IJM Iranian Journal of Microbiology

Volume 15 Number 1 (February 2023) 155-162

ORIGINAL ARTICLE

Phylogenetic characterization of rhinovirus and adenovirus in hospitalized children aged ≤ 18 years with severe acute respiratory infection in Iran

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Received: July 2022**, Accepted:** December 2022

ABSTRACT

Background and Objectives: Human rhinoviruses (HRVs) and human adenoviruses (HAdVs) are among the most prevalent viruses in hospitalized patients with severe acute respiratory infection (SARI). This study aimed to evaluate the molecular characterization of HRV and HAdV in hospitalized patients with SARI, who aged ≤ 18 years in Tehran, Iran.

Materials and Methods: To detect these two viruses, a conventional nested RT-PCR (Reverse transcription-polymerase chain reaction) assay was performed on 264 throat swabs collected from December 2018 to March 2019. The epidemiological data were analyzed and phylogenetic trees were constructed.

Results: Of 264 cases with SARI, 36 (13.6%) and 28 (10.6%) were positive for HAdV and HRV respectively. Of 21 HRV sequenced samples, HRV-A (42.9%), HRV-B (9.5%) and HRV-C (47.6%) and of 36 HAdV sequenced samples, HAdV-C6 (38.9%), HAdV-B7 (22.2%), HAdV-B3 (11.1%), HAdV-B16 (5.6%), HAdV-C5 (13.9%), HAdV-C57 (5.6%), HAdV-E4 (2.8%); were detected in children with SARI. Some viral genotypes appeared to cause more severe disease, which may lead to hospitalization.

Conclusion: Large-scale studies are recommended to investigate the epidemiology and molecular characterizations through surveillance networks to provide useful information on etiology, seasonality, and demographic associations in patients with SARI.

Keywords: Human rhinovirus; Human adenovirus; Respiratory infection; Phylogeny; Iran

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INTRODUCTION

Children are a vulnerable population affected by viruses that cause mild to severe acute respiratory infections (SARI) (1). These viruses are commonly circulating in the general population which include human rhinovirus (HRV), human adenovirus (HAdV), influenza virus, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), parainfluenza viruses, enterovirus, bocavirus, and coronaviruses. Of particular interest are HRV and HAdV, which can frequently result in acute respiratory tract infections (ARTI) in young children requiring hospitalizations (2-4). HRVs (family *Picornaviridae*, genus *Enterovirus*) are nonenveloped viruses, with single-stranded RNA (ssRNA), which are classified into three species (A, B, C) (5). HRV can frequently cause upper respiratory tract infections (URTI) as well as otitis media, sinusitis, and rhinosinusitis which can be associated with acute lower respiratory tract infections (ALRTI) (e.g. severe bronchiolitis). HRV appears to be an important risk factor for patients with underlying diseases, such as chronic respiratory illnesses like asthma or chronic obstructive pulmonary disease (COPD), or those with cystic fibrosis or immunosuppressed patients (1).

HAdVs (family *Adenoviridae*, genus *Mastadenovirus*) are nonenveloped viruses, with a double-stranded DNA genome (dsDNA). Recently more than 84 genotypes have been identified and classified into seven species (A to G) with HAdV species C, B, and E primarily responsible for respiratory tract infections (6). HAdVs are one of the most frequent respiratory pathogens and can cause a wide range of illnesses including URTIs and LRTIs, conjunctivitis, pneumonia, gastroenteritis, tonsillopharyngitis, hepatitis, and hemorrhagic cystitis (7).

The prevalence and the circulating genotypes of these two viruses depend on the population studied varies in different countries. This variation may be due to climatic or geographical properties, socioeconomic and cultural aspects, or alterations in healthcare management (8). Since there are limited studies related to HRV and HAdV prevalence in Iran, we evaluated the prevalence and genotypes of HRV and HAdV in hospitalized patients with SARI under 18 years old (considering the range of prevalence in children lower and upper primary school for comparison) between December 2018 to March 2019 in Tehran, Iran.

MATERIALS AND METHODS

Patients and clinical specimens. For this population study, throat swabs collected in universal transport media (COPAN, Italy) from 264 hospitalized patients of ≤ 18 years with SARI were transferred to the National Influenza Center, School of Public Health, Tehran University of Medical Sciences. These hospitalized patients had symptoms such as fever, cough, dyspnea, sore throat, myalgia, and respiratory distress.

Ethics approval. This study was approved by the Ethical Review Committee of the School of Public Health, Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1397.175).

Preparation of nucleic acids. Total nucleic acid from 200 µl of samples was extracted using the High Pure Viral Nucleic acid kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Primers and RT-PCR. The 5′UTR (the 5′ untranslated region), a conserved region of HRV, was used for the nested RT-PCR (Reverse transcription-polymerase chain reaction) test (9). In addition, the hypervariable part of the 5′UTR, the entire VP4 (viral protein 4) gene, and the 5′ termini of the VP2 gene were targeted for genotyping of HRV (10). The reaction mixture for HRV detection in the first round of nested PCR included 5 µl of genome extraction product, 10 µl of 5 × buffer, 0.2 mM dNTP, 2 µl enzyme, and 25 pmol of each forward (Fa1) and reverse primers (Rb1) (Table 1) in a total volume of 50 µl, using Qiagen one-step RT-PCR kit (Germany) according to the manufacturer's instructions. The PCR condition in thermal cycler was carried out for the first round at 60°C for 1 min, 50°C for 30 min, and 95°C for 15 min, followed by 40 cycles of denaturation step at 94°C for 30s, annealing at 64°C for 30s and extension at 72°C for 1 min and final extension of 72°C for 10 min. Afterward 2.5 µl of the first-round product as a template with 25 µl of Ampliqon 2× master mix (Ampliqon, Odens, Danish master mix), and 12.5 pmol of each forward (Fa2) and reverse primers (Rb2) (Table 1) were used for amplification in the second round. The PCR cycling conditions were 94°C for 5 min, 35 cycles consisting of denaturation at 95°C for 30s, annealing at 66°C for 40s, extension at 72°C for 30s, and final extention at 72°C for 5 min.

Table 1. Properties of HRV (human rhinovirus) & HAdV (human adenovirus) primers

Organism	Primer	Sequence (5' to 3')	Product length (bp)	Target gene	Reference
HRV	Forward (Fa1)	CACTTCTGTTTCCCCGGAGCGAG	388	5' UTR	9
	Reverse (Rb1)	GAAACACGGACACCCAAAGTAGTCGGT			
	Forward (Fa2)	CACTTCTGTTTCCCCGGAGCGAGG	283	5' UTR	9
	Reverse (Fb2)	CCGCATTCAGGGGCCGGAG			
HRV	Forward (FRV1)	GCA TCI GGY ARY TTC CAC CAC CAN CC	549	5' UTR, VP4/VP2	10
	Reverse (RRV2)	GGG ACC AAC TAC TTT GGG TGT CCGTGT			
HAdV	Forward 1	WCT GAA CAG CAT CGT GGG TCT	209-259	Partial hexon	4
	Reverse 1	AGG TAC TCC GAR GCR TCC TG			
	Reverse R2a	CCT GTC CGG CGA TGT GCA TG			
	Reverse R2b	CCT GGC CCG AGA TGT GCA TG			

I=Inoisine; Y=T or C; R=G or A; N= any nucleotide, UTR (untranslated region)

Using forward (FRV1) and reverse primers (RRV2) (Table 1) positive HRV samples were genotyped. The reaction mixture consisted of 8 µl genome extraction product, 10 µl of 5× buffer, 0.2 mM dNTP, 2 µl Enzyme, and 25 pmol of each primer in a total volume of 50 µl, using Qiagen one-step RT-PCR kit. The PCR program was the same as the first round of nested RT-PCR with annealing at 58°C.

Using hemi-nested PCR assay (4) (Table 1) the spanning region of partial hexon was selected for detection and genotyping of HAdV. To run the assay 5 µl of DNA extraction product, 12.5 µl Ampliqon 2× master mix, and 12.5 pmol of each primer in a total volume of 25 µl was used in the first round. In the second round, 5 µl of PCR product from first-round was added as a template to 12.5 µl of `Ampliqon 2× master mix, 10 pmol of each primer (F1, R2a, R2b) and 4.5 µl of RNase-free water in a total volume of 25 µl. PCR cycling conditions for both rounds were 95°C for 5 min, followed by 35 cycles at 94°C for 30s, 56°C for 25s, 72°C for 15s, and final extention at 72°C for 8 min.

The amplicon products were analyzed using 1.5% agarose gel (Invitrogen, USA) and safe stain in TBE (Tris/Borate/EDTA) buffer. Product length for HRV in nested PCR for the first and second rounds were 388 bp and 283 bp (Fig. 1) respectively and for genotype determining was 549 bp (Fig. 1). The product length for HAdV was 208 bp (Fig. 1). The PCR products were cleaned up by a QIAquick PCR purification Kit (Qiagen, Düsseldorf, Germany), then used for sequencing by the Sanger method. Positive controls were prepared from isolated HRV and HAdV on cell culture.

Phylogenetic analysis. To determine HRV and HAdV genoypes, the PCR products of positive samples were sequenced by Genetic Analyzer 3130 (ABI). The sequenced HRV and HAdV were compared with NCBI GenBank sequences using Bioedit version 7.0.9. Phylogenetic analysis was performed by MEGA10 with a bootstrap of 1000 replicates, using Maximum likelihood and Neighbor-joining methods for HRV and HAdV respectively.

Statistical analysis. Statistical analysis of relationships between viruses (HRV, HAdV), age groups, and gender was performed by SPSS version 20 using Pearson's chi-square test. A 2-sided p-value < 0.05 was considered to indicate statistical significance.

RESULTS

Virus identification. From the total of 264 patients with SARI, 28 (10.6%) HRV, and 36 (13.6%) HAdV were detected. HRV and HAdV in 3 (1.1%) samples were co-detected.

Demographic characteristics. Evaluation of the age range in children who were positive for HRV illustrated that 16 (57.1%) of children were ≤1 year of age, followed by 9 (32.2%) between 1≤4, and 3 (10.7%) between 4≤18 years of age. Considering that 9.5% of

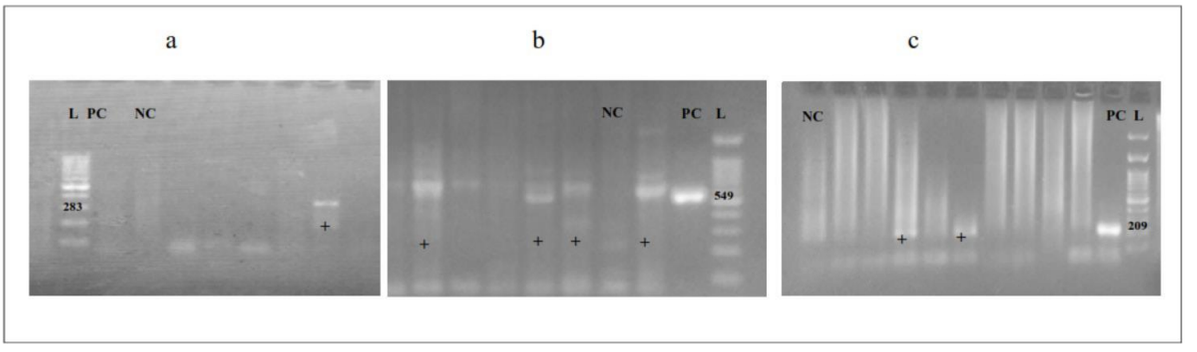

Fig. 1. Gel electrophoresis of PCR products for HRV and HAdV. a: HRV Nested PCR, b: HRV RT- PCR, c: HAdV Nested PCR
L=Ladder PC= Positive Control NC= Negative Control

HRV positive samples were male and 12.4% were female (Table 2). In addition, 15 (41.6%) of HAdV positive samples were ≤1 year, 11 (30.1%) between 1≤4, and 10 (27.7%) between 4≤18 years of age. Males and females of HAdV positive samples were 16.8% and 9.6% respectively (Table 2). Considering $p \leq 0.05$, statistically significant relationship between these two viruses with age groups (p=0.19, p=0.78) and gender (p=0.46, p=0.09) was not detected (Table 2).

HRV and HAdV genotyping and phylogenetic analysis. Of 28 HRV positive samples, 21 samples were successfully sequenced. Of them, 9 cases belonged to species HRV-A, 2 cases to species HRV-B, and 10 cases to species HRV-C (Fig. 2). Of 36 SARI patients infected with HAdV, 21 cases belonged to species C (14 HAdV-C6, 5 HAdV-C5, 2 HAdV-C57), 14 cases to species B (8 HAdV-B7, 4 HAdV-B3, 2 HAdV-B16), and 1 case to HAdV-E4. HAdV-C6 and HAdV-B7 were the most prevalent genotypes in these patients (Fig. 3). HRV and HAdV sequences were submitted to GenBank with accession numbers (MW567705-MW567725), (MN564753-MN564788), respectively.

DISCUSSION

This molecular epidemiology study affords interesting insights into the prevalence and genotypes of two respiratory viruses, HRV and HAdV, in SARI patients aged ≤ 18 years from December 2018 to March 2019. During this period, the highest prevalence of respiratory tract infections by these two viruses was in February and March 2018, the coldest months in Iran.

This is consistent with previous studies, which have shown the seasonal occurrence of respiratory infections during the coldest and driest months (11). Scheidt et al. showed that in southern Brazil, HAdV respiratory infections occurred throughout the year with a higher incidence rate in early spring and winter (12). However, Li et al. and Shatizadeh et.al reported that the main peak of HAdV prevalence was

Table 2. HRV (human rhinovirus) & HAdV (human adenovirus) distribution in different age groups and gender.

Variable	Number of Patients	HRV positive cases	HAdV positive cases	P-Value	
				HRV	HAdV
Total	264	28 (10.8%)	36 (13.6%)		
Age (years)					
≤ 1	124	16 (57.1%)	15 (41.6%)		
1 ≤ 4	75	9 (32.2%)	11 (30.5%)		
4 ≤ 18	65	3 (10.7%)	10 (27.7%)	0.19	0.78
Gender					
Male	149	14 (9.5%)	25 (16.8%)		
Female	115	14 (12.4%)	11 (9.6%)	0.46	0.09

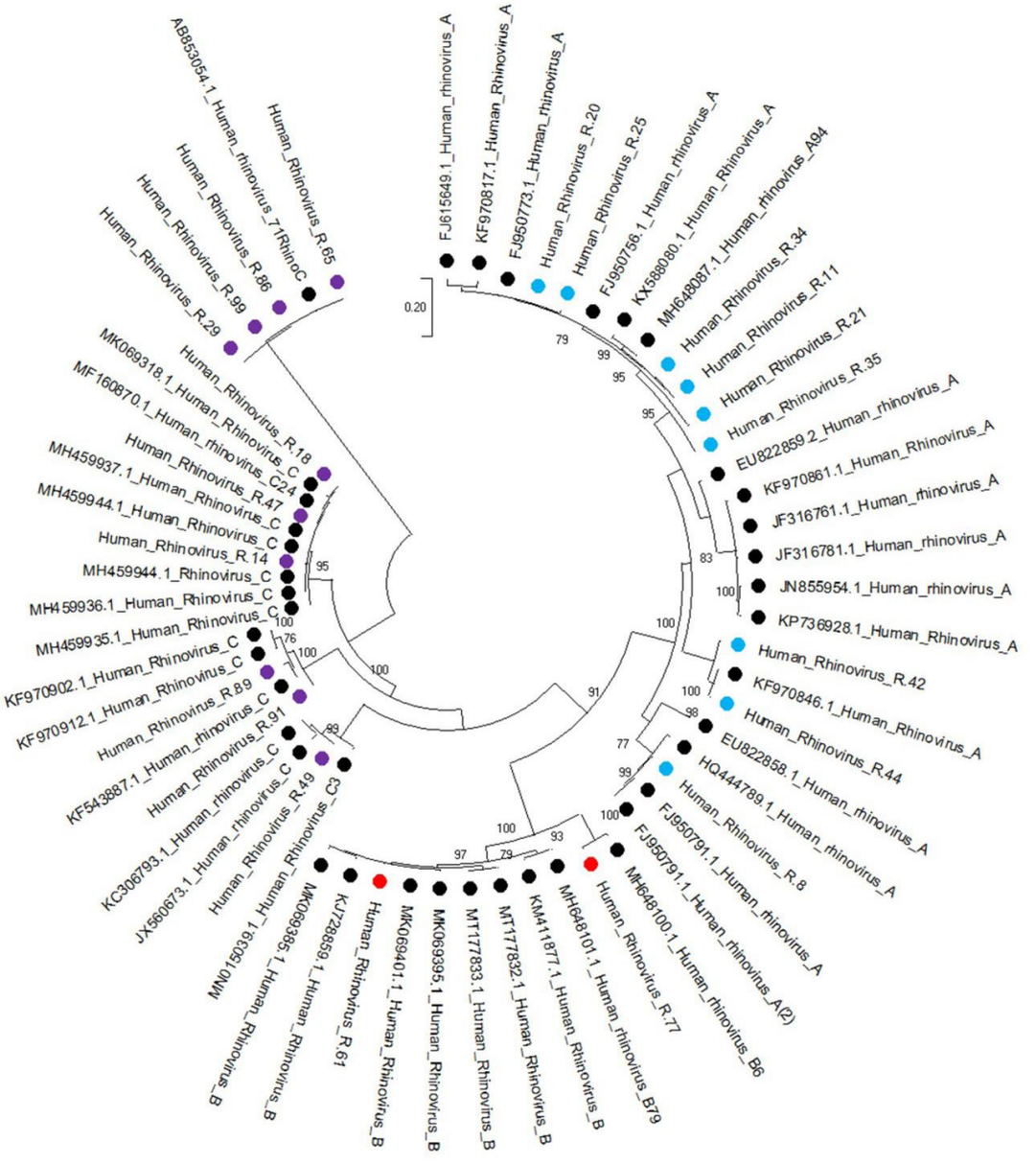

Fig. 2. Phylogenetic tree of HRV (human rhinovirus) (5'NCR, VP4/VP2 549 nucleic acid sequence) compared with GenBank sequences (dark circles), applying a bootstrap 1000 replicates with Maximum likelihood method and Tamura-nei model. The accession numbers for 21 strains are present in GenBank (MW567705-MW567725). HRV species are indicated with colored circles: HRV-A blue circle, HRV-B red circle,and HRV-C purple circle.

in the summer (2, 13).

In this study, the prevalence rate of HRV and HAdV in all age groups was 10.6% and 13.6%, respectively. In children aged ≤ 5 years prevalence rates of HRV and HAdV were 9.5% and 9.8% respectively. These prevalences vary by country in different studies (2, 14). In Brazil, a systematic investigation revealed a high frequency of HRV (37%) in children with SARI between 2012 to 2013 (15). In two epidemiological studies in Nigeria and Egypt, the prevalence of HRV in 200 infected children was 18.5% and 43.5% respectively (16). Also, in a population study of 351 diagnosed viral respiratory infections in Kuwait, HRV (41.6%) was the most detected virus (17).

Herein the results showed no statistical relationship between HRV and HAdV infections with gender, as was also reported by Zhao et al. (18). The phylogenetic analysis of HAdV sequences in the

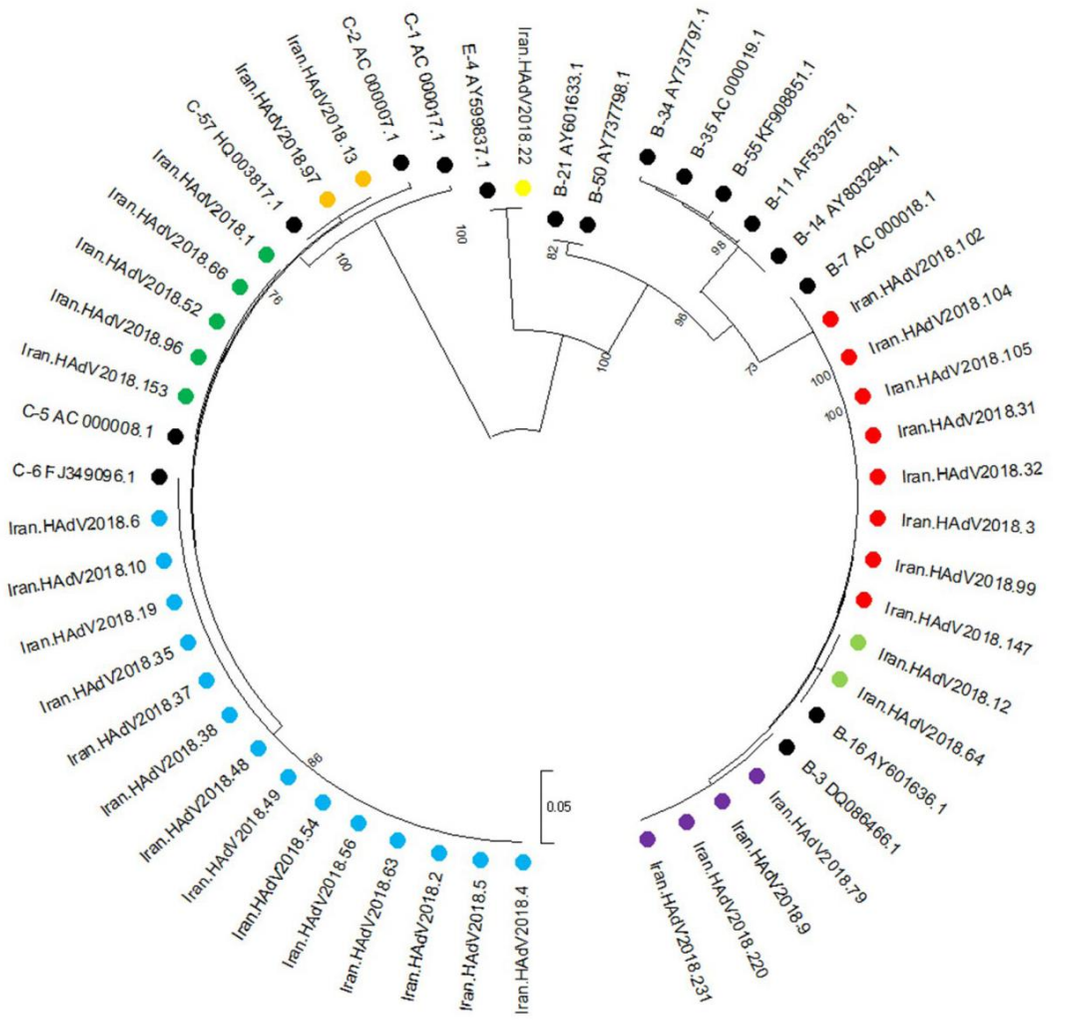

Fig. 3. Phylogenetic tree of HAdV (human adenovirus) (hexon gene 209-250 nucleic acid sequence) compared with GenBank sequences (dark circles), applying a bootstrap 1000 replicates, with Neighbor joining method and Tamura-nei model. The accession numbers for 36 strains are present in GenBank (MN564753-MN564788). HAdV species are indicated with colored circles: HAdV-C6 blue circle, HAdV-C5 green circle, HAdV-B7 red circle, HAdV-B3 purple circle, HAdV-B16 light green circle, HAdV-C57 orange circle, and HAdV-E yellow circle.

current study indicated that the majority of strains (58.3%) belonged to HAdV-C species and among them, 38.8% belonged to HAdV-C6, which was in agreement with an epidemiological study performed by Nakamura et al. during 2013-2015 in Japan (19). Furthermore, 38.9% of cases belonged to HAdV type B, and 22.2% were HAdV-B7. Other species were HAdV-C5 (13.9%), HAdV-C57 (5.6%), HAdV-B3 (11.1%), HAdV-B16 (5.6%), and HAdV-E4 (2.8%) respectively.

Among HAdVs, many studies have mentioned that HAdV-B7 manifested severe illness in infants and military recruits. Likewise, frequency of nosocomial outbreaks with fatal cases, pneumonia, admission to ICU, mechanical ventilation, and longer hospital stays in patients with HAdV-B7 were significantly higher than the other species (20). HAdV-B3 has recently become a serious pathogen of ARI, being responsible for 15% to 87% of all adenoviral respiratory infections worldwide (21).

In this study HAdV-B16 accounts for 5.6% of positive HAdVs samples. HAdV-B16 was first isolated from a patient with conjunctivitis in Saudi Arabia in 1955 (22). HAdV-E4 included 2.8% of positive HAdVs samples in the present study. Prior investigators have reported the circulation of HAdV-E4 in military

recruits but not in young children (23). Furthermore, HAdV-E4 circulation was shown only in one season during a 2-year period study. These results suggested that HAdV-E4 circulates only in a limited time period, induced little herd immunity in children, and left young adults vulnerable to reinfection (19). Despite HAdV-C consisting of only 5 types (1, 2, 5, 6, and 57), however, these types have clinically been more significant than HAdV-B and D in producing severe manifestations in immune-compromised patients, particularly in allogeneic hematopoietic stem cell transplant (HSCT) recipients. Species C type 1 and 2 have been shown to be predominant types of HAdV infections in immune-compromised hosts. After primary infection HAdV-C DNA can persevere in a latent state in lymphoid cells and asymptomatic, recurrent shedding of infectious virus in feces can be observed for many years (24).

In addition, our phylogenetic analysis revealed the presence of three types of HRV among the positive samples, including HRV-A (42.9%), HRV-B (9.5%), and HRV-C (47.6%). A cohort study by Lee et al. provided pieces of evidence that HRV-A and HRV-C cause more severe respiratory illnesses in infants (25). As in our results, the rate of these two types was more prevalent in SARIs. Several studies have revealed the difference between HRV-C virulence in comparison to HRV-A and B species (26, 27).

In a number of these studies, HRV-C viruses appeared to be over signified compared to HRV-A and B in infants and children with LRTIs and in exacerbations of infantile asthma (28). Zlateva et al. in their findings indicated that the prevalence of HRV-A associated LRTI was significantly higher than that of HRV-B and HRV-C, while HRV-B infections were more often asymptomatic (29). Furthermore, HRV-B symptomatic infections had significantly lower viral loads than HRV-A symptomatic infections, which indicated lower pathogenicity of HRV-B viruses. Lower viral loads of HRV-B in comparison with HRV-A and HRV-C in respiratory specimens have formerly been reported for adults with pneumonia. Further, the higher prevalence of HRV-A and HRV-C in hospitalized children confirmed the virulence severity of these two species (30).

This study had some limitations. The lack of patients' clinical data and the specimen collection for a period of less than 1 year, limiting the ability to evaluate long-term temporal variation in HRV and HAdV prevalence and species/type diversity.

CONCLUSION

This study showed that HRV and HAdV infections as influenza-like diseases are important respiratory pathogens in children. However, some viral genotypes appeared to cause more severe disease, which may lead to hospitalization. Therefore, multi-pathogen diagnostic testing through surveillance networks with a larger sample sizes can provide useful information on etiology, seasonality, and demographic associations in patients with SARI especially after COVID-19 pandemics.

ACKNOWLEDGEMENTS

We express our thanks to our colleagues at Virology Department, School of Public Health, Tehran University of Medical Sciences. Also special thanks to Dr. Bruno Lina and Dr. John McCauley in providing positive controls for us. This study was supported by Tehran University of Medical Sciences under grant No. 97-3-27-39246.

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