PŮVODNÍ PRÁCE

Human Rhinoviruses A9, A49, B14 and Echovirus 3, 9 among the patients with acute respiratory infection

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ABSTRACT

Background: Acute respiratory infection result in high mortality and morbidity worldwide. There are several viral factors that originate respiratory diseases among them Enteroviruses(EVs) and Human Rhinoviruses(HRVs) can be mentioned. HRVs and EVs belong to Picornaviridae family and they have been recently classified under Enteroviruses. The pattern of respiratory infections generating organisms varies according to geographical locations. Therefore, it seems necessary to organize an appropriate plan to manage common viral diseases exclusively about Rhinoviruses and Enteroviruses.
Patient and Methods: A total of 100 samples were collected from patients with acute respiratory infections (ARIs) who were hospitalized in Ahvaz city hospitals during December 2012 to November 2013 (one year longitude). Semi-Nested PCR was done on samples for detection of HRVs and EVs using region gene of VP4/VP2. Phylogenetic and molecular evolutionary analyses performed with MEGA version 5 software find out the sequence homology among the detected HRV and EV serotype.
Results: The results of this study revealed that from of 100 cases of ARIs 19 patients (19%) were HRV positive and 3 (3%) patients positive for EVs. Most positive cases of HRVs were observed in the autumn season while 3 positive cases of EVs were equally found in spring, summer and autumn. Phylogenetic analyses showed that the HRV strains were HRV-A9, HRV-A49, HRV-B14 and EV strains were Echo3 and 9.
Conclusion: The results of this study revealed that high prevalence of 19% HRVs, HRV-A9, HRV-A49, HRV-B14 serotypes and low frequency of 3% Echo Viruses, Echo3 and Echo 9 serotypes have been detected in patients with ARI.

KEYWORDS

Human rhinoviruses (HRVs) – Enteroviruses (EVs) – Semi-Nested PCR – acute respiratory infections (ARIs) – Phylogenetic analysis – Khuzestan province

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BACKGROUND

Respiratory system infection is one of those important reasons of hospital referrals for infectious diseases [1]. In developing countries, it has been regarded as one primary reason of death among children with immunological defects [2–4]. There are several viral infections involve in respiratory diseases among them Enteroviruses and rhinoviruses are less reported [4, 5]. Both Rhinoviruses and Enteroviruses genus belong to Picornaviridae family and they have been recently classified under Enteroviruses [6].They are small-sized viruses with 30nm diameter, non-enveloped, having icosahedral capsid and single sense positive RNA genome with 7200nt of length. Despite the fact that HRVs are the cause of upper respiratory mild infections during fall and spring, but recent evidences showed HRV may be accompanied by severe respiratory problems. Primarily, Enteroviruses have tendency to digestive system, but findings revealed that some including EV 68, enterovirus 71 (EV71), coxsackievirus A16 (CA16), Echovirus 3,6,9, coxsackie A12,Coxsakie B, can cause acute respiratory infection [7–10]. Almost 100 different serotypes of HRV have been identified and classified into 3 types (HRV A–C) [6].Throughout present study, the RT-PCR was utilized to screen HRV and HEVs. To do this, we used designed primers which covers for a part of 5'UTR region and (a conserve region among Picornaviridea) and a part of VP_4/VP_2 region to identify enterovirus and rhinovirus serotypes [11, 12]. Thus this study was aimed to investigate the role of Human Rhinoviruses and Enteroviruses among patients with acute respiratory infection Ahvaz city. Ahvaz city is capital of khozestan province is located in south west region of Iran.

PATIENT AND METHODS

The sampling was carried out on hospitalized patients with acute respiratory infections (ARIs) in the Ahvaz city hospitals during December 2012 to November 2013 (one year longitude). The sampling procedure was a cross sectional study. To do this, 100 patients were registered. The physicians recorded history from each ARI patient. All the patients had clinical signs and symptoms including coughing, headache, congestion, runny nose,

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fever, difficult breading, and wheezing. The samples of Nasopharynx swab were obtained from patients. Then, all the samples were transferred to Ahvaz Jundishapour University of Medical Sciences Virology lab and stored at -70 °C until the time of extraction.

RNA extraction
The viral RNA was extracted from throat samples of patients by a High Pure Viral Nucleic Acid Kit (Roche Applied Science, Germany) according to the manufacturer's instructions, and the extracted RNA was stored at -70 °C.

Reverse transcription (RT)
cDNA was synthesized from the extracted RNA using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit, according manufacture instruction. The products were stored at -20 °C until use.

Semi-nested RT-PCR
To do PCR reaction variable region gene of VP4/VP2 of the enteroviruses were assigned as the target sequences to discriminate HRV and enterovirus serotypes. For the first round, 5µl of cDNA was added into 25µl PCR reaction containing 2.5µl of 10 x Buffer MgCl2 (Roche manufacturer's), 0.5 µl of 10 mM dNTP, 1.25 U/µl of Taq DNA polymerase (Roche Diagnostic Systems) and 1 µl (10 pmol) of each primer include; forward primer1 (MD91) [13] and reverse primer(OL68-1) [14] (table 1) and D/W 14.8 µL. The first round PCR was performed Thermo cycler (PQ-lab, Germany), programed for initially at 94 °C for 5 min followed by, 35 cycles, 95° for 60 s, 48 °C for 60s, and 72 °C for 1 min. Final extension was performed at 72 °C for 10 minutes. The primers forward primers 2 (EVP4) [14] and reverse primer(OL68-1) – table 2 – were used for the second round. The 25 µl PCR reactions buffer containing 2.5 µl of PCR product of the first round,2.5 µl of 10 x Buffer MgCl2, 0.5 µl dNTP (10 mM), 1.25 U of Taq DNA polymerase and 1 µl (10 pmol) of each primer and D/W 17.3 µl. The PCR was performed for 30 cycles consisting initially 5 min for 95 °C followed by 95 °C for 1 min, 58 °C for 60 s, 72 °C for 1 min, and finally one cycle at 72 °C for 5 min. Polio virus vaccine was used as positive control and DPEC as negative control. PCR product of 650 bp indicates positive for Enteroviruses and 530 bp for Rhinoviruses. After second rounds PCR, 6 PCR product samples were sequenced (Bioneer Corporation, South Korea) – Figures 1.

Phylogenetic Analysis and compute pair wise distances
The results of sequencing of VP4/VP2 were blasted to determine Enteroviruses and Rhinoviruses serotype and it was compared with sequences retrieved from GenBank using online nucleotide BLAST, National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/).To evaluate nucleotide homology between the detected Enteroviruses and references isolated Enterovirus serotypes recovered from GenBank and the nucleotide homology between the detected Rhinoviruses and references isolated Rhinovirus serotypes retrieved from GenBank, the phylogenetic trees using MEGA5 program was used. The Phylogenetic trees were constructed by the neighbor-joining method with 1,000 bootstrap replicates [15]. To assess nucleotide homology in detected Enteroviruses, Rhinoviruses and related reference strains [16, 17] – Figures 2, 3.

Nucleotide sequence accession numbers: The 6 partial sequences of VP4/VP2 have been submitted to GenBank database to record and received accession numbers (table 3).

RESULTS

Statistical Analysis
For Statistical analyses Chi-square test and descriptive statistics was done by SPSS software version 22. The conventional p-value of ≤ 0.05 was considered as overall significant level.
The following accession numbers have been recorded in GenBank database (table3).
In the present study, 41/100 (41%) and 59/100 (59%) of subjects were women and men respectively.19/100 (19%) individuals showed positive for Rhinovirus which among them 5/100 (5%) were men and 14/100 (14%) women (Odds ratio 0.446 (95%) confidential interval, lower 0.147, upper 1.365, P = 0.148) (Table 4). The results of sequencing and blasting showed the frequency of HRV-A9, HRV-A49, HRV-B14 serotypes and frequency of Enteroviruses were Echo3 and Echo 9 serotypes among the patients with ARI in Ahvaz city. The frequency of rhinoviruses were observed in different age grouping and not found significant (table 5). In present work the seasonal prevalence of Rhinoviruses in summer 5(5%), fall 14 (14%). Low num-

Table 1. Primers used for PCR

Sense	Region	Sequences	Primers
+	Nt 541–560 5'UTR	CTACTTTGGGTGTCCGTGTT	forward primer2 EVP4
–	Nt 1178–1197 VP4/VP2	GGTAA(C/T)TTCCACCACCA(A/T/G/C)CC	Reverse primer OL68-1

Table 2. Primers used for PCR

Sense	Region	sequence	Primer
+	Nt 443–461 5'UTR	TCCTCCGGCCCCTGAATG	Forward primer1 MD91
–	Nt 1178–1197 VP4/VP2	GGTAA(C/T)TTCCACCACCA(A/T/G/C)CC	Reverse primer OL68-1

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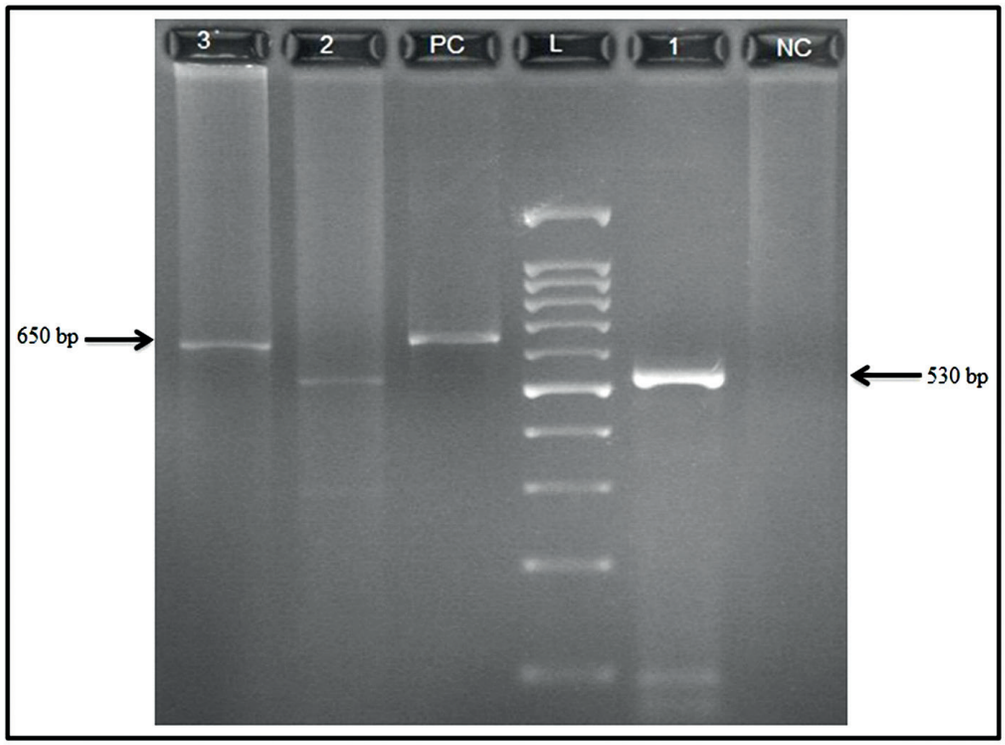

Figure 1. NC (Negative Control). L (Ladder).
PC (Positive Control). 1, 2, 3 (sample) 650bp (Enterovirus). 530bp (Rhinovirus)

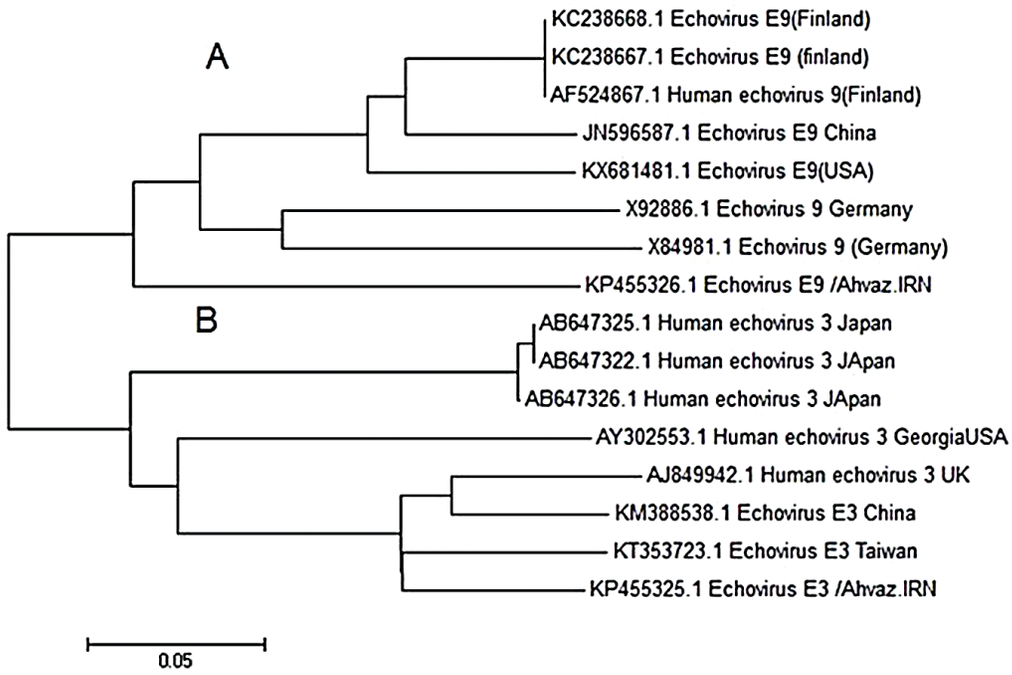

Figure 2. Comprises A and B parts.
(A) A phylogenic tree constructed based on partial nucleotide sequences of the VP2-VP4 regions of the strains of Echoviruses 9, (B) phylogenic tree of Echoviruses 3 isolated from patients with acute respiratory Ahwaz city – Iran. The references sequences retrieved from GenBank. The phylogenetic trees were built with the NJ method using MEGA 5 software and tested with 1000 bootstrap replicates. Scale bar = 5%

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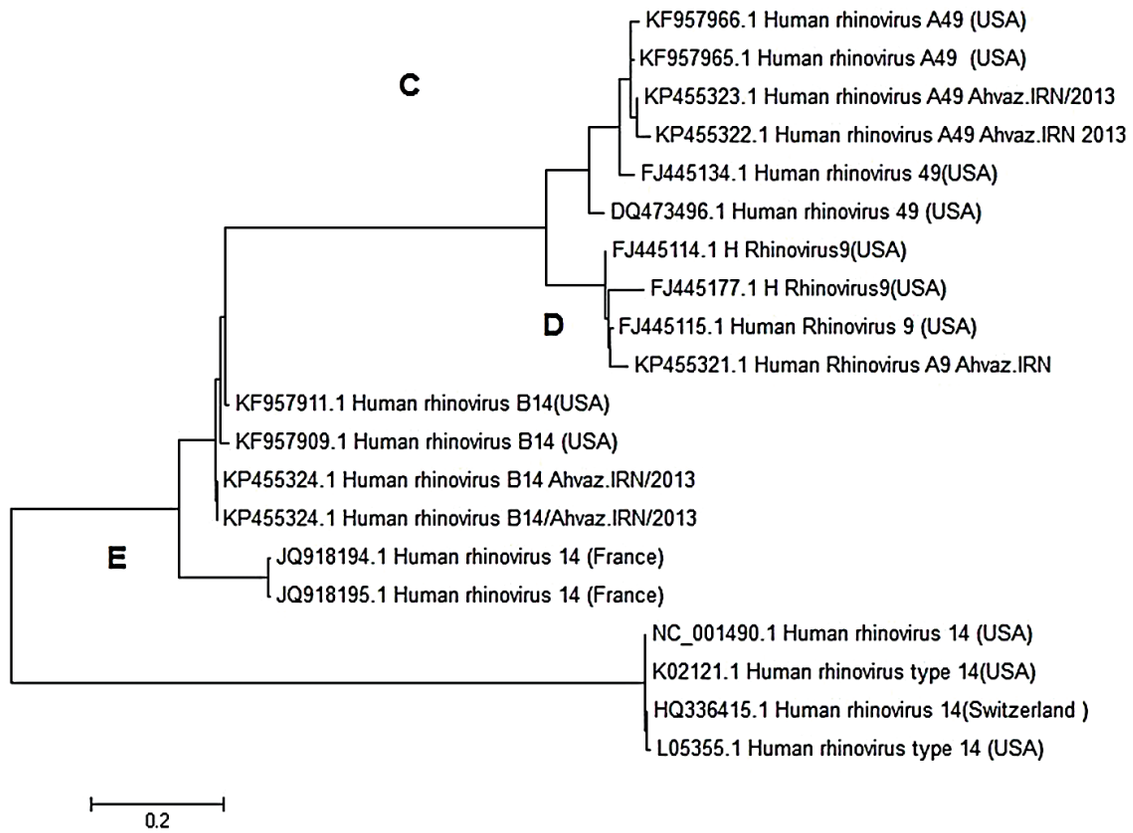

Figure 3. A phylogenic tree constructed based on partial nucleotide sequences of the VP2-VP4 regions of the strains of acute respiratory Human Rhinoviruses A9(C), A49 (D) and B14 (E) isolated from patients with acute respiratory infection in Ahwaz city – Iran.
The references sequences retrieved from GenBank. The phylogenetic trees were built with the NJ method using MEGA 5 software and tested with 1000 bootstrap replicates. Scale bar =2%

ber of Echovirus E3, E9 were detected and belonged to Enteroviruses group B.

DISCUSSION

In our present work 19(19%) samples showed positive for Rhinovirus which HRV-A9, HRV-A49, HRV-B14 serotypes were identified, 3 (3%) samples positive for Enteroviruse which Echo3 and Echo 9 serotypes were detected. Among those with positive Rhinoviruses, 5% (5/100) were male and 14% (14/100) were female. The frequency of rhinoviruses were observed in all group age patients. The prevalence of rhinoviruses were observed in summer 5 (5%), fall 14 (14%). In the present study No Enteroviruses 68 was detected. Kaida et al. reported out of 448 individuals with severe respiratory infections, 15 samples showed positive for Enteroviruses 68, and 140 positive for Rhinoviruses [18]. Khadadah et al. studied on 1014 patients with lower respiratory system infections and found 28.4% of patients (288 individuals) had viral infections which among them 34.4% were infected by Rhinovirus which occurred during fall and winter [4]. Our finding is in agreement with Khadadah et al. results.

In recent study conducted by Timmermans A et al., in Cambodia (2016), have screened 586 patients with Influenza like Illness and observed low prevalence of Echovirus-E6 (2.6%), E9 (3.9%) which is in accordance with our finding [9]. Mizuta et al. in Japan (2001) have detected CVA2(5.53%), CVA4 (1.7%), CVA16 (2.2%), CVB2 (0.1%), CVB3 (1.5%), CoxB5 (0.27%), Echol6 (7.46%), entero71 (0.27%) and polio2 (0.13%) which Echovirus16 showed dominant [19].

Imamura et al. have studied ARI among 816 patients and detected 245 (30%) Rhinoviruses and 21 (2.5%) Enteroviruses EV68, EV70, EV94 and coxsackie virus A16 [20].

In Lauing et al. reported 36/473(7.61%) individuals with acute respiratory infection were infected by Enteroviruses. Among them 18 identified Enteroviruses

Table 3. Rhinoviruses and Echoviruses accession numbers

No	Accession No	Virus typing
1	KP455321.1	Human rhinovirus A9
2	KP455322.1	Human rhinovirus A9
3	KP455323.1	Human rhinovirus A49
4	KP455324.1	Human rhinovirus B14
5	KP455325.1	Echovirus 3
6	KP455326.1	Echovirus 9

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Table 4. Frequency of Rhinovirus according to Genus

Gender	Rhinovirus	P-Value	
	Positive	Negative	
Male	5 (5%)	36 (36%)	0.148
Female	14 (14%)	45 (45%)	
Total	19 (19%)	81 (81%)	

Table 5. *Frequency of Rhinovirus according to Different Age Groups*

Age Group	Rhinoviruses	Odd`s Ratio (95% CI)	P-Value	
	Positive	Negative		
≤ 15	2 (10.5%)	10 (12.3%)	0.835 (0.167–4.170)	0.826
16–30	3 (15.8%)	18 (22.2%	0.656 (0.172–2.506)	0.536
31–45	7 (36.8%)	31 (38.3%)	0.941 (0.334–2.647)	0.908
46–60	5 (26.3%)	16 (19.8%)	1.451 (0.456–4.621)	0.527
≥ 61	2 (10.5%)	6 (7.4%)	1.471 (0.273–7.928)	0.652
Total	19 (19%)	81 (81%)	–	0.931

68 serotype and 18 Enteroviruses but no Rhinoviruses and Echoviruses have been detected (19).

The role of other enteroviruses including Coxsakie A4, A6, A8, A9, A12 and Coxsakie B3, B4 have been reported in patients with acute respiratory infection (40). Chavoshzadeh et al. in Tehran(Iran) investigated on 96 children with acute respiratory infection and have detected RSV (45.8%,), HRV (13.5%) and human Metapneumovirus (HMPV) – 6.5% [21].

Gorjipour et al. have studied on 330 children with ARI and have detected Adenovirus (29.9%) Rhinovirus (23.1%), Influenza (21.6%), RSV (12.7%) and Enteroviruses(9%). The Rhinoviruses detection were in fall (71%), summer (19%), winter (26.7%) and spring (25%) [5]. Messacar et al. have investigated on 2299 patients with ARI and found that 427 (18.6%) were positive for HRV/EV and 202 (8.8%) for influenza virus. In the influenza group, 191 (95%) patients with influenza A (H1N1) virus, 10 (5%) with seasonal influenza A virus, and 1 (0.5%) patient with influenza B. The seasonal prevalence of Rhino/Enteroviruses was found in spring, summer and fall [22].

Wisdom et al. have studied Rhino/Enteroviruses infections among 456 children with ARI. The frequency of Coxsackie virus A (0.6%), B (0.6%), HRV-A (14%), HRV-B (1.18%) and HRV-C (6.8%) were found among the children [23].

Jean Longtin et al. have reported out of 297 patients with ARI and found 174 (59%) positive for Rhinoviruses HRV-A31, HRV-A 33, HRV-A 82, and HRV-C N7 [3].The role of other HRVs including HRV 30, HRV 44 and HRV 49 have been reported among patients with ARI in United States of America [24]. Overall 22% of patients with ARI showed positive for HRV and EV but 78% were negative. The role of other viruses involve in ARI such as RSV, human Metapneumovirus(HMPV), parainfluenza, Influenza A, B Adenovirus should be investigated [4, 21].

In conclusion the results of this study revealed that high prevalence of 19% HRVs, HRV-A9, HRV-A49, HRV-B14 serotypes and low frequency of Echo viruses, Echo3 and Echo 9 serotypes found in the patients with ARI. The distribution of HRVs were found among the all age groups of patients. In the light of aforementioned data the role of other virus including RSV, HMPV, parainfluenza, Influenza A, B Adenovirus should be investigated.

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Authors' Contributions
Manoochehr Makvandi: study design, and manuscript correction. Alireza Samarbafzadeh: funding support. Ahmad Shamsizadeh: study design. Amir Pouremamali: experimental works, data collection, bioinformatics consults, writing manuscript. Niloofar Neisi, Mojtaba Rasti, Rahim soleimani, Roohangiz Nashibi, Shokrallah Salmanzadeh, Roya Nikfar and Abdolnabi Shabani: sample collection. Ali Teimoori: bioinformatic consult. Mehrdad Sadeghi Haj: statistical analysis.

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