Frequent detection and genotyping of human rhinovirus in SARS-CoV-2 negative patients; a study from south of Iran

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ABSTRACT

Background and Objectives: Human rhinovirus (HRV), a major cause of common cold, was associated to the hospitalization of children and adults. This cross-sectional study aimed to determine the prevalence, and genotype distribution of HRV in the patients with mild to severe respiratory infections who were negative for SARS-Cov-2.

Materials and Methods: Nasopharyngeal swab specimens (n = 356) from the patients aged 29 days to 82 years, received for the respiratory virus detection from January to December 2021, were analyzed for human rhinovirus (HRV) by RT- PCR. As a final step, genotyping was performed on obtained sequences.

Results: A total of 37 HRV infections were identified (37/356, 10%). The highest rates of positive HRV tests were observed in February (21.6%), and January (18.9%), compared with June and August (0%). HRV-positive cases mainly appeared in winter. Among the age groups, those 2-<5 years of age had the highest detection rate (21%), however, those >55 years of age had the lowest detection rate (3%). Among HRV-positive samples, 30 (81%) were identified as type HRV-A, 5 (13.5%) as HRV-B, and 2 (5.5%) as HRV-C.

Conclusion: Our results suggested that HRV frequency gradually decreased with the age of patients which is more active in Iran, especially in the cold months.

Keywords: Human rhinoviruses; Prevalence; Respiratory infection; Emerging disease, Genotyping

INTRODUCTION

By a diameter of 28-34 nm, the human rhinoviruses (HRVs) are small, single-stranded positive RNA viruses belonging to the genus *Enterovirus* in the family *Picornaviridae*. Rhinoviruses are the most common cause of colds worldwide. However, HRV infections are now linked to pneumonia in elderly and immunocompromised patients, severe bronchiolitis and asthma exacerbations in children, and chronic obstructive pulmonary disease (COPD). These conditions were previously thought to be caused by relatively mild upper respiratory illnesses (1). To date, more than 160 serotypes of Rhinovirus have been identified, which are classified into three species (A, B, and C) (2). HRV is transmitted in 2 ways: by the

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aerosols of respiratory droplets and contaminated surfaces (fomites) (3). The upper respiratory tract is the entry route for rhinoviruses into the body. HRV uses 2 types of cell receptors: serotypes A and B use intercellular adhesion molecule 1 (ICAM-1) as a receptor, whereas serotype C associates with cadherin-related family member 3 (CDHR3) to enter the epithelial cells (4). Since 2019, when the prevalence of the coronavirus disease 2019 (COVID-19) led to the pandemic, the rapid diagnosis of respiratory rhinovirus infections in the patients suspected of having COVID-19 has become a significant worldwide problem. More specifically, it is critical because similar symptoms of these two viruses in clinical diagnosis, which can be confusing to clinicians, such as cough, fatigue, headache, and fever (5). Hence, it would appear that individuals who require hospitalization need a clear laboratory diagnosis. We sought to identify the prevalence and genotype distribution of HRV in patients with cold symptoms whose PCR test for SARS CoV-2 was negative in this study.

MATERIALS AND METHODS

Study design. Patients with common cold signs (including fever, cough, fatigue, headache, and nausea) and respiratory infections, who were formerly checked for SARS CoV-2 with PCR test and their result was negative, were included in the study. From January to December 2021, a total of 356 respiratory samples (nasal swab or nasopharyngeal aspirate) were collected from Persian Gulf Martyrs Hospital in Bushehr as part of SARS-CoV-2 routine detection in outpatients and inpatients. Patients were allowed to relax while nasopharyngeal swabs were taken and kept in a viral transport medium. Samples were kept at -80°C until use. Written informed consent was obtained from the patients or their guardians. The study was approved by Bushehr University of Medical Sciences ethical committee (IR.BPUMS.REC.1401.166).

Real-time PCR and PCR. RNA was extracted from samples using QIAamp Viral RNA Mini Kit (Qiagen) based on the manufacturer's protocol. A total of 356 patients (29 days infants to 82 years old) were initially checked for *Enterovirus* with a pre-designed exclusive primer and prob (Table 1) for real-time PCR test (6). RealQ Plus RT 5× PCR Mix (RealQ Plus One-step RT-PCR Kit, Ampliqon, Den-

mark) was used for real-time RT-PCR test based on manufacturer's protocol. In brief, the real-time PCR mixture of 20 μL contained: 4 μL of Master Mix, 1 ul of 20× RT Mix, 0.5 μL of each primer and probe (10 pmol), 6 μL of RNA, and 7.5 ul of PCR-grade H_2O. The reaction was performed with following incubation conditions: 50°C for 15 minutes (reverse transcription), 95°C for 15 minutes (Initial heating), 95°C for 5 seconds (degeneration), 45 cycles at 60°C for 30 seconds (Annealing/Elongation). Forty samples out of 356 patients were positive for *Enteroviruses*. In subjects with positive real-time RT-PCR results, a traditional RT-PCR test was run to detect HRV and distinguish it from other respiratory illnesses. RNA transcripts were reverse transcribed into cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV) and 5' untranslated region (5'-UTR) of HRV genome was amplified in a PCR assay as described previously (7), using the primers OL26 and OL27 (Table 1). In brief, the PCR mixture contained 10 ul of Taq DNA Polymerase 2× Master Mix RED (Amplicon, Denmark), 0.5 μL of each primer (10 pmol), and 5 μl of cDNA in a final volume of 20. The 40 cycles in 3 segments of the PCR process were as follows: initial denaturation at 94°C for 5 minutes, followed by 30 seconds at 94°C, 30 seconds at 58°C, and 45 seconds at 72°C, and a final extension at 72°C for 5 minutes. Using electrophoresis in 1% agarose gel, the PCR products (388 base pairs) were confirmed to be accurate.

Sequencing and phylogenic analysis. PCR products were sequenced by a 3130 Genetic Analyzer (Applied Biosystems), and edited using Bioedit software. For phylogenetic analysis, the sequences were compared to reference sequences representing three main HRV strains available in the GenBank database: HRV-A (NC-038311), HRV-B (NC_038312.1), and HRV-C (NC_009996.1). Finally, the phylogenetic tree was made by MEGA-X software. Chi-square test or Fisher's exact test was used to compare the variables. GraphPad Prism 9.00 statistical software was used for data analysis. P-value ≤ 0.05 was considered significant.

RESULTS

From January to December 2021, 356 unique patients were included, without COVID-19 disease, while 210

Table 1. Primer sequences used for real-time RT-PCR and conventional PCR of rhinovirus in the study.

Primers	Sequences (5′ > 3′)
PANEV-F	AGCCTGCGTGGCKGCC
PANEV-R	GAAACACGGACACCCAAAGTAGT
Probe	FAM-CTCCGG CCCCTGAATGYGG CTAA-BHQ1
HRV-F (OL26)	GCACTTCTGTTTCCCC
HRV-R (OL27)	CGGACACCCAAAGTAG

(59%) were males and 146 (41%) were females. In 40 (11.2%) of the specimens from patients who had an acute respiratory infection, enterovirus was found. 37 out of the 356 specimens with enterovirus positivity (about 10%) had rhinovirus confirmed (Table 2). The mean age of the patients who tested positive for rhinovirus was 36.86 ± 19.10 years (range: 29 days - 82.0 years), while the mean age of patients with HRV was 30.15 ± 15.75 years (range: 64 days - 71.0 years). The highest HRV detection rate was observed in February (21.6%), followed by January (18.9%). Furthermore, this detection rate was higher in winter than in summer (56.7 vs. 2.7%) (Fig. 1). The detection rate for the patients 2-<5 years old was the highest (21%; N=4/19), and the detection rate for the patients >55 years old was the lowest (3%; N=2/69) (Fig. 2). In the multinomial logistic regression model, age was significantly ($p = 0.012$) associated to increased odds of rhinovirus detection, after adjusting for age, sex, and disease status. Besides, HRV distribution was not statistically different between inpatients vs. outpatients (Table 2). Among 37 patients who tested positive for rhinovirus, 30 (81%) had rhinovirus A, 5 (13.5%) had
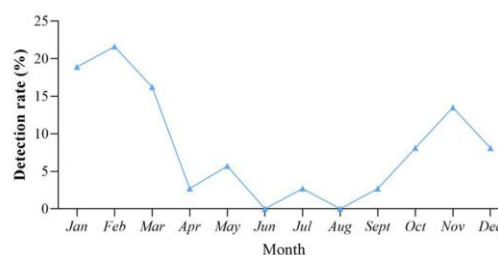
Fig. 1. Monthly positivity rate of HRV infection in Bushehr, Iran, from January to December 2021.
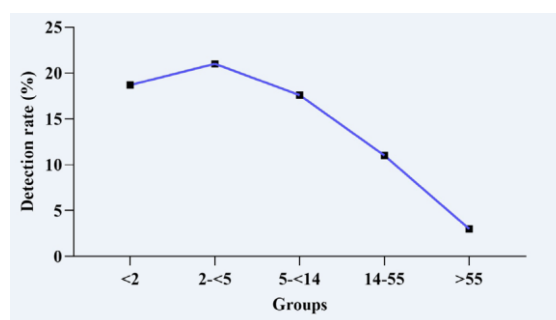
Fig. 2. Detection rate of HRV aggregated by groups in respiratory specimens isolated from Iran, from January to December 2021.

Table 2. Demographic of patients with HRV infection.

Characteristics	Number of HRV negative patients n=319 n (%)	Number of HRV positive patients n=37 n (%)	P value
Age groups			
<2	13 (4%)	3 (8.1%)	0.2249
2-<5	15 (4.7%)	4 (10.8%)	0.1223
5-<14	14 (4.3%)	3 (8.1%)	0.4022
14-55	210 (65.8%)	25 (67.5%)	>0.9999
>55	69 (21.6%)	2 (5.4%)	0.0164
Gender			
Male	188 (59%)	22 (60%)	>0.9999
Female	131 (41%)	15 (40%)	
Disease Status			
Inpatient	98 (30.7%)	11 (29.7%)	>0.9999
Outpatient	221 (69.3%)	26 (70.3%)	

rhinovirus B, and 2 (5.5%) had rhinovirus C (Fig. 3). The average evolutionary divergence over sequence pairs within groups indicated that HRV-C isolates with an intra-species nucleotide p-distance of 0.23, have greater genetic distance than HRV-A and HRV-B with p-distances of 0.14 and 0.10, respectively. Inter-group variability at the nucleotide level was 39.9% between HRV-A, and HRV-B, 28.3% between HRV-A and HRV-C, and 45.1% between HRV-B and HRV-C. GenBank accession numbers for the nucleotide sequences

of 5'-UTR region of HRV are OP900582-OP900618.

DISCUSSION

Recent years have seen an increase in HVR respiratory infection epidemics across various nations. We did a cross-sectional investigation between January and December 2021 and partially sequenced the isolated HRV strains from patients with respiratory illnesses in the south of Iran to evaluate the novel HRV circulating strains. In the present study, 37 (10%) were positive for HRV, which is consistent with a few studies in Iran (10.6%) and other Middle Eastern countries, including UK pilgrims in Mecca (13%) (8) and hospitalized young children in Jordan (11%) (9). However, this result is lower than the rate reported in pre-COVID-19 pandemic seasons from Iran (21%) (10). Based on the literature (11), the frequency of rhinoviruses decreased at the beginning of pandemic as a result of non-pharmaceutical interventions, including masking, and the closure of schools, and many workplaces. However, this rate returned and persisted despite maintaining these interventions. The inherent characteristics of rhinoviruses, such as the lack of a viral envelope, the number of co-circulating strains, and stability on surfaces may be the reason for this rapid return. The application of social distancing for SARS-CoV-2 may have contributed to the decline in rhinovirus rates in our study. In contrast to previous studies (12), which mentioned most frequently in spring and autumn, our results showed that HRV is more active in winter. These results may be due to the fact that HRV infections are more severe in winter and more hospitalizations occur during this season. Although all age groups were included, children aged between 2-<5 years showed a higher rate of HRV infection (21%), which was reported in previous studies (13). Our results, in line with previous reports, indicated that the detection rates gradually decrease with patient age (13, 14). As seen in the results, there was no correlation between the disease status (inpatient and outpatient), and the HRV frequency. Phylogenetic analysis showed that three HRV groups, including HRV-A (30/37, 81%), HRV-B (5/37, 13.5%), and HRV-C (2/37, 5.5%) exist in samples; consistent with previously reported patterns in Iran (15). However, the frequency of the genotypes has been different in other studies from Iran, which may be related to the target population of these
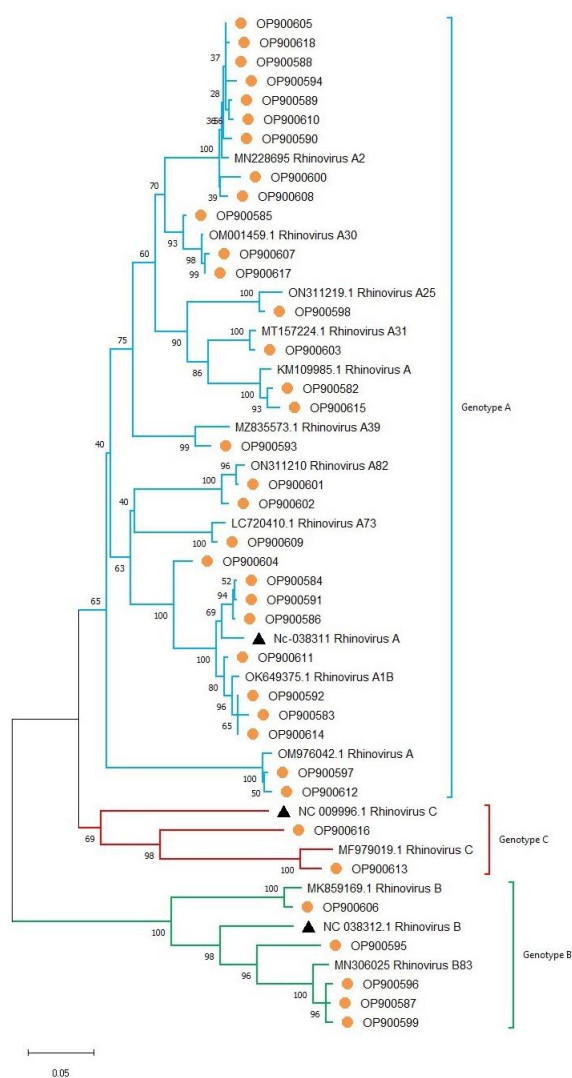

Fig. 3. Phylogenetic tree based on the 5' UTR region of HRV, constructed by the neighbor-joining method with 1000 bootstrap replicates using MEGA-X (http://www.megasoftware.net). The sequences detected in the present study are followed by an orange circle and reference sequences are represented by GenBank ID (Black triangles).

studies (10, 16). Clinical symptoms, including pneumonia, cough, rhinorrhea, sore throat, and fever were common findings in HRV-infected patients, which is based on previous studies (17). There were no significant differences found in clinical symptoms among species, however, some previous studies have reported an association among clinical characteristics, and HRV species (18). Analysis by phylogenetic distance showed that the level of genetic diversity among HRV-C strains is higher than that seen in HRV-A and HRV-B strains, consistent with past studies (19). Study limitations may affect how the findings are interpreted because numerous rhinovirus types are present in a community at the same time and their incidence varies seasonally. This study has some limitations. Since numerous rhinovirus types are present in a community at the same time and their incidence varies seasonally, these parameters should be considered in interpreting the data. Therefore, in future studies, it is important to collect larger sample sizes over a few seasons to analyze the virulence of individual types.

CONCLUSION

To prevent further outbreaks, health organization needs to study the epidemiology of this type of virus at different times of the year. According to the molecular study of HRV, HRV-A predominates in HRV infections. Our research shown that the detection rate of HRV increases during the winter and subsequently declines with aging.

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