Frequency and Detection of Respiratory Syncytial Virus Pneumonia in Children by Nested Reverse Transcriptase Polymerase Chain Reaction

SOHEIR HELAL, M.D.*; GEHAN HUSSEIN, M.D.***; MAHA GAAFAR, M.D. * and AZZA AHMAD ALY, M.D. **

The Department of Clinical and Chemical Pathology, Cairo University* and National Research Centre**, Cairo and the Department of Pediatrics, Faculty of Medicine, Cairo University***.

Abstract

Introduction: Respiratory syncytial virus (RSV) is one of major causes of viral respiratory tract disease in young children and infants. We aimed to study the frequency of respiratory syncytial virus and its subtypes in pneumonia and bronchopneumonia in pediatric patients less than two years old tested by nested polymerase chain reaction with the evaluation of routinely used methods of diagnosis which are clinical manifestations and chest X-ray.

Material and Methods: The study included 70 patients exhibited lower respiratory tract symptoms in the form of pneumonia and bronchopneumonia. Their age ranged from two months to eighteen months. All participants were subjected to clinical examination, chest X-ray examination, nasopharyngeal aspirate (NPA) samples for microbiological examination and nested reverse transcriptase polymerase chain reaction for diagnosis of respiratory syncytial virus.

Results: RSV was highly prevalent infection in our studied patients, as 60 patients (86%) out of 70 were positive. Children from 2-3 months were highly affected (43.3%) and rate of infection decreases with increase of age. RSV infection was statistically significant with some clinical findings and radiological findings.

Conclusion: RSV is the most important etiological agent causing pneumonia and bronchopneumonia in infancy. Nasopharyngeal aspirate is a sensitive method in detecting viral infection during infancy. Nested reverse transcriptase polymerase chain reaction which is rapid and reliable technique may provide important diagnostic information in establishing the etiology and improving management of the patients.

Key Words: RSV – RSV subtypes – Nested reverse transcriptase polymerase chain reaction.

Introduction

RESPIRATORY syncytial virus (RSV) infection in children is associated with more than 90,000 hospital admissions/year nationally and as many as 1,000,000 deaths/year worldwide [1]. RSV bronchiolitis and pneumonia are most common diseases of childhood; virtually all children have been exposed to this virus by age two years. Although essentially incurable, it is generally self-limited to common cold symptoms in most patients. In vulnerable infants and those who are immuno-compromised, RSV can progress to severe pneumonia, with a resulting high likelihood of hospitalization and an increased chance of significant morbidity or death [2].

The rapid diagnosis of RSV may affect patient care by influencing decisions regarding:

i- Initiation of antiviral therapy.
ii- Diminishment or elimination of the use of antibiotics.
iii- Isolation of patients to diminish nosocomial transmission.
iv- Provision of prognostic information for the patient and clinical staff [3].

For appropriate treatment of RSV infection, it is crucial to have an accurate and timely diagnostic method for detection of the virus. A number of techniques are available for detection and identification of RSV, including cell culture, enzyme immunoassay (EIA), immunofluorescence (IF), and conventional reverse transcription (RT)-PCR [4]. Conventional RT-PCR, which has recently been developed for the detection of RSV, is a more sensitive and specific diagnostic method that also allows for the sub grouping of the virus in a single reaction [8]. Nested PCR is up to 100-fold more sensitive than standard single primer pair PCR and
therefore an assay employing outer and inner primers targeted against a region of the highly conserved N gene of human RSV is used [6].

The aim of this work is studying the frequency of respiratory syncytial virus and its subtypes in pneumonia and bronchopneumonia in pediatric patients less than two years old tested by Nested polymerase chain reaction with evaluation of routinely used method of diagnosis which are clinical manifestations and chest X-ray.

Patients and Methods

This study included 70 children exhibited lower respiratory tract symptoms in form of pneumonia and bronchopneumonia. The patients were 37 males and 33 females. Their ages ranged from 2-18 months. Those patients were classified into 5 groups according to their ages. Group I includes those from 2 to 3 months old, they were 31 (44.2%), Group II, from more than 3 to 6 months old and includes 20 patients (28.6%), Group III, more than 6 to 9 months old which were 11 patients (15.7%), Group IV more than 9 to 12 months old which were 6 patients (8.6%) and Group V more than 12 month old and includes 2 patients (2.9%). They were chosen among attendants admitted to Abo-Elrich El-Mounira Pediatric Hospital, Faculty of Medicine, Cairo University. The study period lasted 3 years from 2005-2007, timing of sample collection each year of the study from January to May where these months had the peak incidence of respiratory syncytial virus replication.

Inclusion criteria:
• Paediatric patients with pneumonia and bronchopneumonia.
• Age of paediatric patients less than 2 years.
• Newly admitted children within 24 hours of admission to avoid nosocomial infection.

Exclusion criteria:
• Neonates (1st month of life).
• Cases with congenital cyanotic heart disease.
• Patients with chest symptoms admitted to hospital for more than 24 hours.

All patients were subjected to the followings:

i- Proper history taking and clinical examination for preliminary screening of symptoms (cough, fever, wheezes and tachypnea) and physical signs (fever, intercostal muscles retraction, inspiratory crepitations and cyanosis) of lower respiratory tract infection.

ii- Chest X-ray examination for detection of radiological abnormalities as hyperinflation and abnormal increase of bronchovascular markings.

iii- Laboratory investigations:
1-Routine laboratory investigations as complete blood picture and erythrocyte sedimentation rate.
2- Nasopharyngeal aspirate collection: Nasopharyngeal aspirate (NPA) samples were collected by suction using a sterile pediatric suction catheter size (2.7mm) (Delee Suction Catheter with Mucus Trap, Tyco Health Care England). The nasopharyngeal aspirate was collected automatically in mucus traps (size 20cc) attached to the suction catheter.

Then small part of the NPA was taken for bacterial culture to exclude bacterial pneumonia and the remaining portion of NPA was frozen immediately after collection and stored at -70°C for PCR

Polymerase chain reaction:
Nested RT-PCR was done for detection of RSV.

According to manufacturer's direction the test was done as follows:

Total cellular RNA was extracted from 180-μl aliquots of thawed NPA by the QIA ampe Viral RNA Mini kit (Qiagen, Valencia, Calif). Two microliters of eluted RNA was transferred to a reverse transcriptase PCR (RT-PCR) mixture of 28 μl of RNase-free water, 10 μl of 5x reaction buffer, 1 μl of deoxynucleoside triphosphate mix, 5 μl of primer mix containing 25 pmol of each primer, 2 μl of MgSO4, and 1 μl of each avian myeloblastosis virus RT and Tfl polymerase (all reagents provided in the Promega RT-PCR kit; Promega, Madison, Wis.). The primers used were those described by Stockton et al., 1998 [7] (outer primers: RSV AB15’-GTCTTACAGCCGTGATTAGG-3’ and RSV AB2, 5’-GGGCCTTCTTTTGTTACTTCT-3’). The cycling protocol included one hr of reverse transcription at 48°C, a 5-min activation step of the Tfl polymerase at 95°C and 40 cycles of 15 seconds of denaturation at 95°C, and 30 s of primer annealing at 50°C and 30 s of primer extension at 72°C, followed by a final extension step of 7 min at 72°C in PE Biosystems Gene Amp 2400 thermocyclers. Five microliters of the first-round PCR product was transferred to freshly prepared master mixes
containing \(26 \mu l\) of \(H_2O\), \(5 \mu l\) of \(10x\) reaction buffer, \(5 \mu l\) of primer mix (containing 25pmol of each inner primer), \(4 \mu l\) of 12.5mM MgCl\(_2\), and 2U of hot start Taq polymerase (Amplitaq Gold; PE Biosystems, Foster City, Calif.). Type-specific primer pairs for RSV serotype A (RSV A) and RSV B were used for nested PCR: (inner primers: RSV A1, 5’-GATGTTACGGTGGGGAGTCT-3’ RSV A2 5’-GTACACTGTAGTT-AATCACA-3’; RSV B1, 5’-AATGCTAAGATGG-G-GAGTTC-3’; and RSV B2, 5’-GAAATTGAGTTAATGACAGC-3’).

Cycling conditions of the second round of PCR included a 12-min activation step of the Taq polymerase at 94°C, followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of primer annealing at 50°C, and 90 s of primer extension at 72°C for both serotypes. PCR products (first-run products, 1.1Kb for RSV A and B; second-run products, 0.9Kb for RSV subtype A and 0.78Kb for RSV subtype B, respectively) were detected by gel electrophoresis on 1.5% agarose gels and with ethidium bromide staining. Diluted supernatants of uninfected and RSV A-infected Vero cell cultures served as negative and positive controls and were included in every assay.

Statistical analysis:

Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages). Comparison between the study groups was done using Chi square \((\chi^2)\) test. Yates correction equation was used instead when the expected frequency is less than 5. A probability value \((p\) value\) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel version 7 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) statistical program for Microsoft Windows.

Results

Results of nested polymerase chain reaction for respiratory syncytial virus were positive in nasopharyngeal aspirate of 60 patients (86%) and negative in 10 patients (14%).

RSV subtype A were detected in 12 patients (20%), RSV subtype B in 21 patients (35%) while RSV subtype A & B coinfection were found in 27 patients (45%) as shown in Table (1).

As shown in Fig. (1A,B). (first-run products, 1.1Kb for RSV A and B; second-run products, 0.9kb for RSV A and 0.78kb for RSV B, respectively) patients (8,9) were positive for RSV subtype A, patients (3,5,6) were positive for RSV subtype B.

Table (1): Results of polymerase chain reaction (PCR). Values are expressed as number and percent.

<table>
<thead>
<tr>
<th>Item</th>
<th>Number of patients</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV positive</td>
<td>60</td>
<td>86</td>
</tr>
<tr>
<td>RSV subtype A</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>RSV subtype B</td>
<td>21</td>
<td>30</td>
</tr>
<tr>
<td>RSV subtype A &amp; B coinfection</td>
<td>27</td>
<td>39</td>
</tr>
<tr>
<td>RSV negative</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

RSV = Respiratory syncytial virus.

Fig. (1-A): Agarose gel showing results of RT-PCR on clinical samples (nasopharyngeal aspirates). Lane M (molecular weight marker). Lane 1, negative control; Lane 2, positive control; Lanes 3 to 7, clinical samples. A band of 1.1kb is observed in lanes 2,3,5 and 7.

Fig. (1-B): Agarose gel showing the results of nested PCR on clinical samples. Lane M (molecular weight marker). Lane 1, negative control; lane 2 subtype A positive control, lane 3, subtype B positive control, lane 4 to 9 clinical samples. A band of 0.9kb of subtype A RSV is seen in lanes 2,8, and 9 and a band of 0.78kb of subtype B RSV is seen in lanes 3,5 and 6. No bands were seen in lanes 4,7,10.
As shown in Tables (2,3). There was no statistically significant value between PCR results for respiratory syncytial virus and the different age groups of patients ($p$-value $>0.05$) (Table 2) nor with the sex of the patients ($p$-value $>0.05$) (Table 3).

<table>
<thead>
<tr>
<th>Age group</th>
<th>RSV Positive</th>
<th>RSV Negative</th>
<th>Percent</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-3 months</td>
<td>26</td>
<td>5</td>
<td>43.3</td>
<td>0.961</td>
</tr>
<tr>
<td>&gt;3-6 months</td>
<td>16</td>
<td>4</td>
<td>26.7</td>
<td>0.627</td>
</tr>
<tr>
<td>&gt;6-9 months</td>
<td>10</td>
<td>1</td>
<td>16.7</td>
<td>0.947</td>
</tr>
<tr>
<td>&gt;9-12 months</td>
<td>6</td>
<td>0</td>
<td>10</td>
<td>0.663</td>
</tr>
<tr>
<td>More than 12 months</td>
<td>2</td>
<td>0</td>
<td>3.3</td>
<td>0.660</td>
</tr>
</tbody>
</table>

RSV = Respiratory syncytial virus.
PCR = Polymerase chain reaction.

Table (3): Frequency of PCR results with sex of patients.

<table>
<thead>
<tr>
<th>Sex</th>
<th>RSV Positive</th>
<th>RSV Negative</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>30</td>
<td>7</td>
<td>0.406</td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

RSV = Respiratory syncytial virus.

As shown in Table (4) RSV subtype B and coinfection with subtypes A & B were predominant in age group from 2-3 months (21.6%) while RSV sub type A was predominant in age group from 3-6 months (11.7%).

<table>
<thead>
<tr>
<th>Age group</th>
<th>RSV subtype A</th>
<th>RSV subtype B</th>
<th>RSV subtype A&amp;B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>2-3 months</td>
<td>3</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>&gt;3-6 months</td>
<td>7</td>
<td>11.7</td>
<td>4</td>
</tr>
<tr>
<td>&gt;6-9 months</td>
<td>2</td>
<td>3.3</td>
<td>2</td>
</tr>
<tr>
<td>&gt;9-12 months</td>
<td>2</td>
<td>3.3</td>
<td>1</td>
</tr>
</tbody>
</table>

Frequency of PCR results for RSV with clinical diagnosis of patients were insignificant ($p$-value $>0.05$) as shown in Table (5).

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>RSV Positive</th>
<th>RSV Negative</th>
<th>Percent</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumonia</td>
<td>21</td>
<td>3</td>
<td>35</td>
<td>0.959</td>
</tr>
<tr>
<td>Bronchopneumonia</td>
<td>38</td>
<td>6</td>
<td>63.4</td>
<td>0.880</td>
</tr>
<tr>
<td>Bronchopneumonia with predominant wheezy chest</td>
<td>1</td>
<td>1</td>
<td>1.6</td>
<td>0.660</td>
</tr>
</tbody>
</table>

RSV = Respiratory syncytial virus.
PCR = Polymerase chain reaction.

Table (5): Frequency of PCR results with clinical diagnosis of patients.

<table>
<thead>
<tr>
<th>Chest X-ray findings</th>
<th>RSV Positive</th>
<th>RSV Negative</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prominent bronchovascular markings</td>
<td>30</td>
<td>4</td>
<td>0.733</td>
</tr>
<tr>
<td>Bilateral pneumonic patches</td>
<td>27</td>
<td>3</td>
<td>0.882</td>
</tr>
<tr>
<td>Single pneumonic patch</td>
<td>3</td>
<td>3</td>
<td>0.045*</td>
</tr>
</tbody>
</table>

RSV = Respiratory syncytial virus.

Discussion

It may be difficult to clinically differentiate RSV from other seasonal respiratory viruses. The rapid and accurate detection of RSV facilitates appropriate clinical management, including supportive care, hospital admission, and isolation to decrease nosocomial transmission of the virus, antiviral treatment, and judicious antibiotic usage [8].

In this study during successive 3 years period a total of 70 children were studied for detection of RSV. Among the 70 patients, RSV was detected in nasopharyngeal aspirate of (60) patients (86%). RSV subtype A was detected in 12 patients (20%), RSV subtype B detected in 21 patients (35%) and RSV subtypes A and B coinfection detected in 27 patients (45%).

This result was in agreement with Study done by Grimwood and his colleagues [9] who reported that during the southern hemisphere winter months of 2003-2005, 230 infants aged <24 months hospitalized with bronchopneumonia were recruited in his study and RSV was identified in 141 (61%) of them.

This is also in agreement with Chakravarti [10] who stated that a very high percentage of cases of RSV infection occurred in children below one year of age. Earlier studies in India and abroad have clearly pointed to this age group as a target for RSV infection.
Also, Kalina et al. [3] investigated the subgroup prevalence and genotype distribution patterns of HRSV strains in a community in Belgium during 10 successive epidemic seasons (1996 to 2006). HRSV infections with both subgroups were more prevalent among children younger than 6 months and had a peak incidence in December and this was in agreement with our result as RSV subtypes A and B coinfection detected in 27 patients (45%) and 19 of 27 patients were younger than 6 months.

In our study, positive PCR for RSV in the different age group was as follows in children 2-3 month (43.3%), >3-6 month (26.7%), >6-9 month (16.7%), >9-12 month (10%) and more than 12 month (3.3%). Results of our study showed that with increased age of patients, the rate of RSV infection was reduced. These findings are reported and consistent with the previous study done by Elena et al. [11] who reported that approximately 40% of RSV was infants younger than 3 months, 30% of those aged 3 to 6 months, 20% 7-12 months and 10% 1 to 3 years. Patients older then 3 years were rarely tested (less then 1%).

Also, Chkhaidze [12] Stated that, RSV was the predominant viral pathogen only in children aged from 6 to 12 months and the rate of detection of RSV decreased according to the age.

Regarding the sex of the patients in our study we found that of 60 children with RSV there were (30) males and (30) females, no statistically significant difference was seen in these values. This result was consistent with study done by Chakravarti1 [10], who stated that in children infected with RSV, there was no statistically significant difference in both sex.

In this study, RSV infections resulted in lower respiratory tract involvement including pneumonia (35%) and bronchopneumonia (65%) of our studied patients.

Also, Chakravarti1 [10] reported that RSV has been identified as an important cause of lower respiratory tract infections (17 to 32%) in the pediatric age group. RSV is the most common viral cause of lower respiratory tract infection (pneumonia and bronchopneumonia) in infants and young children worldwide. Approximately 0.5-1% of all children are hospitalized for RSV LRTI during the first year of life. Of these children, 10% will develop respiratory insufficiency necessitating mechanical ventilation.

Also, a study done by Nokes et al. [13] estimated that fifty-five percent to 65% of RSV-associated with bronchopneumonia and occurred in children aged >6 months.

Our results are not coinciding with that of Starliotto et al. [14] who conducted a survey in Brazil, in order to monitor the main respiratory viruses present in bronchiolitis and/or pneumonia and their involvement in the severity of viral respiratory infections. Viral respiratory infection prevalence was 38.7%. In bronchiolitis, respiratory syncytial virus (RSV) was detected in 36% of the cases. In pneumonia, the prevalence rates in RSV (7.7%), which is much lower than its incidence in our cases.

Regarding chest X-ray findings, statistically significant value was shown with lung consolidation of RSV infected patients while statistically insignificant with the other chest X-ray findings. This was in agreement with a study done by Friis et al. [15] who stated that lobar or segmental consolidation was observed more often in children aged <6 months infected with respiratory syncytial virus (RSV).

Also, Lopez and colleagues [16] reported that, the total of 284 patients were included, most were admitted during December and January, 74% had respiratory syncytial virus (RSV) infection. Images of pulmonary consolidation or atelectasis on admission chest X-ray reported in (55%).

Conclusion and recommendations:

RSV was the found to be most prevalent viral infection and important etiological agent causing pneumonia and bronchopneumonia in infancy. The exposure is virtually universal during childhood with a very high percentage of cases occurring in children below one year of age. Nasopharyngeal aspirate is a sensitive method in detecting viral infection in infancy. Positivisty for RSV infection was significantly associated with intercostal retrac- tion and inspiratory crepitation not with other clinical findings.

Tachypnea was the most prevalent clinical findings manifestation in RSV positive cases followed by inspiratory crepitation. Presence of RSV was statistically significant with radiologically detected lung consolidation not with other radiological findings. Though more studies are required to evaluate the various diagnostic modalities available for RSV, our study shows that rapid and reliable techniques used here (nested reversed transcriptase-polymerase chain reaction) may provide important diagnostic information in establishing the etiology and improving management of the patients.
Based on our findings, recommendations for rapid and reliable detection of RSV for efficient patient management would have to be streamlined, in order to prevent nosocomial infections. Also it is recommended that antibiotic use should be restricted for children with pneumonia until final diagnosis reached to prevent the development of antibiotic resistance.

Hyperimmune polyclonal RSV immunoglobulin (RSVIG) derived from blood donations, which was mentioned has shown to reduce the incidence and severity of RSV infections when given prophylactically in some babies and infants at high risk of severe infection. It is recommended to conduct other large scales studies on patients more than 12 months to detect prevalence of RSV at these age groups.

Further studies are needed to clarify the role of RSV subtypes A and B coinfection in infancy patients less than 12 months.

References


