Original Article

Molecular Epidemiology of Human Respiratory Syncytial Virus in Iranian ≥60 Years Old Hospitalized Patients with Acute Respiratory Symptoms

Yaser Arjeyni MSc[1], Ebrahim Faghihloo PhD[2], Katayoun Samimi Rad PhD[3], Vahid Salimi PhD[4], Mahmood Mahmoudi PhD[5], Talat Mokhtari-Azad PhD•[6]

Abstract

Background: Human respiratory syncytial virus (HRSV) is an important cause of acute respiratory infection (ARI) and mortality in ≥60 years old population around the world. The aim of this study was to determine the frequency and circulating genotypes of HRSV in Iranian patients ≥ 60 years old.

Methods: In this cross-sectional molecular epidemiology study, we examined the C-terminal located hypervariable domain of G glycoprotein of HRSV in throat swabs from Tehran, Hormozgan, Boushehr, West Azarbayjan, Ghom and Alborz provinces of Iran which were addressed to national influenza center between October 2013 and March 2015. During these two consecutive years, a total of 225 samples collected from patients older than 60 years were tested using RT hemi-nested PCR and sequencing and the acquired sequences were phylogenetically analyzed.

Results: Sixteen out of 225 samples (7.1%) yielded a positive result. Among the positive samples, 13 cases (81%) pertained to antigenic group A and the remaining 3 cases (19%) belonged to group B. Three genotypes including GA1, GA2 and BA9 were identified in the first year of survey whereas during the second year, only GA1 and GA2 genotypes were detected.

Conclusion: Our study indicates that HRSV genotypes from both A and B antigenic groups which were discovered in pediatric population previously, are circulating among Iranian ≥60 years old population.

Keywords: Frequency, genotype, ≥60 years old, Iran, human respiratory syncytial virus

Cite this article as: Arjeyni Y, Faghihloo E, Samimi Rad K, Salimi V, Mahmoudi M, Mokhtari-Azad T. Molecular Epidemiology of Human Respiratory Syncytial Virus in Iranian ≥60 Years Old Hospitalized Patients with Acute Respiratory Symptoms. *Arch Iran Med*. 2017; 20(6): 368 – 375.

Introduction

Human respiratory syncytial virus (HRSV) infection is considered as one of the most dangerous and lethal respiratory infections in elderly individuals.[1-3] HRSV is an important cause of seasonal respiratory tract infection among adult individuals and accounts for 25% of deaths attributed to these infections in cold seasons, thus resembling the influenza infection. Reports from around the world indicate an HRSV-associated mortality rate of about 26.5 cases per 100,000 persons per year in adults aged 65 years and above.[4] A study in the USA on the consequences of pneumonia among elderly individuals estimated about 687,000 cases of hospitalization and 74,000 deaths occurring annually, of which 2%–9% were due to HRSV.[5]

HRSV has been internationally classified as a member of the *Paramyxoviridae* family. This virus belongs to the *Pneumovirus* genus from the subfamily of *Pneumovirinea*.[6,7] HRSV virion contains a negative-sense, nonsegmented ssRNA genome, composed of 10 genes which encode 11 structural and nonstructural proteins.[8,9] One of these proteins is attachment G glycoprotein, a type II glycoprotein and the most variable protein of the virion which is responsible for the differences between HRSV genotypes. The external domain of this glycoprotein contains two hypervariable regions both of which (especially the second region) are important in genetic variability and are widely used for HRSV molecular epidemiology.[9] HRSV also is divided into two major antigenic groups A and B on the basis of reaction with monoclonal antibodies against the G glycoprotein.[10,11] HRSV group A is subdivided to 11 genotypes including GA1-GA7, ON1, SAA1, NA1 and NA2[9,12,13] and HRSV group B is classified into genotypes GB1-GB4, SAB1-SAB3, SAB4, URU1, URU2, BA1-BA12, and THB.[9,14-22] Continuous variations occurring in amino acid sequence and glycosylation pattern of the G glycoprotein over time results in the virus's evasion from the host's immune responses and annual occurrence of re-infections and frequent epidemics.[23-25] Therefore, determining the circulating genotypes in the community, as well as changes in G glycoprotein over consecutive years is of great importance. The present study is the first report of molecular epidemiology of HRSV among Iranian patients ≥60 years old and intends to determine the frequency and

Authors' affiliations: [1]Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. [2]Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [3]Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. [4]Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. [5]Department of Biostatistics and Epidemiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. [6]Department of Virology - School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.

•Corresponding author and reprints: Talat Mokhtari-Azad PhD, Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Telefax: +98 2188962343, E-mail address: mokhtari@sina.tums.ac.ir

Accepted for publication: 27 April 2017

circulating genotypes of HRSV in this age group.

Materials and Methods

Study population and sample collection

The present cross-sectional study was carried out using throat swab samples received from hospitals of Tehran, Hormozgan, Boushehr, West Azarbayjan, Ghom and Alborz provinces of Iran between October 2013 and March 2015. The target population of the study consisted of adults aged ≥60 years who were referred to hospitals or outpatient centers due to acute respiratory infection. Throat swabs were taken from patients and placed in a virus-specific transport medium. Then, all of these mediums along with ice packs and patient's information sheets were transferred to a Styrofoam container and subsequently referred to the national influenza center (NIC) where we performed the study. After receiving samples, influenza positive samples were excluded from analysis and the remaining specimens were analyzed for HRSV infection.

RNA extraction and cDNA synthesis

Viral RNA extraction was performed on 225 throat swabs using high pure nucleic acid extraction kit (Roche Diagnostic, Manheim, Germany) according to the manufacturer's instructions. The extraction yield was dissolved in 50 µL of elution buffer. In order to synthesize the cDNA, 13.5 µL of this extracted RNA was added to 6.5 µL of RT master mix containing 2 µL RT buffer, 2 µL dNTP, 1 µL random hexamer primer, 1 µL reverse transcriptase enzyme of Moloney murine leukemia virus (M-MuLV) and 0.5 µL RNase inhibitor and then incubated in 3 steps: 10 min at room temperature (25°C), 1 h at 42˚C and 10 min at 70°C.

Primer sequences and hemi-nested RT-PCR

In external step, GPA (nt 511-530, 5'-GAAGTGTTCAACTTTGTACC-3') for subgroup A and GPB (nt 494-515, 5'-AAGATGATTACCATTTTG AAGT-3') for subgroup B were applied as forward primers. Hemi-nested PCR step was performed using nRSAG (nt 539-558, 5'- TATGCAGCAACAATCCAACC-3') as HRSV-A specific forward primer, and nRSBG (nt 512-531, 5'-GTGGCAACAATCAACTCTGC-3') as HRSV-B specific forward primer. In both external and hemi-nested PCR steps, F1 (nt 3-22, 5'-CAACTCCATTGTTATTTGCC-3') was used as common reverse primer for subgroups A and B amplification.[26]

For amplification by PCR in external step, 10 µL of the synthesized cDNA was subjoined to 40 µL of hand-made PCR master mix consisting of 27 µL distilled water, 5 µL 10X PCR buffer, 2 µL dNTP, 2 µL MgCl2, 1.5 µL forward primer, 1.5 µL reverser primer and 1 µL Taq DNA polymerase. Thermal conditions included 5 min at 94°C followed by 30 cycles of 94°C for 30 sec, 50˚C for 30 sec and 72°C for 30 sec and a final extension step at 72°C for 10 min. Subsequently, 5 µL of the product was used for hemi-nested PCR. All conditions for this step were similar to the external step. Amplicons of external and hemi-nested PCR steps were 450 and 400 bps, respectively. Standard HRSV A2 strain as positive control and also negative control were used along with all clinical samples. Subgroup analysis was performed for both A and B subgroups using electrophoresis on 1.5% agarose gel and finally visualized under UV light.

Sequencing

Sequencing was carried out using nRSAG and nRSBG internal forward primers of hemi-nested PCR and F1 as common reverse primer for both A and B subgroups. PCR products after purification were sequenced in forward and reverse directions inside an ABI PRISM 310 genetic analyzer (PE Applied BioSystems Inc., Foster City, CA) using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (PE Applied BioSystems Inc).

Phylogenetic analysis

The sequencing results of second hypervariable region of G protein gene of HRSV were aligned along with reference sequences from GenBank database using CLUSTAL W tool in MEGA6 software. Subsequently, phylogenetic analysis was performed using the neighbor-joining method in the mentioned software. Intragenotypic and intergenotypic pairwise distances were calculated by Kimura 2 parameters. All sequences were submitted to GenBank database and possessed accession numbers of KT347290-KT347296.

Amino acid analysis

After phylogenetic analysis, aligned DNA sequences were translated to their corresponding amino acid sequences using MEGA6 software for highlighting probable mutations involved in HRSV immune evasion and disease burden. Amino acid sequences were also analyzed by two online glycosylation prediction tools including NetOGlyc 4.0 Server and NetNGlyc 1.0 Server for identification of potential O-glycosylation and N-glycosylation sites, respectively.

Statistical analysis

IBM SPSS Statistics software version 22 was used for statistical analysis of data. We analyzed the relation between HRSV abundance and patients' sex by Chi-square test. The relation between HRSV abundance and patients' age was studied by t-test. Also, the relation of HRSV genotypes with patients' sex was evaluated using Chi-square test. After confirming normal distribution using Kolmogorov-Smirnov test, the relation of HRSV genotypes with patients' age was analyzed using ANOVA test. P values <0.05 were considered statistically significant.

Results

HRSV distribution and seasonality

A total of 225 throat swabs were examined in this study all of which belonged to patients ≥60 years old with acute respiratory symptoms. Overall, 16 (7.1%) out of all samples tested positive for HRSV among which 13 (81%) were identified as subgroup A and 3 (19%) were identified as subgroup B, both of which were circulating during the two-year study period. HRSV infection was distributed throughout the cold seasons and early spring during the study period but peaks of infection occurred during middle and late winter in February and March. In terms of geographical distribution, Tehran had 12 positive samples and four other cities including Varamin, Sardasht, Boushehr and Bandarabbas each had one positive sample. Among 16 HRSV positive cases, 10(62.5%) were female and 6(37.5%) were male. Distribution of positive samples in three age groups including 60–70, 70–80, and 80–90 years old consisted of 3(18.75%), 6(37.5%) and 7(43.75%) positive cases, respectively. Our statistical analysis showed

no significant relation between HRSV positive cases and age (*P*-value: 0.165) or sex (*P*-value: 0.164).

Phylogenetic analysis and genotype distribution

Genotypes isolated in our study included GA2 (4 isolates, 57%), GA1 (2 isolates, 29%) and BA9 (1 isolate, 14%). All of these three genotypes were co-circulating during the 2013–2014 cold seasons and GA2 genotype predominated in this period. During 2014–2015 cold seasons, only GA1 and GA2 genotypes were circulating with similar frequencies with GA1 detected in mid-fall and GA2 in mid-winter. The phylogenetic analysis results of group A and B isolates are shown in Figure 1 and Figure 2, respectively. From the four detected GA2 genotypes, two were isolated from 80–90 year old patients and the other two were isolated from patients aged 70–80 years. Two detected GA1 genotypes were isolated from two patients aged 74 and 81 years. The BA9 isolate was from a patient with 80 years of age. From the four GA2 isolates, two were isolated from men and two were from women. Two GA1 isolates were also obtained from one man and one woman and the BA9 genotype was isolated from a woman. We found no significant relation between isolated HRSV genotypes and patients' age (*P*-value: 0.964) or sex (*P*-value: 0.646).

Amino acid and glycosylation pattern analysis

Our analyses revealed 3 amino acid changes which were common among all group A isolates. These conserved residues which may be under positive selective pressure included Leu215Pro, Asn250Ser and Lys257Glu. There were also several GA2 specific amino acid changes at positions 226, 233, 262, 265, 269, 280, 286, 289, 290, 292, 293, 297, and 300. The results for group A and B isolates are shown in Fig. 3 and Figure 4, respectively. Three N-glycosylation sites (NXT, where X is not Pro) were found in group A isolates at amino acid positions 237, 251 and 294. Moreover, two O-glycosylation motifs at amino acid position 219 including TTKP in GA2 isolates and TTKS in GA1 isolates and also one common O-glycosylation motif as TTKP at position 227 were detected which can be potential sites for extensive O-glycosylation of G glycoprotein. Also, we discovered one GA2 genotype specific O-glycosylation motif as TEKP at position 231. In addition, in BA9 genotype three probable N-glycosylation sites at amino acid residues 228 (NPT), 276 (NHT) and 308 (NTT) we detected. Our analyses determined up to 39 O-glycosylation sites in BA9 isolate, three of which at positions 249, 283 and 284 possessed O-glycosylation scores higher than 0.9.

Figure 1. HRSV-A G gene phylogenetic relationships of sequences obtained from the second variable region of HRSV stains isolated in Iran together with those available in GenBank. The MEGA 6 software was used for construction of neighbor-joining tree by Kimura 2-parameter model with uniform rates and bootstrap of 1000 replicates. The numbers cloe to nodes represent bootstrap values in percent. Our isolates are characterized by geographic region, followed by group designation, year of detection and sample number. Also, our GA2 isolates are specified with black squares and isolated GA1 strains are marked with black circles. Reference sequences of different genotypes have been characterized with access number followed by genotype name and are not marked.

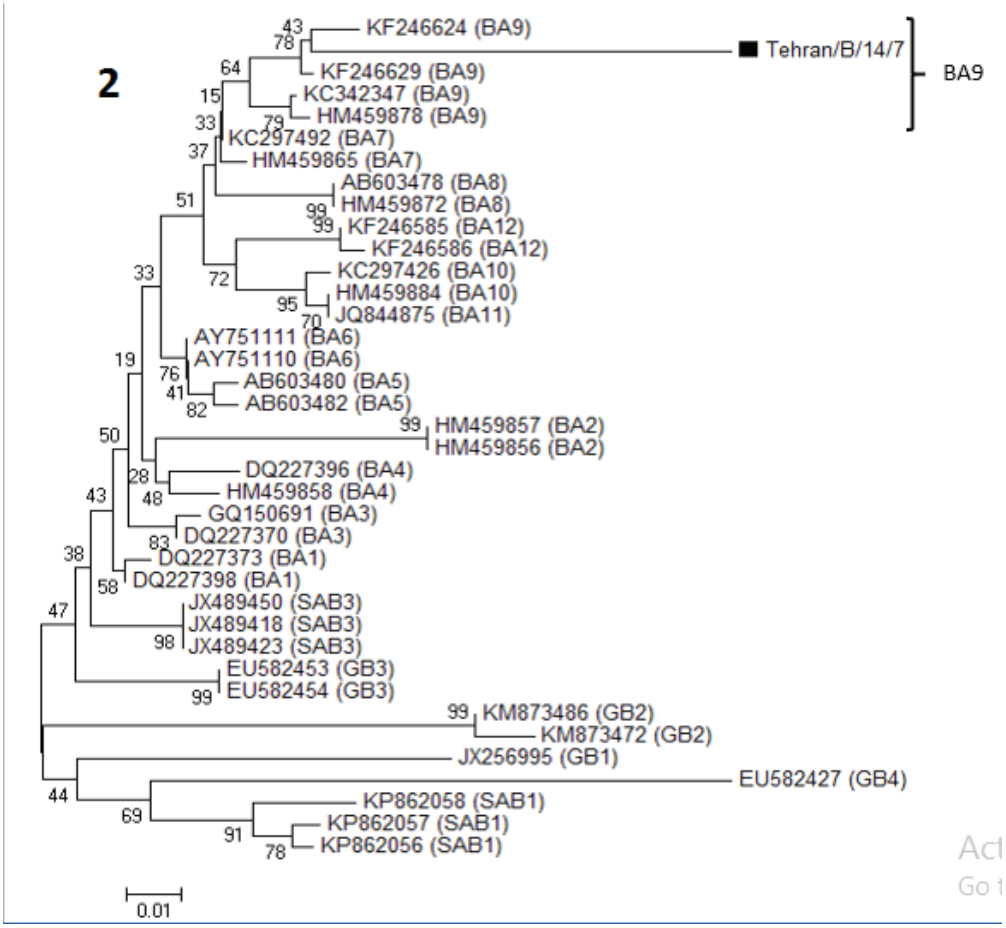

Figure 2. HRSV-B G gene phylogenetic relationships of sequences obtained from the second variable region of HRSV stains isolated in Iran together with those available in GenBank. The MEGA 6 software was used for construction of neighbor-joining tree by Kimura 2-parameter model with uniform rates and bootstrap of 1000 replicates. Numbers close to each node represent bootstrap values in percent. Our BA9 isolate is characterized by geographic region, followed by group designation, year of detection and sample number and is also marked by a black square. Sequences used as reference for different genotypes are characterized with access number followed by genotype name and are not marked.

3

```
                212       221       231       241       251       261       271       281       291       301
                 |         |         |         |         |         |         |         |         |         |
Long             KKDLKPQTT KPKEVPTTKP TEEPTINTTK TNITTTLLTN NTTGNPKLTS QMETFHSTSS EGNLSPSQVS TTSEHPSQPS SPPNTTRQ*L L
Tehran/A/14/32   ...P..... .S..L..... ......I... ...I.....S ......E... .L........ ...P...... ....Y..... .....P.... .
Varamin/A/14/80  ...P..... .S..A..... ...R....S. .K.I.....S ......E..C .......... ...P...... ....Y..... .....PP... .
Tehran/A/14/58   ...P..... .....L.... I.K...D... ...R.....S H.....EH.. .E..L...T. ..Y.....LY ....YL..SP .SS...K*.S .
Tehran/A/14/64   ...P....I ...K.L.... ..K...D... S..R.....S ....I.EH.. .E..L...T. ...PC....Y .....L..SP .SS...K*.S .
Tehran/A/14/65   ...P..... ...K.L.... ..K....... ...R.....S ..P...EH.. .EG.L..NT. ...P.R...Y ....YL.ESP .SS...K*.S .
Tehran/A/15/198  ...P.L... .....L.... ..K...H... .........S I.....EN.. .E..LL..T. ..IP.....Y ...QYL..SP .SS...KS.S .
```

Figure 3. Aligned amino acid sequences of group A isolates in this study along with the Long strain as reference sequence (accession number: AY911262.1).

4

```
                212       221       231       241       251       261       271       281       291       301       311
                 |         |         |         |         |         |         |         |         |         |         |
MAD/2258/98-99   RDPKTLANT LKKETTINPT KKPTPKTTER DTSTSQSTVL DITTSKHTER DTSTSQSTVL DTTTSKHTIQ QQSLHSTTPE NTPNSTQTPT ASEPSTSNST QKL*SYA
Tehran/B/14/7    ......PK. P......... ....R..... H.T.P..... .T......G. ..TATHCIA. ....TN.... ....Y...L. ...K.....S ........T. L..Q...
```

Figure 4. Aligned amino acid sequences of group B isolate in this study along with the MAD/2258/98-99 strain as reference sequence (accession number: GQ150687.1).

Discussion

This molecular epidemiology study analyzed the second variable region of external domain of G gene product of HRSV in throat swab samples addressed to the national influenza center between October 2013 and March 2015. From the 225 throat swab samples, 16 were positive for HRSV infection using hemi-nested RT-PCR which led to 7.1% frequency estimation for HRSV. In an epidemiology study of viral respiratory infections in Switzerland, using RT-PCR, HRSV infection was detected in 12% of upper respiratory specimens and 14% of lower respiratory specimens of elderly patients >65 years of age.[27] A prospective study was performed on healthy working adults aged 18-60 years, of whom 7% acquired HRSV infection during the study period.[28] In another study, HRSV infection was investigated in two prospective cohorts including 608 healthy elderly individuals and 540 high risk adults and a hospitalized cohort of 1388 adults, among whom HRSV infection was detected using culture, RT-PCR and serologic tests in 46 (7.56%), 56 (10.37%) and 142 (10.23%) cases, respectively.[29]

CTL immune responses play a critical role in HRSV clearance and recovery of patients,[30-33] but in elderly adults, CTL responses become nonfunctional due to decreased naïve and functional T cells.[34-37] As mentioned above in the results section, the distribution of positive samples in the three age groups (namely, 60–70, 70–80, and 80–90 years) was 18.75%, 37.5% and 43.75%, respectively. These data show that with increasing age in the population ≥60 years, there is a trend toward increasing HRSV infection abundance, which may be due to impaired CTL response in this age group, although we could not find any significant relation between HRSV abundance and age. Houspie *et al*. investigated the circulation of HRSV in Belgium using HRSV antigen test, RT-PCR and phylogenetic analysis. Their results showed that 18.5% of all HRSV positive patients had an age of 13 months and older and 6.4% of these cases were ≥65 years old but overall, the HRSV frequency decreased with age.[38] In another survey, Berginc and Prosenc reported that in hospitalized patients aged between 3-64 years, the frequency of HRSV infection decreased with age (6.8%, 2.6%, 1.5%, and 2.7% in ages 3–6, 7–14, 15–19, and 20–64 years old, respectively), but in patients ≥65 years old, the frequency increased again to 7.4%.[39] A relation between HRSV abundance and age was reported by Pretorius *et al.* who showed that HRSV frequency decreased with age in patients with aged <1 to >65 years old.[40] In a prospective study about seasonal incidence of HRSV infection among adults ≥50 years old, a relation was found between HRSV incidence and age as incidence increased with patients' age (124, 147 and 199 cases in patients 50-59, 60-69 and ≥70 years old, respectively).[41] However, another survey showed that HRSV incidence among patients aged 3 weeks to 79 years decreased with increasing age.[14]

The majority of our positive cases (62.5%) were female and the rest (37.5%) were male; however, despite the higher rate of positive cases in women, we found no significant relation between HRSV frequency and sex. In contrast to this finding, McClure *et al.* reported a significant relation between higher HRSV incidence and female sex in patients aged ≥50 years old.[41] According to our results among patients ≥80 years old, the rate of HRSV infection was higher in women compared to men (5 cases vs. 2 cases). Falsey *et al.* have reported almost similar results.[29]

HRSV group A was predominant in all seasons during our study. HRSV-A has been also shown to be the predominant group in several epidemics among adult and also pediatric populations. In a study during 2006–2010, 1326 enrolled adults ≥50 years old were tested for HRSV by multiplex RT-PCR among whom 164 (12%) were infected with this virus. The mean age of HRSV positive cases in that study was 64.2 years and 62% of them were female. Seasonal incidence of HRSV group A and B was 80 and 74 cases per 10000 persons, respectively. For HRSV group A, seasonal incidence increased with patients' age while for HRSV group B, there was not any uptrend for seasonal incidence, although overall the group B seasonal incidence was higher in upper ages.[41] HRSV abundance of about 19% was reported by Ohno *et al.* among hospitalized children. In that study, 2150 nasopharyngeal swabs were tested using nested RT-PCR, with 65% of positive cases belonging to the HRSV group A and 35% to group B.[42] Similar results were acquired for children in China where 15.8% of the cases tested positive for HRSV using immunofluorescent assay (IFA). Males had significantly higher rate of HRSV infection than females. Using multiplex PCR and real time RT-PCR, 63.9% and 33.1% of IFA positive samples were identified as group A and B, respectively.[23] HRSV epidemic periods among children were investigated in Fortaleza, Brazil. Immunofluorescence assay (IFA) was used for both screening and subgrouping purposes in 2885 nasopharyngeal aspirates from which 456 samples (15.8%) had a positive result for HRSV infection. Eighty out of all IFA-positive cases were used for subgrouping and genotyping by RT-PCR and sequencing. HRSV subgroups A and B were found in 58 and 28 cases of analyzed samples, respectively.[43] These studies indicate that application of a serological screening method prior to molecular methods like PCR, may enable us to improve the study scale without significant decrease in study cost-effectiveness.

Totally, 7 out of 16 positive samples (44%) were successfully sequenced in our survey which subsequently fell into GA1, GA2 and BA9 genotypes using phylogenetic analysis (Figure 1 and Figure 2). Several other studies have also encountered this problem and failed to sequence a large number of their positive samples.[44-46] This can be secondary to various reasons such as decrease in RNA concentration in the samples due to RNA degradation caused by inappropriate sample transport conditions, decreased viral load in the samples due to late referral of patients (especially ≥60 years old patients) to medical centers, low sample volume etc.

Overall, we did not find any statistically significant relation between detected HRSV genotypes and patients' age or sex. Several studies have reported co-circulation of different HRSV genotypes from both A and B groups in the adult population. A nosocomial outbreak of HRSV infection was surveyed on in adult leukemia/lymphoma ward. Forty-five hospitalized patients aged 27–68 years were tested using immunofluorescence staining, cell culture and RT-PCR, of whom 8 (17.8%) were HRSV positive. Six of HRSV infected patients were men and two were women. Seven out of eight HRSV positive samples were sequenced and genotyped of which six were genotype GB3 from HRSV group B and one was genotype GA5 from HRSV group A.[47] In another study between 2011 and 2013, infection with novel HRSV ON1 genotype was investigated in adult HCT recipients. Among 20 HRSV infected adult HCT recipients aged 22–69 years, 14 (70%) belonged to the HRSV group A and 6 (30%) to the HRSV group B; also, 50% of their positive cases were male. Eighteen positive samples were sequenced and, after phylogenetic analysis, were

placed in 3 genotypes including ON1, NA1 and BA. Genotype frequency was 11 ON1 isolates, 2 NA1 isolates and 5 BA isolates. They also found no relation between HRSV genotypes and age or sex of the infected patients.[48]

There is no previous report on molecular epidemiology of HRSV in the Iranian population ≥60 years old and the present study is the first of its kind in Iran. Nevertheless, several studies performed in Iran have demonstrated the HRSV genotype circulation pattern among children and infants which can be representatives of HRSV genotype circulation pattern in Iran. From October to December 2009, the genetic diversity of G protein gene of HRSV was studied in Iranian children suffering from acute respiratory symptoms. Using RT-PCR, 24 (22.42%) out of 107 throat swab samples tested positive for HRSV infection among which 16 belonged to the HRSV group A and 8 to group B. GA1, GA2 and BA were the isolated genotypes in that study and GA1 was predominant.[49] We also found these three genotypes in our study which may suggest a common source of infection and intermittent circulation of these genotypes between children and adults ≥60 years old, especially regarding the fact that the mutations and glycosylation patterns we found in G glycoproteins of HRSV isolates from adult patients were similar to those were found in HRSV isolates from pediatric studies (discussed below). However, whether or not the circulating genotypes in children and the adults are exactly attributable to each other needs further investigations in comparative studies with more pervasive scales. Also in another survey between 2009 and 2013, 485 throat swabs taken from children were examined by RT-PCR from which 94 (19.38%) were positive for HRSV. After phylogenetic analysis, the researchers identified genotypes GA1, GA2 and GA5 from group A and genotype BA from group B.[50]

As mentioned above in the results section, GA2 had the highest frequency (57%) among detected genotypes and predominated during the first year of the study. In a previous study, molecular characteristics of HRSV strains were surveyed among HSCT recipients and health care workers in a HSCT unit from April 2001 to October 2002. GA2 was identified as the dominant genotype in the first year of the study. During the second year, the predominant genotype changed to SAB3 followed by SAB1 and GA2.[51] An almost similar predominance pattern was reported in pediatric patients with an HRSV frequency of about 24%. HRSV A and B subgroups were found in 91.4% and 5.1% of positive cases, respectively and the remaining cases were co-infected with both subgroups. The only recognized genotypes included GA2 and BA and overall, the GA2 genotype predominated.[52] Co-circulation of GA2 and BA genotypes among children during five consecutive years was also reported by Moura *et al*.[43]

Considering the fact that the majority of nucleotide changes in G gene sequence lead to amino acid changes in its corresponding protein, it has been concluded that there is a positive selective pressure on this glycoprotein.[53,54] Amino acid variations in HRSV group A, including Ser269Thr and Pro289Ser have been reported to be associated with the outcome of the HRSV disease.[55] We also found these two amino acid substitutions in all GA2 genotypes identified in our study.

It has been indicated that different glycosylation patterns occurring in group A and B viruses result in antigenicity variation between their genotypes and thereupon may help the virus to evade host's immune responses.[56] For group A isolates, we found three N-glycosylation sites (NXT, where X is not Pro) at amino acid positions 237, 251 and 294, two O-glycosylation motifs at amino acid position 219 including TTKP in GA2 isolates and TTKS in GA1 isolates and one common O-glycosylation motif as TTKP at position 227 which can be potential sites for extensive O-glycosylation of this glycoprotein. These glycosylation data are in agreement with several studies.[23,57-59] We also discovered one GA2 genotype specific O-glycosylation motif as TEKP at position 231 which was previously reported.[57]

Also in BA9 genotype, we detected three probable N-glycosylation sites at amino acid residues 228 (NPT), 276 (NHT) and 308 (NTT), which are similar to the results reported by Tan *et al*.[60] Our analyses specified up to 39 O-glycosylation sites in BA9 isolate, three of which at positions 249, 283 and 284 had O-glycosylation scores higher than 0.9.

We should mention here that the present study was performed using only samples from some provinces of Iran which were sent to the National Influenza Center, Tehran University of Medical Sciences and is not representative of the overall HRSV prevalence and genotype distribution in Iran. The limitations that we encountered in this study include difficulty of access to samples and financial limitations. Overall, previously mentioned studies, as well as the present study, represent a considerable prevalence for HRSV infection which requires more effective health care programs for both elderly and infant age groups. The majority of our positive samples belonged to the antigenic group A. Three genotypes including GA1, GA2 and BA9 were identified in this study and the GA2 genotype predominated. The discovered genotypes were similar to those previously detected in pediatric population. Continuous studies should be carried out every year in order to survey and monitor the circulation pattern of HRSV genotypes among the population ≥60 years old, and the gathered information should be provided to health centers to take appropriate measures. In addition to its use in decision-making for health care purposes, this epidemiological information can be also useful for developing new HRSV vaccines.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

Here, we wish to thank the entire staff of the National Influenza Center, School of Public Health, Tehran University of Medical Sciences. This work was supported by Tehran University of Medical Sciences under research No. 93-04-27-27355.

References

1. Ellis SE, Coffey CS, Mitchel Jr MS EF, Dittus RS, Griffin MR. Influenza– and Respiratory Syncytial Virus–Associated Morbidity and Mortality in the Nursing Home Population. JAGS. 2003; 51(6): 761 – 767.
2. Widmer K, Zhu Y, Williams JV, Griffin MR, Edwards KM, Talbot HK. Rates of Hospitalizations for Respiratory Syncytial Virus, Human Metapneumovirus, and Influenza Virus in Older Adults. J Infect Dis. 2012; 206(1): 56 – 62.
3. Zambon MC, Stockton JD, Clewley JP, Fleming DM. Contribution of influenza and respiratory syncytial virus to community cases of influenza-like illness: an observational study. Lancet. 2001; 358(9291): 1410 – 1416.
4. Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, et al. Mortality associated with influenza and

respiratory syncytial virus in the United States. JAMA. 2003; 289(2): 179 – 186.
5. Han LL, Alexander JP, Anderson LJ. Respiratory Syncytial Virus Pneumonia among the Elderly: An Assessment of Disease Burden. J Infect Dis. 1999; 179(1): 25 – 30.
6. Van den Hoogen BG, Herfst S, Sprong L, Cane PA, Forleo-Neto E, De Swart RL, et al. Antigenic and genetic variability of human metapneumoviruses. Emerg Infect Dis. 2004; 10(4): 658 – 666.
7. Williams JV, Harris PA, Tollefson SJ, Halburnt RL, Pingsterhaus JM, Edwards KM, et al. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. N Engl J Med. 2004; 350(5): 443 – 450.
8. Falsey AR, Walsh EE. Respiratory Syncytial Virus Infection in Adults. Clin Microbiol Rev. 2000; 13(3): 371.
9. Peret TCT, Hall CB, Schnabel KC, Golub JA, Anderson LJ. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. J Gen Virol. 1998; 79(Pt. 9): 2221 – 2229.
10. Anderson LJ, Hierholzer JC, Tsou C, Hendry RM, Fernie BF, Stone Y, et al. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis. 1985; 151(4): 626 – 633.
11. Mufson MA, Orvell C, Rafnar B, Norrby E. Two distinct subtypes of human respiratory syncytial virus. J Gen Virol. 1985; 66(Pt. 10): 2111 – 2124.
12. Peret TCT, Hall CB, Hammond GW, Piedra PA, Storch GA, Sullender WM, et al. Circulation patterns of group A and B human respiratory syncytial virus genotypes in 5 communities in North America. J Infect Dis. 2000; 181(6): 1891 – 1896.
13. Venter M, Madhi SA, Tiemessen CT, Schoub BD. Genetic diversity and molecular epidemiology of respiratory syncytial virus over four consecutive seasons in South Africa: identification of new subgroup A and B genotypes. J Gen Virol. 2001; 82(Pt 9): 2117 – 2124.
14. Arnott A, Vong S, Mardy S, Chu S, Naughtin M, Sovann L, et al. A Study of the Genetic Variability of Human Respiratory Syncytial Virus (HRSV) in Cambodia Reveals the Existence of a New HRSV Group B Genotype. J Clin Microbiol. 2011; 49(10): 3504 – 3513.
15. Auksornkitti V, Kamprasert N, Thongkomplew S, Suwannakarn K, Theamboonlers A, Samransamruajkij R, et al. Molecular characterization of human respiratory syncytial virus, 2010–2011: identification of genotype ON1 and a new subgroup B genotype in Thailand. Arch Virol. 2014; 159(3): 499 – 507.
16. Baek YH, Choi EH, Song M-S, Pascua PNQ, Kwon H-I, Park SJ, et al. Prevalence and genetic characterization of respiratory syncytial virus (RSV) in hospitalized children in Korea. Arch Virol. 2012; 157(6): 1039 – 1050.
17. Blanc A, Delfraro A, Frabasile S, Arbiza J. Genotypes of respiratory syncytial virus group B identified in Uruguay. Arch Virol. 2005; 150(3): 603 – 609.
18. Dapat IC, Shobugawa Y, Sano Y, Saito R, Sasaki A, Suzuki Y, et al. New Genotypes within Respiratory Syncytial Virus Group B Genotype BA in Niigata, Japan. J Clin Microbiol. 2010; 48(9): 3423 – 3427.
19. Eshaghi A, Duvvuri VR, Lai R, Nadarajah JT, Li A, Patel SN, et al. Genetic variability of human respiratory syncytial virus A strains circulating in Ontario: a novel genotype with a 72 nucleotide G gene duplication. PLoS One. 2012; 7(3): e32807.
20. Khor C-S, Sam I-C, Hooi P-S, Chan Y-F. Displacement of predominant respiratory syncytial virus genotypes in Malaysia between 1989 and 2011. Infect Genet Evol. 2013; 14: 357 – 360.
21. Shobugawa Y, Saito R, Sano Y, Zaraket H, Suzuki Y, Kumaki A, et al. Emerging Genotypes of Human Respiratory Syncytial Virus Subgroup A among Patients in Japan. J Clin Microbiol. 2009; 47(8): 2475 – 2482.
22. Trento A, Viegas M, Galiano M, Videla C, Carballal G, Mistchenko AS, et al. Natural history of human respiratory syncytial virus inferred from phylogenetic analysis of the attachment (G) glycoprotein with a 60-nucleotide duplication. J Virol. 2006; 80(2): 975 – 984.
23. Cui G, Zhu R, Qian Y, Deng J, Zhao L, Sun Y, et al. Genetic Variation in Attachment Glycoprotein Genes of Human Respiratory Syncytial Virus Subgroups A and B in Children in Recent Five Consecutive Years. PLoS One. 2013; 8(9): e75020.
24. Sullender WM, Edwards KG. Mutations of Respiratory Syncytial Virus Attachment Glycoprotein G Associated with Resistance to Neutralization by Primate Polyclonal Antibodies. J Virol. 1999; 264(1): 230 – 236.
25. Zlateva KT, Lemey P, Vandamme AM, Ranst MV. Molecular Evolution and Circulation Patterns of Human Respiratory Syncytial Virus Subgroup A: Positively Selected Sites in the Attachment G Glycoprotein. J Virol. 2004; 78(9): 4675 – 4683.
26. Sato M, Saito R, Sakai T, Sano Y, Nishikawa M, Sasaki A, et al. Molecular epidemiology of respiratory syncytial virus infections among children with acute respiratory symptoms in a community over three seasons. J Clin Microbiol. 2005; 43(1): 36 – 40.
27. Ambrosioni J, Bridevaux P-O, Wagner G, Mamin A, Kaiser L. Epidemiology of viral respiratory infections in a tertiary care centre in the era of molecular diagnosis, Geneva, Switzerland, 2011–2012. Clin Microbiol Infect. 2014; 20(9): O578 – O584.
28. Hall CB, Long CE, Schnabel KC. Respiratory Syncytial Virus Infections in Previously Healthy Working Adults. Clin Infect Dis. 2001; 33(6): 792 – 796.
29. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory Syncytial Virus Infection in Elderly and High-Risk Adults. N Engl J Med. 2005; 352(17): 17.
30. Brandenburg AH, De Waal L, Timmerman HH, Hoogerhout P, De Swart RL, Osterhaus ADME. HLA Class I-Restricted Cytotoxic T-Cell Epitopes of the Respiratory Syncytial Virus Fusion Protein. J Virol. 2000; 74(21): 10240 – 10244.
31. Chang J, Srikiatkhachorn A, Braciale TJ. Visualization and Characterization of Respiratory Syncytial Virus F-Specific CD8 T Cells During Experimental Virus Infection. J Immunol. 2001; 167(8): 4254 – 4260.
32. Domachowske JB, Rosenberg HF. Respiratory Syncytial Virus Infection: Immune Response, Immunopathogenesis, and Treatment. Clin Microbiol Rev. 1999; 12(2): 298 – 309.
33. Simmons CP, Hussell T, Sparer T, Walzl G, Openshaw P, Dougan G. Mucosal Delivery of a Respiratory Syncytial Virus CTL Peptide with Enterotoxin-Based Adjuvants Elicits Protective, Immunopathogenic, and Immunoregulatory Antiviral $CD8^+$ T Cell Responses. J Immunol. 2001; 166(2): 1106 – 1113.
34. De Bree GJ, Heidema J, Van Leeuwen EMM, Van Bleek GM, Jonkers RE, Jansen HM, et al. Respiratory Syncytial Virus–Specific CD8+ Memory T Cell Responses in Elderly Persons. J Infect Dis. 2005; 191(10): 1710 – 1718.
35. Haynes L, Maue AC. Effects of aging on T cell function. Curr Opin Immunol. 2009; 21(4): 414 – 417.
36. Herndler-Brandstetter D. How aging affects T lymphocyte-mediated immunity. Front Immunol. 2013; 4: 296.
37. Lee N, Shin MS, Kang I. T-Cell Biology in Aging, With a Focus on Lung Disease. J Gerontol A Biol Sci Med Sci. 2012; 67A(3): 254 – 263.
38. Houspie L, Lemey P, Keyaerts E, Reijmen E, Vergote V, Vankeerberghen A, et al. Circulation of HRSV in Belgium: from multiple genotype circulation to prolonged circulation of predominant genotypes. PLoS One. 2013; 8(4): e60416.
39. Berginc N, Prosenc K. Monitoring the burden of respiratory syncytial virus in different age groups from 2011 to 2015 at National Influenza Centre in Slovenia. J Clin Virol. 2015; 70: S1 – S126.
40. Pretorius MA, Van Niekerk S, Tempia S, Moyes J, Cohen C, Madhi SA, et al. Replacement and Positive Evolution of Subtype A and B Respiratory Syncytial Virus G-Protein Genotypes From 1997–2012 in South Africa. J Infect Dis. 2013; 208(S3): S227 – S237.
41. McClure DL, Kieke BA, Sundaram ME, Simpson MD, Meece JK, Sifakis F, et al. Seasonal Incidence of Medically Attended Respiratory Syncytial Virus Infection in a Community Cohort of Adults ≥50 Years Old. PLoS One. 2014; 9(7): e102586.
42. Ohno A, Suzuki A, Lupisan S, Galang H, Sombrero L, Aniceto R, et al. Genetic characterization of human respiratory syncytial virus detected in hospitalized children in the Philippines from 2008 to 2012. J Clin Virol. 2013; 57(1): 59 – 65.
43. Moura FEA, Perdigao ACB, Ribeiro JF, Florencio CMGD, Oliveira FMS, Pereira SAR, et al. Respiratory syncytial virus epidemic periods in an equatorial city of Brazil. Influenza Other Respir Viruses. 2013; 7(6): 1128 – 1135.
44. Chu HY, Kuypers J, Renaud C, Wald A, Martin E, Fairchok M, et al. Molecular epidemiology of respiratory syncytial virus transmission in childcare. J Clin Virol. 2013; 57(4): 343 – 350.
45. Chu HY, Englund JA, Podczervinski S, Kuypers J, Campbell AP, Boeckh M, et al. Nosocomial Transmission of Respiratory Syncytial Virus in an Outpatient Cancer Center. Biol Blood Marrow Transplant. 2014; 20(6): 844 – 851.
46. Mosero LM, Ng'ang'a Z, Khamadi S, Oundo J, Ndegwa L. Potential Health-Care Associated Respiratory Syncytial Virus in Three Referral

Hospitals in Kenya, 2009-2011. Afr J Health Sci. 2016; 29(1): 1 – 13.
47. Mazzulli T, Peret TCT, McGeer A, Cann D, MacDonald KS, Chua R, et al. Molecular Characterization of a Nosocomial Outbreak of Human Respiratory Syncytial Virus on an Adult Leukemia/Lymphoma Ward. J Infect Dis. 1999; 180(5): 1686 - 1689.
48. Avadhanula V, Chemaly RF, Shah DP, Ghantoji SS, Azzi JM, Aideyan LO, et al. Infection With Novel Respiratory Syncytial Virus Genotype Ontario (ON1) in Adult Hematopoietic Cell Transplant Recipients, Texas, 2011–2013. J Infect Dis. 2015; 211(4): 582 – 589.
49. Faghihloo E, Salimi V, Rezaei F, Naseri M, Mamishi S, Mahmoodi M, et al. Genetic Diversity in the G Protein Gene of Human Respiratory Syncytial Virus among Iranian Children with Acute Respiratory Symptoms. Iran J Pediatr. 2011; 21(1): 58 – 64.
50. Faghihloo E, Yavarian J, Shafiei Jandaghi NZ, Shadab A, Mokhtari-Azad T. Genotype circulation pattern of human respiratory syncytial virus in Iran. Infect Genet Evol. 2014; 22: 130 – 133.
51. Machado AF, Sallum MAM, Boas LSV, Tateno AF, Machado CM. Molecular Characterization of Strains of Respiratory Syncytial Virus Identified in a Hematopoietic Stem Cell Transplant Outpatient Unit Over 2 Years: Community or Nosocomial Infection? Biol Blood Marrow Transplant. 2008; 14(12): 1348 – 1355.
52. Tran DN, Pham TMH, Ha MT, Tran TTL, Dang TKH, Yoshida LM, et al. Molecular Epidemiology and Disease Severity of Human Respiratory Syncytial Virus in Vietnam. PLoS One. 2013; 8(1): e45436.
53. Cane PA, Matthews DA, Pringle CR. Identification of variable domains of the attachment (G) protein of subgroup A respiratory syncytial viruses. J Gen Virol. 1991; 72(Pt. 9): 2091 – 2096.
54. Sullender WM, Mufson MA, Anderson LJ, Wertz GW. Genetic diversity of the attachment protein of subgroup B respiratory syncytial viruses. J Virol. 1991; 65(10): 5425 – 5434.
55. Goto-Sugai K, Tsukagoshi H, Mizuta K, Matsuda S, Noda M, Sugai T, et al. Genotyping and phylogenetic analysis of the major genes in respiratory syncytial virus isolated from infants with bronchiolitis. Jpn J Infect Dis. 2010; 63(6): 393 – 400.
56. Roca A, Loscertales MP, Quintó L, Pérez-Breña P, Vaz N, Alonso PL, et al. Genetic variability among group A and B respiratory syncytial viruses in Mozambique: identification of a new cluster of group B isolates. J Gen Virol. 2001; 82(Pt. 1): 103 – 111.
57. Aamir UB, Alam MM, Sadia H, Zaidi SSZ, Kazi BM. Molecular Characterization of Circulating Respiratory Syncytial Virus (RSV) Genotypes in Gilgit Baltistan Province of Pakistan during 2011–2012 Winter Season. PLoS ONE. 2013; 8(9): e74018.
58. Crowley B. Molecular Epidemiology of Human Respiratory Syncytial Virus Subgroups A and B Identified in Adults with Haematological Malignancy Attending an Irish Hospital Between 2004 – 2009. J Med Virol. 2010; 83(2): 337.
59. Reiche J, Schweiger B. Genetic Variability of Group A Human Respiratory Syncytial Virus Strains Circulating in Germany from 1998 to 2007. J Clin Microbiol. 2009; 47(6): 1800 – 1810.
60. Tan L, Coenjaerts FEJ, Houspie L, Viveen MC, Van Bleek GM, Wiertz EJHJ, et al. The Comparative Genomics of Human Respiratory Syncytial Virus Subgroups A and B: Genetic Variability and Molecular Evolutionary Dynamics. J Virol. 2013; 87(14): 8213 – 8226.