Isolation and characterization of clinical RSV in South Korea and its immunogenicity evaluation methods for vaccines in development

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Isolation and characterization of clinical RSV in South Korea and its immunogenicity evaluation methods for vaccines in development

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But none of these things move me, neither counts I my life dear unto myself so that I might finish my course with joy and the ministry, which I have received of the Lord Jesus, to testify the gospel of the grace of God. [Acts 20:24, KJV]

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May God bless us! Thank you so much!

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Bonhyang Na

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ABSTRACT

Isolation and characterization of clinical RSV in South Korea and its immunogenicity evaluation methods for vaccines in development

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Respiratory Syncytial Virus (RSV) is a particularly significant viral pathogen in young children, elderly individuals, and immunocompromised adults. Most of the published researches with RSV had been performed on RSV Long and RSV A2, isolated in 1956 and 1961; nevertheless, contemporary RSV isolates are distinct from these prototype strains. These viruses have also undergone serial passages in cell culture, which may have led to modifications that impact virus-host interaction. While many new preclinical and clinical studies are under development for vaccines against RSV, up to date, no studies have established *in vitro* culture system and defined the immunogenicity evaluation based on contemporary clinical RSV strains in Korea. In this study, we isolated and characterized the clinical RSV strains from September 2019 to December 2022. We inoculated clinical RSV into Hep-2 cells and confirmed their characteristics with the capacity for syncytium formation by immunocytochemistry and flow cytometry. Clinical isolated RSV was successfully inoculated, and RSV-infected Hep-2 cells showed the matured and enriched syncytium in an RSV concentration-dependent manner.

Moreover, we also investigated whether significant alterations occurred in the genotypic patterns of RSV strains in Korea before and after the COVID-19 pandemic, utilizing whole-genome sequencing (WGS). This prospective study

was conducted at Severance Children's Hospital in Seoul, Korea from September 2019 to December 2022. WGS analysis was performed using the PacBio Circular Consensus Sequencing platform. samples were collected from 285 children, out of which RNA extracts from 155 samples (66 type-A and 89 type-B) were available for G protein genotyping and whole-genome sequencing (WGS) analysis. The G protein genotyping revealed that the most frequently identified genotypes were ON1 for RSV- A and BA9 for RSV-B. The WGS analysis showed that the genotypes clustered closely with ON1/NA1 for RSV-A and BA for RSV-B, respectively. One mutation was identified in the RSV strain that confers resistance to palivizumab. In addition, mutations associated with nirsevimab resistance were not detected.

Lastly, we also document the methodology of RSV-specific serologically mediated immunogenicity evaluation by focus reduction neutralization test (FRNT). These results will provide the potential effectiveness of vaccine developments and consequently the burden resulting from RSV disease in Korea.

Key words: respiratory syncytial virus, clinical isolate, syncytium formation, genotype, whole-genome sequencing, immunogenicity, foucus reduction neutralization test

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I. INTRODUCTION

1. Respiratory Syncytial Virus (RSV)

In newborns, kids, and adults with immunodeficiency or cardiac disease, Respiratory Syncytial Virus (RSV), recently renamed human orthopneumovirus, is a very important viral respiratory pathogen and is recognized as a major threat to the older population[1-6]. According to estimates, the virus causes 30 million episodes of acute lower respiratory tract infections (RTIs) worldwide and more than 50,000 infant deaths each year[7]. RSV infections consistently occur as annual or biennial epidemics around the world. People of all ages can contract the infection and experience a variety of clinical outcomes, from mild upper respiratory tract infections to severe pneumonia or bronchiolitis[8, 9] (Figure 1).

2. RSV treatments

With the exception of Synagis® or Ribavirin nebulizer, there are no licensed vaccinations or treatments available (Figure 2). Palivizumab inhibits fusion by specifically targeting a highly conserved epitope on the fusion protein, which is only utilized for passive immunization of high-risk newborns owing to

its cost and modest efficacy[10, 11]. Moreover, the effect is limited to reducing the hospitalization rate of severe RSV by 50% rather than RSV infection itself and strains resistant to palivizumab have recently been reported. Currently, only supportive care—such as providing oxygen and nutrition—is used to treat severe lower respiratory tract infections brought on by RSV infections. Due to the absence of an effective vaccine and with limited options for therapeutic interventions, uncontrolled epidemics of RSV occur annually worldwide.

In the past 5–10 years, there have been tremendous efforts with RSV vaccine candidates in clinical or preclinical development. Including passively-acquired maternal neutralizing antibodies[12, 13] and administered human immunoglobulin showed its protective effects against RSV[14] (Figure 2). Furthermore, a humanized anti-RSV-F monoclonal neutralizing antibody was shown to provide significant defense against RSV disease in high-risk infants under 2 years of age in controlled clinical trials[10, 15]. Currently, several pharmaceutical companies, including Pfizer, GSK, and Moderna, are making intense efforts to develop a safe and effective RSV vaccine (Figure 3). These companies are conducting clinical trials in the United States, and Pfizer and GSK are awaiting FDA approval in May 2023, indicating that they are nearing the end of the race to develop an RSV vaccine.

However, despite the progress in RSV vaccine development, the evaluation methods for RSV immunogenicity specific to South Korea are still in their early stages and lagging behind. This suggests that South Korea needs to further develop and establish its own evaluation methods to assess the immunogenicity and effectiveness of RSV vaccines within its population. This is important to ensure that the RSV vaccines are appropriately evaluated and suitable for the South Korean population's specific needs and characteristics.

3. RSV subtypes and structure

RSV has two subtypes, RSV-A and RSV-B, and is a member of the genus Orthopneumovirus in the family Pneumoviridae. It has 10 genes that code for 11 proteins in a non-segmented, negative, single-stranded RNA genome (Figure 4). The viral envelope contains three proteins: the attachment protein (G), the fusion protein (F), and the small hydrophobic protein (SH). The G protein bonds the virus particle to the cell surface by interacting with cellular receptors on the host cell membrane. The protein consists of a central conserved domain, two glycosylated mucin-like regions, and an N-terminal region containing a transmembrane domain and a cytoplasmic domain[16, 17].

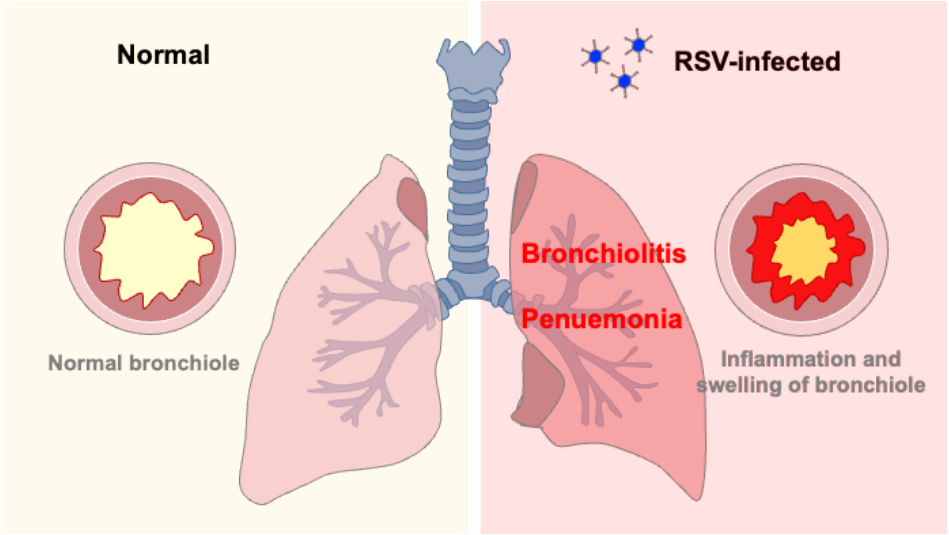
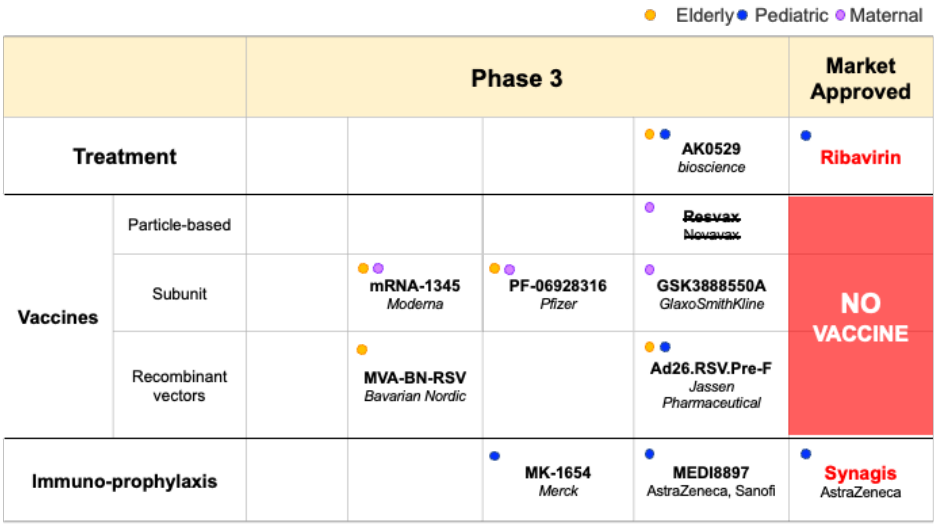


Figure 1. RSV is an important cause of annual epidemics of bronchiolitis and pneumonia. Respiratory syncytial virus (RSV) is the most common pathogen of acute lower respiratory system infection in infants and toddlers worldwide. Almost all infants before the age of 3 develop RSV infection at least once, accounting for the next common cause of malaria (12%) among single causes of death in infants under the age of 1 worldwide (7%).



(Domachowske, J. B. et al., *Infectious Diseases and Therapy*, 2021_updated)

Figure 2. RSV antivirals, vaccines, and mAbs under phase 2/3 clinical development. World Health Organization (WHO) has recently recognized the global importance of RSV and focused on developing vaccines and treatments that are effective for RSV, and many drugs are being studied in stages 2-3 clinical trials.

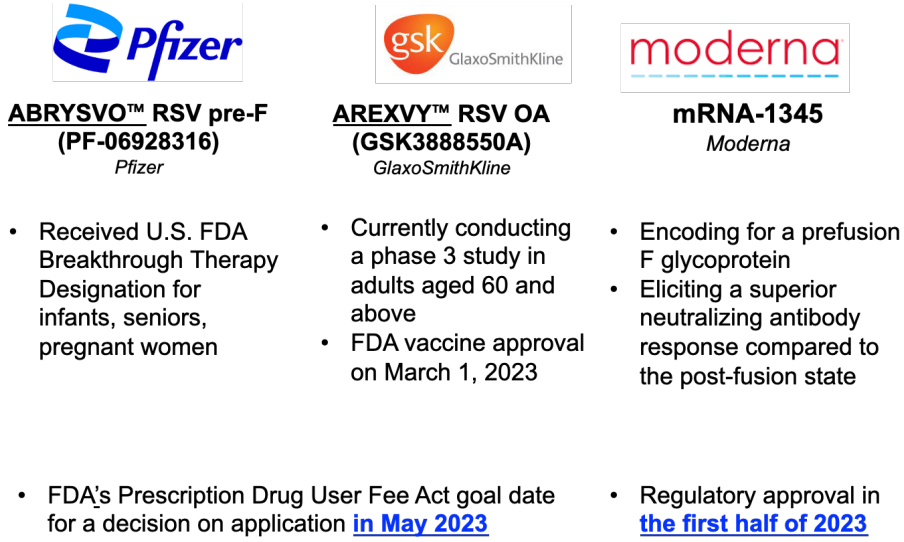
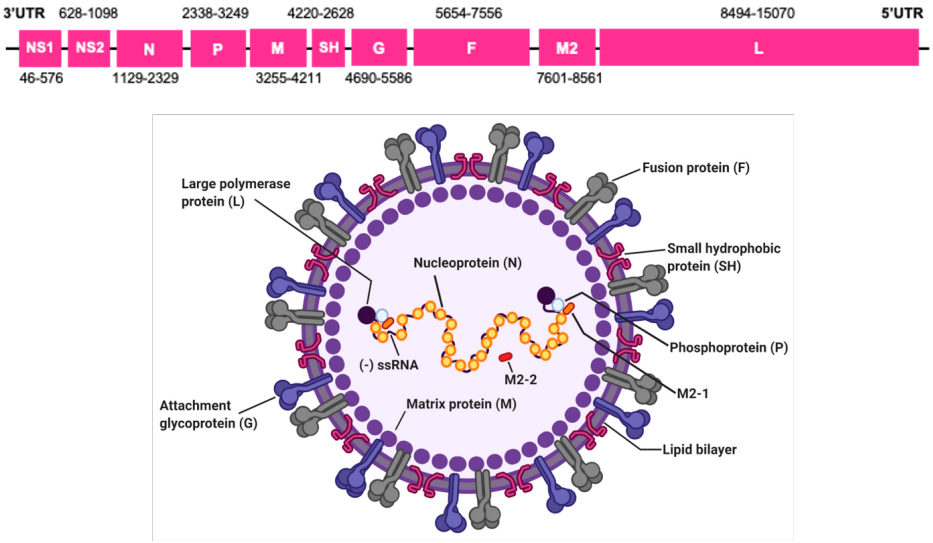


Figure 3. Upcoming RSV vaccines. Nowadays, the intense efforts are underway to develop a safe and effective RSV vaccine from various pharmaceutical companies such as pifzer, GSK, mordena. As RSV vaccines are being actively studied in U.S. trials, the race to an RSV vaccine is nearly over, since Pfizer and GSK are waiting for the FDA's approval in MAY 2023.



(Jung, H. E. et al.,*Viruses*, 2020)

Figure 4. The structure of respiratory syncytial virus (RSV). The RSV genome is 15.2 kb of nonsegmented negative-sense RNA encoding 11 viral proteins. The viral envelope of RSV contains three transmembrane glycoproteins: attachment glycoprotein (G), fusion protein (F), and small hydrophobic protein (SH). Matrix proteins (M) are present on the inner side of the viral envelope. Viral RNA is tightly encapsidated by nucleoproteins (N) and the large proteins (L), phosphoproteins (P), and M2-1 proteins that mediate viral RNA transcription. M2-2 protein regulates viral RNA synthesis.

4. Demand for RSV whole genome sequencing

Accurate RSV genotypes and epidemiological dynamics are critical for selecting the variable and conserved regions of RSV needed for research on RSV pathophysiology and vaccine development, and for determining the treatment and preventive effects of genotypes on the contemporary RSV strains. The two mucin-like sections that flank the core domain of RSV-A and RSV-B only share 67% of their nucleotide sequences and 53% of their deduced amino acid sequences, according to the sequencing of the G gene[18]. The two mucin-like regions are thus excellent research targets for RSV evolution. Based on these genetic variations, both subtypes are further divided into genotypes. The genotypes GA1-7, SAA1-2, NA1-4, and ON1 of RSV-A have been defined, whereas the genotypes GB1-5, SAB1-4, URU1-2, BA1-12, and THB of RSV-B have been reported[19-27]. The G protein together with the fusion (F) protein are important targets of human protective antibody responses[28, 29], and it is believed that alterations in this area are prompted by pressure to evade host immune responses. For this reason, historically RSV molecular epidemiology has concentrated on the 900-bp region encoding the G protein[29, 30] which has clinical and immunologically important implications.

Although studies of RSV sequence variability have focused on G gene variability, transmission studies over short periods require the stronger evolutionary signal provided by the full virus genome sequence due to RSV's rapid infection pace but the relatively low evolutionary rate (15,200 nucleotides [nt]: 11 open reading frames [ORFs] and noncoding regions). Full-genome sequencing is now possible for a number of viruses, including RSV[31-36], norovirus[37], and the Middle East respiratory syndrome (MERS) coronavirus, thanks to advancements in primer design, sequencing technology, and sequence assembly algorithms[38, 39]. To date, most of the RSV genotyping studies have been conducted in the United States and Europe, whereas studies in Asia, including Korea, are peripheral and limited to small-scale studies. Therefore, we intend to

examine the molecular genetic dynamics of RSV infections that occur frequently in children through the WGS method. This can be used as basic data to predict the effectiveness of several RSV-related treatment drugs and preventive substances currently under development in Korea.

5. RSV *in vitro* culture system

RSV-Long and RSV A2, which were isolated from the population in 1956 and 1961, respectively, were used in the majority of published studies on RSV[40, 41]. These strains are not representative of currently circulating genotypes as they have a convoluted passage history which may have resulted in an accumulation of mutations. This may complicate their use in studies on viral pathogenesis and intervention strategies. Acquiring the low-passage, well-characterized clinical strains is very critical for understanding contemporary RSV.

As a result, we recovered 57 clinical samples from RSV-infected patients during the infectious seasons of 2021–2022 and 2022–2023 in Korea. We have raised, amplified, and stocked the clinical-derived RSV and used them to test their cytopathic effects (CPE). An important side effect of RSV-infected cells is the fusion of the cell membranes of an infected cell with adjacent cells, resulting in a giant cell with multiple nuclei, better known as a syncytium[42]. Syncytia are known to form when an infection is effectively spread along epithelial surfaces with the least amount of immune system contact[43]. Here we have also checked the syncytium formation in patient-derived RSV-infected Hep-2 cells.

6.Focus reduction neutralization test (FRNT)

Neutralizing antibody titer measurement is a critical component of vaccine immunogenicity study. The plaque reduction neutralization test (PRNT) has been the preferred technique by most studies to determine the correlates of

protective immunity to RSV[12, 44-47]. However, PRNT is technically demanding, not easy to automate, and has limitations for screening the large numbers of sera needed for epidemiological investigations. For large-scale studies alternative assays which could be performed in 96-well plates would be preferred. Tremendous efforts have been taken to simplify or shorten plaque assay procedures[48-50]. A rapid, simple, and accurate immunoplaque assay was developed using antibodies and diaminobenzidine (DAB) to visualize the plaques at two days post infection[49]. This manuscript describes the development of RSV focus reduction neutralization test (FRNT), compares its performance with PRNT and shows that the developed mumps FRNT is a more convenient alternative to PRNT.

Thus, our RESEARCH GOAL is to establish a clinical RSV *in vitro* culture system and document the phylogenic analysis and RSV-specific serologically mediated immunogenicity evaluation. The CENTRAL HYPOTHESIS of this study is that understanding clinical RSV characters through the optimized virus culture system and its molecular epidemiology and examining RSV-specific immunogenicity will inform future RSV control strategies and provide the platform for evaluating the efficacy of RSV vaccines.

Our specific aims will test the following hypotheses:
Aim 1: Isolate and culture of RSV from clinical samples
Aim 2: Investigate clinical RSV characteristics and its molecular epidemiology
Aim 3: Evaluate RSV-specific serologically mediated immunogenicity

In summary, our study will establish a virus culture system to identify and characterize patient-derived RSV (Figure 5). Moreover, this study will investigate the molecular epidemiology and neutralizing antibody responses to RSV. We expect that this study will provide important information on the

potential effects of future vaccine programs in reducing virus transmission and consequently the burden resulting from RSV disease.

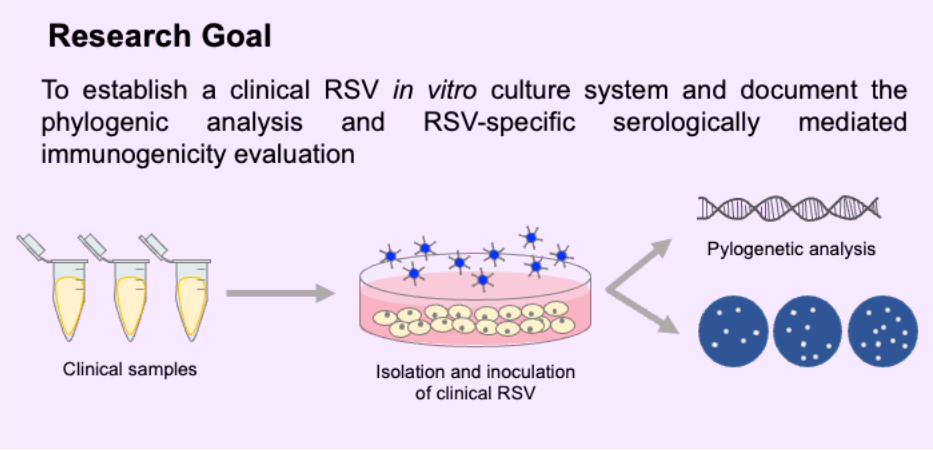


Figure 5. Schematic representation of the research project. The research goal of this study is to establish a clinical RSV *in vitro* culture system and document the phylogenic analysis and RSV-specific serologically mediated immunogenicity evaluation.

II. MATERIALS AND METHODS

Our goal is to establish a clinical RSV *in vitro* culture system and document the phylogenetic analysis. To test the proposed hypothesis, the following materials and methods were utilized:

1. Cell culture

The HEp-2 cell lines were obtained from and cultured to the instructions of ATCC. Cells were cultured in Modified Eagle Medium containing 10% inactivated fetal bovine serum (MEM-10) (Thermo Fisher Scientific, Waltham, The virusA). The virus was isolated from a clinical sample and quantified in a conventional plaque assay on HEp-2 cells. HEp-2 cells were seeded at a concentration of 4 x 10^5 cells/well in clear 6 well plates (Falcon, Corning, NY, USA) one day prior to infection. Cells were washed with PBS and infected with 100 µL of RSV made in DMEM-0. Cells were incubated for 2 hours at 37 °C after which the inoculum was replaced by MEM-2 and incubated for additional days at 37 °C (5% CO_2) until cytopathic effects (CPE) were visible throughout the flask.

2. Virus isolation from clinical samples

This study was approved by the ethical committee of the Yonsei University College of Medicine and Severance Children's Hospital. Clinical samples were collected from children showing symptoms of an RSV- during the winter seasons after written parental consent was given. The secretions were extracted by a nasal swab and/or a nasopharyngeal aspirate. One day prior to inoculation, Hep-2 cells were seeded at a concentration of 4 x 10^5 cells/well in clear 6-well plates. After 2 h of incubation with the 100 µL RSV isolates in DMEM-0 at 37 °C with antibiotics (penicillin/streptomycin (Thermo Fischer Scientific). Plates were incubated for seven days at 37 °C (5% CO_2). After seven

days, the plates were checked for syncytium formation was transferred to a newly seeded plate. A total of 1 mL from wells presenting with syncytia was transferred to a freshly seeded T25, which was incubated until CPE was visible throughout the culture. The supernatant was collected, centrifuged for ten minutes at 1000× *g*, aliquoted, and snap-frozen in liquid nitrogen (passage 0).

3. Immunocytochemistry

Cells were washed with DPBS (CORNING) and fixed in 4% paraformaldehyde (PFA; VWR, Radnor, PA, USA) in the dark for 20 min at room temperature (RT) and permeabilized with 0.5-1% Triton X-100 (Sigma-Aldrich) in DPBS in the dark for an hour at RT. After washing with PBS, the cells were blocked with 1-5% bovine serum albumin (BSA; MACS BSA Stock solution, Miltenyi Biotec) in DPBS in the dark for an hour at RT. The cells were subjected to staining with primary antibodies followed by secondary antibodies. DAPI was used for nuclear staining and the cells were visualized under a fluorescent microscope (Nikon).

4. Flow cytometry

Cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS; CORNING), detached with ACCUTASE (STEMCELL Technologies, Vancouver, BC, Canada), and harvested with autoMACS Running Buffer (Miltenyi Biotec, Bergisch, Gladbach, Germany). The cells were fixed and permeabilized with 4% PFA and 0.5-1% Triton X-100 (Sigma-Aldrich). Cells were incubated with an Anti-Respiratory Syncytial Virus antibody (2F7, ab43812) followed by Alexa Fluor® 488-conjugated Goat Anti-Mouse IgG H&L (ab150113) Alexa 488 secondary antibody. The resulting cells were analyzed on BD FACS Verse II (2-laser, 6-color analyzer, BD Biosciences), and data were evaluated with FlowLogic software (Invai Technologies, Melbourne, Victoria, Australia) using appropriate isotype-matched controls.

5. Scanning Electron Microscopy (SEM)

Specimens were fixed for 24hrs in Karnovsky's fixative (2% Glutaraldehyde, 2% Paraformaldehyde in 0.1M phosphate buffer, pH 7.4) and washed two times for 30min in 0.1M PB. They were postfixed with 1% OsO4 for 2hr and dehydrated in ascending gradual series (50 ~100%) of ethanol and used a Critical Point Dryer(LEICA EM CPD300). They were coated with Platinum by ion sputter (LEICA EM ACE600) and observed with a field emission Scanning Electron Microscopy (MERLIN, ZEISS).

6. Transmission Electron microscopy (TEM)

Specimens were fixed for 12 hours in 2% Glutaraldehyde-2% Paraformaldehyde in 0.1M phosphate buffer(pH 7.4) and washed in 0.1M phosphate buffer, post-fixed with 1% OsO4 in 0.1M phosphate buffer for 2hr and dehydrated with an ascending ethanol series(50, 60, 70, 80, 90, 95, 100, 100%) for 10min each, and infiltrated with propylene oxide for 10min. Specimens were embedded with a Poly/Bed 812 kit (Polysciences), polymerized in an electron microscope oven(TD-700, DOSAKA, Japan) at 65℃ for 12hr. The block is equipped with a diamond knife in the Ultramichrome and is cut into 200 nm semi–thin sections and stained toluidine blue for observation by optical microscope. The region of interest was then cut into 80 ㎚ thin sections using the ultramicrotome, placed on copper grids, double stained with 3% uranyl acetate for 30min and 3% Lead citrate for 7min staining, and imaged with a transmission electron microscopy(JEM-1011, JEOL, Tokyo, Japan) at the acceleration voltage of 80 ㎸ equipped with a Megaview III CCD camera (Soft imaging system-Germany).

7. Primer design

All RSV sequences available with lengths of > 14,000 nt were collected and sorted by group, yielding 138 RSVA and 38 RSVB genomes. The sequences

for each group were pooled and sliced into 33-nt strings with a 1-nt step size. The 33-mers were filtered to remove sequences with ambiguous nucleotides, and the frequency of each sequence within the set was determined. The 33-nt sequences were then trimmed to a calculated melting temperature (T_m) of 58°C, discarding sequences mapping to human rRNA, with GC contents of <30% or >65%, or with a single nucleotide frequency of >60%. The RSV genome was divided into six 3-kb segments overlapping by 300 nt. All sequences were mapped to an RSVA or RSVB reference strain, and the two most frequent primers mapping within 300 nt of the end of each amplicon were selected. The reverse complement of the downstream sequences was prepared. To ensure amplification of the far ends of the genomes, two additional primers were included from the 5=- and 3=-terminal genomic regions. A summary of the primer sequences and their predicted target sequences across all known RSV genomes is presented in Table 1.

8. RNA extraction, RT, and PCR

Viral RNA was extracted with the QIAmp extraction kit (Qiagen, United Kingdom) from a starting NPS specimen volume of 140 μL and a final elution volume of 60 μL. Reverse transcription (RT) of RNA molecules was performed with the forward primers for each of the six amplicons. A separate RT reaction was per- formed for each amplicon. Typically, the 20 μL reaction mixture contained 2 μL of sample RNA. A 5 μL aliquot of the resulting cDNA was used in each 25 μL PCR mixture. The PCR mixture was incubated at 98°C for 30 s, followed by 40 cycles of 98°C for 10 s, 53°C for 30 s, and 72°C for 3.0 min, and a final extension of 72°C for 10.0 min. Following PCR, aliquots of the products were run on a 0.6% agarose gel to monitor amplification success, and the products from the 6 reactions for each sample were pooled for the PacBio Circular Consensus Sequencing platform.

Table 1. Summary of RSV primers used in this study[51]

[a] Primer mapping position in RSVA (GenBank accession number FJ948820) or RSVB (GenBank accession number JQ582843).
[b] T_m (melting temperature) calculated using a Python script that approximates the method of Breslauer et al.[52]
[c] Percentage of full-length RSVA genomes (n = 290) or full-length RSVB genomes (n -=102) showing perfect homology to primer, i.e., 0 mismatches (MM).
[d] Percentage of full-length RSVA genomes (n = 290) or full-length RSVB genomes (n = 102) showing the target sequence for the primer with up to 3 mismatches.

Target	Primer	Sequence (5' to 3')	Strand	Position [a]	T_m (°C) [b]	% with 0 MM [c]	% with 0–3 MM [d]
RSVA	rsvas	ACGCGAAAAAATGCGTACAAC	Plus	1	57.13	18.28	18.97
RSVA	rsva52	TGTGCATGTTATTACAAGTAGTGATATTTG	Plus	266	56.96	95.52	98.97
RSVA	rsva50	GCATGTTATTACAAGTAGTGATATTTGCC	Plus	269	57.51	95.17	98.97
RSVA	rsva117	ATAAGAGATGCCATGGTTGGTTTAAGA	Plus	2849	58.44	95.86	100.00
RSVA	rsva86	AAGAGATGCCATGGTTGGTTTAAGA	Plus	2851	58.43	95.86	100.00
RSVA	rsva175	TTCTCTTAAACCAACCATGGCATCT	Minus	2878	58.43	95.86	100.00
RSVA	rsva39	CTTCTCTTAAACCAACCATGGCATC	Minus	2879	58.22	95.86	100.00
RSVA	rsva1820	GCAGCATATGCAGCAACAATC	Plus	5207	56.95	93.79	98.97
RSVA	rsva1914	CAGCATATGCAGCAACAATCCAA	Plus	5208	58.32	93.10	98.62
RSVA	rsva1644	CAACTCCATTGTTATTTGCCCC	Minus	5674	56.05	89.66	100.00
RSVA	rsva1688	CAACTCCATTGTTATTTGCCCCA	Minus	5674	57.54	89.66	100.00
RSVA	rsva704	ATGTGTTGCCATGAGCAAACTC	Plus	7893	57.95	91.03	100.00

RSVA	rsva731	GCCATGAGCAAACTCCTCACT	Plus	7900	58.49	71.38	99.31
RSVA	rsva341	TTGTCAGGTAGTATCATTATTTTTGGCATG	Minus	8196	58.53	98.97	99.31
RSVA	rsva312	AGGATATTTGTCAGGTAGTATCATTATTTTTGG	Minus	8203	58.08	98.97	100.00
RSVA	rsva374	AAGAGAACTCAGTGTAGGTAGAATGTTT	Plus	10360	57.89	96.55	100.00
RSVA	rsva350	AGAACTCAGTGTAGGTAGAATGTTTG	Plus	10363	56.64	96.55	100.00
RSVA	rsva497	GCTTGATTGAATTTGCTGAGATCTGT	Plus	10620	58.44	95.52	100.00
RSVA	rsva539	ATGCTTGATTGAATTTGCTGAGATCTG	Minus	10622	58.68	95.52	100.00
RSVA	rsva1220	GATTGGGTGTATGCATCTATAGATAACAAG	Minus	12386	57.94	95.86	99.31
RSVA	rsva1232	ATTGGGTGTATGCATCTATAGATAACAAG	Plus	12387	57.17	95.86	99.31
RSVA	rsva364	TTATATATCCCTCTCCCCAATCTTTTTCAAA	Minus	13070	58.32	96.21	100.00
RSVA	rsva385	ATCAGTTATATATCCCTCTCCCCAATCTT	Minus	13075	58.46	96.21	100.00
RSVA	rsva4066	GTTGTATAACAAACTACCTGTGATTTTAATCAG	Minus	14983	57.95	88.97	99.31
RSVA	rsva5632	TAACTATAATTGAATACAGTGTTAGTGTGTAGC	Minus	15063	57.95	29.31	95.17
RSVA	rsvae	ACGAGAAAAAAAAGTGTCAAAAACTAATA	Minus	15223	55.09	17.59	18.28
RSVB	rsvbs	ACGCGAAAAAATGCGTACTACA	Plus	1	57.56	43.14	43.14
RSVB	rsvb3	TGGGGCAAATAAGAATTTGATAAGTGC	Plus	44	58.58	48.04	54.90
RSVB	rsvb1021	GGGGCAAATAAGAATTTGATAAGTGCTATT	Plus	45	58.75	47.06	54.90
RSVB	rsvb33	ATATTAGGAATGCTCCATACATTAGTAGTTG	Plus	2777	57.21	88.24	100.00

RSVB	rsvb71	TAAGAGATGCTATGGTTGGTCTAAGAGA	Plus	2841	58.69	90.20	100.00
RSVB	rsvb50	AGTCTTGCCATAGCCTCTAACCT	Minus	2937	58.57	93.14	100.00
RSVB	rsvb95	CCATTTTTTCGCTTTCCTCATTCCTA	Minus	2963	58.14	95.10	100.00
RSVB	rsvb7884	AGTATATGTGGCAACAATCAACTCTG	Plus	5202	57.48	81.37	100.00
RSVB	rsvb7996	TATGTGGCAACAATCAACTCTGC	Plus	5206	57.70	81.37	100.00
RSVB	rsvb7442	GATGTGGAGGGCTCGGATG	Minus	5548	57.92	75.49	100.00
RSVB	rsvb7423	CCATGGTTATTTGCCCCAGATTTAAT	Minus	5662	57.87	77.45	99.02
RSVB	rsvb3762	AGAGGTCATTGCTTGAATGGTAGAA	Plus	7642	57.98	93.14	100.00
RSVB	rsvb3712	AAGAGCATAGACACTTTGTCTGAAATAAG	Plus	7762	57.89	77.45	100.00
RSVB	rsvb3652	GCTTATGGTTATGCTTTTGTGGATATCTAAT	Minus	8130	58.41	89.22	98.04
RSVB	rsvb3660	GCAATCATGCTTTCACTTGAGATCAA	Minus	8247	58.67	64.71	98.04
RSVB	rsvb32	AAGAAGAGTACTAGAGTATTACTTGAGAGATAA	Plus	10236	57.04	90.20	100.00
RSVB	rsvb52	AAATCCAAATCTTAGCAGAGAAAATGATAG	Plus	10412	56.70	96.08	100.00
RSVB	rsvb47	CCATGCAGTTCATCTAATACATCACTG	Minus	10673	58.13	90.20	99.02
RSVB	rsvb168	TGCATGTCTATATGTACATATTATTGTGACAAG	Minus	10746	58.25	91.18	99.02
RSVB	rsvb651	ATCGACATTGTGTTTCAAAATTGCATAAG	Plus	12640	58.40	81.37	100.00
RSVB	rsvb165	TTCAAAATTGCATAAGTTTTGGTCTTAGC	Plus	12653	58.06	88.24	100.00
RSVB	rsvb27	TTAATGAACATATGATCAGTTATATACCCCTCT	Minus	13088	57.88	79.41	100.00

RSVB	rsvb60	AACTTAAAACTGTGACAGCCTTTTATTCT	Minus	13325	58.08	89.22	100.00
RSVB	rsvb1199	ATAGTACACTACCTGTTATTTTAATCAGCTTCT	Minus	14977	58.56	88.24	100.00
RSVB	rsvb989	TATAGTACACTACCTGTTATTTTAATCAGCTTC	Minus	14978	57.57	88.24	100.00
RSVB	rsvbe	ACGAGAAAAAAAAGTGTCAAAAACTAATGT	Minus	15216	57.47	5.88	6.86

9. PacBio Circular Consensus Sequencing

Sequencing of the pooled amplicons was performed with the PacBio Circular Consensus Sequencing platform. Samples were multiplexed at 15 to 20 per MiSeq run and processed as paired-end reads (2 x 149 nt), generating approximately 1.5 million reads per sample. Raw sequence data were processed with QUASR to remove low-quality (< median Phred 35) and adapter-containing reads, and *de novo* assembly with SPAdes was performed. RSV contigs were identified by BLASTN analysis, and low-coverage contigs were excluded. Where necessary, partial but overlapping genome contigs were combined using Sequencher (v5.2.4). All final viral genomes were examined for appropriate assembly based on the length and the presence of the expected intact RSV open reading frames.

10. Reference data set

A comprehensive RSV genome data set was generated from the GenBank database using as a starting set all reported RSV genomes. The search was conducted on 28 September 2014 using the search term “txid11250 [Organism]) AND 13500[SLEN]: 17000[SLEN].” Genomes with multiple ambiguous bases, lacking country of detection or date of collection (year), or from patent depositions were excluded. The newly sequenced Kilifi RSV genomes for each group were combined with those from GenBank in the subsequent analysis. Thinned representative reference sets were prepared by using the usearch algorithm.

11. Phylogenetic analyses

Phylogenetic trees of the genome sequences and selected genomic regions were constructed using the Bayesian methods in MrBayes program v3.2.1 (http://mrbayes.sourceforge.net/index .php) under the general time reversible

model of evolution. RSVA and RSVB were analyzed separately using both the total data set and the thinned data sets. The viruses within the groups were assigned to genotypes based on the clustering pattern of the G ORF portion sequences with reference sequences representative of the previously described RSV genotypes: for RSVA, strains representing GA1-7, SAA1, and ON1, and for RSVB, strains to represent GA1-4, SAB1-SAB4, and BA (11, 33–35). Phylogenetic trees were visualized in FigTree v1.4.2.

12. Focus reduction neutralization test (FRNT)

The FRNT procedure for determining the titer of RSV-neutralizing antibodies has been described previously[46]. The method incorporated a step in which serum samples were incubated at 56°C in a heat block for 30 mins to inactivate complement cascade proteins. Each serum sample was repeatedly diluted 2 or 10-fold over ten consecutive dilutions and mixed with an equal volume of 50 plaque-forming units (pfu) of RSV. The virus-serum mixture (200 μL per well) was dispensed over a confluent monolayer of Hep2 cells in a 6-well culture plate, incubated at 37°C for 1 hour. Inocula were then removed, and 3 mL of Methylcellulose overlay medium was added. After incubating the plates for four days at 37 °C, the overlay medium was removed, and the cell monolayers were fixed with 10% formalin solution. The RSV monoclonal antibody used in the focus assay was the same as the one used in previous immunocytochemistry (ICC) experiments.

13. Statistical analyses

Statistical analysis and calculation of sample size were performed using Prism 8 software (GraphPad Software, La Jolla, CA, USA). All data demonstrated a normal distribution and similar variation between groups; hence, only parametric tests were required. The exclusion criterion was set to be 1.5 s.e.m. from the mean. No data met this criterion, and thus all data points were

included in the analysis. In each experiment, the sample size was determined using the expected difference and estimated standard deviation, with a two-sided significance of 0.05 and a power of 0.90. Standard unpaired student t-test (two-tailed, 95% confidence intervals) was performed for comparisons between two groups in flow cytometry analyses. Repeated Measures ANOVA followed by multiple comparisons with Bonferroni-post comparison was used for the results comparing three groups. Flow cytometry experiments were performed at least in triplicate with three independent experiments. Investigators were blind to the assessment of the analyses of cell and molecular biological experiments when comparisons between groups were made. All data were presented as mean ± standard error of the mean (s.e.m). A *P* value of less than 0.05 (* $P < 0.05$) was considered statistically significant.

III. RESULTS

1. Isolate and culture of RSV from clinical samples

The general strategy of aim 1 is to isolate and culture RSV from clinical samples. Residual samples of diagnostic respiratory samples of patients who visited the Yonsei University College of Medicine and Severance Children's Hospital for lower respiratory tract infections were collected. Samples for the 2021-2022 season (10 cases) and 2022-2023 season (47 cases) were collected during the respiratory virus epidemic (October to February).

In order to optimize the RSV culture system, the protocols of papers using clinical samples over the past 10 years were referred to (Table 2). Our experimental conditions were established by referring to the methods of those papers. First, the most paper used the Hep-2 cell line for RSV inoculation. For the culture media, the majority of papers used 10% FBS MEM. Regarding clinical samples, most of them used Nasopharyngeal aspirates or nasal swap and for the virus transportation and time, some of them used special virus transport media, but most of them used ice only. The virus inoculation condition varies a lot, however, they usually used serially diluted clinical isolates. Dates for detecting CPE after RSV inoculation range from day 2 to 3 and up to 7 days.

After optimizing and establishing the culture protocol according to the reference, we purify the RSV from samples and inoculate them into Hep-2 cells. HEp-2 cells were seeded in 4.5 x 10^5 cells/well concentrations in a 6-well culture plate and infected with 100-300 uL virus inoculum along with MEM with 2% FBS. The virus was incubated in a 37°C 5% CO2 incubator for 60 minutes, an additional 1 mL of 2% FBS MEM was added, and further incubated in a 37°C CO2 incubator for 4 to 5 days (Figure 6A). After virus inoculation, we followed up on morphological changes of cells confirming that it showed the typical syncytium formation and also the cytopathic effects (CPE) (Figure 6B, C).

There were 9 RSV (+) only groups without duplicate infections of other viruses in the 21-22 season, and two samples showed cytologic effects (CPE) of viruses. Of the 47 samples in the 22-23 season, a total of 34 were RSV (+) groups, and 7 were samples showing CPE. To sum up, a total of 9 RSVs were amplified (Figure 7) and are currently being stored in a -80°C deep freezer.

Table 2. List of papers on clinical RSV *in vitro* culture system

Cell	Culture media	Clincal sample	Virus transportation/ time	Virus inoculation condition	Reference
Hep-2	5% FBS Opti-MEM	Clinical swab samples	N/A	Serial dilutions of virus stock	Jo, W. K. et al., PNAS, 2021
Vero	10% FBS DMEM				
Hep-2	2% FBS DMEM	Nasopharyngeal aspirates	N/A	Treated with penicillin (100 units/mL) and streptomycin (100 ug/mL) before being inoculated	Zhao, T. et al., Brazilian Journal of Infectious Diseases, 2020
Hep-2	10% iFBS DMEM	Nasal swab and/or nasopharyngeal aspirate	Stored at 4 ◦C for less than 10 h	50 µL of a 1 dilution series of virus in DMEM w/o FBS	Van der Gucht, W. et al., Viruses, 2019
Hep-2	N/A	Nasopharyngeal aspirates	On ice/ within <3 h after collection	HRSV-positive NPAs	Gagliardi, T. B. et al., Intervirology, 2017
Hep-2	10% iFBS DMEM	Obtained from BEI resources	N/A	N/A	Van der Gucht, W. et al., Virology journal, 2017
Hep-2	10% FBS MEM with L-glutamine, Earle's salts and a penicillin G-streptomycin sulfate-amphotericin B solution	HEp-2 supernatants of RSV clinical isolates	N/A	Tenfold serial dilutions of P1 supernatants	Stokes, K. L., et al., Journal of virology, 2011
Hep-2	10% FCS DMEM with 50 µg/mL Gentamicin	Nasal aspirates	Virus transport medium (DMEM, 25 mM HEPES, 50 µg/ml gentamicin, 0.22 M sucrose, 30 mM MgCl2, 0.5 mg/ml fungizone)	Vortexed & sonicated for 10 mins in an ultrasonic water bath and centrifugated at 440 × g for 10 min at 4°C	Villenave, R. et al., Virology Journal, 2011

Cell	Cytopathic effects (CPE) detection	Experiments	Reference
Hep-2 Vero	90% confluent HEp-2 cells in 96-well plates and incubated for 5 days	Multistep Growth Curve Analysis,Sequencing and Phylogenetic Analyses, Cell-to-Cell Fusion Assay, Microneutralization Assay (palivizumab)	Jo, W. K. et al., PNAS, 2021
Hep-2	CPE negative samples at the seventh day -inoculated for 2 more passages CPE positive samples recorded and stored at −80 ◦ C for future use	Indirect immunofluorescence assay, RT-PCR, Sequencing and Phylogenetic Analyses, Analysis of deduced amino acid sequences	Zhao, T. et al., Brazilian Journal of Infectious Diseases, 2020
Hep-2	After 2 hr of incubation at 37 ◦C 50 µL of 2% FBS DMEM, antibiotics (penicillin/streptomycin, moxifloxacin) and anti-fungals (Fungizone) ~ 7 days	RSV-A and RSV-B Subtyping, Sequencing and Phylogenetic Analysis,Viral Replication Kinetics, Infectious Virus Production, Thermal Stability Assay, Cell-to-Cell Fusion Assay, Plaque Reduction Assay	Van der Gucht, W. et al., Viruses, 2019
Hep-2	Of 238 primary HRSV isolates, only 13 (5.5%) propagated in HEp-2 cells No cytopathic effect in P2 and IF negative: unsuccessful 70% confluent HEp-2 cell in a small volume of DMEM and left to adhere for 2 hr on 37 °C	Cell-to-Cell Fusion Assay, RT-PCR	Gagliardi, T. B. et al., Intervirology, 2017
Hep-2	70% confluent HEp-2 cell in a small volume of DMEM and incubated for 2 hr on 37 °C Afterwards, 2% iFBS DMEM and iFBS were added CPE detection: 2–3 days after inoculation	Single infection cycle, Drug treatment, Viability testing, Fluorescence microscopy and image analysis	Van der Gucht, W. et al., Virology journal, 2017
Hep-2	HEp-2 cells in six-well plates >50% cytopathic effect (CPE) was harvested for sequential passage in HEp-2 cells	BALB/c Mice in vivo study	Stokes, K. L., et al., Journal of virology, 2011
Hep-2	Supernatants: either snap frozen in liquid nitrogen or used directly to inoculate HEp-2 cells (Stock) Viruses were harvested after 48 – 72hr by scraping attached cells into the medium,	RSV G gene sequencing,Primary pediatric bronchial epithelial cells (PBECs) infection,	Villenave, R. et al., Virology Journal, 2011

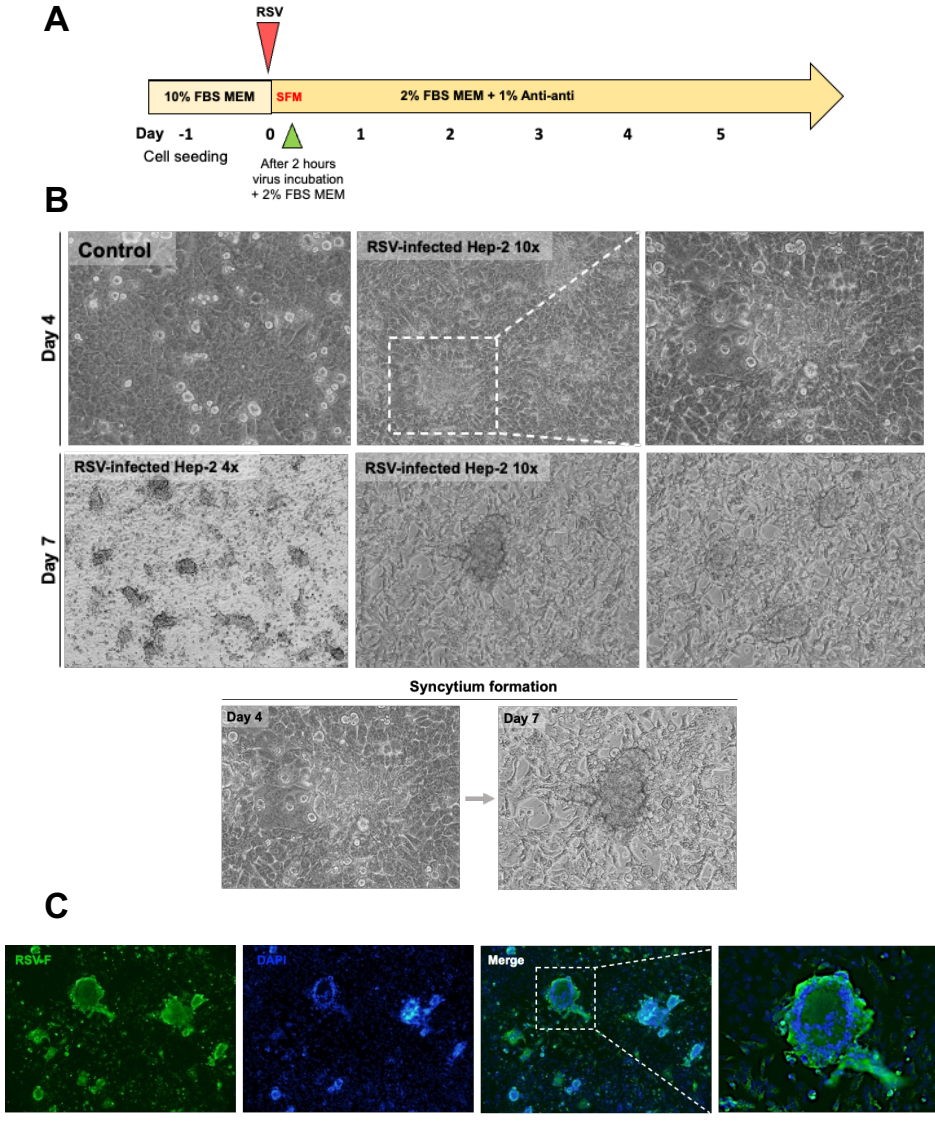


Figure 6. Isolation and culture of clinical RSV. (A) Schematic representation of clinical RSV inoculation protocol. Cells were inoculated by clinical RSV isolates for 2 hours at 37 ˚C after which the inoculum was replaced by MEM-2 and incubated for additional days at 37 ˚C (5% CO_2) until cytopathic effects (CPE) were visible throughout the flask. (B) Morphological changes showing the emergence of syncytium in RSV-infected Hep-2 cells at days 4 to 7. (C) The expression of RSV F protein was determined by Immunocytochemistry. DAPI was used as a nuclear counterstain. The boxes in the figure are enlarged to show increased details, respectively.

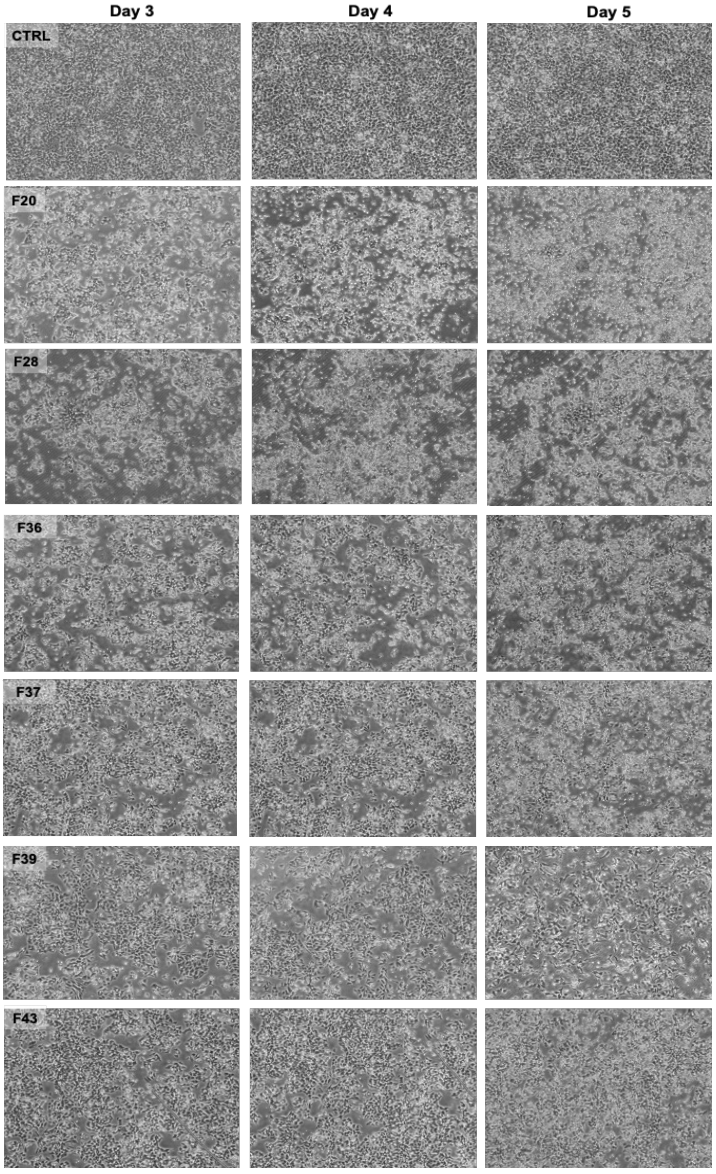


Figure 7. RSV (+) only groups without duplicate infections showed cytologic effects (CPE). Cells were inoculated by clinical RSV isolates for 2 hours at 37 °C after which the inoculum was replaced by MEM-2 and incubated for additional days at 37 °C (5% CO_2) until cytopathic effects (CPE) were visible throughout the flask. Of the 47 samples in the 22-23 season, a total of 34 were RSV (+) groups, and 7 were samples showing CPE. Totally, 9 RSVs were amplified and are currently being stored in a -80°C deep freezer.

2. Investigate clinical RSV characteristics and its capacity for syncytium formation

The objective of this aim is to investigate clinical RSV characteristics and its capacity for syncytium formation. We examined RSV-specific F protein expression by immunocytochemistry and flow cytometry. Furthermore, cell-to-cell fusion was checked to demonstrate the infectibility of clinical RSV.

Syncytium formation has long been considered a typical characteristic of RSV infection in immortal cell lines, and it has been used as a measure of the activity of the fusion protein[43]. HEp-2 cells were infected with 100 uL of RSV inoculum and incubated for 4-5 days. Syncytium of RSV-infected HEp-2 cells was observed through scanning electron microscope (SEM) that can observe changes in cell shape and transmission electron microscope (TEM) that can observe internal organelles, respectively (Figure 8, 9). Observations of RSV-infected Hep-2 cells with electron microscopy showed that poly nucleases (syncytium) containing multiple nuclei specifically expressed in RSV-inoculated cell lines.

Characteristic patterns in the cytoplasm and nucleus were also observed through ICC, which stained infected viral antigens using monoclonal antibodies specific to the Fusion protein (F-protein) of RSV. Unlike cells cultured in a single layer, confocal microscopy was used to observe the three-dimensional polynucleate formed after viral infection, and the cytoplasm and nucleus shape of RSV-infected Hep-2. As shown in Figure 10, the virus was distributed around the cell membrane, with several nuclei clustered within the poly nucleus. Through orthogonal projection analysis and the Z-stack technique, which could observe monolayers of cells, the condensed nucleus is surrounded by the cell membrane. In addition, the cell membrane showed that apoptosis is occurring, in which the cell membrane is ruptured due to RSV infection.

Next, we confirmed whether CPE and syncytium formation occur in a virus inoculum-dependent manner (Figure 11). Cells were inoculated with 100, 200,

300, 400, and 500 uL of clinical made in DMEM-0. On day 4, cells were fixed, fluorescently stained, and analyzed with fluorescence microscopy or flow cytometry. As the concentration of RSV increased, DAPI, which indicates the number of cells, decreased, and Syncytium formation increased. This means that CPE and syncytium formation is working in RSV-concentration dependent manner. Next, RSV-infected Hep-2 cells were further examined its infectibility by FACS analysis (Figure 12). FACS analysis of cells showed that more than 70% of cells were infected at concentrations of 100 and 200 uL, 80% at concentrations of 300 and 400 uL, and 90% at concentrations of 500 uL of clinical RSV inoculum.

Comprehensively observed, it was confirmed that clinical RSV was successfully infected and proliferated in HEp-2 cell lines, as the cell lines infected with patient-derived RSV showed CPE and syncytium formation during RSV infection.

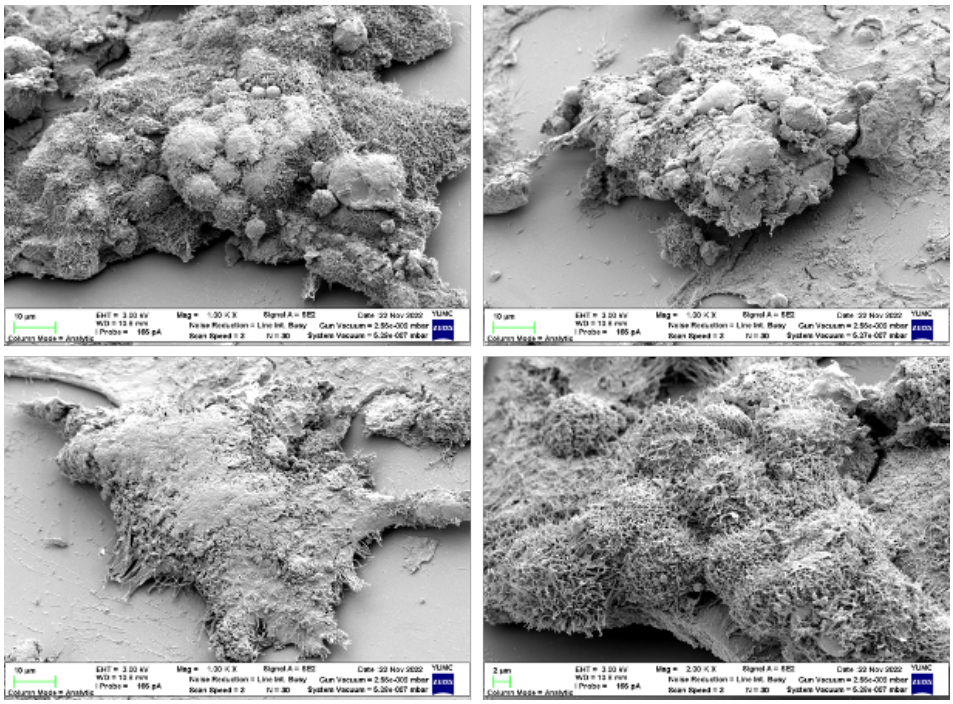


Figure 8. Morphological changes of clinical RSV-infected Hep-2 cells by scanning electron microscope (SEM). Cells were inoculated by clinical RSV isolates for 2 hours at 37 ˚C after which the inoculum was replaced by MEM-2 and incubated for additional 4 days at 37 ˚C (5% CO_2) until syncytium formation was visible throughout the flask.

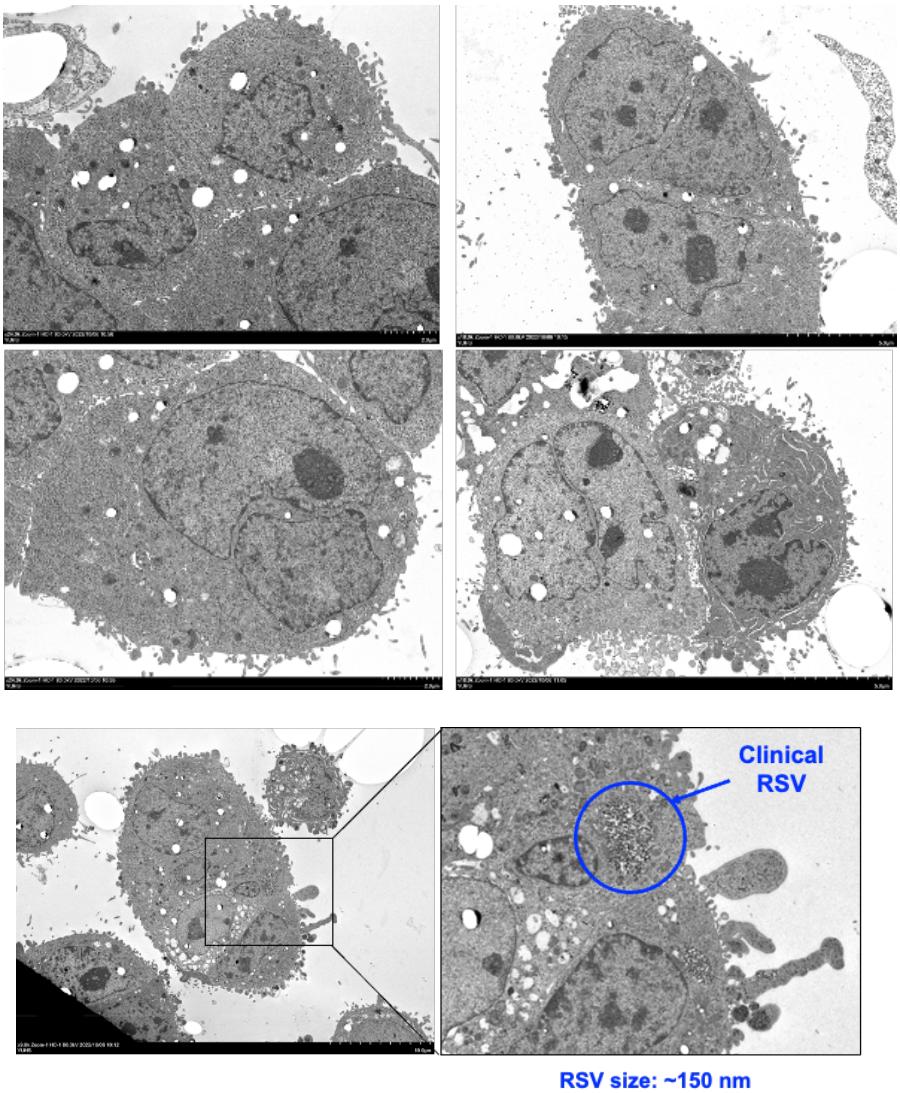


Figure 9. Ultrastructural features of RSV-induced syncytium were documented by Transmission Electron Microscope (TEM). Cells were inoculated by clinical RSV isolates for 2 hours at 37 ˚C after which the inoculum was replaced by MEM-2 and incubated for additional 4 days at 37 ˚C (5% CO_2) until syncytium formation was visible throughout the flask.

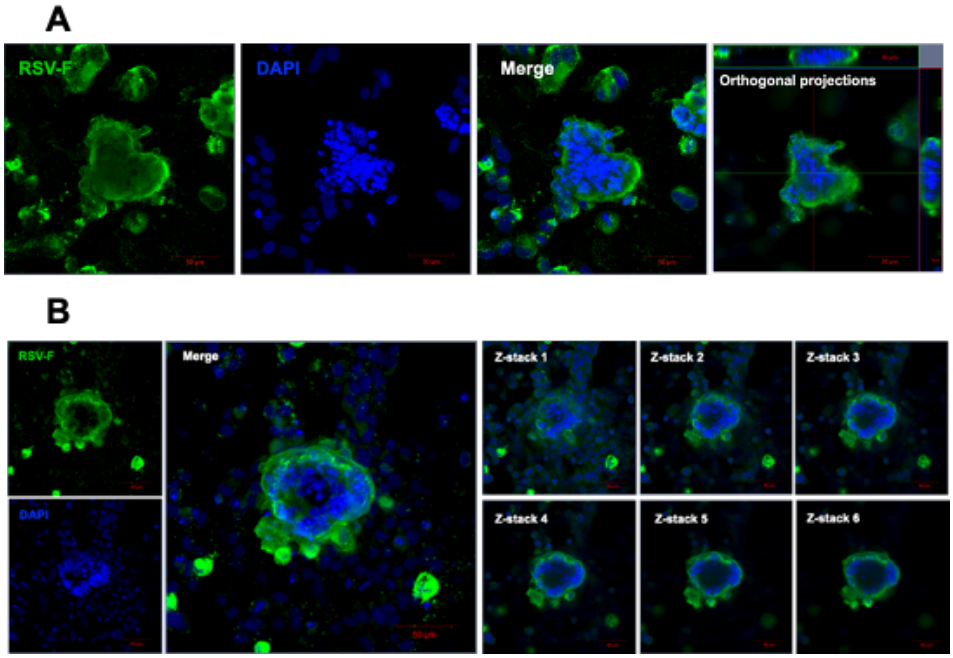


Figure 10. RSV- induced syncytia were detected by confocal images. Cells were inoculated by clinical RSV isolates for 2 hours at 37 ˚C after which the inoculum was replaced by MEM-2 and incubated for additional 4 days at 37 ˚C (5% CO_2) until syncytium formation was visible throughout the flask. Confocal images were taken syncytia at 20× magnification. Cells were visualized with an Alexa Fluor 488-labeled anti-F protein antibody (green) and DAPI nuclei staining (blue). The orthogonal projection and z-stacks show details respectively.

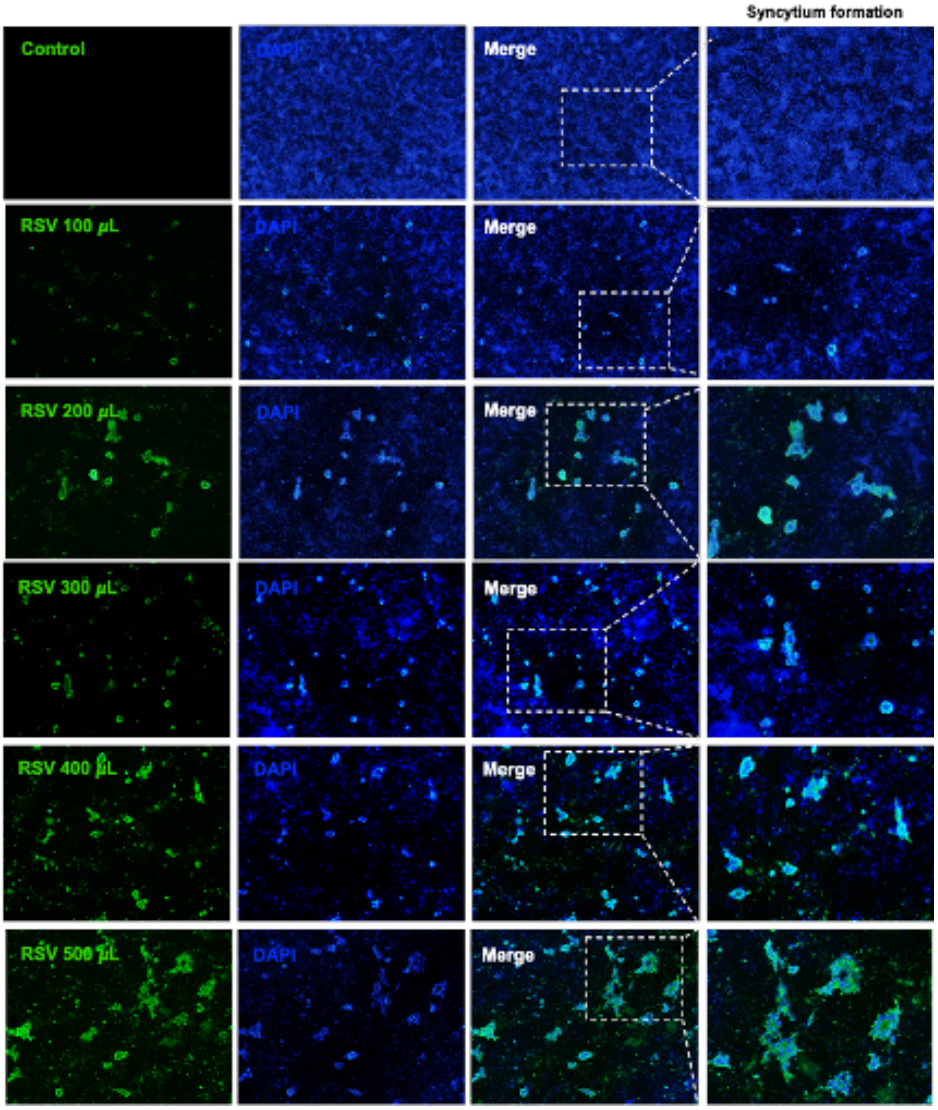


Figure 11. Syncytium formation induced in clinical RSV isolates dependent manner. Cells were inoculated by 100, 200, 300, 400, and 500 uL of clinical RSV isolates for 2 hours at 37 ˚C after which the inoculum was replaced by MEM-2 and incubated for additional 4 days at 37 ˚C (5% CO_2) until syncytium formation was visible throughout the flask. Confocal images were taken syncytia at 20× magnification. Cells were visualized with an Alexa Fluor 488-labeled anti-F protein antibody (green) and DAPI nuclei staining (blue). The boxes in the figure are enlarged to show increased details, respectively.

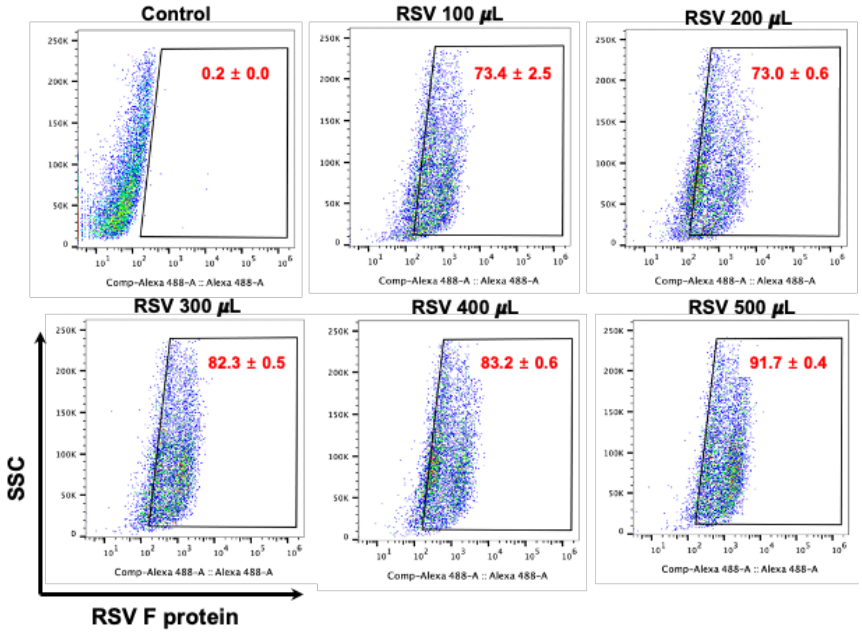


Figure 12. Infectibility of clinical RSV by flow cytometry. Cells were inoculated by 100, 200, 300, 400, and 500 uL of clinical RSV isolates for 2 hours at 37 ˚C after which the inoculum was replaced by MEM-2 and incubated for additional 4 days. All results were expressed as mean ± s.e.m., with 3 independent experiments. Statistical analysis was performed by a one-way ANOVA for comparisons followed by Dunnett's multiple comparisons tests (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

3. Clinical RSV phylogenetic analysis in South Korea

The molecular epidemiology study conducted in South Korea aimed to investigate the trends of RSV epidemics before and after the COVID-19 pandemic. The occurrence of RSV was nearly zero during the COVID-19 pandemic but became prevalent again from the 2021-2022 season onwards. The study collected RSV nucleic acid samples before and after the pandemic and performed whole genome sequencing and phylogenetic analysis. In the pre-pandemic seasons, 32 RSV-A and 52 RSV-B samples were used for analysis, while in the post-pandemic seasons, 34 RSV-A and 37 RSV-B samples were used. In total, 66 RSV-A and 89 RSV-B samples were analyzed. The success rate of amplicon amplification was over 50%, with a total of 155 successful amplicons out of 285 samples.

Two sets of reverse transcription and PCR primers were selected from all available RSVA and RSVB genomic sequence data based on frequency, location, and predicted PCR function (see Table 1 for further details). The general pattern of primer sites and the locations of primer targets in RSVA and RSVB genomes is shown in Figure. 13B. Actual PCR results are shown in Figure. 13C for RSVA and RSVB samples, with PCR products of the expected size obtained for all 6 amplicons. These primers were used as part of a deep-sequencing process for RSV combining the full cDNA preparation and genome amplification, sequencing with PacBio SMRT, and *de novo* assembly.

The RSV G gene sequence is known to be highly variable, and strains are classified into different genotypes based on the second hypervariable region of the G gene. Fifteen genotypes of RSV-A and 30 genotypes of RSV-B have been identified to date. The phylogenetic analysis of the G gene showed that globally, the same predominant clades of RSV circulated, and the distribution of old clades could change with the emergence of different RSV strains. The analysis of RSV-A G protein sequences revealed clustering with the ON1 genotype in both pre- and post-pandemic samples (Figure 14). For RSV-B G protein sequences, the pre-

pandemic and post-pandemic samples were divided into two large clusters but both clustered with the BA9 strain (Figure 15).

The RSV fusion protein (F) plays a crucial role in virus entry by mediating viral and host membrane fusion. Antibodies targeting the F protein can prevent viral entry and reduce RSV-related disease (Figure 16). Prefusion F-specific antibodies have been found to have stronger RSV-neutralizing activity compared to post-fusion antibodies. Palivizumab is currently the only pharmaceutical agent for prophylaxis against RSV infection, and it interacts with the RSV F protein's epitope in site 2. However, continual exposure to palivizumab has led to the emergence of RSV mutations conferring resistance, particularly at amino acid positions 272, 275, or 276 in the F protein (Figure 17).

The study presented an example of a 1-year-old boy with RSV bronchiolitis who was planned for a liver transplant but developed severe RSV pneumonia (Figure 18). Treatment with oral ribavirin and palivizumab led to a full recovery, but after transplantation, pneumonia recurred, and palivizumab-resistant RSV mutants at position 272 of the F protein were detected. The analysis of RSV-A and RSV-B F protein sequences showed clustering with the ON1 genotype and BA9 strain, respectively, in both pre- and post-pandemic samples (Figure 19, 20).

In whole genome sequencing analysis of RSV-A, genotypes showed close clustering with ON1 in pre-pandemic samples and ON1/NA1 in post-pandemic samples, respectively (Figure 21). In RSV-B WGS sequences, both samples were assigned to the BA genotype (Figure 22).

There have been no significant changes detected in the RSV strain during the pandemic period. Nevertheless, one case of palivizumab mutation has been identified, which emphasizes the significance of monitoring genotypic trends. Our NGS method can not only quickly track these genotypic trends, but also provide information on the clinical effectiveness of existing and/or recently developed RSV-specific agents.

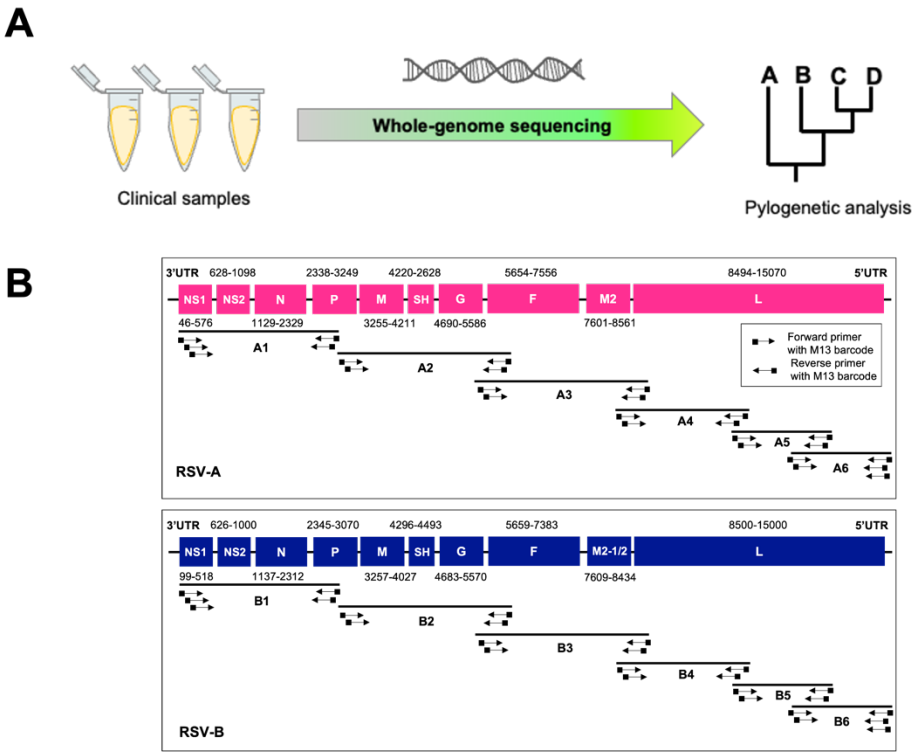


Figure 13. The pattern of primer sites and actual PCR results. (A) Flowchart of the RSV sequencing process. (B) PCR primer target sites in RSVA and RSVB[51]. The primer target sequences in representative RSVA (left) and RSVB (right) viruses were determined. Circular markers indicate the positions of primer target sites in the test genome color-coded by a number of mismatches with the primer; gray bars indicate the lengths and positions of the predicted products. (C) Six examples of reverse transcription-PCR function. The DNA products of reverse transcription and PCR amplification of six samples were resolved by agarose gel electrophoresis. Sizes of some of the molecular size markers (in base pairs) are indicated to the left of the gel. Lane m, molecular size markers; lanes 1 to 6, individual 2- to 3-kb RSV amplicons 1 to 6, respectively.

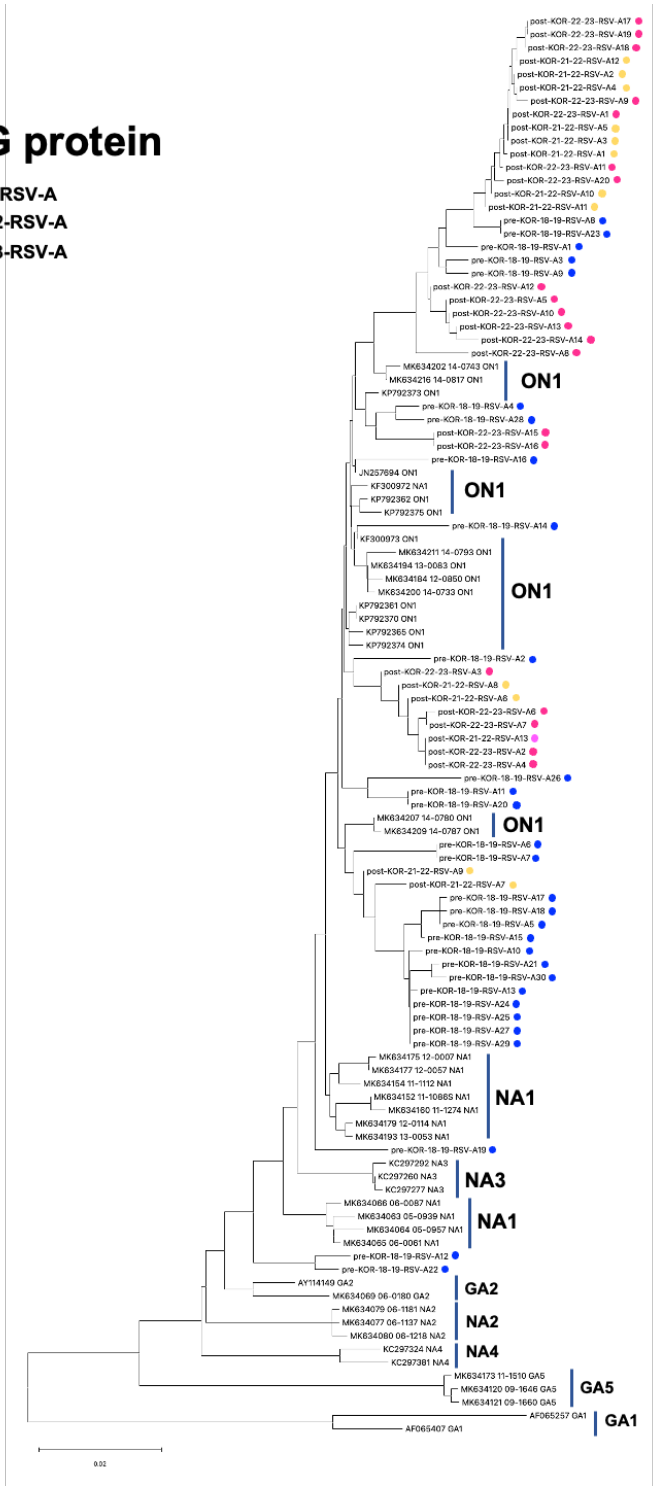


Figure 14. Phylogenetic trees for RSV-A G protein in the pre/post-pandemic era. The phylogenetic trees were constructed with maximum likelihood with 1000 bootstrap replicates using MEGA 11 software. The trees are based on the fragment of the G protein of RSV-A strains, consisting of the second hypervariable region. Nucleotide sequences of the clinical isolates (indicated with SEV) were compared to reference strains found on GenBank (indicated with genotype and accession number). Bootstrap values greater than 70% are indicated at the branch nodes and the scale bar represents the number of substitutions per site.

RSV-B G protein

- pre-KOR-19-20-RSV-B (n = 52)
- post-KOR-21-22-RSV-B (n = 37)

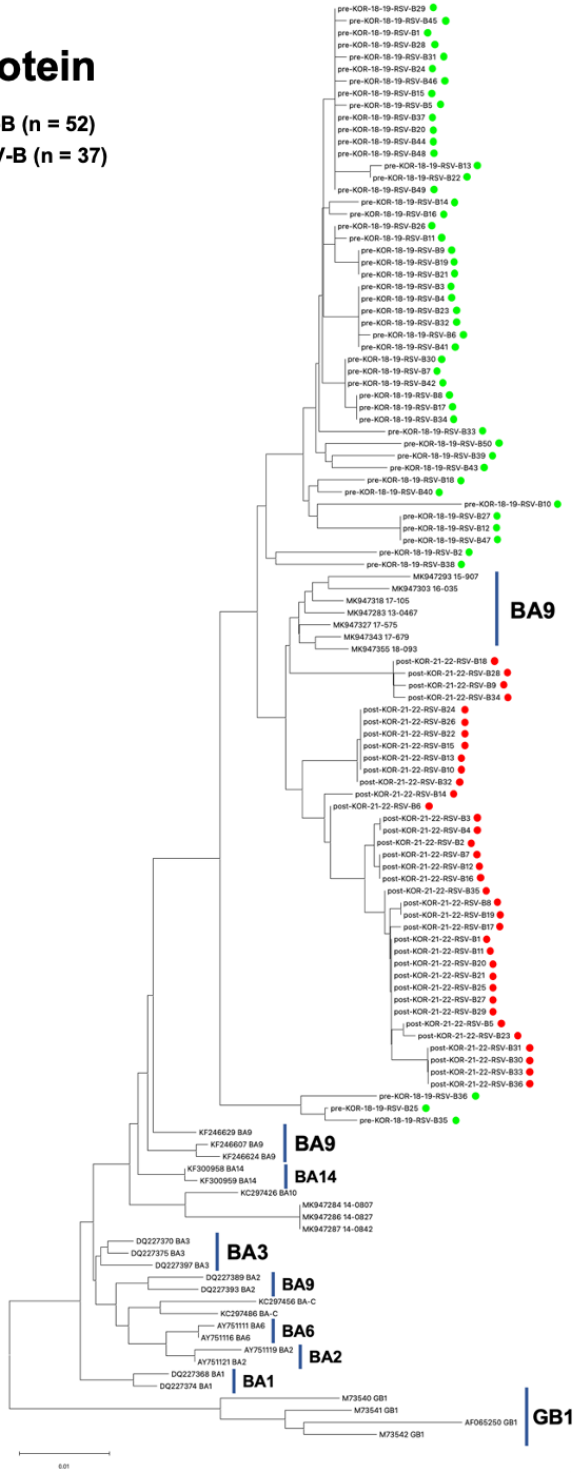


Figure 15. Phylogenetic trees for RSV-B G protein in the pre/post-pandemic era. The phylogenetic trees were constructed with maximum likelihood with 1000 bootstrap replicates using MEGA 11 software. The trees are based on the fragment of the G protein of RSV-B strains, consisting of the second hypervariable region. Nucleotide sequences of the clinical isolates (indicated with SEV) were compared to reference strains found on GenBank (indicated with genotype and accession number). Bootstrap values greater than 70% are indicated at the branch nodes and the scale bar represents the number of substitutions per site.

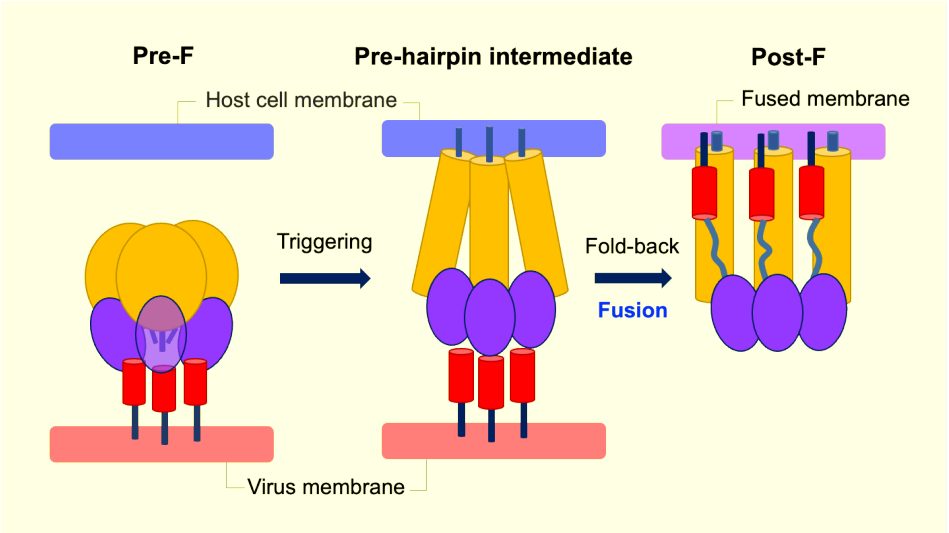
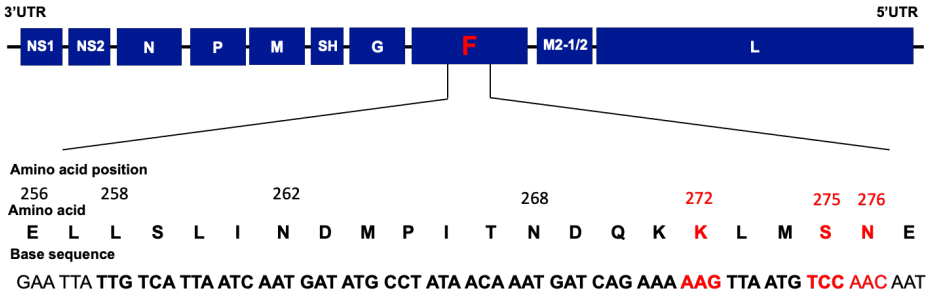


Figure 16. RSV F glycoprotein in the pre-fusion, intermediate, and post-fusion states.



Palivizumab mutation site: K272E, S275F, N276S

(Yasui, Y. et al., *Journal of virological methods* , 2016)

Figure 17. Palivizumab-binding and mutation sites. Continual exposure in presence of palivizumab induced RSV mutations for resistance (aa from 258 to 275 indicate palivizumab-binding site). Single amino acid substitution at 272,275 or 276 in RSV F protein conferred resistance to palivizumab and those mutations have been increasing in recent years.

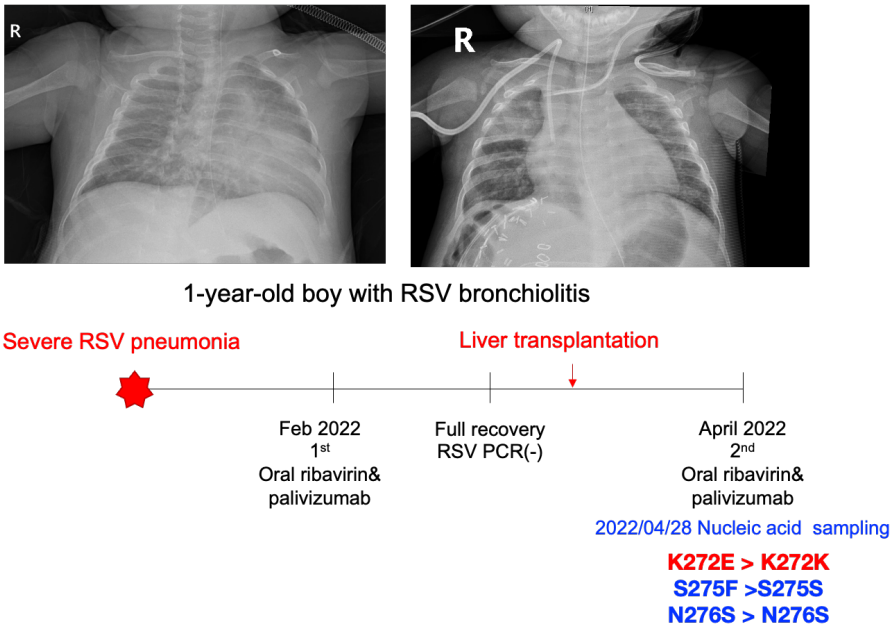


Figure 18. Palivizumab mutation case. The study presented a case of a 1-year-old boy with RSV bronchiolitis who recovered after treatment with ribavirin and palivizumab. However, after a liver transplant, the boy developed pneumonia again, and palivizumab-resistant RSV mutants were detected. This highlights the challenges in treating RSV and the need for further research on alternative treatments and vaccines.

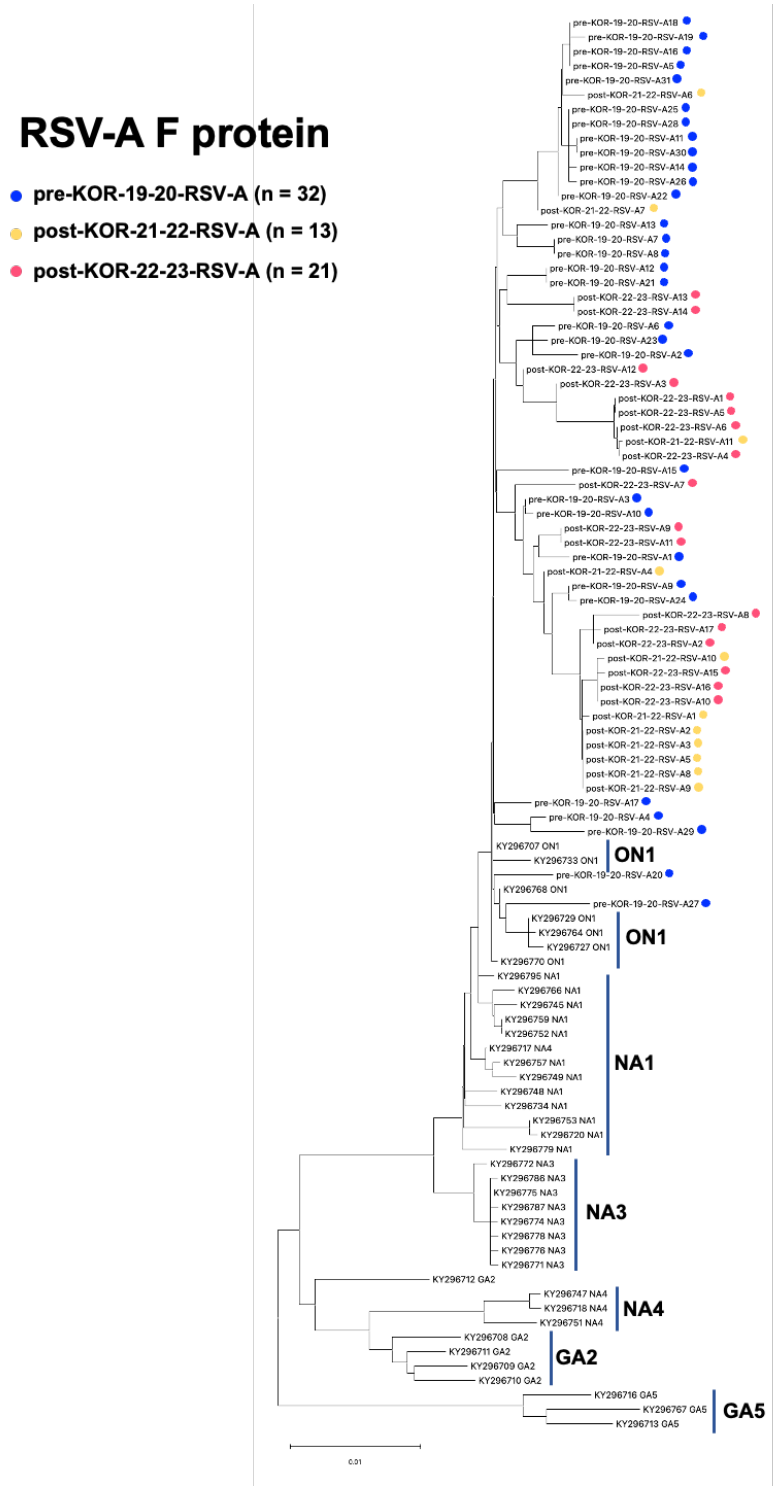


Figure 19. Phylogenetic trees for RSV-A F protein in the pre-pandemic era. The phylogenetic trees were constructed with maximum likelihood with 1000 bootstrap replicates using MEGA 11 software. The trees are based on the fragment of the F protein of RSV-A strains, consisting of the second hypervariable region. Nucleotide sequences of the clinical isolates (indicated with SEV) were compared to reference strains found on GenBank (indicated with genotype and accession number). Bootstrap values greater than 70% are indicated at the branch nodes and the scale bar represents the number of substitutions per site.

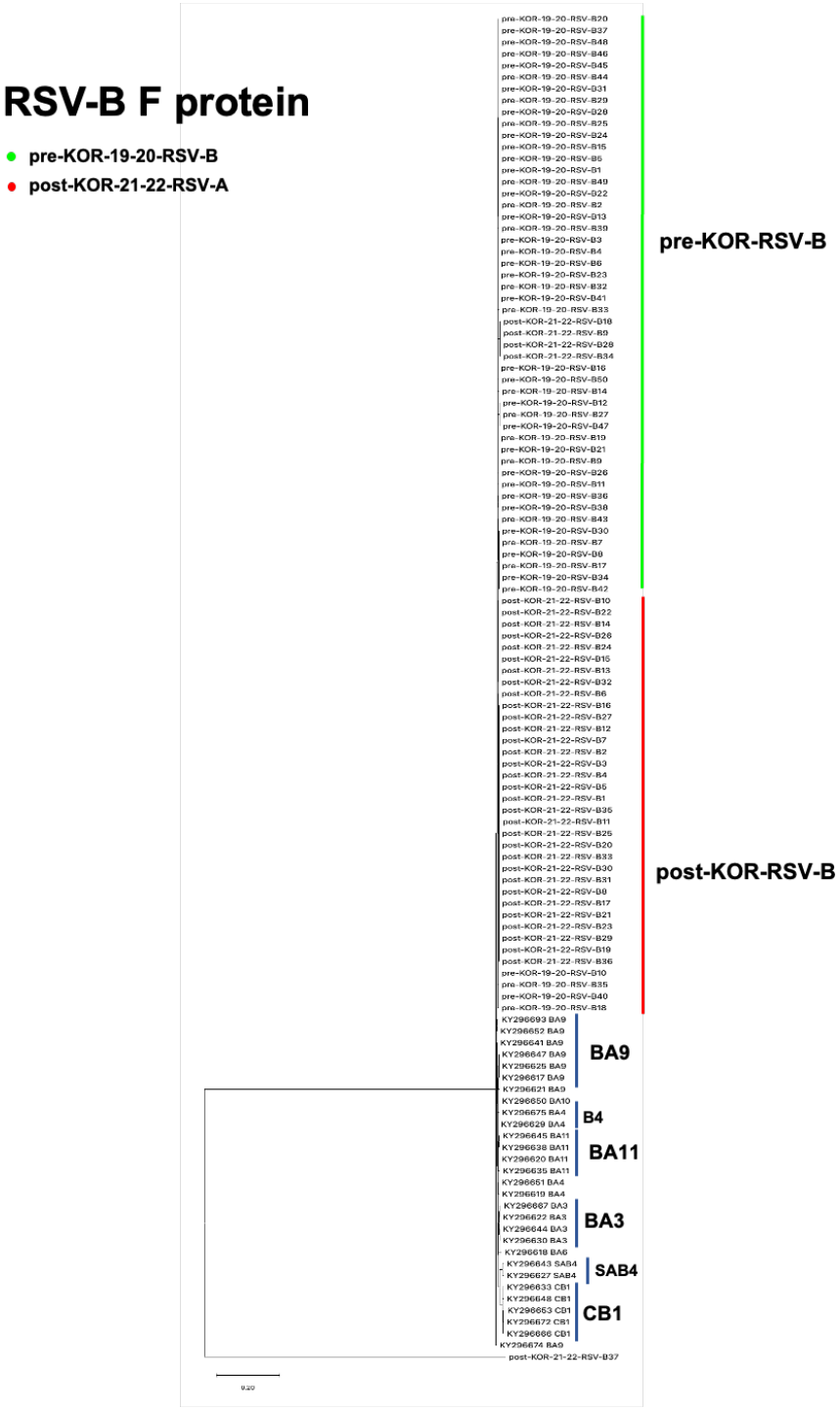


Figure 20. Phylogenetic trees for RSV-B F protein in the pre-pandemic era. The phylogenetic trees were constructed with maximum likelihood with 1000 bootstrap replicates using MEGA 11 software. The trees are based on the fragment of the F protein of RSV-B strains, consisting of the second hypervariable region. Nucleotide sequences of the clinical isolates (indicated with SEV) were compared to reference strains found on GenBank (indicated with genotype and accession number). Bootstrap values greater than 70% are indicated at the branch nodes and the scale bar represents the number of substitutions per site.

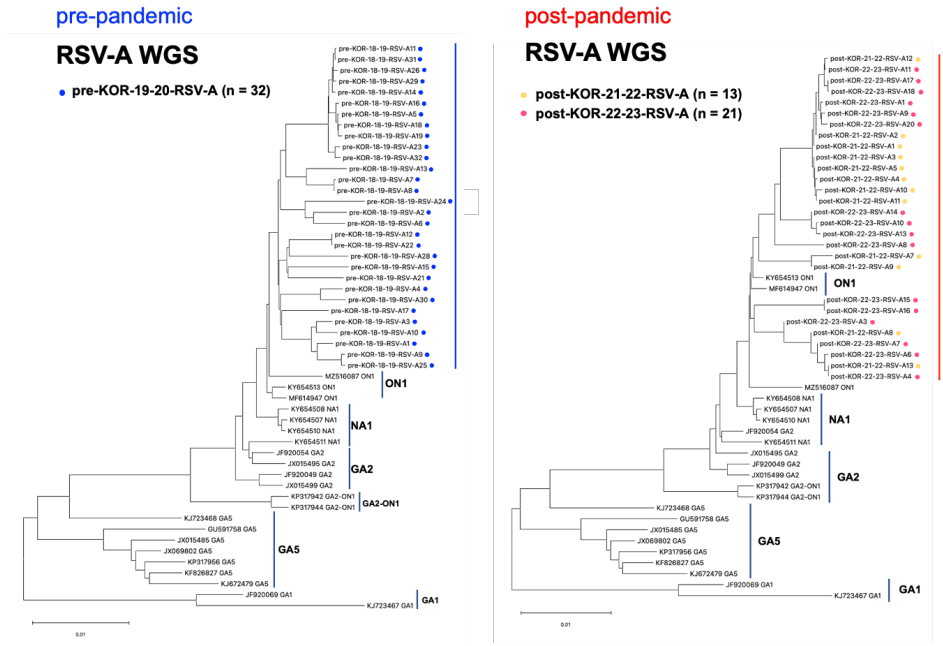


Figure 21. Phylogenetic trees for RSV-A in the pre/post-pandemic era. The phylogenetic trees were constructed with maximum likelihood with 1000 bootstrap replicates using MEGA 11 software. The trees are based on the fragment of pre-pandemic(left) and post-pandemic(right) strains respectively, consisting of the second hypervariable region. Nucleotide sequences of the clinical isolates (indicated with SEV) were compared to reference strains found on GenBank (indicated with genotype and accession number). Bootstrap values greater than 70% are indicated at the branch nodes and the scale bar represents the number of substitutions per site.

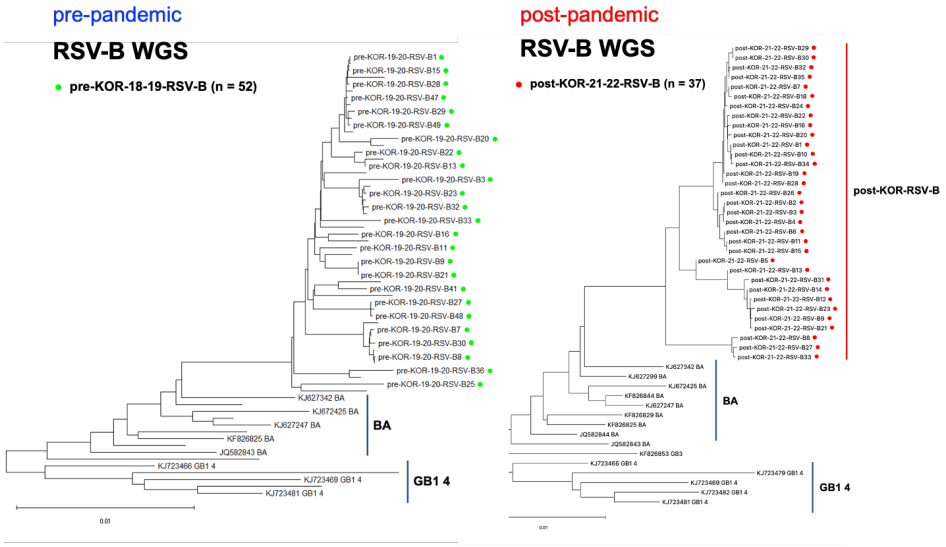


Figure 22. Phylogenetic trees for RSV-B in the pre/post-pandemic era. The phylogenetic trees were constructed with maximum likelihood with 1000 bootstrap replicates using MEGA 11 software. The trees are based on the fragment of pre-pandemic(left) and post-pandemic(right) strains respectively, consisting of the second hypervariable region. Nucleotide sequences of the clinical isolates (indicated with SEV) were compared to reference strains found on GenBank (indicated with genotype and accession number). Bootstrap values greater than 70% are indicated at the branch nodes and the scale bar represents the number of substitutions per site.

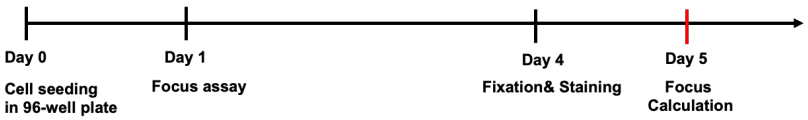
4. Evaluate RSV-specific serological-mediated immunogenicity

The general strategy of aim is to check the serological immunogenicity of RSV in Hep-2 cells. For quantifying the level of neutralizing antibodies, we optimize the condition of the focus reduction neutralization test (FRNT) assay.

To develop a high-throughput FRNT assay against RSV, we first examined the optimal experimental conditions. First, we tested six different conditions after virus inoculation to determine the appropriate time and focus shape: 1) Suction + 2% FBS, 2) Suction + 10% FBS, 3) RSV + 2% FBS, 4) RSV + 10% FBS, 5) Only methylcellulose, 6) RSV+ methylcellulose. Methylcellulose (M/C) is a compound derived from cellulose and used in cell culture to study viral replication by dissolving it in the growth medium and infecting a single layer of cells on a flat surface with a virus briefly. With M/C groups (groups 5 and 6) was found to result in clearer focus shapes compared to groups 1-4, which exhibited a spread pattern indicative of secondary infection. Furthermore, regarding the focus counts, the group with M/C had the highest number of foci, while the RSV+M/C group had the least count. For future experiments, M/C overlay will be used. Issues identified included low-tier detection of foci and the need to optimize the dilution ratio and time for checking focus shape. Clear control conditions will be established to address variations in false positives and experimenter conditions. These findings will help establish optimal conditions for the RSV focus assay and ensure reliable results in future experiments.

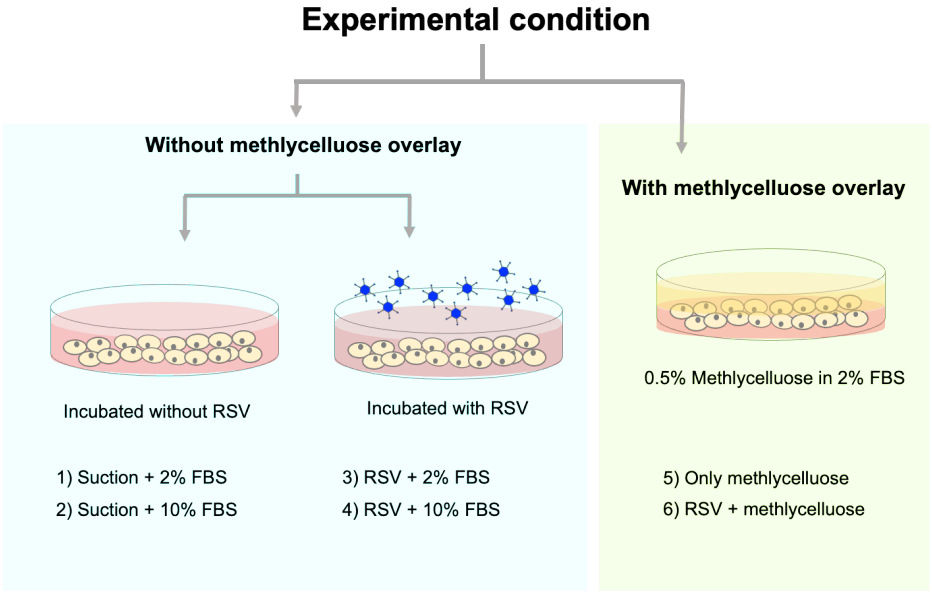
Moreover, with the above results, we have passed the first step of FRNT against RSV and plan to standardize RSV immunogenicity evaluation methods for the vaccine in development by continuing to optimize experimental conditions.

	1	2	3	4	5	6	7	8	9	10	11	12
A	No virus	No virus	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}
B	No virus	No virus	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}
C	No virus	No virus	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}
D	No virus	No virus	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
E	No virus	No virus	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}
F	No virus	No virus	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}
G	No virus	No virus	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}
H	No virus	No virus	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}



(Vanderheiden, A et al., *Current protocols in immunology*, 2020) -**SARS-CoV-2 FRNT**
(Jorquera, P. A., Anderson, L., & Tripp, R. A, *Human Respiratory Syncytial Virus: Methods and Protocols* 2016)

Figure 23. Example of limiting dilution and microtitation plate layout. Example of template for plaque assay in 96-well plate format to determine the amount of infectious RSV in each sample. Each dilution of virus stock, from 1:10 to $1:10^8$, is assayed in ten wells from column 3 through column 12. Columns 1 and 2 are for negative controls for infection.



(Vanderheiden, A et al., *Current protocols in immunology*, 2020) **-SARS-CoV-2 FRNT**
(Jorquera, P. A., Anderson, L., & Tripp, R. A, *Human Respiratory Syncytial Virus: Methods and Protocols* 2016)

Figure 24. RSV-FRNT experimental condition. We tested six different conditions after virus inoculation to determine the appropriate time and focus shape: 1) Suction + 2% FBS, 2) Suction + 10% FBS, 3) RSV + 2% FBS, 4) RSV + 10% FBS, 5) Only methylcellulose, 6) RSV+ methylcellulose.

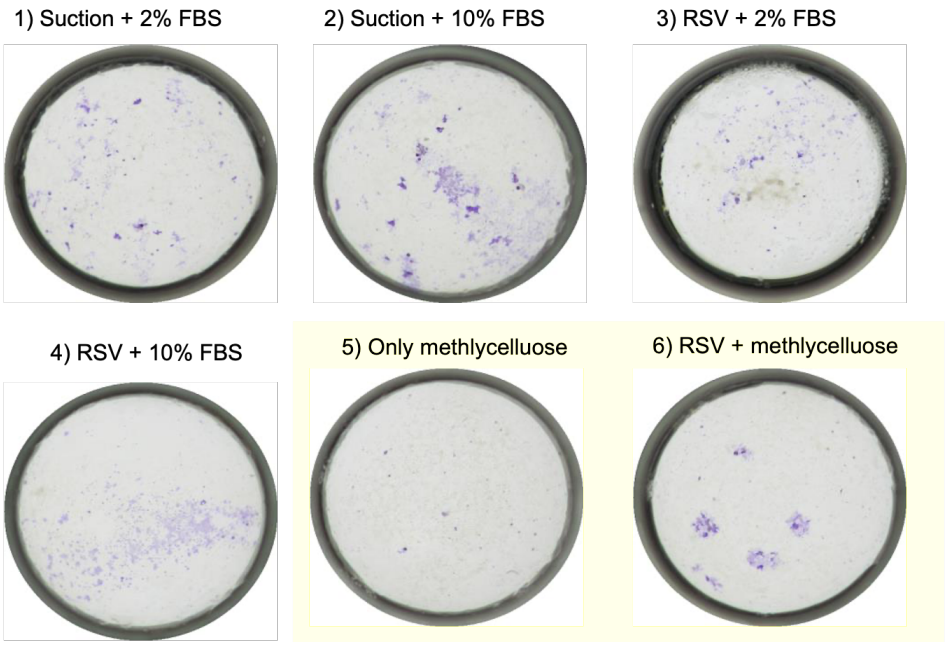
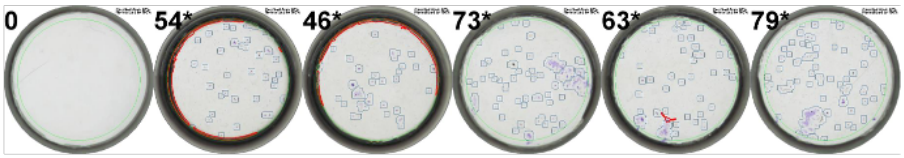
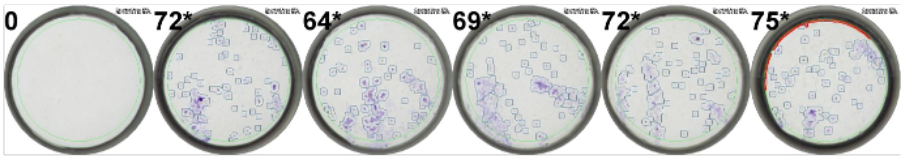


Figure 25. RSV focus morphology. With M/C groups (groups 5 and 6) was found to result in clearer focus shapes compared to groups 1-4, which exhibited a spread pattern indicative of secondary infection.

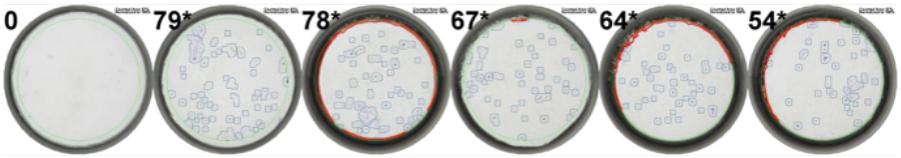
1) Suction + 2% FBS :



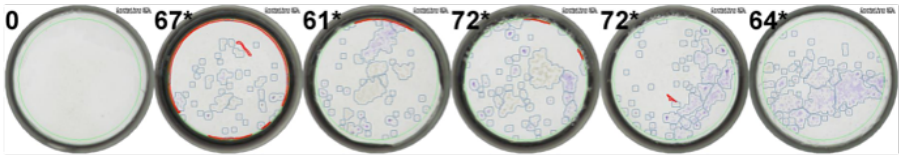
2) Suction + 10% FBS



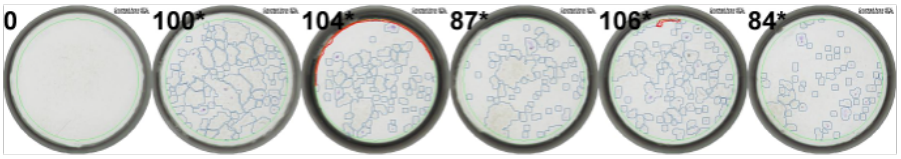
3) RSV + 2% FBS



4) RSV + 10% FBS



5) Only methlycelluose



6) RSV + methlycelluose

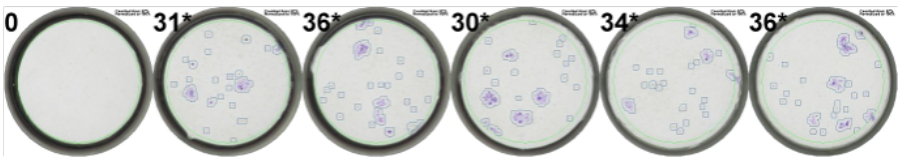


Figure 26. RSV focus assay in six different experimental condition. The group with M/C had the highest number of foci, while the RSV+M/C group had the least count.

IV. DISCUSSION

We have isolated RSV from nasal samples from patients in the winter seasons of 2021–2022 and 2022–2023 and found that isolating infectious viruses from nasal samples is most efficient from nasopharyngeal aspirates. However, yield may also depend on the timing of sampling compared to the onset of symptoms and the time between sampling and inoculation of cell culture. HEp-2 cells were used for isolation since they are permissive for most RSV strains and isolates described.

In a 6-well culture plate, HEp-2 cells were seeded at a density of 4.5 x 10^5 cells per well. MEM containing 2% FBS was also added, and a viral inoculum of 100–300 uL was added. The virus was incubated for 60 minutes in a 37°C, 5% CO2 incubator before being given an additional 1 mL of 2% FBS MEM and incubated for a further 4 to 5 days in the same temperature-controlled environment. We monitored cell morphological changes following virus inoculation to ensure that it manifested both the normal syncytium formation and the cytopathic effects (CPE). In the 21–22 season, there were 9 RSV (+) only groups without duplicate infections of other viruses, and two samples revealed viral cytologic impact (CPE). 34 of the 47 samples from the 22–23 season were RSV (+) groups, and 7 of those samples displayed CPE. In total, 9 RSVs were amplified, and they are currently being kept in a deep freezer at -80°C.

Electron microscope observations of RSV-infected Hep-2 cells revealed syncytium that was exclusively expressed in RSV-injected cell lines. ICC, which uses monoclonal antibodies specific to the Fusion protein (F-protein) of RSV to stain infected viral antigens, also revealed distinctive patterns in the cytoplasm and nucleus. We verified whether the development of CPE and syncytium is reliant on the presence of viral inoculum. 100, 200, 300, 400, and 500 uL of clinical produced in DMEM-0 were used to inoculate the cells. On day 4, cells were fixed, fluorescently stained, and examined using flow cytometry or

fluorescence microscopy. DAPI, which counts the number of cells, has reduced and syncytium production has grown as the quantity of RSV has increased. This indicates that RSV concentration is a factor in how CPE and syncytium development function.

Additionally, we looked into whether the genotypic patterns of RSV strains in Korea considerably changed prior to and following the COVID-19 pandemic. The analysis of RSV-A G protein sequences showed clustering with the ON1 genotype in both pre- and post-pandemic samples, while RSV-B G protein sequences clustered with the BA9 strain. The RSV fusion protein (F) is crucial for viral entry, and antibodies targeting it can reduce RSV-related disease. Palivizumab is the main prophylactic agent, but RSV mutations at amino acid positions 272, 275, or 276 in the F protein have led to resistance. A case study highlighted the recurrence of RSV pneumonia post-liver transplant with palivizumab-resistant mutants. Clustering with ON1 genotype and BA9 strain was observed in both RSV-A and RSV-B F protein sequences. Whole genome sequencing analysis showed clustering with ON1/NA1 genotypes for RSV-A and the BA genotype for RSV-B. No significant changes were detected in the RSV strain during the pandemic, but one case of palivizumab mutation was identified, underscoring the importance of monitoring genotypic trends. NGS methods can track these trends and provide insights into the effectiveness of RSV-specific agents.

To develop a high-throughput FRNT assay against RSV, we examined several experimental conditions such as virus incubation time, the volume of medium, number of cells (1.5/2 x 10^4 cells/ 96- well plate) and overlay composition. This is only the beginning, to perform FRNT, we will explore various experimental conditions such as plaque formation, neutralization time of the virus and serum.

V. CONCLUSION

This study documents established the clinical RSV *in vitro* culture system and cultivated the phylogenic analysis and RSV-specific cell mediated immunogenicity evaluation methods. We administered clinical RSV to Hep-2 cells and used immunocytochemistry and flow cytometry to check the cells' syncytium-forming abilities. Successful inoculations of clinically isolated RSV resulted in the maturation and enrichment of the syncytium in Hep-2 cells in a concentration-dependent manner. Additionally, we investigated whether the genotypic patterns of RSV strains in Korea considerably changed prior to and following the COVID-19 pandemic. 285 samples were collected from 285 children, out of which RNA extracts from 155 samples (66 type-A and 89 type-B) were available for G protein genotyping and whole-genome sequencing (WGS) analysis. The G protein genotyping revealed that the most frequently identified genotypes were ON1 for RSV- A and BA9 for RSV-B. The WGS analysis showed that the genotypes clustered closely with ON1/NA1 for RSV-A and BA for RSV-B, respectively. One mutation was identified in the RSV strain that confers resistance to palivizumab. In addition, mutations associated with nirsevimab resistance were not detected. Finally, we present the procedure for the focus reduction neutralization test for evaluating RSV-specific serological-mediated immunogenicity (FRNT). By enhancing an acceptable overlay medium composition, a solid base for FRNT testing was created. We expect that this study will provide important information on the potential effectiveness of future vaccine programs in reducing virus transmission and consequently the burden resulting from RSV disease.

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ABSTRACT (IN KOREAN)

국내 임상 호흡기세포융합바이러스의 분리 및 특징 분석과 면역원성평가법 개발

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나 본 향

호흡기세포융합바이러스(Respiratory syncytial virus, RSV)는 전 세계 영유아의 급성 하부 호흡기계 감염의 가장 중요하고 흔한 병원체이다 RSV에 관련되어 발표된 연구의 대부분은 1956년과 1961년에 각각 분리된 RSV Long과 RSV A2을 사용하여 수행되었다. 이 바이러스들은 연속적으로 배양되었고 이 과정에서 바이러스와 숙주의 상호 작용에 영향을 미치는 변이를 초래했을 가능성이 있다. 현재, RSV백신 또는 치료제에 대한 비임상 및 임상 연구가 계속해서 개발되고 있지만, 현재까지 국내에서 유행하는 바이러스의 *in vitro* 배양 시스템을 구축하거나 이를 기반으로 면역원성 평가를 수립한 연구는 아직 없는 실정이다. 따라서, 본 연구에서는 2019년 9월부터 2022년 12월까지 환자 유래 RSV를 *in vitro* 배양 및 증식 시스템을 통해 분리 및 증식하였고 바이러스 특징을 분석 하였다. 또한, Hep-2 세포에 배양한 RSV를 접종하여 세포 면역염색법(Immunocytochemistry) 또는 유세포분석 (Flow cytometry)기법 활용하여 세포 감염률을 측정하였다. 그 결과, 환자 유래 RSV를 감염시킨 세포주에서 RSV 감염 특징인 세포병변효과

(Cytopathic effect, CPE) 와 다핵질(Syncytium)이 형성됨을 관찰할 수 있었다.

다음으로, 본 연구에서는 전장유전체 분석법 (Whole genome sequencing, WGS)를 활용하여 코로나19 유행 전후로 국내 RSV 균주의 유전자형 패턴의 변화를 조사하였다. G 단백질의 유전자형 분류의 결과, RSV-A에서 ON1이었고, RSV-B에서는 BA9에 포함되었다. WGS 분석에서는 RSV-A와 RSV-B의 유전자형은 각각 ON1/NA1과 BA와 밀접하게 군집함을 확인할 수 있었다. Palivizumab에 관해 한개의 돌연변이가 RSV 균주에서 확인되었고 nirsevimab에 대한 저항성과 관련된 돌연변이는 검출되지 않았다.

마지막으로, 초점 감소 중화시험 (Focus reduction neutralization test, FRNT)은 초점 감소를 통해 혈청 검체 내 중화 항체(neutralization antibody)를 측정하는 방법이다. 본 연구에서는 RSV에 특이적으로 바이러스 적정 감염 시간 및 초점 형성과 계수에 대한 기준을 확립함으로 FRNT 분석을 위한 중요한 기초를 확립할 수 있었다. 본 연구를 통해 구축된 RSV 면역 원성 평가법은 개발 중인 백신의 효능평가에 활용될 수 있으며, 국내 환자 유래 RSV 특징 분석 결과를 국외 유행 RSV와 비교·분석함으로써 향후 국내에 도입될 예정인 RSV 백신의 예방 효과를 예측하여 백신 도입 적정성 평가에 중요한 자료로 기여할 것이다.

핵심되는 말 : 호흡기세포융합바이러스, 배양 시스템, 다핵질 형성, 유전자형 분석, 전장유전체분석법, 면역 원성 평가법, 초점감소중화시험법