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Short Communication

Viral etiology of acute respiratory infections in children in Southern Iran

Mehrdad Halaji[1]*, Tayebeh Hashempour[1]*, Javad Moayedi[1]*, Gholam Reza Pouladfar[1]*, Behzad Khansarinejad[2], Reza Khashei[3], Afagh Moattari[3], Zahra Musavi[1], Farzaneh Ghassabi[1] and Neda Pirbonyeh[3]

[1]. Clinical Microbiology Research Center, Nemazee Teaching Hospital, Shiraz University of Medical Sciences, Shiraz, Iran.
[2]. Department of Microbiology & Immunology, Arak University of Medical Sciences, Arak, Iran.
[3]. Department of Bacteriology & Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.

Abstract

Introduction: Prevalence of influenza A virus (Flu-A), respiratory syncytial virus (RSV), and human metapneumovirus (hMPV) was assessed in children with acute respiratory infections (ARIs). **Methods:** Nasopharyngeal aspirates and throat swabs were subjected to real-time polymerase chain reaction (PCR) to detect RSV and Flu-A and to conventional PCR to detect hMPV. **Results:** Of the 156 children assessed, 93 (59.6%) carried at least one virus, with 35.9% positive for RSV, 14.1% for hMPV, and 9.6% for Flu-A. The prevalence of co-infections was 2.6%. **Conclusions:** The high detection rate may reflect increased sensitivity of real-time PCR compared to traditional PCR and viral culture.

Keywords: Acute respiratory infections. Respiratory syncytial virus. Influenza A virus. Human metapneumovirus. Real-time polymerase chain reaction.

Acute respiratory infections (ARIs) are a significant cause of acute illness in the general population and a leading cause of morbidity and mortality in children and elderly people worldwide. ARIs are commonly caused by viruses or bacteria and are classified into upper respiratory tract infections (URTIs) and lower respiratory tract infections (LRTIs). The most common diseases associated with ARIs include common cold, bronchitis, and pneumonia[1]. Viral ARIs present the second most common cause of morbidity and mortality in children under the age of 5[2]. The epidemiology and etiologies of ARIs vary in different geographic areas, and hence early determination and rapid and correct diagnosis of viral agents are important to develop appropriate ARI management strategies[3]. Several diagnostic methods are employed to detect viruses including virus cultures and serological detection of antigens; these procedures are time-consuming and exhibit low sensitivity in certain cases. In contrast, molecular techniques such as polymerase chain reaction (PCR) and real-time PCR assays are sensitive and specific tools for virus detection[4]. The predominant viruses causing ARIs are influenza A and B viruses (Flu-A, Flu-B), rhinoviruses (RV), human metapneumovirus (hMPV), human adenoviruses (hAdV), respiratory syncytial virus (RSV), and coronaviruses[5]. Information regarding viral ARIs in children in Shiraz (Southern Iran) is limited, and we therefore aimed to determine the frequency of Flu-A, RSV, and hMPV in children under 15 years of age in this area using real-time or conventional PCR.

This cross-sectional study was performed between December 2014 and April 2015. A total of 156 nasopharyngeal aspirates (NPAs) and throat swab samples were collected from in- and out-patients who had presented with symptoms of ARIs and were admitted to the hospital (Nemazee Teaching Hospital, Dastgheib Hospital, or Emam Reza clinic affiliated with Shiraz University of Medical Sciences, all in Shiraz, Iran). The inclusion criteria were an age under 15 years and presence of one or more ARIs symptoms such as fever, cough, sore throat, tachypnea, crackles (rales), stridor, and wheezing or upper respiratory tract symptoms (rhinorrhea and sneezing). Throat swabs and NPAs were collected by trained personnel or physicians using standard operating methods from patients who agreed to participate in the study. Demographic and clinical information was collected from

*These authors contributed equally to this work.
Corresponding author: Tayebeh Hashempour.
e-mail: thashem@sums.ac.ir
Orcid: 0000-0002-5249-209X
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each patient using a standardized data form. The study protocol was approved by the ethics committee of Shiraz University of Medical Sciences and was in accordance with the declaration of Helsinki. Written informed consent was obtained from the parents or legal guardians of the children.

Viral nucleic acid was extracted from 0.2 mL of each respiratory specimen using the High Pure Viral Nucleic Acid extraction kit (Roche Diagnostics, Mannheim, Germany). Complementary DNA (cDNA) was synthesized using the First Strand cDNA synthesis kit (Thermo Scientific, Waltham, MA USA). RNA integrity was confirmed by amplification of a genomic β-globin sequence as a reference gene.

We developed a highly sensitive and specific real-time PCR using TaqMan technology, virus-specific primers, and probes designed to detect Flu-A and RSV (**Table 1**). In brief, 2 µL of cDNA, 12.5 µL of TaqMan Universal PCR Master Mix 0.8 µmol of each primer, and 0.4 µmol of the respective probe were combined and the volume adjusted to 20 µL with DNase-free water. The PCR reactions were performed with an ABI 7500 PRISM real-time PCR system according to the manufacturer's protocol (Applied Biosystems, Waltham, MA, USA). Thermal cycling was initiated with uracil-N-glycosylase incubation at 50°C for 2 min and polymerase activation at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and annealing at 60°C for 60 seconds.

Moreover, a conventional PCR procedure was used for detection of hMPV. The reaction was carried out in a final volume of 25 µL containing 1 pmol of each primer, 2.5 µL of 10× PCR buffer, 0.2 mM of deoxynucleotide triphosphates (sNTPs), 2 mM of $MgCl_2$, 0.4 µL of *Taq* DNA polymerase (CinnaGen, Tehran, Iran), and 2 µL of cDNA. The amplification was started with an initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec, an extension step at 72°C for 45 sec, and final extension at 72°C for 5 min, yielding a 169 base-pair (bp) product. The PCR products were separated using gel electrophoresis on a 1% agarose gel.

Statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS version 21, IBM, Chicago, USA). The association between categorical variables was assessed using Chi-square test and given as both the number and percentage. The significance level was set to 5%.

A total of 156 eligible children, 92 boys (59%) and 64 girls (41%), with clinical symptoms of ARIs were enrolled in this prospective cross-sectional study. Out of these patients, 96 (61.5%) were outpatients and 60 (38.5%) inpatients. The mean age was 3 years (SD=2.5 years, range: 1 month to 15 years). The medical diagnoses after physical examination included common cold (51.9%), pneumonia (25.6%), nasopharyngitis (4.5%), pharyngitis (3.8%), bronchitis (3.8%), sinusitis (2.6%), and croup (1.3%). Most patients had no other underlying disease (**Table 2**). The most common clinical symptoms were cough (91.7%) and sore throat (85.3%, **Table 3**).

Specimens of 93 patients (59.6%) were positive for at least one virus, with 89 single-infection cases (57.6%) and 4 co-infection cases (2.6%). Of these patients, 60 (38.5%) were under 2 years while 44 (28.2%) and 52 (33.3%) were in the age groups of 2-5 years and 6-15 years, respectively. Although RSV was most frequently detected in children under 2 years (42%), viral infections were not associated with the age of patients and distributed evenly among all age groups ($p>0.05$).

RSV was also the most common virus overall (35.9%, 56/156), followed by hMPV (14.1%, 22/156) and Flu-A (9.6%, 15/156). Laboratory analyses revealed the absence of Flu-A and RSV or Flu-A and hMPV co-infections, while co-infection with hMPV and RSV was detected in 4 patients that were all under 1 year of age. The remaining 67 specimens (42.9%) were negative for all respiratory viruses tested in this study. Flu-A ($p=0.033$) was more frequently detected in girls (10/5), while RSV was more prominent in boys (17/39, $p=0.031$). Nonetheless, clinical signs and symptoms did not significantly differ between infected and non-infected patients.

hMPV infection typically exhibits a clear seasonality with obvious peaks in January ($p=0.012$), while Flu-A infections are normally not observed in a particular season. Nevertheless, the detection rate of Flu-A was highest in the winter months with January as the most critical month with the most patients.

To the best of our knowledge, the current study is the first report to determine the frequency of Flu-A, RSV, and hMPV in children with ARIs in Southern Iran using real-time PCR.

TABLE 1: Sequences of primers and probes designed for the detection of Flu A, RSV, and hMPV.

Virus	Primer	Sequence (5' to 3')	Length (bp)
Flu-A	Forward	tggartggctaaagacaagrcc	120
	Reverse	cgtctacgctgcagtcctcg	
	Probe	JOE-agtcctcgctcactgggcacggt-BHQ1	
RSV	Forward	gtaacagaattgcagttgctcatg	178
	Reverse	cgattgcagatccaacacctaac	
	Probe	FAM-cacaccagcagccaacaatcgagcca-BHQ1	
hMPV	Forward	ttacctgcgagcctracwatatgg	169
	Reverse	gtacagacattgcwgcaccytg	

BHQ1: black hole quencher 1, **FAM:** 6-carboxyfluorescein, **JOE:** 4',5'-dichloro-2',7'-dimethoxy-5(6)-carboxyfluorescein.

TABLE 2: Distribution of background disease in patients with acute respiratory infection.

Background disease	Frequency (number, n=156)	Frequency (percentage)
No Background disease	114	73
Asthma	24	15.4
Cerebral palsy	7	4.5
Favism	3	1.9
Heart Disease	2	1.3
Lung Disease	2	1.3
Kidney disease	2	1.3
Liver Disease	2	1.3

TABLE 3: Clinical manifestations of patients with acute respiratory infection.

Symptoms of ARIs	Frequency (number, n=156)	Frequency (percentage)
Cough	143	91.7
Sore Throat	133	85.3
Rhinorrhea	88	56.4
Fever	72	46.2
Wheezing	42	26.9
Dyspepsia	37	23.7
Fatigue	33	21.2
Sneezing	28	17.9
Tachypnea	20	12.8
Chills	10	6.4
Diarrhea	7	4.5
Chest Pain	1	0.6

According to our results, 59.6% of our ARI patients were positive for at least one respiratory virus in agreement with other studies performed in similar settings[6,7]; nonetheless, the prevalence was higher than that previously reported in 2014 from Iran (17.2%)[8].

The high rate of positive samples observed in this study probably reflects the higher sensitivity of the real time-PCR assay compared to conventional PCR and the use of NPAs rather than nasal swabs in some cases. RSV was the predominant virus in patients of this study accounting for 35.9% (56/156) of all cases, which is similar to findings of previous studies from Pakistan and the Middle East region[9,10]. Moatari et al. reported an RSV rate of 30% in patients with ARIs in Southern Iran, while another study in this region achieved an RSV detection of 9% by conventional PCR using NPA samples from children hospitalized with ARI symptoms[11,12]. A similarly low RSV rate of 24% was detected by Shamsudeen et al. using real time-PCR on specimens from children presenting with ARI in Iran[13]. Overall, the frequency of RSV was higher in our study compared to other studies conducted in Iran[8, 12]. This may be explained by the use of real-time PCR and a low patient age with most children under 2 years old. Most RSV cases (53.5%, 30/56) were associated with the common cold and the detection rate showed peaks between December and February. These observations may be attributed to the cold weather in our region during this period that increases the risk of respiratory infections. A study conducted in Pakistan revealed a highly seasonal pattern of RSV infections with most cases occurring between July and September, while Shatizadeh et al. reported that most RSV cases occurred in February[9,14].

hMPV was the second most common virus with a prevalence of 14.1% (22/156). This figure agrees with those obtained in other studies; Moatari et al. reported an hMPV prevalence of 15.7% in hospitalized children with ARIs in Southern Iran, and Asad et al. detected hMPV in 14.2% of children hospitalized

with pneumonia[9,11]. Nonetheless, most comparable studies reported a lower frequency of hMPV. For instance, a study conducted in Teheran, Iran, detected hMPV in 10% of the participants[15].

The seasonal distribution of hMPV is not well-defined but the incidence of hMPV infections appears to experience a peak in January (p=0.012).

Our findings showed that hMPV infection coincided with RSV infections in agreement with previous reports[16]. The peak season of these viruses was winter in studies performed in Central Iran and Turkey but spring in studies conducted in Southern Iran[11]. The rate of co-infection with RSV and hMPV was 2.6% in our study, which is consistent with the study from Shiraz identifying 3.5% of patients as infected with both viruses[11]. We detected less Flu-A infections than hMPV and RSV infections. The prevalence of Flu-A in our patients was 9.6% (15/156), which is similar to many other studies conducted worldwide[8,9,14]. We observed a slight peak in the incidence of Flu-A infection in January, coinciding with the cold weather in this area during this month. We did not identify any significant differences in the clinical manifestation between infected and non-infected patients, but the common cold and pneumonia were more frequent in infected patients.

In conclusion, we discovered that RSV and hMPV are highly prevalent in children with ARI and demonstrated that real-time PCR is a sensitive and rapid approach for the diagnosis of these infections.

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Conflict of Interest

The authors have no conflict of interest to declare.

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