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MOLECULAR CHARACTERISTICS OF RESPIRATORY SYNCYTIAL VIRUS (RSV) ISOLATED IN THE NORTH VIETNAM, 2017-2018

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ABSTRACT

Respiratory syncytial virus (RSV) is one of the most interesting respiratory viruses in the world. This virus causes symptoms of illnesses like influenza and imposes a heavy burden on medical services and the economy. However, studies on RSV in Vietnam are limited, while most of the RSV research has primarily been done before 2015. We collected the clinical respiratory samples from severe acute respiratory infection (SARI) patients to screen for RSV by real-time RT-PCR and study their molecular characteristics. RSV-positive specimens with Ct value < 25 collected between 2017–2018 in north Vietnam were inoculated on the Hep2 cell line. The results showed that 25 (22.32%) RSV virus strains were harvested from the inoculation procedure with 18 RSV A and 7 RSV B. The whole genomes of four representative strains were sequenced with the Illumina iSeq 100. Phylogenetic trees analysis of each subtype were classified as two RSV A and two RSV B sequences as genotype ON1 lineage 1.2 and genotype BA9 respectively. These genotypes were identified as typical Vietnamese strains from 2009–2012. Nevertheless, the RSV strains before 2015 and those in this study had significant differences in the G gene, with 34–35 amino acids in RSV A and 4 amino acids in RSV B. Moreover, the first whole genome of Vietnamese RSV since 2016 may give more understanding of the molecular characteristics of RSVs in Vietnam.

Keywords: Genotype, isolation, molecular characteristic, next-generation sequencing, RSV.

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INTRODUCTION

The human respiratory syncytial virus (RSV) is one of the most important pathogens which causes severe acute respiratory infection (SARI) among infants and young children all over the world (Vianna et al., 2021). It was estimated that 95% of children under two years old had been infected with RSV at least once time and 2% of those require hospitalization. RSV can also regularly infect adults and cause several illnesses in the elderly. Besides, RSV can cause re-infection throughout life with milder symptoms, which indicates that the immune response to RSV infection is unstable (Battles & McLellan, 2019).

RSV is classified in the family *Pneumoviridae*, genus *Orthopneumovirus*. The genome has a non-segmented, negative, single-strand RNA, and has ten genes encoding 11 proteins (Cao et al., 2021). The most interesting and important proteins are G (attachment glycoprotein) and F (fusion protein) which are the major surface antigens of RSV. The G protein is involved in virus attachment to cell receptors and the F protein is related to cell membrane fusion (Fall et al., 2021). RSV is classified into two major subtypes RSV A and RSV B, based on genetic variability and antigenic characterization of the G gene. RSV A virus has been divided into 12 genotypes (GA1-GA7, SAA1, NA1-2, ON1-2) and RSV B virus has been divided into 32 genotypes (GB1-5, BA1-14, SAB1-4, URU1-2, NZB 1-2, BA-CCA. BA-CCB, BA-C, CBB, and CB1). This classification is based on the difference in the G gene's second hypervariable region (HVR) in both RSV A and RSV B (Yun et al., 2020).

Of all the RSV genotypes, RSV A ON1 and RSV B BA9 are the dominant types in various regions worldwide. RSV A genotype ON1 emerged in Canada in 2010 and after four years this became the most popular RSV in many countries. In RSV B, BA genotypes (BA1-14) have been the most predominant over the past 20 years, and since 2015, the only common genotype was BA9 (Yu et al., 2021).

There was limited research on RSV in Vietnam. Previous studies have been mainly on the prevalence of RSV or its co-infection with other viruses and bacteria. Most of the research was done in Ho Chi Minh City (Do et al., 2016; Tran et al., 2013; Tuan et al., 2015), and several in Nha Trang City (Yoshihara et al., 2016b). The data on RSV in Vietnam has not been updated since 2015 in both prevalence and genetic characterization. In this study, we examined the prevalence of RSV and analyzed the characteristics of the RSV genotype in pediatric SARI patients in the North of Vietnam during 2017–2018 to gain a better understanding of the molecular epidemiological aspect of RSV in Vietnam.

MATERIALS AND METHODS

Clinical samples

Throat swabs were collected from pediatric patients with severe acute respiratory infections at the National Children's Hospital and Quang Ninh General Hospital in the two years 2017–2018. The specimens were collected following decision number 4608/QD-BYT (October 11th, 2017). Clinical samples were kept in the virus transport medium at -80 ^{o}C until transported for testing at the National Influenza Center-National Institute of Hygiene and Epidemiology (NIC-NIHE). The patients' samples were extracted and screened for the presence of RSV by real-time RT-PCR. RSV-positive samples with Ct < 25 were then isolated to collect RSV strains.

Virus isolation

The selected samples were isolated following the protocol of Other Respiratory Viruses of the Influenza Laboratory, Department of Virology, NIHE. The summary of the procedure was as follows: 500 µL of the clinical sample was mixed with 20 µL of antibiotics (Kanamycin sulfate and Cipro-HCl) in a new tube and incubated at 33 ^{o}C for 30–60 minutes. Then, 1.5 mL of D-MEM medium (Dulbecco's Modified Eagle Medium - Gibco, USA) was added to the sample. The single layer Hep2 cell line on a culture tube/plate was used to inoculate these samples. Incubation was at 35 ^{o}C, and the

cells were checked for cytopathogenic effect (CPE) after 5–7 days. Samples with no CPE at the first and second inoculations were sub-cultured. The procedure of sub-culture was similar to the first inoculation.

Identification of RSV strains

Samples, that had CPE, were checked for the presence of RSV by real-time RT-PCR. The primers and probes to detect RSV were conducted following the protocol of the US-CDC (Centre for Disease Control and Prevention, 2015). In the case of the positive result, these samples were examined and imaged with the transmission electron microscope (TEM). Supernatant inoculation was dyed with phosphotungstic acid (PTA) 0.25% for the negative staining method, which showed the shape of viruses with envelopes.

Whole-genome sequencing of RSV

RSV virus isolation was selected by year of collection, location, and subtype for sequencing. Next-generation sequencing (NGS) of whole-genome RSV was followed by NGS sequencing of the RNA virus protocol of the Influenza Laboratory, Department of Virology, NIHE (Trang et al. 2020). cDNA was generated from single strain RNA by Superscript IV VILO Master Mix (Thermo Fisher, USA). Then, the NebNext® Ultra II Non-Directional RNA Second Strand Synthesis Module (New England BioLabs, USA) helped to make double-strain DNA. This DNA was purified with Exosap-IT PCR Product Cleanup (Thermo Scientific Fisher, USA) and diluted to 0.2 ng/µL. The library for sequencing was done by the Nextera XT DNA Library Preparation Kit (Illumina, USA) followed by the manufacturer's instructions. Finally, a library with a concentration of 60 pM was run on the Illumina iSeq 100 (Illumina, USA).

Analysis of sequencing data

Sequencing data were processed on the software system incorporated into the Illumina iSeq 100 sequencer. The fastq files were generated for downstream analysis and integrated into Qiagen's commercial software for NGS - CLC Genomics Workbench version 11.0 (CLC bio, Aarhus, Denmark). Nucleotide sequences were assessed by sequencing quality control (QC report), then trimming the noise ends of sequencing reads, and deleting the short reads (Trims). The trimmed reads were assembled and then aligned with reference sequences of the representative RSV subtype. The consensus was generated from the comparison between reads and references. The reference strains for RSV A and RSV B were EPI_ISL_412866-hRSV/A/England/397/2017 and EPI_ISL_1653999-hRSV/B/Australia/VIC-RCH056/2019, respectively.

Based on the sequence and annotation of each gene on references, studied whole genome sequences were annotated and identified mutations by CLC Genomics Workbench version 11.0 and AliView version 1.28 (Larsson, 2014). The sequences of virus strains were registered for identification on the GISAIDS (Global Initiative on Sharing Avian Influenza Data, which now includes the Sars-CoV-2 and RSV data).

Phylogenetic analysis

Maximum-likelihood phylogenetic trees of RSV were constructed and the relationship to sequences of representative genotypes was > 70% (with bootstrap 1,000 resamplings). The second HVR (amino acid position 210–322) in the G gene of RSV was chosen for generating a phylogenetic tree. 339 nucleotides in the second HVR of RSV A and RSV B were aligned separately and edited using Clustal W with MEGA version X (Kumar et al., 2018). To study the relationship between the Vietnamese RSV strain and other genomes available on GISAID, we chose several sequences in neighboring countries and other continents. Reference sequences for each genotype and lineage were based on Myanmar sequences (Phyu et al., 2021).

RESULTS

RSV isolation

From the initial screening results, there were 112/294 clinical samples with Ct < 25

which met the isolation criteria. These samples were inoculated and transferred 3 times, then examined for CPE by electronic microscope and real-time RT-PCR test results. Finally, there were 25 (22.32%) RSV strains detected from 112 clinical samples. The image of CPE was shown in Figure 1 and the results of RSV isolation were described in Table 1.

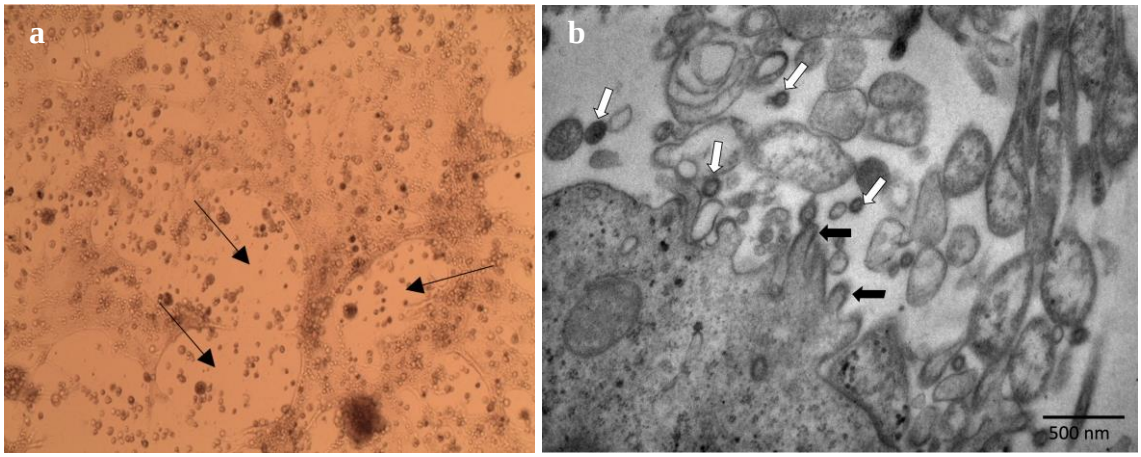

Figure 1. RSV isolation in Hep2 cell line in Vietnam, 2017–2018. Clinical specimens from SARI pediatric patients in viral transport medium were inoculated in Hep2 cell line. After three times culture or cytopathogenic effect (CPE) occurred, supernatant inoculation was took a photo with the microscopy and the transmission electron microscope (TEM). (a) CPE image in microscopy. The arrows show the CPE areas; (b) Image of virus in the supernatant. The RSV viral particles with spherical morphology is marked by the white arrows. The budding viral are shown with the black arrows

Table 1. Results of RSV isolation

	National Children's Hospital n/N (%)	Quang Ninh General Hospital n/N (%)	Sub-Total n/N (%)
RSV A			
2017	9/21 (42.86)	1/17 (5.88)	10/38 (26.32)
2018	6/16 (37.5)	2/5 (40.0)	8/21 (38.1)
2017–2018	15/37 (40.54)	3/22 (13.64)	18/59 (30.51)
RSV B			
2017	4/25 (16.0)	0/6 (0.0)	4/31 (12.9)
2018	3/19 (15.79)	0/3 (0.0)	3/22 (13.64)
2017–2018	7/44 (15.91)	0/9 (0.0)	7/53 (13.21)
Total	22/81 (27.16)	3/31 (9.68)	25 (22.32)

The numbers of RSV A and RSV B clinical samples isolated in this research were 59 and 53, respectively. However, the number of harvested strains of each subtype was not the same. RSV A had 18 strains (30.51%) and the RSV B had 7 strains (13.21%). The difference in successful isolation rates was significant with $p < 0.05$. By year, this rate was 20.29% (14/69) in 2017 and 25.59% (11/43) in 2018. Overall, the rate of inoculation RSV was 22.32%.

Sequencing of RSV strains

Among 25 isolates, four representative samples were selected based on year, collected location, and subtype. The Ct value of real-time RT-PCR of these strains was between 14–15. The sequencing results also had good data with 1.8Gb. The percentage of Cluster Passing Filter (%Cluster PF) was 94% and the average percentage of Q30 was 82.19%. Even though the whole genome of RSV was done without a gap, the average coverage was quite low, from 56.54 to 76.26 reads. The reads that were mapped to reference were only around 8.5–10% trimmed reads. It means that under 300,000 reads were from the RSV genome, and the remaining were sequences of HEP2 cells, bacteria, and viruses that were contaminated during isolation.

The complete Vietnamese RSV genome of each strain was from 15,091 to 15,214 nucleotides. These were identified on GISAID as EPI_ISL_12544916 - EPI_ISL_12544919. These sequences became the first complete genomes of RSV in Vietnam after 2016 when they were uploaded to GenBank.

Molecular epidemiology of RSV

Based on sequence alignment and analysis, all RSV A strains possessed a 72-nucleotide duplication in the HVR of the G gene, which is characteristic of the ON1 genotype. The phylogenetic tree revealed that two Vietnamese RSV A sequences were similar in the ON1 genotype, ON1.2 lineage (Fig. 2a). This cluster includes RSV sequences from China, Myanmar, and Germany during 2016−2018.

For RSV B, all sequences were characterized as BA9 genotypes (Fig. 2b). The Vietnamese strains in the past and the two studied strains also had significant genetic differences. On the phylogenetic tree, they were far from each other. The strain SARI02/CC/17/015 was placed in the Myanmar cluster while the SARI02/CC/18/136 was in the global cluster with China, Australia, Switzerland, Argentina, and others.

When comparing sequences of Vietnamese RSV with reference strains in GISAID, some mutations were revealed. The G protein sequence showed the most significant change in amino acids, with 34–35 differences in RSV A and 4 differences in RSV B (Table 2). Positions 261–284 in G gen of Vietnamese RSV A sequences had deleted 24 amino acids compared with the reference. In contrast, in the protein sequences from the remaining genes, there were 0–2 changes, except for the L protein.

Table 2. The difference in amino acid of Vietnamese RSV sequences

Protein	No. of mutation (% identity)			
	RSV A		RSV B	
	SARI-18-NH-17-18	SARI-18-HH-18-96	SARI-02-CC-17-15	SARI-02-CC-18-136
NS1	0 (100%)	0 (100%)	0 (100%)	1 (99.3%)
NS2	0 (100%)	0 (100%)	0 (100%)	1 (99.2%)
N	1 (99.7%)	1 (99.7%)	1 (99.7%)	1 (99.7%)
P	1 (99.6%)	1 (99.6%)	1 (99.6%)	0 (100%)
M	0 (100%)	0 (100%)	0 (100%)	0 (100%)
SH	0 (100%)	0 (100%)	0 (100%)	1 (98.5%)
G	35 (96.3%)	34 (96.6%)	4 (98.7%)	4 (98.7%)
F	0 (100%)	0 (100%)	0 (100%)	0 (100%)
M2-1	1 (99.5%)	1 (99.5%)	0 (100%)	0 (100%)
M2-2	2 (97.7%)	2 (97.7%)	2 (97.8%)	1 (98.9%)
L	9 (99.6%)	6 (99.7%)	3 (99.9%)	4 (99.8%)

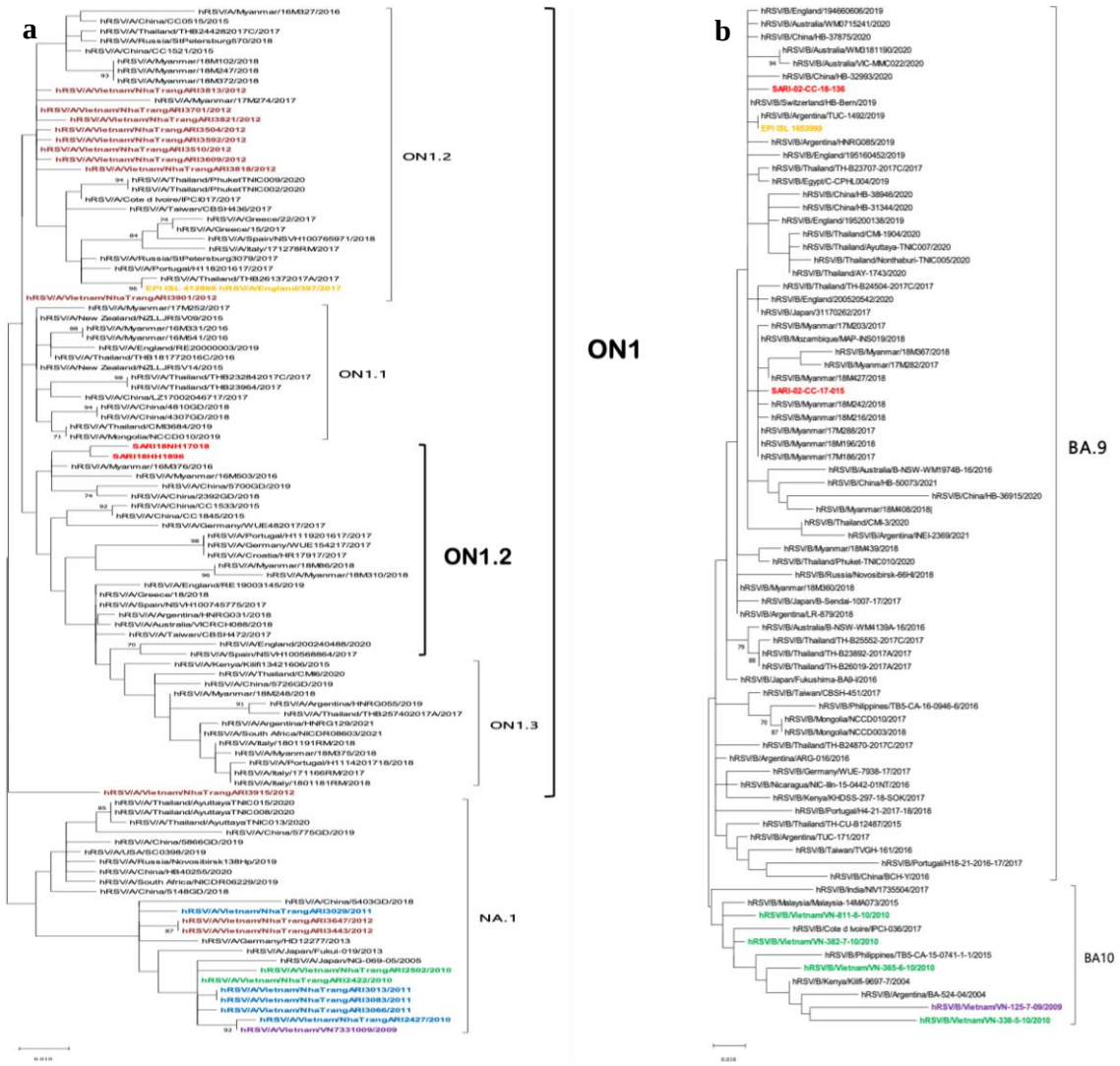

Figure 2. The phylogenetic tree of 339 nucleotides in the second hypervariable region in the G gene of Vietnamese RSV sequences. (a) RSV A phylogenetic tree; (b) RSV B phylogenetic tree. The tree was constructed by the Maximum likelihood (ML) method and Tamura-Nei model. Bootstrap value is determined by 1000 replications and greater than 70% is displayed at the branch nodes. The scale bars represent the number of nucleotide substitutions per site. The RSV sequences in this study were red. The RSV sequences collected in Vietnam in 2009, 2010, 2011, and 2012 were purple, blue, green, and brown, respectively. Reference strains were yellow, and sequences from other countries were black

DISCUSSION

RSV was one of the seven other respiratory viruses that have been under laboratory surveillance for the SARI pathogen in children from 2017 up to now in the NIC-NIHE. At the NIC-NIHE laboratory, the RSV was isolated on cell lines such as Vero, Hep2, and A549. However, Hep2 cells are the most commonly used due to their efficiency in comparison to others (Van Der Gucht et al., 2019). Therefore, this cell line was chosen for isolation RSV in NIC-NIHE. RSV isolation efficiency has been low when

compared to other common respiratory viruses such as Adeno, influenza, and rhinoviruses. The successful isolation percentage in this study was 22.32% (25/112) compared to the rate of about 50–60% for influenza A/H1N1pdm09 (data recorded in NIC-NIHE). The overall rate of RSV isolation in respiratory specimens with Ct < 25 was about 20% in this study, which is in the range of 10%–50% of samples isolated and obtained strains (Griffiths et al., 2017). The study design, type of specimen, storage temperature of clinical specimens, and freezing-thaw process before the experiment all affected the isolation results. In addition, if all real-time RT-PCR positive samples were isolated, regardless of viral load (Ct value), the success rate of isolation would be greatly reduced. Therefore, it was difficult to give a general success rate of RSV isolation for all studies.

The number of clinical inoculated samples of the two subtypes was almost similar, but the number of RSV A and RSV B strains successfully isolated was a statistically significant difference ($p < 0.05$). This showed that RSV A isolation had a higher rate than RSV B. However, more studies are needed to prove this hypothesis or that this is just a characteristic result of this study.

With the development of science, research recently on the molecular influenza virus could be performed directly from clinical specimens since 2016 (Seong et al., 2016). Furthermore, errors in the isolation process can lead to genetic changes. During replication on susceptible cell lines, similar to viral replication in the host, crossover, integration, or replication errors can occur. This fact has shown whether we continue to isolate all the positive samples to obtain strains or if the sequencing is done first, analyzing the genetic characteristics and then selecting the representative strains for isolation and storage. This strategy has been applied very successfully in the fight against the current COVID-19 pandemic (Hussen et al., 2022). With RSV research, scientists have also begun to move in this general trend, so virus isolation will be based on direct sequencing results of clinical samples.

Using random primers that are not specific for RSV gene sequencing on the Illumina iSeq 100 system, we had good results with four samples in one session. The number of reads available for RSV analysis should not exceed 10% of the total reads. The remaining reads were sequences of Hep2 cells, viruses, and bacteria in the culture fluid. This indicated that the sequencing efficiency was not high when using this method. Since 2015, several studies have used specific primers to clone the whole genome of RSV. The size of PCR fragments ranges from 2kb to 4.5kb depending on the study (Di Giallonardo et al., 2018). On average, there are 4–8 primer pairs for each subtype. Thus, to solve this problem, increasing both the number of sequenced samples in one performance and the method of sequencing to achieve higher efficiency is the way to go. Therefore, the primer design and selection of suitable primer sets to amplify the sequenced DNA fragments of the RSV genome are very important.

In terms of genotype, the two RSV A strains belong to the genotype ON1 lineage 1.2 (ON1.2). This lineage includes sequences from China, Myanmar, the UK, Germany, etc. These strains were collected from 2015 to 2020. Thus, the genetics of the RSV A strain circulating in Vietnam has similarities with other strains in the world at the same time. Although belonging to the same sub-group ON1.2 on the phylogenetic tree, the RSV A strains circulating in Vietnam in this study formed a different branch compared to those in 2012.

Besides, RSV B in this study all belonged to genotype BA9, which was discovered in 2009 from samples collected in southern Vietnam. To date, this genotype has been still the dominant genotype of RSV B strains. Genotype BA9, although not divided into many lineages, includes many branches, and the two Vietnamese strains in this study belonged to two different branches. On the other hand, the two RSV B strains are also

different from the strains circulating in Vietnam in 2009 and 2010.

In the previous study, Tuan et al reported on circulating RSV groups in pediatric patients in Ho Chi Minh City in 2010, in which RSV A had genotypes NA1 and GA5; RSV B had genotypes BA3, BA9, and BA10 Tuan et al. (2015). Another study at the same time showed the genotype of RSV A consisting of GA only and no other genotypes. RSV B belongs to two genotypes BA9 and BA10 (Tran et al., 2013). Yoshihara et al collected samples from Nha Trang in 2010–2012 and showed a change in the RSV genotypes circulating in this area. According to the study, 2010 and 2011 were dominated by RSV A genotype NA. Samples in the year 2012 had a change when part of the genotype belonged to NA1 and the majority belonged to ON1 (Yoshihara et al., 2016a). Sampling studies since 2012 reported the presence of the RSV A ON1 genotype. This genotype continued to be found in the 2013, 2014 and 2015 samples (Lu et al., 2020). It shows that the genotype ON1 has been circulating since 2012 and the genotype BA9 has been circulating since 2010 in Vietnam.

CONCLUSION

This study showed the first whole genome sequencing of RSV in Vietnam since 2016. The percentage of virus isolated was 22.32% and the isolation rate of RSV A was higher than RSV B. The RSV strains in the north of Vietnam during 2017–2018 were classified as ON1.2 (RSV A) and BA 9 (RSV B).

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