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ORIGINAL ARTICLE

Epidemiological and phylogenetic assessment of human respiratory syncytial virus among pediatric patients presenting acute respiratory infections in Shiraz, Iran during 2015-2016

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ABSTRACT

Background and Objectives: The pediatric population worldwide bears a significant morbidity and death burden due to acute respiratory infections (ARIs). Human Orthopneumovirus, sometimes referred to as the Human Respiratory Syncytial Virus (HRSV), is one of the main causes of ARIs in infants. The main goal of this study was to identify the genetic diversity of HRSV strains that were circulating in the Iranian population at a certain time of year.

Materials and Methods: Two hundred youngsters less than 12 years old with acute respiratory infections had samples taken from their throat and pharynx secretions. Then, external and hemi-nested PCR were employed, using specific primers targeting the G gene region to detect HRSV. Subsequently, nine randomly selected positive samples were subjected to sequencing. The results were then compared with reference strains cataloged in GeneBank, and phylogenetic tree was constructed using Chromes and MEGA7.

Results: Out of 200 samples, 34 were identified as containing HRSV. Subgroup A was predominant, accounting for 61.76% of cases, followed by subgroup BA (35.29%) and subgroup B (2.94%). Phylogenetic analysis revealed five samples associated with subtype B and four with genotype A. Genomic analysis showed three samples under the GA2 subgroup and one under GA1 for subtype A, and four samples in subgroup BA and one in GB2 for subtype B.

Conclusion: In this study, subgroup A strains, particularly genotype GA2, exhibited a higher prevalence compared to subgroup B strains during the specific period under investigation, shedding light on the genetic landscape of HRSV in this region.

Keywords: Respiratory syncytial virus; Seasonal infection; Respiratory tract infections; Genotype; Pediatrics

INTRODUCTION

Among the pediatric population worldwide, human Orthopneumovirus, sometimes referred to as human respiratory syncytial virus (HRSV), is a major cause of severe acute respiratory illnesses, particularly in newborns and early children (1). This virus belongs to the Orthopneumovirus genus and the Pneumoviridae family. This virus has an RNA genome with negative-sense orientation and spanning approximately 15,200 nucleotides. Within this genome, there are 10 genes responsible for encoding 11 proteins. Despite having a single serotype, RSV may be further classified into two antigenic groups, A and B, based on differences in the attachment glycoprotein (G)'s epitopes (2, 3). Interestingly, the RSV G gene dis-

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plays unique genetic variants, such as a duplication of 72 nucleotides in group A and a duplication of 60 nucleotides in group B (4-6). It has been noted by researchers that strains of RSV from distinct lineages and groups can coexist for years in a row within the same geographic area. The preponderance of groups A and B, as well as their corresponding lineages, do, however, follow a cyclical pattern that lasts for one to two years (7, 8).

Common symptoms of RSV encompass a congested nose, reduced appetite, cough, sneezing, fever, and wheezing. Although most RSV infections tend to clear up naturally within one to two weeks without requiring medical treatment, there are instances of more serious complications like bronchiolitis and pneumonia. Nearly all children contract RSV by the age of two, and subsequent infections are common. Despite its widespread occurrence in most children, particular attention must be paid to protection of preterm infants and those under six months of age. Furthermore, given the current Coronavirus disease 2019 (COVID-19) pandemic, toddlers who have not previously been exposed to RSV may be more susceptible to infection (9, 10). RSV causes over 2.1 million outpatient visits and 58,000-80,000 hospital admissions for children under the age of five each year in the United States. Additionally, each year it results in 60,000-160,000 hospitalized cases and 6,000-10,000 adult fatalities aged 65 and beyond. Interestingly, RSV is the main cause of pneumonia and bronchiolitis in American infants under the age of one (11).

The examination of HRSV prevalence is of paramount importance for gaining insights into its implications, influencing public health policies, and steering the direction of preventative and therapeutic approaches. This study's main goal was to investigate the most common HRSV genotypes in Iran. This was accomplished by utilizing (reverse transcriptase polymerase change reaction) RT-PCR to specifically focus on the G protein's second variable region. Finding trends in the distribution of these HRSV genotypes has great potential to progress the creation of effective vaccines and antiviral treatments.

MATERIALS AND METHODS

Study population. In the period of October 2015 to February 2016, two hundred swab samples of the throat and pharynx secretions were taken from children under the age of 12 who were admitted to Shiraz facilities in southern Iran. The ethics committee of Shiraz University of Medical Science authorized this study (IR.SUMS.REC.1394.S599), and the patients' parents or legal guardians provided written informed permission.

Samples were gathered from various healthcare facilities, such as Namazi, Imam Reza, Ghadir Mother and Child, and Al-Zahra hospitals, all of which either catered exclusively to children or possessed specialized departments for pediatric care. The inclusion criteria were patients who experienced acute respiratory symptoms including fever, cough, sneezing, wheezing, and runny nose.

Complete clinical records were kept at the time of admission, and all affected cases' parents or guardians gave their approval as required. The respiratory samples were then transported to the Department of Virology at Shiraz University of Medical Sciences, where they were preserved at a temperature of -80°C until they could undergo a thorough examination. The Shiraz University of Medical Sciences' medical faculty ethics committee gave its clearance for this study (IR.SUMS.REC.1394.S599). Additionally, individuals with underlying medical conditions, including co-infections with HIV, HBV, HCV, diabetes, and any compromised immune situations were excluded from the study.

RNA extraction, cDNA synthesis, and RT-PCR. Using a high-purity nucleic extraction kit with LOT# 25796300 (Roche Diagnostic, Manheim, Germany), we extracted the viral genome from the swabs. To determine the RNA concentration in the extracted sample, a 1/100 dilution of the extracted RNA sample was necessary. The quality of the RNA sample for PCR was assessed by Nanodrop, ensuring that the ratio of optical absorption at 260 to 280 was greater than 1.8.

The extracted RNA was subsequently reconstituted using 50 µL of elution buffer. For the next step, cDNA synthesis was done based on the kit instruction (Yekta Tajhiz Azma, Iran). For the external PCR amplification of the G-protein, 10 µL of synthesized cDNA was combined with a 40 µL reaction mixture. This mixture consisted of 23 µL distilled water, 5 µL 10× PCR buffer, 4 µL dNTP, 2.5 µL $MgCl_2$, 2.5 µL of each primer (10 pmol/mL), and 0.5 µL Taq DNA polymerase. The study's primer sequences are shown in Table 1 (13). A hemi-nested PCR was subsequently

performed using 5 µL of the external PCR product under the same conditions. The amplification process is shown in Table 1. All PCR runs included both positive and negative controls. UV light was used to view the PCR products after they had been electrophoresed on a 1.5% agarose gel. The external PCR amplicons were 450 bp, while the hemi-nested PCR amplicons were 400 bp. Figs. 1A and B show that all PCR runs contained both positive and negative controls.

DNA sequencing. To ascertain the genetic sequence, we employed hemi-nested primers, nRSAG, and nRSBG, serving as forward primers for subgroups A and B, respectively. For sequencing, the reverse primer utilized was R2 (Table 2) (12). Following purification, the PCR products underwent sequencing in both forward and reverse directions. This sequencing process was conducted using an ABI PRISM 310 genetic analyzer from PE Applied BioSystems Inc., headquartered in Foster City, CA. The ABI PRISM BigDye Terminator cycle sequencing ready reaction kit, also from PE Applied BioSystems Inc., facilitated and supported the sequencing procedure.

Table 1. PCR conditions for amplification of the G-protein of HRSV.

PCR steps	Temperature (°C)	Time	Cycles
First- Denaturation	95	2’	1
Denaturation	94	1’	
Annealing	50	1’	30
Extension	72	2’	
Final extension	72	7’	1

Phylogenetic analysis. A comparison was made between nucleotide sequences acquired from the G protein gene's second variable region and HRSV sequences found in the GenBank database. Subsequently, we conducted a phylogenetic analysis employing the neighbor-joining, with MEGA 7 software and a bootstrap value of 1000. To calculate the pairwise distances, both within genotypes and between them, we applied the Kimura 2 parameters model.

Statistical analysis. This study was based on the total number of patients hospitalized in the respiratory care departments of the respective hospitals. The patients' data, including age, gender, and recorded clinical symptoms, were collected. The study data were entered into Microsoft Excel 365 and crosschecked for accuracy. Descriptive analysis and chi-square and Fisher’s exact test were done by IBM SPSS Statistics version 27.

RESULTS

Demographic data. 86 (43%) girls and 114 (57%) boys made up the 200 respiratory samples taken from children under the age of 12 who had acute respira-

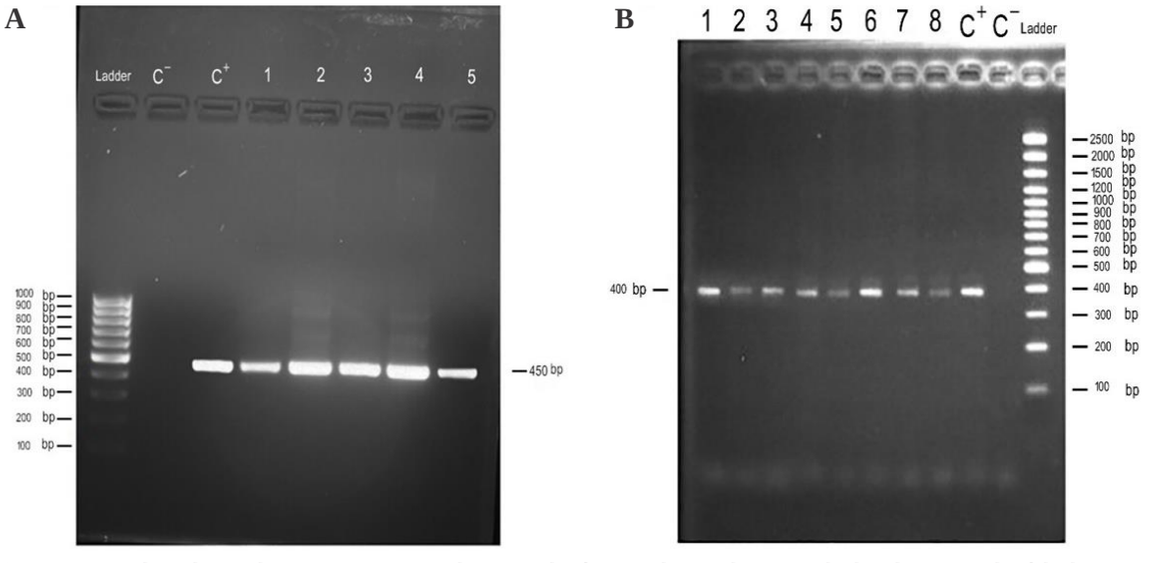

Fig. 1. PCR product electrophoresis on agarose gel. A: Result of external PCR shows 450 bp bands. B. Result of the hemi-nested PCR detects 400 bp bands.

Table 2. The primers with associated sequences and TM used in the study (13).

PCR Step	Primer Name	Primer Sequence	Tm (ºC)
External PCR	Forward (A)	5'-GAAGTGTTCAACTTTGTACC-3' (nt511-530)	56.8
	Forward (B)	5'-AAGATGATTACCATTTTGAAGT-3' (nt494-515)	56.9
	Reverse (R1)	5'-CAACTCCATTGTTATTTGCC-3' (nt3-22)	57.6
Hemi-nested PCR	Subgroup A (nRSAG)	5'-TATGCAGCAACAATCCAACC-3' (nt539-558)	61.1
	Subgroup B (nRSBG)	5'-GTGGCAACAATCAACTCTGC-3' (nt512-531)	61.9
	Reverse (R2)	5'-CAACTCCATTGTTATTTGCC-3' (nt3-22)	57.6

tory symptoms such as fever, coughing, rhinorrhea, and wheezing. Among them, 34 (22.24%) tested positive for HRSV, including 19 (56%) girls and 15 boys (44%).

The age range of those infected with HRSV was 1 to 12 years old, with a mean age of 3 ± 1. The average age for females and males was 2 ± 0.25 and 4 ± 0.75, respectively (estimated). Conversely, individuals without the infection, classified as negative cases, had an average age of 8 ± 2. For this group, the mean age was 7 ± 1.25 for males and 9 ± 0.75 for females.

Clinical manifestations. In this study, the prevalence of each symptom is examined within both positive and negative patient groups based on HRSV positive/negative and gender (Tables 3 and 4). Fever was observed in 63.5% of the patients (127 out of 200). Within the negative group, 64% (106 out of 166) experienced fever, while in the positive group, 62% (21 out of 34) had fever. There was no statistically significant difference in the occurrence of fever between positive and negative patients ($p = 0.846$). Cough was reported by 85 individuals (42.5%) in total. Among negative patients, 45% (75 out of 166) had a cough, whereas among positive patients, 29% (10 out of 34) had it. The p-value for cough was 0.127, indicating no statistically significant difference between positive/negative patients.

Sneezing was reported in 63% (126 out of 200) of all patients. Among negative patients, 66% (109 out of 166) experienced sneezing, while in the positive group, the prevalence was 50% (17 out of 34). The two patient groups did not appear to vary significantly in terms of sneezing, as indicated by the p-value of 0.118.

A total of 168 patients (84%) showed wheezing. Within the negative group, 88% (146 out of 166) had wheezing, compared to 65% (22 out of 34) in the positive group. There was a statistically significant ($p = 0.003$) difference in the prevalence of wheeze between positive and negative patients.

A runny nose was observed in 66.5% of all patients (133 out of 200). Among negative patients, 72% (120 out of 166) had a runny nose, whereas among positive patients, 38% (13 out of 34) showed this symptom. A statistically significant difference was seen between patients who tested positive and negative for runny nose, as shown by the p-value of less than 0.001.

Genotype distribution. In the context of our research, we conducted a detailed examination of the distribution of HRSV genetic subgroups among the pediatric patient cohort. Our findings highlight the prevalence and composition of these subgroups, shedding light on the dynamics of HRSV infection within this population. Out of the total cases studied, 21 instances, constituting a majority at 61.76%, were found to be linked with subgroup A. This indicates the noteworthy predominance of this genetic variant among the affected pediatric patients. Subgroup BA, while less prevalent, was still significantly present, comprising 12 cases or 35.29% of the total. Notably, subgroup B, though relatively rare in occurrence, played a notable role, with a lone instance accounting for 2.94% of cases. Our study also delved into the broader picture of HRSV prevalence within this pediatric cohort, revealing that the overall prevalence of HRSV infection was substantial, standing at 22.24%. This data provides a comprehensive overview of the distribution and prevalence of HRSV genetic subgroups within the pediatric patient population, thereby enhancing our understanding of the impact of the virus in this specific demographic feature.

Phylogenetic analysis. We used a thorough phylogenetic analysis in our investigation to examine the genetic diversity and relatedness of the HRSV's isolated samples. A total of 9 samples were subjected to

Table 3. Comparison of clinical manifestations based on HRSV positive and negative patients.

Variable	Negative N=166	Positive N=34	Overall N=200	P-value
Fever				
Negative	106 (64%)	21 (62%)	127 (63.5%)	0.846
Positive	60 (36%)	13 (38%)	73 (36.5%)	
Cough				
Negative	75 (45%)	10 (29%)	85 (42.5%)	0.127
Positive	91 (55%)	24 (71%)	115 (57.5%)	
Sneezing				
Negative	109 (66%)	17 (50%)	126 (63%)	0.118
Positive	57 (34%)	17 (50%)	74 (37%)	
Wheezing				
Negative	146 (88%)	22 (65%)	168 (84%)	0.003
Positive	20 (12%)	12 (35%)	32 (16%)	
Runny nose				
Negative	120 (72%)	13 (38%)	133 (66.5%)	<0.001
Positive	46 (28%)	21 (62%)	67 (33.5%)	

Note: The statistical significance level is $p < 0.05$.
Abbreviation: HRSV, human respiratory syncytium virus.

Table 4. Clinical symptoms of individuals with HRSVs are compared, broken down by gender.

Variable	Female N=98	Male N=102	Overall N=200	P-value
Fever				
Negative	60 (61%)	67 (66%)	127 (63.5%)	0.558
Positive	38 (39%)	35 (34%)	73 (36.5%)	
Cough				
Negative	45 (46%)	40 (39%)	85 (42.5%)	0.391
Positive	53 (54%)	62 (61%)	115 (57.5%)	
Sneezing				
Negative	56 (57%)	70 (69%)	126 (63%)	0.108
Positive	42 (43%)	32 (31%)	74 (37%)	
Wheezing				
Negative	77 (79%)	91 (89%)	168 (84%)	0.053
Positive	21 (21%)	11 (11%)	32 (16%)	
Runny nose				
Negative	55 (56%)	78 (76.5%)	133 (66.5%)	0.003
Positive	43 (44%)	24 (23.5%)	67 (33.5%)	

Note: The statistical significance level is $p < 0.05$.

sequencing analysis, revealing valuable insights into the viral subtypes and genetic variations within our dataset. From the analysis, we found that five of these samples were associated with subtype B, highlighting the significance of this subtype in our sample set. Conversely, four samples exhibited genotype A, indicating a considerable presence of this genotype among the isolated samples. Notably, none of the nine samples collected from the patients was classified as subtype A1 or B1, underscoring the unique genetic compo-

sition of our sample cohort. Further delving into the phylogenetic analysis, we scrutinized the genomic synonyms of the G gene region in the A isolates of the HRSV virus. Within this subgroup, our analysis revealed that three of the samples were categorized under the GA2 subgroup, and one sample was classified as belonging to the GA1 subgroup (Fig. 2).

Turning our attention to the B isolates of the HRSV virus, we found that four samples could be attributed to the BA subgroup, while one sample was identified within the GB2 subgroup (Fig. 3). This detailed genetic characterization enhances our understanding of viral diversity and provides valuable information regarding the specific subtypes and genotypes present in our study population.

DISCUSSION

In developing nations, a significant percentage of mortality among young children arises from acute respiratory infections, with HRSV being among the most important one (13). Within these cases in our study, subgroup classification revealed 21 (61.76%) instances associated with subgroup A, 12 (35.29%) within subgroup BA, and a lone occurrence (2.94%) attributed to subgroup B. The total prevalence of HRSV was 22.24% in pediatric patients.

In comparison to other investigations, a study focused on RSV distribution and genetic diversity among Italian adults during the 2021/22 winter season. Researchers analyzed over 1,200 samples from

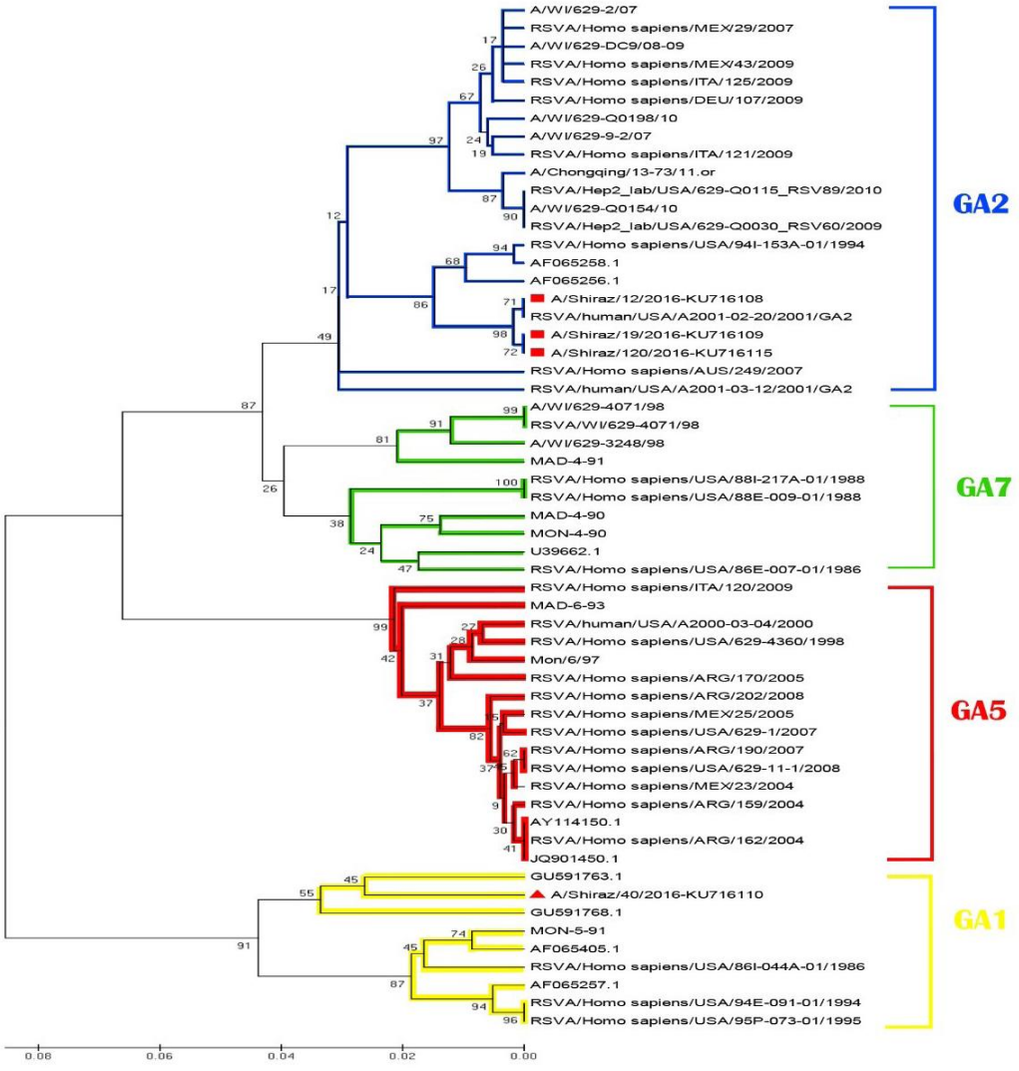

Fig. 2. Nucleotide sequences pertaining to the second variable region of the G protein are displayed in the phylogenetic tree for RSV-A (A), which was created with MEGA 7 and the neighbor-joining technique.

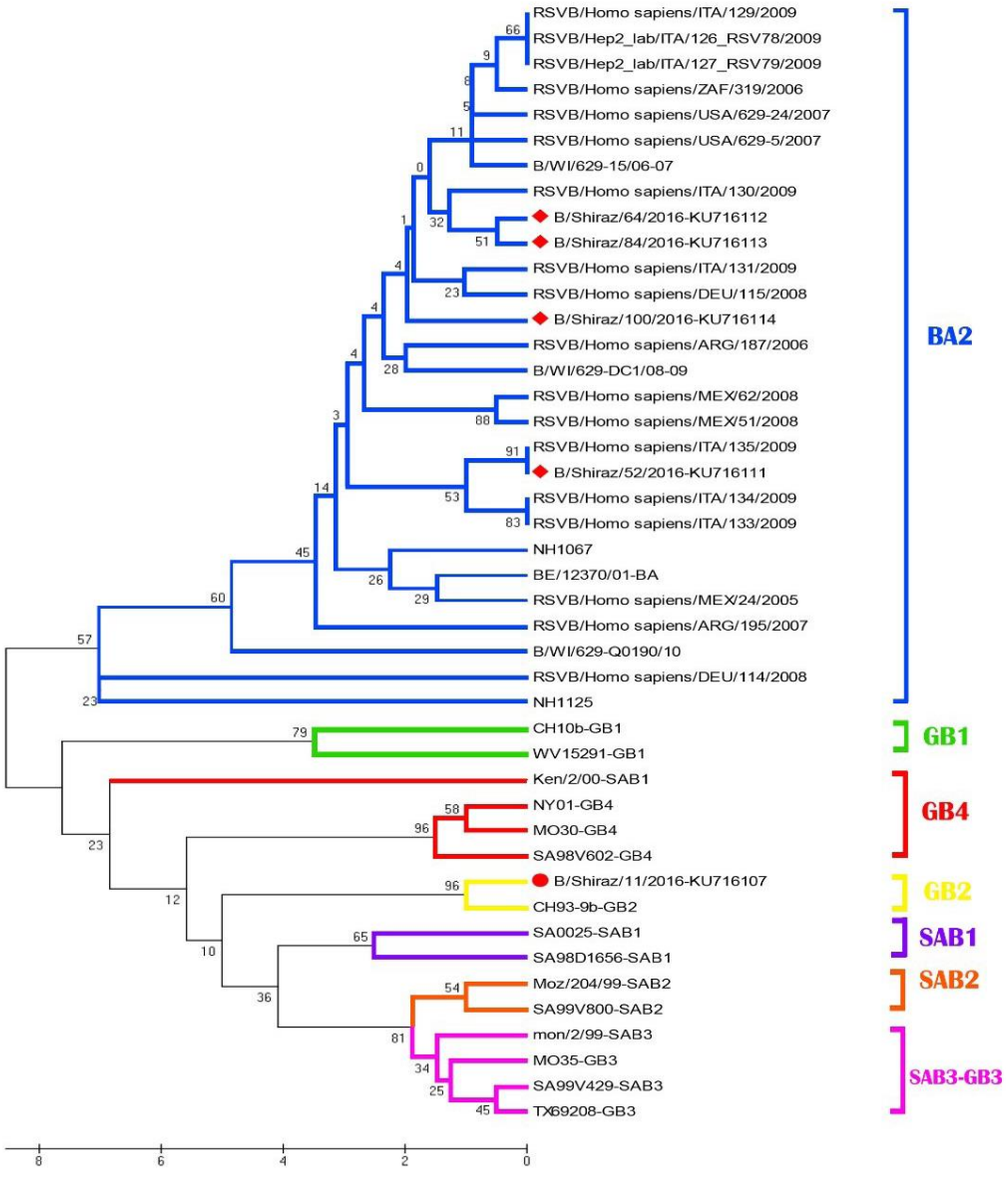

Fig. 3. Nucleotide sequences pertaining to the second variable region of the G protein are displayed in the phylogenetic tree for RSV-B (B), which was created with MEGA 7 and the neighbor-joining technique.

adults with respiratory symptoms. Among the subgroups A (44.4%) and B (55.6%), they discovered that 1.6% were RSV-positive. RSV prevalence peaked in December 2021, similar to influenza. RSV strains belonged to ON1 and BA genotypes for A and B subgroups, respectively. Most RSV-positive cases (72.2%) also had other pathogens, like SARS-CoV-2. The findings highlighted the need for a National RSV surveillance system, especially with upcoming vaccines (14).

In contrast to the study carried out in Italy, our research found a higher prevalence of RSV subgroup A, which appears to suggest a different pattern compared to Italy.

Another study in South Korea between 2008-2010 examined HRSV prevalence and genetics by analyzing the patients' records and the G-protein gene from nasopharyngeal aspirates. Higher HRSV rates were observed in two consecutive winter seasons. Among 297 positive samples from infants and children, 67% were HRSV-A and 33% belonged to HRSV-B. Subgroup dominance shifted from B to A strains. Novel

genotypes were identified in both HRSV-A (genotype CB-A) and B (genotypes BA11 and CB-B) subgroups. Variability in the G protein's C-terminal section suggested selective pressure affecting immune recognition. These variants were the first for South Korea, indicating ongoing HRSV genetic evolution (15). The study's findings about the predominance of Subgroup A are consistent with our own.

In another study, scientists looked at nasopharyngeal swabs from 206 sick kids who were sent to the pediatric unit of Bahrami Children's Hospital in Iran because they had a respiratory illness. The presence of RSV was confirmed using Nested RT-PCR. The glycoprotein gene was then sequenced, and MEGA X software was used to perform phylogenetic analysis and confirm the viral genotypes. RSV was detected in 74 (35.92%) of the total samples. Sequence analysis was conducted on a subset of specimens, specifically 10 samples from 2018 (RSV-A: RSV-B = 4:6) and 19 samples from 2019 (RSV-A: RSV-B = 16:3). According to the phylogenetic study, all RSV-A strains were of the ON1 genotype, whereas RSV-B strains were classified as BA9 genotype. Notably, the Iranian BA9 genotype had a newly discovered N-glycosylation site, while the ON1 genotype showed evidence of positive selection (16). Another study in 2017 was an exploration to decipher the molecular epidemiology, genotypic diversity, and phylogenetic traits of RSV strains among individuals afflicted with respiratory infections. Among 400 Iranian military trainees exhibiting respiratory symptoms, 2.75% (11/400) were confirmed with RSV infection via RT-PCR. The sequencing revealed a notable prevalence of type A (2.5%, n=10), overshadowing type B (0.25%, n=1). Patients infected with RSV most frequently reported having a sore throat as their symptom. Phylogenetic scrutiny indicated a significant alignment of the strains from the samples with those originating from the Philippines and the United States (17). Furthermore, a study carried out in 2020 in Iran indicated that Iranian samples containing the Human RSV ON-1 genotypes showed grouping within three separate lineages. Noteworthy amino acid alterations were detected, such as X218Q, I240S, L289P, Y304H, and L310P (18).

Our study demonstrated a prevalence of 17% for HRSV among patients who presented respiratory symptoms. This percentage differs from those of the studies conducted at Bahrami Hospital, where they have reported a higher prevalence. This observation may indicate a higher prevalence of this virus among children in Tehran. In the study on military personnel, subgroup A showed a significantly higher prevalence compared to subgroup B, which is consistent with our findings. In our study, subgroup A also had a percentage of 61.76%, while subgroup B had a percentage of 2.94%. It is worth noting that the lower prevalence of HRSV in this study, compared to ours, is entirely expected as the prevalence of this virus is generally higher in children and infants compared to adults.

Amidst the COVID-19 pandemic, particularly in its early stages, there was a noticeable decline in the incidence and mortality attributed to other respiratory infections. This reduction was followed by a subsequent surge in infections and fatalities linked to the causative agent of COVID-19, namely the SARS-CoV-2 virus. HRSV, similarly affected by adherence to quarantine measures and health guidelines, demonstrated a marked decrease in infection rates as well. Notably, a compelling example of this phenomenon is illustrated in the study conducted by Letafati et al. where no instances of HRSV infections were reported during the winter of 2021 amidst the pandemic (19). There was indications of a delayed rebound of RSV during the COVID-19 pandemic in another research by Tavakoli et al. In this study, due to the delay in the occurrence of infected cases and this change and the pandemic happened during spring (not the expected winter), the rate of HRSV patients was reported to be 50% (20). A separate investigation conducted by Zendehrouh et al. further reinforced the trend of diminished HRSV prevalence, accounting for approximately 2%, amid the backdrop of the COVID-19 pandemic (21). These observations underscore the notion that the ascendancy of the respiratory virus SARS-CoV-2 has been associated with a substantial reduction in the incidence of HRSV infections during its anticipated seasonal period.

Our results demonstrate how important HRSV is as a major viral infection in newborns and early children. This study also emphasizes how effective it is to use RT-PCR to target the G protein's second variable region in future research on HRSV genotyping in Iran. Moreover, these outcomes hold potential relevance for the prospective design of HRSV vaccines and the formulation of innovative strategies for prevention and treatment. However, further investigation with a larger sample size is recommended.

CONCLUSION

The genetic variety of HRSV strains that were prevalent in the pediatric Iranian population during a certain seasonal time was clarified by this work. Among children exhibiting acute respiratory symptoms, our data indicate a significant incidence of HRSV infections. Subgroup A strains prevailed, with a notable presence of genotype GA1, while subgroup B strains were less prevalent, primarily associated with genotype BA. The concurrent circulation of multiple genotypes within a single season underscores the dynamic nature of HRSV transmission and highlights the importance of continuous monitoring to understand viral epidemiology. The dominance of genotype GA1 in subgroup A and genotype BA in subgroup B suggests potential variations in virulence, transmissibility, or immune evasion strategies among different genotypes. These insights have significant implications for public health strategies, vaccine development, and therapeutic interventions. Our study contributes to the growing body of knowledge aimed at combating HRSV-related morbidity and mortality, particularly among the vulnerable pediatric population. Continued surveillance and genomic studies will be crucial in adapting and refining strategies to mitigate the impact of HRSV infections in Iran.

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