Department of Pediatrics and Microbiology in University College of Medical Sciences and Guru Teg Bahadur hospital, Delhi from August 2011 - February 2012. The study was approved by the Institutional Ethical Committee and informed consent was taken from the guardians of patients before collection of samples. A detailed questionnaire was filled for each patient including relevant clinical history.

Flocked nasal swabs were collected from 130 children attending the pediatric outpatient department and ward of our hospital with signs and symptoms of ALRTI. Study subjects included children of either sex from 2 months to 5 years of age. Children with clinical or radiological features consistent with bronchiolitis and pneumonia based on standard case definitions were enrolled in the study [9,10]. Bronchiolitis was diagnosed in infants presenting with cough and episodes of tachypnea with respiratory distress; auscultation revealed wheeze with or without Crepitation. There was no significant response to bronchodilators. Chest radiographs usually revealed only hyperinflation. Pneumonia was characterized by presence of fever and tachypnea, with or without chest wall retractions. Crepitations were present on auscultation and bronchial breathing in case of consolidation. Those subjects who are not fast breathing and on auscultation revealed wheeze was diagnosed as a case of wheeze associated lower respiratory tract infection (WALRI). For labeling the child as tachypneic, the WHO guidelines were followed (age <2 months: respiratory rate >60/min; age 2-12 months: respiratory rate >50/min; age >1yr: respiratory rate >40/ min). Subjects with a clinical diagnosis of bronchial asthma (based on wheeze, history of repeated similar episodes, positive family history of bronchial asthma and rapid response to bronchodilator therapy) were excluded from the study.

The nasal swab was obtained from the right nostril from a depth of 2 to 3 cm by using a sterile flocked swab that was then inserted into a vial containing 2.5 ml of viral transport medium (5% tryptose phosphate broth, 0.5% bovine serum albumin, and antibiotics in phosphate-buffered saline) and transported in ice to laboratory within one hour and processed within 48 hours of collection.

Sample was processed immediately on receipt at the laboratory for viral RNA isolation. Viral RNA was extracted using ZR Viral RNA kit™ (Zymo Research) following manufacturer's instructions. The RNA was stored in aliquots at - 80 °C until use.

The rapid RSV antigen detection was done using Binax NOW RSV test kit based on immunochromatographic principle. Briefly, RSV antigen present in the sample reacts to bind anti-RSV conjugated antibody. The resulting antigen-conjugate complexes are captured by immobilized anti-RSV antibody forming the Sample Line [11]. The gold standard was defined as any sample tested positive by Rapid antigen detection method. All the 130 samples were amplified by 1 step RT- PCR using the following RSV G- protein specific primers with an expected band size of 287bp using forward Primer- (nt542-564, 5'-3' GCAGCAACAATCCAACCTGCTGG) and reverse Primer- (nt806-828, 5'-3' ATCGGAGGAGGTTGAGTGGAGGG). Primers were designed using Primer3 software and custom synthesized. Conventional RT-PCR was performed on Thermal cycler (Eppendorf). The Novagen Toyobo one-step RT PCR kit was used for 1 step conversion of viral RNA to cDNA and then amplification with in the same tube according to manufacturer's instruction. In brief, 25µl of 2x RT- PCR Quick Master Mix, 2.5µl of 50 mM Mn(OAc)₂, 1µl of reverse and forward primer each, 2µl of extracted RNA and remaining water was added to make reaction mixture of final volume 50µl. The polymerase activation was carried at 90°C for 30 sec, reverse transcription at 60°C for 30 min, denaturation at 94°C for 1 min, followed by 35 cycles of amplification (denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min), final extension at 72°C for 7 min and held at 4°C. The PCR products were analyzed on 1.5 percent agarose gel and the amplicon size compared with the appropriate DNA ladder. A Negative and Positive control was run with each batch of PCR reactions. Positive control consisted of known sample of RSV RNA isolated from cell culture samples (Figure.1).

Sensitivity and specificity of the RT- PCR was calculated.

OBSERVATIONS AND RESULTS

Of the 130 patients studied, maximum (51) were between 7-12 months of age followed by patients in age group 2-6 months (47). In this study, we noticed maximum patients presented with pneumonia (63/130) followed by bronchiolitis (40/130) and rest (27/130) diagnosed as Wheezing associated lower respiratory infection (WALRI). In the present study, the bronchiolitis patients were predominantly distributed in the lower age group, especially in children 2-6 months of age (>72 %) while pneumonia was found to have wider age distribution.

RSV was detected in 14 samples out of 130 by rapid method. However, by RT-PCR, 16 out of 130 were positive for RSV. Total 17 samples were positive for RSV by at least one technique. Out of the 14 positive cases by rapid antigen method, 13 subjects were also positive by RT-PCR but one was negative. There were 3 more subjects, positive only by RT-PCR (Table 1).

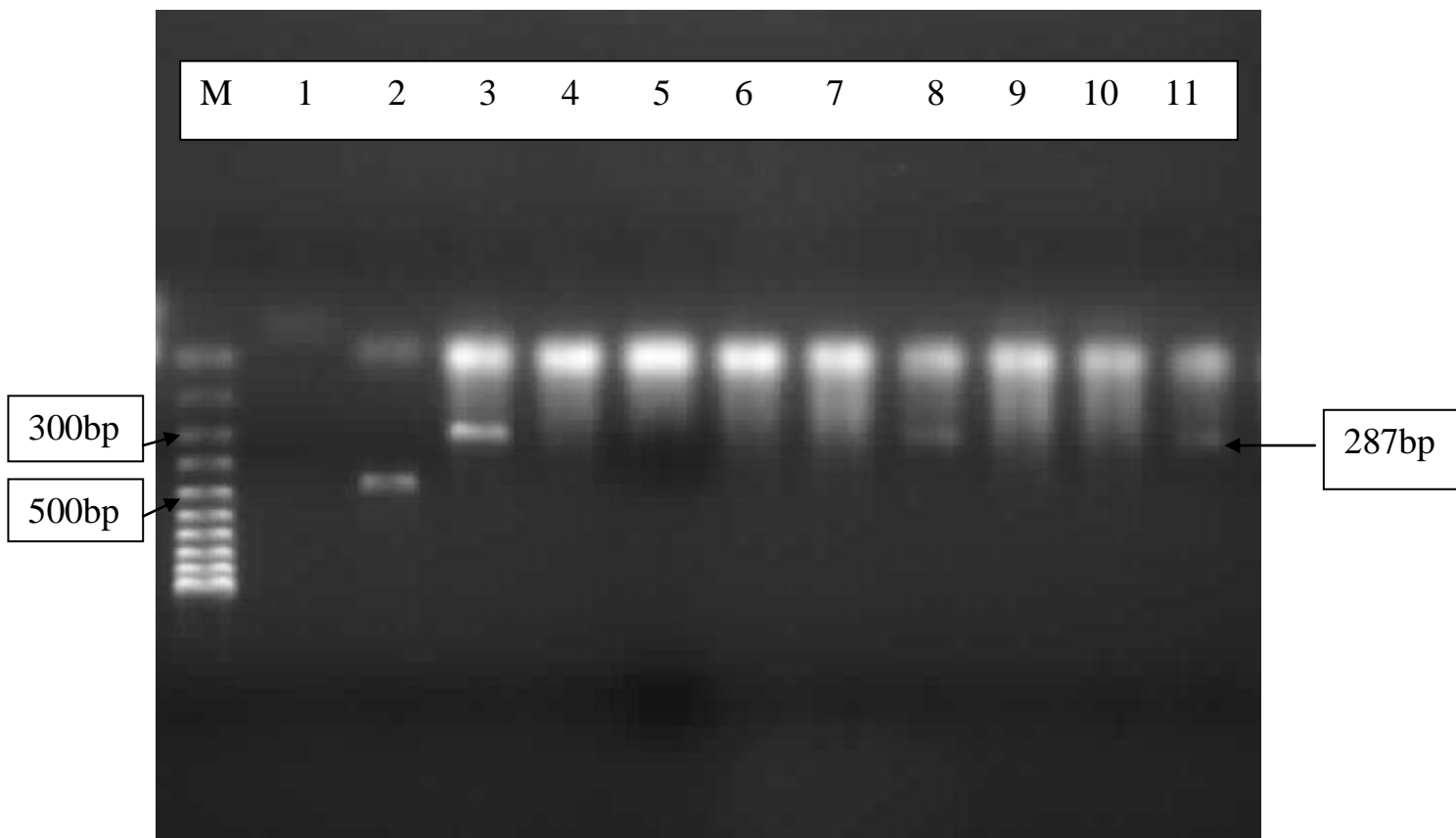


Figure 1: RT-PCR product for detection of RSV as shown by agarose gel electrophoresis

Lane M represents 100bp DNA Ladder
 Lane 1 represents Negative Control
 Lane 2 shows Kit (internal) Control of size 450bp
 Lane 3 shows RSV Positive Control of size 287bp
 Lane 8, 11 shows Positive amplification of a 287bp fragment
 Lane 4, 5, 6, 7, 9, 10 show Negative results

Table 1: Clinical and Laboratory details of RSV Positive patients

Month	Sex (M/F)*	Age (months)	Clinical Diagnosis	RAM†	RT-PCR‡
October	M	7-12	WALRI [§]	Positive	Positive
November	M	2-6	Bronchiolitis	Positive	Positive
November	F	2-6	Bronchiolitis	Positive	Negative
November	F	2-6	Bronchiolitis	Positive	Positive
December	M	2-6	Bronchiolitis	Positive	Positive
December	M	2-6	Bronchiolitis	Positive	Positive
December	M	2-6	Bronchiolitis	Positive	Positive
December	M	2-6	Bronchiolitis	Positive	Positive
December	F	2-6	Pneumonia	Positive	Positive
December	F	2-6	Pneumonia	Positive	Positive
December	F	2-6	Bronchiolitis	Positive	Positive
December	M	13-60	Pneumonia	Negative	Positive
December	F	2-6	Bronchiolitis	Negative	Positive
December	M	2-6	Bronchiolitis	Negative	Positive
December	M	7-12	Pneumonia	Positive	Positive
January	M	7-12	Bronchiolitis	Positive	Positive
January	F	7-12	Pneumonia	Positive	Positive

The sensitivity and specificity of RT-PCR were 92.85 percent and 97.41 percent respectively. The Positive Predictive value (PPV) and Negative Predictive Value (NPV) were 81.24 percent and 99.12 percent. In this study,

RSV infection could be demonstrated in much higher percentage of bronchiolitis. 22.5 percent (9/40) RSV positivity observed in patients who had been clinically diagnosed as bronchiolitis, 6.34 percent (4/63) in pneumonia and 3.7 percent (1/27) in WALRI.

DISCUSSION

Rapid detection of RSV infection in children and infants can be critical for effective patient management by focusing appropriate drug treatment, reducing unnecessary use of antibiotics, and preventing nosocomial spread. Diagnosis of RSV infection can be made by observation of clinical signs and symptoms, characteristic chest radiographs, rapid antigen detection, viral culture, or RT-PCR of clinical specimens [12].

Conventional viral culture is often time consuming with low sensitivity. Poor specimen quality and / or inappropriate specimen handling, markedly decreases the sensitivity of cell culture, giving rise to false negative results [13]. In addition, isolation in cell culture is not sufficiently rapid to influence patient management [14]. Analysis of clinical specimen by Enzyme-immunoassay or Immunochromatography (ICT) technology represents an alternative method for virus detection which has high specificity and good sensitivity with shorter time requirements compared to culture techniques. However, the moderate sensitivity of the antigen capture assays restricts the method to acute-phase samples from children, who shed virus in significant high titer than do adults [15]. Based on ICT technology, a rapid test for RSV detection (Binax NOW RSV) was developed and shown to have satisfying sensitivity (89%) and specificity (100%) in comparison with cell culture isolation [16]. This rapid test is largely used for testing for RSV at admission to the hospital and for bedside testing.

Several publications have reported the use of RT-PCR for detecting RSV [17,18]. The main benefit of molecular methods is their extreme sensitivity and a high specificity depending on appropriate primer selection. One of their drawbacks up to now has been that the majority of PCR protocols target only a single virus for identification. In addition, these methods are expensive and extremely prone to contamination, thus requiring high technical laboratory standards. Therefore, nucleic acid amplification methods are not yet routine in clinical diagnostic laboratories. The lack of conformity in technology between individual laboratories and the missing availability of external quality controls permits only a generalized assessment of their efficacy and usefulness.

In this study, RT-PCR was evaluated and compared with the rapid antigen assays which is FDA approved for RSV detection using 130 nasal swabs. RSV was detected in 10.76% and 13% of the studied patient by rapid antigen assay and RT-PCR respectively. This result is consistent with other investigators who found that 13% samples positive by antigen detection method [19]. In addition RT-PCR detected additional three positive samples which would otherwise have been missed by antigen assay. These results highlight the superiority of RT-PCR in RSV detection compared with that of antigen assay. The finding of rapid antigen method negative but RT-PCR positive sample may be due to low titer of the virus present in clinical samples. However, one sample positive by rapid antigen method was tested to be negative by RT-PCR. There could be possibility of PCR inhibitors or degradation of RNA template [20].

Since there are specific limitations for RSV detection by culture, RT-PCR offers several advantages: (i) detection at a low titre (ii) the potential stability of the assay after specimen freezing and thawing and (iii) an assay that is unaffected by therapeutic passive neutralizing antibodies or antiviral agents. These potential advantages would facilitate multicenter studies of pathogenesis and therapeutic trials.

We conclude that rapid and reliable detection of RSV infection should be streamlined for efficient patient management. The rapid test with its satisfying sensitivity and specificity represents a useful tool for routine testing in emergency rooms. While PCR is more expensive and need technical skills and time, increased accuracy of the results satisfies the extra resources required particularly for the inpatient. In inpatients the accuracy in the diagnosis of RSV infection is more critical rather than time which could eliminate the need for testing and antimicrobial use. However, in outpatient time is more critical which justifies use of rapid antigen assays.

CONCLUSION

In conclusion, we recommend the use of RT-PCR assay for detection of RSV as it is a highly sensitive method for investigating RSV in clinical specimen obtained in diagnostic, prophylactic and therapeutic setting. If an appropriate turnaround time can be offered, molecular detection should be the gold standard offered at all times for RSV diagnosis. Overall, it may help to reduce the incidence of nosocomial transmission, and improve clinical management by earlier diagnosis.

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