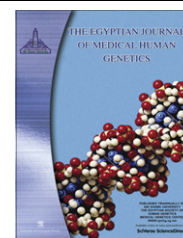




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## ORIGINAL ARTICLE

# Simultaneous detection of respiratory syncytial virus types A and B and influenza virus types A and B in community-acquired pneumonia by reverse transcription-multiplex PCR

Tharwat Ezzat Deraz <sup>a</sup>, Mervat Gamal Eldin Mansour <sup>a,\*</sup>, Sherin Albendary <sup>b</sup>,  
Ahmed Mohamed Abdelwahab <sup>a</sup>

<sup>a</sup> Department of Pediatrics, Faculty of Medicine, Ain Shams University, Cairo, Egypt

<sup>b</sup> Department of Microbiology, Faculty of Medicine, Ain Shams University, Cairo, Egypt

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**KEYWORDS**

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RSV;  
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**Abstract** *Background:* Respiratory syncytial virus (RSV) types A and B and influenza A and B cause about 80–90% of viral lower respiratory tract infections. It is impossible to distinguish the cause of viral respiratory infections by their clinical presentation. Multiplex RT-PCR has a significant advantage in that it permits the simultaneous amplification of several viruses in a single reaction facilitating cost-effective diagnosis and perhaps improved clinical management.

*Objectives:* In this study, our aim was to determine the prevalence of influenza A and B, and RSV types A and B among children with CAP, by the use of the newly developed rapid, accurate, and pathogen-specific technique of multiplex RT-PCR in order to accomplish the greatest positive effect on patient care and health care costs.

*Study methodology:* This study is a cross-sectional study involving 24 children admitted to the Children's Hospital of the Ain Shams University due to severe lower respiratory tract infection (LRTI). Clinical and radiological assessment of all patients was performed followed by the molec-

\* Corresponding author. Address: Abbas Elakad, Nasr City, Cairo, Egypt. Tel.: +20 1000837437.  
E-mail address: [mervatgmansour@gmail.com](mailto:mervatgmansour@gmail.com) (M.G.E. Mansour).



ular analysis of both respiratory and blood samples of all enrolled patients simultaneously by the use of the newly available reverse transcription-multiplex PCR technique (RT-m PCR).

**Results:** Viral pneumonia was detected in one-third of enrolled patients (8/24), with the predominance of respiratory syncytial virus A (4/8), followed by influenza A virus (3/8) and influenza B virus (1/8) while no cases of respiratory syncytial virus B was detected. The same results were identified in both blood and respiratory specimens.

**Conclusion:** Reverse transcription-multiplex PCR technique – multiplex has a significant advantage in that it permits the simultaneous amplification of several viruses in a single reaction making it well suited for use in epidemiological studies and to improve etiology-directed clinical management of viral pneumonia.

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## 1. Introduction

Respiratory syncytial virus (RSV) types A and B, influenza A and B, and human para-influenza virus (HPIV) types 1, 2, and 3 cause about 80–90% of viral lower respiratory tract infections [1]. It is impossible to distinguish the cause of viral respiratory infections by their clinical presentation; therefore, laboratory diagnosis is needed. Viral culture has been thought to be the “gold standard” for their testing; however, this method is generally slow, often taking up to 14 days before the results are available [2]. Viral antigen detection by immunofluorescence (IF) provides rapid results, but it often lacks sensitivity in detecting some viruses and further confirmation by viral culture may sometimes be required. Although the combination of both of these techniques can provide an increase in the proportion of positive results, it has been reported that a significant number of specimens still remains negative, despite clinical and epidemiological suspicions of viral infection [3,4]. To overcome these limitations, there has been a keen interest in the development of new nucleic acid-based assays. Reverse transcription-PCR (RT-PCR) assays have been shown to be rapid, sensitive, and specific for the detection of respiratory viruses [2]. However, mono-specific RT-PCR assays requiring the separate amplification of each virus of interest are potentially expensive and resource intensive, especially since respiratory pathogens may cause similar clinical syndromes. Multiplex RT-PCR has a significant advantage in that it permits the simultaneous amplification of several viruses in a single reaction facilitating cost-effective diagnosis and perhaps improved clinical management [5]. In this study, our aim was to determine the frequency of influenza A and B, and RSV types A and B among children with CAP, by the use of the newly developed rapid, accurate, and pathogen-specific technique of multiplex RT-PCR in order to accomplish the greatest positive effect on patient care and health care costs.

## 2. Patients and methods

### 2.1. Study setting

This is a cross-sectional observational study performed in the Children’s Hospital of Ain Shams University.

### 2.2. Patient enrollment

Children with clinical or radiological suggestions of community-acquired pneumonia and admitted to the Children’s Hospital of the Ain Shams University were enrolled from December 2008 to

April 2009. We excluded patients with bronchial asthma, chronic lung diseases, pulmonary tuberculosis (TB) and hospital-acquired pneumonia.

### 2.3. Clinical samples

Both blood and respiratory samples were collected from all eligible patients after having an informed consent signed by the parents of the child.

### 2.4. Sample collection and processing

Respiratory specimens were obtained by the gentle suction of gastric contents early in the morning, from infants and young children less than 5 years, by placing a sterile nasogastric tube of proper size after lubrication with paraffin oil. In the case of difficult suctioning or minimal amount (<2 ml), 5 ml of normal saline was added for liquefaction, and suction was repeated. In children more than 5 years, expectorated sputum was collected into sterile disposable plates. Both serum and respiratory samples were placed separately in 4 ml of M4 viral transport medium in a sterile universal container, and were transported to the laboratory, on wet ice and stored at  $-4^{\circ}\text{C}$  until processed within 24 h. Blood samples were centrifuged at  $2030\times g$  for 10 min. The supernatant serum was removed and stored at  $-80^{\circ}\text{C}$  until further testing.

### 2.5. Reverse transcription-multiplex PCR

Collected specimens were assessed for both RSV and influenza viruses simultaneously. These viruses are sharing some physical properties during their processing for PCR. RNA extraction: Total RNA was subsequently extracted using a MagNA Pure Compact system with MagNA Pure Compact NA isolation kit 1 according to the manufacturer’s instructions (Roche Applied Science, Mannheim, Germany; Cat. No. o3730964001). Amplification by RT-PCR was done by Light Cycler-RNA Amplification Kit SYBR Green I (Cat. No. 2015137). The kit is for one-step RT-PCR using the Light Cycler 2.0 System (Roche, Germany). Primers were non-labeled forward primers and biotin labeled reverse primers with horseradish peroxidase-labeled probes according to Liolios et al. [1].

### 2.6. Statistics

Statistical presentation and analysis of the present study was conducted, using the mean, standard deviation, analysis of variance (ANOVA) test and chi-square test by SPSS V.16.

### 3. Results

#### 3.1. Patients' cohort characteristics

Forty-six percent of the enrolled patients' ages ranged between 1 and 5 years, 37% of them was in their first year of life, while the minority (17%) was older than 5 years. Fifty-four percent of enrolled patients was male.

#### 3.2. Viral community-acquired pneumonia and its presentations

The presenting symptoms of patients with viral pneumonia were indistinguishable from those with non-viral pneumonia. Cough, expectoration, dyspnea and wheezes were common presenting symptoms in all studied patients. On the other hand, high grade fever and toxemia ( $p = 0.03$ ) were significantly present in patients with non-viral pneumonia, while diffuse auscultatory findings of fine crepitations ( $p = 0.026$ ) was a significant finding in patients with viral pneumonia. Preceding upper respiratory tract infections were evident in the majority of patients with viral pneumonia (87.5%,  $p = 0.02$ ). All patients had evidence of radiological findings on chest roentgenogram. Patients with viral pneumonia had significantly more diffuse areas of consolidation while patients with non-viral pneumonia had more localized forms of consolidation ( $p = 0.03$ ). Total leucocytes count was comparable in both groups of patients ( $p = 0.81$ ). While lymphocytes count showed markedly significant elevation in patients with viral pneumonia ( $p = 0.001$ ), and marked neutrophilia was found in patients with non-viral pneumonia ( $p = 0.001$ ) (Table 1).

#### 3.3. Multiplex reverse transcriptase polymerase chain reaction and viral pneumonia

Multiplex RT-PCR was positive for the examined viruses in 16 specimens out of 48 (33%), collected from 24 eligible patients. The results of multiplex RT-PCR were identical for both respiratory and blood samples collected from the same patient. RSV type A was the most frequent virus among the detected viruses (8/16 specimens, 50%). Influenza A virus was found in 37.5% of specimens (6/16) followed by RSV type B which was found in two specimens only (12.5). No evidence of Influenza B virus was found among the tested specimens. No obvious significant differences in clinical findings were found between patients with RSV and influenza viral pneumonia.

### 4. Discussion

Common community-acquired respiratory viruses cause significant morbidity and mortality in all demographic subsets of the population. Children are considered among the groups at highest risk for viral pneumonia-associated morbidity and mortality. Rapid, accurate, inexpensive point-of-care testing that is able to detect the majority of clinically indistinguishable respiratory viruses was a real challenge for physicians to diagnose this large group of respiratory pathogens. The main problems have been the lack of 'gold standard' methods for obtaining viral etiology [6].

In this study, simultaneous detection of aforementioned viruses by viral multiplex reverse transcriptase PCR was able

to diagnose viral pneumonia in nearly one-third (33%) of children admitted to the hospital due to severe acute lower respiratory tract infections (LRTI) and pneumonia. RSV type A was the most frequent virus (4/24 patients, 17%) among the detected viruses followed by influenza A virus (3/24 patients, 12.5%) and RSV type B was found in one patient only (4%). We believe that the identification of these viruses as causes of respiratory disease in these patients is the first step in determining how frequently they may cause serious problems and, hence, how hard we should push with accepted treatments such as those for influenza virus infection (either empirical or targeted treatment) and more controversial treatments such as those for RSV infections (Ribavirin and RSV hyper-immune globulin).

The molecular diagnosis of viral respiratory infections has become commonplace and widely accepted in major medical centers. This acceptance has been partly due to the significant evidence of dramatic improvements in sensitivity compared to older methods [7].

Comparable results were found in Nepal, where at least one viral pathogen was isolated using PCR from the nasopharynx of 40% of the children with community-acquired pneumonia during their first 3-years of age. However, RSV and influenza viruses account for only 26.3% of their patients [8]. Study of 515 Korean children less than 5 years of age; detected 11 respiratory viruses in 312 cases (60.6%) by multiplex PCR assays [7]. However, respiratory syncytial virus, influenza virus types A and B proved to represent 30% of all the study cases of lower respiratory tract infection encountered in the first year of life by Kusel et al. [9]. In Mozambique, almost half of the hospitalized children (394/807) with clinically severe pneumonia had at least one respiratory virus detected and the most prevalent ones were rhinovirus (41%), adenovirus (21%), and respiratory syncytial virus (11%) [10].

Comparing studies is difficult because pneumonia case definitions, study settings and method of case ascertainment, as well as the age group of the study populations differ. Many hospital-based studies have had limited observation time, and some were performed during one or only a few seasons. In addition, various clinical specimens and diagnostic assays with different sensitivity and specificity have been used in order to identify a varying number of viral agents. This has contributed to great variations in the reported overall frequency of viruses detected in children with pneumonia. Moreover, seasonal variations of RSV and influenza infections in temperate regions in the southern hemisphere, seemingly differ from that in sub-tropical and tropical locations with seasonal rainfall. RSV tended to occur in relation to the rainy season; however, in locations closer to the equator with perennial rainfall, RSV activity was almost continuous. Influenza is also reported to be detectable throughout the year in tropical and sub-tropical regions with less predictable timing of outbreaks, although there are reports of a biannual pattern of outbreaks with considerable activity between epidemic periods [11].

As known for most of the viral infections, our results showed a male predominance for viral pneumonia with male to female ratio 1.2:1 which raised the question of whether males are more susceptible to the progression of illness and more vulnerable to severe viral illness. Some researchers consider male sex to be one of the several important host risk factors for severe viral lower respiratory tract infections [11]. This

**Table 1** Clinical and demographic implications of viral pneumonia.

Characteristic	Viral pneumonia ( <i>n</i> = 8)		Non-viral pneumonia ( <i>n</i> = 16)		Chi-square	
	<i>n</i>	%	<i>n</i>	%	$\chi^2$	<i>p</i> -Value
<i>History</i>						
Upper respiratory infection	7	87.5	6	37.5	5.371	0.020*
Previous admission	4	50	3	18.8	2.521	0.112
Previous pneumonia	2	25	2	12.5	0.600	0.439
<i>Clinical symptoms</i>						
Cough	7	87.5	16	100	2.087	0.049*
Expectoration	5	62.5	12	75	0.403	0.445
Dyspnea	6	75	7	43.8	2.087	0.148
Wheeze	5	62.5	10	62.5	0.963	0.055
<i>Clinical signs</i>						
Fever	7	87.5	16	100	2.087	0.033*
Rales (sibilant rhonchi)	7	87.5	12	75	0.505	0.477
Bronchial breathing	1	12.5	15	93.8	15.844	0.001*
Fine crepitations	8	100	14	87.5	1.091	0.026*
Decreased air entry	3	37.5	14	87.5	6.435	0.013*
Respiratory distress	8	100	16	100	–	–
<i>Radiological pulmonary infiltrate</i>						
Localized infiltrate	1	12.5	11	69	4.325	0.033*
Diffuse infiltrates	7	87.5	5	31	–	–
<i>Hematological findings</i>						
WBC; mean $\pm$ SD	10.25 $\pm$ 1.85		10.43 $\pm$ 1.77		0.234	0.816
Lymphocyte %; mean $\pm$ SD	62.25 $\pm$ 7.59		30.75 $\pm$ 6.83		10.267	0.001*
Neutrophil %; mean $\pm$ SD	37.25 $\pm$ 7.24		66.37 $\pm$ 6.71		9.767	0.001*

\* *p* is significant if less than 0.05.

was in agreement with many comprehensive studies of viral acute lower respiratory tract infections [12–15].

It is noted in our study that 46% of patients admitted due to severe viral LRTI were less than 12 months and this may be explained by the vulnerability of this age group to life threatening diseases. Primary infection is rarely asymptomatic and re-infections are frequent. In a prospective study in the United States, around two-thirds of children were infected during their first year of life, and by the age of two, nearly all children had experienced one infection and nearly half had been infected twice. Level of passively acquired maternal antibody to RSV could be an underlying factor in the age of acquisition [16].

High grade fever and cough were the chief presenting clinical findings in patients with non-viral (bacterial) etiology of LRTI in this study, while upper respiratory tract infections were more common in patients with viral LRTI. Korppi et al. [17] found that wheezing, rales, hypoxemia, dyspnea and cough are seen in 60–80% of cases with viral and bacterial pneumonia. Yet, it is difficult to distinguish clinically between viral and bacterial causes of pneumonia. Fever may fail to settle in primary viral LRTI, while cough becomes more prominent. In contrast, where influenza or RSV infection is complicated by bacterial pneumonia, fever may settle initially but then re-credence [18]. On the other hand, we could not discriminate clinically between RSV or Influenza infections. Many studies report no differences in clinical symptoms or signs between illness episodes caused by type A and B viruses [19]. While Korppi et al. [17] found that RSV is more often associated with rhinorrhea, sputum production, and wheezing and less often associated with gastrointestinal complaints and fever compared with influenza [17]. In our study, radiological

findings of viral pneumonia showed diffuse areas of interstitial infiltrate, while patients with non-viral pneumonia had more localized forms of consolidation. In a study of 128 ambulatory children with pneumonia, three observers interpreted a lobular or segmental consolidation in children with bacterial pneumonia, and diffuse nodular densities or a disseminated reticular pattern in children with viral pneumonia [20]. Though lymphocyte and neutrophil count seemingly differentiated viral from non-viral pneumonia in our study, total leukocyte count showed no difference between the two groups. This was in agreement with Virkki et al. [20], who found that the proportion of patients with an increased WBC ( $> 15.0 \times 10^9/L$ ) was similar in bacterial and viral pneumonia (48% vs. 47%).

The clinical syndromes or hematological changes associated with respiratory viruses are frequently indistinguishable from one another or from bacterial pathogens. Viral illness often exacerbates underlying conditions and complicating diagnosis. So multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridization assay (MRT-PCR) can be used to detect and quantitate RNA of respiratory syncytial viruses A and B, influenza viruses A and B, in respiratory specimens in a rapid single test with less cost. This was suggested early by Liolios et al. [1], who suggested that this MRT-PCR can provide a highly sensitive and specific means of the diagnostic detection of major respiratory viruses compared to viral culture and immunofluorescence techniques.

## 5. Conclusion

Multiplex RT-PCR constitutes a rapid diagnostic tool for the simultaneous detection of more than one viral agent, making

this multiplex assay well suited for use in epidemiological studies and beneficial for a respiratory disease diagnostic service especially in critical clinical setting. Thereby it limits unnecessary antibiotic use, and improves clinical management as a result of the use of appropriate and directed therapy following the diagnosis of infection with a specific virus.

### Conflict of interest

The authors have no conflict of interest to declare.

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