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International Journal of Infectious Diseases 90 (2020) 5–17

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Epidemiological characteristics and phylogenic analysis of human respiratory syncytial virus in patients with respiratory infections during 2011–2016 in southern China

Hong-jiao Luo[a,b,c,d], Xu-bin Huang[e], Hui-ling Zhong[a,b,c,d], Cong-xiu Ye[f], Xin Tan[a], Kai Zhou[b,c,d], Lei Yuan[b,c,d], Su-fen Zhang[b,c,d], Xun Zhu[b,c,d], Cui-ji Lin[b,c,d], Wen-jun Wang[a], Lin Xu[b,c,d,*], Kai-yuan Cao[b,c,d,*]

[a] Department of Obstetrics and Gynecology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, 510120, China
[b] Department of Microbiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, 510080, China
[c] Key Laboratory of Tropical Disease Control, Ministry of Education, Sun Yat-sen University, Guangzhou, 510080, China
[d] Sun Yat-sen University — University of Hong Kong Joint Laboratory of Infectious Disease Surveillance, Sun Yat-sen University, Guangzhou, 510080, China
[e] Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, 510080, China
[f] Department of Dermatovenereology, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, 510630, China

ARTICLE INFO

Article history:
Received 10 May 2019
Received in revised form 6 October 2019
Accepted 11 October 2019
Corresponding Editor: Eskild Petersen, Aarhus, Denmark.

Keywords:
Human respiratory syncytial virus
Respiratory virus
Subtype
Epidemiology
Phylogenic analysis

ABSTRACT

Background: Human respiratory syncytial virus (RSV) is one of the most important pathogens that cause acute respiratory infections in children and immunocompromised adults. This work was conducted to understand the epidemiological and phylogenetic features of RSV in southern China during 2011–2016.
Methods: A total of 16 024 nasopharyngeal swabs were collected from patients with respiratory infections in 14 hospitals, and screened for RSV and seven other respiratory viruses using real-time PCR. Six hundred and twenty-three RSV-positive samples from 13 hospitals were further analyzed for subtypes. G gene sequencing and phylogenetic analysis were performed based on 46 RSV-A and 15 RSV-B strains.
Results: RSV was detected in 9.5% of the 16 024 specimens, the highest among the eight respiratory viruses screened. Most of these specimens came from inpatients and children under 3 years of age. The incidence of RSV-A (9.4%) was higher than that of RSV-B (4.4%) in children (<15 years), but not in adults (0.64% vs. 0.58%). A 2-year RSV-A dominance followed by a 1-year RSV-B dominance pattern was found. The co-detection rate of RSV was 25.1%. The main prevalent genotypes were NA1, ON1, and BA9. The prevalent RSV-A genotype in 2011–2012 was NA1, close to Chongqing and Brazil, but a new Hong Kong ON1 genotype was introduced and became the prevalent genotype in Guangzhou in 2014–2015. Deduced amino acid sequence analysis confirmed the ongoing evolution and a high selection pressure of RSV-A and B strains, especially in RSV-A ON1 and NA1 genotypes.
Conclusions: This study demonstrated the molecular epidemiological characteristics of RSV in patients with respiratory infections in southern China.

Introduction

Human respiratory syncytial virus (RSV), an *Orthopneumovirus* belonging to the *Pneumoviridae* family, is one of the most important pathogens causing severe acute lower respiratory infections (ALRI) in children (Borchers et al., 2013). Studies have shown that RSV is the most common cause of hospitalization among children under 2 years of age and is associated with significant morbidity and mortality (Borchers et al., 2013; Nair et al., 2010; Shi et al, 2017). In elderly and immunocompromised adults or patients with preexisting diseases, the morbidity and mortality rates have been shown to be significantly higher in

* Corresponding authors at: Department of Microbiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, 510080, China
E-mail addresses: rora691318@163.com (H.-j. Luo), huangxubin00@163.com (X.-b. Huang), 815290239@qq.com (H.-l. Zhong), yecongxiu@126.com (C.-x. Ye), jessietansysu@163.com (X. Tan), 444096499@qq.com (K. Zhou), ourchina1995@qq.com (L. Yuan), 1060839333@qq.com (S.-f. Zhang), zhuxun8@mail.sysu.edu.cn (X. Zhu), cherie1011@126.com (C.-j. Lin), wangwenjunsys@163.com (W.-j. Wang), xulin@mail.sysu.edu.cn (L. Xu), caoky@mail.sysu.edu.cn (K.-y. Cao).

https://doi.org/10.1016/j.ijid.2019.10.009

RSV-infected patients (Colosia et al., 2017). Therefore, RSV-related respiratory tract infection is a major public health issue worldwide.

The clinical manifestations after RSV infection range from a mild upper respiratory tract infection to severe life-threatening lower respiratory tract involvement such as bronchiolitis, pneumonia, and croup, together with some common symptoms including fever, rhinorrhea, cough, and wheezing (Borchers et al., 2013), which are not readily distinguished from those of other common respiratory virus infections. Although efforts to develop an RSV vaccine for all age groups began in the 1960s, no safe and effective vaccine is yet available, and antiviral treatment for RSV infection is currently also very limited, with treatments usually being supportive and symptomatic (Colosia et al., 2017).

Although RSV has only one single serotype, it can be divided into two antigenic groups – RSV-A and RSV-B – according to the antigenicity of the G protein (Mufson et al., 1985). To date, 14 genotypes of RSV-A and 25 genotypes of RSV-B have been confirmed (Ren et al., 2015; Shobugawa et al., 2009), suggesting that genetic variability and evolution of RSV exists. However, data concerning the molecular epidemiological characteristics of RSV subtypes are limited, and there are few reports about the epidemiological status and genotypic characteristics of RSV prevailing in patients with acute respiratory infections (ARI) in southern China (Liu et al., 2016). This study was, therefore, performed to investigate the presence of RSV in pediatric (<15 years) and adult (≥15 years) patients with ARI in Guangzhou. Specimens were collected from ARI patients during 2011–2016 and RSV was analyzed along with seven other common respiratory viruses. A phylogenetic analysis of RSV-A and RSV-B strains was performed in order to better understand the molecular epidemiological characteristics of RSV circulating in southern China.

Materials and methods

Patients and samples

Nasopharyngeal swabs were collected from pediatric and adult patients with ARI from January 2011 to December 2016 in 14 hospitals covering Guangdong Province, southern China. Inclusion criteria were as follows: fever (body temperature ≥37.5 °C within 3 days, accompanied by one or more symptoms of ARI including cough, runny nose, sputum, and sore throat. Information including demographic data, case history, symptoms, and clinical results of each patient was collected simultaneously. RSV and seven common respiratory viruses were screened: influenza virus (Flu), parainfluenza virus (PIV), human metapneumovirus (HMPV), human coronavirus (HCoV), human adenovirus (HADV), human rhinovirus (HRV), and human bocavirus (HBoV).

Nucleic acid extraction and reverse transcription

Viral RNA and DNA were extracted from 200 μl nasopharyngeal swab using a QIAamp MiniElute Virus Spin Kit (Qiagen, Germany) according to the manufacturer's instructions. Reverse transcription of viral RNA was performed using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

Screening for respiratory viruses

Common respiratory viruses and RSV were screened by real-time PCR as described previously (Bonroy et al., 2007; Van de Pol et al., 2007; Xu et al., 2012; Zhang et al., 2018). The primers and probes used in the screening are listed in the Supplementary Material Table S1. The RSV-specific primers and probes (synthesized by Invitrogen, Life Technology, Shanghai, China) are listed in Table 1. To detect RSV, samples were assayed in a 25-μl reaction mixture containing 5 μl of cDNA, 12.5 μl of 2 × Universal Master Mix (Applied Biosystems, USA), 300 nM of the forward and reverse primers, respectively, and 200 nM of the probe, with the following procedure: 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. For detection of the RSV subtypes, samples were assayed in a 20-μl reaction mixture containing 2 μl of cDNA, 10 μl 2 × LightCycler 480 Probes Master (Roche, Switzerland), 100 nM of forward and reverse primer, respectively, and 100 nM of probe, with the procedure of 95 °C for 10 min and 45 cycles of 95 °C for 15 s, 55 °C for 1 min.

Table 1
Real-time PCR primers and probes used for the detection of the RSV subtypes.

Primer	Sequence (5′–3′)
RSV-F	GCTCTTAGCAAAGTCAAGTTRAATGATACA
RSV-R	GTTTTTGCACATCATAATTRGGAGT
RSV-Probe	VIC-CTGTCATCYAGCAAATACACTATCCAACGTAGCACAGG-TAMRA
RSVA-F	GCTCTTAGCAAAGTCAAGTTGAATGA
RSVA-R	TGCTCCGTTGGATGGTGTATT
RSVA-Probe	FAM-ACACTCAACAAAGATCAACTTCTGTCATCCAGC-TAMRA
RSVB-F	GATGGCTCTTAGCAAAGTCAAGTTAA
RSVB-R	TGTCAATATTATCTCCTGTACTACGTTGAA
RSVB-Probe	FAM-TGATACATTAAATAAGGATCAGCTGCTGTCATCCA-TAMRA

RSV, respiratory syncytial virus.

G gene sequencing of RSV-A and RSV-B

The RSV-positive samples were selected and further analyzed for genotypes using a nested PCR. Four pairs of primers were designed for G gene amplification, as listed in Table 2. The procedure for the first and the second round amplification of RSV-A was as follows: 95 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The following procedure was used for the first and second round amplification of RSV-B: 95 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 7 min. The PCR products were purified by DNA purification kit (Takara, China) and cloned into vector PMD19-T (Takara, China). Sequencing was performed using a dideoxy chain termination method in both forward and reverse directions by Invitrogen Co., China. All PCR products used for cloning and sequencing were from three independent reactions.

Nucleotide sequence analysis

A total of 61 strains from the years 2011–2015 were selected for G gene sequencing and phylogenetic analysis. The selection criteria were (1) the sample was available; (2) the sample was well preserved and the G gene could be successfully amplified. The G gene sequences were compared with those of the representative sequences of RSV-A and RSV-B from other countries or regions in GenBank, and were aligned by the Clustal X program. The phylogenetic tree of the RSV G gene was constructed by maximum-likelihood method with the Kimura 2-parameter model using MEGA 5.0 software. Bootstrap values were decided by 1000 replicates. The deduced amino acid sequences were analyzed with Bioedit software. Potential N-glycosylation sites (Asn–Xaa–Ser/Thr, where Xaa is not a proline) were predicted using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

Statistical analysis

IBM SPSS Statistics version 20.0 (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. The incidence was calculated as the percentage of positive samples in the indicated total number of samples. The Chi-square test or Fisher's exact test was used to

Table 2
Primers used for RSV G gene amplification[a].

Primers	Sequence (5′–3′)	Location	Size of product (bp)
RSV-A-1F	5′-GAAGTGTTCAACTTTGTACC-3′	5147–5633	487
RSV-ABR	5′-CAACTCCATTGTTATTTGCC-3′		
RSV-A-2F	5′-TATGCAGCAACAATCCAACC-3′	5175–5633	459
RSV-ABR	5′-CAACTCCATTGTTATTTGCC-3′		
RSV-B-1F	5′-AAGATGATTACCATTTTGAAGT-3′	5167–5673	507
RSV-ABR	5′-CAACTCCATTGTTATTTGCC-3′		
RSV-B-2F	5′-GTGGCAACAATCAACTCTGC-3′	5215–5673	459
RSV-ABR	5′-CAACTCCATTGTTATTTGCC-3′		

RSV, respiratory syncytial virus.
[a] Sense primer: RSV-A-F or RSV-B-F; antisense primer: RSV-ABR.

evaluate the difference between rates. The odds ratio was calculated using the risk estimate of crosstabs Chi-square test in SPSS. The trend line was drawn by moving average method. A p-value of < 0.05 was considered statistically significant.

Results

Epidemiological surveillance of RSV and seven common respiratory viruses

Among the 16 024 patients, 5818 (36.3%) were found to be positive for at least one virus and 771 (4.8%) were infected by more than one virus. The male to female ratio was 1.63 (9923/6106), and the inpatient to emergency/outpatient ratio was 2.95 (11 964/4060). Analysis of the RSV subtypes was performed on 623 RSV-positive swabs from 13 hospitals except Guangzhou Institute of Respiratory Diseases. The demographic and clinical characteristics of the 16 024 patients are shown in the Supplementary Material Table S2. The patient composition and surveillance results for RSV and the seven other common respiratory viruses are shown in Table 3. The monthly distributions are shown in Figure 1 and Supplementary Material Figure S1.

Influenza virus was prevalent mainly in the spring, especially in February to May. Similarly, the epidemic of HMPV was mainly in the spring, especially in March to May, but in 2012, the epidemic season appeared earlier in the winter. PIV was mainly epidemic in spring and autumn, especially in 2011–2012. HRV was epidemic throughout the year; however, as most of the hospitals did not test for HRV in 2011, the incidence in that year was low. HBoV was epidemic mainly in summer during 2011–2014, but in 2015–2016, the epidemic peak appeared in autumn. HCoV and HADV were prevalent throughout the year. RSV was mainly epidemic in winter and spring, but in the summer of 2011 and 2013, the epidemic level was also high (Figure 1A–H).

The age of the enrolled patients ranged from 0 days to 103 years, with a median age of 7 years. Among them, 33.8% (5419/16 024) were infants (<1 year) and toddlers (1–2 years). The age distributions of the patients with RSV and the seven other common respiratory viruses are shown in Figure 2. For most of the viruses screened, the incidence in young children (3–5 years) was much higher than that in older children (6–14 years) and adults (≥15 years), as shown in Fig. 2B–H, except for Flu, which was mostly detected in the 15–35 years group (row percentage 14.9%, Fig. 2A). PIV and RSV mostly infected infants of 0–1 year (6.5% and 27.6%, respectively), and HBoV mostly infected those in the 1–2 years group (3.5%). For HADV and HMPV, the group with the highest incidence was 4–6 years (10.6%) and 2–3 years (4.0%), respectively. Similarly, HRV and HCoV tended to infect infants, with a high incidence of 7.4% and 4.0% respectively, and also children in the 3–4 years group (5.6% and 4.3%, respectively).

Clinical and epidemiological characteristics of RSV-positive cases

Among the 16 024 samples, 1514 (9.5%) were found to be RSV-positive, the highest among the eight respiratory viruses tested. The RSV-positive patients included 1389 inpatients and 125 outpatients, and the incidence was significantly higher in inpatients (91.7% vs. 8.3%, Chi-square = 257.86, $p < 0.01$). The odds ratio of infection with RSV resulting in severe disease or admission was 4.14 (95% confidence interval (CI) 3.43–4.98). Among the 1514 RSV-positive patients, 1039 (68.6%) were male and 475 (31.4%) were female; the difference was significant (Chi-square = 31.84, $p < 0.01$).

The common symptoms of RSV infection were cough (94.8%), fever (71.7%), runny nose (35.6%), and sputum (30.5%), which were not distinguishing from those of other common respiratory virus infections. Most of the RSV-positive patients (1432 cases, 94.6%) were children, including children not in nursery (59.5%), school

Table 3
Surveillance results for RSV and seven other common respiratory viruses in acute respiratory infection patients during 2011–2016 in southern China.

Hospital group[a]	Number of cases	Number positive (incidence %)							
		Flu	PIV	HCoV	HADV	HBoV	HMPV	HRV	RSV
Outpatients	4060	609 (15.00)	117 (2.88)	78 (1.92)	175 (4.31)	17 (0.42)	72 (1.77)	114 (2.81)	125 (3.08)
Infants and toddlers	696	89 (12.79)	54 (7.76)	12 (1.72)	42 (6.03)	7 (1.01)	26 (3.74)	27 (3.88)	72 (10.34)
Young and older children	1181	169 (14.31)	41 (3.47)	20 (1.69)	100 (8.47)	5 (0.42)	32 (2.71)	40 (3.39)	28 (2.37)
Adults	2183	351 (16.08)	22 (1.01)	46 (2.11)	33 (1.51)	5 (0.23)	14 (0.64)	47 (2.15)	25 (1.15)
Inpatients	11 964	880 (7.36)	433 (3.62)	367 (3.07)	504 (4.21)	158 (1.32)	257 (2.15)	523 (4.37)	1389 (11.61)
Infants and toddlers	5659	470 (8.31)	329 (5.81)	241 (4.26)	321 (5.67)	138 (2.44)	187 (3.30)	361 (6.38)	1273 (22.50)
Young and older children	1622	206 (12.70)	46 (2.84)	57 (3.51)	137 (8.45)	9 (0.55)	39 (2.40)	73 (4.50)	59 (3.64)
Adults	4683	204 (4.36)	58 (1.24)	69 (1.47)	46 (0.98)	11 (0.23)	31 (0.66)	89 (1.90)	57 (1.22)
Total	16 024	1489 (9.29)	550 (3.43)	445 (2.78)	679 (4.24)	175 (1.09)	329 (2.05)	637 (3.98)	1514 (9.45)
Children	9158	934 (10.20)	470 (5.13)	330 (3.60)	600 (6.55)	159 (1.74)	284 (3.10)	501 (5.47)	1432 (15.64)
Adults	6866	555 (8.08)	80 (1.17)	115 (1.67)	79 (1.15)	16 (0.23)	45 (0.66)	136 (1.98)	82 (1.19)

Flu, influenza virus; PIV, parainfluenza virus; HCoV, human coronavirus; HADV, human adenovirus; HBoV, human bocavirus; HMPV, human metapneumovirus; HRV, human rhinovirus; RSV, respiratory syncytial virus.
[a] Infants and toddlers: age <3 years; young and older children: age 3–15 years; children: <15 years; adults: ≥15 years.

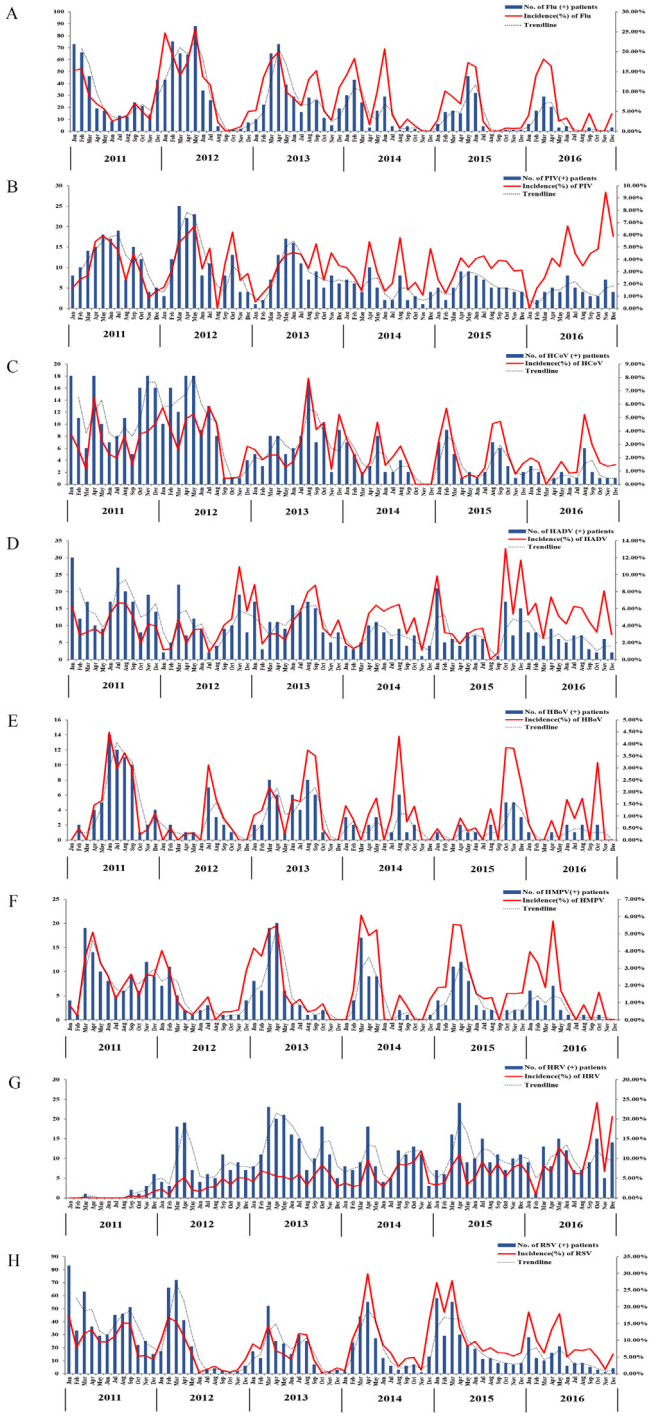

Figure 1. Monthly distribution of RSV and seven other common respiratory viruses from 16 024 acute respiratory infection patients in southern China, from January 2011 to December 2016. The monthly incidence (percentage of positive patients in the total samples for the indicated month) and the number of virus-positive cases are shown. Trend lines are indicated by dotted lines. (A) Influenza virus (Flu); (B) parainfluenza virus (PIV); (C) human coronavirus (HCoV); (D) human adenovirus (HADV); (E) human bocavirus (HBoV); (F) human metapneumovirus (HMPV); (G) human rhinovirus (HRV); (H) respiratory syncytial virus (RSV).

nursery children (17.5%), and primary and junior high school students (1.6%); the adult group were mainly retirees (1.3%). Infants and toddlers (<3 years) were the population with the highest risk of RSV infection, especially infants <1 year (Figure 2H).

RSV-positive cases were distributed in eight hospitals. Guangzhou Institute of Respiratory Health showed the highest incidence in 2011–2013, but a notable epidemic peak appeared in Zhu Jiang Hospital in 2014 (Figure 3A). The annual distributions of RSV from 2011 to 2016 in Guangzhou are shown in Figure 3B. The RSV incidence was significantly lower in 2012, 2013, and 2016, and higher in 2011, 2014, and 2015 (Chi-square = 70.06, $p < 0.01$), showing epidemic peaks in 2011 and 2014–2015 in Guangzhou, respectively.

Epidemiological distribution of RSV subtypes

Among the 1514 RSV-positive samples, 623 from 13 hospitals were further analyzed for RSV-A/RSV-B subtypes; RSV subtyping was not performed at Guangzhou Institute of Respiratory Diseases. Among the 623 cases, 394 (63.2%) were RSV-A and 196 (31.5%) were RSV-B; both RSV-A and RSV-B were co-detected in three cases (0.5%), and 30 (4.8%) failed to be determined for specimen reasons. Among the 8936 ARI patients from 13 hospitals (excluding samples from Guangzhou Institute of Respiratory Diseases), the incidence of RSV-A was 4.4% and RSV-B was 2.2%. The three cases of RSV-A and B co-detection were all children (two were 2 years old and one was 5 months old) who were inpatients with ALRIs. The RSV-A incidence was 4.7% (258/5467) in males and 4.0% (139/3469) in females (Chi-square = 2.54, $p > 0.05$). However, the RSV-B incidence was 2.5% (139/5467) in males and 1.7% (60/3469) in females, with a significant difference (Chi-square = 6.44, $p < 0.05$). Both RSV-A and RSV-B mainly infected infants and toddlers, and the incidence of RSV-A (9.4%) was higher than that of RSV-B (4.4%) in children (Chi-square = 63.46, $p < 0.01$); however there was no difference in adults (0.64% vs. 0.58%, Chi-square = 0.15, $p > 0.05$; Figure 3C).

The annual distribution trend for RSV-A was similar to that of the total RSV, with epidemic peaks in 2011 and 2014–2015 (Chi-square = 194.03, $p < 0.01$; Figure 3D). In contrast, RSV-B was predominant in 2013 and 2016 (Chi-square = 168.19, $p < 0.01$; Figure 3E), when RSV-A circulated at a low level. There was an interesting RSV year distribution trend, with RSV-A dominant in 2011 and 2012, followed by RSV-B dominant in 2013, and then RSV-A dominant in 2014 and 2015, followed by RSV-B dominant in 2016 (Figure 3B), showing a 2-year RSV-A and 1-year RSV-B dominant pattern.

RSV-A mainly circulated in winter and spring, with a peak in January to April and a lower peak in July to September (Figure 4A). RSV-B showed a 1-month stagger in epidemic peaks, with a later high peak in March to May and an earlier small peak in June to August (Figure 4B). RSV-A was the predominant subtype in most of the months except July, when RSV-B slightly exceeded (Figure 4C). On analysis of the detailed monthly distribution for 2011–2016 (Figure 5), it was found that most of the time, RSV-A and RSV-B had dominance alternately, except for January to June in 2014, when both RSV-A and RSV-B were epidemic at a high level.

Co-detection

Among the 16 024 patients, 4.8% (771/16 024) had co-detected viruses, and among the 5818 positive patients, 13.3% (771/5818) had more than one virus co-detected. Consistent with Soudani et al. (2019), RSV was the most common co-detected virus (49.3%, 380/771), followed by Flu (33.7%, 260/771), HRV (26.3%, 203/771), PIV (24.4%, 188/771), HADV (24.3%, 187/771), HCoV (22.8%, 176/771), HBoV (13.1%, 101/771), and HMPV (12.6%, 97/771). Among the 1514 RSV-positive samples, 25.1% (380/1514) were co-detected with other viruses, most commonly with Flu, followed by HRV and HADV (Table 4); 91.1% were double infections (346/380) and 8.9% were triple infections (34/380). No correlation was found between co-detection and clinical symptoms. Among the 380 patients with co-detection, 232 was diagnosed as ALRI, not statistically higher

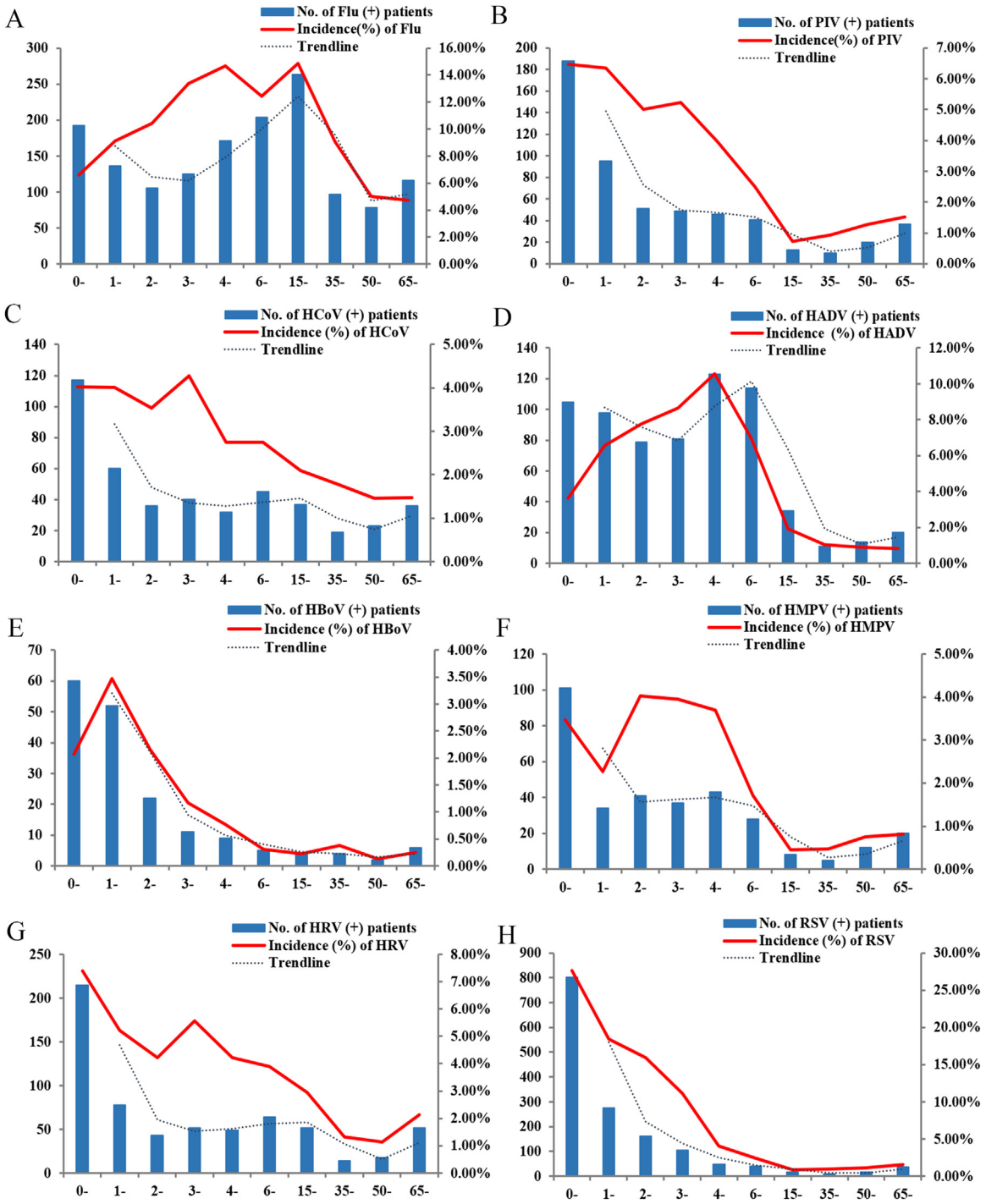

Figure 2. Age distribution of RSV and seven other common respiratory viruses from 16 024 acute respiratory infection patients in southern China during 2011–2016. The number of virus-positive patients and incidence (% of positive cases in the indicated age group) are shown. Trend lines are indicated by dotted lines. (A) influenza virus (Flu); (B) parainfluenza virus (PIV); (C) human coronavirus (HCoV); (D) human adenovirus (HADV); (E) human bocavirus (HBoV); (F) human metapneumovirus (HMPV); (G) human rhinovirus (HRV); (H) respiratory syncytial virus (RSV).

than RSV single positive patients (232/380 vs. 745/1134, Chi-square = 2.68, $p > 0.05$). The age of patients with co-detection ranged from 0 to 87 years. Children <3 years were the most likely population to have RSV as a co-detection, accounting for 77.4% (294/380) of the co-detection cases, and 44.2% (168/380) were infants (<1 year). The co-detection rates were 25.6% in inpatients (355/1389) and 20.0% (25/125) in outpatients, with no significant difference between inpatients and emergency/outpatients (Chi-square = 1.88, $p > 0.05$). However, the co-detection rate in females was 28.6% (136/ 475), which was higher than the rate of 23.5% in males (244/1039, Chi-square = 4.59, $p < 0.05$).

Phylogenetic analysis of RSV-A and RSV-B sequences

To better understand the phylogenetic characteristics of RSV in southern China, 61 RSV strains, including 46 RSV-A and 15 RSV-B strains obtained in 2011–2015 in Guangzhou, were selected for G gene sequencing. Among the 46 RSV-A strains, 24 were selected

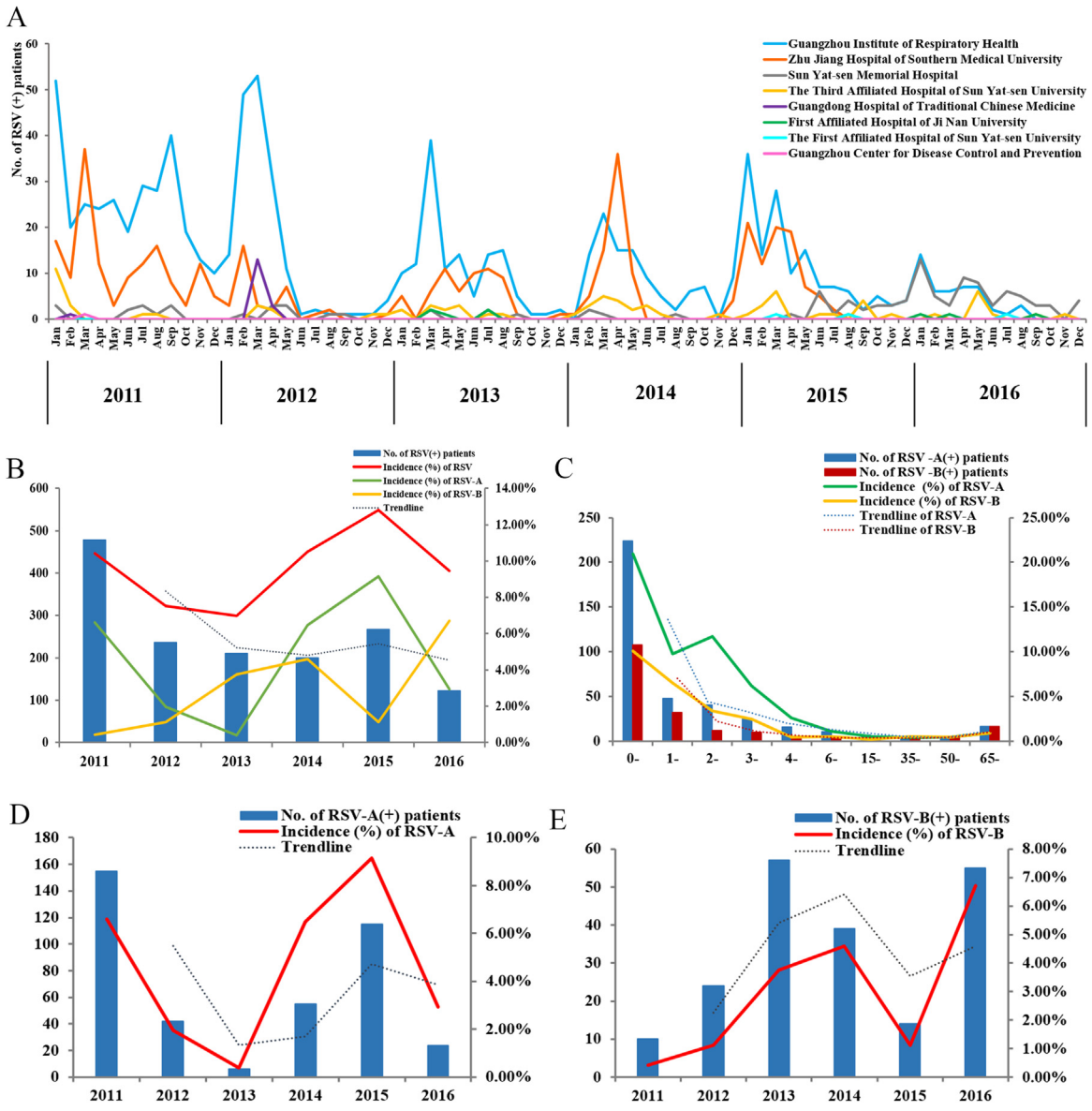

Figure 3. Regional distribution, age distribution, and annual distribution of RSV and the subtypes RSV-A and RSV-B in southern China during 2011–2016. A total 1514 cases from 14 hospitals were detected as RSV-positive; among these, 623 were analyzed for RSV-A/RSV-B subtypes. Among the 14 hospitals, eight detected RSV-positive cases. The positive number of RSV and the subtypes and the corresponding incidence (% of positive patients in the indicated year) are shown. Trend lines are indicated by dotted lines. (A) Regional distribution of RSV-positive cases during January 2011 to December 2016; (B) annual distribution of total RSV; (C) age distribution of RSV-A and RSV-B; (D) annual distribution of RSV-A; (E) annual distribution of RSV-B.

from 2011 (out of 155 RSV-A in that year; 24/155), six from 2012 (6/42), one from 2013 (1/6), six from 2014 (6/55), and nine from 2015 (9/115). Among the 15 RSV-B strains, one was from 2011 (1/10), four from 2012 (4/24), one from 2013 (1/57), eight from 2014 (8/39), and one from 2015 (1/14). Accordingly, two to four of the representative strains for each RSV-A and RSV-B genotype were selected from GenBank as reference strains.

Phylogenetic analysis showed that among the 46 Guangzhou RSV-A strains, there were 29 genotype NA1, 15 genotype ON1, one genotype NA3, and one genotype GA5 (Figures 6 and 7). The 29 NA1 strains included 24 strains from 2011 and five strains from 2012. Among these, 25 were close to Chongqing NA1 strain **GU550471**, and the remaining four strains were in another cluster, closer to the Brazil strain **JX513285**. The 15 ON1 strains included eight 2015strains, five 2014 strains, one 2012 strain, and one 2013 strain, and all of them were close to Hong Kong ON1 strain **KP221572**. The NA3 strain was a 2015strain which was close to Beijing strain **KC297277** and the GA5 strain was a 2014 strain close to Japan strain **AB175815**. All 15 Guangzhou strains of RSV-B belonged to the BA9 genotype and were close to Chengdu strain **KT765074**. Therefore, the main genotypes of RSV-A circulating in Guangzhou from 2011 to 2015 were NA1 and ON1, whereas the prevalent RSV-B genotype was BA9. Moreover, the dominant RSV-A genotype in 2011–2012 was NA1, but ON1 became the dominant genotype in 2014–2015 (Figure 6).

Amino acid sequence analysis

The deduced amino acid sequences (according to the second hypervariable region of the G protein) of the 46 RSV-A and 15 RSV-B Guangzhou strains were aligned and compared with the RSV-A prototype strain A2, representative NA1, ON1, NA3, GA5 strains and

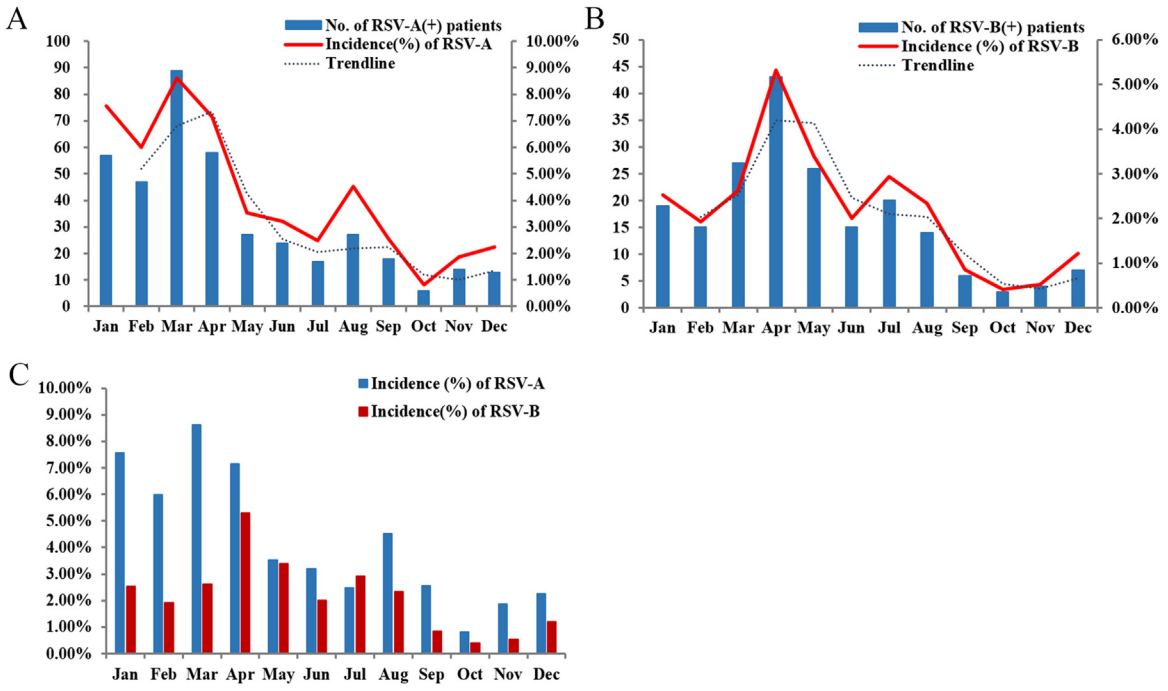

Figure 4. Cumulative monthly distribution of RSV-A and RSV-B in southern China from January 2011 to December 2016. Analysis of RSV subtypes based on 623 RSV-positive cases. The number of RSV subtype-positive patients and the cumulative monthly incidence (% of positive patients in the indicated month) are shown. Trend lines are indicated by dotted lines. (A) Positive number and incidence of RSV-A; (B) positive number and incidence of RSV-B; (C) incidence of RSV-A and RSV-B.

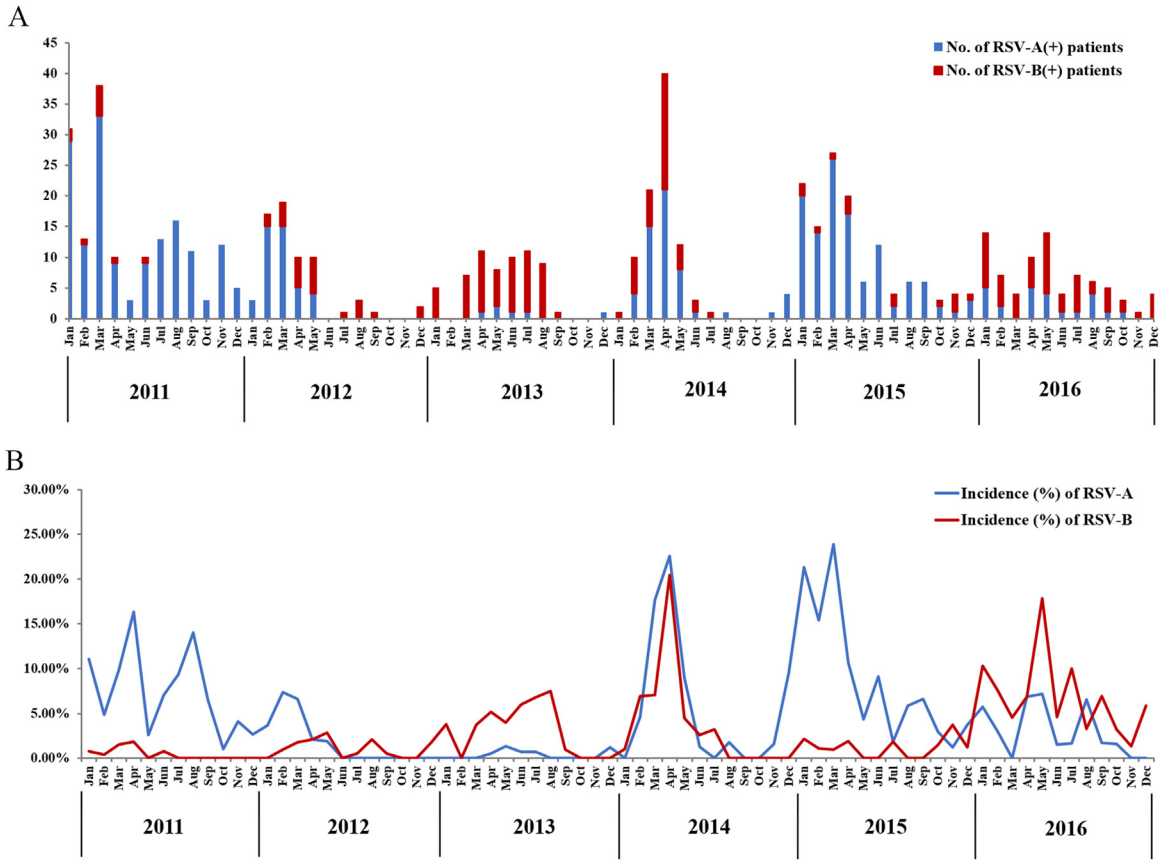

Figure 5. Monthly distribution of RSV-A and RSV-B from January 2011 to December 2016 in Guangzhou. The number of positive patients and the monthly incidence (% of positive patients in the indicated month) are shown. (A) The positive numbers of RSV-A and RSV-B; (B) the monthly incidences of RSV-A and RSV-B.

Table 4
Cases of co-detection of RSV and seven other common respiratory viruses (Flu, PIV, HMPV, HCoV, HADV, HRV, and HBoV).

Co-detection viruses	Number of cases	Constituent ratio
RSV + Flu	93	24.47%
RSV + PIV	40	10.53%
RSV + HADV	57	15.00%
RSV + HRV	64	16.84%
RSV + HCoV	44	11.58%
RSV + HMPV	24	6.32%
RSV + HBoV	24	6.32%
RSV + Flu + PIV	4	1.05%
RSV + Flu + HADV	2	0.53%
RSV + Flu + HRV	1	0.26%
RSV + Flu + HCoV	4	1.05%
RSV + PIV + HADV	3	0.79%
RSV + PIV + HRV	4	1.05%
RSV + PIV + HCoV	3	0.79%
RSV + HADV + HRV	2	0.53%
RSV + HADV + HCoV	1	0.26%
RSV + HADV + HBoV	4	1.05%
RSV + HRV + HCoV	2	0.53%
RSV + HRV + HMPV	2	0.53%
RSV + HMPV + HBoV	2	0.53%
Total	380	100.00%

RSV, respiratory syncytial virus; Flu, influenza virus; PIV, parainfluenza virus; HMPV, human metapneumovirus; HCoV, human coronavirus; HADV, human adenovirus; HRV, human rhinovirus; HBoV, human bocavirus.

RSV-B BA1 and BA9 strains in GenBank, respectively. The loss of a 24-amino acid fragment in the duplication region of ON1 genotype was observed in three Guangzhou strains (GZ/2014/03, GZ/2014/04, and GZ/2015/43). GZ/2014/03 and GZ/2014/04 lost amino acids 261–284 (i.e., QEETLHSTTSEGYLSPSQVYTTSG) and GZ/2015/43 showed a shift of two amino acids and lost amino acids 263–286 (i.e., ETLHSTTSEGYLSPSQVYTTSGQE). Compared to the A2 prototype, ON1 subtype-specific substitutions (S222P, T253K, and S314L) were observed in all of the ON1 subtypes, and E232G was observed in most of the ON1 strains except GZ/2014/05. M286E, P310L, and S314P were observed in all NA1 strains. Other commonly observed substitutions for RSV-A strains included L208I, P215S, S222P, N237D/Y, T249I, N251Y, E262K, N297Y, P298L, and P320T. There were two deletions (L265 and E308) observed in GZ/2015/16. The analysis of potential N-glycosylation sites revealed that due to the N237Y substitution of ON1 and NA3 genotype, GZ/2015/16 and GZ/2015/10 lost potential N-glycosylation sites at residue 237, but interestingly, due to the D237N substitution, 10 Guangzhou NA1 strains regained this N-glycosylation site. Moreover, due to N318Y, T319A, or T320S/I/P mutation, eight Guangzhou NA1 and one Guangzhou NA3 strains lost the potential N-glycosylation site at residue 318 (Figure 8). Compared to the sequences of the representative BA1 strain (BA4128/99B; Fan et al., 2017; Trento et al., 2003), all BA9 strains had K218T, L223P, and S247P substitutions. I281T and H287Y existed in most of the Guangzhou BA9 strains except GZ2012/03 and GZ/2012/15, respectively. Besides the I281A substitution, GZ/2012/03 strain showed a loss of amino acids 254–273 (i.e., TTTSKHTERDTSTSQSIALD). Other commonly observed substitutions for RSV-B strains included P216S/L, I229T, T254I, T275I, and S304P. Due to T312P substitution, three Guangzhou BA9 strains lost potential N-glycosylation sites at residue 310 (Figure 9).

Discussion

RSV is one of the most common pathogens that cause ALRI, especially in young children (Shi et al., 2017). Since there is no specific drug and no effective vaccine for RSV infection, the long-term monitoring of RSV is important for RSV prevention and control – by continuously collecting and analyzing data related to RSV introduction, transmission, distribution, and variation, and by understanding more about RSV epidemic and variation patterns, we can predict the prevalent level and scale of RSV and help to produce control and prevention strategies. This study investigated the molecular epidemiological characteristics of RSV and its subtypes in ARI patients during 2011–2016 in Guangzhou, southern China. Since RSV usually co-infects with other respiratory viruses, seven common respiratory viruses including Flu, PIV, HCoV, HADV, HBoV, HMPV, and HRV were detected simultaneously.

The results showed that among the eight common respiratory viruses, RSV was the one most commonly detected, with a detection rate of 9.5% in 16 024 patients with ARI from 14 hospitals during 2011–2016 in southern China. Flu was the second most frequently detected respiratory virus (9.3%). The age characteristics and monthly distribution of RSV and the seven respiratory viruses in Guangzhou during 2011–2016 were consistent with those in previous reports (Liu et al., 2014; Zhang et al., 2014). A total of 1514 patients were RSV-positive, 91.7% of whom were inpatients, significantly higher than outpatients ($p < 0.01$). The odds ratio of hospitalized patients with RSV infection to outpatient/emergency was 4.14, indicating that RSV infection has a considerable risk of resulting in severe disease. Infants and toddlers (<3 years) not in nursery (59.5%) seemed to have the highest risk of RSV infection, and the reason may lie in the decrease in maternal protective antibodies and the increase in exposure opportunities to crowds. The monthly distribution confirmed that RSV was mainly prevalent in winter and spring, consistent with other regions (Bashir et al., 2017; Slovic et al., 2016). The annual distribution of RSV during 2011–2016 in Guangzhou revealed high epidemic years for 2011, 2014, and 2015, with year 2015 as an epidemic peak. There may be a biennial high incidence pattern of RSV in Guangzhou, and surveillance over a longer period will confirm the annual distribution characteristics (Esposito et al., 2015; Xiang et al., 2013).

To better understand the epidemiological characteristics of RSV subtypes, RSV-A and RSV-B subtypes were determined based on 623 RSV-positive samples from 13 hospitals. The results showed that RSV-A was the dominant subtype circulating in Guangzhou during 2011–2016, consistent with reports from other regions (Hu et al., 2017; Slovic et al., 2016). The three patients with co-detection were all infants and toddlers and inpatients with ALRI, and none of them died. It cannot be deduced whether the co-detection of both subtypes exacerbates the disease based on the symptoms. Both RSV-A and RSV-B mainly infected young children under 3 years of age, and the incidence of RSV-A was higher than that of RSV-B in children ($p < 0.01$), but not in adults ($p > 0.05$), indicating that RSV-A may have a higher pathogenicity to children.

The annual distribution of RSV subtypes revealed an interesting year distribution, as a 2-year RSV-A dominance followed by a 1-year RSV-B dominance pattern was found. A similar year distribution pattern has been reported previously in Beijing and Belgium, among others (Xiang et al., 2013; Coggins et al., 1998; Zlateva et al., 2007; Scott et al., 2004). In addition to this pattern, RSV-A has been reported to show an epidemic for 2–4 years followed by RSV-B for 1 year pattern, as reported in Uruguay (Arbiza et al., 2005); and also, an RSV-A epidemic for 4 years followed by RSV-B for 1 year pattern, as reported in Japan (Shobugawa et al., 2009). It appears that this is the first time such a year distribution characteristic of RSV subtypes has been reported for Guangzhou. Most of the time, RSV-A and RSV-B took dominance alternately, but in January to June 2014, both RSV-A and RSV-B were epidemic at a high level. The co-detection rate of RSV was as high as 25.1% in Guangzhou from 2011 to 2016, and 91.1% of them were double infections and 8.9% were triple infections.

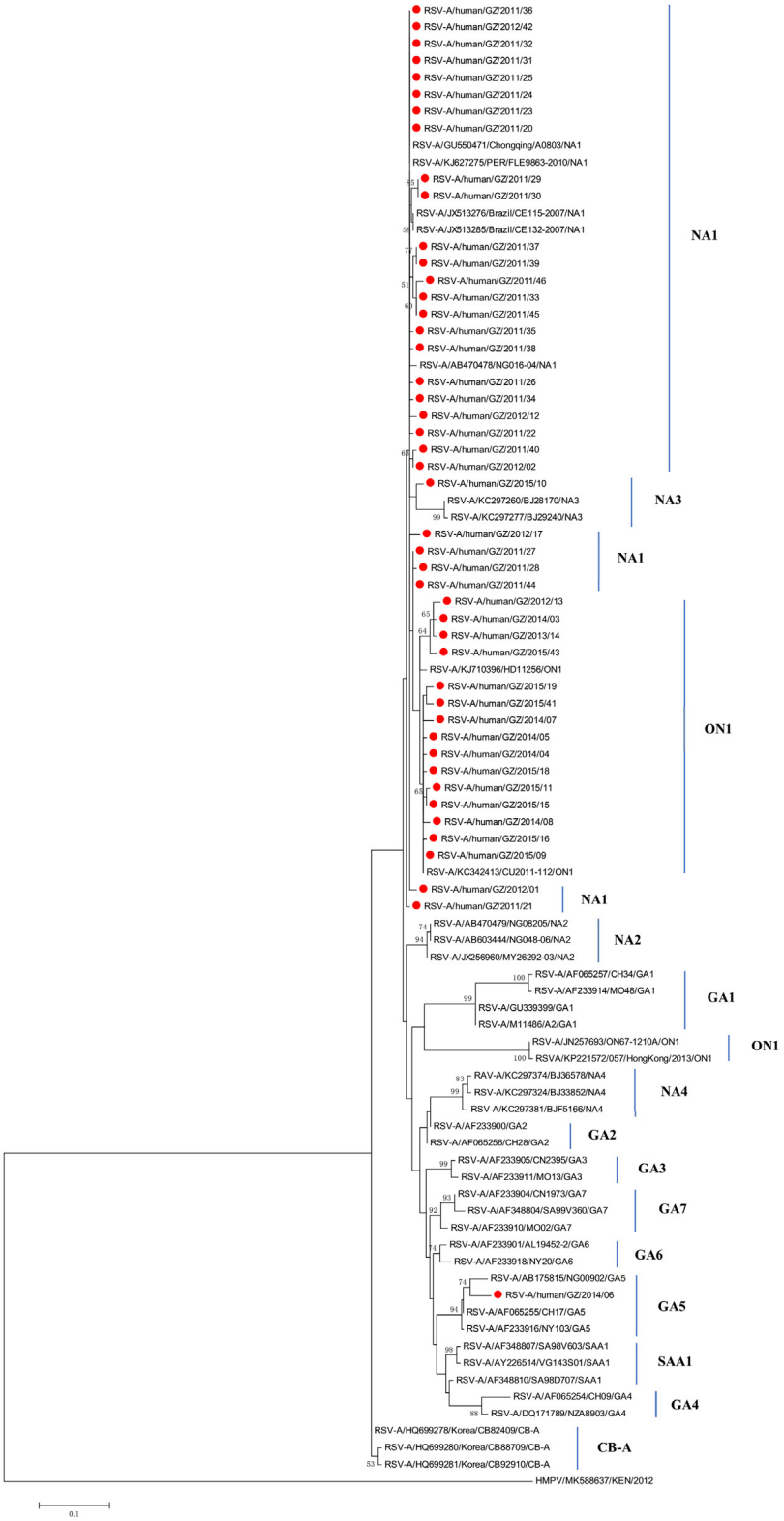

Figure 6. The G gene phylogenetic tree of RSV-A strains from acute respiratory infection patients in southern China during 2011–2015. A total of 46 RSV-A strains from the years 2011–2015 were selected for G gene sequencing and genotypic analysis. The phylogenetic tree was constructed by maximum-likelihood method with the Kimura 2-parameter model using MEGA 5.0 software. Bootstrap values were decided by 1000 replicates. Genotype analysis results for the 46 strains identified in this study are presented with red circles. Of the 46 Guangzhou RSV-A strains, 29 strains belonged to genotype NA1, 15 belonged to genotype ON1, one belonged to genotype NA3, and one belonged to genotype GA5.

Influenza virus was the most common co-detected virus. No correlation was found between clinical symptoms and co-detection ($p > 0.05$), indicating that co-detection may not aggravate the disease or enhance the mortality of patients. However, this finding may be limited by the small sample size and the heterogeneity of co-detected viruses, and more studies will be needed to elucidate the relationships between RSV co-infection and disease severity.

Figure 7. The G gene phylogenetic tree of RSV-B strains from acute respiratory infection patients in southern China during the years 2011–2015. A total of 15 RSV-B strains from the years 2011–2015 were selected for G gene sequencing and phylogenetic analysis. The phylogenetic tree was constructed by maximum-likelihood method with the Kimura 2-parameter model using MEGA 5.0 software with 1000 bootstrap replicates. Genotype analysis results of the 15 strains identified in this study are presented with red circles. All of the 15 Guangzhou strains of RSV-B belonged to the BA9 genotype.

The genome of RSV encodes 11 proteins, including NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2, and L (Cane, 2001). According to the genetic diversity, RSV is genotyped mainly by the second high-variant region of the G protein C-terminal (HVR2) (Schobel et al., 2016). Therefore, to better understand the phylogenetic characteristics of RSV in southern China and to gain more insight into RSV variation, we further analyzed the sequences of the G genes of the Guangzhou RSV strains. Most of the RSV-A genotypes from Guangzhou were close to Chongqing and Hong Kong strains and the RSV-B was close to a Chengdu strain. The prevalent RSV-B

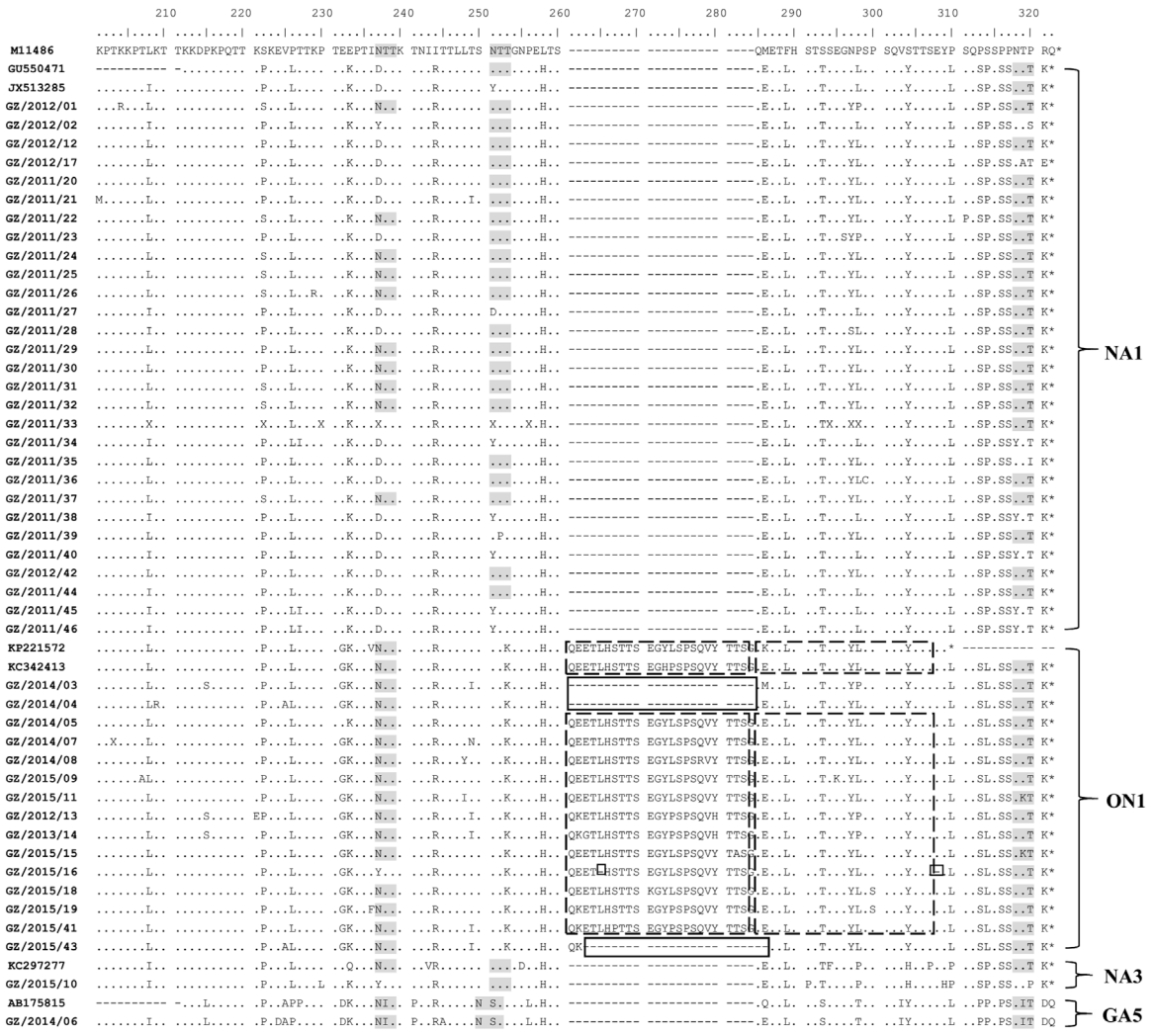

Figure 8. Alignment of the deduced amino acid sequences of the 46 Guangzhou RSV-A strains. Amino acid sequences of the second variable region of the G protein were aligned with the RSV-A prototype A2 strain (GenBank accession number M11486), representative NA1 Chongqing and Brazil strains (GenBank accession numbers GU550471 and JX513285, respectively), ON1 Hong Kong and Thailand strains (GenBank accession numbers KP221572 and KC342413, respectively), a representative NA3 strain (GenBank accession number KC297277), and a representative GA5 strain (GenBank accession number AB175815). The results show amino acids 201–322. Identical residues are indicated by dots and stop codons by asterisks; N-linked glycosylation sites (NXT, where X is not a proline) are indicated in light gray. The duplicate region is framed by a dashed rectangle. The deleted regions are framed by a solid rectangle.

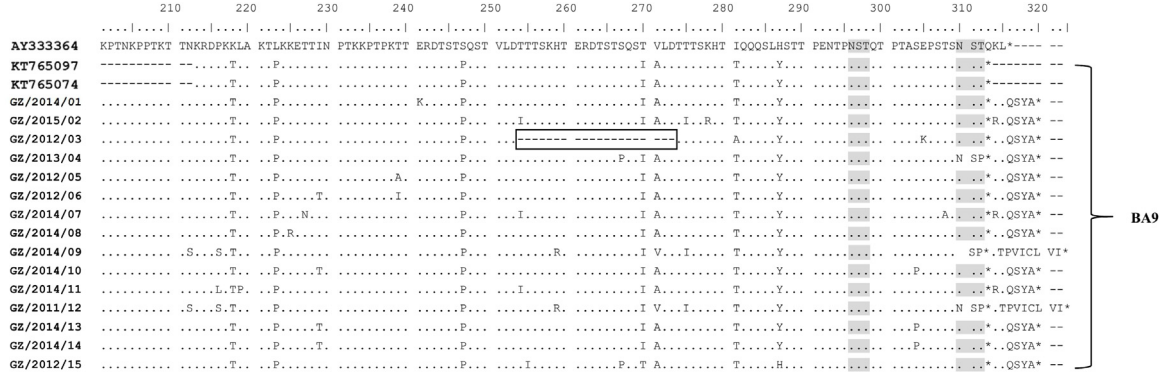

Figure 9. Alignment of the deduced amino acid sequences of the 15 Guangzhou RSV-B strains. Amino acid sequences of the second variable region of the G protein were aligned with the RSV-B representative BA1 strain (GenBank accession number AY333364) and representative BA9 strains (GenBank accession numbers KT765097 and KT765074). The results show amino acids 201–322. Identical residues are indicated by dots and stop codons by asterisks; N-linked glycosylation sites (NXT, where X is not a proline) are indicated in light gray. The deleted regions are framed by a rectangle.

genotype in Guangzhou during 2011–2016 was BA9, whereas the prevalent RSV-A genotypes were more diverse. Among the 30 strains from the years 2011–2012, 29 were NA1 and one was ON1. Among the 15 strains from the years 2014–2015, 13 were ON1 and the other two were GA5 and BA9, respectively. The 2013 strain belonged to ON1. It can be seen that the NA1 genotype of RSV-A was prevalent in 2011–2012, while ON1 became the prevalent genotype in 2014–2015, which is consistent with the report of Fan et al. (2017).

Deduced amino acid sequence analysis confirmed the ongoing evolution of RSV-A and B strains, especially in RSV-A ON1 and NA1 genotypes. Multiple highly variable substitutions were observed, and moreover, three Guangzhou ON1 strains showed the loss of a 24-amino acid fragment in the duplication region, and one BA9 strain had a 20-amino acid deletion. N-glycosylation site changes were also commonly observed in Guangzhou RSV-A strains, including the loss of the 251 and 318 glycosylation sites and regain of the 237 glycosylation site in most of the NA1 strains, and the loss of the 237 glycosylation site and two single amino acid deletions in an ON1 strain GZ/2015/16. Glycosylation site changes were also observed in three RSV-B BA9 strains. These mutations indicated a high selection pressure.

Since 2014–2015 was also the highest epidemic year for RSV-A, it was deduced that before 2012, the prevalent RSV genotype was NA1 from Chongqing, but in 2014, due to staff turnover, a new Hong Kong strain of ON1 genotype was introduced (Song et al., 2017), evolved, and became the dominant strain. Furthermore, because the population lacked antibody protection, the new genotype spread rapidly and caused the high incidence of RSV-A in 2014–2015 in Guangzhou. Combined with the monthly distribution by site, we believe that the epidemic peaks in Zhu Jiang Hospital and Guangzhou Institute of Respiratory Diseases in 2014 reflected the prevalence of the new genotype. Although Zou et al. (2016) also investigated the evolution and transmission of RSV-A in Guangdong in 2008–2015, the present study is the first to report the introduction, evolution, and transmission of the new ON1 genotype in Guangzhou, southern China. Further studies should be conducted to reveal the mechanisms behind the emergence of new genotypes and RSV epidemic.

In conclusion, this study investigated the molecular epidemiological characteristics of RSV and its genotypes in children and adult patients with ARI in southern China during 2011–2016. The findings may have significance in guiding policy-making for RSV prevention and control, and may provide important information for RSV variation studies.

Ethical approval

All studies involving human participants were approved by the Medical Ethics Review Board of Zhongshan School of Medicine, Sun Yat-sen University, in accordance with the guidelines for the protection of human subjects. Written informed consent was obtained from all participants or their guardians after being informed about the aims of the research and the right to keep their information confidential.

Conflict of interest

All of the authors declare that there is no conflict of interest.

Acknowledgements

All participants involved in this monitoring project are appreciated. We owe our special thanks to the medical staff at the 14 hospitals: the First Affiliated Hospital of Sun Yat-sen University, Sun Yat-sen Memorial Hospital, The Third Affiliated Hospital of Sun Yat-sen University, Guangdong Provincial Center for Disease Control and Prevention, Guangzhou Center for Disease Control and Prevention, Guangdong Provincial People's Hospital, Guangdong Hospital of Traditional Chinese Medicine, Zhu Jiang Hospital of Southern Medical University, First Affiliated Hospital of Ji Nan University, Guangzhou Woman and Children's Medical Center, Guangzhou Red Cross Hospital, First Affiliated Hospital of Guangzhou Medical University, Guangzhou Institute of Respiratory Health, and Li Wan Affiliated Hospital of Guangzhou Medical University, for their great help in collecting clinical information and samples. This research was supported by the National Major Projects of Major Infectious Disease Control and Prevention, the Ministry of Science and Technology of the People's Republic of China (grant numbers 2009ZX10004-213, 2012ZX10004213-001, and 2017ZX10103011). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.10.009.

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