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Original article

# Comparison of multiplex reverse transcription-PCR-enzyme hybridization assay with immunofluorescence techniques for the detection of four viral respiratory pathogens in pediatric community acquired pneumonia

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ABSTRACT

The burden of illness due to viral respiratory pathogens in the pediatric population is increasingly being recognized. Children are considered among the groups at highest risk for viral pneumonia-associated morbidity and mortality. Clinical discrimination between different causative agents is extremely difficult. The main problems have been the lack of 'gold standard' method for obtaining viral etiology (Liolios et al., 2001). 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. In this study, our aim was to compare between two modalities for diagnosis of viral illness among children with community-acquired pneumonia (CAP). Multiplex reverse transcription-PCR-enzyme hybridization assay and immunofluorescence antigen detection techniques for the detection of four viral respiratory pathogens (Influenza viruses A & B and Respiratory Syncitial Viruses A & B) were targeted to evaluate their diagnostic yield for these patients in our study. Among 56 respiratory samples were evaluated from children with clinical and radiological criteria of CAP; twenty-one patients had viral pneumonia proved by multiplex RT-PCR and/or IF technique with disease prevalence 35% (95% CI: 23:49). All 21 specimens were positive by multiplex RT-PCR, while 20 out of them were positive by IF. All results showed no discordance of detected viral pathogen. Initial comparison of IF results to those of RT-PCR generated a sensitivity 100% (95% CI: 83:100), a specificity 97.2% (95% CI: 85:99.9), a positive predictive value 95% (95% CI: 23:49.6), and a negative predictive value of 100% (95% CI: 74:99).

*Conclusion:* Multiplex reverse transcription PCR has an excellent potentials for diagnosis of viral pneumonia with a cost effective advantage in assessing simultaneously multiple clinically significant viruses. Rapid antigen tests for diagnosis of variable respiratory viruses, can be useful in etiological diagnosis of community acquired lower respiratory tract infection as well specially with the proved high sensitivity and predectivity in our study.

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## 1. Introduction

Viral pathogens are increasingly recognized as an important etiology of lower respiratory tract infections and are considered the predominant pathogens in community-acquired pneumonia (CAP) in preschool children [4].

Respiratory syncytial virus (RSV), influenza viruses, and rhinoviruses are highly prevalent agent affecting the lower airways and may progress with pulmonary infiltrates. These viruses might progress from upper respiratory tract infections to CAP in childhood [1].

It is impossible to distinguish the cause of viral respiratory infections by their clinical presentation. Rapid and accurate diagnostic techniques may reduce the need for empirical antibiotics which may contribute directly and indirectly to increased antimicrobial resistance and costs of hospitalization [2].

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Isolation of Influenza viruses and RSV in tissue culture was considered as the gold standard for confirmation of presumed viral infection. However besides the technical expertise in appropriate specimen handling for efficient virus recovery, this technique requires an average of 3–6 days until viral cytopathic effect appears.

A positive rapid test result for viruses might decrease the need for further testing or for starting antibiotic therapy; it may also give the opportunity for early start of antiviral therapy [5].

Multiplex RT-PCR has a significant advantage in that it permits simultaneous amplification of several viruses in a single reaction facilitating diagnosis and perhaps improved clinical management. Rapid antigen detection tests by immunofluorescence (IF) technique provide faster results because the test is performed directly on specimens obtained from patients without complicated sample processing but has questionable sensitivity.

In this study, our aim was to compare the diagnostic yield of multiplex RT-PCR and immunofluorescence antigen detection for Influenza A, B, Respiratory Syncytial virus A and B.

## 2. Patients and methods

### 2.1. Patient selection

Children with clinical or radiological suggestions of lower respiratory tract infection and admitted to Children's Hospital of Ain Shams University were enrolled from January 2015 to June 2015. We excluded patients with chronic lung diseases, or hospital-acquired pneumonia.

### 2.2. Sample collection and processing

Fifty-six specimens (40 nasopharyngeal aspirate and 15 morning gastric lavage specimens) from 56 patients were screened against all four viruses by RT-PCR and immunofluorescence (IF). A total of 0.5–1 ml of specimen was added to viral transport medium (minimal essential medium with 2% fetal bovine serum, penicillin [100 U/ml], streptomycin [100 mg/ml], amphotericin B [20 mg/ml], neomycin [40 mg/ml], $NaHCO_3$ buffer), and the mixture was frozen at −70 °C for subsequent analysis by RT-PCR-EHA. Another 1–2 ml of each sample was used for IF testing.

### 2.3. Viral antigen immunofluorescence

Clinical specimens underwent IF by standard methods. Briefly, the specimens were diluted in phosphate-buffered saline (PBS) and centrifuged at 2000 3*g* for 10 min. The pellets were re-suspended in PBS, dotted onto Teflon-coated microscope slides, and then dried and fixed in acetone. 25 μL of FITC-(Fluorescin isothiocyanate)-labeled monoclonal antibody specific for the nucleoprotein of respiratory syncytial virus and Influenza viruses, were added to each well. Slides were then incubated for 45 min at 37 °C in a humid chamber in the dark. The slides were read with an IF microscope. Wells showing characteristic apple-green fluorescence as that seen in the positive control were considered positive.

### 2.4. Reverse Transcription-Multiplex PCR

Collected samples were assessed for both RSV and Influenza viruses simultaneously. **RNA extraction:** Total RNA was subsequently extracted using a MagNA Pure Compact system with MagNA Pure Compact NA isolation kit 1 according to the instructions of the manufacturer (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. [8].

### 2.5. Primer and probe specificities

To assess the integrities of the primers and probes used in the RT-PCR, positive RNA controls from all four viruses were assayed in the presence of all primer pairs and screened against all four probes. Typical optical density readings for negative controls and for positive controls, were achieved with the specific probes. No cross-reactivity was detected among examined respiratory pathogens, demonstrating the high degree of specificity of this assay.

### 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

We have surveyed 123 patients attending the emergency department and having CAP. Sixty- seven patients have been excluded because they proved to have bacterial pneumonia. Therefore, fifty-six patients with community acquired pneumonia were enrolled and completed the study. Their ages ranged from 1 to 6 years, 55% of them were below one year. Fifty- three percent of enrolled patients were males.

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

All patients presented with variable degrees of respiratory distress. Patients were presented by cough and expectoration (98%), dyspnea (66%) and wheezes (55%). On the other hand, mild to moderate grade fever in 93%, while diffuse pulmonary rales in 92%. None of the patients had respiratory failure. Preceding upper respiratory tract infections were evident in the majority of patients (92%). All patients had evidence of increased broncho-vascular markings on chest roentgenogram, while 71% had evidence of consolidation (Table 1).

**Table 1**
Patients' characteristics.

| Characteristic | Value |
|---|---|
| Total patients number | 56 |
| Gender; male (%) | 30 (53%) |
| Age; years (mean ± SD) | 1 ± 1.3 |
| Age distribution: | |
| Below one year: | 31 (55%) |
| From 1:5 years | 21 (37%) |
| Above 5 years | 4 (7%) |
| Clinical presentation | |
| Preceding URTI | 52 (92%) |
| Cough and expectoration | 55 (98%) |
| Dyspnea | 37 (66%) |
| Wheezes | 30 (55%) |
| Fever | 52 (93%) |
| Respiratory distress | 56 (100%) |
| Chest X-ray findings | |
| Increased bronchovascular markings | 56 (100%) |
| Diffuse areas of pulmonary infiltrates | 39 (71%) |

**Table 2**
Summary of positive results of viral detection by either RT-PCR or IF of collected respiratory specimens.

| Sample number | Sample type | RT-PCR | IF |
|---|---|---|---|
| 1 | NPA | RSV (A) | ND |
| 2 | NPA | RSV (A) | RSV (A) |
| 3 | NPA | FLU (A) | FLU (A) |
| 4 | NPA | RSV (A) | RSV (A) |
| 5 | NPA | FLU (A) | FLU (A) |
| 6 | NPA | RSV (A) | RSV (A) |
| 7 | GL | FLU (A) | FLU (A) |
| 8 | GL | FLU (A) | FLU (A) |
| 9 | GL | RSV (A) | RSV (A) |
| 10 | NPA | FLU (A) | FLU (A) |
| 11 | NPA | RSV (A) | RSV (A) |
| 12 | NPA | RSV (A) | RSV (A) |
| 13 | NPA | RSV (A) | RSV (A) |
| 14 | GL | FLU (A) | FLU (A) |
| 15 | NPA | RSV (A) | RSV (A) |
| 16 | NPA | FLU (A) | FLU (A) |
| 17 | GL | FLU (A) | FLU (A) |
| 18 | GL | RSV (A) | RSV (A) |
| 19 | GL | RSV (A) | RSV (A) |
| 20 | NPA | RSV (B) | RSV (B) |
| 21 | NPA | RSV (A) | RSV (A) |

NPA: Nasopharyngeal aspirate, GL: Gastric lavage, RSV (A): Respiratory syncytial virus type A. RSV (B): Respiratory syncytial virus type B, FLU (A): Influenza virus type A, ND: Not detected.

#### *3.2.1. Viral detection by Multiplex RT-PCR and IF technique*

Amongst 56 respiratory samples collected from 56 patients, 21 samples proved to be positive by multiplex RT-PCR and/or IF technique with Disease prevalence 35% (95% CI: 23:49). All 21 specimens were positive by multiplex RT- PCR, while 20 out of them were positive by IF. All results showed no discordance of detected viral pathogen (Table 2).

#### *3.2.2. Multiplex RT-PCR*

Amongst 21 positive samples; Respiratory syncytial virus type A was detected in 12 samples (57%), Influenza virus type A in 8 samples (38%), Respiratory syncytial virus type B was detected in one sample (4.7%).

#### *3.2.3. Viral antigen immunofluorescence*

Amongst 20 positive samples; Respiratory syncytial virus type A was detected in 11 samples (55%), Influenza virus type A in 8 samples (40%), Respiratory syncytial virus type B was detected in one sample (5%).

#### *3.2.4. Sensitivity and specificity*

Initial comparison of IF results to those of RT-PCR generated a sensitivity 100% (95% CI: 83:100), a specificity 97.2% (95% CI: 85:99.9), a positive predictive value 95% (95% CI: 23:49.6), and a negative predictive value of 100% (95% CI: 74:99).

## 4. Discussion

The burden of illness due to viral respiratory pathogens in the pediatric population is increasingly being recognized. Children are considered among the groups at highest risk for viral pneumonia-associated morbidity and mortality. Influenza and respiratory syncytial virus (RSV), in particular, have most commonly been found to be the leading culprits of community acquired viral lower respiratory illness. Accurate, [11] and inexpensive point-of-care testing that is able to detect the majority of clinically indistinguishable respiratory viruses is a real challenge for physicians. The main problems have been the lack of 'gold standard' methods for obtaining viral etiology [8]. Traditionally, culture has been the gold standard for diagnosis of viral respiratory disease. But definitive identification of a viral pathogen may take days to even weeks. Yet, no single cell culture line can grow all medically important viruses. Moreover, viral culture is most useful for relatively hardy viruses, such as influenza virus, which can survive transportation to a laboratory, whereas more labile viruses like RSV cannot [6].

In this study, simultaneous detection of aforementioned viruses by viral multiplex reverse transcriptase PCR was able to diagnose viral pneumonia in nearly 11% 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 (12/21 patients, 57%) among detected viruses followed by Influenza A virus (8/21 patients, 38%) and RSV type B was found in one patient only (4.7%). 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 significant evidence of dramatic improvements in sensitivity and diagnostic accuracy compared to older methods [13]. On the other hand; Multiplex RT-PCR has a significant advantage in that it permits simultaneous amplification of several viruses in a single reaction [7,10], facilitating cost-effective diagnosis and perhaps improved clinical management over monospecific RT-PCR assays which requires separate amplification of each virus of interest. Monospecific RT-PCR, thought to have a higher specificity than Multiplex RT-PCR, but it is potentially expensive and resource intensive, in a resource limited countries especially since respiratory pathogens may cause similar clinical syndromes.

In the current study; viral antigen detection by immunofluorescence showed a relatively comparable sensitivity and specificity to the molecular diagnosis. We can attribute this surprisingly results to the probable high plaque-forming units of viruses in collected samples and the high viral load in the positive cases who were having more severe respiratory illness. Rapid antigen tests are simple, straightforward tests that can be performed at the point of care, with results available in 15 min. Thereafter, antigen detection had showed great success in the diagnosis of influenza and RSV infection in children. Unfortunately, similar results have not been noted in older adults [3,14]. Steininger et al. [12] found that the sensitivity of EIA for the diagnosis of influenza decreases with increasing patient age and can be as low as 8–22% in patients aged 80 years. Despite the low sensitivities associated with EIA, the test does has good specificity in the older adult population. Therefore, a positive EIA result is likely a true positive test result. However, a negative test result in older adults does not rule out influenza.

## 5. Conclusion

Rapid antigen tests for diagnosis of variable respiratory viruses, can be useful in etiological diagnosis of community acquired lower respiratory tract infection as long as clinicians are mindful of test limitations. Erroneous negative results may lead to delays in timely administration of antiviral treatment and institution of appropriate isolation precautions. The increasing availability of new, rapid, and sensitive molecular diagnostics, such as polymerase chain reaction especially with the ability to detect multiple viruses simultaneously, should provide more accurate and timely diagnoses of viral respiratory infections in children in the near future.

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## Further reading


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