ORIGINAL ARTICLE

Analysis of genetic variability of respiratory syncytial virus groups A and B in Kuwait

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Abstract

Respiratory syncytial virus (RSV) is the most frequently identified viral agent in infants, children, and elderly people with acute respiratory tract infections (ARTIs). This study is the only one of its kind in Kuwait, and its purpose was to investigate the genetic variability of the G protein gene in RSV strains prevalent in Kuwait. Respiratory samples were collected from patients with ARTIs in various hospitals in Kuwait and subjected to reverse transcription PCR (RT-PCR) amplifying a fragment of the G gene of RSV. A total of 305 samples were collected between January and mid-December 2016, and 77 (25.2%) were positive for RSV. Group A viruses were predominant over group B viruses; the RSV-A group was detected in 52 (67.5%) of the positive samples, while the RSV-B group was detected in 25 (32.5%) of the positive samples. Phylogenetic analysis showed that all RSV-A strains grouped into eight clusters of identical sequences of untyped strains. Twelve RSV-B strains, on the other hand, belonged to the RSV-B/BA10 genotype, while the rest were untyped. These data suggest that new and untyped strains of RSV-A group likely predominated in Kuwait and that the BA10 genotype of the RSV-B group became the dominant genotype in the 2016 season.

Introduction

Respiratory syncytial virus (RSV) is the major pathogen of lower respiratory tract infections (LRTIs), which can be severe in infants, young children, and the elderly [1]. RSV infection is associated with a high rate of morbidity and mortality in children aged < 5 years with acute lower respiratory infections (ALRIs) in both industrialised and developing countries [2–4]. RSV also is an important virus in adults, causing 25% of lethal respiratory infections in cold seasons, similar to the rate for seasonal influenza [5]. Globally, studies have shown an RSV-associated mortality rate of about 26.5 cases per 100,000 persons/year among adults aged > 65 years [6].

RSV is a member of the genus *Orthopneumovirus*, family *Pneumoviridae*. It has a nonsegmented, negative-sense RNA genome of approximately 15,200 nucleotides encoding 11 structural and non-structural proteins [7]. Of these proteins, the G protein, a type of II attachment glycoprotein of about 300 amino acids in length, is the most variable and is responsible for the differences between RSV genotypes. The G protein has two hypervariable regions, and the C-terminal region (the second hypervariable region) contains strain-specific epitopes [7–9]. According to the reactivity of RSV with monoclonal antibodies against G glycoprotein, the virus is divided into two major antigenic groups: group A and group B [10, 11]. The RSV-A group is further subdivided into 11 genotypes, GA1-GA7, SAA1, NA1-, NA2 and ON1 [12–14], and the RSV-B group is subdivided into 23 genotypes: GB1-GB4, SAB1-SAB3, SAB4, URU1, URU2, BA1-BA12, and THB [12, 15–21]. This strain variation allows the virus to evade the host's immune responses, which contribute to the capability of the virus to cause frequent re-infections and epidemics and creates a challenge for vaccine development

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Table 1 RSV group A G protein gene GenBank Reference Sequences

Strain	GenBank accession no.	Strain	GenBank accession no.
A2	M11486	sal/173/99	AY472094
NY108	AF2339171	AL/9452-2	AF233901
DEL/2 W/04/A	DQ248923.1	NY20	AF233918
LLC235-267	AY114149.1	SA99V1239	AF348808
MO16	AF33913.1	WUE/16397/12	JX912364
TX68481	AF233920.1	MO02	AF233910
NY/CH09	AF065254.1	Cam2008-7262	JN119952.1
DEL/138/021A	DQ248894.1		

Table 2 RSV group B G protein gene GenBank Reference Sequences

Strain	GenBank accession no.	Strain	GenBank accession no.
WV10010	M73541.1	NG-147-02	AB603480
CH93-9b	AF065251	BE/210/03	AY751111
NY97	AF233932	NG-095-05	HM459865
BA/3018/98	AY672691	NG-046-06	AB603478
Ken/2/03	AY660684	CU2011/205	KC342347
SA99V800	AF348821	BJ/20240	KC297426
Mon/7/99	AY488800.1	RSV/PUNE/ NIV0931702/09/B	KF246586
Cam2009-2145	JN119987	CU2011/81	KC342343
Mon/7/01	AY488804	SA98V602	AF348824.1
Mon/6/01	lY488803	TX69208	AF233933.1
BA/770/02	DQ227373	SA0025	AF348825.1
NG-010-09	HM459857	SAQ98V192	AF348811.1
MAD/3743/02-03	GQ150691	AL19734-4	AF233924.1
BA/524/04	DQ227396		

[22]. Therefore, it is of great importance to identify the genotypes circulating in the population.

In Kuwait, information regarding the molecular epidemiology of RSV is lacking. The present study is the first one to investigate the genetic diversity and patterns of circulating genotypes of groups RSV-A and RSV-B collected from hospitalised patients with RTIs in comparison with other RSV genotypes circulating worldwide. This information will be beneficial for RSV vaccine development and implementation.

Materials and methods

Study population and sample collection

Retrospectively, we analysed patients admitted to different hospitals in Kuwait between January and mid-December 2016 with clinical symptoms of acute upper or lower RTI. Different respiratory samples were collected in the hospital from the patients, including nasopharyngeal aspirates/washes, tracheal aspirates, and throat swabs. The collected samples were first screened for respiratory viruses, including RSV, using multiplex real-time PCR at the Virology Unit, Faculty of Medicine, Kuwait University. RSV-positive samples were stored frozen at -70 °C for further molecular analysis by specific RSV PCR and phylogenetic analysis after nucleic acid sequencing. Detailed clinical data of the patients could not be obtained from their medical records.

Nucleic acid extraction and diagnosis of RSV infection

Viral RNA was extracted from clinical samples using a Roche ®MagNA Pure LC System (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions with the addition of RNase inhibitor (Applied Biosystems, USA) after extraction. Extracted RNA from all samples was tested for RSV and other respiratory viruses using a multiplex real-time PCR assay. This assay can detect influenza A virus, influenza A (H1N1) swl virus, influenza B virus, human rhinovirus (HRV), human coronavirus NL63 (HCoV-NL63), HCoV-229E, HCoV-OC43, HCoV-HKU1, parainfluenza virus (PIV) types 1, 2, 3, and 4, human

metapneumovirus (HMPV) A/B, bocavirus, respiratory syncytial virus (RSV) A/B, adenovirus (AdV), enterovirus, parechovirus, mycoplasma pneumonia, and an internal control, using a Fast Track Kit (Fast Track Diagnostics, Luxembourg). This PCR assay is a routine diagnostic test for the detection of respiratory viruses performed at the Virology Unit, Faculty of Medicine, Kuwait University.

RT-PCR for the G protein gene of RSV

A One-Step RT-PCR Kit (QIAGEN, GmbH, Hilden, Germany) was used to synthesise cDNA from the extracted RNA according to the manufacturer's instructions. The second hypervariable region of the G protein gene of RSV was amplified using external and semi-nested PCR. External PCR was performed using the forward primer ABG490 and the reverse primer F164. Semi-nested PCR was performed using the forward primer AG655 for group A and forward primer BG517 for group B along with reverse primer F164 as described previously [23]. PCR products of 450/585 and 645 bp for group A/B and BA viruses, respectively, were analysed by 1% agarose gel electrophoresis.

DNA sequencing

The PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions. Subsequently, the purified products were sequenced in the forward and the reverse directions using the forward primer AG655 for group A and BG517 for group B, together with the reverse primer F164. Sequencing was performed using an ABI 3500/3500xL Genetic Analyzer (PE Applied Biosystems Inc., Foster City, CA, USA) with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems Inc., USA).

Phylogenetic analysis

The obtained nucleic acid sequences of the second hypervariable region of the G protein gene of RSV were edited manually and aligned with the available RSV sequences from the GenBank database (https://www.ncbi.nlm.nih.gov/) using the Clustal W method in MEGA software version 7 [24]. Phylogenetic trees were constructed using the neighbourjoining method, and bootstrap values with 1,000 replicates were calculated in MEGA software version 7. Pairwise distances between and within the genotypes of groups RSV-A and RSV-B at the nucleotide level were calculated using the Tamura-Nei Model. Reference strains representing each known genotype of RSV group A and group B were retrieved from GenBank and included in the trees. Fifteen published reference sequences of RSV group A and 27 reference sequences of RSV group B were used for comparison

to the sequences obtained in this study. Online Resources 1 and 2 contain the GenBank accession numbers of RSV group A and RSV group B reference sequences used in the current study.

Results

Detection of RSV

Between January and mid-December 2016, a total of 305 respiratory samples from hospitalised infants, children and adults were analysed for respiratory viruses by multiplex real-time PCR, resulting in 77 (25.2%) RSV-positive samples. Among the RSV-positive patients, 44 were females, and 33 were males, and their age ranged from five days to 79 years (median, two months). All RSV-positive samples were sequenced and further analysed to determine their genotypes. Of the viruses identified in the RSV-positive patients, 52 (67.5%) were subgrouped as RSV-A and 25 (32.5%) as RSV-B. Coinfections with RSV and other respiratory viruses were detected; RSV and rhinovirus were detected in 15 (19.5%) patients; RSV, enterovirus and parainfluenza virus type 4 were detected in one (1.3%) patient; and RSV and parainfluenza virus type 3 were detected in one (1.3%) patient. The occurrence of RSV infection in the various age group, as shown in Figure 1, was as follows: < 12 months, 51 (66%); 1-5 years, 13 (17%); 6-13 years, 2 (3%); 14-28 years, 1 (1%); 28-60 years, 2 (3%); > 60 years, 8 (10%). The seasonal distribution of RSV-A and RSV-B in Kuwait is shown in Figure 2, with the peak of positivity being detected in January 2016 and the lowest positivity detected in February 2016.

Phylogenetic analysis

The sequences from 46 RSV group A and 22 group B Kuwaiti strains collected from the patients were included in a phylogenetic analysis, which revealed that the 46 RSV-A strains were untyped and did not belong to any of the known group A genotypes (Fig. 3A). Among these untyped Kuwaiti strains, 18 (39%) were found to form eight clusters with identical sequences, with two to three sequences per cluster, while the remaining 28 (61%) strains were sporadic single strains (Fig. 3A). The divergence between prototype strain A2 and the Kuwaiti strains ranged from 6% to 22% at the nucleotide level, while differences of up to 19% were observed among all group A Kuwaiti strains. Moreover, strains in clusters 5 and 6 were genetically closer to the ON1 reference strain than strains in other clusters; the differences in the nucleotide sequence between strains in clusters 5 and 6 and ON1 ranged from 0.5% to 3%, while the differences in the nucleotide sequences between strains from other

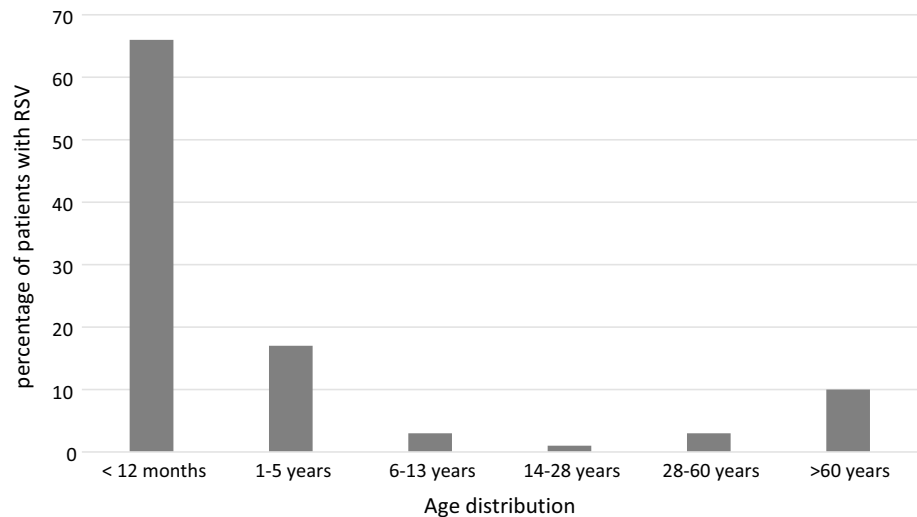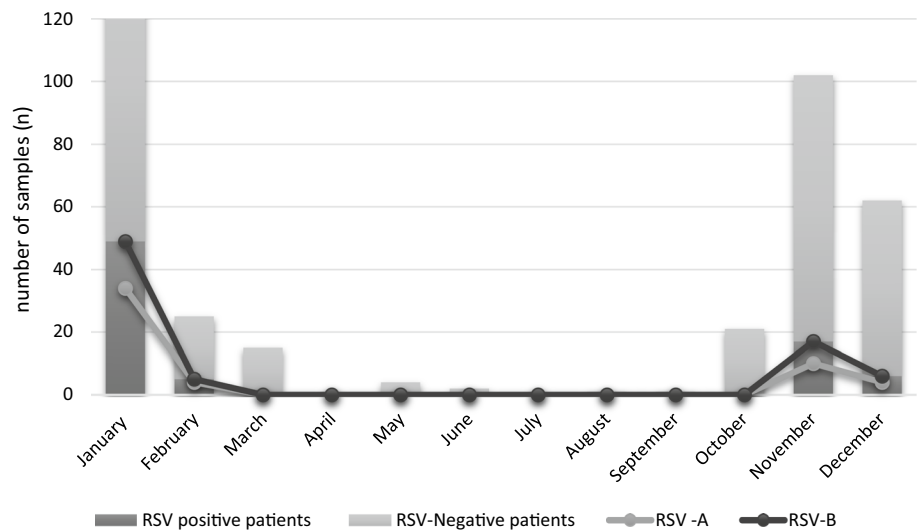**Fig. 1** Age distribution of RSV-positive patients

Fig. 2 Seasonal trends of subgroups RSV-A and RSV-B in patients with acute RTI in Kuwait from January to mid-December 2016

clusters and ON1 ranged from 0.5% to 16%. Furthermore, the KW-827 and KW-1411 strains were genetically closer to the NA1 reference strain than other Kuwaiti strains. The nucleotide sequence difference between the NA1 reference strain and the KW-827 and KW-1411 strains was 3% and 2%, respectively. However, the differences in the nucleotide sequences between the NA1 reference strain and the rest of Kuwaiti strains ranged from 16% to 19%.

The 22 RSV group B Kuwaiti strains clustered into four groups. Twelve (55%) strains clustered with the BA10 genotype with bootstrap values of 65% to 85%, and the divergence in the nucleotide sequences ranged from 0% to 4% between the Kuwaiti strains and BA10 references strains. The remaining 10 (45%) Kuwaiti strains were clustered into three groups of untyped strains that do not belong to any of the known group B genotypes (Fig. 3B). Among these clusters, cluster 2 contained three identical sequences and one

divergent strain with nucleotide sequence differences ranging from 0.4% to 1%. Moreover, there was 3.5% to 7% divergence at the nucleotide level between the Kuwaiti strains and the prototype BA strain, while up to 7% differences in the nucleotide sequences were observed among all group B Kuwaiti strains.

Discussion

RSV is the most common viral pathogen causing acute lower respiratory tract infections in infants, children and older people. The present study is the first to investigate the frequency and genetic variability of RSV group A and group B in patients with ARTI in Kuwait. A total of 305 samples from ARTI patients, including infants, children and adults, were collected, and 77 (25.2%) of the patients

Fig. 3 Phylogenetic tree for RSV-A (A) and RSV-B (B) nucleotide ▶ sequences from the second variable region of the G protein gene, constructed using the neighbor-joining method in MEGA version 7. Solid triangles indicate the sequences of the reference strains. Details for the reference GenBank sequences used for phylogenetic analysis are given in Table 1 and Table 2 for RSV-A and RSV-B strains, respectively. The scale bar indicates the proportion of nucleotide substitutions, and the numbers at the branches are bootstrap values determined for 1,000 repetitions. Bootstrap values greater than 50% are shown at the branches. The evolutionary distances were computed using the Tamura-Nei method and are in units of base substitutions per site

were found to be positive for RSV by multiplex real-time PCR. Our results, which demonstrated the predominance of the RSV-A group over the RSV-B group in the clinical samples, are in agreement with the findings of the majority of the studies on the epidemiology of RSV genotypes [23, 25–30]. We found that most of the patients had RSV single infections, while 17 (22%) had mixed infections of RSV and other respiratory viruses. Furthermore, we found that coinfections with RSV and rhinovirus were the most commonly detected mixed infections among the patients – a finding consistent with those of previous studies [31–34]. These coinfections may influence the severity of respiratory disease in patients with RSV infection. Calvo *et al.* showed that coinfection with RSV and other respiratory viruses did not result in greater severity of disease, but did result in mixed clinical features [31]. Goka *et al.* on the other hand, demonstrated an increased risk of admission to the intensive care unit (ICU) and death as a result of coinfection with RSV and other respiratory viruses [35]. In this study, however, we were unable to investigate the association between RSV groups or coinfections on the severity of the RSV respiratory diseases due to the lack of clinical data for the patients.

Since little is known about the distribution of RSV infection in different age groups, in this study we analysed the age distribution of the patients with RSV infections. Our results showed that infants less than one year of age represented that largest proportion of patients with RSV infections (66%), followed by children between one and five years old (17%). The prevalence decreased to 3% in children between six and 13 years old and further decreased to only 1% in children and adults between 14 to 28 years old. Many other studies have also demonstrated a high incidence of RSV infection and disease among infants less than one year old [36, 37]. a recent epidemiological meta-analysis study implicated RSV as one of the most significant pathogens of early childhood and placed it as the second-most-likely single pathogen to cause death in children less than one year of age [38–40]. Although there is evidence that maternal antibodies in infants are likely to be important in protection against RSV disease, severe disease was noted in children under six months of age, indicating that maternal RSV-specific antibodies are protective during the first 6-7 months, but
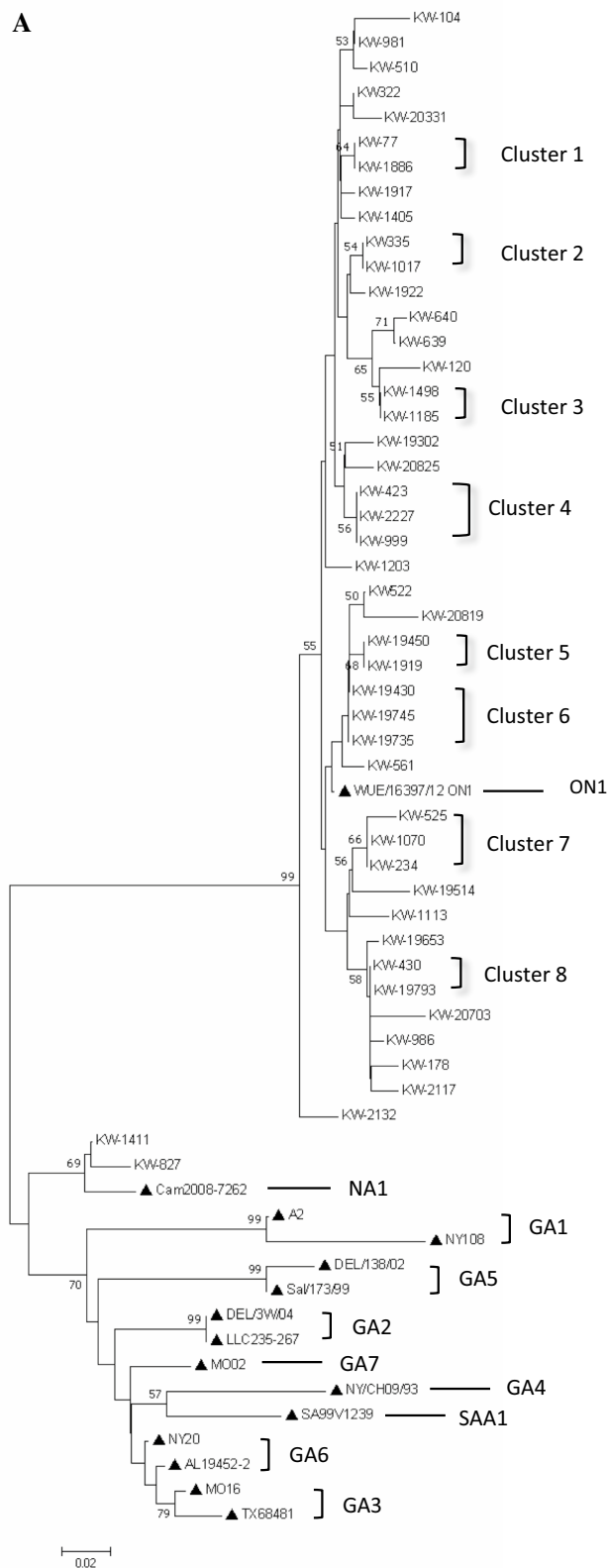
this protection may then wane [41]. There are other risk factors known to predispose infants to RSV-associated disease, including physical and immunological immaturity, which
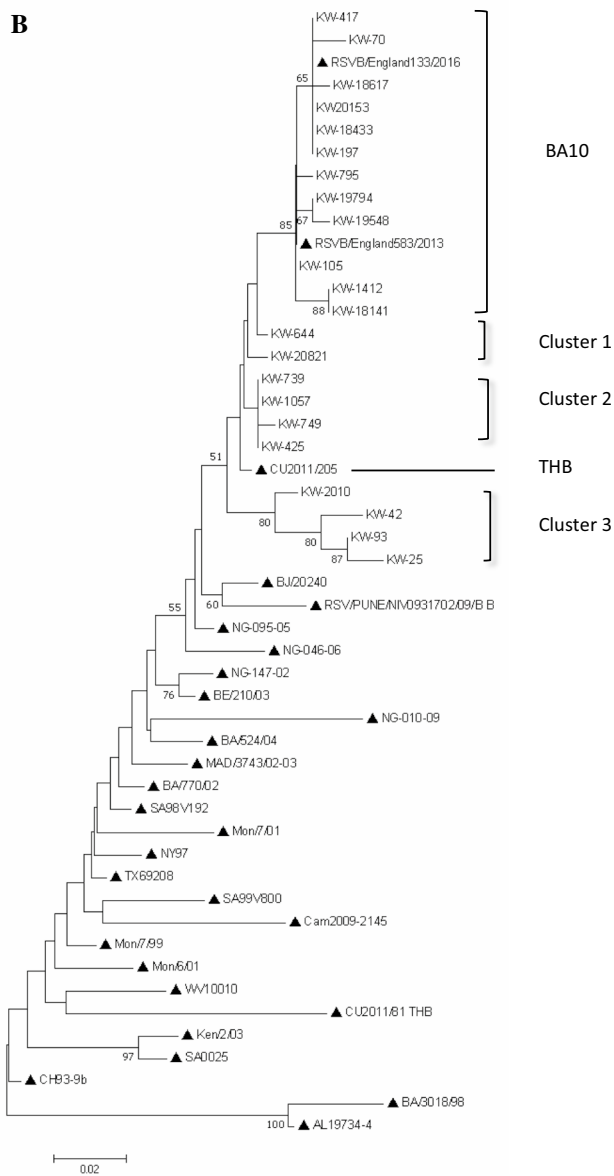
B

KW-417
KW-70
▲ RSVB/England133/2016
65 — KW-18617
KW20153
KW-18433
KW-197   BA10
KW-795
KW-19794
57 — KW-19548
85 ▲ RSVB/England583/2013
KW-105
KW-1412
88 KW-18141

KW-644   } Cluster 1
KW-20821

KW-739
KW-1057   Cluster 2
KW-749
KW-425

51 ▲ CU2011/205 ——— THB

KW-2010
KW-42
80 KW-93   Cluster 3
80 KW-25
87

▲ BJ/20240
55 60 ▲ RSV/PUNE/NIV0931702/09/B B
▲ NG-095-05
▲ NG-046-06
▲ NG-147-02
76 ▲ BE/210/03
▲ NG-010-09
▲ BA/524/04
▲ MAD/3743/02-03
▲ BA/770/02
▲ SA98V192
▲ Mon/7/01
▲ NY97
▲ TX69208
▲ SA99V800
▲ Cam2009-2145
▲ Mon/7/99
▲ Mon/6/01
▲ WV10010
▲ CU2011/81 THB
▲ Ken/2/03
97 ▲ SA0025
▲ CH93-9b
▲ BA/3018/98
100 ▲ AL19734-4

0.02

Fig. 3 (continued)

causes infants younger than three months to be the most susceptible to severe disease. Also, the size of the airway is specifically problematic in neonates and young infants because obstruction from sloughed epithelium, fibrin, inflammatory debris, and mucus is a significant factor in disease pathogenesis. In fact, the period after birth is critical in the development of the lung architecture and cellular functions, including the development of immunological effector mechanisms to deal with commensal and pathogenic microorganisms [42–44]. It has been shown that an immune system that is weak in the early years will mature sooner when children are re-infected for the second or third time [45]. Our results showed that the prevalence of RSV infection reached 10% in patients > 60 years old, whose immune response is weakened

by age. Similar observations have been made in other studies [46–50]. Researchers have found that infants infected with RSV develop maternal antibodies that recognise both the F and G proteins. However, in older infants, there is a dramatic increase in antibodies recognizing the G protein, while infection provokes only a modest increase in antibodies against the F protein. It was also shown that antibodies against the F protein continue to increase with age, whereas those against the G protein decrease [51].

RSV infection is characterised by a seasonality that may be associated with region and climate. Many studies have shown that RSV infection in non-tropical countries occurs almost year-round and peaks mainly during winter [47, 52–54], which is different from what has been reported in tropical countries [55]. In our study, however, RSV infection was detected in patients in autumn to spring (November to February) but peaked in winter (January), a result that is in coordinance with studies conducted in non-tropical countries. The cold and sometimes dry conditions of the winter season in Kuwait favour survival of RSV in the environment and encourage people to crowd together indoors, which may increase the chance of parents and older siblings acquiring RSV infection and passing it on to infants in the home. Also, the cold and relative humidity conditions in winter might delay the drying of large respiratory droplets, which could favour RSV survival and transmission, since RSV is predominantly transmitted through large droplets [56].

Interestingly, phylogenetic analysis showed that all RSV-A strains collected from the patients in this study were untyped strains that did not belong to any of the known group A genotypes in the Genbank database. Furthermore, the data showed that these Kuwaiti strains formed different clusters of identical sequences. These data indicate that there is heterogeneity among the Kuwaiti strains, which differ from the known RSV-A genotypes. One can speculate that these are new strains or that they originate through recombination of other known strains. Whole-genome sequencing of these isolates is necessary to address this question. Although they were untyped, RSV-A Kuwaiti strains in cluster 5 (Fig. 3A) were more closely related to the new RSV-A/ON1 genotype than the rest of the Kuwaiti strains according to the percentage of differences in the nucleotide sequences. RSV-A/ON1 is novel genotype that was first described by Eshaghi *et al.* in Ontario, Canada [57], and this strain later emerged and became the dominant genotype in different countries, including northern Italy, Germany, Thailand, Turkey, and Saudi Arabia [16, 19, 30, 47, 54, 58–61]. A further significant result is that the two Kuwaiti strains (KW-1411 and KW-827) were more closely related to the RSV-A/NA1 strain than other Kuwaiti strains based on pairwise nucleotide distances. The RSV-A/NA1 genotype and the NA2 genotype are new variants of A2 genotype that caused large epidemics in Japan during the 2005-2006

and 2006-2007 seasons because of the lack of immunity in the children [62]. Also, the AN1 genotype was a dominated genotype in India among children < 6 years old during the period 2009-2012 [28] and in Italy among children < 3 years old during 2006-2012 [63]. Moreover, between October and December 2014, NA1 and ON1 genotypes of group A RSV dominated in Riyadh, Saudi Arabia, among children less than five years of age with ARI symptoms [30].

In this study, the RSV-B/BA10 genotype was the predominant genotype among Kuwaiti RSV-B strains, while the rest of the Kuwaiti RSV-B strains formed three clusters of untyped strains. RSV-B/BA genotypes emerged in the late 1990s and then spread globally and became the predominant genotypes [64]. Many studies have divided members in the BA clade into 13 different genotypes. However, other studies disagree on the total number of genotypes because they use different reference sequences for the genotypes. Therefore, there is overlap between genotypes, as evidenced by a partial CDS tree [65]. In our study, in the phylogenetic tree, we used different BA reference sequences that represented BA1 to BA10, and it was easily to identify the BA10 strains in particular. Previous studies have also demonstrated the predominance of RSV-B/BA genotypes, which is in line with our findings [17, 19, 25, 66, 67]. Interestingly, some reports have shown that genotype BA had replaced other prevailing genotypes in certain places, such as Argentina and India [21, 23]. It has been speculated that antigenic changes in the G protein and the evasion of host immune responses had led to the selective advantage of genotype BA viruses over other RSV-B genotypes [21].

Conclusion

In summary, the findings of this study provide vital information regarding the frequency of RSV infection among patients of different age with ARTI in Kuwait. Sequence analysis of the circulating strains demonstrated the predominance of the RSV-A group over the RSV-B group in these patients. Additionally, the circulating RSV-A strains were untyped strains, indicating heterogenicity among group A RSV strains. On the other hand, most of the strains of the RSV-B group belonged to the BA10 genotype, showing the predominance of this genotype among patients with ARTI in Kuwait. We are firmly recommending whole-genome sequencing of the untyped Kuwaiti strains for full strain characterisation, which will be accomplished in the next project. Further surveillance and sequencing of RSV strains are required to determine if these untyped Kuwaiti strains are still circulating in this region and if the dominance of certain RSV genotypes over others is sustained over time or fluctuates from year to year. The knowledge obtained in

the study may be beneficial for the development of a future vaccine against RSV.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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