Original Article

ISSN: 1735-0344 *Tanaffos 2024; 23(1): 30-37*

TANAFFOS

Prevalence and Phylogenetic Analysis of Rhinoviruses in Patients with Acute Respiratory Infections and Community-Acquired Pneumonia in a Referral Hospital, Tehran, Iran

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Received: 24 April 2023
Accepted: 9 January 2024

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Background: Rhinoviruses are known as the leading pathogens of respiratory diseases. Determining the prevalence and phylogeny of rhinoviruses plays a pivotal role in producing vaccines and medications and preventing virus complications. This study investigated the frequency, and genetic variation of rhinoviruses detected in patients referred to Masih Daneshvari Hospital.

Materials and Methods: It was a descriptive cross-sectional study. The samples were from all ages whose information was recorded in 2017 according to a clinical diagnosis of acute respiratory infection (ARI) and in 2015 based on a clinical diagnosis of community-acquired pneumonia (CAP) within the HIS system of Masih Daneshvari Hospital. Using a random number table, 202 patients diagnosed with ARI and 51 patients diagnosed with CAP were selected. The real-time PCR method was used for primary screening; nested PCR was performed in the VP2/VP4 gene region for phylogenetic investigations, and MEGA software drew a phylogenetic tree.

Results: The highest level of rhinovirus infection was seen in subjects under 18 years of age, males, and during the spring season. In this study, the genotypes of HRV-A (including A15, A29, A40, A47, A58, A67, and A80) and HRV-C (including C39 and C44) and two samples of enterovirus D68 were found.

Conclusion: Like other studies conducted in Asia, the most detected genotypes were HRV-A and HRV-C. Conducting further studies with a larger sample size and in different geographical regions of Iran will provide us with more comprehensive information about the frequency of rhinoviruses and common genotypes.

Keywords: Rhinovirus; Acute respiratory infection; Community-acquired pneumonia; Genotype; Phylogeny

INTRODUCTION

About 4 million people worldwide die prematurely from chronic respiratory diseases annually (1). Studies have shown that rhinoviruses (RVs) are known as the leading pathogens of respiratory diseases, including the common cold in all ages, the most common pathogen in people with mild upper respiratory tract diseases, the leading cause of COPD exacerbation in adults, worsening of asthma symptoms in adults and especially in children, causing acute respiratory infections and hospitalization of patients, and as one of the leading causes of death in children under five years, especially in poor areas (2). According to available evidence, human rhinoviruses (HRVs) cause more than billions of dollars annually to

governments and individuals due to medical visits and absenteeism at work (3).

HRVs were first detected in 1950 (3). Tropism to respiratory tracts and better replication at 33 °C to 35 °C make them replicate better in the upper airways(4). RV is transmitted through close contact with the sick person, contaminated hands, and infected respiratory secretions (5, 6). Moreover, studies have shown that RV transmission from children to adults and transmission among children is more common than from adults to children (7, 8). The circulation season of this virus is different in various countries; for example, in Asia, RVs are more contagious in temperate climates, and in Europe, in cold seasons such as autumn and winter (9, 10).

RVs belong to the *Picornaviridae* family and the genus *Enterovirus*. RV is a non-envelop spherical virus with an approximate size of 30 nm and icosahedron symmetry that contains 60 copies of the virus's envelope protein. It also has an organized capsid structure and genome. The RV genome has a positive polarity single-stranded RNA of approximately 7200 base pairs and has a single ORF, expressed as a polyprotein (11). The virus capsid comprises four proteins (VP1, VP2, VP3, and VP4) covering genomic RNA. VP1, VP2, and VP3 proteins are responsible for antigenic diversity, while the VP4 protein connects the RNA core to the capsid. RVs have a remarkable genome diversity, and the high genetic variation mainly resulted from frequent co-infections caused by rhinoviruses (12). Based on the genetic sequence of the genome, RV is divided into three different types A (HRV-A), B (HRV-B), and C (HRV-C). Based on the results of previous surveys, the likelihood of a relationship between the severity of the disease and the type of RV is suggested; for example, HRV-C plays a significant role in causing severe lung diseases, asthma, and hospitalizations in children (13). The RV has more than 100 different serotypes. Genetic recombination has chiefly been seen in the 5' untranslated region (5' UTR) and internal ribosomal entry sites (IRESs) and rarely in P2-P3 gene regions. New sequences of RVs from various samples demonstrate that HRV-A and HRV-C have been repeatedly recombined (14, 15). Epidemiologically, genotypes A and C are the most frequently seen genotypes in Asia(9, 10). However, in Iran, few genetic and phylogenetic studies have been conducted on RVs. On the other hand, knowledge about RV phylogeny plays a pivotal role in the production of vaccines and drug development, as well as in preventing the complications caused by the virus. The objective of the present study was to investigate the prevalence and genetic diversity of RVs diagnosed in patients referred to Masih Daneshvari Hospital, a major referral center for respiratory diseases.

MATERIALS AND METHODS

Study Design, Sample Size, Sample Collection

The present study is a descriptive cross-sectional study. The samples were made up of men and women in all age groups whose information was recorded in 2017 with a clinical diagnosis of acute respiratory infection (ARI) and in 2015 with a clinical diagnosis of community-acquired pneumonia (CAP) in Masih Daneshvari Hospital's HIS system. The sample size in this study was determined based on the budget allocated for the research. The patients were first selected from acute respiratory patients in 2017 and patients with CAP in 2015, and their information was extracted from the HIS system. Then the clinical samples were recruited from the Virology Research Center archive and selected using a random number table. A total of 253 samples from patients referred to Masih Deneshvari Hospital (202 samples from ARI patients and 51 samples from CAP patients) were analyzed.

RV Screening

The nucleic acid extraction from clinical samples was conducted using a column base-extraction kit according to the manufacturer's instructions (SinaPure TM Viral kit, Cat. No.: EX6061, SinaClon, Tehran, Iran). Then, Real-Time PCR was used for rhinovirus screening. The primers and probe sequences used to perform the screening test were as follows: 5'-CTA GCC TGC GTG GC-3', 5'-GAA ACA CGG ACA CCC AAA GTA-3', and 5'-Fam TCC TCC GGC CCC TGA ATG YGG C-BHQ-1-3'. The total reaction volume was 25 µl, and the reaction mixture contained 0.9 µmol/L (each) of the primers, 0.15 µmol/L of the probe, the Add-Probe RT-PCR Master kit (Cat. No. 74201, AddBio Inc., Korea) components, and ten µl of the extracted sample.

The profile real-time PCR reaction was set up for 20 minutes at 50 °C, with initial denaturation for 10 minutes at a temperature of 95 °C, denaturation for 10 seconds at 95 °C, and in the last phase, annealing and extension for 60 seconds at 56 °C, and was conducted by using the CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Inc., USA).

Sequencing and Phylogenetic Analysis

After screening, some positive samples were selected for phylogenetic studies using primers targeting the VP2/VP4 gene region and nested PCR amplified a 450-bps region. The primer sequences used to perform nested PCR were as follows: 5'-CCG GCC CCT GAA TGY GGC TAA-3' and 5'-ACA TRT TYT SMC CAA ANA YDC CCA T-3' as outer primers and 5'-ACC RAC TAC TTT GGG TGT CCG TG-3' and 5'-TCW GGH ARY TTC CAM CAC CAN CC-3' as inner primers. For both rounds, the profile PCR reaction was set up in 20 minutes at 42°C, 1st denaturation for 10 minutes at 95°C, amplification for 20 seconds at 95°C, 35 seconds at 52°C and 45 seconds at 72°C by 30 times repetitions, and in the last phase, final extension for 5 minutes at 72°C. Amplification of PCR products was verified by 1.5% Agarose gel electrophoresis. The samples that showed acceptable bands on gel electrophoresis were then subjected to sequencing.

The obtained partial sequences were analyzed by BioEdit version 7.0 software (Tom Hall); after editing, the sequences in the NCBI database were checked by BLASTn software to determine the authenticity of the viral sequences (16). All sequences were blasted using MEGA X with reference sequences (17). Phylogenetic trees were constructed using the maximum composite likelihood nucleotide substitution pattern and bootstrap 1000 by MEGA X.

To determine the RV genotypes, along with referring to the phylogenetic tree, the genetic distance of the isolates compared to the reference sequences was calculated using MEGA software.

All the statistical analysis results in this study were reported using SPSS software (version 10) with a P-value significance level of 0.05.

Ethical Issues

The present study was approved by the Iran Ministry of Health's Ethics Committee and Shahid Beheshti University of Medical Sciences (IR.SBMU.NRITLD.REC.1398.051). In all stages of the project, the confidentiality of participants' information was maintained.

RESULTS

Out of 253 selected samples, 51 (20.16%) belonged to CAP patients and 202 (79.84%) to patients with ARI (Table 1). Sixty-seven samples belonged to outpatients (OP) (26.5%), and 186 samples (73.5%) belonged to inpatients (IP) (Table 1). The sample type included 245 nasopharyngeal swabs (96.8%), two Broncho alveolar lavages (BAL) (0.8%), four sputa (1.6%), and two tracheal secretion aspirates (0.8%) (Table 1).

Among all the subjects, 150 cases (59.3%) were male, and 103 cases (40.7%) were female patients (Table 1). The mean age of the studied subjects was 42.56±26.25 years, with a range of 4 months to 94 years, while the median was 45. Most samples were related to patients over 18 years old; 183 samples (72.6%) and Sixty-nine samples (27.4%) were related to those less than 18 years old (Table 1). The highest rate of received samples was related to the autumn (107 samples (42.3%), Table 1), and the lowest rate was associated with the summer (24 samples (9.5%), Table 1). In spring and winter, the number of samples was 39 (15.4%) and 83 (42.8%), respectively (Table 1).

The general prevalence of HRVs in studied samples was obtained at 11.50% (29 out of 253 patients, Table 1). Results of the present study demonstrated a significant difference between the rhinovirus prevalence in patients with ARI and CAP (ARI 13.4% vs. CAP 3.9%, P=0.04, Table 2). The rhinovirus prevalence was not significantly different between males and females (P=0.303, Table 2), but the number of males infected with rhinovirus was higher. In addition, there was no statistically significant difference in the rhinovirus prevalence in outpatients and inpatients (P= 0.521, Table 2).

The highest number of received samples occurred in autumn; however, there was a significant difference between the prevalence of HRVs in different seasons (P=0.006, Table 2); the prevalence of rhinovirus was reported more in spring (23.1%) and summer (20.8%) (Table2). Besides, HRVs were most prevalent in the age group under 18 years (P=0.001, Table 2).

Genotyping of HRV

Out of 29 rhinovirus-positive samples by real-time PCR test, 16 were positive by the nested PCR test, of which 11 were of good quality for Sanger sequencing. Then, the samples were sequenced for phylogenetic analyses.

Based on the blast study, two of the 11 sequences obtained in this study belonged to Enterovirus D68. Considering the phylogenetic tree, out of 9 RV sequences, 7 were genotype A (63.63%), and 2 were genotype C (18.18%) (Figure 1). Genotype B was not found in this study. Among the HRV-A, genotypes A15, A29, A40, A47, A58, A67, and A80 were detected; while genotypes C39 and C44 were identified among the HRV-C samples.

Table 1. The demographic characteristics of study subjects, sample type, clinical status, and prevalence of RVs

Variable		Number	Percent
HRV status	Positive	29	11.50
	Negative	224	88.50
Clinical diagnosis	Acute respiratory infection (ARI)	202	79.84
	Community-acquired pneumonia (CAP)	51	20.16
Patient status	Outpatients (OP)	67	26.5
	Inpatients (IP)	186	73.5
Sample type	Nasopharyngeal swabs	245	96.8
	Broncho alveolar lavages	2	0.8
	Sputa	4	1.6
	Tracheal secretion aspirates	2	0.8
Sex	Male	150	59.3
	Female	103	40.7
Age	18≤	183	72.6
	18>	69	27.4
Season	Spring	39	15.4
	Summer	24	9.5
	Autumn	107	42.3
	Winter	83	42.8

Table 2. Relationships between the presence of RHV in the study subjects and characteristic parameters

Variable		Rhino		P-value
		Positive N (%)	Negative N (%)	
Clinical diagnosis	Acute respiratory infection (ARI)	27(13.4%)	175(86.6%)	0.041
	Community-acquired pneumonia (CAP)	2(3.9%)	49(96.1%)	
Patient status	Outpatients (OP)	8(11.9%)	59(88.1%)	0.521
	Inpatients (IP)	21(11.3%)	165(88.7%)	
Sex	Male	19(12.7%)	131(87.3%)	0.303
	Female	10(9.7%)	93(90.3%)	
Age	18≤	13(7.1%)	171(92.9%)	0.001
	18>	16(23.2%)	53(76.8%)	
Season	Spring	9(23.1%)	30(76.9%)	0.006
	Summer	5(20.8%)	19(79.2%)	
	Autumn	12(11.2%)	95(88.8%)	
	Winter	3(3.6%)	80(96.4%)	

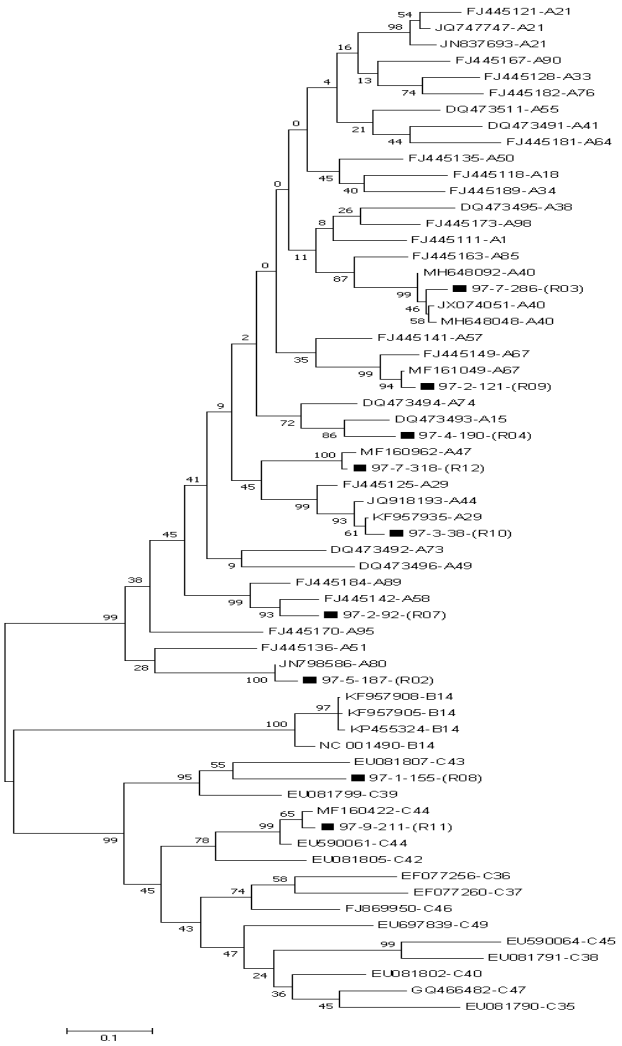

Figure 1. The image of the Phylogenetic tree using MEGA software. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model with 1000 bootstraps(18, 19).The studied isolates are marked by black squares

DISCUSSION

Rhinoviruses are among the most widespread human respiratory viruses and infect billions annually. They are also responsible for at least half of the causes of acute upper respiratory infections (3). Along with the common cold, HRV infection causes many other clinical consequences, from asymptomatic infections to critical conditions, including severe lower respiratory tract diseases such as bronchitis, pneumonia, and worsening of asthma symptoms (20). This study aimed to investigate the prevalence and genetic diversity of rhinoviruses identified among patients referred to Masih Daneshvari Hospital, a major referral center for respiratory diseases.

The results of the present study suggested that the most collected samples belonged to the cold seasons, while the least belonged to the summer; however, the prevalence of rhinoviruses was higher in spring and summer, respectively (P=0.006). It indicated the association between the season and the level of RV surges (Table 2). The seasonality results of the present study were not aligned with the findings of other studies. In 2016, in a review study by Aslam et al. on the prevalence of rhinovirus and its genotypes in Asia, including Iran, the majority of HRV was reported in autumn and winter (21). Moreover, in 2015, L'Huillier et al. from Africa suggested that the highest HRV infection was in autumn(22). In another study by Jacobs et al. in the United States, the full seasons for HRV infection were autumn, winter, and late spring, respectively (23). Evidence has shown that climate changes caused by shifting seasons provide an ideal situation for viruses' growth and increase their infectivity; their implications were caused by seasons turning through several years and various geographical regions (24, 25). In addition, surveys conducted on the prevalence of other viral respiratory infections, including influenza and COVID-19, have affirmed that the prevalence of HRV increased after the New Year's holidays (26-28). In Iran, the transmission of RV will also increase due to the Iranian New Year (Nowruz) and trips.

The present study's findings indicated a significant difference in the prevalence of RVs based on the manifestation of the disease (ARI and CAP) (3.9% CAP vs. 13.4% ARI, *P=0.041*, Table 2), and the prevalence of RVs was higher in patients with ARI. In a study in 2017 in China, out of 438 patients, 42 were infected with RV, of which 27 patients had URTI and 15 had CAP (29). RVs better replicate at 33-35 °C, and It seems the lower temperature of the upper respiratory tract is one of the reasons for the higher prevalence of the virus in patients with acute and upper respiratory infections (30).

Our results show a significant difference in RV prevalence among young and adult subjects, and the RV was higher in patients under 18 years old (P=0.001, Table 2). In 2016 a study in Asia provided that the rate of RV infection among subjects aged 3 to 18 years was 51%, of which 34.5% were under two years old (21). In 2015, an African study investigated the samples of 1005 subjects aged from 2 months to 10 years, of which 379 samples (38%) had positive RV tests (22). According to the available evidence, people under 18 years of age are more likely to get RV infections. Lack of memory T lymphocytes at a young age can be one of the reasons for the higher prevalence of RVs in children (31).

We found that the incidence of HRV infections in men was more than in women. However, there was no significant difference in the prevalence of rhinoviruses based on gender (males 12.7% vs. females 9.7%, P = 0.303, Table 2). In 2016, Sonia Aslam's study stated that the RV infection rate in men was higher than in women (21). Men are more likely to be susceptible to RV infections, and this may be due to biological and genetic differences between males and females and environmental factors (32).

Although the number of positive cases of rhinovirus was higher among inpatients in the present study, there was no significant difference between inpatients and outpatients (P=0.521) (Table 2). Previous studies conducted in other countries have shown that the prevalence of RV infection was higher in inpatients. Ren et al. examined 438 patients with respiratory symptoms for respiratory pathogens (147 CAP inpatients and 291 URTI outpatients) in their study in China. In this study, 42 patients were positive for HRV, of which 15 cases (10.2%) were inpatients with CAP and 27 cases (9.2%) were outpatients with URTI (29); likewise, Aslam et al. investigated the prevalence of HRV and its genotypes in Asia in a review study. In their study, 94 articles were investigated and analyzed. In total, 126.026 subjects were examined for HRV, among which 21156 (16.8%) were infected with HRV. Besides, the number of positive HRV cases was 16353 (21.6%) in inpatients and 1573 (2.07%) in outpatients (21). These findings explained the ability of RVs to cause severe infections of the lower respiratory tract (33).

Our study reported seven samples with genotype A, including A29, A15, A67, A58, A47, A40, and A80, and two with genotype C, including C39 and C44; however, genotype B was not found. These findings are similar to other studies, especially those conducted in Asia. The results of a review study in 2016 suggested that in Asian countries, the HRV-A genotype had the highest majority, and the HRV-B genotype had the lowest one (21). A study in Shanghai conducted from 2013 to 2015 on 1003 tracheal samples from hospitalized patients with severe respiratory infections showed that 280 samples (27.9%) were positive for RVs. Based on the VP2/VP4 gene region genotyping assay, 140 cases of HRV-A (14%), 56 cases of HRV-C (5.6%), and 21 cases of HRV-B (2.1%) were detected among the positive samples. Genotypes A58, A29, A80, and C39 were among the studied subjects (34).

Another study in 2016 found that the most common genotypes detected in Singapore were HRV-A and HRV-C (35). A study in 2015 in Africa revealed that the most common RV genotypes were HRV-A (64%), HRV-C (23%), and HRV-B (13%) (22). Furthermore, in 2020 a survey in the United States investigated 768 infants, of which 92 were infected with HRV-A and 92 were infected with HRV-C (36). In 2019, Vandini et al. (37) from Italy reported results on 229 nasal samples from the PICU ward, in which 41% were infected with RVs, while the most common genotypes were genotype C (22.3%) and genotype A (17.5%).

Previous studies from other parts of the world (4, 13, 38, 39) have suggested that HRV-C may cause more severe illness and is more prevalent in lower respiratory tract infections (LRTI) than HRV-A and HRV-B. This was not evident in our study; however, HRV-A and HRV-C were detected among the studied subjects and had different prevalences among the cases.

Our Study limitations included the low sample size due to the limited research budget and the partial sequence data. In this study, the low prevalence of genotype C might

be attributed to the small number of samples from infants and children. On the other hand, just one genetic region of VP2/VP4 was studied. Regarding genetic recombination, exploring more expansive genetic areas, more authentic information was acquired about the genetics and epidemiology of the HRVs.

CONCLUSION

This study aimed to investigate the prevalence and phylogenetic analysis of rhinoviruses detected in patients referred to Masih Daneshvari Hospital. The findings of our study exhibited that the prevalence of rhinoviruses was higher in spring and summer and for ages less than 18 years. The most common genotypes detected in our study were HRV-A and HRV-C. No genotype B was seen in this study. One of the research limitations is the sample size; thus, it is recommended to carry out further studies with larger sample sizes and in the wider geographical region to detect and perceive the genetics of rhinoviruses in Iran thoroughly.

Data Availability

The data is available, and there is no restriction on additional information.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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