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ORIGINAL ARTICLE

Molecular epidemiology of human respiratory syncytial virus (HRSV) in Iranian military trainees with acute respiratory symptoms in 2017

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ABSTRACT

Background and Objectives: Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection in many populations, including military recruits receiving basic training. Therefore, this study was set out to determine the molecular epidemiology, genotype and phylogenetic features of RSVs in patients with respiratory infection as a case study.
Materials and Methods: In this study, military barracks of Tehran, Iran, between January to March 2017 exposed to respiratory diseases were used for sampling. Throat swabs were taken, a reverse transcriptase-polymerase chain reaction (RT-PCR) assay was performed to identify RSV and then the genotyping and phylogenetic analyses of RSVs in patients with a respiratory infection.
Results: Among 400 Iranian military trainees with respiratory symptoms, RSV infection was identified in 2.75% (11/400) using RT-PCR. Sequencing showed the incidence of type A (2.5%, n=10) to be much higher than type B (0.25%, n=1); Sore throat was the most common symptom among RSV patients. Phylogenetic analysis revealed that the majority of strains from the studied samples were more consistent with those from the Philippines and the US strains.
Conclusion: This study is the first to document RSV as a major cause of acute respiratory illness among military trainees in Iran. The prevalence of RSV is substantial in the cold season and the prevalence of genotype A is dominant in the country, leading to take essential steps in preparing a preventive vaccine against this viral infection.

Keywords: Respiratory tract infection; Human respiratory syncytial virus; Military trainees; Reverse transcription polymerase chain reaction; Genotyping

INTRODUCTION

Military trainees are at increased risk of acute respiratory infection (ARI) outbreaks that could intervene in training and induce a delay in the military schedule, thus, influencing military capability and undergoing a tremendous loss. Immunity is easily compromised in a condition of high physical exercise, psychological stress and the crowded popula-

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tion such as the military training camp (1).

According to the studies, the primary viral infections, including adenoviruses, Epstein-Barr virus, rhinoviruses, influenza A and B viruses and coronavirus, are associated with acute respiratory infections (2). Studies further reveal that respiratory syncytial virus (RSV) would be an important etiologic pathogen that causes respiratory infections in the military trainees (1, 3).

Human respiratory syncytial virus (HRSV) is an enveloped, single-stranded, negative-sense RNA virus and a member of the recently defined Pneumoviridae family, Orthopneumovirus genus. RSV is classified into two major A and B groups, according to antigenic and genetic analysis (4, 5). It initially described as a significant pathogen in pediatric populations and the most common cause of moderate to severe respiratory infection in infants, young children, military recruits receiving training, older people, and immunocompromised patients (6, 7). RSV is responsible for about 34 million infections, with up to 200,000 deaths annually worldwide (8, 9). Generally, RSV is responsible for up to 90% of episodes of bronchiolitis and up to 50% of pneumonia in human (10).

Studies indicate that RSV is a global pathogen that will become critical in developed countries with elderly populations. Clinical symptoms are similar to that of other viral respiratory infections; also, lower respiratory tract infectionis common, often resulting in respiratory failure (8-13%) or death (2-5%) (11), especially in premature infants, as well as those with chronic lung and congenital heart disease (12, 13). RSV has a global distribution, with yearly outbreaks of disease in late fall, winter, or spring. The infection incidence of infants can be as high as 50%. more than 90% of young children are infected by the virus at first five years of age (14). For older adults, the annual attack rates of RSV reported in the USA range from 5-10% (11).

Unfortunately, immunity into either strains of RSV is incomplete because of the interference with the host's immune, and infections in children and adults recur throughout life (15-17). A possible deficiency in IgA memory, particularly in childhood while IgA appears to provide crucial protective immunity, may cause to recurrent infections. In contrast, in aging patients, it is a deduction in circulating serum neutralizing antibodies, predisposing them to RSV infection (18).

There are no clear instructions for the treatment of RSV except supportive care; there is no licensed vaccine product. Palivizumab is the only approved intervention to prevent RSV infections. It is a monoclonal antibody against the RSV fusion protein recommended only for the prevention of acute lower respiratory tract disease caused by RSV in preterm infants and children at high risk of RSV infections (19, 20). The estimated average 2016-2017 seasonal cost of palivizumab treatment ranged from $3221 to $12,568 (21). No licensed vaccine currently exists for RSV infection. However, 14 candidate vaccines are being tested in clinical trials now (4, 22).

This study aims to assessment the prevalence of RSV genome among military trainees in their early twenties just admitted to the Iran military training camp. Subjects with acute respiratory infection outcomes are prospectively observed for molecular epidemiology of RSV during the military basic training period.

MATERIALS AND METHODS

Subjects and sample collection. This study plans to describe the prevalence, clinical features and molecular epidemiology of RSV in respiratory infections in Military trainees in Tehran, Iran. Four hundred respiratory specimens (including throat swabs) were taken from troops stationed in military barracks with clinical symptoms such as the oral temperature of ≥38°C, sore throat, cough and runny nose or recently infected with acute respiratory disease in winter season from January to March 2017. Prior history of chronic respiratory disease such as asthma, chronic obstructive pulmonary disease (COPD), recent antiviral therapy, and military personnel were the exclusion criteria of this study. Specimens were transported to the laboratory in viral transport media under the cold chain conditions and stored at -70°C until the start of the experiment.

RNA extraction and cDNA synthesis. Viral RNA was extracted from collected specimen (200 µl) using viral RNA extraction kits (Roche Applied Science, Mannheim, Germany). Before cDNA synthesis, RNase-free-DNase I was used to remove DNA, and agarose gel electrophoresis for 18S and 28S rRNA was used to evaluate RNA quality. Then viral RNA was immediately used in cDNA synthesis (8.2 µl) and

the rest was stored at -80°C for later examination. RNA was reversely transcribed into cDNA using first-strand cDNA synthesis kit (Thermo Scientific RevertAid) and random hexamers according to the manufacturer's instructions. The concentration and purity of nucleic acids were evaluated using the Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). 0.1 ng to 5 µg of RNA was used to generate cDNA as the initial step of the two-step RT-PCR protocol.

2 µl synthesized cDNA was subsequently was used in PCR reaction. Gene Runner software and sequences, available in GeneBank (with Accession number KP317928.1, KF246618.1 for RSV-A and KY249674.1 for RSV-B), were used to design the primers. For the RSV-A PCR, two oligonucleotides (forward primer [ACAAACCACCAAACAAACCC] and reverse primer [GTTATGACACTGGTATACCAACC]) were used as primers enclosing a 599 or 689 or 761-bp fragments of the G protein coding region of the RSV genome. For the RSV- B PCR, another set of oligonucleotides (forward primer [ACAAACCAAAGGCAGAACCTCTA] and reverse primer [GATGCTGTGGGTGTCTGTGT]) were applied as primers enclosing a 513-bp fragment. The specificity of each set of primers used in this study was investigated.

The PCR mixture (25 µl) contained 2.5 U *pfu* polymerase (Fermentas Life Sciences, Vilnius, Lithuania), 0.2 µM each primer, 10× buffer, 200 µM each dNTP and 2.5 µl cDNA. Acording to optimized PCR program the mixture in the microtubes were initially denatured at 95°C for 5 min and then subjected to 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The final extension step was carried out at 72°C for 5 min. Amplicons were separated on 2% agarose gel electrophoresis with TAE buffer and Safe DNA Gel Staining for visualization of DNA and 100-bp DNA ladder (marker XIV [Roche Applied Science, Mannheim, Germany]) as molecular weight marker.

Nucleotide sequencing and phylogenetic analysis. The PCR products of the HRSV virus G gene were sequenced, and the results compared to Reference Sequences via Clustal X Software Version (Version 1.83) and analyzed using BioEdit and Treecon software to draw a phylogenetic tree. This program has Kimura Nj method with bootstrap value 1000 Rep that a phylogenetic tree with bootstrap above 70%, is reliable.

RESULTS

A total of 400 military trainees with clinical symptoms of respiratory infections were entered in the study. All subjects were male, with an age range of 20 years. The most frequent blood group of participants were A (32.25%) and O (34.75%) and the lowest frequency belongs to the AB blood group (8%). From the Clinical symptoms, patients with sorethroat (n=233; 58.25%) were the most frequent, followed by cold (n=41; 10.25%), cough (n=33; 8.25%) and rhinorrhea (n=10; 2.5%), respectively (Table 1).

Using specific primers designed for RSV-A and RSV-B types, a polymerase chain reaction was performed on all samples of the population and the PCR amplicons were separated in 2% agarose gel electrophoresis. Each amplicon generated a sharp band at 599, 689 or 761-bp for RSV-A and 513-bp for RSV-B types, respectively (Fig. 1).

Among 400 specimens 11 (2.75%) were detected RSV positive by RT-PCR method. Of them, 10 (90.9%) were RSV-A and 1 (0.9%) was RSV-B. Comparison between symptoms and RSV-A positive individuals showed that 80% of people with sorethroat had the same symptoms ($p<0.05$), and 10% had a sore throat with cold and the remaining 10% had cold ($p>0.05$). In RSV-B positive sample, only sore throat was observed (Table 1).

Regarding the presence of individuals inside and outside of the military camp, the ratios were close

Table 1. Frequency of clinical symptoms of disease (n=400)

Symptoms	Frequency n (%)	RSV Positive	
		Type A n (%)	Type B n (%)
Fever>38.5°C	1 (0.25)	-	-
Cough	33 (8.25)	-	-
Rhinorrhea	10 (2.5)	-	-
shortness of breath	4 (1)	-	-
Sore throat	233 (58.25)	8 (18.6)	1 (2.3)
Sore throat/Cough	6 (1.5)	-	-
Humor/Cough	3 (0.75)	-	-
Sore throat/Cold	38 (9.5)	1 (3.8)	-
Sore throat/Headache	4 (1)	-	-
Sore throat/Rhinorrhea	3 (0.75)	-	-
Cough/Sorethroat	22 (5.5)	-	-
Cold	41 (10.25)	1 (2.4)	-
Sore throat/Fever	2 (0.5)	-	-
Total	400	10 (2.25)	1 (0.25)

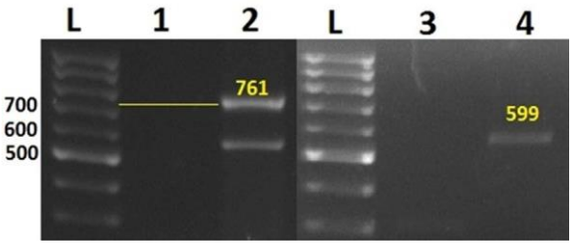

Fig. 1. RT-PCR products for the presence of RSV in the studied samples. Lanes 2 and 4 have 761 and 599 bp bands which are RSV positive. Negative controls (non-template control (NTC)) were always negative (lanes 1 and 3). A 100 bp ladder as DNA size marker (lane L).

to one another, with 51.2% being in addition to the outside of the camp and 48.8% having a permanent presence in the collection. In this study, it was found that 70% (n=7; 3.6%) of most of the RSV-A positive individuals were from individuals inside the camp ($p<0.05$) and 30% (n=3; 1.5%) included people outside the camp. A positive RSV-B sample was also for those who travelled outside the camp (Table 2).

Comparison of the mean age and RSV-A infection showed that patients under 19 years had a frequency of 30% of patients (n=3), and 20 to 21 years 40% (n=4), 41 to 51 years 10% (n=1) and over 51 years 10% (n=1) respectively. A positive RSV-B sample was in average age between 20 to 21 years old (Table 3). There was not any significant relationship between different variables and presence of RSV infection.

Phylogenetic analysis of sequencing data of RT-PCR products revealed that strains Number 36 and 19 isolated from the studied samples were more consistency with those from the Philippines and the US strains. The rest of the strains identified are more similar to sequences isolated from the US (Fig. 2).

Table 2. The relationship between the presence of patients inside and outside the garrison and types of RSV virus

Residency	RSV-A		RSV-B	
	Negative	Positive	Negative	Positive
Inside	188 (96.4%)	7 (3.6%)	195 (100.0%)	0 (0.0%)
Outside	202 (98.5%)	3 (1.5%)	204 (99.5%)	1 (0.5%)
Total	390	10	399	1

Table 3. Relationship between age range and RSV type A and type B prevalence

Age Category	N	RSV-A		RSV-B	
		Negative	Positive	Negative	Positive
<19	78	75	3	78	0
20-21	211	207	4	210	1
21-31	97	96	1	97	0
31-41	4	4	0	4	0
41-51	8	7	1	8	0
>51	2	1	1	2	0
	n=400	n=390	n=10	n=399	n=1

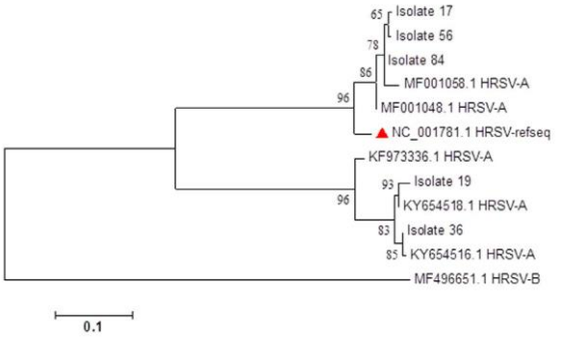

Fig. 2. Phylogenetic tree drawn with maximum likelihood method for some positive samples. The red triangles represent the reference sequences taken from the GeneBank. The numbers in each branch indicate how close the branches are to each other.

DISCUSSION

RSV is a common cause of respiratory disease and often results in outbreaks in young and adults, particularly military trainees (23, 24). RSV was first identified as a potential military-relevant pathogen in 1959 among recruits at Fort Ord, CA (25). Later, in the investigation by Dutch military researchers from 1967 to 1968, RSV was identified in 3% of radiographically confirmed cases of pneumonia (26). US military researchers also identified serologic association of RSV infection in 9% of 97 soldiers with sustained acute undifferentiated febrile illness after adenovirus (21%) as a cause of such diseases (27).

In the Naval Health Research Center (NHRC)-led surveillance of recruits from 2011 to 2013, RSV was found to be an important pathogen together with other respiratory viruses, accounting for upwards of 6% to 8% of febrile respiratory illnesses (FRIs) in the winter (28). RSV was also reported to cause

FRIs among 10% to 14% of US Army recruits at Fort Benning, GA and 14% of FRIs among Royal Navy recruits in the United Kingdom (1, 3). In troops stationed in Iraq and Afghanistan (2004 to 2007), asymptomatic infections were common, affecting 46% of nonimmune persons (29).

In the study of Matthew K et al. on the etiology of acute respiratory illness in Royal Navy trainees in 2001, among 54 Royal Navy recruits with respiratory symptoms, adenovirus, influenza viruses and RSV was identified in 35%, 19%, and 14% respectivily (3). Park WJ et al. showed that the seroprevalence of RSV IgG in Basic Training for the Republic of Korea Air Force was 98.4% among Healthy Young Adults (30).

RSV has the potential to spread rapidly under conditions of overpopulation or deficient personal sanitation, e.g., military camps (1, 3). The mean incubation period for RSV is almost five days; the period varies from 3 to 7 days (31). In most cases, in the military trainees, RSV infection causes low-grade fever, nonproductive cough, pharyngitis, sore throat, an acute onset of coryza, nasal congestion, and wheezing in >60% of patients with the lower respiratory tract (1, 3). Other symptoms, including acute bronchitis, influenza-like illness (ILI), pneumonia, severe asthma, and chronic bronchitis, are also observed in adults with RSV infection (32). In the present study, 80% of RSV-A positive individuals had a sore throat ($p<0.05$), 10% had a sore throat with colds, and the remaining 10% had colds.

Mehta J et al. indicated race, sex, age, smoking, exposure to children, home oxygen and steroid utilization, instrumental activities of daily living (IADL) rates, and underlying diseases as the main risk factors for RSV illness in chronic obstructive pulmonary disease (COPD). Also, congestive heart failure was the only important risk factor for developing RSV infection among adult patients with COPD (24).

RSV infections occur especially in a seasonal epidemic, mostly in the fall and winter (33, 34). Studies have shown that rainfall (relative humidity, especially during November or December) and temperature are the two main meteorological parameters separately associated with RSV activity in hospitalized children with acute lower respiratory infections (ALRIs) (35-37). The present study shows that the prevalence of RSV in Iran is similar to other temperate regions of the world with rainy and cold seasons.

The study of Laurel M et al. on the British Army recruits showed that more than one-third of participants do not meet sleep duration recommendations while training (7-9 h per night). Moreover , those who described sleeping less than six h per night were four times more probably to be diagnosed with an upper respiratory tract infection (URTI) and missed more training days due to URTI. Since sleep restriction is considered a essential part of military training, it can disturb the immunity and host defense (38). Korzeniewski et al. studying soldiers of the Polish Military Contingent deployed to Iraq and Afghanistan, showed the prevalence of respiratory infections to be nearly related to the environmental factors, such as sand and dust storms, extreme temperature changes, poor sanitary conditions and common neglect of basic principles of infection prevention (39). The main factors associated with increased respiratory infection among deployed US military in Iraq and Afghanistan described were the female sex, advancing age, higher-ranking, lack of well latrine facilities, navy branch of service, and deployment to Operation Iraqi Freedom (OIF) (40).

Long-term molecular epidemiological studies of HRSV in consecutive years showed the variation of common genotypes of this virus between subgroups A and B. For example, a study in 2007 in Belgium for ten successive epidemic seasons showed that subgroup A was predominant for two consecutive years. Then, subgroup B became dominant for third year and this period is being repeated every 10-year in Belgium (41).

A study by Marietjie Venter et al. on Subtyping of RSV strains in hospitalized children in South Africa showed that PCR amplicons for G protein significantly reduced in RSV-B strains, lacking nearly the entire G protein ectodomain in 2 out of 209 clinical specimens (one HIV-positive and one HIV-exposed child hospitalized with pneumonia) screened over four years (42). According to the study of Tabatabai et al. on immunocompromised patients with prolonged RSV-A shedding, the G gene sequencing in two patients, with particularly prolonged viral shedding, revealed the appearance of mutations, leading to premature stop codons (37 and 70 amino acids truncated) in the G gene (43).

In the present study, we used specific primers for amplification of RSV-A and RSV-B G gene via RT-PCR. Positive products were carried out for direct sequencing. Genotype differences were discovered by sequence alignment and phylogenetic tree construction.

Based on our results RSV was identified more in 20-21 age group (38%) and low in over 46 years. These results were in agreement with those of Wenther et al. who reported the most prevalent RSV virus in the age group of 15 to 20 years (44).

The present study showed that the highest prevalence of RSV among the blood groups was related to those of A and O, somehow similar to the results of the study by Parveen et al. (45) and Lu et al. (46), reporting the highest frequency of RSV in the blood group O with 43% prevalence.

In the current work, the prevalence of RSV-A among patients with respiratory symptoms was 2.5% and higher than RSV-B, similar to the previous studies; however, the target populations of these studies were different. Rezai et al. studied the genotypes of the RSV in children under five years old with acute respiratory infection in Iran in 2011. They showed that A genotype (GA1 and GA2) were more prevalent than genotype B (47). Salimi et al. in a meta-analysis study on the prevalence of the RSV circulating in Iran, showed that genotypes GA1, GA2, GA5, and BA co-circulated in Iran in 2007-2013 (48).

The lower prevalence of B subtypes than subtype A could be due to genetic alterations and lower mutations in subtype B strains than subtype A, leading to reduced viral immunity and lower prevalence. Studies have shown that people infected with subgroup B viruses have more stable immunity to subgroup A strains, which reduces the prevalence of these viruses (49). However, in some studies, RSV-B has been shown to be the most prevalent type of virus (50). Phylogenetic analysis reveals that the positive strains in the studied samples are very similar to the strains registered in GenBank from the US and the Philippines.

To sum up, the results of molecular epidemiology in Iranian military trainees with respiratory infection show that most of the infections are due to the presence of RSV, and most of the contaminations are also related to RSV-A. Given the limited volume of positive samples taken in the first six months of the year, similar and broader studies are suggested to be conducted in the second half of the year, which is the predominant season for these diseases. Also, sampling in different military barracks from four geographical areas of Iran to determine the exact prevalence and probable origin of the strains present in Iran is essential. Although advances in molecular diagnostics have facilitated rapid identification of RSV infection, development an effective antiviral druges and vaccines remain a considerable medical necessity.

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