Research Article

Detection of Common Respiratory Viruses in Patients with Acute Respiratory Infections Using Multiplex Real-Time RT-PCR

Niloofar Neisi [1, 2], Samaneh Abbasi [3], Manoochehr Makvandi [1, 2, *], Shokrollah Salmanzadeh [1], Somayeh Biparva [4], Rahil Nahidsamiei [1, 2], Mehran Varnaseri Ghandali [5], Mojtaba Rasti [1, 2] and Kambiz Ahmadi Angali [6]

[1]Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]Virology Department, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[3]Abadan Faculty of Medical Sciences, Abadan, Iran
[4]Department of General Courses, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[5]Deputy of Health, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[6]Biostatistics Department, School of Health, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Corresponding author: Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Email: manoochehrmakvandi29@yahoo.com

Abstract

Background: Acute respiratory infection (ARI) is caused by human metapneumovirus (HMPV), respiratory syncytial virus type A and B (RSV-A, RSV-B), human parainfluenza viruses 1, 2, and 3 (HPIV-1, HPIV-2, and HPIV-3), influenza viruses A and B (IfV-A, IfV-B), and human coronaviruses (OC43/HKU1, NL63, 229E) worldwide.

Objectives: This study was conducted to assess the causative agents of viral ARI among hospitalized adults by real-time PCR.

Methods: Clinical nasopharyngeal swabs of 112 patients including 55 (49.1%) males and 57 (50.89%) females with ARI were analyzed using multiplex real-time RT-PCR.

Results: Out of 112 specimens, 61 (54.46%) were positive including 10 (8.9%) for influenza H3N2, one (0.89%) for influenza B, 28 (25%) for RSV-A, 18 (16.07%) for HMPV-A, two (1.78%) for HPIV-1, and three (2.67%) for HPIV-3. Two (1.78%) specimens were positive for two agents, RSV-A/HMPV-A and RSV-A/HPIV-3. The distribution of viral infections was 30 among males (26.78%) and 31 (27.67%) among females (P = 0.862). High frequency of RSV-A infection (25%) and the low frequency of influenza B virus infection (0.89%) were detected among patients. The remaining 51 (45.53%) samples were negative for RSV-B, HMPV-B, IfV-A, HPIV2-4A-4B, and HCoVs (OC43/HKU1, NL63, 229E).

Conclusions: The role of other viruses such as human adenoviruses rhinovirus/enterovirus (RV/EV), human bocavirus (HBoV), and human parechovirus (HpeV) was not investigated. Multiplex PCR can be used as a rapid test for the diagnosis of viruses causing acute respiratory infection, which results in decreased length of hospitalization.

Keywords: Acute Respiratory Infection, Real-Time Polymerase Chain Reaction, Genotype

1. Background

Human metapneumovirus (HMPV), human parainfluenza viruses (HPIV-1, HPIV-2, HPIV-3, and HPIV-4), respiratory syncytial virus type A (RSV-A, RSV-B), influenza A and B viruses and HCoVs (OC43/HKU1, NL63, 229E) are common viruses to cause acute respiratory infection (ARI) worldwide (1). Human metapneumoviruses (HMPVs) are the members of the genus *Metapneumovirus* and family *Pneumoviridae* (2). Human metapneumoviruses are causative agents of ARI worldwide (3, 4). Human metapneumovirus is classified into two distinct groups A and B. Based on nucleotide sequence variations, each group is further divided into two subgroups (A1 and A2 in group A and B1 and B2 in group B). In addition, subgroup A2 is further classified into two distinct clades A2a and A2b (5).

Human respiratory syncytial virus (HRSV) types A and B belong to genus *Orthopneumovirus* and family *Pneumoviridae* (2). The human respiratory syncytial virus is responsible for bronchiolitis and pneumonia in infants and young children (6, 7). Human respiratory syncytial virus group A is subdivided into 11 genotypes such as GA1-GA7, ON1, SAA1, NA1, and NA and HRSV group B is categorized into genotypes GB1-GB4, SAB1-SAB3, SAB4, URU1, URU2, BA1-BA12, and THB (8, 9). Human parainfluenza virus (HPIV) 1-4 are belong to family *Paramyxoviridae* HPIV-1 and HPIV-3 belong to genus Respirovirus, and HPIV-2 and HPIV-4 to genus Rubulavirus (10). Human parainfluenza viruses are considered common causes of acute upper and lower respiratory

infections among children and adult populations worldwide (11). Parainfluenza viruses (HPIVs) are associated with clinical illnesses such as otitis media, pharyngitis, conjunctivitis, croup, tracheobronchitis, and pneumonia based on genetic and antigenic variations (polymerase, matrix, and nucleoprotein). Human parainfluenza viruses are classified into four types HPIV-1 to HPIV-4, among which HPIV-4 is regarded as less important (11).

There are four types of influenza viruses A, B, C, and D. Human influenza A (H1N1, H3N2) and B viruses can cause seasonal epidemics of diseases including ARI among all age-groups in different regions of the world (12, 13). Four types of HCoVs (HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1) have been associated with a wide range of respiratory illnesses, including the common cold, pneumonia, and bronchiolitis (14). Concerning clinical manifestations of ARI, it is difficult to distinguish between RSV, HMPV, influenza virus (A, B), HPIVs (1-4) and HCoVs (OC43/HKU1, NL63, 229E) infections. Therefore, the primary diagnosis is important to ease the early management and control of ARIs (15).

To date, the standard detection of viral ARI has been achieved through the application of molecular methods. Among them, the real-time PCR technique provides a productive solution for early diagnosis of specific respiratory DNA or RNA viruses (16). To compare real-time PCR with traditional virus culture and immunofluorescence detection methods, real-time PCR has advantages of highly sensitive, rapid, and simultaneous detection of multiple pathogens. Multiplex real-time PCR allows detecting multiple pathogens simultaneously in a single reaction as a cost-effective method (17).

2. Objectives

This study was conducted to evaluate the detection of HMPV, RSV-A, RSV-B, HPIV-1, HPIV-2, HPIV-3, HPIV-4, influenza A and B viruses, and HCoVs (OC43/HKU1, NL63, 229E) in hospitalized patients using multiplex real-time PCR.

3. Methods

3.1. Study Design and Sample Collection

The samples for this cross-sectional study included throat swabs received from hospitals of Ahvaz city, Iran, from September 2015 to December 2016. The target population comprised adults above 40 years of age who referred to the hospitals with clinical ARI. Most patients had symptoms, including congestion, runny nose, coughs, sore throat, body aches, fatigue, fevers over 39°C, chills, difficulty in breathing, dizziness, loss of consciousness, bronchitis, pneumonia, and bronchiolitis. Throat swabs were

obtained from patients, put in viral transport medium with ice packs, and transferred to a Virology Department. They were kept at -80°C before RNA extraction.

3.2. RNA Extraction and cDNA Synthesis

Viral RNA was extracted from 112 throat swabs using a high-pure nucleic acid extraction kit (Roche Diagnostic, Manheim, Germany) following the manufacturer's instructions. The cDNA was synthesized from the extracted RNA samples using a thermos-scientific cDNA synthesis kit following the manufacturer's instructions.

3.3. Primers and Real-Time PCR

Based on *Taq*Man technology, real-time PCR was used to detect viruses associated with ARI. Table 1 shows primers and probes used for different viral respiratory infections including influenza A (IfA) H1N1 and H3N2 and influenza B (IfB) viruses, parainfluenza viruses (HPIV-1, HPIV-2, HPIV-3, and HPIV-4), respiratory syncytial viruses (RSVs) A and B, human coronavirus HCoVs (OC43/HKU1, NL63, 229E), and human metapneumoviruses A and B. The PCR reaction mixture contained 2X reaction buffer, 10 μM of each of forward and reverse primers, 2 μL of cDNA template, and D/W water up to 20 μL. All the samples including positive and negative controls were subjected to ABI one-step thermal cycling with the following conditions: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds and annealing at 60°C for 20 seconds.

3.4. Statistical Analysis

The data were analyzed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). The chi-square test was used for the analysis of categorical variables including gender, distribution of viral infections among genders and different age groups. P values of $\leq$ 0.05 were considered statistically significant.

4. Results

Within 15 months, 112 ARI patients were enrolled in the study. Among them, 55 (49.1%) were males and 57 (50.89%) females. Out of 112 samples, 61 (54.46%) were positive for viruses causing respiratory infections. Among them, there were 30 (26.78%) males and 31 (27.67%) females (P = 0.862). The frequency of viruses among ARI patients was as follows: Influenza A H3N2, n = 9 (8.03%), Influenza B, n = 1 (0.89%), RSVA, n = 28 (25%), MPVA, n = 18 (16.07%), HPIV1, n = 2 (1.78%), and PIV3, n = 3 (2.67%) (Table 2). Two (1.78%) specimens were simultaneously positive for two agents: RSV$_A$/MPV$_A$ and RSV$_A$/PIV$_3$. The remaining 51 (45.53%) patients were negative for RSVB, MPVB, IfV H1N1, PIV2-4A-4B,

Table 1. Primers and Probes Used to Detect Viruses Associated with ARI

Virus (Gene Target)	Amplicon Size (bp)	Function (5/3 Probe Dye)	Oligonucleotide Sequence
RSV polymerase	94	Forward, L gene, 13907 - 13934 (MH447960.1)	AATACAGCCAAATCTAACCAACTTTACA
		Reverse,14000 - 13980	GCCAAGGAAGCATGCAATAAA
		Probe A (FAM/MGB)	TGCTAYTGTGCACTAAAG[a]
		Probe B (VIC/MGB) (MK534512.1)	CACTATTCCTTACTAAAGATGTC
MPV fusion (F)	A = 69	Forward F, 3580 - 3602, (KJ627430.1)	GCCGTTAGCTTCAGTCAATTCAA
		Reverse F, 3627 - 3648	TCCAGCATTGTCTGAAAATTGC
		Probe A (FAM/MGB)	CAACATTTAGAAACCTTCT
	B = 74	Forward, 3597 - 3619 (KF530178.1)	GCTGTCAGCTTCAGTCAATTCAA
		Reverse, 3648 - 3670	GTTATCCCTGCATTGTCTGAAAACT
		Probe B (VIC/MGB)	CGCACAACATTTAGGAATCTTCT
P1V1 (L protein)	84	Forward, L,12605 - 12634 (MH892404.1)	ACAGATGAARTTTTCAAGTGCTACTTTAGT[a]
		Reverse, 12660 - 12688	GCCTCTTTTAATGCCATATTATCATTAGA
		Probe B (FAM/BHQ) 12638 - 12658	ATGGTAATAAATCGACTCGCC
PIV2 (L protein)	78	Forward L, 10773 - 10802 (KY674972.1)	TGC ATGTTTTATAACTACTGATCT TGCTAA
		Reverse, 10828 - 10850	GTTCGAGCAAAATGGATTATGGT
		Probe B (VIC/MGB)	ACTGTCTTCAATGGAGATAT
PIV3 (matrix)	66	Forward M, 4172 - 4190	TGCTGTTCGATGCCAACAA
		Reverse, 4210 - 4237	ATTTTATGCTCCTATCTAGTGGAAGACA
		Probe B (FAM/MGB)	TTGCTCTTGCTCCTCA
PIV4A (nucleoprotein)	72	Forward 4A, 365 - 389	GRGCATTATTATCTCTGCTTTCCTT[a]
		Reverse 4A, 415 - 435 (KF878965.2)	GGCTCTGGCAGCAATCATAAG
		Probe A (VIC/MGB), 400 - 416	CACATCAATGCAGAATC
		Forward 4B, 416 - 436	GGAGCGTTATTATCTCTGCTTTCTTT
		Reverse 4Bm, 397 - 413 (EU627591.1)	GGCTCTGGCAGCAATCATAAG
		Probe B	CACATCAATGCAGAATC
FluA (matrix)	111/112	H1N1, matrix, 123 - 148. (HE584759.1, H1N1)	TCTYATGGAATGGCTAAAGACAAGAC[a]
		Forward H3N2, matrix, 137 - 161 (CY202867.1, H3N2)	CTYATGGAATGGCTAAAGACAAGAC[a]
		Reverse A	SCGTCTACGCTGCAGTCCTC[a]
		Probe H1N1 (VIC/MGB)	CTGGGCACGGTGAGC
		Probe H3N2 (FAM)	ACTGGGCACGGTGAG
Flu B (matrix)	76	Forward B, matrix, 25 - 46, (MK676289.1)	CACAATTGCCTACCTGCTTTCA
		Reverse B, & 3 - 100	CCAACAGTGTAATTTTTCTGCTAGTTCT
		Probe B (VIC/MGB)	CTTTGCCTTCTCCATCTT
C0V (polymerase)	OC43/HKU1 = 96	Forward 1, 15068 - 15087, OC43 (MK303625.1) HKU1, MK167038.1	TGGTGGCTGGGATGATATGT
		Reverse 1, 15141 - 15163	GGCATAGCACGATCACACTTAGG
	NL63 = 100	Forward 2, NL63, (MG428707.1)	TTTATGGTGGTTGGAATAATATGTTG
		Reverse 2	GGCAAAGCTCTATCACATTTGG
		Probe 1A (FAM/MGB)	ATAATCCCAACCCATRAG[a]
	229E = 99	Forward 3, 13973 - 13992, (KF293666.1)	TGGCGGGTGGGATAATATGT
		Reverse 3, 14046 - 14071	GAGGGCATAGCTCTATCACACTTAGG
		Probe 2 (VIC/MGB)	ATAGTCCCATCCCATCAA

[a] R = A or G, K = G or T, Y = C or T, M = A or C, and S = G or C.

and HCoVs (OC43/HKU1, NL63, 229E). Table 2 shows the distribution of viruses causing respiratory infection among different age groups. High frequency of viruses causing respiratory infections were observed in winter 55/76 (49.1%), while low detection of viruses were observed in autumn 6/36 (5.35%) (Figure 1). No virus infection was detected

Table 2. Distribution of Respiratory Virus Infections in Genders and Age Groups[a]

Category	Male	Female	P Value
Gender	30 (26.78)	31 (27.67)	0.862
RSV	15 (13.39)	13 (11.6)	0.877
Influenza A2	5 (4.46)	4 (3.57)	
Influenza B	-	1 (0.89)	
MPVA	8 (7.14)	10 (8.92)	
HPIV1	1 (0.89)	1 (0.89)	
HPIV3	1 (0.89)	2 (1.78)	
Total	30/55 (25.89)	31/57 (27.68)	
Age			0.521
40 - 49	5 (4.46)	6 (5.35)	
50 - 59	9 (8.03)	14 (12.5)	
60 - 69	12 (10.71)	9 (8.03)	
> 70	4 (3.57)	2 (1.78)	

[a] Values are expressed as No. (%).

in spring and summer (P = 0.000). Figure 1 illustrates the distribution of positive and negative viruses causing respiratory infection in different seasons.

5. Discussion

To date, viruses causing respiratory infections have been easily detectable in clinical laboratories by multiplex PCR tests. Thus, it will be helpful to acquire epidemiological data to ameliorate patient management. In the present study, 54.46% of patients were positive for viruses causing ARI. Among them, the most frequent viruses were RSV-A (n = 28; 25%) and HMPV-A (n = 17; 15.17%). Previous studies indicated that RSVA was the most frequent etiologic agent in patients with ARI [18-20]. Arjeyni et al. reported a high frequency of RSVA (81%) and RSVB (19%) in adult patients with ARI in Iran [21]. Birger et al. detected 85/168 (50.6%) human rhinovirus (HRV), 65 (38.7%) coronavirus (CoV), and 18 (10.2%) other viruses (human adenovirus, human metapneumovirus, influenza virus, and parainfluenza virus) in patients with ARI in the USA [22]. In our survey, the positivity of other viruses causing respiratory infections was as follows: H3N2 n = 9 (8.03%), Influenza B n = 1, (0.89%), HPIV-1 n = 2 (1.78%), and HPIV-3 n = 3 (2.67%).

Han et al. in South Korea detected high frequency of Rhinovirus (n = 17; 37%), followed by parainfluenza virus (n = 14; 30.4%), respiratory syncytial virus (n = 10; 21.7%), coronavirus (n = 4; 8.7%), human metapneumovirus (n = 3; 6.5%), adenovirus (n = 2; 4.3%), Influenza A virus (n = 1; 2.2%) while no Influenza B virus and human bocavirus were detected in patients with ARI [23]. Han et al.'s findings are not consistent with our results. In our survey, 51 (45.53%) ARI patients were negative for RSVA, RSVB, Influenza A2,
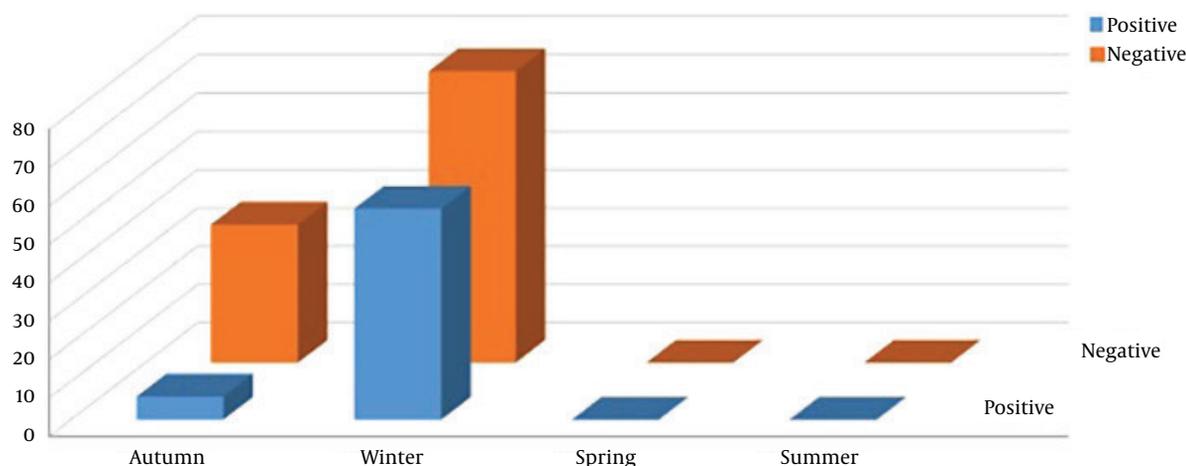
Influenza B, HPIV1, HPIV3, and HCoVs (OC43/HKU1, NL63, 229E). In this survey, we did not investigate the detection of human adenoviruses rhinovirus/enterovirus (RV/EV), human bocavirus (HBoV), and human parechovirus (HpeV) in patients with ARI.

In Iran, the circulation of Adenovirus (AdV) has been reported in patients with ARI. Pourakbari et al. reported that the frequency of AdV was 3.4% in patients with ARI in Tehran [24]. Tabasi et al. described that the rate of the Human Boca virus was 10.7% in children with ARI in Tehran [25]. In our study, HCoVs (OC43/HKU1, NL63, 229E) were not detected in ARI patients. Mehdi et al. reported that 5.5% of ARI patients were positive for Coronavirus E229 in Tehran [26]. In our study, two (1.78%) patients had two mixed viral infections, RSV_A/MPV_A and RSV_A/PIV_3. Many researchers have described the co-infection of viruses causing respiratory tract infections. Yoshida et al. in Japan detected the co-infection of human rhinovirus, human metapneumovirus, and parainfluenza virus-3 in patients with ARI [27]. Petrarca et al. in Italy reported the co-infection of HRSV and HRV in patients with lower respiratory tract infection [28]. The distribution of viruses causing ARI were 30 (26.78%) in males and 31 (27.67%) in females (P = 0.862).

The influenza vaccination is not mandatory in Iran although high-risk groups need to receive the vaccine. In this experiment, the outbreaks of respiratory virus infections were observed in autumn and winter, which are consistent with other reports [29, 30]. No virus causing respiratory infection was detected in spring and summer seasons in this region of Iran. This might be due to that the temperature exceeds 40°C in the spring and remains constant above 48°C during summer. Ribavirin and oseltamivir antiviral therapy can reduce the progression of URI to LRI and mortality rate due to RSV and influenza virus infections [31]. Currently, no specific antiviral therapy is available against human rhinoviruses, human coronaviruses, human metapneumovirus, and human bocavirus. Therefore, new treatment and preventive options are required. Sensitive molecular methods like multiplex real-time PCR in clinical practice can help the rapid diagnosis of viruses causing respiratory infection and reduce improper antibiotic therapy [32]. Overall, several investigations have described that multiplex real-time PCR as a non-invasive method is faster and more sensitive than other standard methods such as serology and culture [33, 34].

5.1. Conclusions

In conclusion, in this study, RSVA (n = 28, 25%) and MPVA (n = 18, 16.07%) were the most common causes of RI in adults. Considering the low diagnostic cost of clinical presentation and laboratory findings in ARI, multiplex real-time PCR as a rapid diagnostic test can be consid-

Figure 1. The chart showing a high frequency of viral respiratory infections observed in winter (49.1%), followed by autumn (5.35%); no sample was observed in spring and summer (P = 0.000).

ered efficient in reducing the length of patients' stay in hospitals. The results indicated that 49 (43.75%) patients were negative for RSVA, RSVB, Influenza A2, Influenza B, HPIV2, HPIV4, HCoVs (OC43/HKU1, NL63, 229E); however, it requires further investigation to disclose the role of human adenoviruses rhinovirus/enterovirus (RV/EV), human bocavirus (HBoV), and human parechovirus (HpeV).

Acknowledgments

Footnotes

Authors' Contribution: Study concept and design: Manoochehr Makvandi; collection of data: Niloofar Neisi, Manoochehr Makvandi, and Rahil Nahid Samiei; anlysis and interpretation of data: Manoochehr Makvandi and Samaneh Abbasi; drafting of manuscript: Samaneh Abbasi, Somayeh Biparva Haghighi, and Manoochehr Makvandi; critical revision of the manuscript for important intellectual concept: Niloofar Neisi and Manoochehr Makvandi; statistical analysis: Kambiz Ahmadi Angali; administrative, technical, and material support: Shokroallah Salmanzadeh and Mehran Varnaseri Ghandali; supervosion: Manoocher Makvandi and Mojtaba Rasti.

Conflict of Interests: No conflict of interest is declared by the authors.

Ethical Approval: This study with code number ajums.REC.1393.126 was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Iran. All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

Patient Consent: Informed consent was obtained from each patient.

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