



Impact of Respiratory Syncytial Virus season evolution on antibody responses

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PLAGIARISM DECLARATION

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PRESENTATIONS ARISING FROM THIS STUDY

1. Poster Presentation at Wits MBRT (Molecular Biosciences Research Thrust) 2023 - “Tools to assess impact of RSV F escape: successful production of anti-RSV monoclonal antibodies and RSV F pseudoparticles”
2. Poster Presentation at WITS Faculty of Healthy Science research day, 2023 - “Development of immunoreagents and assays for immunological surveillance of respiratory syncytial virus”
3. Poster Presentation at the 13th international RSV Symposium (Iguazu Falls, Brazil), 2025 - Futter, J., Lambson BE., Mhlanga, D., van Graan, S., Ntozini, B., Mahlangu, B., Moore, PL., Wolter, N., Kitchin, D., Bhiman, JN “Low incorporation of RSV fusion protein on pseudotyped VSV particles precludes their use in neutralization assays”

PUBLICATIONS ARISING FROM THIS STUDY

Futter, J., Lambson BE., Mhlanga, D., van Graan, S., Ntozini, B., Mahlangu, B., Moore, PL., Wolter, N., Kitchin, D., Bhiman, JN. Low incorporation of RSV fusion protein on pseudotyped VSV particles precludes their use in neutralization assays. Manuscript in preparation.

ABSTRACT

Respiratory Syncytial Virus (RSV) is a leading cause of infant and child mortality, in low-and-middle-income countries. The RSV fusion (F) protein mediates viral entry by undergoing a conformational change from the prefusion (preF) to postfusion (postF) states. Stabilised preF serves as a target for prophylactic or vaccine-elicited neutralising antibodies, and deployment of these RSV preventative interventions may increase selective pressure on the F gene. Immunological surveillance to monitor the functional impact of this diversity on the efficacy of these interventions is crucial. We analysed 569 RSV-A and 370 RSV-B F genomes, identifying 52 antigenic site-specific mutations, 14 of which were frequent across multiple years, increased in frequency in recent years, and/or had predicted antigenic impact. We aimed to develop a F-pseudotyped vesicular stomatitis virus (VSV)-based neutralisation assay to evaluate the impact of these mutations on neutralisation. We produced RSV F-pseudotyped viruses by optimising more than 200 conditions, including multiple cell types and densities, incubation times, F protein C-terminal (CT) modifications, and freeze-thaw parameters. CT modifications increased pseudovirus infection between 9 to 20-fold, with binding of preF and postF mAbs in cell surface expression experiments maintained. However, neutralisation by anti-RSV monoclonal antibodies was not observed likely due to low levels of RSV F protein, mostly in the postF conformation on budded virions. This demonstrates the challenge of establishing an RSV neutralisation assay using pseudotyped VSV particles. Future studies, including a live-virus neutralisation assay using a reverse genetics system and ELISA binding assays, will assess the functional impact of emerging mutations.

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TABLE OF CONTENTS

PLAGIARISM DECLARATION.....	ii
PRESENTATIONS ARISING FROM THIS STUDY.....	iii
PUBLICATIONS ARISING FROM THIS STUDY.....	iv
ABSTRACT.....	v
ACKNOWLEDGEMENTS.....	vi
LIST OF FIGURES.....	xi
LIST OF TABLES.....	xiii
LIST OF ABBREVIATIONS.....	xv
1. INTRODUCTION	1
1.1. Respiratory syncytial virus and its disease burden.....	1
1.2. RSV genome, virion structure and pathogenesis	1
1.3. RSV diversity	3
1.4. The fusion protein synthesis, processing and function	5
1.5. RSV F sites of vulnerability.....	6
1.6. Historic and current RSV preventative interventions.....	7
1.7. The need for RSV immunological surveillance.....	11
2. OBJECTIVES	13
2.1. Overall aim.....	13
2.2. Specific aims.....	13
2.2.1. <i>Identify key mutations in circulating South African variants and introduce these into RSV pseudoviruses.....</i>	13
2.2.2. <i>Express and characterise anti-RSV monoclonal antibody controls</i>	13
2.2.3. <i>Establish and optimise a vesicular stomatitis virus (VSV)-based RSV pseudovirus neutralisation assay.....</i>	13
3. MATERIALS AND METHODS	14
3.1. Anti-RSV mAb expression and purification.....	14
3.1.1. <i>Digestion of codon optimised anti-RSV neutralising mAb constructs.....</i>	14
3.1.2. <i>Purification of DNA fragments.....</i>	14
3.1.3. <i>Ligation into pCMVR expression vector.....</i>	15
3.1.4. <i>Transformation of XL-10 Gold cells.....</i>	15

3.1.5. Plasmid purification.....	16
3.1.6. Sequence validation of plasmid constructs.....	16
3.1.7. Maxiprep plasmid purification.....	18
3.1.8. Culture and maintenance of mammalian 293F cells for mAb expression.....	19
3.1.9. Expression of anti-RSV mAbs by transfection of HEK 293F cells.....	19
3.1.10. Protein A affinity chromatography for mAb purification...	20
3.1.11. SDS-PAGE for anti-RSV mAb purity and size verification.....	20
3.1.12. ELISA for anti-RSV mAb functionality.....	21
3.2. RSV pseudovirus production.....	22
3.2.1. RSV gene construct design for pseudovirus expression.....	22
3.2.2. RSV F gene construct cytoplasmic tail (CT) modifications.....	22
3.2.3. Plasmid preparation of unmodified and modified pseudovirus constructs.....	25
3.2.4. Sequence confirmation of the unmodified and modified pseudovirus constructs.....	25
3.2.5. Pseudovirus plasmid purification.....	26
3.2.6. Culture and maintenance of mammalian cells for pseudovirus expression.....	26
3.2.7. Generation of RSV pseudoviruses.....	26
3.2.8. Validation of cell surface expressed RSV F protein.....	27
3.3. RSV pseudovirus infection optimisation	29
3.3.1. Culture and maintenance of adherent cell lines for pseudovirus infection.....	29
3.3.2. RSV pseudovirus titrations.....	29
3.3.3. Optimisation of RSV pseudovirus infection parameters...	30
3.4. Neutralisation assay.....	32
3.4.1. VSV-RSV pseudovirus neutralisation assay.....	32
3.5. Whole-virion ELISA.....	33

3.5.1. Validation of RSV F protein expression at the pseudotyped virion surface by ELISA.....	33
3.6. RSV F gene mutation identification.....	34
3.6.1. Phylogenetic analysis – Determining appropriate RSV F gene reference sequences.....	34
3.6.2. RSV F protein mutation analysis.....	34
4. RESULTS	35
4.1. RSV-A and -B F protein diversity.....	35
4.1.1. RSV GISAID dataset.....	35
4.1.2. Identification of appropriate reference sequences.....	35
4.1.3. Analysis of South African RSV-A and RSV-B genomic surveillance data and F gene mutation prioritisation.....	39
4.1.3.1. RSV F gene antigenic site diversity.....	39
4.1.3.2. Mutations increasing in frequency within circulating strains.....	40
4.1.3.3. High frequency mutations.....	41
4.1.3.4. Mutations with likely impact on conformation or structure of preF.....	42
4.1.3.5. Priority mutations.....	43
4.2. Anti-RSV mAb production and validation.....	46
4.2.1. Anti-RSV mAb size and purity.....	46
4.2.2. Anti-RSV mAb functionality.....	48
4.3. RSV pseudovirus production and infection optimisation.....	53
4.3.1. Optimisation of RSV pseudovirus infection parameters...	53
4.3.1.1. Cell type, density and incubation period optimisation.....	53
4.3.1.2. Optimisation of pseudovirus freezing conditions.	56
4.3.1.3. Optimisation of pseudovirus plasmid transfection amounts.....	56
4.3.1.4. Optimisation of pseudovirus thawing conditions..	57
4.3.1.5. Effect of RSV F protein CT modification on pseudovirus infectivity.....	57

4.3.1.6. Reproducibility of modified RSV pseudovirus infection.....	58
4.3.2. <i>Confirmation of modified RSV F protein cell surface expression</i>	60
4.4. Neutralisation assay.....	63
4.4.1. <i>Neutralisation assay optimisation</i>	63
4.5. Whole pseudovirus ELISA.....	70
5. DISCUSSION.....	72
APPENDIX A1.....	78
APPENDIX A2.....	81
REFERENCES.....	102

LIST OF FIGURES

Figure 1.1. Structure of the RSV genome and viral particle.....	3
Figure 1.2. Lineage classification of RSV-A and RSV-B.....	4
Figure 1.3. RSV F protein structure and key features.....	6
Figure 1.4. Antigenic sites of the RSV F protein in the prefusion and postfusion conformations.....	7
Figure 1.5. Landscape of all RSV immuno-prophylactics and vaccines.....	11
Figure 3.1. Strategies for RSV F gene CT modifications.....	23
Figure 3.2. Plate map of virus titrations.....	30
Figure 3.3. RSV pseudoparticle optimisation conditions overview.....	31
Figure 4.1. Phylogenetic tree for RSV-A reference gene identification, All African dataset 1 st iteration (1956 USA excluded).....	37
Figure 4.2. Phylogenetic tree for RSV-B reference gene identification, All African dataset 1 st iteration (1956 USA excluded).....	38
Figure 4.3. Identification of South African RSV-A F protein antigenic site mutations putatively of relevance.....	44
Figure 4.4. Identification of South African RSV-B F protein antigenic site mutations putatively of relevance.....	45
Figure 4.5. Size and purity of anti-RSV monoclonal antibodies by SDS-PAGE.....	46
Figure 4.6. Anti-RSV monoclonal antibody binding to prefusion RSV-A (A2) and RSV-B (B18537).....	49
Figure 4.7. Anti-RSV monoclonal antibody binding to post fusion RSV-A (A2) and RSV-B (B18537).....	51
Figure 4.8. Optimisation of RSV-A and RSV-B pseudovirus infection.....	55
Figure 4.9. Batch to batch reproducibility of CT modified RSV pseudoviruses.....	59
Figure 4.10. Anti-RSV-Binding to Expi293 cell surface expressed RSV F protein.....	61
Figure 4.11. Viral infection shows a dose response relationship.....	63
Figure 4.12. Neutralisation assay of RSV-B Δ 21 in the presence of anti-RSV mAbs.	65
Figure 4.13. Optimisation of assay mAb sample input.....	66
Figure 4.14. Optimisation of sample-virus incubation time and temperature.....	67

Figure 4.15. Monitoring infection in the absence of mAb samples.....	68
Figure 4.16. Bald Virus titration verses modified RSV pseudoviruses.....	69
Figure 4.17. RSV F and VSV-G protein expression on the pseudoparticle surface.....	71
Figure A2.1 Phylogenetic tree for RSV-A reference gene identification, all African dataset 2 nd iteration (1956 USA included).....	83
Figure A2.2 Phylogenetic tree for RSV-A reference gene identification, all African dataset 3 rd iteration (1956 USA as outgroup).....	84
Figure A2.3 Phylogenetic tree for RSV-A reference gene identification, South African dataset 1 st iteration (1956 USA excluded).....	85
Figure A2.4 Phylogenetic tree for RSV-A reference gene identification, South African dataset 2 nd iteration (1956 USA included).....	86
Figure A2.5 Phylogenetic tree for RSV-A reference gene identification, South African dataset 3 rd iteration (1956 USA as outgroup).....	87
Figure A2.6 Phylogenetic tree for RSV-B reference gene identification, all African dataset 2 nd iteration (1956 USA included).....	88
Figure A2.7 Phylogenetic tree for RSV-B reference gene identification, all African dataset 3 rd iteration (1956 USA as outgroup).....	89
Figure A2.8 Phylogenetic tree for RSV-B reference gene identification, South African dataset 1 st iteration (1956 USA excluded).....	90
Figure A2.9 Phylogenetic tree for RSV-B reference gene identification, South African dataset 2 nd iteration (1956 USA included).....	91
Figure A2.10 Phylogenetic tree for RSV-B reference gene identification, South African dataset 3 rd iteration (1956 USA as outgroup).....	92
Figure A2.11. Unmodified RSV-A pseudoparticle infectivity evaluated in different cell lines and infection periods.....	93
Figure A2.12. Unmodified RSV-B pseudoparticle infectivity evaluated in different cell lines and infection periods.....	94
Figure A2.13. Optimisation of unmodified RSV-A and RSV-B infection parameters.....	95
Figure A2.14. RSV-B $\Delta 21$ pseudoparticle infectivity evaluated in different cell lines and infection periods.....	96

Figure A2.15. RSV-B VSV-G CT pseudoparticle infectivity evaluated in different cell lines and infection periods.....	98
Figure A2.16. RSV-A $\Delta 21$ pseudoparticle infectivity evaluated in different cell lines and infection periods.....	99
Figure A2.17. RSV-A VSV-G CT pseudoparticle infectivity evaluated in different cell lines and infection periods.....	100
Figure A2.18. Reproducibility of modified RSV infection.....	101

LIST OF TABLES

Table 3.1. Sanger Sequencing thermocycling parameters.....	18
Table 3.2. Pseudovirus reference sequence identifier information.....	23
Table 3.3. PCR cycling parameters – large/backbone fragments.....	25
Table 3.4. PCR cycling parameters – small/Insert fragments.....	25
Table 4.1. RSV-A mutation selection criteria and prioritisation.....	41
Table 4.2. RSV-B mutation selection criteria and prioritisation.....	41
Table A1.1. Anti-RSV mAb sequences.....	78
Table A1.2. Primers for assembly of RSV-A F gene CT modification constructs..	79
Table A1.3. Primers for assembly of RSV-B F gene CT modification constructs..	79
Table A1.4. Unmodified and modified RSV pseudovirus construct sequencing primers.....	80
Table A1.5. Reference modified pseudovirus plasmid sequences.....	81

LIST OF ABBREVIATIONS

6HB – 6 Helix bundle	LAV – Live attenuated virus
a.u.- Arbitrary units	LB – Luria-Bertani
ALRTI – Acute lower respiratory tract infection	LMIC - low-and-middle-income countries
ARV – Antiretroviral	M – Matrix protein
CC – Cell control	mAb – Monoclonal antibody
CT – Cytoplasmic tail	MF – Mr Frosty
ddNTP – Dideoxynucleotide triphosphate	MPER – Membrane proximal external region
dH ₂ O – Distilled water	N – Nucleoprotein
DMEM - Dulbecco's Modified Eagle's Medium	NaOAc – Sodium acetate
DNA – Deoxyribonucleic acid	NB – No Binding
dNTP – Deoxynucleotide triphosphate	NEAA – Non-essential amino acids
EC ₅₀ – Effective concentration 50	NS1 – Non-structural protein 1
ELISA – Enzyme linked immunosorbent assay	NS2 – Non-structural protein 2
EtOH - Ethanol	ORF – open reading frame
EU – European union	P – Phosphoprotein polymerase cofactor
F – Fusion protein	PBS – Phosphate buffered saline
FBS – Fetal bovine serum	pCMVR – Cytomegalovirus replication plasmid
Fc – Fragment crystallisable	PCR – Polymerase chain reaction
FDA – Food and Drug administration	PEG – Polyethelyne glycol
FI-RSV – Formalin-inactivated respiratory syncytial virus	PEI-MAX – Polyethyleneimine max
Fw – Forward	PIV5 – Parainfluenza virus 5
G – Attachment protein	postF – postfusion
GAGs – Glycosaminoglycans	preF – prefusion
GISAID – Global Initiative on Sharing All Influenza Data	RLU – Relative light units
GMFI – Geometric mean Fluorescence intensity	RSV - Respiratory syncytial virus
H ₂ SO ₄ – Sulphuric Acid	S.O.C – Super Optimal media with Catabolite repression
HEK – Embryonal human kidney	SD – Standard deviation
HIC – High income country	SDS – Sodium dodecyl sulphate
HIV – Human immunodeficiency virus	SDS-PAGE – Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
hMPV – Human metapneumovirus	SFM – Serum free media

HR1 – Heptad repeat region 1
HR2 – Heptad repeat region 2
ICAM1 – Intracellular adhesion molecule 1
IGF1R – Insulin-like growth factor-1 receptor
IgG – Immunoglobulin G
Kb – Kilobase
kDa – Kilodalton
L – large polymerase subunit

SH – Small hydrophobic protein
TAE – Tris-acetate-EDTA
TBE – Tris-borate-EDTA
T_m – Melting temperature
US – United states
VC – Virus control
VSV – Vesicular stomatitis virus
VSV-G – Vesicular stomatitis virus glycoprotein

1. INTRODUCTION

1.1. Respiratory syncytial virus and its disease burden

Respiratory syncytial virus (RSV) causes a substantial global disease burden in key populations including infants, young children, immunocompromised and the elderly¹. It is the leading cause of acute lower respiratory tract infections (ALRTI) and hospitalisations in children under the age of two². Global estimates from 2019 reported 33 million cases of RSV ALRTI, 3.6 million RSV-associated ALRTI hospital admissions, and 101 400 RSV-related deaths in children under the age of five¹. Low- and middle-income countries (LMICs) are disproportionately affected by the RSV burden, accounting for more than 95% of reported RSV cases and more than 97% of RSV-associated deaths across all age groups¹, with many of these deaths occurring not within hospitals, but rather within households³. In infants less than 6 months of age, an estimated 6.6 million cases of RSV ALRTI, 1.4 million hospital admissions, and 45 700 RSV-related deaths were reported globally¹. Severe RSV disease has been linked to conditions like chronic lung disease, congenital heart disease, and prematurity in infants^{4,5}. Early RSV infection has also been associated with a higher risk of impaired lung function and childhood asthma⁶. The elderly population and immunocompromised individuals with comorbidities are also impacted by severe RSV, due to waning and impaired immunity⁷⁻¹⁰.

As with influenza, RSV transmission during the COVID-19 pandemic was influenced by changes in human behaviour, particularly stay-at-home orders that reduced social interactions and, in turn, viral transmission. As a result, reported cases of RSV infection and hospitalisations significantly decreased, especially in the paediatric population, where RSV primarily spreads through close contact in schools¹¹. In South Africa, following the end of pandemic-imposed measures, RSV transmission peaked in 2021 and has since remained at high levels comparable to pre-pandemic years¹².

1.2. RSV genome, virion structure and pathogenesis

RSV is a filamentous, enveloped, negative-sense single stranded RNA virus belonging to the family *Pneumoviridae*¹³ and genus *Orthopneumoviridae*¹⁴. The 15.2 kb RNA genome contains ten genes (Figure 1.1 A) that encode 11 proteins including two non-

structural proteins (NS1 and NS2), which inhibit apoptosis¹⁵ and modulate interferon responses¹⁶. The genome encodes five structural proteins including the envelope glycoproteins (fusion (F), attachment (G), and small hydrophobic (SH) proteins) which are displayed within the virion lipid bilayer¹⁷ and matrix (M) protein which provides structural support to the viral envelope and associates with the F protein cytoplasmic domain¹⁸. The nucleoprotein (N), associates with and encapsulates the RNA genome, providing structural support¹⁹. The genome further encodes four regulatory proteins, including the M2-1 and M2-2, proteins transcribed from two open reading frames (ORFs) of the M2 gene, which act as a transcription factor and mediate switching from transcription to replication, respectively^{20,21}. The regulatory large polymerase subunit (L) and phosphoprotein polymerase cofactor (P) form the RNA-dependent polymerase complex, which associates with the ribonucleoprotein complex comprising the viral genome and nucleoprotein^{19,22}.

The major surface glycoproteins, G and F mediate viral entry and while these were previously named according to their presumed roles of attachment and fusion respectively, recent literature has demonstrated that the F protein is capable of mediating attachment independently of G or SH¹⁴. The G protein interacts with heparin sulphate glycosaminoglycans (GAGs) on the surface of ciliated epithelial cells to mediate attachment²³. The CX3C-chemokine receptor 1 (CXCR1), which binds fractalkine, serves as an additional host cell attachment factor for the G protein. Like the G protein, the F protein interacts with host cell cellular heparin sulphate^{23,24} but also interacts with host insulin-like growth factor-1 receptor (IGF1R)²⁵, which upon engagement triggers a signal cascade that recruits other co-receptors, including intracellular adhesion molecule 1 (ICAM1)²⁶, and nucleolin²⁵.

A



B

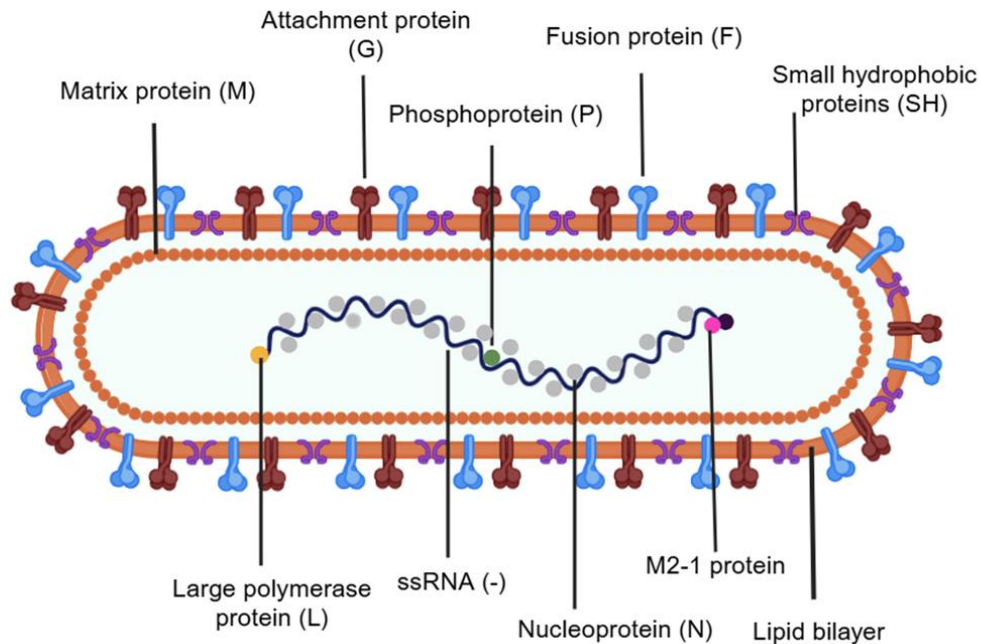


Figure 1.1 Structure of the RSV genome and viral particle. (A) The RSV genome comprising ten genes NS1, NS2, P, M, SH, G, F, M2, and L. M2 contains two ORFs encoding for proteins M2-1 and M2-2. **(B)** RSV virion structure. RSV is an enveloped negative-sense single stranded RNA virus which comprises ten proteins encoded by its genome. Structural proteins include the envelope glycoproteins (G, F, and SH), nucleocapsid (N), and matrix (M) protein and regulatory proteins include (M2-1, M2-2, Phosphoprotein polymerase cofactor (P) and the large polymerase subunit (L). Non-structural proteins (NS1 and NS2) are not shown. Generated using BioRender 2024.

1.3. RSV diversity

RSV is classified into two groups, RSV-A and RSV-B, based on antigenic variability²⁷. A recently described standardised nomenclature system for RSV genomic diversity³⁰ describes 24 RSV-A and 16 RSV-B genotype lineages²⁸(Figure 1.2), with much of the sequence diversity located in the mucin-like domains of the G gene ²⁹.

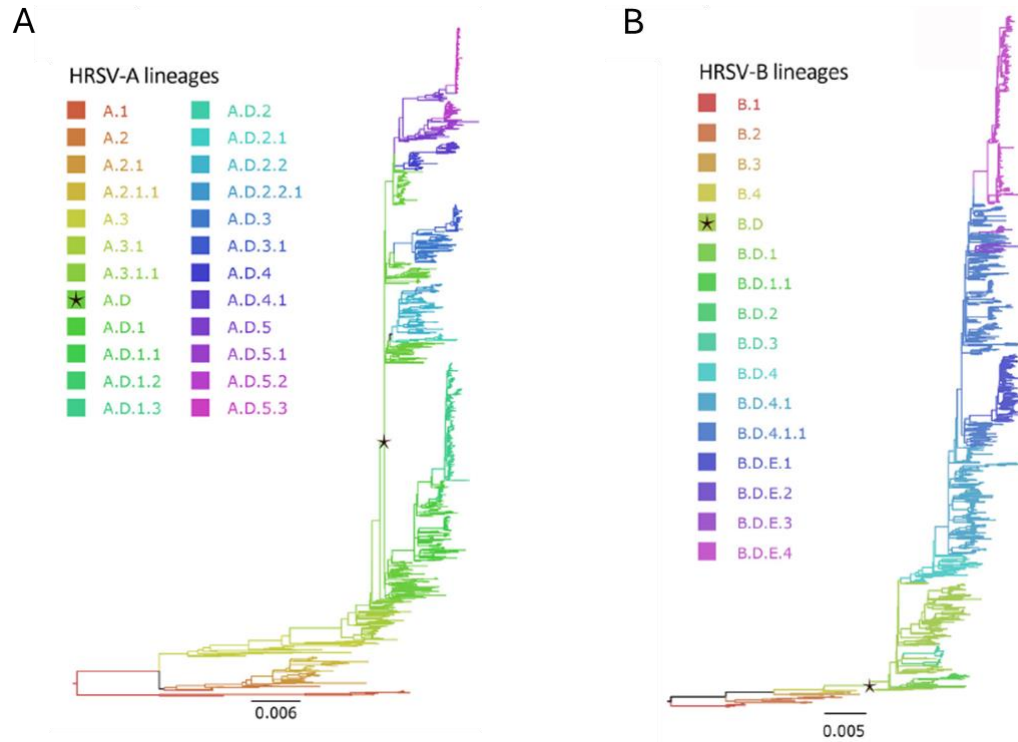


Figure 1.2. Lineage classification of RSV-A and RSV-B. Maximum likelihood phylogenetic trees defining **A)** 24 RSV-A and **B)** 16 RSV-B lineages. The trees are coloured according to lineage classification. The black star marks the A.D. and B.D. lineages of RSV-A and RSV-B, respectively, which are characterised by a 72 and 60 amino acid duplication in the G gene. Figure adapted from Goya et al., 2024.

The 24 RSV-A lineages are distinguished according to those with or without a 72 amino acid duplication and similarly the RSV-B lineage nomenclature reflects presence or absence of a 60 amino acid duplication^{30,31} (Figure 1.2). RSV strains co-circulate annually during annual RSV seasons, with the RSV-A subtype occurring most commonly³². Unlike the G gene which differs by more than 50% between RSV-A and RSV-B, the F gene is highly conserved, differing by approximately 5% between RSV groups¹⁴.

1.4. The fusion protein synthesis, processing and function

The RSV F protein is expressed at the plasma membrane of infected cells in late-stage infection where it mediates fusion forming syncytia with adjacent cells. The RSV F protein, a type I viral fusion protein, is synthesised as a single 547 amino acid polypeptide precursor (F₀) (Figure 1.3 A) with 5-6 post-translational N-linked glycans³³. In contrast to other viral type I fusion proteins, including the coronavirus spike (S) protein and HIV-1 envelope, RSV F protein trimerization occurs only after proteolytic cleavage^{34,35}. F₀ is cleaved at two polybasic sites by a furin-like host protease in the trans-Golgi network^{36,37}, resulting in three polypeptides: a smaller F2 N-terminal subunit (amino acids 26-109), a larger F1 C-terminal subunit (amino acids 137-574), and a 27-amino acid peptide (p27) located between the cleavage sites³⁸. The F2 subunit is approximately 18 kDa, while the F1 subunit is 45-55 kDa¹⁴. The p27 peptide is cleaved during the processing of F₀³⁹. The mature F protein trimer comprises three heterodimeric protomers, each formed by covalently linked F2 and F1 subunits connected by disulfide bonds⁴⁰.

The trimeric F protein undergoes a functionally essential pre- to postfusion (postF) conformational change upon binding to a host cell receptor, which facilitates successful fusion of virus and host cell membranes (Figure 1.3 B)¹⁴. The prefusion (preF) conformation is metastable with a low energy barrier to refolding and postF triggering occurs rapidly, with a greater proportion of postF present on the viral surface than preF¹⁷. As is typical with type I fusion proteins, upon interaction with the host cell, secondary structural elements of the F1 subunit refold into a long α -helix³³ (Figure 1.3 B). When the host cell membrane is in close proximity, the fusion peptide comprising of a stretch of 20 hydrophobic residues at the N terminus of F1, inserts into the membrane³³. This results in the formation of the prehairpin intermediate spanning both membranes. Heptad repeat regions 1 and 2 (HR1 and HR2) at the N and C termini of the F1 subunit associate forming a stable hairpin 6-helix bundle (6HB) which pulls the two membranes together to facilitate fusion with the host cell membrane⁴¹.

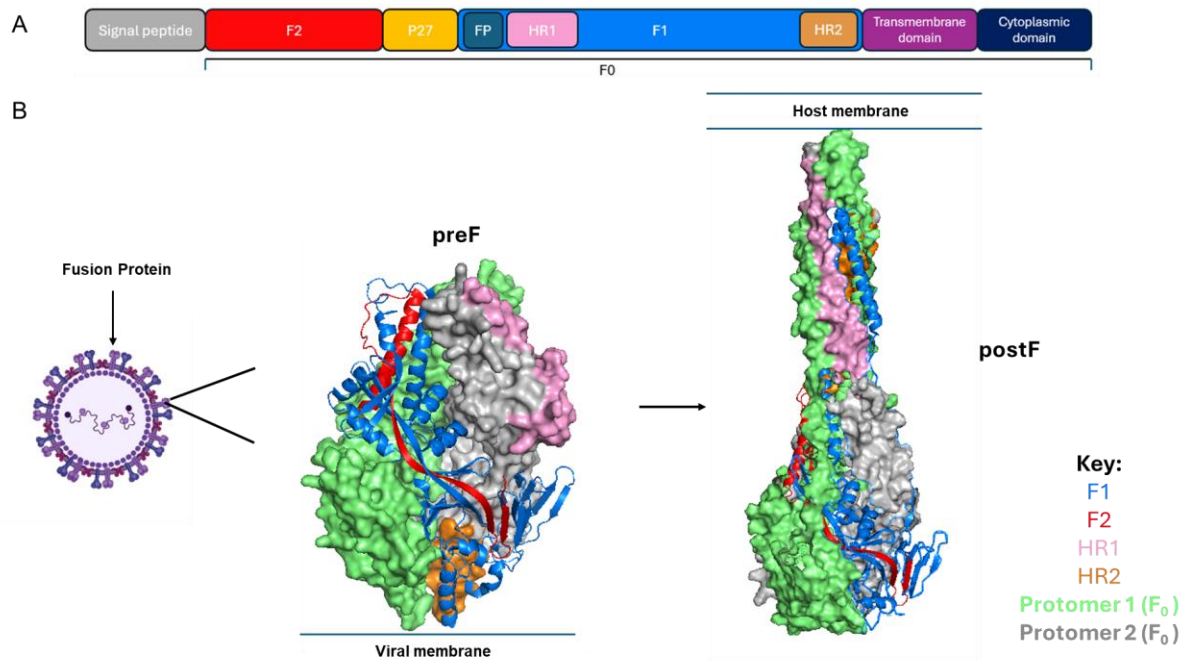


Figure 1.3 RSV F protein structure and key features. (A) Key features of the RSV F protein. The RSV F protein inactive precursor (F_0) is cleaved by host furin-like protease giving rise to mature F1 and F2 subunits. The fusion protein (FP) is located at the N-terminus of F1 and is flanked by HR1. The small peptide 27 (p27) is cleaved following F_0 processing. A signal peptide is located at the N terminus of the F protein. HR2 is located before the transmembrane domain and cytoplasmic domain at the C terminus of the F protein. **(B)** PreF and postF trimers. Surface representation of one protomer (F_0) in lime green and a second protomer (F_0) in grey are shown. HR1 (pink) and HR2 (orange) of this second protomer are highlighted. A third protomer (F_0) is shown as a ribbon with F1 (blue) and F2 (red) subunits highlighted. Surface and ribbon representations were generated in Pymol.

1.5 RSV F sites of vulnerability

Given its sequence conservation and central role in mediating viral entry, the F protein is a promising target for prophylactic antibodies and vaccines⁴². There are seven known antigenic sites (sites Ø (located at the Apex), I, II, III, IV, V⁴³ and VI⁴⁴ (membrane-proximal external region (MPER)) within the F protein (Figure 1.4). Apart from site II, all of these sites are non-linear but highly conformational (Figure 1.4 A and B). Sites I, II, III, and IV remain unchanged following the irreversible conformational change and are thus shared between both preF and postF conformations⁴⁵ (Figure 1.4 A). In contrast, the apex site Ø and site V are conformation-specific and are

exclusively found in the preF conformation⁴⁰ (Figure 1.4 A). These preF specific sites in particular are highly neutralisation sensitive^{43,46,47}

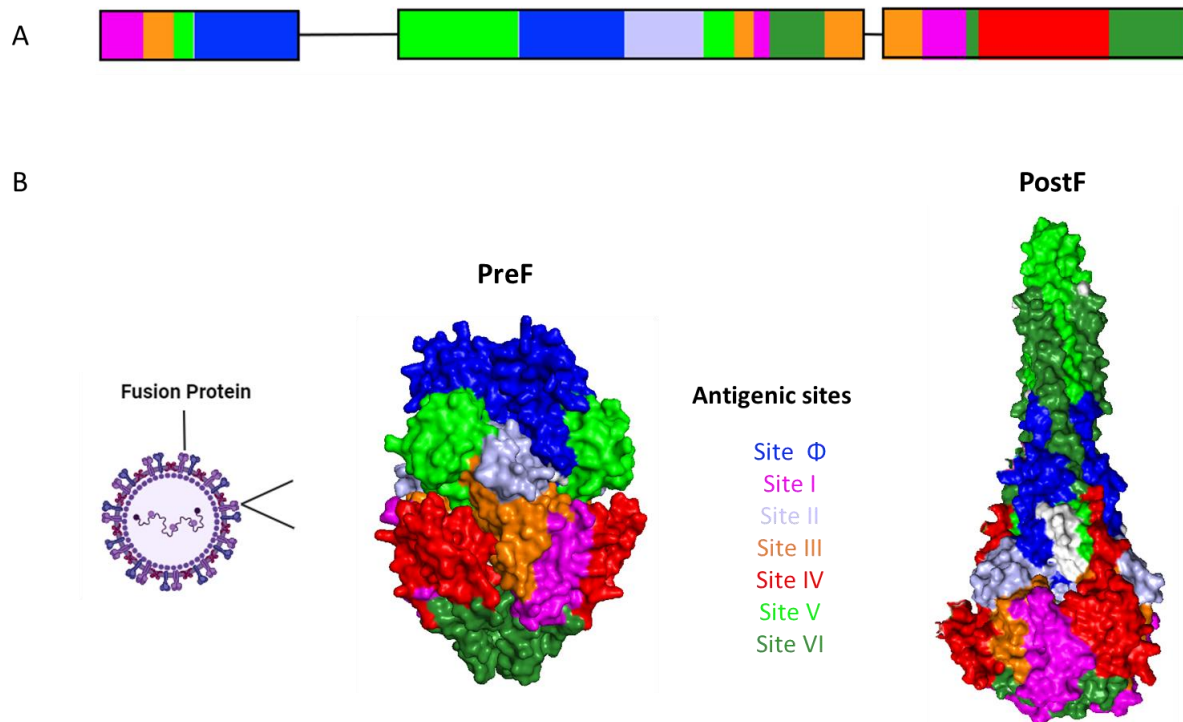


Figure 1.4. Antigenic sites of the RSV F protein in the prefusion and postfusion conformations. (A) Schematic representation of the antigenic sites (coloured by key) and their position in the linear protein. **(B)** Seven known antigenic sites; Ø (blue), I (magenta), II (cyan), III (orange), IV (red), V (green), and VI (tan) exist within the RSV F protein. Sites Ø and V are present exclusively in the preF conformation while all other sites are shared between both conformations. The surface representation of the 3D structure of the preF and postF conformations was generated using PyMOL.

1.6 . Historic and current RSV preventative interventions

The development of an RSV vaccine faced a major setback shortly after the virus's discovery, when a formalin-inactivated RSV (FI-RSV) vaccine candidate led to enhanced respiratory disease in children upon natural infection in vaccinees⁴⁸. As a result, 80% of the immunised children were hospitalised, and two fatalities were recorded⁴⁸. The failure of this vaccine provided many valuable lessons that have

shaped the ongoing development of RSV vaccines, including the need to avoid Th2-based CD4+ T cell and suboptimal neutralisation responses, both of which were linked to the enhanced disease outcome⁴⁹. The identification of the preF⁴² and postF^{50,51} conformations and structural characterisation of these has provided additional insight to why this FI-RSV vaccine failed to provide protection⁴⁵. The FI-RSV vaccine together with several other historic F-based subunit vaccines were revealed to be postF vaccines which lacked critical neutralisation sensitive sites⁴⁵. The FI-RSV vaccine, which lacked preF on its surface, directed immune responses away from infectious virus, ultimately leading to enhanced disease upon subsequent exposure⁴⁵.

This completely shifted the focus from historic postF vaccines lacking sites of vulnerability to novel vaccines which retain neutralisation sensitive preF sites. The first preF stabilised subunit vaccine immunogen, DS-CaV1, was developed on this basis and preserved the key neutralisation sensitive sites Ø and V⁵². It provided proof of concept for the elicitation of enhanced neutralising activity of antibodies targeting the preF conformation in phase I clinical trials^{53,54}. The success of this and other F-based subunit vaccines that maintain the preF conformation has driven the development of novel vaccines displaying important epitopes preserved in the preF conformation, as well as monoclonal antibodies (mAbs) targeting preF⁴⁵.

The present RSV preventative landscape is divided into four categories, including protein-based vaccines, mRNA (nucleic acid-based) vaccines, live-attenuated or chimeric vector vaccines and monoclonal antibody (mAb) prophylactics (Figure 1.5). (<https://www.path.org/our-impact/resources/rsv-vaccine-and-mab-snapshot/>). The preF antigen forms the basis of the three licensed vaccines, two licensed mAb prophylactics, and 11 out of 30 RSV candidates currently in clinical development (Figure 1.5)⁵⁵

Protein based vaccines are focused primarily on displaying vulnerable antigenic sites which are most susceptible to neutralisation, included in these are the preF subunit based vaccines⁵⁵. Three of these preF subunit vaccines have received market approval while an additional five are in clinical development (Figure 1.5).

RSVpreF3 (licensed as Arexvy)⁵⁶, a monovalent preF protein based vaccine (Figure 1.5) became the first RSV vaccine approved for preventing RSV disease in adults aged 60 and older⁴⁵. It was approved by the European Union (EU) together with other

high-income countries (HIC) including the United States, Canada, Australia, Japan and the United Kingdom (May, 2023 - January 2024). Concerns surrounding statistically significant instances of increased preterm births recorded in South African clinical trials however halted its approval as a maternal vaccine and particularly in LMICs⁵⁷. RSVpreF3 is further undergoing phase 2 clinical trial (NCT05921903) for its use in immunocompromised young adults older than 18 years⁵⁵.

RSVpreF vaccine (licensed as Abryvso)⁵⁸, a non-adjuvanted bivalent preF protein based vaccine (Figure 1.5) demonstrated significant protection in phase 3 clinical trials against RSV LRTI in infants after birth^{58,59}. This vaccine was approved by the United States Food and Drug Administration (FDA), EU and other HICs including Canada and Japan (March-August, 2023) for use as a maternal vaccine in pregnant women between 32 and 36 weeks of pregnancy^{45,55}. Abryvso was also approved for administration in the elderly population in August 2023^{45,55}. RSVpreF is in late-stage clinical trials for its use in children aged 2-18 years (NCT05900154) and young adults older than 18 years (NCT05842967)⁵⁵.

Two other particle-based vaccines are in clinical development, including V306-SVLP (Figure 1.5), a particle based vaccine preserving site II of the F protein and IVX-A12, which combines human metapneumovirus (hMPV)-preF with stabilised RSVpreF⁵⁵. There are additional vaccines undergoing phase 2 clinical trials which focus on antigens other than the RSV F protein including the G protein (BARS13)⁶⁰ and the Daichii Sankyo VN-0200 vaccine which utilises a VAGA-9000a antigen (Figure 1.5)⁵⁵.

In addition to particle-based vaccines, mRNA technology has been utilised for RSV vaccine development. The Moderna mRNA-1345 (licensed as mRESVIA) (Figure 1.5) received market approval by the US FDA in the older population in May 2024⁵⁵. This vaccine adapted the same nanoparticle technology as the SARS-CoV-2 Spike Vax to elicit immunity against the stabilised RSV preF protein⁵⁵. The same mRNA-1345 vaccine is undergoing phase 2 clinical trials for use as a maternal vaccine (NCT06143046) and for administration in seropositive children (NCT06097299)⁵⁵. Sanofis SP0256 vaccine (Figure 1.5) a mRNA for administration in older adults is in phase 2 clinical trials.

Chimeric vectors and live-attenuated vaccine (LAV) candidates are also in clinical development. There are three assigned RSV LAV families based on the non-essential

viral proteins which they target including NS1, NS2, and M2-2. The lead LAV candidate (RSVt, Sanofi) which is part of the Δ NS2 family is undergoing phase 3 clinical trial stages for its use in the paediatric population (Figure 1.5)⁵⁵. and is followed by RSV 6120/ Δ NS2/1030s another member of the Δ NS2 family⁶¹. A chimeric vaccine based on live parainfluenza 5 (PIV5) encoding the RSV F antigen is in its early stages of clinical development⁶² (Figure 1.5). The development of an LAV with attenuated NS1, NS2, and G protein deletion (MV-012-968) was recently stopped due to strategic reasoning of the manufacturing company (Figure 1.5)⁵⁵.

Apart from vaccines, two anti-RSV prophylactic mAbs are used for protecting infants from severe RSV disease (Figure 1.5). The first, is Palivizumab, a mAb targeting site II of the F protein⁶³. However, in LMICs, its use is restricted to high-risk infants⁶⁴ because it requires multiple doses and is costly⁶⁵. The alternative, Nirsevimab, targets the apex site Ø and is preferred over Palivizumab providing more than 50-fold higher neutralising activity in clinical trials^{65,66}. It has been engineered with a triple amino acid substitution (M252Y/S254T/T256E), known asYTE, in its heavy chain fragment crystallizable (Fc) region, which extends its half-life⁶⁴. As a result, Nirsevimab requires only a single dose, making it more cost-effective and accessible than Palivizumab⁶⁴. Nirsevimab (licensed Beyfortus) (Figure 1.5) has been approved for use by the US FDA and European Medical Agency since July 2023⁴⁵. It has also been approved by the Medicines and Healthcare products Regulatory Agency (MHRA) in the United Kingdom. Merck's Clesrovimab (MK-1654)⁶⁷, which targets site IV, is another mAb with an extended half-life that is in late-stage clinical development (Figure 1.5). This mAb binds a quaternary epitope with a preference for the preF conformation and has demonstrated potent *in vivo* protection against RSV⁶⁷.

RSV Vaccine and mAb Snapshot

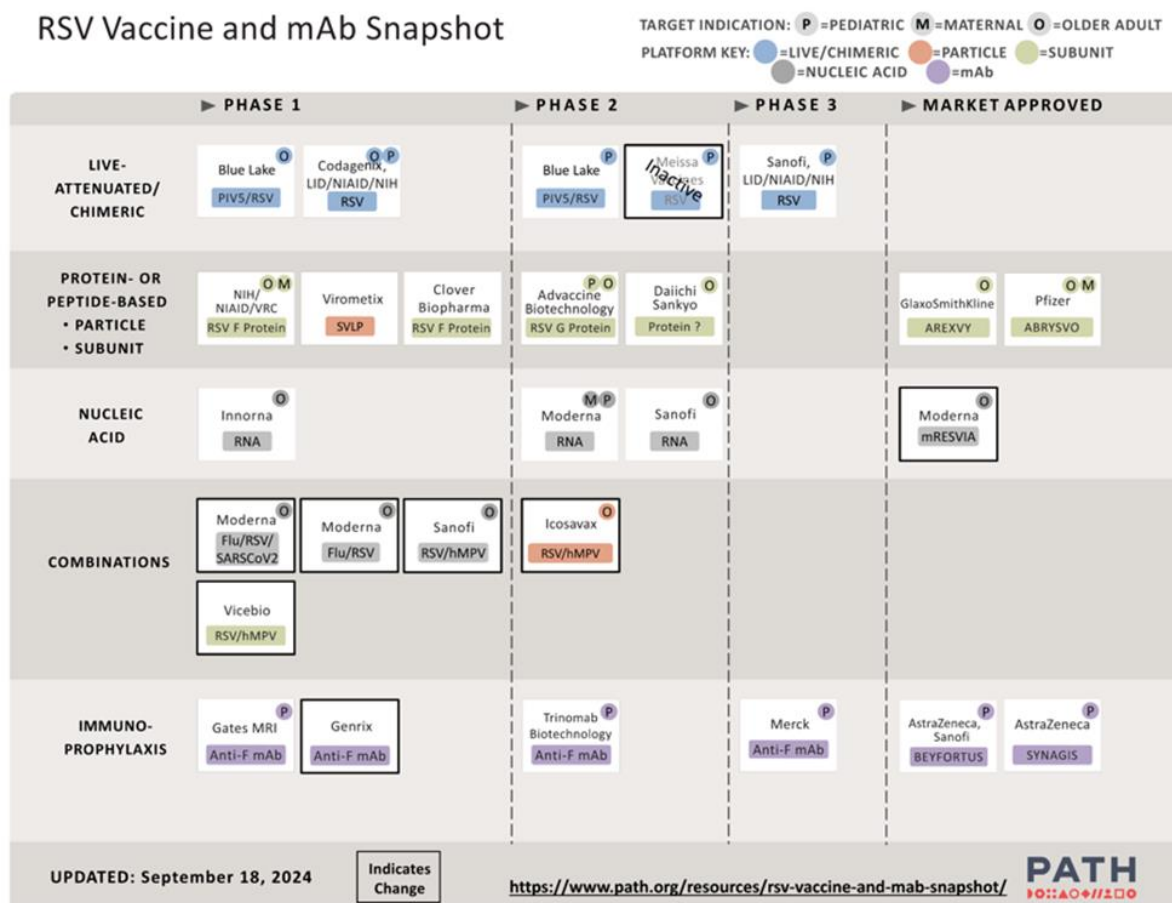


Figure 1.5. Landscape of all RSV immuno-prophylactics and vaccines. A summary of RSV vaccines and mAbs which are in use, undergoing development or those that have been discontinued. Protein based or preF vaccines have progressed the furthest with two vaccines, Arexvy and Abryvso being approved for market use. Arexvy has been approved for use in the elderly population while Abryvso has been approved as both a maternal vaccine and for use in the elderly population. Two licensed mAb prophylactics are also in use, namely Nirsevimab (marketed as Beyfortus) and Palivizumab (marketed as Synagis). Image obtained from PATH on the 7th of February 2025 <https://www.path.org/our-impact/resources/rsv-vaccine-and-mab-snapshot/>).

1.7. The need for RSV immunological surveillance

While novel vaccines and monoclonal antibody (mAb) prophylactics are being implemented there is a pressing need for increased immunological surveillance, especially in LMICs where the translation of genomic sequencing data into functional data is limited^{68,69}. The importance of monitoring the impact of RSV evolution became

apparent after the failure of the late-stage clinical trial of the site IV prefusion F protein targeting mAb Suptavumab (REGN2222)⁷⁰. A two amino acid substitution (L172Q/S173L) in the F protein of the predominant RSV-B strain circulating during the trial period, rendered the antibody ineffective⁷⁰. The REGN2222 trial failure, coupled with the global experience of reduced mAb efficacy against emerging SARS-CoV-2 variants⁷¹, underscores the critical need for continuous RSV immunological surveillance.

Historically, RSV genomic surveillance has focused on the G gene due to its diversity, but sequencing of the F gene, which is functionally important, has only recently become common with the application of whole genome sequencing and the establishment of the World Health Organization's RSV reference laboratories⁷²⁻⁷⁵. Recent sequencing efforts have identified rare Nirsevimab escape variants⁷⁶⁻⁷⁸. Their prevalence highlights the natural variability in key antigenic sites, though the functional relevance of these variations remains unclear.

The current immunological toolkit for assessing the functional relevance of RSV variants relies on whole replication-competent viruses⁷⁹⁻⁸² and lentivirus^{83,84} pseudovirus packaging systems⁸⁵. While these systems have been useful, replication-competent viruses are complex, and lentivirus pseudovirus systems are limited to monitoring samples from individuals living without HIV, due to the background caused by antiretroviral drugs. The vesicular stomatitis virus (VSV) pseudovirus packaging system, which has not yet been utilised in RSV assays, offers a promising alternative. Key advantages of VSV packaging systems are their ability to rapidly form pseudoviruses and their flexibility in incorporating various types of glycoproteins into the viral envelope⁸⁶. VSV pseudotyped virions may also be used in a BSL-2 laboratory⁸⁷. These systems have already been successfully implemented for other respiratory pathogens, such as SARS-CoV-2⁸⁸. Additionally, VSV packaging systems provide significant benefits over lentiviral systems, particularly in regions with high antiretroviral drug usage, like South Africa.

This study therefore aims to establish an VSV-based RSV pseudovirus neutralisation assay to monitor the impact of seasonal RSV evolution on mAb prophylactics and population immunity.

2. OBJECTIVES

2.1. Overall Aim

Evaluate the impact of seasonal variation detected in South African RSV genomes on the efficacy of neutralising antibodies.

2.2. Specific Aims

2.2.1. Identify key mutations in circulating South African variants and introduce these into RSV pseudoviruses.

- Prioritise ten fusion gene mutations, located in antigenic sites of South African RSV-A and RSV-B genomic surveillance data from the 2023 RSV season.
- Use prioritised mutations to generate the ten VSV-RSV-F mutant pseudoviruses.

2.2.2. Express and characterise anti-RSV monoclonal antibody controls

- Clone and express six anti-RSV neutralising mAbs, targeting each prefusion F antigenic site.
- Clone RSV-A (strain A2) and RSV-B (strain 18537) fusion genes and express these as recombinant proteins for use as controls to evaluate anti-RSV mAb binding.
- Assess anti-RSV mAb binding using the recombinant RSV-A and RSV-B fusion proteins.

2.2.3. Establish and optimise a vesicular stomatitis virus (VSV)-based RSV pseudovirus neutralisation assay

- Generate VSV-RSV-F reference pseudoviruses for RSV-A (strain A2) and RSV-B (strain 18537) and verify RSV fusion protein expression.
- Identify and culture suitable cell lines to be used in the pseudovirus neutralisation assay
- Optimise the RSV neutralisation assay by determining suitable cell culture conditions, cell and virus input, incubation times and seeding conditions.

3. MATERIALS AND METHODS

3.1. Anti-RSV mAb expression and purification

3.1.1. Digestion of codon optimised anti-RSV neutralising mAb constructs

pUC57 plasmids containing codon optimised sequences (optimised for expression in mammalian culture) for the heavy and light chain variable regions of nine anti-RSV mAbs (Nirsevimab (MED18897) (site Φ)⁶⁴, ADI-15610 (site I)⁴³, Palivizumab (site II)⁸⁹, MPE8 (site III)⁹⁰, Clesrovimab (MK-1654) (site IV)⁶⁷, CR9501 (site V)⁹¹, 2.4K, 2.4B, and ADI-15558 (site VI)⁴⁴ (Appendix A1, Table A1.1) were obtained from GenScript Biotech (Netherlands) and cloned into a pVRC8400 expression vector⁹². Restriction digest reactions were performed using FastDigest enzymes (ThermoScientific™, USA) – *AgeI* and *Sall*, *AgeI* and *BsiWI*, and *AgeI* and *XhoI* for heavy, kappa and lambda chain variable regions respectively. All variable regions were coupled with the IgG1 constant region for the heavy chains and the lambda or kappa constant region for the light chains. Accordingly, for each digestion reaction, 1 µg of plasmid DNA was combined with 1 µl of *AgeI* (*BshTI* used as an isoschizomer of *AgeI*) and 1 µl of the appropriate 2nd restriction enzyme together with 5 µl of 10X FastDigest buffer (ThermoScientific™, USA) and made up to a final volume of 50 µl with distilled water (dH₂O) (Merk Millipore, USA). The reactions were digested at 37°C for 1.5 hours in a Bio-Rad T100™ thermal cycler (Bio-Rad laboratories Inc. USA) and subsequently resolved on a 1% Tris-acetate-EDTA (TAE) (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) agarose gel supplemented with 0.5 µl/ml of ethidium bromide (ThermoScientific™, USA) alongside appropriate molecular weight markers. Digests were confirmed by visualizing the gels using an iBright™ gel imaging system (Invitrogen™, USA). Following this confirmation, the remaining 46 µl of the digest reactions were resolved on an additional 1% TAE agarose gel at 120 V for one hour in preparation for gel extraction of the desired DNA fragments.

3.1.2. Purification of DNA fragments

Digested insert and backbone DNA fragments of the desired sizes were excised from the agarose gel and purified using the QIAquick Gel extraction kit (Qiagen®, Germany) per the manufacturer's instructions described briefly. Gel slices were weighed, and three volumes of buffer QG were added to one volume of the gel. The solution was incubated for 10 minutes at 50°C to solubilize the agarose. Buffer QG contains a pH

indicator which ensures optimal pH for DNA binding to the silica membrane of the QIAquick spin column. To bind the DNA, the sample was transferred to a QIAquick spin column and centrifuged at 13 000 rpm for one minute. This step was repeated for samples exceeding the 800 µl volume capacity of the column reservoir as needed. The column was washed by the addition of 750 µl of buffer PE and centrifugation at 13 000 rpm for one minute. The flow through was discarded and the column was centrifuged for an additional minute to dry the column and remove any residual ethanol. The DNA was eluted in 50 µl of buffer EB (10 mM Tris-Cl, pH 8.5) by centrifugation for two minutes at 9000 rpm into a new microcentrifuge tube. The concentration of the DNA was measured using a NanoDrop ND-1000 Spectrophotometer (ThermoScientific™, USA).

3.1.3. Ligation into the pCMVR expression vector

Recombinant anti-RSV mAb plasmid constructs were generated by ligation of purified insert and vector (pVRC8400) DNA fragments using the rapid DNA ligation kit (Roche, Switzerland). Accordingly, 2 µl of DNA dilution buffer (5X) was added to an appropriate volume of vector and insert DNA (determined according to a standard ligation reaction molar ratio of vector DNA to insert DNA of 1:3) and made up to a final volume of 10 µl with dH₂O (Merck Millipore, USA). This mixture was supplemented with 10 µl of T4 DNA ligation buffer and 1 µl of T4 DNA ligase and incubated in a Bio-Rad T100™ thermal cycler (Bio-Rad laboratories Inc. USA) for 30 minutes at 25°C for ligation to take place.

3.1.4. Transformation of XL-10 Gold cells

XL-10 Gold® ultracompetent cells (Agilent Technologies, USA) were transformed with recombinant mAb plasmid DNA. Briefly, 4 µl of β-mercaptoethanol (Merck Millipore, USA) was added to 50 µl of XL-10 Gold® ultracompetent cells. After incubation on ice for 5 minutes, 2 µl of recombinant mAb plasmid DNA was added and incubated for an additional 10 minutes. Following a heat shock at 42°C for 30 seconds in a water bath to permeabilize the cell membrane and allow entry of DNA, 450 µl of S.O.C media (Super Optimal media with Catabolite repression, 20 mM Glucose, 10 mM Magnesium chloride, 10 mM Magnesium sulphate, 2.5 mM Potassium chloride, 10 mM Sodium chloride, 20 g/L Tryptone, 5 g/L yeast extract) (Invitrogen™, USA) was added. Cells were incubated while shaking at 200 rpm for one hour at 37°C. After incubation, 100

µl of each transformation mixture was plated on Luria-Bertani (LB) (Invitrogen™, USA) agar plates containing kanamycin (100 µg/ml) antibiotic and incubated at 37°C for 16 hours overnight shaking at 200 rpm.

3.1.5. Plasmid purification

Anti-RSV mAb plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen®, Germany). This kit allows for purification of up to 20 µg of high-copy plasmid DNA and is based on the alkaline lysis of bacterial cells and subsequent adsorption of DNA onto silica in the presence of high salt concentrations. Individual colonies (see section 3.1.4) were cultured in 5 ml of LB broth supplemented with kanamycin (100 µg/ml) antibiotic. The bacterial cultures were incubated at 37°C overnight shaking at 200 rpm and were harvested 16 hours later by centrifugation at 5000 rpm for 15 minutes. The bacterial pellets were resuspended in 250 µl of buffer P1 (containing RNase A and lyseBlue reagent (serves as a visual indicator during the lysis step by staining dead or disrupted cells)). The resuspensions were vortexed, transferred to new microcentrifuge tubes, lysed with 250 µl of buffer P2 (containing sodium dodecyl sulphate (SDS)) before inverting. In addition to complete lysis of the cells this facilitates release of all intracellular material including genomic DNA. These were neutralised with 300 µl of Buffer N3 and pelleted at 13 000 rpm for 10 minutes at room temperature. The supernatants were transferred to a QIAprep spin column and centrifuged at 13 000 rpm for 30 seconds to bind the plasmid DNA to the column. The columns were washed with 750 µl of buffer PE and centrifuged at 13 000 rpm for 30 seconds. The flow through was discarded and any residual wash buffer was removed by an additional centrifugation step at 13 000 rpm for one minute. The QIAprep column was placed into a new 1.5 ml microcentrifuge tube. DNA was eluted from the column in 50 µl of buffer EB by centrifugation at 9000 rpm for 2 minutes at room temperature. The concentration of the purified plasmids was measured using a NanoDrop ND-1000 Spectrophotometer (ThermoScientific™, USA).

3.1.6. Sequence validation of plasmid constructs

Purified mAb constructs cloned into the pVRC8400 expression vector were sequence confirmed using sanger sequencing. In brief, sanger sequencing reactions consist of a DNA template, template specific primer, DNA polymerase, regular deoxynucleotide triphosphates (dNTPs), and modified dideoxynucleotide triphosphates (ddNTPs). The

ddNTPs are fluorescently labelled and act as chain terminators, lacking the 3'-OH group needed to form a phosphodiester bond between nucleotides. Upon incorporation, they terminate DNA strand elongation, forming strands of varying lengths. A standard polymerase chain reaction (PCR) is followed, DNA is denatured, primers are annealed to the DNA template, and DNA polymerase extends the DNA strand by incorporation of dNTPs and ddNTPs. Automated next generation sequencing machines can detect fluorescently labelled ddNTPs. The emission wavelengths of incorporated ddNTPs enables detection of bases at a specific position. Sequencing results are visualised as chromatograms

The ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosciences™ Thermo Fisher Scientific™, USA) was used to sequence the anti-RSV mAb plasmids. A PCR was set up for each reaction comprising of 1 µl of pink ready reaction mix, 1.5 µl sequencing buffer (5X), 3.5 µl dH₂O, template DNA (300 µg) and 2 µM CMVR forward (Fw) primer (CTAGTTAACGGTGGAGGGCAGTGT) specific for the DNA template. The single CMVR Fw primer, specific for the region of the pVRC8400 backbone adjacent to the start of the insert fragment was sufficient for sequencing of the entire constant and variable regions of the light chains but only beginning of the constant region of the heavy chain for the respective anti-RSV mAbs. The reactions were performed under the following thermocycling conditions (Table 3.1)

Table 3.1. Sanger Sequencing thermocycling parameters:

Reaction step	Number of cycles	Temperature °C	Time (minutes)
Denaturation	25	96	0.10
Annealing		50	0.05
Extension		60	4

Upon completion of the PCR reactions, A sodium acetate clean-up was performed to ensure removal of residual sequencing reaction components such as unincorporated primers and dNTPs which may interfere with final sequencing data. A fresh ethanol and sodium acetate mix (EtOH/NaOAc) was made up by combining 1 ml of anhydrous ethanol (EtOH) with 320 µl of sodium acetate (3M pH 4.6). 50 µl of the prepared EtOH/NaOAc solution was mixed into each well/tube of the PCR reactions and

centrifuged for 30 minutes at 2000 x g. The samples were then centrifuged inverted for 1 minute at 150 x g. The pellets were rinsed with 70% cold ethanol while avoiding resuspension of the pellet and then centrifuged for 5 minutes at 2000 x g. The samples were centrifuged inverted again for 1 minute at 150 x g and then heated at 65°C for 3 minutes to get rid of any residual liquid. Finally, the cleaned reactions were resolved using a 3500 XL genetic analyser (Applied Biosciences™ Thermo Fisher Scientific™, USA) and the resultant nucleotide sequence outputs were analysed using the Geneious Prime® 2024.0.7 software (www.geneious.com). The sequences were trimmed of any unresolved sequences, and chromatograms were checked for any false nucleotide call outs. These final sequences were compared to reference sequences (Appendix A, Table A1) of the respective light and heavy chain anti-RSV mAbs and assessed for matching full sequence identity.

3.1.7. Maxiprep plasmid purification

The anti-RSV mAb plasmids from sections from 3.1.5 and 3.1.6 were purified using the ZymoPURE™ II Plasmid Maxiprep kit (ZymoResearch, USA). This kit uses alkaline lysis and spin-columns for purifying transfection grade plasmid DNA. XL-10 Gold® ultracompetent cells (Agilent Technologies, USA) were retransformed with 100 ng/μl of sequence confirmed recombinant mAb plasmid DNA as described in section 3.1.4. Single colonies were used to inoculate 5 ml starter cultures of LB broth supplemented with Kanamycin (100 μg/ml) antibiotic. The bacterial cultures were incubated at 37°C shaking at 200 rpm for 4 hours and then scaled up to 200 ml cultures in a 1L baffled conical flask. These were incubated at 37°C overnight shaking at 200 rpm. The following day, the overnight cultures containing plasmid DNA were pelleted by centrifugation at 5000 rpm for 15 minutes in a microcentrifuge. The cells were resuspended in 14 ml of ZymoPURE™ P1 red buffer and then lysed by adding 14 ml of ZymoPURE™ P2 blue buffer to the suspension and incubating 3 minutes. Samples were neutralised with 14 ml of ZymoPURE™ P3 yellow buffer and centrifuged at 5000 rpm for 5 minutes. Following filtering with the ZymoPURE™ Syringe Filter-X, 14 ml of ZymoPURE™ binding buffer was added to the cleared lysate and mixed thoroughly by inversion. The sample was loaded into the Zymo-Spin™ V-PX column using a vacuum manifold. The column assembly was washed with 10 ml of ZymoPURE™ wash 1. This was followed by two additional wash steps with 10 mL of ZymoPURE. The 15 ml Reservoir-X was discarded and the Zymo-Spin™ V-PX column was placed

in a collection tube. To remove any residual wash buffer, the sample was centrifuged at 13000 rpm for 1 minute. The column was placed in a clean 1.5 ml microcentrifuge tube and the plasmid DNA was eluted with 400 µl of ZymoPURE™ elution buffer and centrifugation at 9000 rpm for 2 minutes. The concentration of the plasmid was measured using a NanoDrop ND-1000 Spectrophotometer (ThermoScientific™, USA). The plasmids were sequence confirmed as described in section 3.6.

3.1.8. Culture and maintenance of mammalian 293F cells for mAb expression.

The FreeStyle™293-F (293F) cell line was used for the expression of all anti-RSV mAb plasmids. This cell line was obtained from Thermo Fisher Scientific™ (USA) and is a variant of the parental 293 cell line adapted for growth media in suspension of FreeStyle™ 293 expression medium. The 293 parental cell line originates from primary human embryonic kidney (HEK) cells transformed with sheared human adenovirus type 5 DNA. All HEK 293F cells used in this study were maintained in FreeStyle™ 293 expression medium (Thermo Fisher Scientific™, USA) were passaged every 48 hours with seeding at 5×10^5 cells/ml in a 500 ml vented flask. The cells were incubated under 37°C and 8% CO₂ shaking at 135 rpm.

3.1.9. Expression of anti-RSV mAbs by transfection of HEK 293F cells

Recombinant mAb constructs were expressed by transient transfection in 400 ml of mammalian HEK 293F cell culture. On the day prior to transfection, 400 ml of cells were seeded at 1×10^6 cells/ml in a 1L sterile vented Erlenmeyer flask and incubated overnight as described in section 3.8. For each transfection reaction, a DNA to transfection reagent ratio of 1:3 was ensured. In brief, 1200 µl of freshly prepared sterilised PEI MAX (1 mg/ml) transfection reagent (Kyfora Bio, USA) was mixed with 10 ml of OptiPRO™ serum free medium (SFM) (Thermo Fisher Scientific™, USA) in a 50 ml falcon tube. SFM was used to reduce the potential of interfering proteins such as antibodies or growth media factors which are typically contained in serum. In a separate 15 ml falcon tube, 200 µg of heavy and 200 µg light chain anti-RSV mAb plasmid was mixed with 10 ml of OptiPRO™ SFM. Both the PEI MAX and DNA mixtures were incubated at room temperature for 5 minutes. The DNA mixture was filtered directly into the PEI MAX mixture using a 2 µm filter and incubated for a further 20 minutes at room temperature. Following incubation, the transfection mixture was added in a dropwise manner to the 400 ml HEK 293F cell culture cell. After incubation

at 37°C and 8% CO₂ in a shaking incubator for six days, the culture was harvested by centrifugation at 4000 rpm for 1 hour at 4°C. The culture supernatant was collected and filter sterilized (0.22 µm filter) using a vacuum pump.

3.1.10. Protein A affinity chromatography for mAb purification

The expressed mAbs were purified from filter-sterilised culture supernatants using protein A affinity chromatography. This method relies on the specific binding of immobilised protein A, derived from *Staphylococcus aureus* to the Fc portion of immunoglobulin G (IgG) antibodies.

A protein A column (2.5mL settled matrix) was prepared using a 10 ml Bio-Rad Econo-Pac® disposable chromatography column (Bio-Rad laboratories Inc, USA). The culture supernatants were passed through the column (1 drop every 2-3 seconds) to bind antibodies to the column. Ten column volumes of 1 x phosphate buffered saline (PBS) (Thermo Fisher Scientific™, USA) was used to wash the columns. Antibodies were eluted with 12.6 ml of elution buffer (0.1 M glycine, 0.15 M NaCl, pH 4.2) and immediately neutralised with 1.4 ml of neutralisation buffer (1M Tris pH 8.0). An additional wash step was performed to return the matrix to neutral pH and the columns were stored in 0.02% Azide for future use. Eluted antibody dialysed overnight into PBS using a Pur-A-Lyzer™ Maxi 3500 dialysis kit (Merck Millipore, USA) according to the manufacturer's instructions. The concentration of the purified antibodies was measured using a NanoDrop ND-1000 spectrophotometer (ThermoScientific™, USA) at an extinction coefficient ($\epsilon_{\text{percent}}$) in the range of 12 to 15.

3.1.11. SDS-PAGE for anti-RSV mAb purity and size verification

Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to verify the purity and size of the purified mAbs. NuPAGE™ Bis-Tris (Invitrogen™, USA) polyacrylamide gels (4-12%) were used for separating and resolving small- to medium-sized proteins (1.5-300 kDa) under denaturing electrophoresis conditions. mAb samples were resolved under non-reducing conditions to confirm the structural integrity of the intact antibody (disulfide-linked fragments) and under reducing conditions to separate the individual light and heavy chains, verifying their sizes. Samples were prepared by adding 10 µg of antibody, 5 µl of NuPAGE™ LDS sample buffer (4X) (Thermo Fisher Scientific™, USA), 3 µl of β -mercaptoethanol (Merck Millipore, USA) for reducing conditions and dH₂O made up to

a final volume of 20 μ l. The same reaction was set up for the samples run under non-reducing conditions without the addition of β -mercaptoethanol. Gels were run with freshly prepared NuPAGE™ MOPS SDS running buffer (1X) (Thermo Fisher Scientific™, USA) and 10 μ l of the prepared sample mixture was loaded alongside the Novex® Sharp pre-stained protein standard (Thermo Fisher Scientific™, USA) resolved at 150 V for one and a half hours. Gels were stained for 1 hour in PageBlue™ protein staining solution (ThermoScientific™, USA), followed by three rinses with dH₂O and destaining overnight shaking. The antibody DNA fragments were visualized on the iBright™ gel imaging system (Invitrogen™, USA).

3.1.12. ELISA for anti-RSV mAb functionality

The functionality of the anti-RSV mAbs was assessed using enzyme linked immunosorbent assay (ELISA) with commercially acquired RSV-A, strain A2 prefusion glycoprotein (Sino Biological, China) and RSV-B, strain B18537 fusion glycoprotein F₀ (Sino Biological, China) as antigens. Accordingly, Corning® (USA) 96-well EIA/RIA ELISA high-binding assay plates were coated with 100 μ l/well of 2 μ g/ml diluted RSV F antigen. The plates were sealed with Corning® (USA) microplate sealing tape and incubated overnight at 4°C. The following day, the plates were washed three times with 250 μ l/ well of wash buffer (1X PBS (Gibco 10X PBS, Thermo Fisher Scientific™, USA), 0.05% Tween 20 (Merck Millipore, USA)) using an ELx405 automated plate washer (Bio-Tek instruments Inc, USA) and then blocked with 200 μ l/well blocking buffer (5% (w/v) skimmed milk powder (local grocery) in wash buffer) for 2 hours. A wash step was performed to remove any residual blocking buffer following this incubation. Three-fold anti-RSV mAb serial dilutions at a starting concentration of 1 μ g/ml were performed and 100 μ l/well of each diluted antibody was transferred in duplicate to the blocked 96-well assay plates. Commercial Palivizumab (1 μ g/ml starting concentration) (AbbVie pty Ltd, South Africa) and in-house VRC01 (anti-HIV mAb) (5 μ g/ml) were included as positive and negative controls, respectively. The plates were sealed, incubated at room temperature for one hour and then washed three times in wash buffer as described. 100 μ l/well of diluted (1: 3000) anti-human IgG peroxidase secondary antibody (Merck Millipore, USA) was added to the samples and incubated for one hour at room temperature. The plates were washed to remove any residual secondary antibody and 100 μ l/well of 1-Step Ultra TMB-ELISA substrate (ThermoScientific™, USA) was added to all wells. After a 5-minute incubation in the

dark, the reactions were stopped by adding 50 µl/well 1 M sulphuric acid (H₂SO₄) (Merck, South Africa) to all wells and read immediately at a wavelength of 450 nm in a SpectraMax® ABS Plus Microplate Reader (Molecular Devices LLC, USA).

3.2. RSV pseudovirus production

3.2.1. RSV F gene construct design for pseudovirus expression.

Pseudovirus constructs for RSV-A and RSV-B were designed using reference RSV F gene sequences (Table 3.2) from isolated RSV lineages obtained from the Global Initiative on Sharing All Influenza Data (GISAID) EpiRSV database (<https://gisaid.org/>). The sequences were codon optimised for expression in RSV F DNA was synthesised and cloned into pcDNA3.1 (+) by GenScript (Netherlands) mammalian cells and inserted into a pcDNA3.1 (+) vector. An additional Kozak (GCCACC) sequence for initiating protein translation in eukaryotic cells was added to the 5' end, before the start codon. ready to be transformed and purified for transformation and purification as described in section 3.2.5.

Table 3.2. Pseudovirus reference sequence identifier information

Virus isolate name	RSV subtype	GISAID accession number
hRSV/A/England/397/2017	A	EPI_ISL_412866
hRSV/B/Australia/VIC-RCH056/2019	B	EPI_ISL_1653999

3.2.2. RSV F gene construct cytoplasmic tail (CT) modifications.

The pseudovirus constructs (described in section 3.2.1) were modified by Gibson assembly to truncate or modify the CT regions of the F gene. Based on similar modifications by Oomens et al. (2006), two additional pseudovirus constructs, one with a complete CT truncation (Δ 21) and the other a chimera which replaces the RSV F CT with the vesicular stomatitis virus glycoprotein CT (VSV-G CT) were assembled. The constructs were designed for both RSV-A and RSV-B. Three key steps including primer design, amplification and assembly of constructs were carried out as follows.

For the Δ 21 constructs, a reverse and forward primer were designed to amplify portions of the F gene without the CT region with overlapping regions of the vector and the vector with overlapping regions of the F gene prior to the start of the CT region,

respectively (Figure 3.1 A). For the VSV-G CT chimeras forward and reverse primers were designed to amplify the VSV-G CT and F gene/vector fragments. For the 20 bp VSV-G CT fragment, a forward and reverse primer were designed to amplify a portion of VSV-G CT with overlapping regions into the F gene region prior to the start of the CT, and the vector, respectively (Figure 3.1 B). For the F gene/vector fragments, a forward primer was designed to amplify a portion of the vector with overlapping regions into VSV-G CT and a reverse primer was designed to amplify a portion of the F gene overlapping regions into VSV-G CT (Figure 3.1 B).

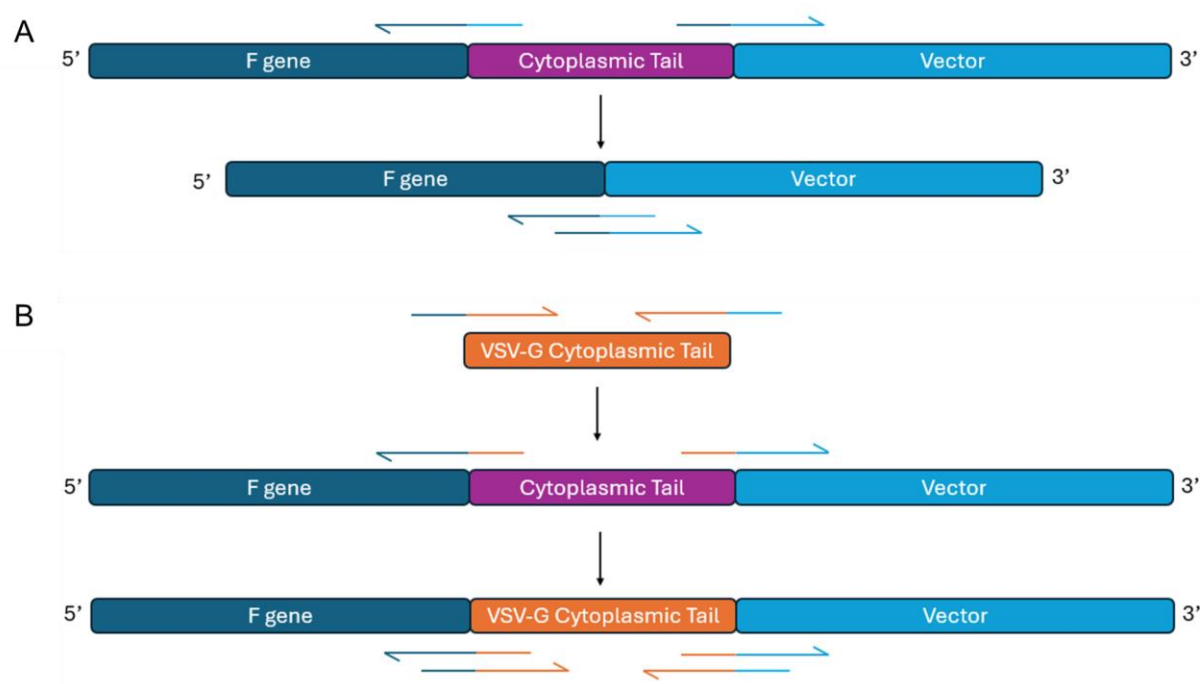


Figure 3.1. Strategies for RSV F gene CT modifications. Schematics of the **(A)** RSV F gene CT truncation and **(B)** RSV F gene CT replacement strategy. Primers are shown with overlapping regions coloured according to the overlapping region of the respective gene regions. The RSV F gene (dark blue), F gene CT (purple), vector (light blue) and VSV-G CT regions are shown (orange).

To ensure efficient assembly of PCR fragments, all primers were designed with a 15 bp overlap, appropriate GC content, and a melting temperature (T_m) equal to or greater than 48°C, according to the manufacturer's instructions. All primers (Appendix A1, table A1.2 and A1.3) were synthesised as designed by Inqaba Biotech Inc (South Africa).

All fragments were amplified by PCR using the Invitrogen™ Platinum Superfi II PCR Master Mix. For the RSV-A and RSV-B $\Delta 21$ constructs, one PCR reaction was set up, respectively to amplify the truncated RSV F gene/vector fragment (Table 3.1). In each reaction, 25 μ l of 2X Platinum Superfi II PCR master mix was combined with 2.5 μ l of the appropriate forward and reverse primer (0.5 μ M) (Appendix A1, table A1.2 and A1.3), 0.1 – 10 ng of template DNA and made up to a final volume of 50 μ l with nuclease free water. For the RSV-A and RSV-B VSV-G CT chimeras, two PCR reactions were set up, respectively. The first reaction to amplify the VSV-G CT fragment (Table 3.4) and the second reaction to amplify the F gene/vector fragment (Table 3.3). The reactions were performed under the following thermocycling conditions (Table 3.3 and 3.4).

Table 3.3. PCR cycling parameters – large/backbone fragments

Cycle Step	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	98	30 sec	1
Denaturation	98	10 sec	25
Annealing	60	10 sec	
Extension	72	4 min (30 sec / kb fragment length)	
Final Extension	72	5 min	1

Table 3.4 PCR cycling parameters – small/Insert fragments

Cycle Step	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	98	30 sec	1
Denaturation	98	10 sec	25
Annealing	60	10 sec	
Extension	72	30 sec (30 sec / kb fragment length)	
Final Extension	72	5 min	1
	4	Hold	

Amplified PCR products were resolved on 1 or 2 % agarose gels to confirm sizes of the larger 7075 bp fragments and smaller 90 bp VSV-G CT fragments respectively. All agarose gels were prepared using 1X TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) buffer supplemented with 0.5 µl/ml of ethidium bromide (ThermoScientific™, USA). The fragments of the expected size were excised and purified using the QIAquick Gel extraction kit (Qiagen®, Germany) as described in section 3.1.2.

Finally, the modified RSV F gene CT constructs were assembled from the purified PCR products using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs® Inc, USA) according to the manufacturer's instructions. A DNA molar ratio of vector to insert of 1:2 was applied in all assemblies. For the RSV-A and B Δ 21 assembly reactions, 7.8 µl and 8 µL, respectively of appropriate truncated RSV F gene/vector was combined with 10 µl of NEBuilder HiFi DNA assembly master mix and H₂O to a final volume of 20 µl. For the RSV-A and B VSV-G CT assembly reactions, 1.5 µl and 1.6 µl of the VSV-G CT fragment respectively, was combined with 1.2 µl of the appropriate F gene/vector fragment, 10 µl of NEBuilder HiFi DNA assembly master mix and H₂O to a final volume of 20 µl. The reactions were incubated at 50°C for 15 minutes in a thermocycler to produce assembled plasmids ready for transformation and purification

3.2.3. Plasmid preparation of unmodified and modified pseudovirus constructs

XL-10 Gold® ultracompetent cells (Agilent Technologies, USA) were transformed with unmodified and modified pseudovirus constructs as described in section 3.1.4. Plasmids were purified from transformed colonies using the QIAprep Spin Miniprep Kit (Qiagen®, Germany) as described in section 3.1.5.

3.2.4. Sequence confirmation of the unmodified and modified pseudovirus constructs

The purified pseudovirus constructs were sequence confirmed using sanger sequencing as described in section 3.1.6. In each PCR reaction, 1 µl of pink ready reaction mix was combined with 1.5 µl sequencing buffer (5X), template plasmid DNA (300 µg), 2 µM of the appropriate Fw and reverse (Rv) sequencing primers (Appendix A1, Table A1.4), and dH₂O to a final volume of 10 µl.

Final sequencing of cleaned-up products was performed using a 3500 XL genetic analyser (Applied Biosciences™ Thermo Fisher Scientific™, USA) and the resultant

nucleotide sequence outputs were analysed using the Geneious Prime® 2024.0.7 software (www.geneious.com). The sequences were trimmed of any unresolved sequences, and chromatograms were checked for any false nucleotide call outs. These final sequences were compared to reference unmodified (Table 3.2) and modified pseudovirus plasmid sequences (Appendix A1, Table A1.5) and assessed for matching full sequence identity.

3.2.5. Pseudovirus plasmid purification

XL-10 Gold® ultracompetent cells (Agilent Technologies, USA) were retransformed with 100 ng/μl of sequence confirmed pseudovirus plasmids as described in section 3.1.4. Plasmids were purified from selected colonies and purified using the ZymoPURE™ II Plasmid Maxiprep kit (ZymoResearch, USA) as described in section 3.1.7 to generate sufficient DNA yield to perform downstream pseudovirus expression in mammalian cells.

3.2.6. Culture and maintenance of mammalian cells for pseudovirus expression.

The Expi293F™ cell line, obtained from Thermo Fisher scientific™ (USA) was used for the transfection of all RSV F pseudovirus constructs. This cell line is derived from the 293F cell line, is grown in suspension culture, and has been optimised to grow to high density in Expi293F™ expression medium (Thermo Fisher Scientific™, USA). Expi293F™ are preferred over standard 293 cell lines as they are highly transfectable and generate higher protein yields. All Expi293F cells used in this study were maintained in serum-free Expi293™ expression media (Thermo Fisher Scientific™, USA) and passaged every 3 days, with seeding $0.3 - 0.5 \times 10^6$ cells/ml. The cells were maintained such that they did not exceed 5×10^6 cells/ml ensuring optimal performance and were incubated at 37°C and 8% CO₂ shaking at 135 rpm.

3.2.7. Generation of RSV pseudoviruses

Expi293F cells were seeded in 25 mL at a density of 3×10^6 cells/ml in a 125 ml sterile vented Erlenmeyer flask and incubated overnight as described in section 3.2.6. Cells were transfected per the manufacturer's instructions using the ExpiFectamine™ 293 Transfection Kit. Twenty-four hours after seeding 3.9 ml of OptiPRO™ Serum free medium (Thermo Fisher Scientific™, USA) was combined with 30 μg RSV F plasmid DNA in a 15 ml Falcon tube. The DNA mixture was filtered into a separate 15 ml falcon

tube containing 80 µl of ExpiFectamine™ 293 transfection reagent using a 2 µm filter and incubated for 10 minutes at room temperature. The transfection mixture was added in a dropwise manner to the 25 ml Expi293F cell culture diluted to a final density of 3×10^6 viable cells/ml and incubated as described in section 3.2.6. Twenty hours post-transfections, expression enhancers (150 µl of Expifectamine™ 293 transfection enhancer 1 and 1.5 ml of Expifectamine™ 293 transfection enhancer 2) were added. Transfected Expi293F cells would express RSV F protein on their cell surface.

At 48 hours post-transfection, the cells were infected with a recombinant envelope gene-deficient vesicular stomatitis virus ($G^*\Delta G$ -luciferase) rVSV⁸⁸ obtained from Dr Theodor Hadziioannou at Rockefeller University to serve as the backbone for the engineered RSV F pseudoviruses. As determined by titration experiments, transfected cells were infected with 3.125 µl per 1×10^6 cells of ($G^*\Delta G$ -luciferase) rVSV and incubated for 2 hours at 37°C and 8% CO₂ shaking at 135 rpm. The cells were pelleted by centrifugation and washed three times with pre-warmed Expi293™ expression medium to remove any residual ($G^*\Delta G$ -luciferase) rVSV. The cells were resuspended to ensure a final concentration of 5×10^6 cells/ml and incubated again at 37°C and 8% CO₂ shaking at 135 rpm. After 24 hours, sufficient for a full round of viral replication, the culture was collected by centrifugation at 1000 x g for 5 minutes at room temperature. The supernatant was filter sterilised using a 2 µm filter, 10% FBS was added, and 2 ml aliquots were stored at - 80°C.

3.2.8. Validation of cell surface expressed RSV F protein

RSV-A and B, $\Delta 21$ and VSV-G CT constructs were expressed by transfection of 800 µl of Expi293F cell cultures in a 96-well format. The transfection protocol described in section 3.2.7 was adapted as follows for small-scale transfections. Expi293F cells were seeded in 25 mL at a density of 3×10^6 cells/ml in a 125 ml sterile vented Erlenmeyer flask and incubated as described in section 3.2.6. Twenty-four hours after seeding, 100 µl of OptiPRO™ Serum free medium (Thermo Fisher Scientific™, USA) was combined with 0.8 µg of purified RSV-A and B, $\Delta 21$ CT and VSV-G CT plasmid DNA in respective wells of the 96-well plate. The DNA mixture was filtered by centrifugation at 400 rpm for 5 minutes using a 96-well filter plate into a 96-well V-bottom collection plate. In each respective well, the DNA mixture was combined with 2.5 µl of ExpiFectamine™ 293 transfection reagent and incubated for 10 minutes to

initiate the plasmid DNA complexation reaction. The Expi239F cell culture was diluted to a final density of 3×10^6 viable cells/ml and 700 μ l of the cells was added to each transfection mixture. The reactions were incubated at 37°C and 8% CO₂ on a Vibramax100 shaker (Heidolph, Germany) shaking at 1100 rpm. After 20 hours, 5 μ l of Expifectamine™ 293 transfection enhancer 1 was combined with 50 μ l of Expifectamine™ 293 transfection enhancer 2 and added to the transfections.

Transfected cells were pelleted by centrifugation at 210 x g for 10 minutes, resuspended in 10 ml of PBS wash buffer, and centrifuged again at 210 x g for 10 minutes. This wash step was repeated three times to remove any residual transfection reagents and enhancer. In the final wash, the cells were resuspended in 998 μ l of PBS and 2 μ l of AquaVital live/dead stain, making a 1 ml final resuspension. After incubation in the dark for 10 minutes, the cells were washed as described previously and resuspended in PBS/BSA (0.1%). The volume added was adjusted to ensure a final concentration of 100 000 cells/well and 50 μ l of the suspension was added to each well of a 96-well V-bottom plate. Five-fold anti-RSV mAbs serial dilutions at starting concentration of 200 μ g/ml were prepared and 50 μ l/well of each diluted antibody was transferred in duplicate to a 96-well V-bottom plate containing cells. The mixture was incubated at room temperature for 30 minutes. The cells were then pelleted at 1500 rpm for five minutes, decanted and washed three times with 200 μ l/well PBS/BSA (0.1%) wash buffer to remove any residual primary antibody. All transfected cells were resuspended in 100 μ l of secondary APC anti-human IgG Fc antibody (1:100) (Thermo Fisher Scientific™, USA) diluted in PBS/BSA (0.1%) wash buffer. Untransfected control samples with no secondary APC stain were also included. The samples were incubated at room temperature for 30 minutes and washed three times with 200 μ l/well PBS/BSA wash buffer to remove any residual secondary antibody. The samples were fixed by resuspension in 250 μ l/well of 4% paraformaldehyde in PBS and stored overnight in the dark at 4°C. The samples were acquired on a CytoFLEX benchtop flow cytometer (Beckman Coulter, USA) to evaluate cell surface mAb binding.

3.3. RSV pseudovirus infection optimisation

3.3.1. Culture and maintenance of adherent cell lines for pseudovirus infection

Hep-2 (Human laryngeal carcinoma cells), Vero (African green monkey kidney cells), and A549 (Lung carcinoma cells) adherent cells obtained from Cellonex (PTY) LTD (South Africa) were selected for use in RSV pseudovirus infection and neutralisation trials, based on their successful use in published RSV culture assays⁸³⁻⁸⁵. To achieve optimal growth media and cell viability for each cell line, the culture conditions, including type of growth media media, growth media media supplement, and antibiotic/fungal agents were optimised. All cell lines were grown in Dulbecco's Modified Eagle's Medium (1X) (DMEM) (Thermo Fisher Scientific™, USA) supplemented with 10% FBS. In addition, Hep-2 culture media was supplemented with 1% 1000 U/ml penicillin/streptomycin/25 µg/ml antibiotic and 1% L-Glutamine (200 mM). A549 culture media was supplemented with 2.5% HEPES buffer and 0.5% Gentamycin antibiotic. Vero culture media was supplemented with 1% anti-anti, L-Glutamine (200 mM), and non-essential amino acids (NEAA). All cells were incubated in adherent cell T75 culture flasks at 37°C and 8% CO₂.

3.3.2. RSV pseudovirus titrations

Infectivity of the RSV pseudoviruses described in section 3.2.7 and a bald virus control (Infection of (G*ΔG-luciferase) rVSV without RSV F transfection) was determined by viral titration. Hep-2, Vero, and A549 cells were seeded 24 hours prior to infection in a white corning® 96-well flat bottom treated culture plate (The Scientific group, South Africa). On the day of infection, in a clear Corning® (USA) round bottom 96-well dilution plate, 120 µl of appropriate cell growth media (see section 3.3.1) was added to each well and 30 µl of RSV pseudovirus was added in duplicate to column 1 (Figure 3.3). A 5-fold dilution series was performed by transferring 30 µl of mixed virus to the next column and repeated through to column 11 (Figure 3.2). No virus was added to column 12 (cell control) (Figure 3.2). All media was removed from the pre-seeded cell plate and 50 µl of the virus dilution was added to the corresponding wells on the cell plate. The plates were covered and incubated for one hour at 37°C in a 5% CO₂ incubator. After incubation, all media was removed from the assay plates and 50 µl of Bright Glo luciferase substrate was added to all wells and incubated shaking for three minutes on an orbital shaker at 600 rpm. The plates were read immediately using a

GloMax luminometer (Promega, USA) and analysed to determine whether infection had occurred as indicated by an increase in relative light units (RLU).

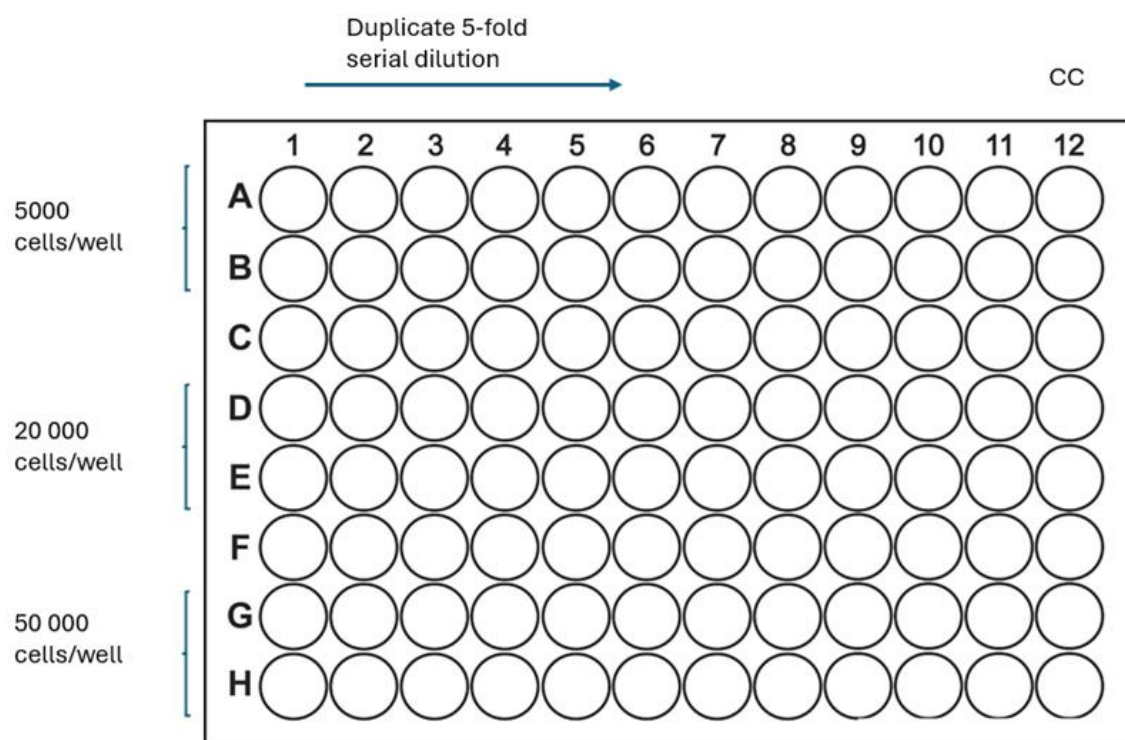


Figure 3.2. Plate map of virus titrations. Unmodified and CT modified RSV titrations were performed in a 96-well plate. Duplicate 5-fold dilutions were performed for each virus. Three cell densities, 5 000 cells/well, 20 000 cells/well, and 50 000 cells/well are shown. Rows C and F contain empty wells. A cell control (CC) column where no virus was added is shown

3.3.3 Optimisation RSV pseudovirus infection parameters

To establish optimal infection of the RSV pseudoviruses, four infection parameters were evaluated: cell type, cell density, cell seeding conditions, and incubation periods (Figure 3.3). Hep-2, Vero, and A549 cells as described in section 3.3.1 were seeded either on the day of infection or a day prior to infection at 5 000 cells/well, 20 000 cells/well, and 50 000 cells/well and then infected with a 5-fold dilution of unmodified or CT modified RSV pseudoviruses (11 duplicate wells per dilution) (Figure 3.2). A cell only control was also included (Figure 3.3). Luminescence was measured as a function of pseudovirus infection using the Bright-Glow luciferase reagent with a GloMax luminometer (Promega, USA) following 24-, 48-, and 72-hour infection periods. Pseudovirus infection was also optimised across varied freeze-thaw conditions, plasmid transfection amounts and CT modifications (Figure 3.3)

Figure 3.3. RSV pseudoparticle optimisation conditions overview. A schematic showing the flow of RSV pseudovirus infectivity optimisation conditions. A total of 252 conditions were tested across unmodified (top) and modified (Δ 21 and VSV-G CT) (bottom) RSV pseudovirus constructs varying cell type (Vero, A549, and Hep-2), cell density (5000, 20 000, and 50 000 cells/well) either pre-seeded (right) or not (left), and incubation periods (24-, 48-, and 72-hour). Unmodified RSV-A pseudoparticles were also subjected to varied freezing conditions, without FBS, with 10% FBS, with Mr Frosty (MF), and with both 10% FBS and MF. Unmodified pseudoparticles were also subjected to varied plasmid transfection amounts (15 μ g, 30 μ g, and 50 μ g), and thawed at either 4°C or at 37°C.

3.4. RSV neutralisation assay

3.4.1. VSV-RSV pseudovirus neutralisation assay

Neutralisation assays were adapted from the Nexelis VSV-SARS-CoV-2 neutralisation assay protocol⁹³. Vero cells were seeded at 0.5×10^6 cells/plate in a white Corning® (USA) 96-well flat bottom culture plate on the day prior to the assay. After 24 hours, 50 μ l of growth media (DMEM, 5% FBS, 1% anti-anti, 1% L-glutamine, and 1% NEAA) was added to each well in a clear Corning® (USA) round bottom 96-well dilution plate, followed by a 12.5 μ l top-up of growth media to the sample wells (columns 3-12) in row H. Anti-RSV mAbs described above were added to the sample wells, with commercial Palivizumab and VRC01 (anti-HIV mAb) used as positive and negative controls, respectively. The samples were added in duplicate at 50 μ g/ml starting concentration and 5-fold serial dilutions were made by transferring 12.5 μ l to each subsequent row, through to row A where the 12.5 μ l was removed and discarded. 50 μ l of each serially diluted mAb sample was transferred from the dilution plate to corresponding wells on a new 96-well falcon plate (Corning®, USA), followed by a 100 μ l and 50 μ l top up of growth media to all wells in column 1 (cell control) and column 2 (virus control), respectively. RSV pseudovirus was thawed at 37°C, diluted in growth media to achieve greater than 50 000 RLU (approximately 1000-fold higher than the cell control), and 50 μ L of the diluted virus was added to all wells except column 1. Following incubation of virus and antibody sample for one hour at 37°C in a 5% CO₂ incubator, 50 μ L from each well was transferred to the corresponding wells of the cell plate (with media removed), from row H to A. After 24 hours of incubation at 37°C in a 5% CO₂ incubator, all media was removed from the assay plates and 50 μ l of Bright Glo luciferase substrate was added to all wells. The plates were shaken for 3 minutes

at 600 rpm on an orbital shaker and then read immediately using a GloMax luminometer (Promega, USA). Variations to key parameters in this neutralisation assay were evaluated. Viral and mAb input dose, as well as virus and sample incubation times and temperature were optimised

3.5. Whole-virion ELISA

3.5.1. Validation of RSV F protein expression at the pseudotyped virion surface by ELISA

A whole-virion ELISA was performed to validate RSV F protein expression at the pseudotyped virion surface. In brief, the remaining 10 ml of RSV-A Δ 21 and VSV-G CT and 12 ml of RSV-B Δ 21 and VSV-G CT pseudovirus cultures harvested as described in section 3.2.8 were subjected to 10% polyethylene glycol (PEG)-6000 precipitation on ice for 90 minutes with moderate stirring. Precipitated pseudoviruses were pelleted by centrifugation at 4200 x g at 4°C for 30 minutes. The supernatants were discarded, and the virions were resuspended in 1.1 ml of 1X PBS (Gibco 10X PBS, Thermo Fisher Scientific™, USA). Appropriate wells of a Corning® (USA) 96-well EIA/RIA ELISA high-binding plates were coated with 100 µl/well of resuspended virions, sealed and incubated at 4°C for 2 hours. The plates were washed three times with 250 µl/well of wash buffer (1X PBS (Gibco 10X PBS, Thermo Fisher Scientific™, USA), 0.05% Tween 20 (Merk Millipore, USA)) using an ELx405 automated plate washer (Bio-Tek instruments Inc, USA) and blocked with 200 µl/well blocking buffer (5% (w/v) skimmed milk powder in (local grocery) in wash buffer) for 2 hours. A wash step was performed to remove any residual blocking buffer following this incubation. Single 5 µg/ml dilutions of Palivizumab, Nirsevimab, and ADI-14359 were performed and 100 µl/well of each diluted antibody was transferred in duplicate to the blocked 96-well assay plates. An anti-VSV-G mAb, 8G5F11, was included as a control mAb to control for the presence of (G* Δ G-luciferase) rVSV described in section 3.2.8. The plates were sealed, incubated at room temperature for one hour and then washed three times in wash buffer as described. Following the addition of 100 µl/well of diluted (1: 3000) anti-human IgG peroxidase secondary antibody (Merck Millipore, USA), the samples were incubated for one hour at room temperature. The plates were washed to remove any residual secondary antibody and 100 µl/well of 1-Step Ultra TMB-ELISA substrate (ThermoScientific™, USA) was added to all wells. After a 5-minute

incubation in the dark, the reactions were stopped by adding 50 µl/well 1 M sulphuric acid (H₂SO₄) (Merck, South Africa) to all wells and read immediately at a wavelength of 450 nm in a SpectraMax® ABS Plus Microplate Reader (Molecular Devices LLC, USA).

3.6. RSV F gene mutation identification

3.6.1. Phylogenetic analysis - Determining appropriate RSV F gene reference sequences

Appropriate RSV F gene reference sequences were determined by phylogenetic analysis of the 335 RSV-A and 370 RSV-B curated F gene sequences described in section 4.1.1. The curated nucleotide sequences were aligned using the MAFFT alignment plugin on Geneious Prime® 2024.0.7 software (www.geneious.com). Maximum likelihood trees were constructed in Geneious Prime® 2024.0.7 based on the appropriate model suggested by the online FindModel tool. (<http://hiv.lanl.gov/content/sequence/findmodel/findmodel.html>)⁹⁴. These trees were imported to the Tree rate tool, (<https://www.hiv.lanl.gov/content/sequence/TREE/RATEv2/treerate.html>) in order to determine the most appropriate sequence place at the root of each phylogenetic tree for^{90,91}. The sequences used to root the phylogenetic trees were selected as the reference sequences for RSV-A and RSV-B, respectively.

3.6.2. RSV F protein mutation analysis

Curated RSV-A (n=569) and RSV-B (n=374) sequences described in section 4.1.1 were aligned using the MAFFT alignment plugin on Geneious Prime® 2024.0.7 software (www.geneious.com) to the reference RSV F gene sequences EPI-ISL-2582791 (Kenya, 2013 isolate), and EPI-ISL-2582791 (South Africa, 2016 isolate) identified through phylogenetic analysis. The aligned F gene sequences were translated to amino acid sequences and novel amino acid substitutions present RSV antigenic sites were identified. The percentage frequency of these amino acid substitutions out of the total RSV F gene sequences in each year was determined.

4. RESULTS

4.1 RSV-A and -B F protein diversity

4.1.1. RSV GISAID dataset

As of May 31, 2023, a total of 639 and 374 RSV-A and -B full genome sequences, respectively were downloaded from the GISAID EpiRSV (<https://gisaid.org/>) database, filtered by nucleotide sequence length (sequences >14 000 nt in length and no smaller than 1725 nt), continent (Africa) and the year of sample collection (2013-2024). The whole genome sequences were aligned to the GISAID EpiRSV F gene reference sequence (GISAID accession ID: EPI_ISL_412866 (RSV-A) and EPI_ISL_1653999 (RSV-B)) using the MAFFT alignment plugin on Geneious Prime® 2024.0.7 software (www.geneious.com) and trimmed to include only the F gene, from the start codon to the stop codon. All sequences with nucleotide ambiguities, including partial sequences, premature stop codons, and sequences mistaken for the incorrect RSV subtype on the GISAID database were removed. Curated RSV-A (n=569) and RSV-B (n=370) sequences, including duplicate sequences with matching nucleotide identity were reserved for mutation analysis but excluded by dereplication for the phylogenetic analysis. Dereplication of identical sequences in Geneious Prime resulted in a final set of 355 RSV-A and 202 RSV-B F from all African countries, with 256 RSV-A and 201 RSV-B F genes from South Africa.

4.1.2. Identification of appropriate reference sequences

To identify novel amino acid substitutions and eliminate identifying reversion in circulating variants from African RSV genomic surveillance data we performed a comprehensive phylogenetic analysis to identify suitable RSV-A and -B sequences.

Three phylogenetic iterations were performed for each of the RSV groups (RSV-A and RSV-B). The iterations were defined by whether an evolutionarily distant 1956 USA sequence was included (1st iteration), excluded (2nd iteration), or set as the outgroup (3rd iteration) in the analysis (Appendix A2, Table A2.1). For both subtypes, the three iterations were conducted on two datasets: an all-African dataset (RSV-A: 2013–2023, RSV-B: 2014–2023) and a South African only dataset (RSV-A: 2017–2023, RSV-B: 2015–2023) (Appendix A2, Table A2.1). While tracing global sequences of RSV-A and RSV-B evolution would have provided more comprehensive lineage representation,

this study aimed to assess the functional impact of South African RSV diversity on antibody responses, so only these sets were included. The inclusion and comparison of these datasets to the evolutionary distant 1956 USA sequence contextualised the African and South African sequences used, with the earliest RSV sequence (1956 USA) available.

For RSV-A, in all three iterations across the all-African dataset, the same root was identified: a Kenyan 2013 sequence (GISAID accession number EPI-ISL-2582791). (Figure 4.1 and Appendix A2, Figure A2.1-A2.2). Similarly, for RSV-B, the same root was identified in all three iterations: a South African 2016 sequence (GISAID accession number EPI-ISL-17220593) (Figure 4.2, Appendix A2, Figure A2.6-A2.7). These sequences were therefore selected as reference sequences for downstream sequence alignment and RSV F protein mutation analysis.

Across the South African only data set, for RSV-A, the first (1956 USA included) (Appendix A2, Figure A2.3) and second (1956 USA excluded) (Appendix A2, Figure A2.4) iterations presented with the same root: a 2019 sequence (GISAID accession number EPI-ISL-17218601), while in the third iteration (1956 USA sequence as outgroup) (Appendix A2, Figure A2.5) a different 2019 sequence (GISAID accession number EPI-ISL-17219369) was identified. All three iterations across the South African dataset for RSV-B presented the same root: a 2016 sequence (GISAID accession number EPI-ISL-17220593) (Appendix A2, Figure A2.8-A2.10).

Therefore, the RSV-A, Kenyan (EPI-ISL-2582791), and RSV-B, South African (EPI-ISL-2582791) sequences from the all-African dataset were selected as reference sequences for downstream sequence alignment and RSV F mutation analysis. However, due to the absence of African RSV sequences prior to 2013, resulting from limited historic genomic surveillance, the ability to fully assess mutations from earlier years is not possible. As a result, some mutations identified using these reference sequences may have been present prior to 2013.

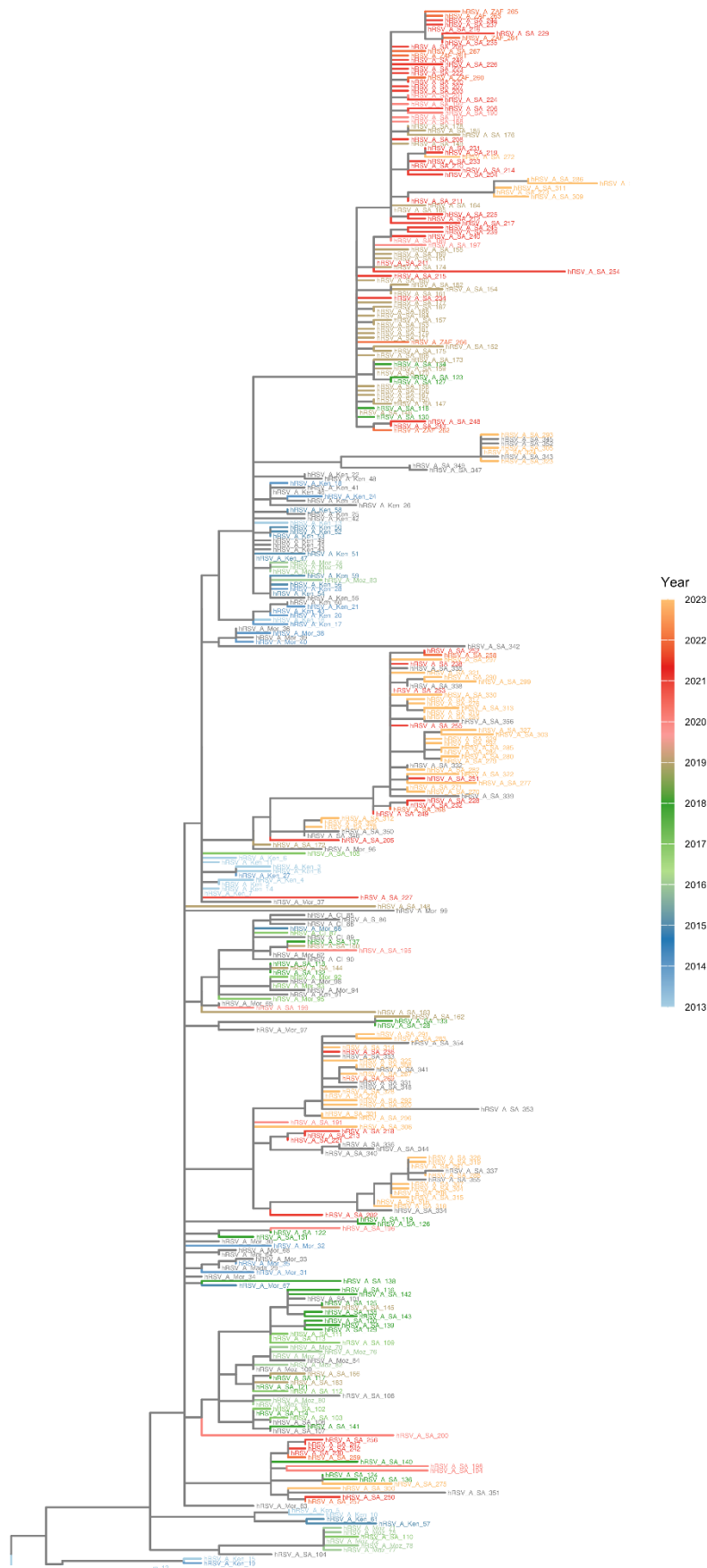


Figure 4.1. Phylogenetic tree for RSV-A reference gene identification, 1st iteration, All African (1956 USA excluded). Maximum likelihood phylogenetic tree of South African and African RSV-A F gene sequence sampled between 2013 – 2023 (colour gradient as per legend) is shown. Tree root is indicated by the blue star and is enlarged. Trees were plotted using the ggtree package in the R software environment for statistical computing and graphics

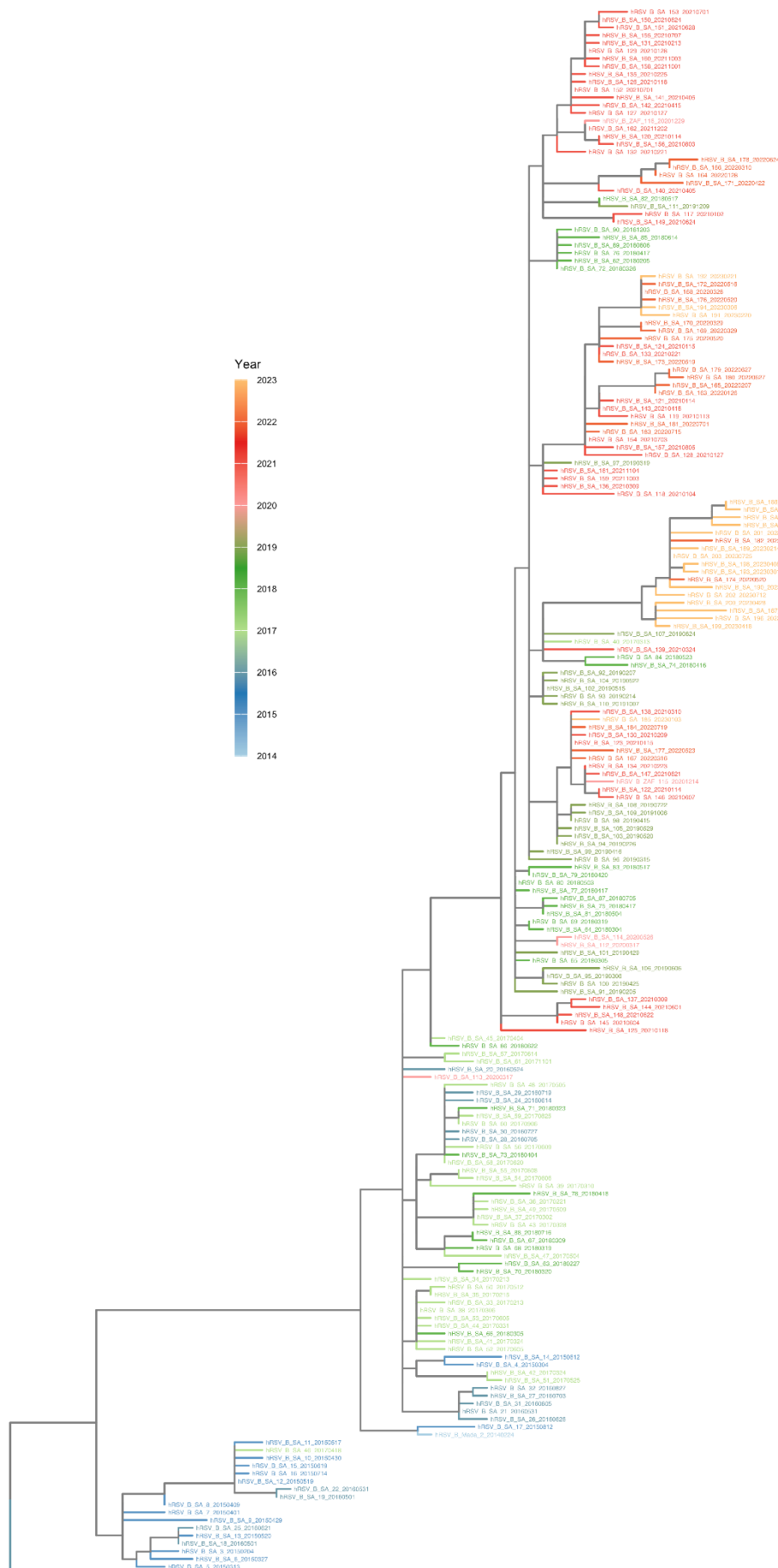


Figure 4.1. Phylogenetic tree for RSV-B reference gene identification, 1st iteration, All African (1956 USA excluded). Maximum likelihood phylogenetic tree of South African and African RSV-B F gene sequence sampled between 2013 – 2023 (colour gradient as per legend) is shown. Tree root is indicated by the blue star and is enlarged. Trees were plotted using the ggtree package in the R software environment for statistical computing and graphics

4.1.3. Analysis of South African RSV-A and RSV-B genomic surveillance data and F gene mutation prioritisation

With the recent approval of RSV vaccines for use in maternal immunisation and the elderly population, increased selective pressure on the virus, particularly within the F gene (which codes for the vaccine target), is anticipated. Monitoring the functional impact of F gene diversity on antibody responses—whether from vaccines, prophylactics or infection—is crucial for maintaining the efficacy of these interventions.

To support this, a mutation analysis was conducted on African RSV-A (n=569) and RSV-B (n=370) genomes from surveillance data collected between 2013-2023 and 2014-2023, respectively. The genomes were aligned using the reference sequences identified in section 4.1.2, and F gene sequences were extracted to identify nonsynonymous mutations contributing to F gene diversity. Mutations were numbered as per GISAID numbering and were prioritised based on the following scoring or selection criteria (Table 4.1 and 4.2): 1) presence in RSV F gene antigenic sites, 2) location within sites targeted by approved RSV prophylactics, 3) emergence as novel mutations, 4) high frequency across multiple years, and 5) amino acid changes with the potential to cause alterations to the conformational or structure of preF. Mutations meeting three or more of these criteria (priority score >3) were deemed significant and likely to contribute to viral escape (Table 4.1 and 4.2).

4.1.3.1. RSV F gene antigenic site diversity

Mutations observed in the seven antigenic sites of the RSV F protein likely to undergo selective pressure by humoral immune response were given a higher score than those outside of antigenic sites. A total of 30 RSV-A and 23 RSV-B mutations were identified across these sites (Appendix A2, Table A2.2). These included the identification of mutations in site Ø (targeted by Nirsevimab) and site II (targeted by Palivizumab). In site Ø, six RSV-A mutations (N63S, I64V, K65R, S215L, V293I, D200N) and six RSV-B mutations (N63S, I206M, Q209R, S211N, V220I, I233V) were identified. In site II, two RSV-A mutations (T245A, S276N) and one RSV-B mutation (S276N) were observed.

Table 4.1 RSV-A mutation selection criteria and prioritisation

	Ø						I	II	III					IV				V								VI					
Selection Criteria	N63S	I46V	K65R	S215L	V239I	D200N	K42R	I384T	T245A	S276N	V301A	T337I	S362L	S377N	K419E	S443T	V459I	L467I	S173T	G151S	N165D	N189F	S190N	V187I	E294K	S169N	T326I	A504E	V495I	A504T	
1. Present in antigenic site	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2. Present in the site of approved prophylactics	1	1	1	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3. Occur at high frequency over several years	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4. Occur at high frequency in recent years	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
5. Amino acid change not conserved e.g. Charge change, Bulk and / or Polarity change	1	0	0	1	0	0	0	1	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	1	0	1	1	0	1	1
Priority score	4	2	3	3	2	2	2	3	3	5	1	1	2	3	2	1	1	1	1	1	1	1	3	1	2	1	2	2	1	2	2

Table 4.2 RSV-B mutation selection criteria and prioritisation

	Ø						I					II	IV	V				VI				
Selection Criteria	N63S	I206M	Q209R	S211N	V220I	I233V	R42K	D385E	I386M	S389P	K390E	S276N	K419E	E463D	K191R	K168N	S190N	V187I	L321Q	N325T	S330T	D486G
1. Present in antigenic site	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2. Present in the site of approved prophylactics	1	1	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
3. Occur at high frequency over several years	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
4. Occur at high frequency in recent years	1	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0
5. Amino acid change not conserved e.g. Charge change, Bulk and / or Polarity change	1	0	1	1	0	0	0	1	0	1	0	1	1	0	0	0	1	0	1	1	1	1
Priority score	4	3	4	4	2	2	2	2	1	3	1	4	2	1	2	1	3	1	2	2	2	2

4.1.3.2. Mutations increasing in frequency within circulating lineages

Emerging mutations, especially those not observed prior to 2021, were also considered significant (Table 4.1 and 4.2). Mutation frequencies were normalised against frequency per year and expressed as percentages. Among RSV-A mutations, N63S, K65R, K42R, T245A, S276N, S377N, and S190N were observed and all increased in frequency in the period assessed here. N63S increased from 1% of sequences in 2021 (n=94) to 14% in 2023 (n=133) (Figure 4.3 A). K65R emerged in 2023 and was present in 18% of the sequences analysed (Figure 4.3 A). K42R was observed in 2% of sequences in 2019 (n= 65) which increased to 5% in 2023 (n=133) (Figure 4.3 B). T245A emerged in 2022, appearing in 6% of sequences analysed (n= 35) (Figure 4.3 C). S276N, previously seen in 4% of sequences in 2013 (n=25), rose to 19% in 2023 (n=133) (Figure 4.3 C). S377N emerged in 2022 (n=35) in 20% of

sequences but decreased to 8% in 2023 (n=133) (Figure 4.3 D). Similarly, S190N appeared in 2022 (n=35) in 29% of sequences, dropping to 7% in 2023 (n=133) (Figure 4.3 F), with both decreases likely due to the larger sample size in 2023.

Emerging RSV-B mutations; N63S, S211N, R42K, S389P, S276N, and S190N were also observed to increase in prevalence. N63S emerged in 2021 at 2% of sequences in 2021 (n=64) and increased to 7% in 2022 (n=46) (Figure 4.4 A). Similarly, S211N emerged in 2022 (n=46), rising from its presence in 2% of sequences to 71% in 2023 (n=28) (Figure 4.4 A). Although these mutations are being selected for, the increase in 2022/2023 may be due to the increase in sample size in 2023 compared with 2022. R42K increased from 12% in 2017 (n=33) to 21% in 2023 (n=28) (Figure 4.4 B), the increase again is likely due to the difference in sample size. S389P, a novel mutation, rose from 35% in 2022 (n=35) to 93% in 2023 (n=28) (Figure 4.4 B). S276N emerged in 2020 (n=9) in 11% of sequences but decreased to 3% in 2021 (n=64) (Figure 4.4 C) likely due to the larger sample size in 2023. A novel S190N mutation emerged in 2023 (n=28) present in 68% of the sequences (Figure 4.4 E).

Although this analysis identified mutations of increased frequency, it did not distinguish between random viral variation and immune-driven selection and future studies will include the use of the ratio between non-synonymous and synonymous mutations as the cut-off for this. For example, the modest increase of K42R (2% to 5%) is likely attributable to the virus's intrinsically error-prone replication, whereas the marked rise of S211N (2% to 71%) more plausibly reflects immune-mediated selection. Modest increases such as K42R were still noted as changes in frequency, although no statistical analyses were performed.

4.1.3.3. High frequency mutations

Antigenic site mutations that have been observed at a high frequency over several years were also considered important in the mutation identification process. While these mutations are not novel, their frequent occurrence in circulating African RSV lineages—alongside emerging novel mutations—could contribute to viral escape and represent the effect of current selection pressures on the virus.

The RSV-A I384T mutation was observed in all years since 2018, with its prevalence across sequences analysed ranging from 5% to as high as 86% (Figure 4.3 B). This pattern of sustained high frequency was more common in RSV-B, with three

mutations— I206M, Q209R (Figure 4.4 A), and K191R (Figure 4.4 E)— observed in every year since 2016/2017, with their prevalence ranging from 3% to 100% across analysed sequences. The persistence of these mutations at high frequencies suggests that they are more likely driven by natural immune selection rather than random variation.

4.1.3.4. Mutations with likely impact on conformation or structure of preF

Nonsynonymous mutations in antigenic sites, particularly those that induce conformational or structural changes in the RSV F protein, are likely to impact function and may contribute to viral resistance. These mutations can include the introduction or loss of charged residues, shifts from positively charged to negatively charged residues (or vice versa), changes in polarity, or the introduction of bulky residues. Such biophysical changes, particularly charge shifts, have been shown to facilitate immune evasion in enveloped viruses, as evidenced by HIV-1 evolution during chronic infection⁹⁵ and SARS-CoV-2 variant evolution⁷¹ reported in the literature. However, these changes are predictive and still need to be confirmed empirically in neutralisation assays.

For RSV-A, mutations that alter polarity were identified, including S215L, I384T, S326L, A384T, T362I, and A504T (Table 4.1). The K419E and E294K mutations represent changes from positively charged residues to negatively charged residues. Additionally, K419N and A504T mutations were identified, with K419N resulting in a loss of charge and A504T resulting in a gain of charge (Table 4.1). Mutations that introduce bulk were also found, such as N63S, S276N, S377N, and S190N (Table 4.1). Note that while the RSV-A T245A mutation occurred at high frequency (described in 4.1.2.3), structurally, this residue is not exposed at the protein surface. It is not likely to be at the direct contact site of any antibody, and for the purpose of this study was not investigated further although it has a priority score of >3.

For RSV-B, mutations identified that alter polarity include S389P and L321Q (Table 4.2). The Q209R mutation results in a gain of charge, while K168N and D486G mutations lead to a loss of charge (Table 4.2). The K390E and K419E mutations are changes from positively charged residues to negatively charged residues (Table 4.2). Apart from these changes in polarity and charge, mutations introducing bulk amino

acids were also identified, including N63S, S211N, S276N, S190N, N325T, and S330T (Table 4.2)

4.1.3.5. Priority mutations

Based on the selection criteria, the following mutations, which scored > 3 were selected as priority: RSV-A mutations (N63S, K65R, S215L, I384T, S276N, S377N, and S190N) (Figure 4.3 H) and RSV-B mutations (N63S, I206M, Q209R, S211N, S389P, S276N, and S190N) (Figure 4.4 H). Although extensive global analysis was beyond the scope of this study, several of these priority substitutions are also observed in global isolates. For instance, RSV-A I384V, S276N, and S377N, along with RSV-B S190N and S389P, have been reported in isolates from the USA. Similarly, RSV-A S377N and RSV-B S190N, S211N, and S389P have been identified in isolates from France. These mutations are prioritised for future assessment of their functional impact on antibody responses.

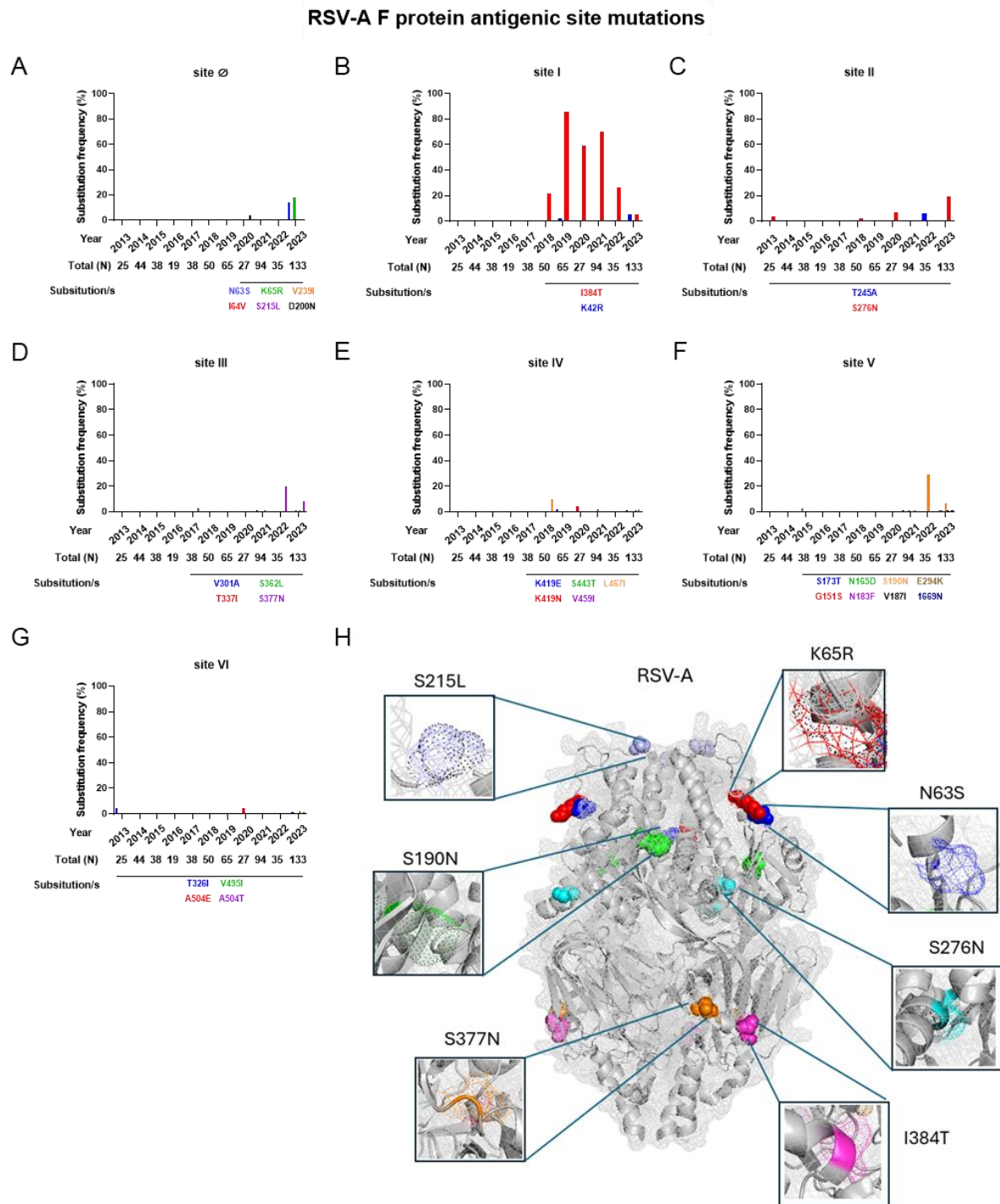


Figure 4.3. Identification of South African RSV-A F protein antigenic site mutations putatively of relevance. (A-G) RSV-A F protein amino acid substitution frequency plots identified from F gene sequences (2013-2024) (n=569) aligned to the EPI-ISL-2582791 reference sequence. The frequency (%) of identified substitutions (y-axis) by year (2013-2023, x-axis) is plotted, with substitutions coloured as per the key shown. **(H)** A cartoon and mesh representation of the 3D structure of the preF protein, displaying secondary structural elements and the surface of the protein. Priority substitutions are highlighted (inlet) – K65R

(red), N63S (dark blue), S276N (cyan), I384T (pink), S377N (orange), S190N (green), and S215L (light blue). Generated using PyMOL.

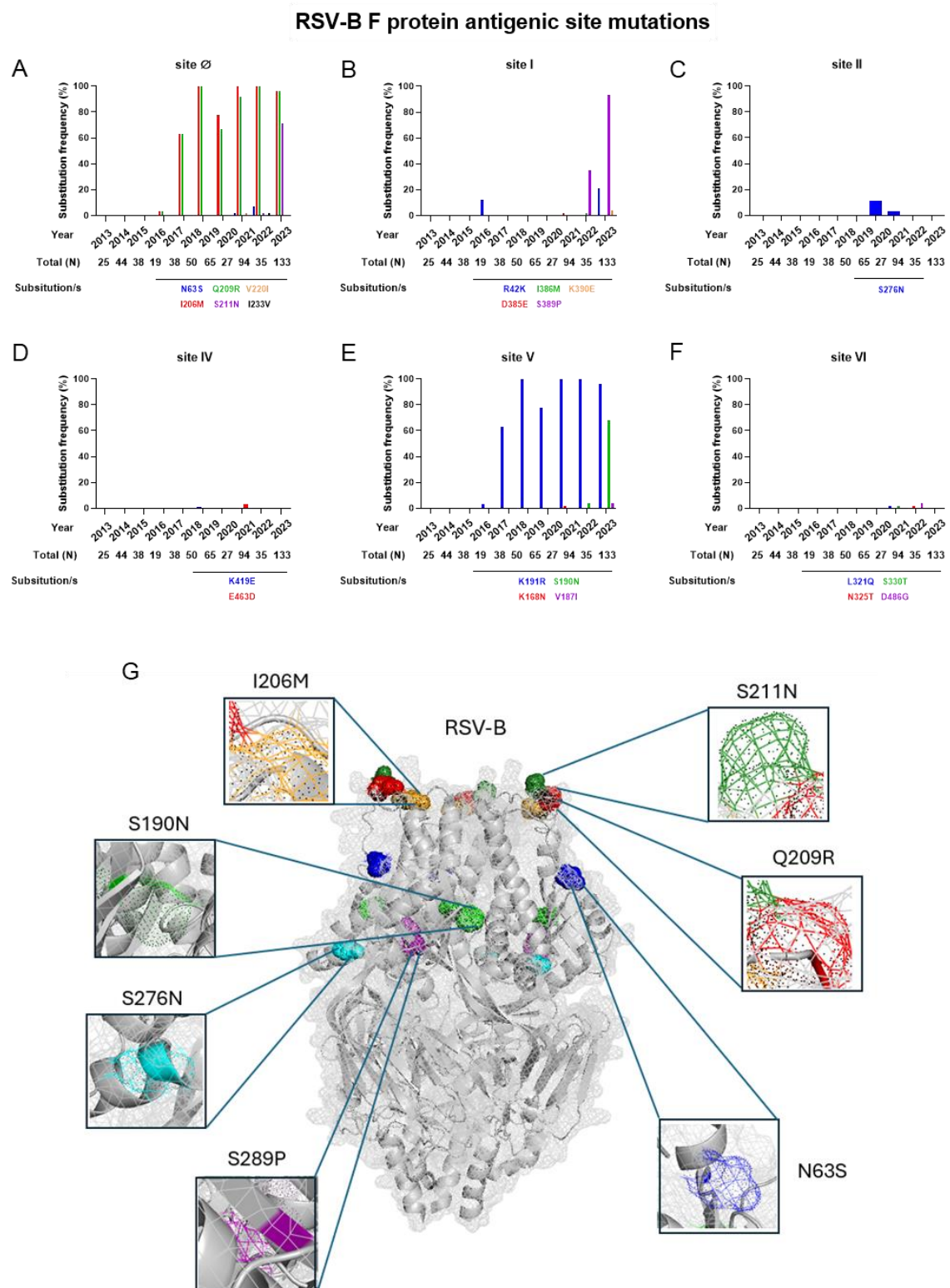


Figure 4.4. Identification of South African RSV-B F protein antigenic site mutations putatively of relevance. (A-H) RSV-B F protein amino acid substitution frequency plots

identified from F gene sequences (2014-2024) (n=370) aligned to the EPI-ISL-2582791 reference sequence. The frequency (%) of identified substitutions (y-axis) by year (2014-2023, x-axis) is plotted, with substitutions coloured as per the key shown. **(G)** A cartoon and mesh representation of the 3D structure of the preF protein, displaying secondary structural elements and the surface of the protein. Priority substitutions are highlighted (inlet) – S211N (dark green), Q209R (red), N63S (blue), S289P (purple), S276N (cyan), S190N (light green), and I206M (orange) – is shown. Generated using PyMOL.

4.2 Anti-RSV mAb production and validation

4.2.1. Anti-RSV mAb size and purity

The nine in-house mAbs (Nirsevimab (site Φ)⁶⁴, ADI-15610 (site I)⁴³, Palivizumab (site II)⁸⁹, MPE8 (site III)⁹⁰, Clesrovimab (site IV)⁵⁸, CR9501 (site V)⁶⁷, 2.4K, 2.4B, and ADI-15558 (MPER, site VI)⁴⁴ were expressed independently based on published sequence data and were assessed for size and purity by SDS-PAGE. These mAbs were expressed as IgG which has a molecular mass of approximately 150 kDa, consisting of two heavy chains ($\pm$ 50 kDa each) and two light chains ($\pm$ 25 kDa each), which are connected by disulfide bonds⁹⁶. Under reducing conditions, these disulfide bonds are broken, resulting in two fragments that represent the individual polypeptide chains. The SDS-PAGE reduced conditions displayed mAb heavy chains at approximately 50 kDa and light chains at approximately 25 kDa (Figure 4.5) as expected.

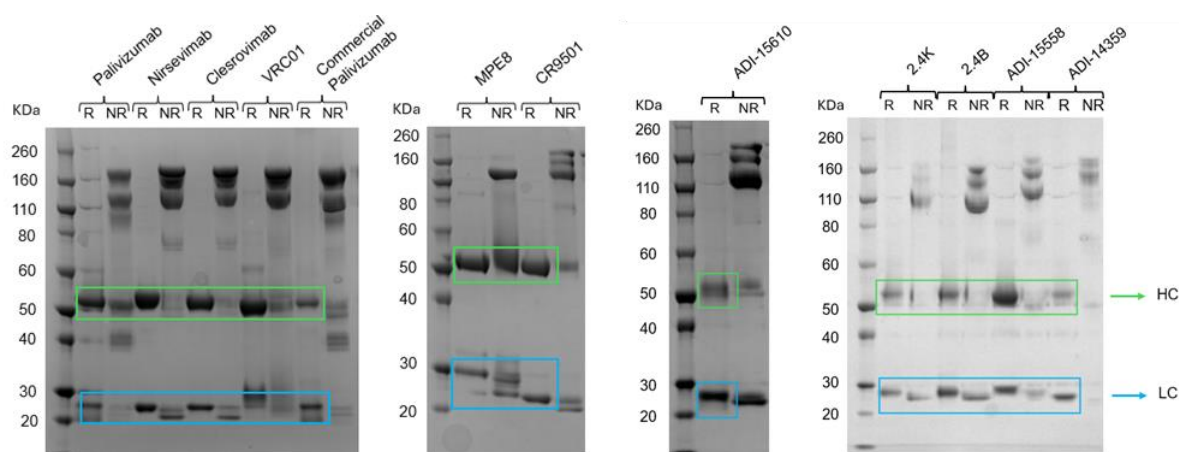


Figure 4.5. Size and purity of anti-RSV monoclonal antibodies by SDS-PAGE. Expressed anti-RSV mAbs, Palivizumab, Nirsevimab, Clesrovimab, MPE8, CR9501, ADI-15610, 2.4K, 2.4B, ADI-15558, and ADI-14359 (PostF specific mAb) as well as control mAbs, VRC01 and commercial Palivizumab were resolved under reducing (R), and non-reducing (NR) conditions. Fragments corresponding to the expected size of IgG heavy chain (HC, green box) and light

chain (LC, blue box) of ± 50 kDa and 25 kDa are shown under reducing conditions, respectively. Molecular weight markers were included in lane 1 of each gel and the sizes (in kilodaltons) of each fragment is indicated.

Under non-reducing conditions, the intact 150 kDa mAbs, with disulfide bonds and the heavy and light chains still bonded, were observed (Figure 4.5). This suggests that all expressed anti-RSV mAbs were of the expected size for IgG antibodies. Partially dissociated IgG antibodies may explain the additional banding patterns observed in most non-reducing lanes. For example, bands for heavy chain dimers (± 100 kDa) and loosely bound light chains (± 25 kDa) would still be seen. This is supported by the same pattern seen for the commercial Palivizumab control (Figure 4.5). Variations in mAb glycosylation may account for variation in heavy or light chain fragments on the gel, with some appearing slightly larger than 50 or 25 kDa as expected. The same is observed with the intact mAbs in the non-reducing lanes appearing larger than 150 kDa. Apart from some molecular weight marker spill-over observed in the lanes for Palivizumab, 2.4K, 2.4B, ADI-1558, and ADI-14359, the single fragments observed under each of the reducing conditions suggested that the expressed mAbs are pure, containing no other non-specific proteins.

4.2.2. Anti-RSV mAb functionality

The anti-RSV mAbs were assessed for functionality by ELISA, targeting representative antigenic sites on the RSV F protein (Figure 4.6 I). All mAbs were validated for binding to commercially acquired RSV preF (A2) (RSV-A antigen) and RSV F B18537 (RSV-B antigen). A2 is stabilised in the trimeric prefusion conformation, while B18537 is not. Furthermore, B18537 is subject to dissociation into F1 and F2 subunits.

Palivizumab bound strongly to both A2 and B18537, with EC₅₀ values of 0.02280 µg/ml and 0.04422 µg/ml, respectively (Figure 4.6 A and J), comparable to commercial Palivizumab (EC₅₀ values, A2: 0.01421 µg/ml, B18537: 0.01823 µg/ml) (Figure 4.6 B and J). Similarly, Clesrovimab with EC₅₀ values of 0.03143 µg/ml and 0.04869 µg/ml (Figure 4.6 C and J) and MPE8 with EC₅₀ values of 0.02511 µg/ml and 0.02679 µg/ml (Figure 4.6 D and J) bound tightly to A2 and B18437 respectively. As B18537 can undergo subunit dissociation, the binding of these mAbs to both antigens suggests that they are not selective for quaternary conformation. In contrast, CR9501 and the MPER mAbs showed higher binding to A2 than B18537. CR9501 bound with an EC₅₀ of 0.04632 µg/ml to A2 compared to 0.0177 µg/ml for B18537 (Figure 4.6 E and J). It is likely that in the presence of dissociated B18537 trimer, these mAbs lose their binding, suggesting a preference for the quaternary conformation. Nirsevimab bound A2 (EC₅₀ of 0.05855 µg/ml) but not B18537 (EC₅₀ of 2 µg/ml) (Figure 4.6 H and J) indicating a preference for quaternary conformation as well. ADI-15610 bound tightly to A2 (EC₅₀ of 0.01324 µg/ml) but did not bind B18537 (EC₅₀ of 2 µg/ml) (Figure 4.6 G and J), consistent with Gilman et al., where this mAb bound exclusively to RSV-A⁴³.

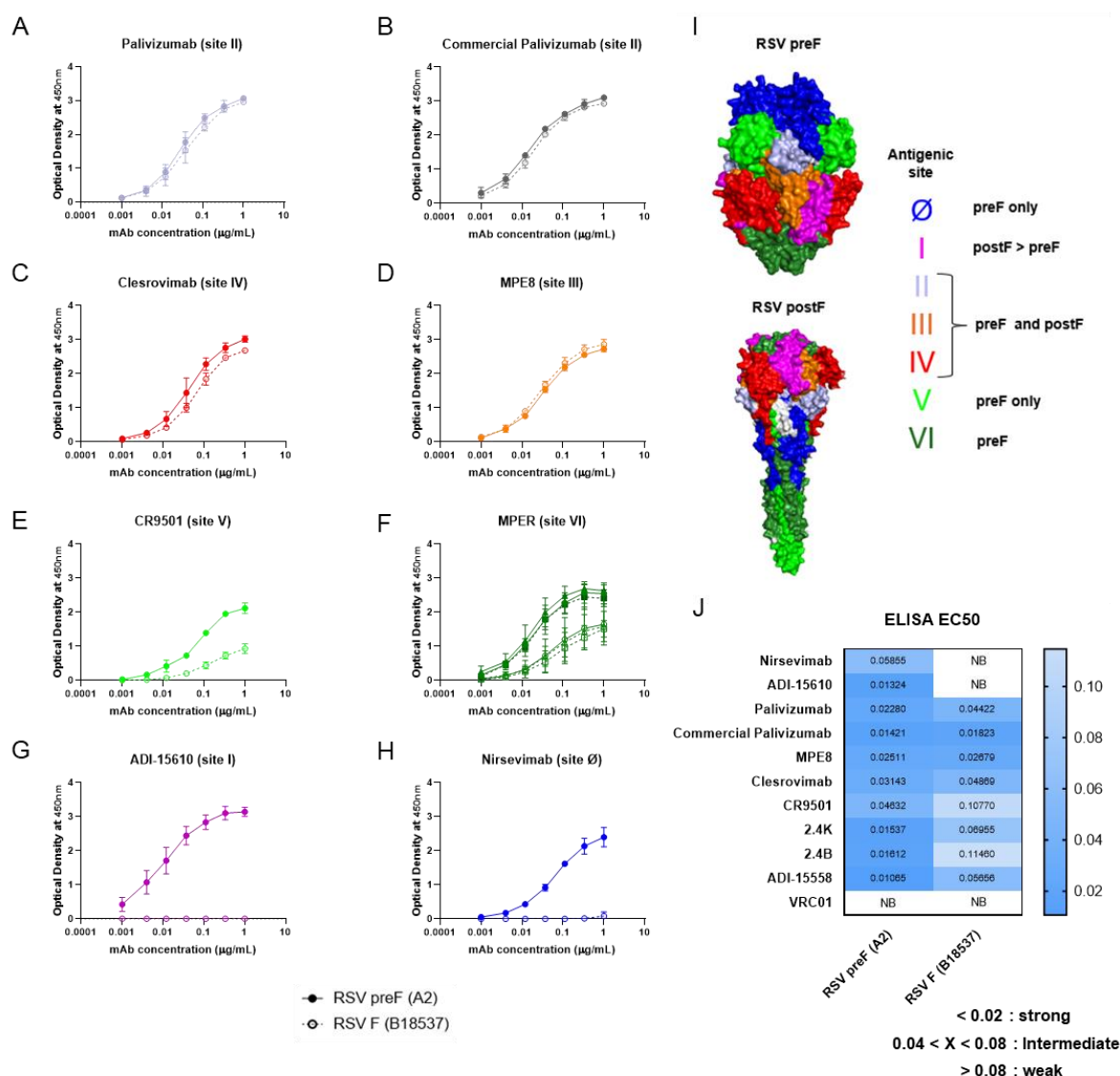


Figure 4.6. Anti-RSV monoclonal antibody binding to prefusion RSV-A (A2) and RSV-B (B18537). (A-H) Anti-RSV mAbs, Palivizumab (blue grey), commercial Palivizumab (grey), Clesrovimab (red), MPE8 (orange), CR9501 (green), 2.4K (circle), 2.4B (square), ADI-15558 (triangle) (MPER) (dark green), ADI-15610 (purple), and Nirsevimab (Blue) ELISA binding to RSV preF (A2) (solid line) and RSV F (B18537) (dotted-line). Error bars indicate the standard deviation between replicate runs (n=2). (I). Pre- and post-fusion conformations of the F protein with its seven antigenic sites, site Ø (blue), site I (pink), site II (grey blue), site III (orange), site IV (red), site V (green), site VI (dark green). PreF versus postF preference per site is indicated. The reconstructions were generated using PyMOL. (J) Heat map displaying the effective concentration 50 (EC₅₀) for anti-RSV-And control (VRC01 and commercial Palivizumab) mAbs against RSV preF (A2) (left) and RSV F (B18537) (right). The blue colour gradient indicates the potency of the mAbs against the respective antigen. Darker shades represent higher

potency, while lighter shades indicate lower potency per the key. NB indicates no binding. Weak, intermediate and strong binding is shown. All EC_{50} values were determined in GraphPad Prism using non-linear regression analysis.

Although the preference for quaternary conformation is hypothesised, a more likely explanation for the differential mAb binding to A2 and B18537 is that RSV antigenic sites are not shared across the preF and postF conformations (Figure 4.6 J and 4.7 J). To control for this, the anti-RSV mAbs were validated for binding to postF A2 and B18537 antigens (Figure 4.7). These antigens are restricted to the postF confirmation, thus excluding all binding preferences for the preF confirmation.

ADI-14359 was included in the analysis as a positive control. It recognizes postF through a recurrent CDR H3 motif together with germline encoded regions of the IGKV1-39 light chain⁴⁶. While successful in binding to postF A2 (EC_{50} of 0.02296 μ g/ml), it did not bind B18537 (Figure 4.7 C and J). Key recognition sites for this interaction include the postF residues N380, S35 and K390 in RSV-A⁴⁶. While S35 and K390 are retained in B18537, the N380 residue exists as S380 in B18537 and this change may account for the lack of binding observed.

postF preference per site is indicated. The reconstructions were generated using PyMOL. **(J)** Heat map displaying the log of the half maximal effective concentration ($\log EC_{50}$) for anti-RSV- And control (VRC01, 946-A6, and commercial Palivizumab) mAbs against RSV postF (A2) (left) and RSV postF (B18537) (right). The blue colour gradient indicates the potency of the mAbs against the respective antigen. Darker shades represent higher potency, while lighter shades indicate lower potency per the key. NB indicates no binding. Weak, intermediate and strong binding is shown. All EC_{50} values were determined in GraphPad Prism using non-linear regression analysis.

Palivizumab and Clesrovimab bound both postF A2 and B18537 respectively; Palivizumab binding strongly with an EC_{50} of 0.02254 $\mu\text{g/ml}$ and 0.02516 $\mu\text{g/ml}$ (Figure 4.7 A and J), and Clesrovimab binding with an EC_{50} of 0.05154 $\mu\text{g/ml}$ and 0.04001 $\mu\text{g/ml}$ (Figure 4.7 C and J). The sites targeted by Palivizumab (Site II) and Clesrovimab (Site IV) are present in both the preF and postF conformation^{14,43,97}, therefore binding to postF antigen is expected. Although relatively lower binding was observed for MPE8 compared to Palivizumab and Clesrovimab, MPE8 still bound both A2 and B18537, with EC_{50} of 0.11120 $\mu\text{g/ml}$ and 0.1136 $\mu\text{g/ml}$, respectively (Figure 4.7 D and J). Site III which is the target for MPE8, is exposed more in the preF conformation than in the postF conformation⁹⁷, explaining the observed but reduced binding for MPE8 to postF antigen. In contrast, CR9501 and Nirsevimab did not bind either postF A2 or B18537 ($EC_{50} > 1 \mu\text{g/ml}$) (Figure 4.7 E,H, and J) owing to the presence of sites V and $\emptyset$ exclusively in the preF conformation⁴². The MPER mAbs also showed overall lower binding to the postF antigens (Figure 4.7 F and J). While the presence of this site in the different conformations has not been fully elucidated in literature⁴⁴, the data here suggests that the preF conformation is favoured over the postF confirmation for the MPER site. ADI-15610 tightly bound A2 (EC_{50} of 0.01324 $\mu\text{g/ml}$) but did not bind B18537 (Figure 4.7 G and J) consistent with Gilman et al., showing exclusive specificity of this mAb to RSV-A⁴³.

Overall, while differences in anti-RSV binding were observed, these differences may be explained by quaternary and conformational preferences and mAbs produced were functional.

4.3. RSV pseudovirus production and infection optimisation

4.3.1. Optimisation of RSV pseudovirus infection parameters

Unmodified and CT modified RSV F pseudoviruses were generated for use in a VSV-based RSV pseudovirus neutralisation assay. To establish optimal and reproducible infectivity of these RSV F pseudoviruses infection was evaluated across 252 conditions, including multiple cell types and densities, incubation times, RSV F cytoplasmic tail (CT) modifications, and freeze-thaw parameters. Across all conditions tested, infectivity was measured as a function of increased luminescence signal compared to the cell control measured in RLU. A baseline threshold of 50 000 RLU for virus infectivity was set to establish positive infection, ensuring a 1000-fold increase compared to cell control.

4.3.1.1 Cell type, density and incubation period optimisation

To identify the appropriate target cell for unmodified RSV-A and RSV-B pseudovirus infection, infection was evaluated in Hep-2, Vero, and A549 cells, all of which have been used in conventional lentiviral-based and replication competent RSV neutralisation assays⁸⁵. Infection was evaluated at varied cell concentrations (5 000, 20 000, and 50 000 cells/well) either pre-seeded or not, and across three infection incubation periods (24-, 48-, and 72-hour).

Unmodified RSV-A and RSV-B infection was not observed in A549 and Hep-2 cells at any of the cell densities or infection periods evaluated (Appendix A2, Figure A2.11 and A2.12). Neither was infection observed for RSV-A in Vero cells at any cell density or infection period evaluated. Infection was lower than the baseline threshold of 50 000 RLU across all of these (Appendix A2, Figure A2.11). In pre-seeded Vero cells, particularly at 5 000 cells/well, detectable but inconsistent infection above the 50 000 RLU threshold was observed for unmodified RSV-B after 48 and 72 hours, however no infection was observed after 24 hours (Appendix A2, Figure A2.12). RSV-B infection was 2-fold higher after 48 hours (Figure 4.8 A) compared 72 hours (Appendix A2, Figure A2.12).

Repeated infections under these conditions (Vero cells at 5,000 cells/well after 48 hours) for additional unmodified RSV-A and B batches were performed (Appendix A2, Figure 2.13 A and B). RSV-A remained below the 50 000 RLU threshold at four days

through 35 days post-harvest (Figure 4.8 A). Infection approximately 4-fold higher than threshold was recorded for RSV-B five days post-harvest but this infection dropped below the threshold after 33 days (Figure 4.8 B). Unmodified RSV-A and RSV-B Infection varied considerably between viral lots. Inconsistent infection within individual virus batches produced from the same transfection was observed, with standard deviations greater than 50,000 RLU between duplicate samples of unmodified RSV-A (Appendix A2, Figure 2.13 A) and RSV-B (Appendix A2, Figure 2.13 A). This combined data suggested the presence of variable non-uniform unmodified RSV-A and B preparations lacking reproducible infectivity.

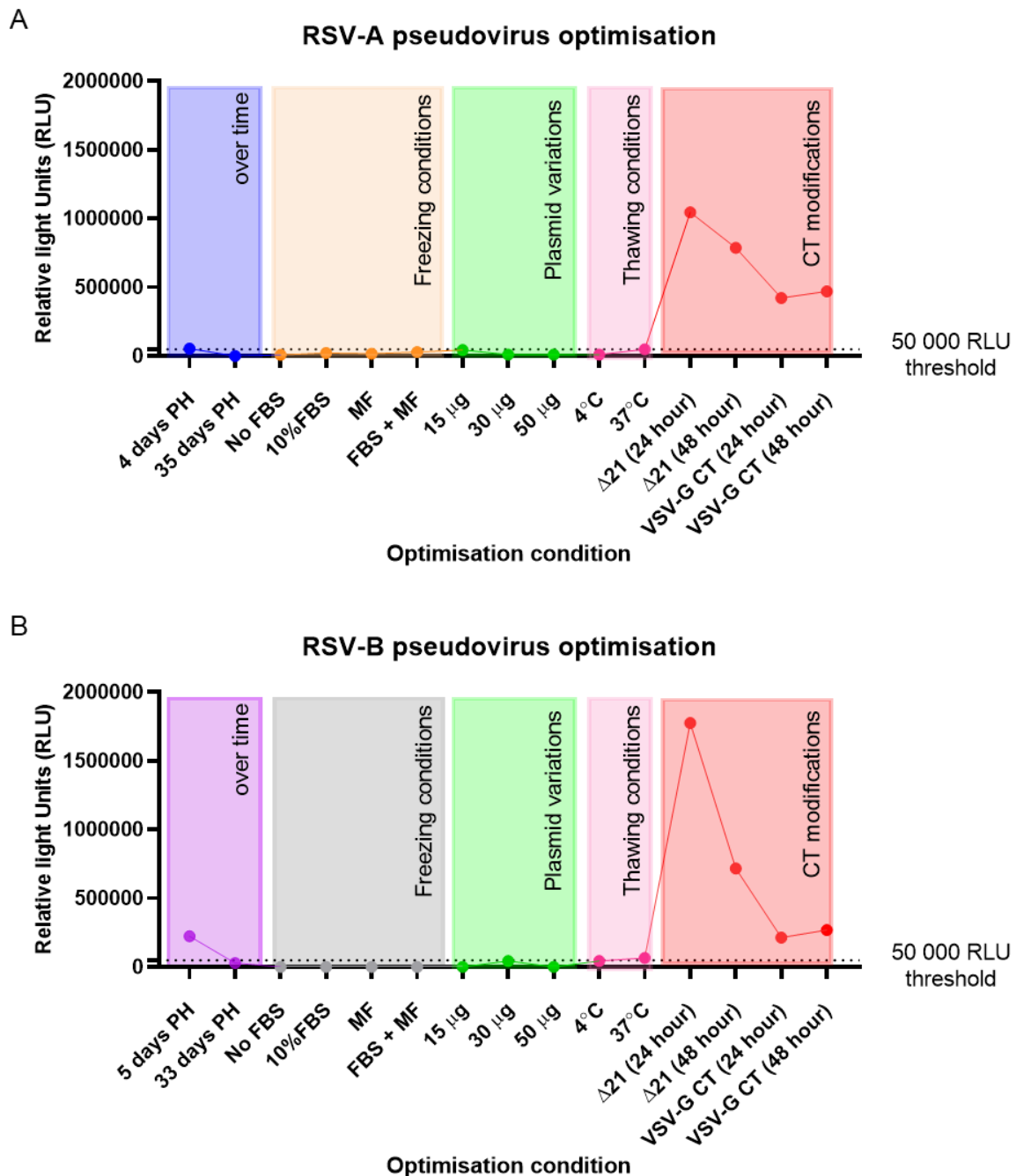


Figure 4.8. Optimisation of RSV-A and RSV-B pseudovirus infection. Summary of **(A)** RSV-A and **(B)** RSV-B pseudovirus infection optimisation parameters based on infection recorded at single point dilutions (1:8 and 1:5 for unmodified and modified virus, respectively) across the unmodified (Appendix A2, Figure A2.14) and modified pseudovirus (Appendix A2, Figure A2.19) titration curves. The infection optimisation conditions (infectivity monitored over time (blue and purple) where PH refers to post-harvest, freezing conditions (orange) where MF refers to Mr Frosty and FBS refers to Fetal Bovine Serum, plasmid transfection amounts (green), thawing conditions (pink), and CT modifications (red) are shown on the x-axis, colour

coded as represented in the titration curves. The freezing conditions were not performed for RSV-B (shown in grey). Mean infection between duplicate wells (n=2) measured in RLU (y-axis) recorded at the 1:8 and 1:5 dilutions for unmodified and modified virus is plotted. The baseline threshold of 50 000 RLU for positive infection is indicated by the dotted line.

4.3.1.2. Optimisation of pseudovirus freezing conditions

The non-uniformity and lack of reproducible infection in the unmodified pseudoviruses suggested possible degradation of the particles during freezing. To investigate this, we tested various freezing conditions, including the addition of 10% FBS as a cryopreservant before freezing. Freezing was carried out using isopropanol in an insulated container (Mr. Frosty), which provides controlled freezing due to its low freezing point. This method ensures efficient heat transfer and prevents direct contact of the sample with extreme cold temperatures, thus minimising ice crystal formation. Additionally, we evaluated the combination of 10% FBS and Mr Frosty on RSV-A pseudovirus infectivity. Neither freezing condition showed any overall positive effect on unmodified RSV-A infectivity, as RLUs remained below the threshold for positive infection (Figure 4.8 A, Appendix A2, Figure A2.13 C). Nevertheless, the addition of 10% FBS was selected as a cryopreservant for all pseudoviruses moving forward. This is because FBS stabilises viral envelopes and structural proteins, prevents aggregation, and reduces ice crystal formation that can cause virion damage.

4.3.1.3. Optimisation of pseudovirus plasmid transfection amounts

Low level or disrupted expression of RSV F protein at the surface of the RSV pseudoviruses was hypothesised to be a contributor to the suboptimal infectivity. To address this, RSV F plasmid transfection amounts were either increased or decreased as an additional parameter for pseudovirus infectivity optimisation. Increasing plasmid transfection amounts is thought to boost transcription and translation, enhancing cell surface protein expression, which in turn improves pseudovirus assembly and budding. However, overexpression of protein at the cell surface can induce cellular stress leading to apoptosis. It can also cause protein aggregation and misfolding, resulting in the incorporation of non-infectious proteins into the virions, providing a rationale for decreased plasmid transfection.

Infectivity of unmodified RSV-A and RSV-B pseudovirus produced from an increased 50 µg RSV F plasmid transfection variation and a decreased 15 µg RSV F plasmid

variation was thus evaluated (Figure 4.8 A and B, Appendix A2, Figure A2.13 D and E). Once again, RLUs remained below the threshold for positive infection suggesting that neither of these varied infection plasmid transfection amounts were beneficial to pseudovirus infection (Figure 4.8 A and B). With no additional benefit from these plasmid variations, the original 30 µg transfection plasmid amount was retained for the production of all pseudoviruses moving forward.

4.3.1.4. Optimisation of pseudovirus thawing conditions

To investigate optimal virus thawing conditions, unmodified RSV-A and -B pseudoviruses produced from 30 µg of RSV F plasmid were subjected to a 4°C and 37°C thaw. The RSV F protein exists in a metastable preF conformation, which undergoes rapid triggering to the postF conformation. Compared to a longer thaw at 4°C thaw, thawing at higher temperatures is hypothesised to minimise triggering, resulting in a higher proportion of virus particles in the infectious preF conformation, thus enhancing infectivity⁸⁴.

Recorded RLUs for unmodified RSV-A infection remained below the infection threshold when thawed at 4°C and 37°C (Figure 4.8 A, Appendix A2, Figure A2.13 F) and for RSV-B thawed at 4°C (Figure 4.8 B, Appendix A2, Figure A2.13 G). However, infection 1.3-fold above threshold was recorded for unmodified RSV-B thawed at 37°C (Figure 4.8 B, Appendix A2, Figure A2.13 G). While this was not a substantial improvement to virus infectivity, the 37°C was chosen as the preferred thawing method for all pseudoviruses based on the published findings of others⁸⁴ (Appendix A2, Figure A2.11).

4.3.1.5. Effect of RSV F protein CT modification on pseudovirus infectivity

Unmodified RSV-A and RSV-B had failed to reach optimal infection levels, consistently falling below the 50 000 RLU threshold, despite varying infection parameters (Figure 4.8 A and B). RSV F CT modifications (Δ 21 and VSV-G CT) were thus introduced into RSV-A and -B pseudovirus constructs, resulting in the generation of RSV-A and B Δ 21, as well as RSV-A and B VSV-G CT pseudoviruses. In HIV, SARS-CoV-2 and RSV pseudoviruses, CT modifications have been shown to improve the localisation of envelope proteins to the cell surface, enhancing glycoprotein incorporation and increasing pseudovirus yield in the VSV-packaging systems^{87,98-100}.

Infectivity of the modified pseudoviruses was evaluated across the same infection parameters tested for the unmodified RSV viruses above, including the type of target cell (Vero, A549, and Hep-2), optimal target cell density (5 000, 20 000, and 50 000 cells/well) and infection incubation periods (24 , 48-, and 72-hour) (Appendix A2, Figure A2.14- A2.17).

Hep-2 cells and A549 cells were again not susceptible to infection by these pseudoviruses at any of the evaluated cell densities or infection periods (Appendix A2, Figures A2.14–A2.17). In Vero cells, particularly at 5 000 cells/well after 24 and 48 hour incubation periods, CT modified RSV-A and B pseudoviruses showed considerable improvements in infection (Appendix A2, Figure A2.14- A2.17). Infection levels for the CT-modified pseudoviruses were approximately 20 to 30-fold higher than the 50 000 RLU threshold, far surpassing the infectivity of unmodified RSV pseudoviruses (Figure 4.8 A and B). The CT modifications resulted in up to a 20-fold increase in infectivity for modified RSV-A pseudoviruses compared to unmodified RSV-A pseudoviruses (Figure 4.8 A). Similarly, modified RSV-B pseudoviruses exhibited up to a 9-fold increase in infectivity compared to their unmodified counterpart (Figure 4.8 B).

Overall CT modifications enhanced pseudovirus infection, yielding higher and more uniform infection levels compared to unmodified pseudovirus. Infection in Vero cells at 5 000 cells/well after 24 and 48 hour incubation periods was therefore considered optimal for modified pseudovirus infection.

4.3.1.6 Reproducibility of modified RSV pseudovirus infection

With the modified viruses capable of yielding infection suitable for neutralisation assays, batch-to-batch reproducibility was assessed to ensure infection consistency (Appendix A2, Figure A2.18). Infection was evaluated across four separate batches of each modified pseudovirus (RSV-A and RSV-B Δ 21, and VSV-G CT) (Appendix A2, Figure A2.18). Uniform titration and high-level infection was observed across all batches, with RLUs ranging from 170 000 to 1.7 million after 24 and 48 hour infection periods (Appendix A2, Figure A2.18). Virus infection was maintained across all batches after 24 and 48 hours of infection (Figure 4.9), highlighting the reproducibility across the different virus batches at both time points.

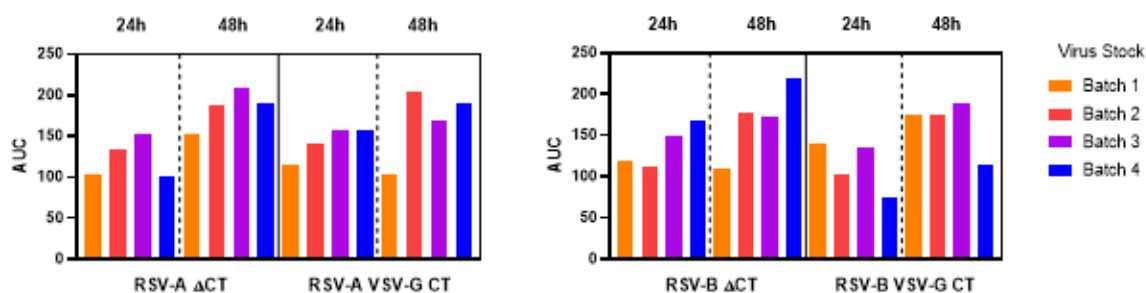


Figure 4.9. Batch to batch reproducibility of CT modified RSV pseudoviruses. RSV pseudovirus infection titrations represented as area under the curve (AUC) of the modified **(A)** RSV-A Δ 21 and RSV-A VSV-G CT, and **(B)** RSV-B Δ 21 and RSV-B VSV-G CT pseudovirus titrations (Appendix A2, Figure A2.19) performed across four virus batches (orange, red, purple and blue, respectively), shown at 24- and 48- hour incubation periods. AUC was determined in GraphPad Prism.

Overall, reproducibility of the modified RSV pseudoviruses at both 24 and 48 hours of infection was confirmed with 1.3 to 1.5-fold range in variability between virus lots. Given the improved infection with CT modified RSV pseudoviruses compared to unmodified RSV pseudoviruses (described in 4.3.1), along with reasonable reproducibility under the tested infection parameters, the effect of the CT modifications on conformational integrity was assessed.

4.3.2. Confirmation of modified RSV F protein cell surface expression

Truncations or modifications to envelope CTs may alter the native structure of the protein thereby impacting the functional phenotype of the pseudovirus⁸⁷. The conformational integrity of the RSV F protein produced by the modified CT plasmid constructs was therefore assessed using a cell surface binding assay. Expi293F cells expressing RSV-A and B Δ 21 and RSV-A and B VSV-G CT F protein were evaluated for binding to seven anti-RSV control mAbs. Expi293F cells were gated as single, live and IgG⁺ (Figure 4.10). The geometric mean fluorescence intensity (GMFI) measured by flow cytometry of positive APC secondary antibody (IgG) was recorded.

The conformational integrity of the F protein was confirmed by the controls, which performed as expected. The anti-HIV mAb VRC01, used as a negative control, yielded GMFIs of around 10 000 arbitrary units (a.u.). The binding of the APC-conjugated secondary antibody to untransfected cells was also controlled to ensure that the fluorescently labelled antibody was binding specifically to the primary test mAbs on the surface-expressed F protein, rather than to the cells themselves. Palivizumab the positive control, which targets a site shared in both preF and postF conformations showed a > 5-fold increase in binding compared to the negative controls.

For RSV-A Δ 21, ADI-15610 demonstrated the highest binding with a GMFI of 81 328 a.u., followed by Palivizumab with a GMFI of 66 036 a.u (Figure 4.10 B). Clesrovimab also exhibited high binding with a GMFI of 52 014 a.u (Figure 4.10 B). The other mAbs — Nirsevimab, MPE8, CR9501, and 2.4K — showed weaker binding, with GMFIs of 33 260 a.u., 32 403 a.u., 31 278 a.u., and 22 364 a.u., respectively (Figure 4.10 B). Similarly, for RSV-A VSV-G CT, ADI-15610 had the highest binding (GMFI of 70 301 a.u.), followed by Palivizumab (63 915 a.u.) (Figure 4.10 C). Clesrovimab also showed high binding with a GMFI of 51 425 a.u. (Figure 4.10 C). The other mAbs — CR9501, Nirsevimab, MPE8, and 2.4K— showed lower binding with GMFIs of 33 259 a.u., 26 186 a.u., 25 041 a.u., and 21 911.5 a.u., respectively (Figure 4.10 C).

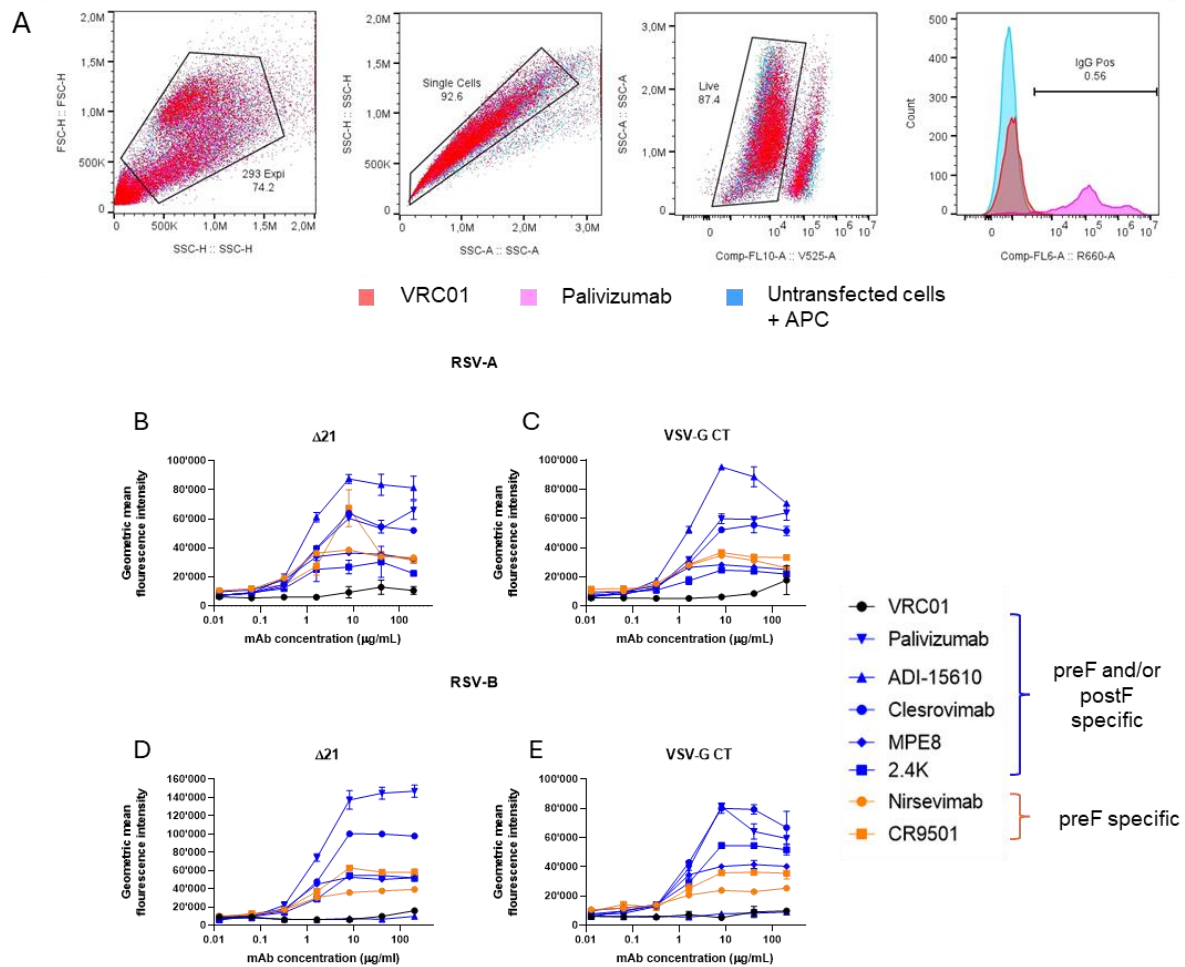


Figure 4.10. Anti-RSV-Binding to Expi293 cell surface expressed RSV F protein. (A) Representative gating strategy for the cell surface binding assay. Expi293F cells were gated as single, live and IgG⁺. Palivizumab, a positive control (pink), is overlaid with VRC01 (red), and untransfected cell + APC (blue) negative controls. **(B)** Geometric mean fluorescence intensity of anti-RSV mAbs with pre and/or postF specificity, ADI-15610, Clesrovimab, MPE8, and Palivizumab (blue), and anti-RSV mAbs with preF specificity only, CR9501 and Nirsevimab (orange) as well as a VRC01 (black) negative control, binding to Expi293F cells expressing RSV-A $\Delta 21$ (top left), RSV-A VSV-G CT (top right), RSV-B $\Delta 21$ (bottom left), and RSV-B VSV-G CT F protein.

For RSV-B $\Delta 21$, ADI-15610 showed no binding, with a GMFI of 9 769 a.u. which was below the 10 000 a.u. cutoff based on the negative controls (Figure 4.10 D). This was expected as ADI-15610 binds exclusively to RSV-A. Palivizumab had the highest binding (GMFI of 146 966 a.u.) followed by Clesrovimab (GMFI of 97 846 a.u.) (Figure 4.10 D). The other mAbs — CR9501, MPE8, 2.4K, and Nirsevimab — showed relatively weaker binding, with GMFIs of 58 054 a.u., 51 748 a.u., 52 208 a.u., and 39

302 a.u., respectively (Figure 4.10 D). Similarly, for RSV-B VSV-G CT, ADI-15610 did not bind (GMFI of 9 055 a.u.) (Figure 4.10 E). Clesrovimab had the highest binding (GMFI of 66 860 a.u.) followed by Palivizumab (GMFI of 59 424 a.u.) (Figure 4.10 E). The other mAbs — 2.4K, MPE8, CR9501, and Nirsevimab — showed relatively weaker binding, with GMFIs of 51 748 a.u., 40 343 a.u., 35 637 a.u., and 25 355 a.u., respectively (Figure 4.10 E).

The reduced binding observed for mAbs such as CR9501 and Nirsevimab, which target preF specific sites, along with the increased binding of ADI-15610 (targeting a epitope exclusively on the postF conformation), and the high binding of Palivizumab and Clesrovimab (binding to sites shared in both preF and postF conformation), supports a hypothesis that the F protein on the cell surface may be in the postF conformation as is observed on live virus as well¹⁷. However, the different levels of mAb binding may also reflect variations in the location and accessibility of each RSV F epitope. For example, MPER sites, which are less accessible, could have reduced binding, as seen with the 2.4K MPER-targeting mAb, compared to Palivizumab and Clesrovimab. F protein stoichiometry—i.e., the number of mAbs per trimer—may additionally contribute to the differences observed.

Overall, while differences in binding were observed amongst the different mAbs tested, all anti-RSV mAbs bound to the cell surface expressed RSV F with CT modifications suggesting that the CT modifications did not significantly alter RSV F conformational integrity.

4.4. Neutralisation assay

4.4.1. Neutralisation assay optimisation

Thus far, we had established enhanced and reproducible infection of Vero cells by the CT modified RSV pseudoviruses after 24 and 48 hour incubations. The conformational integrity of RSV F with both variations of CT modification at the cell surface was intact. With the F protein preserved at the cell surface, successful viral budding and glycoprotein incorporation was assumed, providing the basis for trialling neutralisation assays.

Neutralisation of RSV-B $\Delta 21$ and VSV-G CT by anti-RSV mAbs was evaluated across various conditions, including multiple viral input doses, sample concentrations, and virus-sample incubation times and temperatures. Although modified virus infection occurred after both 24 and 48 incubations (described in section 4.3.1.5 and 4.3.1.6), 24 hours was preferred to maximize assay throughput with a shorter turnaround time.

To determine the viral input dose needed for optimal neutralisation assay sensitivity, we trialled 1:5, 1:10, 1:20, 1:50, 1:100, and 1:1000 dilutions of RSV-B $\Delta 21$ and VSV-G CT (Figure 4.11 A and B). The goal was to identify a viral input that would result in sufficient viral infection, while still allowing for effective neutralisation by the test samples.

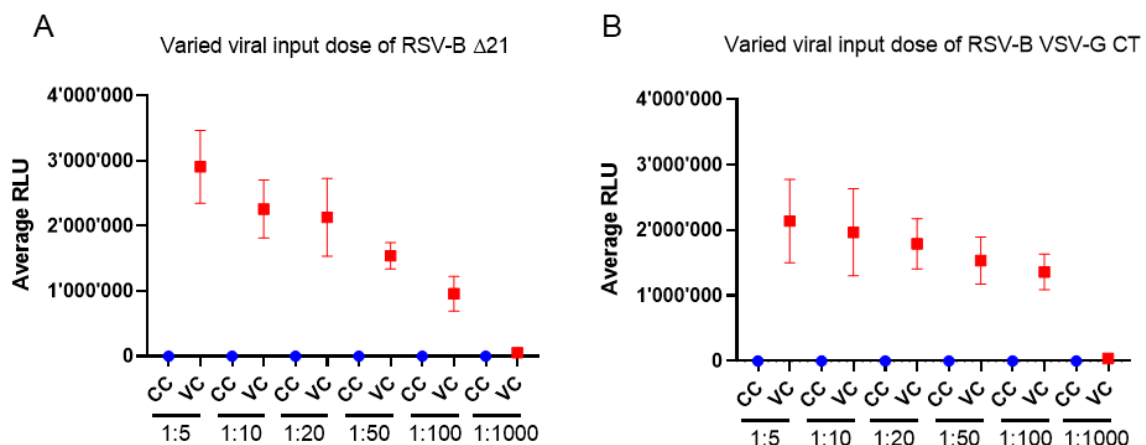


Figure 4.11. Viral infection shows a dose response relationship. (A) RSV-B $\Delta 21$ and (B) RSV-B VSV-G CT infection measured in RLU (y-axis) at varying viral input doses (1:10, 1:20, 1:50, 1:100, 1:1000) in the absence of sample. Cell control (blue) and Virus control (red) for each dilution is shown. Data are shown as the mean $\pm$ SD (n=8), where all wells are included for each duplicate sample per assay plate.

Infection was observed across all input doses in the absence of sample (virus control in Figure 4.11 A and B) apart from 1:1000 where the virus controls were below 50 000 RLU. As anticipated, infection was highest at the 1:5 input dose, exceeding 2 000 000 RLU, and decreased with lower viral input, reaching around 900 000 RLU at 1:100 (Figure 4.11 A and B).

However, despite the viral RLU displaying a dose response relationship with dilution, neutralisation by commercial Palivizumab (positive control) and seven other neutralising anti-RSV mAbs—including Palivizumab, Nirsevimab, Clesrovimab, MPE8, CR9501, 2.4K, and ADI-15558—was not observed. Strikingly, a consistent pattern of reduced infection at the center of the assay plates was observed, regardless of the mAb sample (Figure 4.12 A). An approximate 2-fold decrease in RSV-B Δ 21 infection was observed, for instance, between CR9501 at the plate edge and Clesrovimab at the plate center. The same fold difference was noted when comparing infection in the presence of commercial Palivizumab to 2.4K (Figure 4.12 B). The wells at the edge of the assay plate were subject to the “edge effect”, potentially due to non-specific contributors like temperature differences and sample evaporation.

RSV-B Δ21 at an input dose of 1:5 in the presence of neutralising mAbs

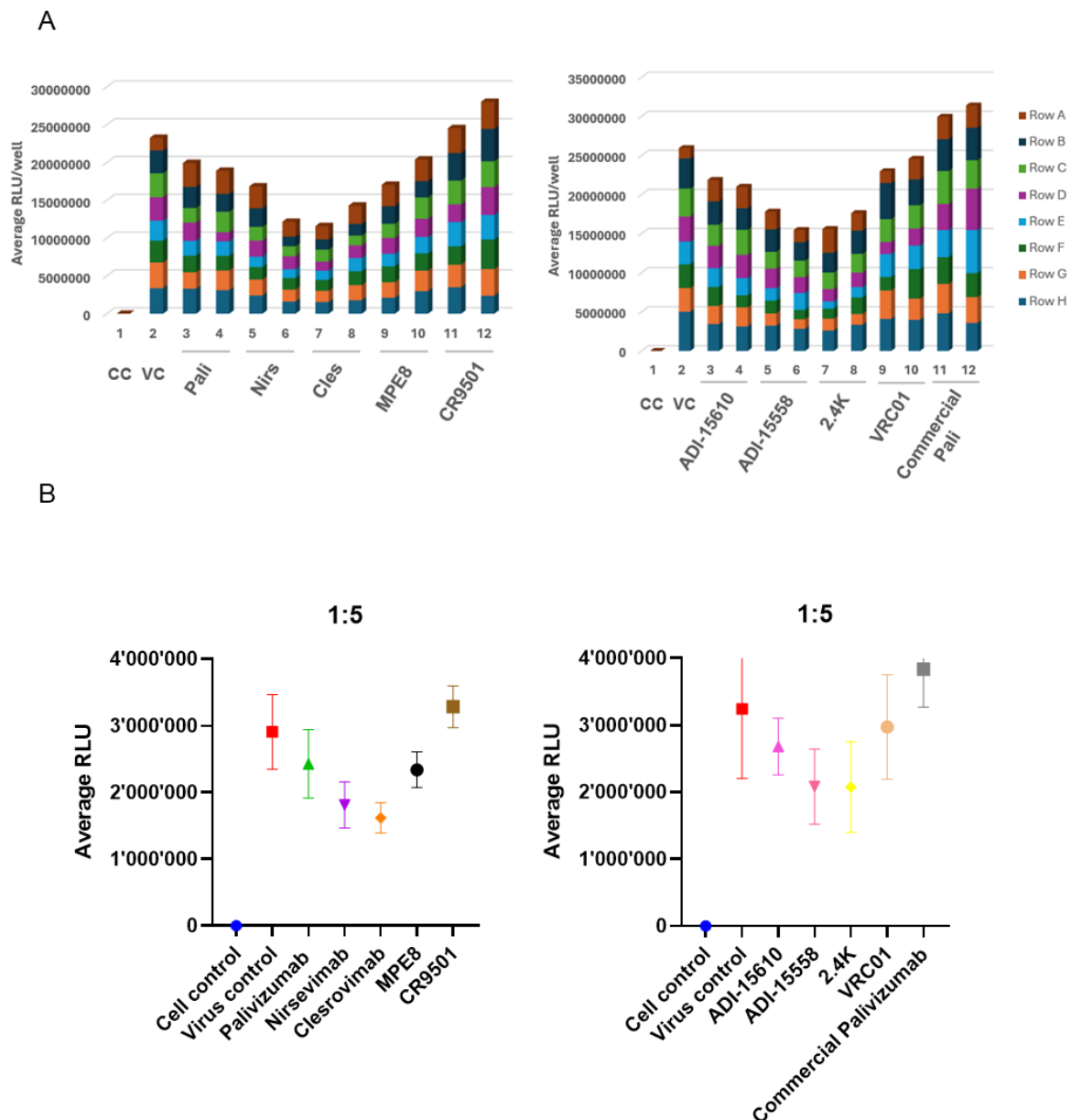


Figure 4.12. Neutralisation assay of RSV-B Δ21 in the presence of anti-RSV mAbs. (A) Graphical representation of the neutralisation assay plate arrangement. Luciferase activity in relative light units (RLU) of RSV-B Δ21 in each well (y-axis) measured 24 hours post-infection is shown. Each row of the plate is represented by a different colour as per the key. The x-axis shows the cell control (CC) and virus control (VC), as well as the duplicate antibody samples in their corresponding column positions. **(B)** Average RLU (y-axis) of RSV-B Δ21 at an input dose of 1:5 in the presence of 50 µg/ml of Palivizumab (green), Nirsevimab (purple), Clesrovimab (orange), MPE8 (Black), CR9501 (brown) (Left), ADI-15610 (pink), ADI-15558 (salmon), 2.4K (yellow), VRC01 (anti-HIV control, wheat), and commercial Palivizumab (grey)

(Right). Data are shown as the mean $\pm$ SD ($n = 8$ rows), where all wells are included and for each duplicate sample per assay plate.

Concerning the sample-virus interaction, the lack of neutralisation suggests a suboptimal virus to test sample input ratio. To investigate sample-virus input dosage that would achieve optimal assay sensitivity, a lower 1:50 input dose with mAb sample concentrations increased to 200 $\mu\text{g/ml}$ (Figure 4.13 A) was evaluated. This increased mAb concentration at a lower virus input did not offer any improvement. No neutralisation occurred and the same pattern of reduced viral infection at the center of the assay plates was observed (Figure 4.13 A). To rule out the potential influence of mAb positioning on the plate effect, the mAbs were rearranged, with those previously placed at the plate edges moved to the center. This rearrangement aimed to confirm that the observed plate effect was not due to differences in sensitivity to the various mAbs. Despite this change, the plate effect remained, suggesting that the pattern was not related to neutralisation by mAbs, but was a non-specific effect resulting in decreased RLU in the centre of the plates.

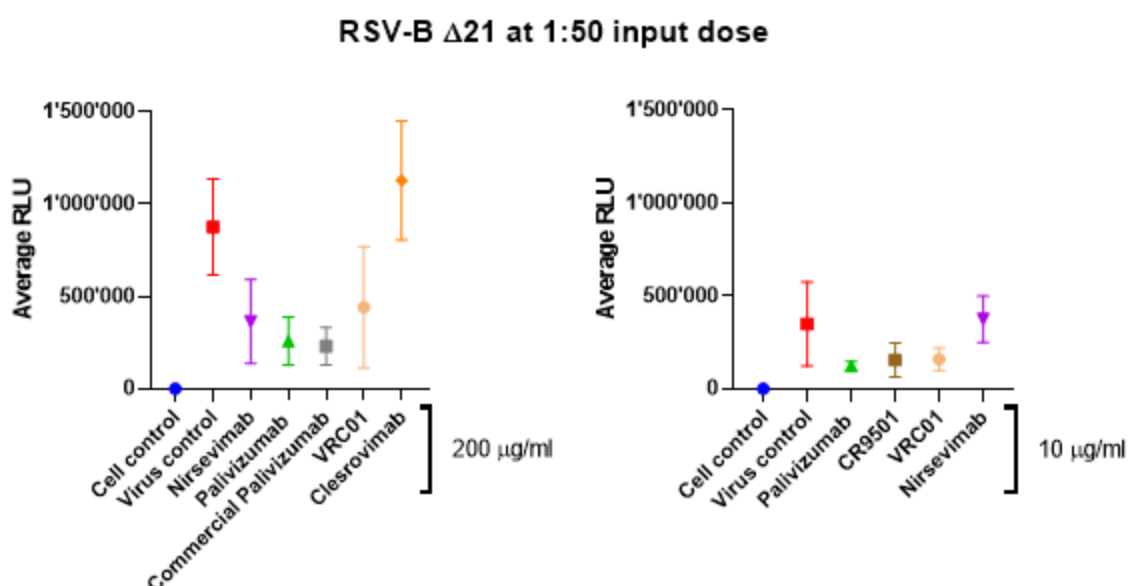


Figure 4.13. Optimisation of assay mAb sample input. RSV-B $\Delta 21$ infection dose was lowered to 1:50, and infection measured in RLU (y-axis) was determined in the presence of **(A)** 200 $\mu\text{g/ml}$ of Nirsevimab (purple), Palivizumab (green), commercial Palivizumab (grey), VRC01 (wheat), and Clesrovimab (orange) or **(B)** 10 $\mu\text{g/ml}$ of Palivizumab (green), CR9501 (brown), VRC01 (wheat), and Nirsevimab (purple) (x-axis). The position of the mAbs on the x-

axis coincide with the position on the assay plate. Data are shown as the mean $\pm$ SD ($n = 8$ rows), where all wells are included for each duplicate sample per assay plate.

As increased mAb concentration had no effect on assay sensitivity, at the same 1:50 virus input, mAb sample concentrations were decreased to 10 $\mu\text{g/ml}$ (Figure 4.13 B), this time with the outer wells also excluded given the hypothesised effect of temperature differences and evaporation at the outer wells. Again, no neutralisation was observed. The plate effect persisted, and this was not due to sample evaporation or differences in test sample concentrations at the outer wells.

Since none of the changes in the neutralisation assay parameters improved the results, we next manipulated the virus-sample incubation times and temperatures. The "edge effect" was thought to be related to temperature changes, so we adjusted the incubation temperatures and the duration of virus-sample exposure to these specific temperatures in an attempt to reduce the effect. The variations tested included a one-hour incubation at room temperature (Figure 4.14 A), a 30-minute incubation at room temperature (Figure 4.14 B), and a 15-minute incubation at 4°C (Figure 4.14 C). However, none of these adjustments showed any improvement, and the lack of neutralisation along with the plate pattern remained the same (Figure 4.14).

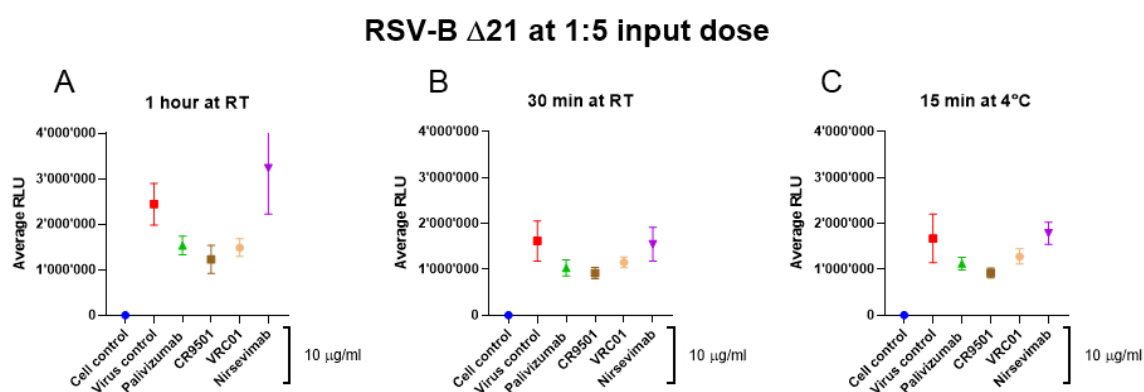


Figure 4.14. Optimisation of sample-virus incubation time and temperature. Infection of RSV-B Δ 21 measured in RLU (y-axis) was determined in the presence of 10 $\mu\text{g/ml}$ of Palivizumab (green), CR9501 (brown), VRC01 (wheat), and Nirsevimab (purple). Virus and sample were incubated at room temperature for **(A)** one hour and **(B)** 30 minutes or at **(C)** 4°C for 15 minutes. Data are shown as the means $\pm$ SD ($n = 6$ rows) where outer wells are excluded, for each duplicate sample per assay plate.

As no improvement had been observed up to this point. No mAb control plates were tested for both the 1:5 and 1:50 virus inputs. Again, the same reduced infection pattern at the centre of the plate was evident, even in the absence of mAbs (Figure 4.15 A and B), suggesting again that the observed plate effect is likely not a result of neutralisation by the mAbs.

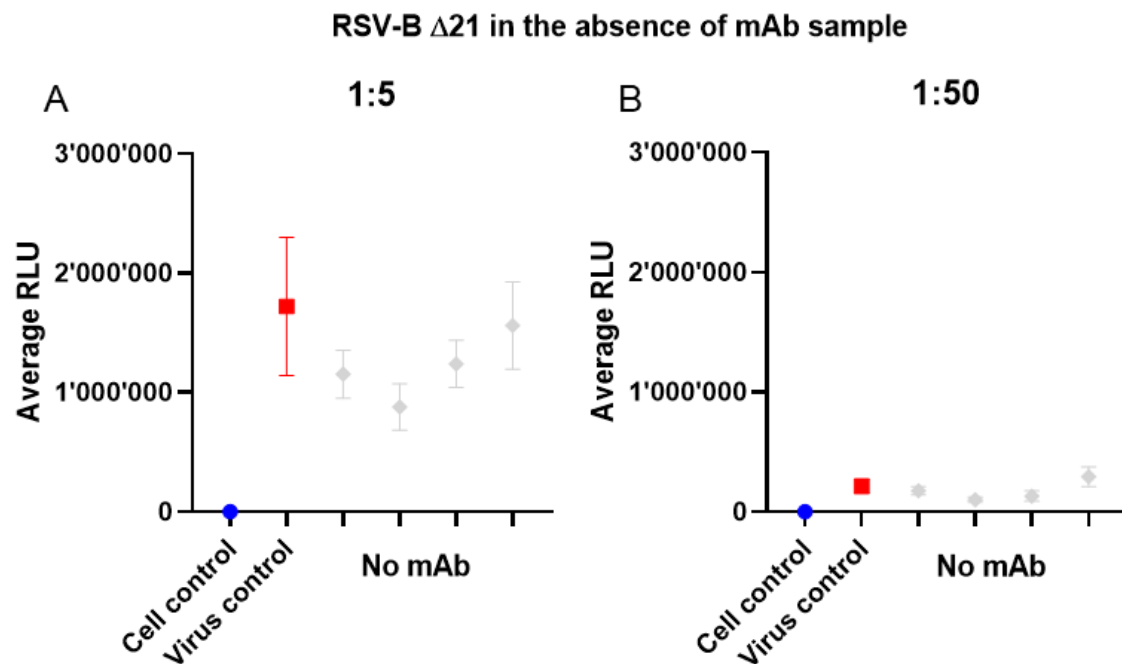


Figure 4.15. Monitoring infection in the absence of mAb samples. Infection of RSV-B Δ 21 at an input dose of **(A)** 1:5 and **(B)** 1:50 measured in RLU (y-axis) was determined in the absence of mAb sample. Data are shown as the mean $\pm$ SD ($n = 6$ rows) where outer wells are excluded, for each duplicate sample per assay plate.

One potential contributor to the persistent ‘non-specific’ plate effect that we did not investigate is the brand of plate used. We exclusively used white Corning® 96-well flat-bottom treated culture plates (Cat no. 3917), which have been reliably used in our SARS-CoV-2 Nexelis VSV pseudovirus assays without any plate effect. However, this reliance on a single plate type is a limitation that should be considered in future studies

The lack of virus neutralisation was unexpected, prompting an additional investigation involving a bald virus control. As previously conducted with both unmodified and modified pseudoviruses, the infectivity of ($G^*\Delta G$ -luciferase) rVSV without RSV F transfection (bald virus) was evaluated by titration. Notably, bald virus infection

showed comparable infectivity to that of the modified RSV pseudovirus infection (Figure 4.16), suggesting potential carryover of (G* Δ G-luciferase) rVSV in the virus preparations, which could interfere with neutralisation. In this case, while infection mediated by the RSV F pseudovirus would be expected to undergo neutralisation, any carryover virus would still facilitate infection. Due to the non-specificity of the anti-RSV mAbs to (G* Δ G-luciferase) rVSV, this carryover-mediated infection would likely be indistinguishable from background levels contributing to the lack of neutralisation observed.

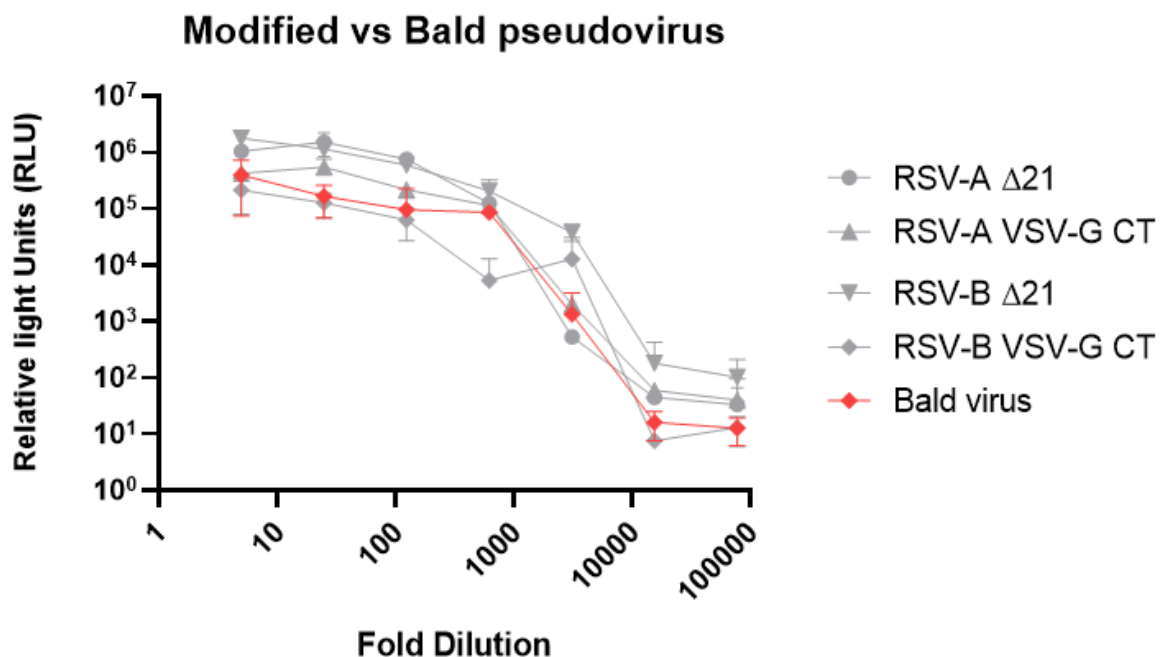


Figure 4.16. Bald Virus titration verses modified RSV pseudoviruses. Infectivity of modified RSV pseudovirus (RSV-A and -B Δ 21, and RSV-A and RSV-B VSV-G CT, batch 2 (grey)) compared to bald virus (red). Infectivity was measured in relative light units (RLUs) (y-axis) for each virus dilution (x-axis)

Overall, while RSV pseudoviruses with improved infectivity upon CT modification were produced, these viruses were not able to be neutralised, despite the RSV F protein being neutralisation sensitive. Neutralisation was assessed only for RSV-B Δ 21 and VSV-G CT, given time constraints; however, future work will also evaluate RSV-A. Variation in multiple neutralisation assay conditions, including viral input doses, mAb concentrations, incubation times, and plate rearrangements, displayed no detection of neutralisation. These data suggest that multiple factors inherent to the pseudoparticle contributed to the lack of neutralisation. These may include background infection

mediated by bald virus carryover, preF to postF triggering, and a sub-optimal ratio of infectious to non-infectious particles. Virions with a high proportion of non-infectious postF particles could still bind and saturate mAbs able to target preF and postF conformations, disrupting neutralisation. Additionally, unsuccessful incorporation of intact RSV F protein at the budded virion surface, leading to disrupted F protein expression, may also contribute to the lack of neutralisation, as mAbs would be unable to effectively bind to the virion.

4.5. Whole pseudovirus ELISA

While RSV F (with modified CT) protein conformational integrity was previously confirmed at the cell surface, expression at the pseudoparticle surface had not yet been validated. In addition, we wanted to quantify whether bald virus carryover could account for the viral infection observed in the CT modified viruses. To investigate this, whole-virus ELISAs were performed on PEG-precipitated CT modified RSV pseudovirus. Precipitated virus input in these ELISAs however could not be controlled, given this limitation, any differences in mAb binding observed may not be exclusively linked to F protein expression, but rather to the varying amounts of precipitated virus. Therefore, high versus low F protein incorporation could not be defined in this experiment.

Palivizumab, which is able to bind both pre- and postF conformations, bound to RSV-A Δ 21 with an OD₄₅₀ of 0.365 (Figure 4.17 A). While Nirsevimab (a preF discriminator) did not bind (OD₄₅₀ < 0.01), ADI-14359 a postF specific mAb bound to RSV-A Δ 21 with OD₄₅₀ of 0.341 (Figure 4.17 A), suggesting a greater proportion of RSV F protein in the triggered postF conformation incorporated at the RSV-A Δ 21 virion surface. The handling of these viruses during the precipitation process may also be a contributor to this triggering. A similar pattern was observed for RSV-A VSV-G CT, where only Palivizumab and ADI-14359 showed any binding, with OD₄₅₀ values of 1.612 and 1.646, respectively (Figure 4.17 B). Nirsevimab did not bind RSV-A VSV-G CT (OD₄₅₀ < 0.01) (Figure 4.17 B) suggesting a predominance of postF protein on RSV-A VSV-G CT particles or, more likely, the triggering of all preF molecules to the postF conformation during the precipitation process.

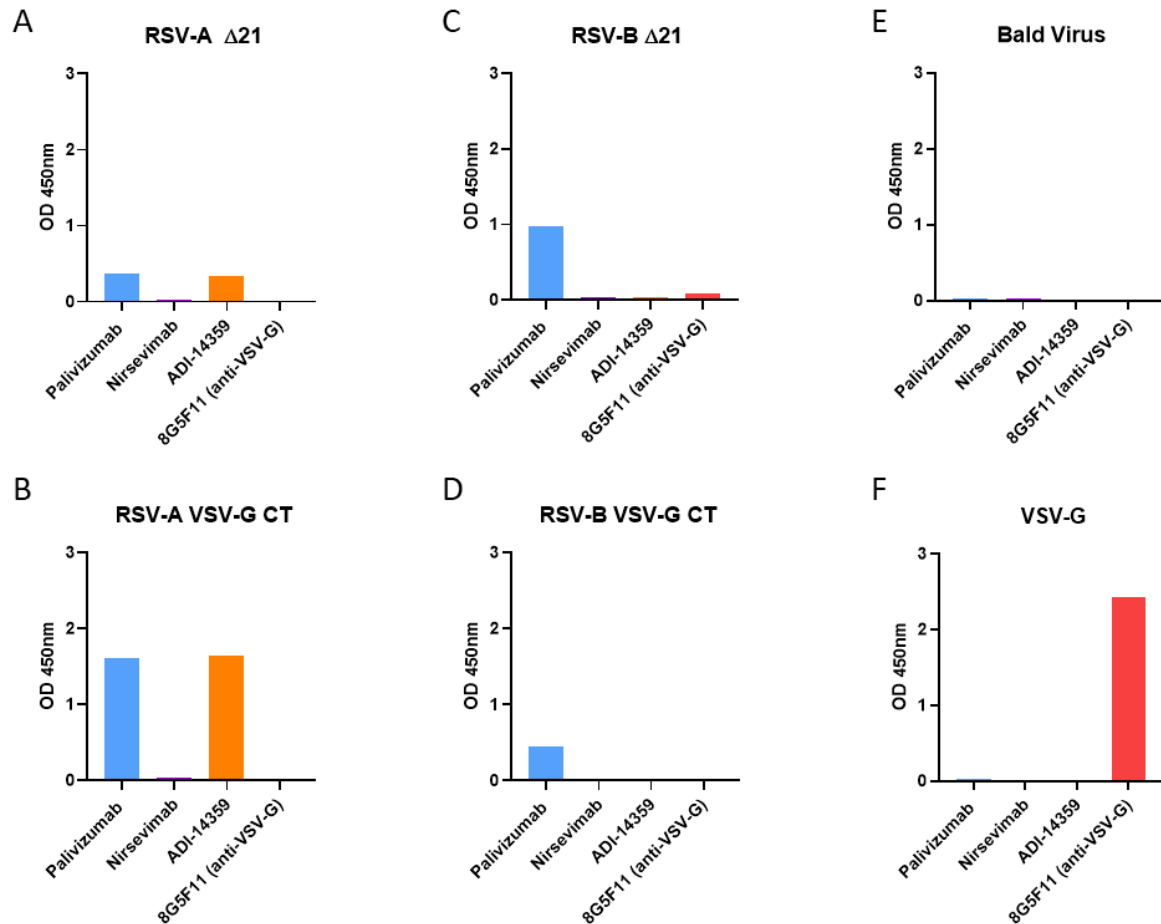


Figure 4.17. RSV F and VSV-G protein expression on the pseudoparticle surface. Monoclonal antibody binding to PEG-precipitated (A) RSV-A $\Delta 21$, (B) RSV-A VSV-G CT, (C) RSV-B $\Delta 21$, (D) RSV-B VSV-G CT (E) VSV-G and (F) Bald pseudoparticles. Palivizumab (blue), Nirsevimab (purple), ADI-14359 (postF specific) (orange), and anti-VSV-G (8G5F11) mAb are shown on the x-axis, while optical density at 450nm is shown on the y-axis.

For RSV-B $\Delta 21$ and VSV-G CT, only Palivizumab showed any binding with OD₄₅₀ values of 0.974 and 0.365, respectively (Figure 4.17 C and D). ADI-14359 did not bind RSV-B (Figure 4.17 C and D), but the same was observed in the mAb ELISA described in section 4.4.2. The lack of Nirsevimab binding to RSV-B points to a predominance of RSV postF protein on RSV-B virions or, more likely, the triggering of all preF molecules to the postF conformation during the precipitation process.

Additionally, the presence of (G* Δ G-luciferase) rVSV in the virus preparations was hypothesised previously as a contributor to lack of virus neutralisation. This was addressed by investigating binding of an additional anti-VSV-G mAb (8G5F11) to

pseudotyped RSV virions. The 8G5F11 mAb bound with high affinity (OD₄₅₀ of 2.42) only to the VSV-G control virion (Figure 4.17 F), but not to any of the modified RSV virions suggesting that there is in fact no (G*ΔG-luciferase) rVSV carry-over as hypothesised.

Overall, the binding of Palivizumab (preF and postF binding mAb), together with ADI-14359 (postF specific) and the lack of Nirsevimab (preF specific) binding observed across the RSV pseudoviruses suggests a predominance of postF protein in budded pseudoparticles or more likely triggering of preF to postF during virus precipitation. There is also no VSV-ΔG-G carry over in the virus preparations.

5. DISCUSSION

Respiratory syncytial virus is responsible for a substantial disease burden across the globe, with LMICs disproportionately affected and accounting for more than 98% of RSV mortality. The recent approval of novel RSV vaccines and mAb prophylactics highlights the need for increased immunological surveillance particularly in Africa where the linkage of genomic sequencing data to functional data and a general understanding of anti-RSV immunity in populations is limited^{68,69}. Here, we conducted analysis of the RSV F gene diversity in circulating South African RSV-A and RSV-B lineages and attempted to establish an F-pseudotyped VSV-based neutralisation assay to evaluate the impact of this sequence diversity on neutralisation by serum and mAbs.

From 569 RSV-A and 374 RSV-B F-gene sequences isolated from 2013 to 2023, we identified 52 antigenic-site specific mutations, 14 of which were frequent across multiple years, increased in frequency in recent years, and/or had structural relevance. Of these, seven were identified in RSV-A (N63S, K65R, S215L, I384T, S276N, S377N, and S190N) and seven in RSV-B (N63S, I206M, Q209R, S211N, S389P, S276N, and S190N). These were identified as mutations with the potential to contribute to viral escape and with implications on RSV interventions based on our selection criteria. Although we applied stringent thresholds, prioritizing mutations that met three or more criteria, it is important to note that even a single factor—such as increased frequency—or the combination of two criteria may also play a significant

role in escape from neutralization. This consideration will be important for future studies.

A potential limitation of this mutation analysis is that we focused primarily on mutations within the RSV F antigenic sites, excluding those outside these sites or those not exposed on the protein surface, which may not be in direct contact with antibodies. In other entry proteins, including the envelope of HIV, it is well-documented that mutations outside of defined epitopes can mediate escape through alteration of the prefusion conformation¹⁰¹. The importance of assessing such mutations should not be overlooked and requires further study. Furthermore, we did not distinguish between random viral diversity and immune-driven diversity. For future work, incorporating the ratio of non-synonymous to synonymous mutations as a threshold will be explored. This measure may reveal a stronger signal of immune-selected nucleotide substitutions, while reducing the contribution of background mutations that naturally occur. Within the context of our study—focused on the impact of viral evolution on antibody responses—immune-driven changes are particularly relevant, as they are responsible for facilitating viral escape from neutralisation.

Additional strategies for stratifying mutations could have strengthened this analysis, such as examining mutation frequencies by HIV status and their potential impact on F gene selection pressure. While patient HIV status is not reported or uploaded to GISAID, other MSc projects in our laboratory are investigating the frequency of RSV F gene mutations in individuals of different ages, genders, locations, and within those with mild versus severe respiratory illness. Given that RSV disproportionately affects different age groups, age may represent an important factor for analysis. We also note that an analysis of genomes at a global scale may have produced a more representative set of references, therefore we will incorporate global sequences in the selection of a reference in future studies as well.

Of particular interest were the RSV-A N63S and K65R and RSV-B I206M, Q209R, and S211N mutations, located in sites Ø and II, the epitopes of approved prophylactics Palivizumab and Nirsevimab. Regarding site II and the impact of mutations on Palivizumab sensitivity, the S276N mutation has been shown to confer palivizumab resistance in RSV-A but not in RSV-B^{69,102} while the K65R mutation acts in combination with I64M (a mutation not observed in the sequencing data used in this

study) to confer resistance⁷⁷. In our analysis, we observed an I64V mutation instead, which substitutes a smaller hydrophobic side than I64 or M64, and may suggest maintenance of the sensitive phenotype, however this will need to be confirmed experimentally.

Ahani et al. (2023) characterised site Ø mutations identified in RSV isolates from the MELODY and phase 2b Nirsevimab clinical trials⁷⁸. RSV-B isolates containing I206M, Q209R, and S211N mutations, which we also identified in circulating South African lineages, were still susceptible to Nirsevimab. In fact, they demonstrated that I206M and Q209R, in combination or alone, increased susceptibility to Nirsevimab, likely through enhanced electrostatic interactions with E55 and D101 in the Nirsevimab paratope⁷⁸. This crucial finding highlights that not all epitope mutations confer monoclonal antibody resistance, but without monitoring the impact of key mutations on antibody binding and/or neutralisation, this remains unknown.

Of the 14 mutations we identified, only the S276N and K65R, which are rare in the sequences we analysed, have evidence supporting viral escape. Functional data detailing the impact of the N63S, S215L, I384T, S389P and S190N mutations remains unknown, which was the reason we sought to establish a VSV-based RSV pseudovirus neutralisation assay.

We selected the VSV backbone due to its benefit over lentivirus-based assays in regions with high antiretroviral (ARV) usage. We produced RSV F-pseudotyped viruses and performed extensive optimisation for infection across more than 200 conditions including multiple cell types and densities, incubation times, RSV F CT modifications, and freeze-thaw parameters. Infection was only reproducible with the introduction of CT modifications, which increased infection by 9 to 20-fold compared to unmodified RSV pseudovirus. These CT modifications likely enhance pseudovirus infectivity by increasing the incorporation of F protein into the virion particle and facilitating membrane fusion processes as demonstrated in previous studies for multiple enveloped viruses^{87,98-100}. The CT region of RSV glycoproteins, including the F protein, associates with the matrix protein, playing a crucial role in virus assembly and budding¹⁸. In live virus systems, specific M gene variants are more compatible with efficient virion formation, further supporting the importance of these modifications⁹⁸. These modified pseudoviruses, which demonstrated enhanced

infection, therefore formed the basis for the neutralisation assay that was trialled. Despite the improved infectivity of these modified RSV pseudoviruses and antibody binding to F protein at the transfected cell surface, neutralisation was not observed by the panel of neutralising anti-RSV mAbs.

A few hypotheses were investigated to identify the factors contributing to this lack of observed neutralisation. An initial hypothesis considered that the F protein conformational integrity was disrupted through CT modification and that structural alterations to antibody epitopes would impede neutralisation. F protein expression at the cell surface was investigated using the CSB assay. The assay confirmed that CT modifications did not compromise F protein integrity as antibodies targeting different epitopes retained their binding. Differences in the observed mAb binding, overall showed that those preferring the preF conformation bound at lower levels than those able to bind both the preF and postF conformations. While this may suggest that a higher proportion of postF may be present in CT-modified constructs, antigenic site accessibility and mAb per trimer stoichiometry may also affect this. While structural analysis may have improved our analysis of epitope structural integrity. This was not performed; however, transmission electron microscopy would be used in future studies to assess the presentation of F on virion surfaces.

We further investigated RSV F protein expression in budded pseudovirus particles, to clarify two major hypotheses 1) neutralisation was thought to be masked by (G*ΔG-luciferase) rVSV carry-over in virus preparations and 2) while F was successfully expressed on the surface of transfected cells, this did not guarantee successful incorporation into budded virions. In our whole virion ELISA experiments, the absence of binding by the anti-VSV-G mAb (8G5F11) to precipitated virus preparations ruled out (G*ΔG-luciferase) rVSV carry-over, confirming that only RSV F were present on the RSV F pseudoparticles. Secondly, the ELISA confirmed that F protein is present on the virions but, given the limited or lack of binding of Nirsevimab (preF specific) and the relatively strong binding of ADI-14359 (postF specific), was mostly in the postF conformation. This was supported by the binding of Palivizumab which although has higher affinity for preF can still bind postF. However, because live virus, also displays this bias in preF:postF ratios and is perfectly capable of mediating successful infection¹⁷, this is not the sole contributor for lack of neutralisation.

Upon consolidation of these results, some of these results remain contradictory. RSV-A and RSV-B infection was improved by CT modification, with mAb binding retained to F expressed at the cell surface, thereby validating the use of our pseudoparticles in the neutralisation assay. The whole virus ELISA confirmed no (G*ΔG-luciferase) rVSV carry-over and the presence of F on pseudoparticles predominantly in the postF conformation. However, a major caveat here is that the precipitation of these viruses may drive preF to postF triggering and therefore this observation may not reflect the actual pre:postF ratio. In addition, RSV virions with a postF predominance would not be able to mediate the high levels of infection observed after CT modifications, as RSV infection depends on the presence of the preF conformation thereby excluding this as a possible conclusion. While bald virus carry over was observed following titration, only in isolation could it be considered to mask neutralisation as this was ruled out by the whole virion ELISA.

This study therefore highlights the challenges and non-trivial nature of establishing RSV neutralisation assays for immunological surveillance, using pseudotyped VSV particles. A preprint however by Simonich et al. has suggested that both the F and G enhances infectivity in RSV pseudovirus systems¹⁰³ which, we will explore in future studies. While RSV can still infect cells without G, there is additional support that with G included infectivity is enhanced¹⁰⁴. These challenges associated with setting up the VSV-based RSV neutralisation assay precluded its use in monitoring the impact of the identified RSV F gene mutations on antibody responses as per the initial aim of the study. Future studies will therefore seek to carry out the intended functional assessment of RSV F gene diversity on antibody responses. These studies could include the establishment of a live-virus neutralisation assay using a reverse genetics system that allows for successful incorporation of variation while maintaining suitable infection. However, this live virus approach is limited by the complexity of replication-competent viruses, which are often challenging to grow and maintain at high virus yields.

Given this limitation, an additional consideration for evaluating functional impact would be through an ELISA binding assay. Variation can be incorporated into soluble F protein that could be used as antigens in the ELISA and therefore assess impact on antibody binding using the established panel of anti-RSV mAbs. Two notable limitations exist for this approach: 1) antibody binding data does not always correlate

with virus neutralisation, in some cases antibodies may bind but not neutralise virus and 2) the introduction of certain mutations may negatively affect soluble F protein expression, potentially leading to insufficient protein to conduct binding studies.

In conclusion, seven RSV-A and seven RSV-B F mutations in circulating South African lineages that may impact RSV interventions and population immunity were identified. However, although the VSV pseudovirus system offers advantages over lentiviral pseudoviruses in regions with high ARV usage, we were unable to produce VSV pseudoviruses compatible with RSV F neutralisation. This precluded us from assessing the impact of the identified F mutations on serum and mAb neutralisation. But, given that the importance of immunological surveillance for RSV remains, the impact of these mutations will be assessed using F protein binding assays in future studies.

APPENDIX A1

Table A1.1 Anti-RSV mAb sequences

Anti-RSV mAb	Antigenic site	Light chain sequence	Heavy chain sequence
Nirsevimab	Ø	DIQMTQSPSSLSAAVGDRVITITCQASQDIVNYLNWYQKPGKAPKLLIYVASNLETGVPSRFSGSGSGTDFSLTISSLQPEDVATYYCQYDNLPFTFGGGTKVEIK	QVQLVQSGAEVKKPGSSVMVSCQASGGLEDYIINWVRQAPGQGPEWMGGIIPVLGTVHYGPKFQGRVTITADESTDAYMELSSLRSEDTAMYYCATETALVVSEYLYPHYFDNWGQGLTVTVSS
ADI-15610	I	EIVMTQSPSSLSASVGDRVITICRASQSVSIYLNWYQKPGKTPPELLIYGASRLQSGVPSRFSGSGSGTDFLTISLQPEDFATYYCLQTYSTPLTFGGQGTKVEIK	EVQLLESQAEVKKPGSSVKVSCASGGTFSSYAITWVRQAPGQGLEWMGGIIPILGTTTYAQRFFQGRVTITADKSTTTAYMELSLRSEDTAVYYCAKTVSQYPNTYNYGMDVWVGQTTVTVSS
Palivizumab	II	DIQMTQSPSTLSASVGDRVITITCKQLSVGYMHWYQKPGKAPKLLIYDTSKLASGVPSRFSGSGSGTAFTLTISLQPDDEFATYYCFQSGSGYPFTFGGGTKLEIK	QVTLRESGPALVKPTQTLTLTCTFSGFSLTSGMSVGVIRQPPGKALEWLADIWWDDKKDYNPSLKSRLTISKDTSANQVVLKVTNMDPADTATYYCARSMITNWYFDVWGAGTTVTVSS
MPE8	III	QSVVTQTPSVSGAPGQRTISCTGSSSNIGAGYDVHWYQQLPGTAPKLLIYDNNNRPSGVPDRFSASKSGTASLAITGLQAEDEADYYCQSYDRNLSGVFGTGKVTVL	EVQLVESGGGLVKPGGSLRLSCAASGFTFSSYSMNWVRQAPGKGLEWVSSISASSSYSDYADSAKGRFTISRDNAKTSLFLQMNSLRSEDATYIFCARARATGYSSITPYFDIWGQGLTVTVSS
Clesrovimab	IV	DIQMTQSPSSLSASVGDRVITICRTSQDVRGALAWYQKPGKAPKLLIFDASSLETGVPSRFSGSGSGTVFTLTISLQPEDFAAAYCQQFLDFPFTFGQGTREIK	EVQLVESGGGLVLRPGRSLRLSCTVSGFSFDDSSAMSWVRQAPGKGLEWISFIKSKTYGGTKEYAASVKGRFTISRDDSKNIAYLQMNSLKTEDTAVYYCTRGPYGGNSDYGYGLDVWGQGTITVTVSS
CR95091	V	EIVMTQSPSSLSASIGDRVITITCQASQDISTYLNWYQKPGQAPRLLIYGASNLETGVPSRFTGSGYGTDFSVTISLQPEDATYYCQYQYLYPYTFAPGKVEIK	QVQLVQSGPGLVKPSQTLALTCTVSGASINSNDNYUNMODIFIEDWIRQRPGGGLWIGHISYTGNTYYTPSLKSRLMSLETQSQFSLRLTSVTAADSAYVFCAACGAYVLISNCGWFDSSWGQGTQVTVSS
2.4K	VI	DIQLTQSPDSLAVSLGERATINCKSSQSVLYRPNNKNFLAWYQQKPGQPPKLLIYWASTRQSGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQYHTTPLTFGGGTVDIK (ADI-14409 sequence)	QVQLVQSGGEVKKPGASVKVSCASGYTFTYYGISWVRQAPGQGLEWMGWISAYNGNTNYAQKFQGRVTMTTDTGTSTAYMELTSLRSDDTAVYYCARDRIVVVTGANYGMDVWGQGTITVTVSS
2.4B	VI	DIQLTQSPDSLAVSLGERATINCKSSQSVLYRPNNKNFLAWYQQKPGQPPKLLIYWASTRQSGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQYHTTPLTFGGGTVDIK (ADI-14409 sequence)	QVQLVQSGAEVKKPGASVKVSCASGYTFSYYGISWVRQAPGQGLEWMGWISASNGNTEDAQKFQGRVTMTTDTSTSTAYMELSLRSDDTAVYYCARDRIVVVVGANYHGMVWGQGTITVSVSS
ADI-15558	VI	QSVLTQPPSLSVSPGQTASISCSGDQLGNKYVCWYQKPGQSPVLVIYQDSRRPSGVPERFSGSNSGNTATLTGGTQPMDEADYYCQAWDSSIRVFGGGTKVTVL	QVQLVQSGGEVKKPGASVKVSCASGYTFTTYGISWVRQAPGHGLEWLGWISPKNGNTKYAQKQVQGRVTMTIDPTTSTAYMELSLRSDDTAMYYCARDYIVAIVAALPHGMDVWGQGLTVTVSS

Table A1.2. Primers for assembly of RSV-A F gene CT modification constructs

Primer	Forward / Reverse	Template	Overhang	Sequence
1	Reverse	RSV-A F Δ 21	Vector	AGACTCGAGTTATCATCTGGCCTTGCAATACAGCA
2	Forward	Vector	RSV-A F Δ 21	TATTGCAAGGCCAGATGATAACTCGAGTCTAGAGGGC
3	Reverse	RSV-A F Δ 21	VSV-G CT	TCTGGCCTTGCAATACAGCA
4	Forward	VSV-G CT	RSV-A F Δ 21	TATTGCAAGGCCAGATTAAAGCACACCAAGAAAAGAC
5	*Reverse	VSV-G CT	Vector	AGACTCGAGTTATCACTTTCCAAGTCGGTTCATCTCT
6	*Forward	Vector	VSV-G CT	TGATAACTCGAGTCTAGAGGGC

*Same primer used for RSV-A and RSV-B constructs

Table A1.3. Primers for assembly of RSV-B F gene CT modification constructs

Primer	Forward / Reverse	Template	Overhang	Sequence
1	Reverse	RSV-B F Δ 21	Vector	AGACTCGAGTTATCACTTGGCCTTGCACTACAGCA
2	Forward	Vector	RSV-B F Δ 21	TACTGCAAGGCCAAGTGATAACTCGAGTCTAGAGGGC
3	Reverse	RSV-B F Δ 21	VSV-G CT	CTTGGCCTTGCACTACAGCA
4	Forward	VSV-G CT	RSV-B F Δ 21	TACTGCAAGGCCAAGTTAAAGCACACCAAGAAAAGAC
5	*Reverse	VSV-G CT	Vector	AGACTCGAGTTATCACTTTCCAAGTCGGTTCATCTCT
6	*Forward	Vector	VSV-G CT	TGATAACTCGAGTCTAGAGGGC

*Same primer used for RSV-A and RSV-B constructs

Table A1.4 Unmodified and modified RSV pseudovirus construct sequencing primers

Subtype	Primer	Direction	Nucleotide binding location	length	Tm	%GC	Sequence
RSV-A	F1	Forward	1228-1247	20	63.3	60	GCCGTGACAGAACTGCAGC T
	F2	Forward	1732-1751	20	60.1	55	CTGCTGAGCCTGATCAACG A
	F3	Forward	2202-2221	20	60.1	55	CGTGAGCTGCTACGGAAAG A
	F4	Forward	2632-2651	20	59.9	55	GTGACACTGAGCAAGGACC A
	R1	Reverse	1440-1459	20	60.3	55	TGTTCACTTCGCCTTCCAGG
	R2	Reverse	1919-1938	20	60	55	ATTGGTGGTGCACAGAGGA G
RSV-B	F1	Forward	1458-1477	20	51.3	40	CAAGATTAAGAATGCTCTCC
	F2	Forward	2247-2265	19	57.6	57.9	CCGGGGAATCATCAAGACC
	R1	Reverse	1646-1664	19	56.6	52.6	GTGATTTCAGCAGTCTGC
	R2	Reverse	2319-2338	20	60.7	60	CGTAGTACAGGGTGTGCC G

APPENDIX A2

Table A2.1. RSV-A and -B phylogenetic iterations summary

Subtype	Curated F gene subset	Iteration	Adaptation
RSV-A	All African (2013-2023)	1 st	1956 USA sequence included
	All African (2013-2023)	2 nd	1956 USA sequence excluded
	All African (2013-2023)	3 rd	1956 USA sequence set as out group
	South African (2017-2023)	1 st	1956 USA sequence included
	South African (2017-2023)	2 nd	1956 USA sequence excluded
	South African (2017-2023)	3 rd	1956 USA sequence set as out group
RSV-B	All African (2014-2023)	1 st	1956 USA sequence included
	All African (2014-2023)	2 nd	1956 USA sequence excluded
	All African (2014-2023)	3 rd	1956 USA sequence set as out group
	South African (2015-2023)	1 st	1956 USA sequence included
	South African (2015-2023)	2 nd	1956 USA sequence excluded
	South African (2015-2023)	3 rd	1956 USA sequence set as out group

Table A2.2. RSV-A and RSV-B F gene antigenic diversity

Antigenic site	RSV-A mutations	RSV-B mutations
Ø	N63S I64V K65R S215L V239I D200N	N63S I206M Q209R S211N V220I I233V
I	K42R I384T	R42K D385E I386M S389P K390E
II	T245A S276N	S276N
III	V301A T337I S362L S377N	N/A
IV	K419E K419N S443T V459I L467I	K419E E463D
V	S173T G151S N165D N183F S190N V187I E294K S169N	K191R K168N S190N V187I
VI	T326I A504E V495I A504T	L321Q N325T S330T D486G

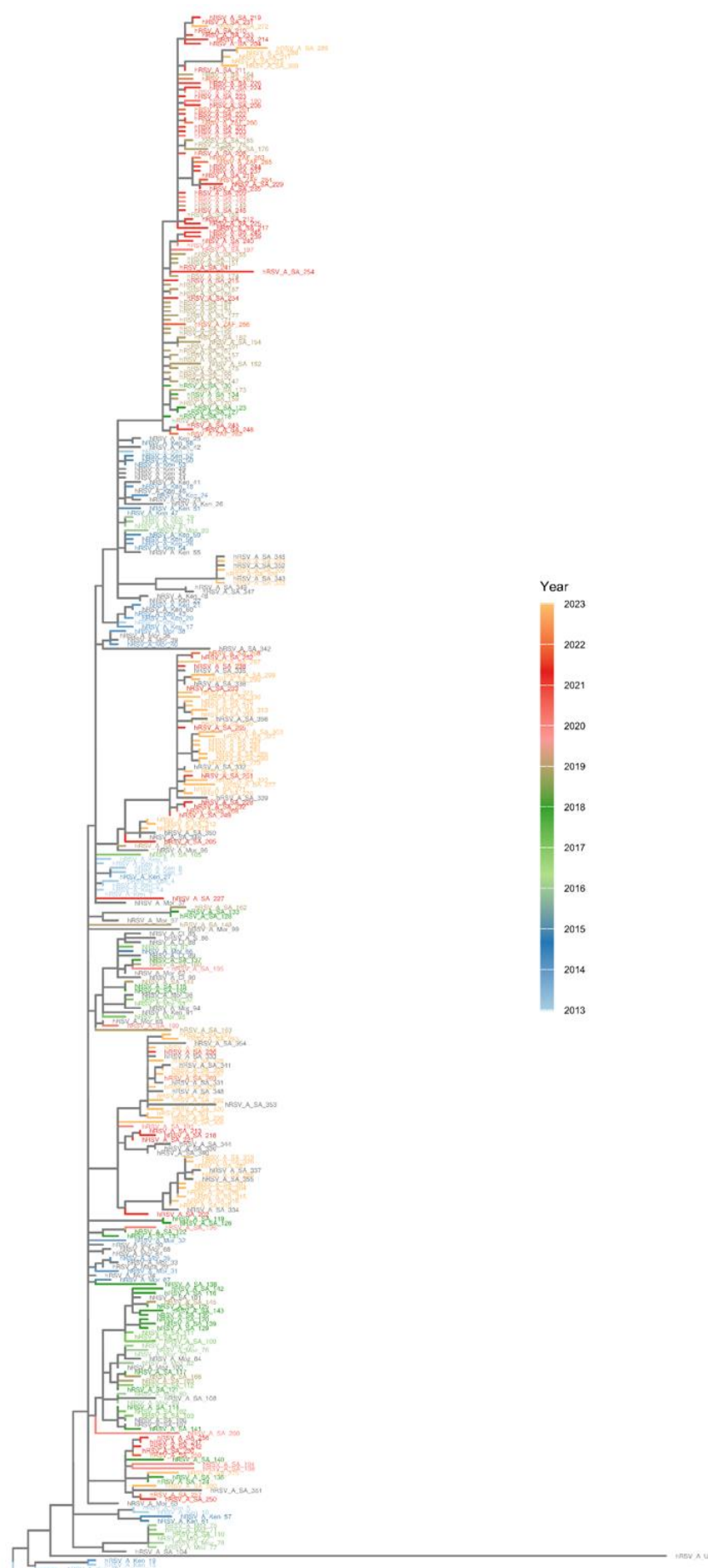
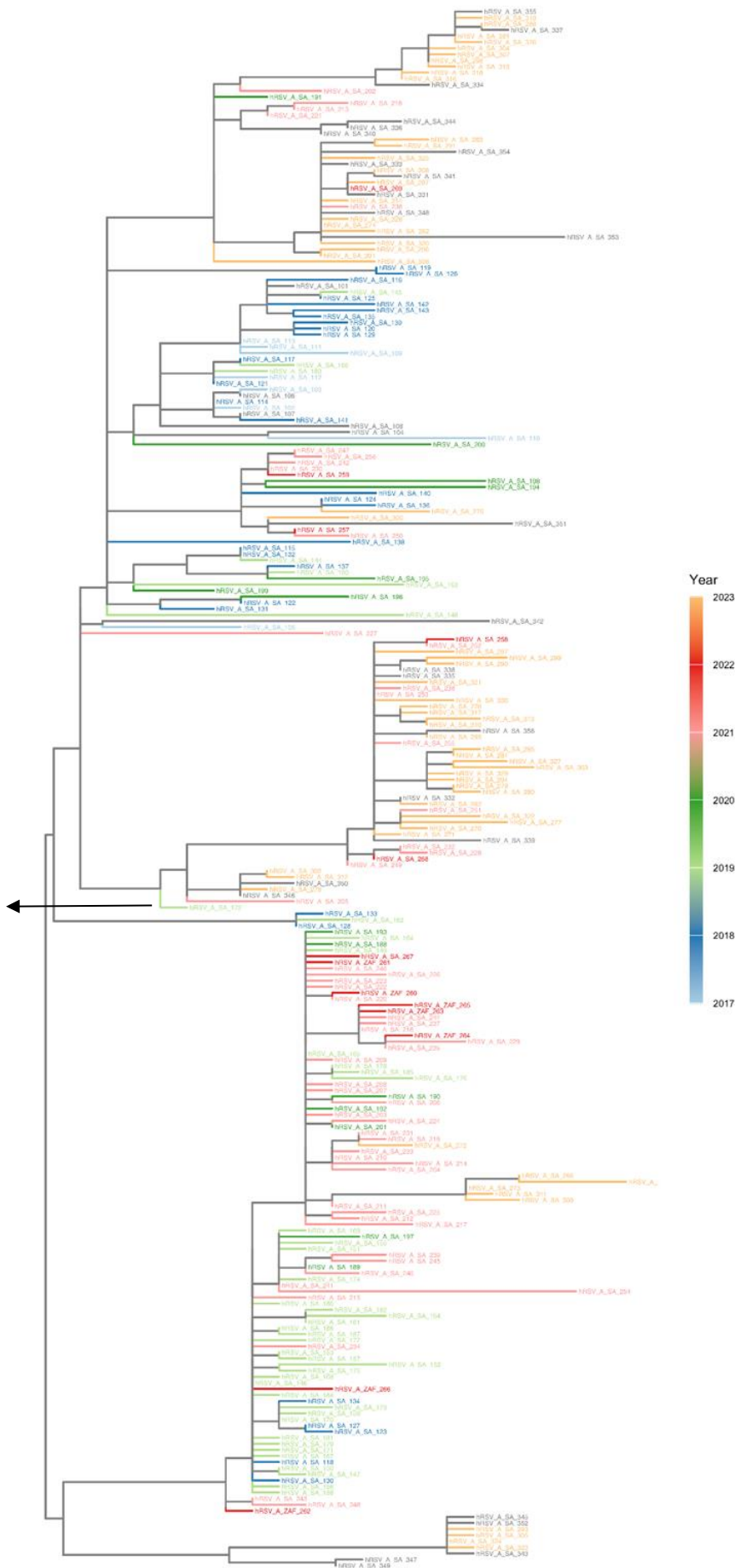


Figure A2.1 Phylogenetic tree for RSV-A reference gene identification, all African dataset 2nd iteration (1956 USA included). Maximum likelihood phylogenetic tree of South African and African RSV-A F gene sequence sampled between 2013 – 2023 (colour gradient as per legend) is shown. Tree root is indicated by the blue star and is enlarged. Trees were plotted using the ggtree package in the R software environment for statistical computing and graphics.



Figure A2.2 Phylogenetic tree for RSV-A reference gene identification, all African dataset 3rd iteration (1956 USA as outgroup). Maximum likelihood phylogenetic tree of South African and African RSV-A F gene sequence sampled between 2013 – 2023 (colour gradient as per legend) is shown. Tree root is indicated by the blue star and is enlarged. Trees were plotted using the ggtree package in the R software environment for statistical computing and graphics.



★ EPI-ISL-17218601

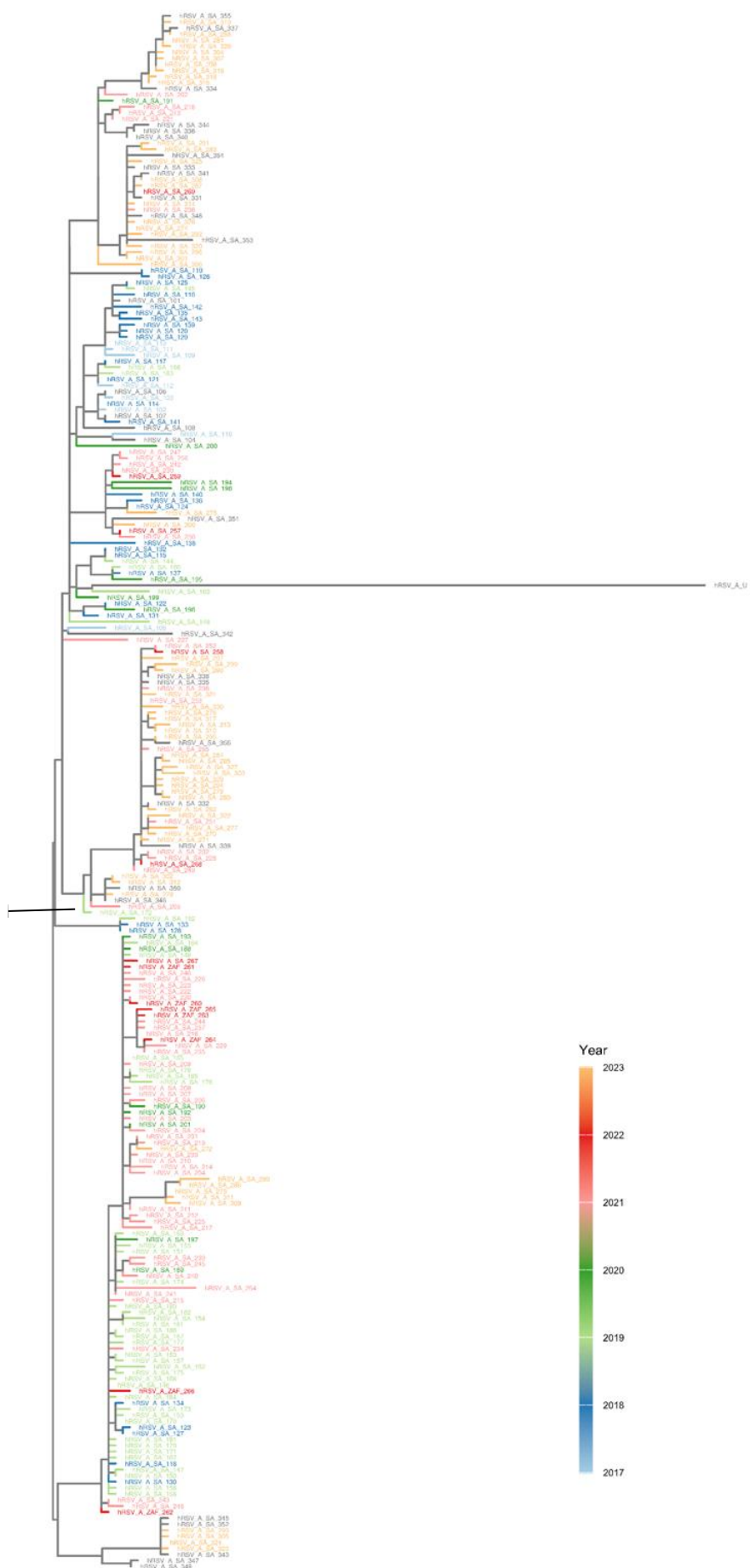


Figure A2.4 Phylogenetic tree for RSV-A reference gene identification, South African dataset 2nd iteration (1956 USA included). Maximum likelihood phylogenetic tree of South African RSV-A gene sequence sampled between 2017 – 2023 (colour gradient as per legend) is shown. Tree root is indicated by the blue star and is enlarged. Trees were plotted using the ggtree package in the R software environment for statistical computing and graphics.



Page | 88

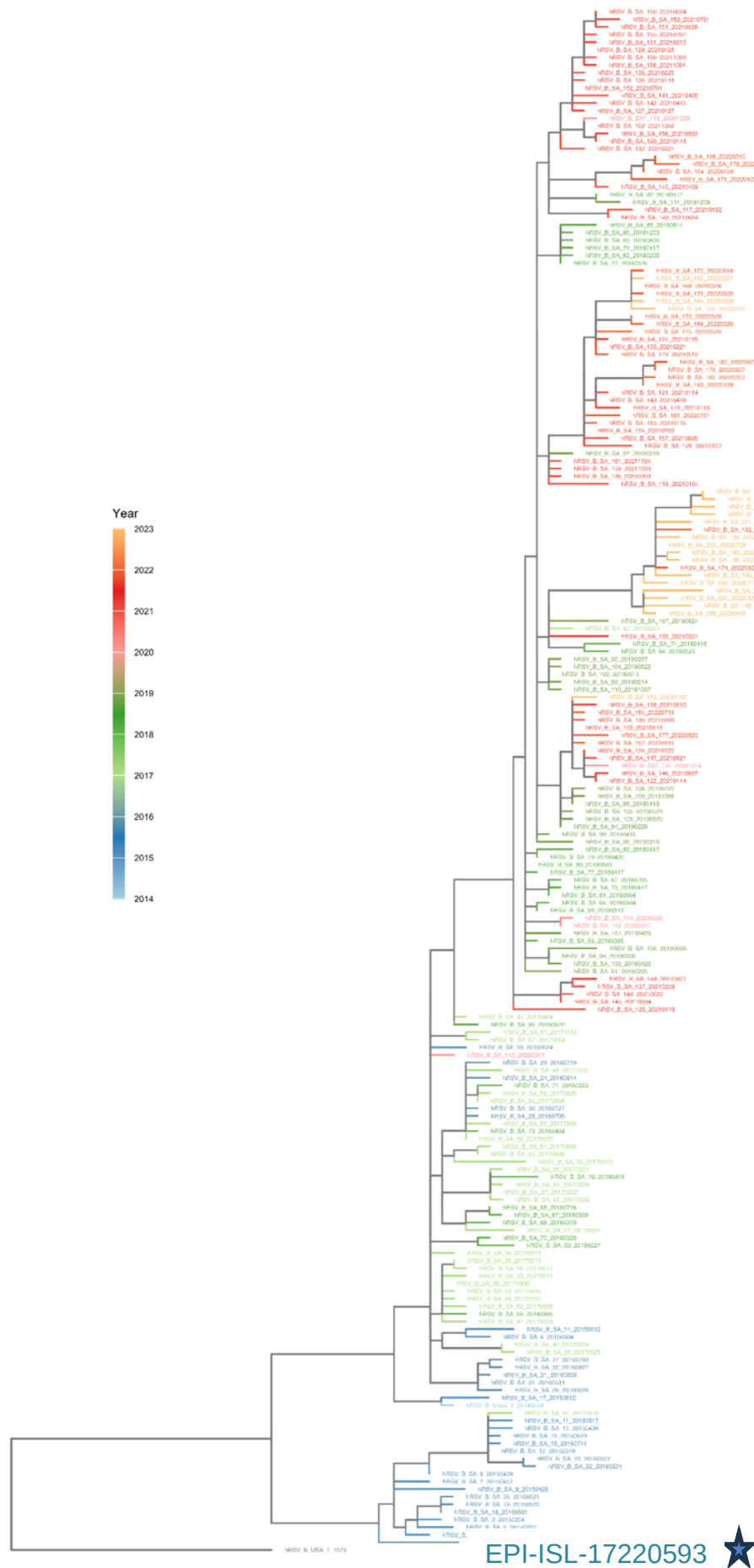


Figure A2.7 Phylogenetic tree for RSV-B reference gene identification, all African dataset 3rd iteration (1956 USA as outgroup). Maximum likelihood phylogenetic tree of South African and African RSV-A F gene sequence sampled between 2014 – 2023 (colour gradient as per legend) is shown. Tree root is indicated by the blue star and is enlarged. Trees were plotted using the ggtree package in the R software environment for statistical computing and graphics.

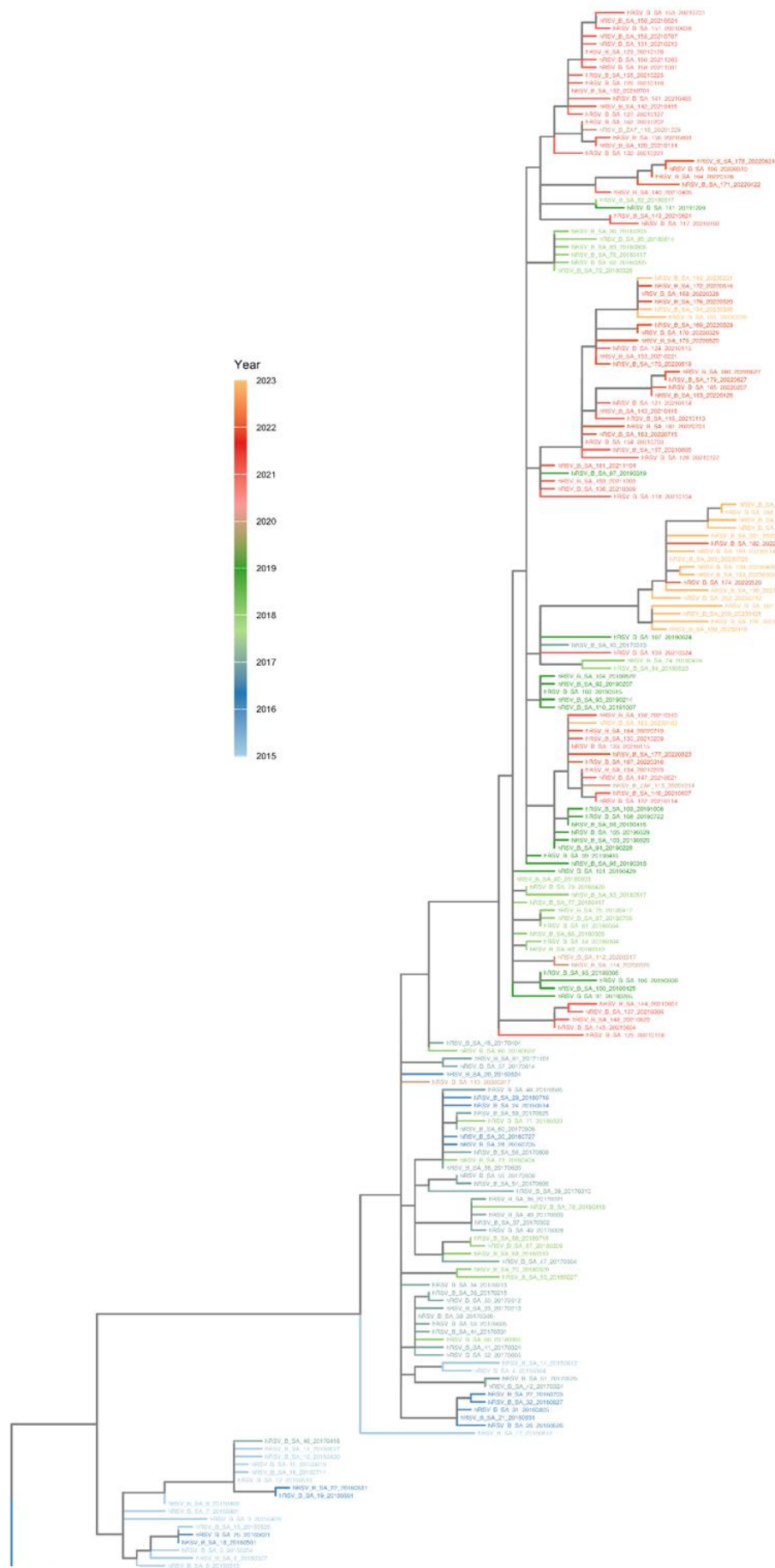


Figure A2.8 Phylogenetic tree for RSV-B reference gene identification, South African dataset (1956 USA excluded). Maximum likelihood phylogenetic tree of South African RSV-B F gene sequence sampled between 2015 – 2023 (colour gradient as per legend) is shown. Tree root is indicated by the blue star and is enlarged. Trees were plotted using the ggtree package in the R software environment for statistical computing and graphics.

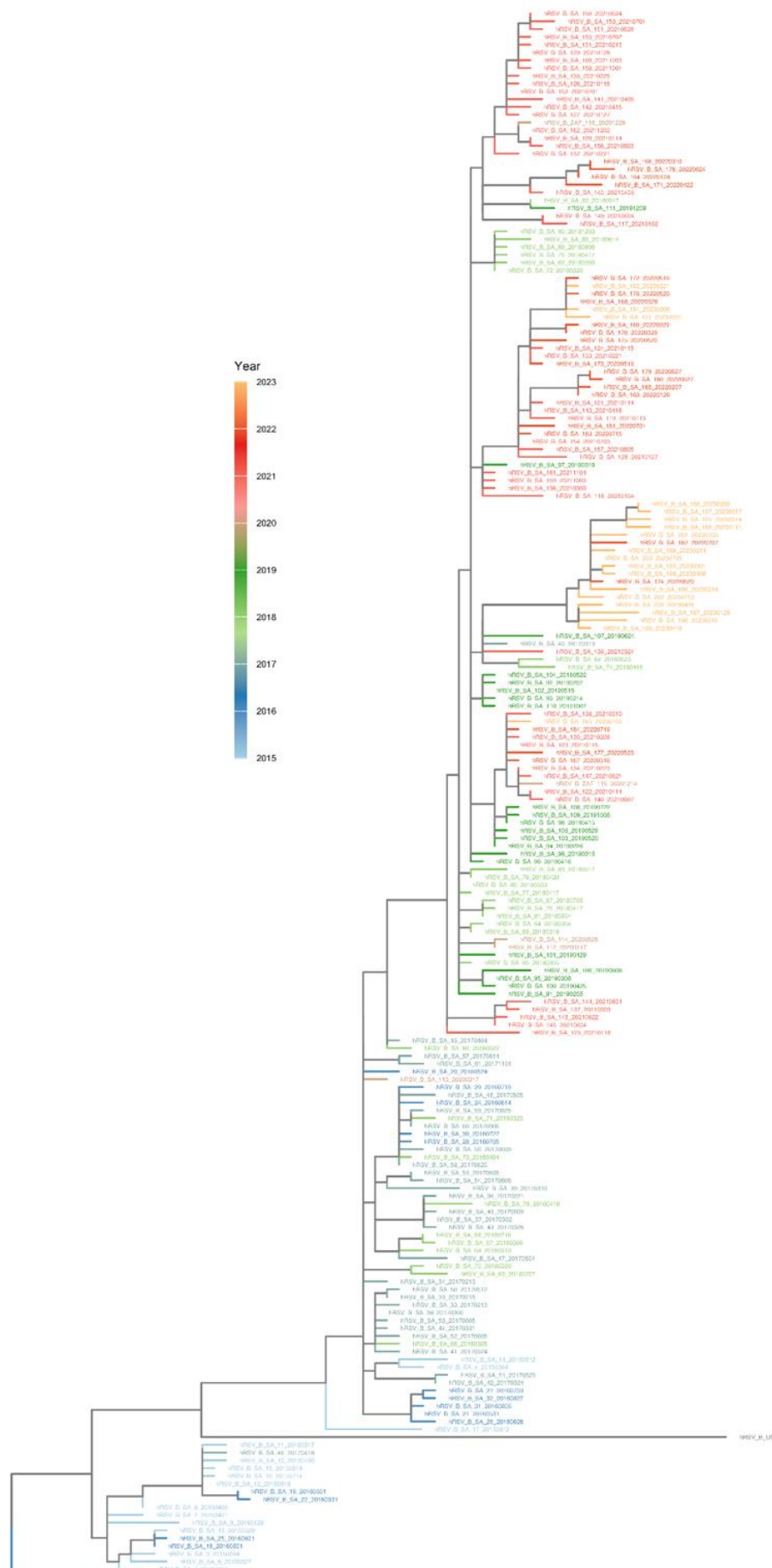


Figure A2.9 Phylogenetic tree for RSV-B reference gene identification, South African dataset 2nd iteration (1956 USA included). Maximum likelihood phylogenetic tree of South African RSV-B gene sequence sampled between 2015 – 2023 (colour gradient as per legend) is shown. Tree root is indicated by the blue star and is enlarged. Trees were plotted using the ggtree package in the R software environment for statistical computing and graphics.

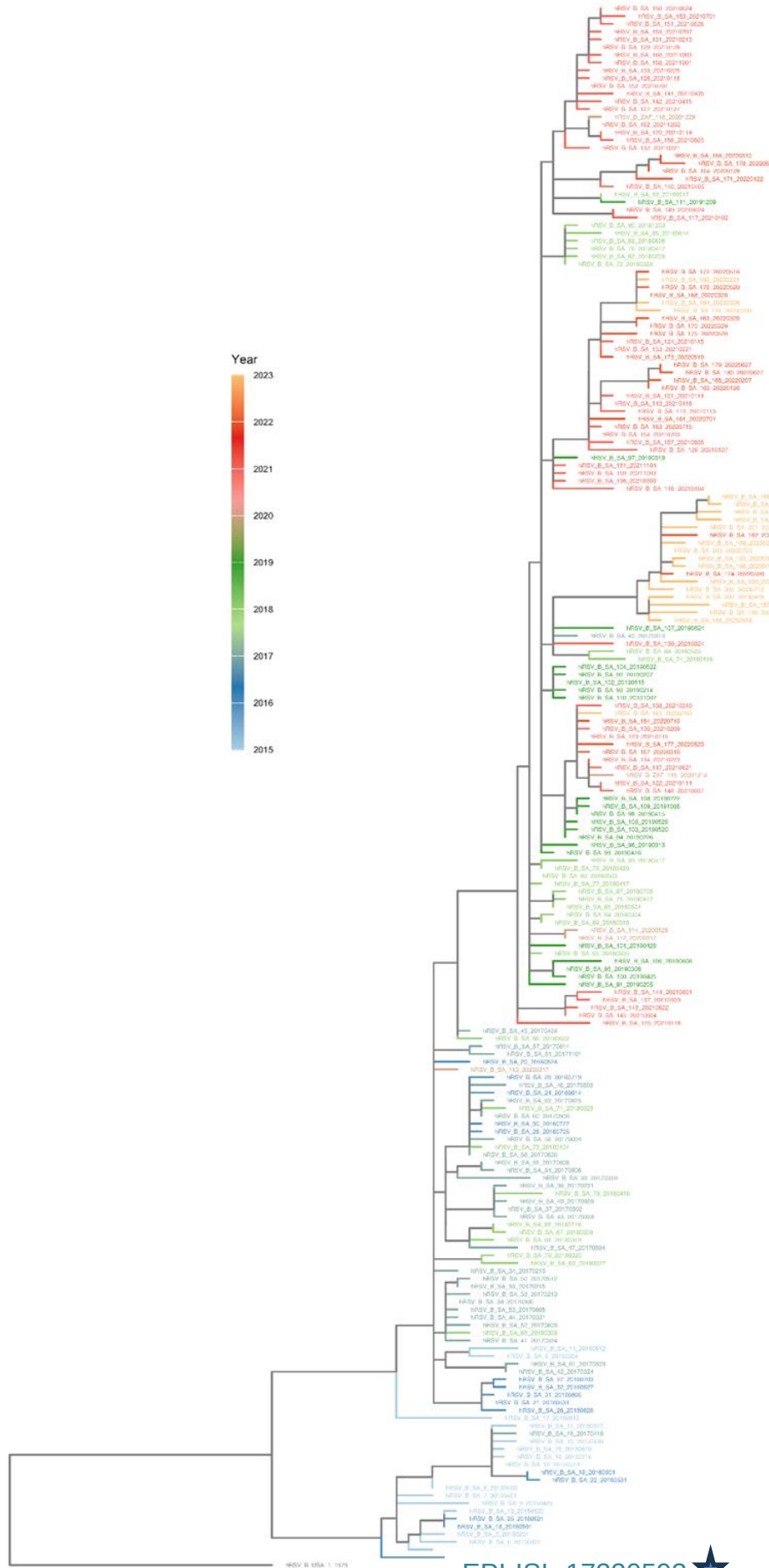


Figure A2.10 Phylogenetic tree for RSV-B reference gene identification, South African dataset 3rd iteration (1956 USA as outgroup). Maximum likelihood phylogenetic tree of South African RSV-B gene sequence sampled between 2015 – 2023 (colour gradient as per legend) is shown. Tree root is indicated by the blue star and is enlarged. Trees were plotted using the ggtree package in the R software environment for statistical computing and graphics.

Unmodified RSV-A cell type, density and incubation period optimisation

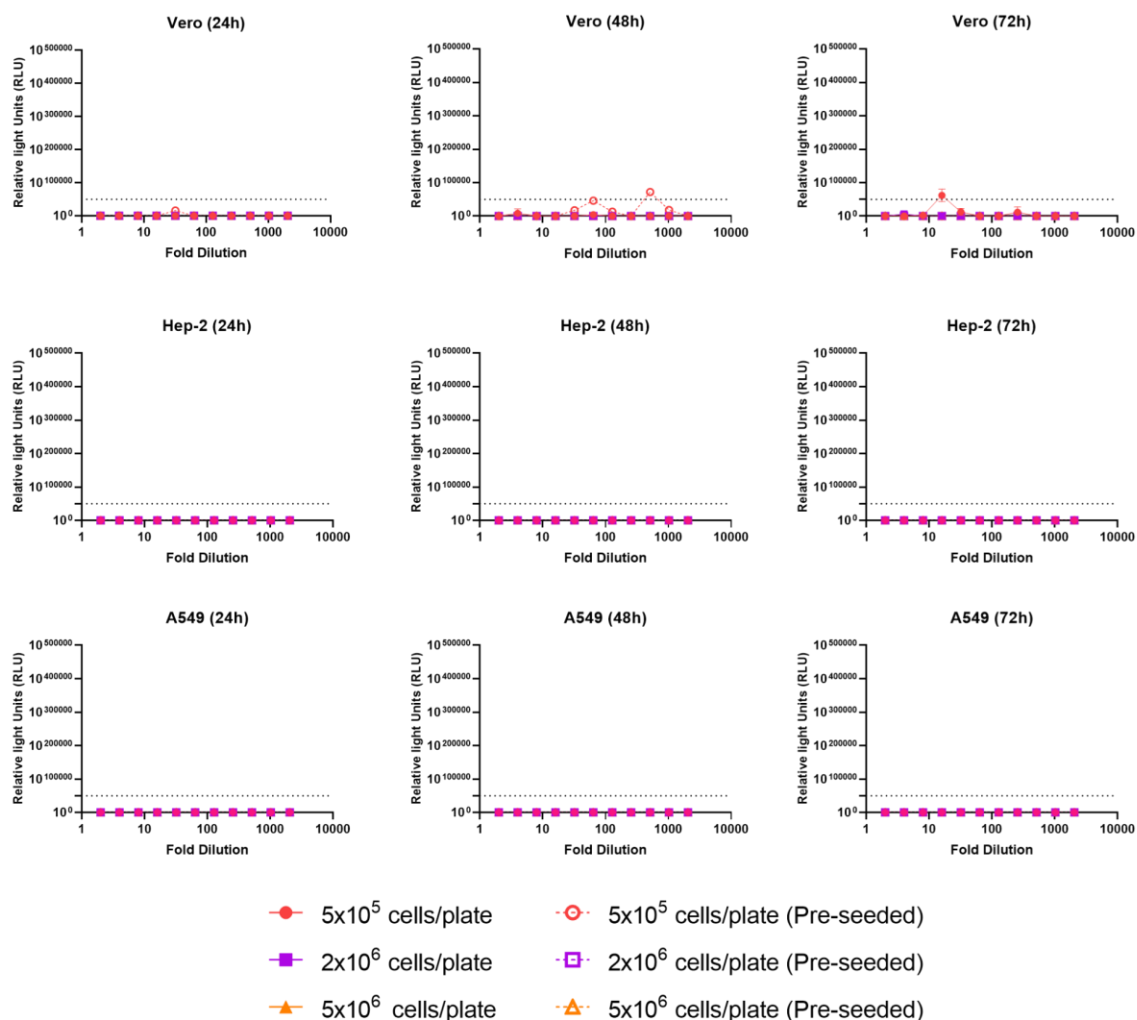


Figure A2.11. Unmodified RSV-A pseudoparticle infectivity evaluated in different cell lines and infection periods. RSV-A pseudoparticle infectivity was evaluated in Vero (top panel), Hep-2 (middle panel), and A549 (bottom panel) at varying cell concentrations (5 000 (red) 20 000 (purple), and 50 000 (pink) cells/well) either pre-seeded (dotted-line) or not (solid line). In each case, infectivity was evaluated following a 24-, 48-, and 72- hour incubation (shown in the respective graph titles). Infectivity per virus dilution (x-axis) was reported as a function of light detected and is measured in RLU (y-axis). The 50 000 RLU threshold for positive infection is indicated by the dotted line.

Unmodified RSV-B cell type, density and incubation period optimisation

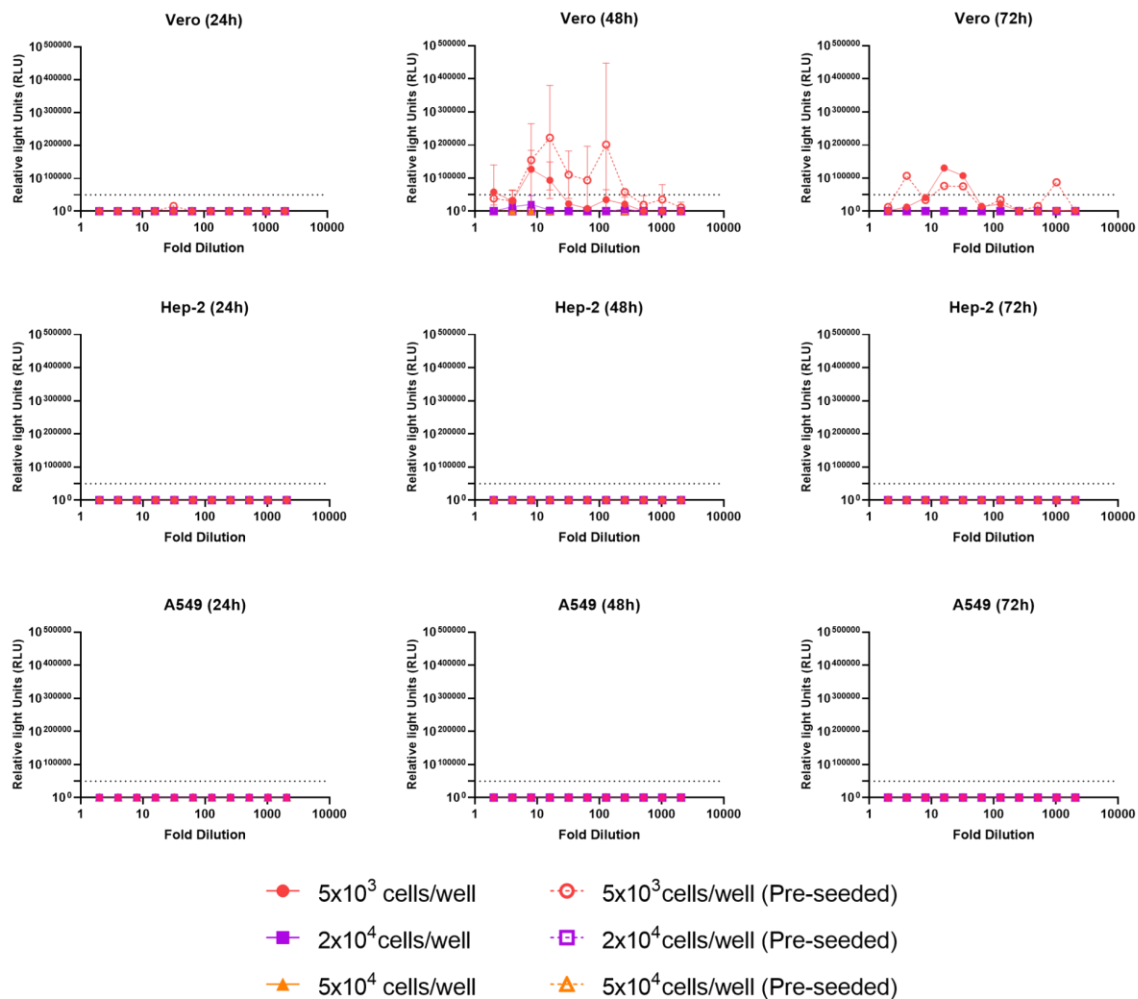


Figure A2.12. Unmodified RSV-B pseudoparticle infectivity evaluated in different cell lines and infection periods. RSV-B pseudoparticle infectivity was evaluated in Vero (top panel), Hep-2 (middle panel), and A549 (bottom panel) at varying cell concentrations (5 000 (red) 20 000 (purple), and 50 000 (pink) cells/well) either pre-seeded (dotted-line) or not (solid line). In each case, infectivity was evaluated following a 24-, 48-, and 72- hour incubation (shown in the respective graph titles). Infectivity per virus dilution (x-axis) was reported as a function of luminescence detected and is measured in RLU (y-axis). The 50 000 RLU threshold for positive infection is indicated by the dotted line.

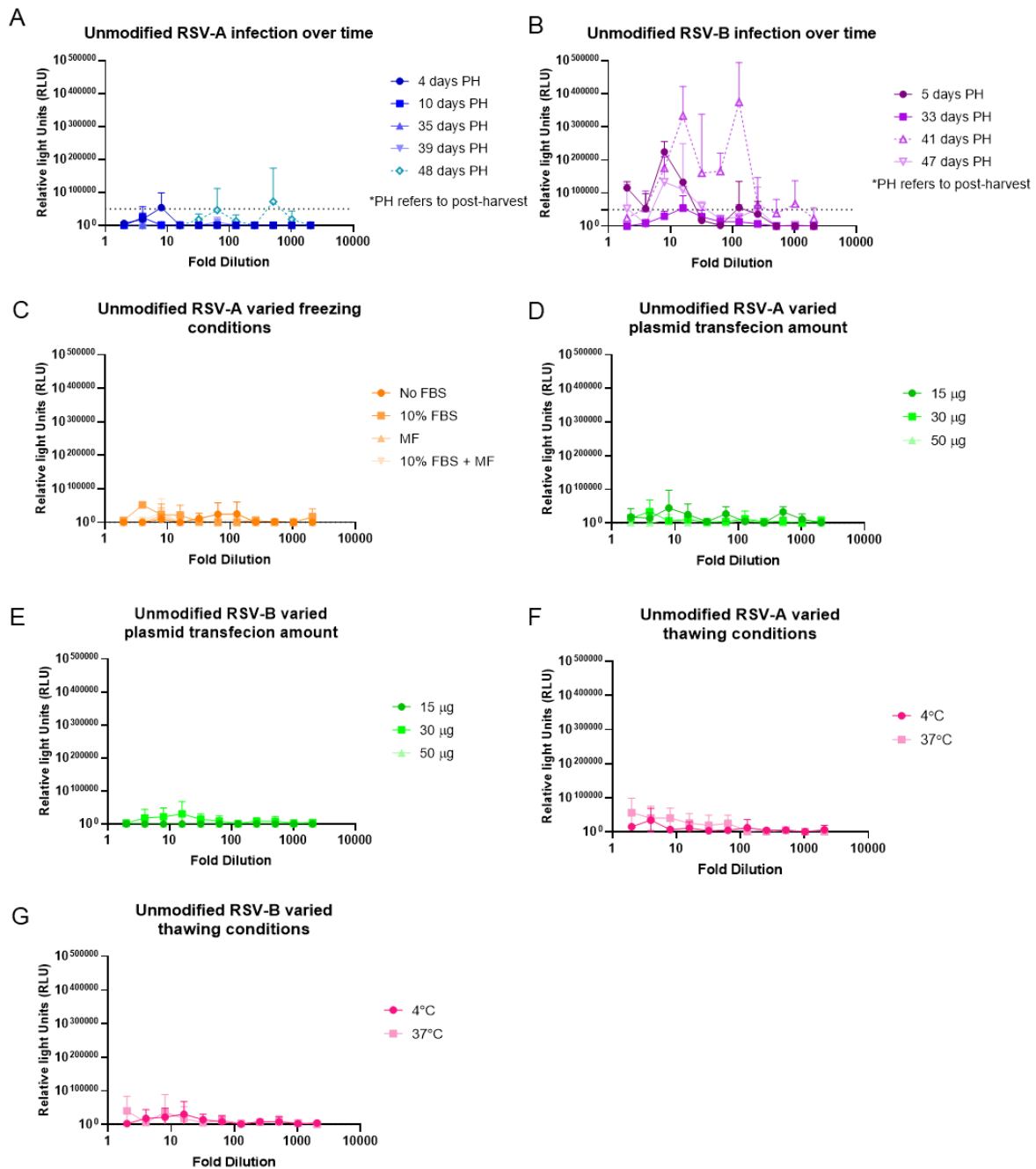


Figure A2.13. Optimisation of unmodified RSV-A and RSV-B infection parameters. **(A)** Unmodified RSV-A and **(B)** RSV-B infectivity was monitored over time in Vero cells (5 000 cells/well) after a 48-hour incubation period evaluated at 4-,5-,10-,33-,35-,39-,41-,47-, and 48-days post-harvest (PH) (shades of blue and purple, respectively; either pre-seeded (solid line) or not (dotted-line). Error bars indicate the standard deviation between duplicate samples in each run. **(C)** Effect of varied freezing conditions (without FBS, with 10% FBS, with Mr Frosty (MF), and with both 10% FBS and Mr Frosty (Shades of orange as per key)) on RSV-A pseudoparticle stability and infectivity. Error bars indicate the standard deviation between replicate runs (n=2). Effect of varied RSV F plasmid amounts (15 µg, 30 µg, and 50 µg (shades

of green as per key)) on unmodified RSV-A **(D)** and RSV-B **(E)** infectivity. These pseudoparticles generated with the respective amounts of RSV F plasmid were evaluated for infectivity in Vero cells (5 000 cells/well) after a 48-hour incubation. Error bars indicate the standard deviation between replicate runs (n=2). Effect of varied thawing conditions (thawing at 4°C or at 37°C (shades of pink)) on unmodified RSV-A **(F)** and RSV-B **(G)** infectivity in Vero cells (5 000 cells/well) after a 48-hour incubation. Error bars indicate the standard deviation between replicate runs (n=2). Across all graphs, infectivity was measured in RLU (y-axis) for each virus dilution (x-axis). The 50 000 RLU threshold for positive infection is indicated by the dotted line.

RSV-B Δ 22 cell type, density and incubation period optimisation

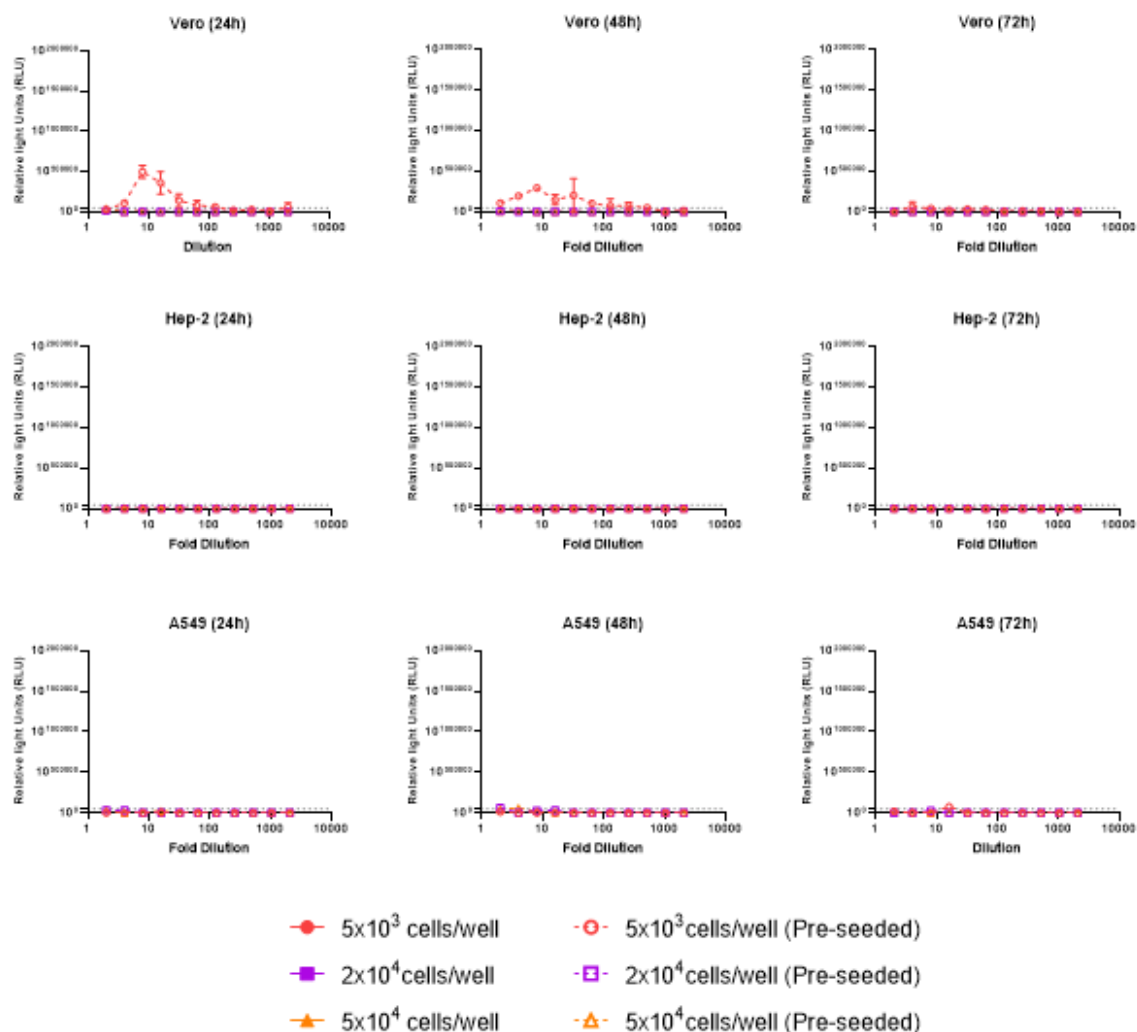


Figure A2.14. RSV-B Δ 21 pseudoparticle infectivity evaluated in different cell lines and infection periods. RSV-B Δ 21 pseudoparticle infectivity was evaluated in Vero (top panel), Hep-2 (middle panel), and A549 (bottom panel) at varying cell concentrations (5 000 (red) 20 000 (purple), and 50 000 (pink) cells/well) under pre-seeding conditions (dotted-line). In each case, infectivity was evaluated following a 24-, 48-, and 72- hour incubation (shown in the respective graph titles). Infectivity per virus dilution (x-axis) was reported as a function of light detected and is measured in RLU (y-axis).

RSV-B VSV-G CT cell type, density and incubation period optimisation

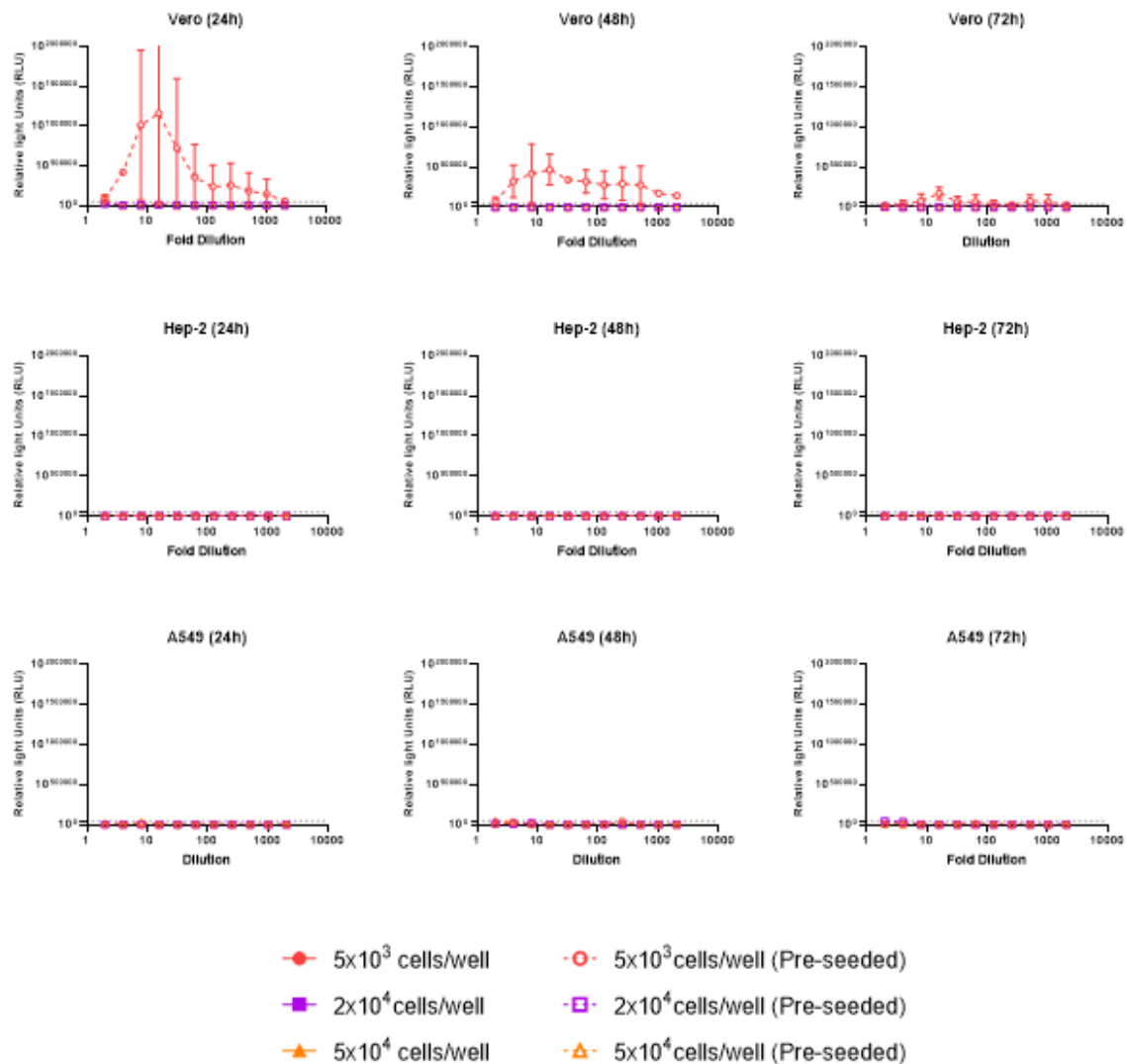


Figure A2.15. RSV-B VSV-G CT pseudoparticle infectivity evaluated in different cell lines and infection periods. RSV-B VSV-G CT pseudoparticle infectivity was evaluated in Vero (top panel), Hep-2 (middle panel), and A549 (bottom panel) at varying cell concentrations (5 000 (red) 20 000 (purple), and 50 000 (pink) cells/well) under pre-seeding conditions (dotted-line). In each case, infectivity was evaluated following a 24-, 48-, and 72- hour incubation (shown in the respective graph titles). Infectivity per virus dilution (x-axis) was reported as a function of light detected and is measured in RLU (y-axis).

RSV-A Δ 21 cell type, density and incubation period optimisation

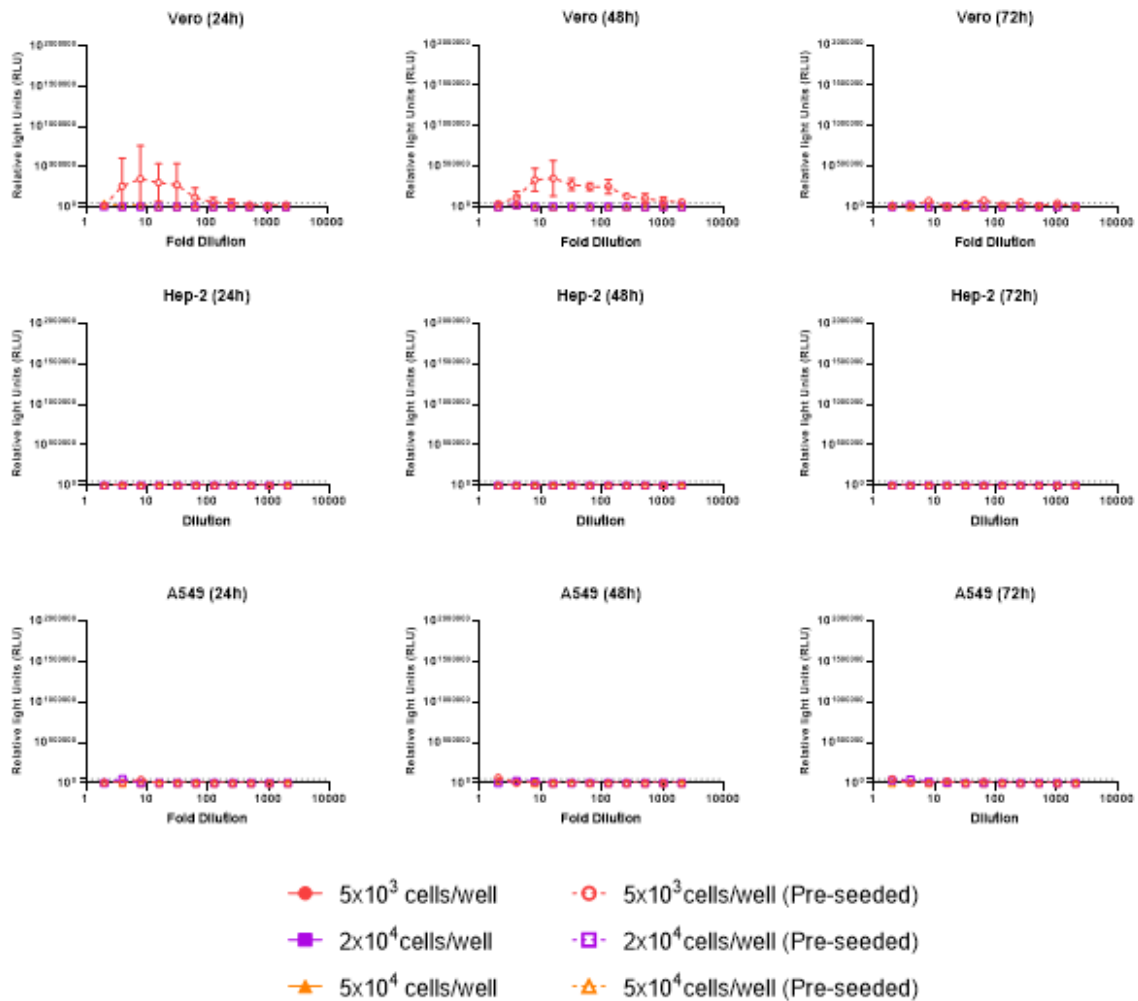


Figure A2.16. RSV-A Δ 21 pseudoparticle infectivity evaluated in different cell lines and infection periods. RSV-A Δ 21 pseudoparticle infectivity was evaluated in Vero (top panel), Hep-2 (middle panel), and A549 (bottom panel) at varying cell concentrations (5 000 (red) 20 000 (purple), and 50 000 (pink) cells/well) under pre-seeding conditions (dotted-line). In each case, infectivity was evaluated following a 24-, 48-, and 72- hour incubation (shown in the respective graph titles). Infectivity per virus dilution (x-axis) was reported as a function of light detected and is measured in RLU (y-axis).

RSV-A VSV-G CT cell type, density and incubation period optimisation

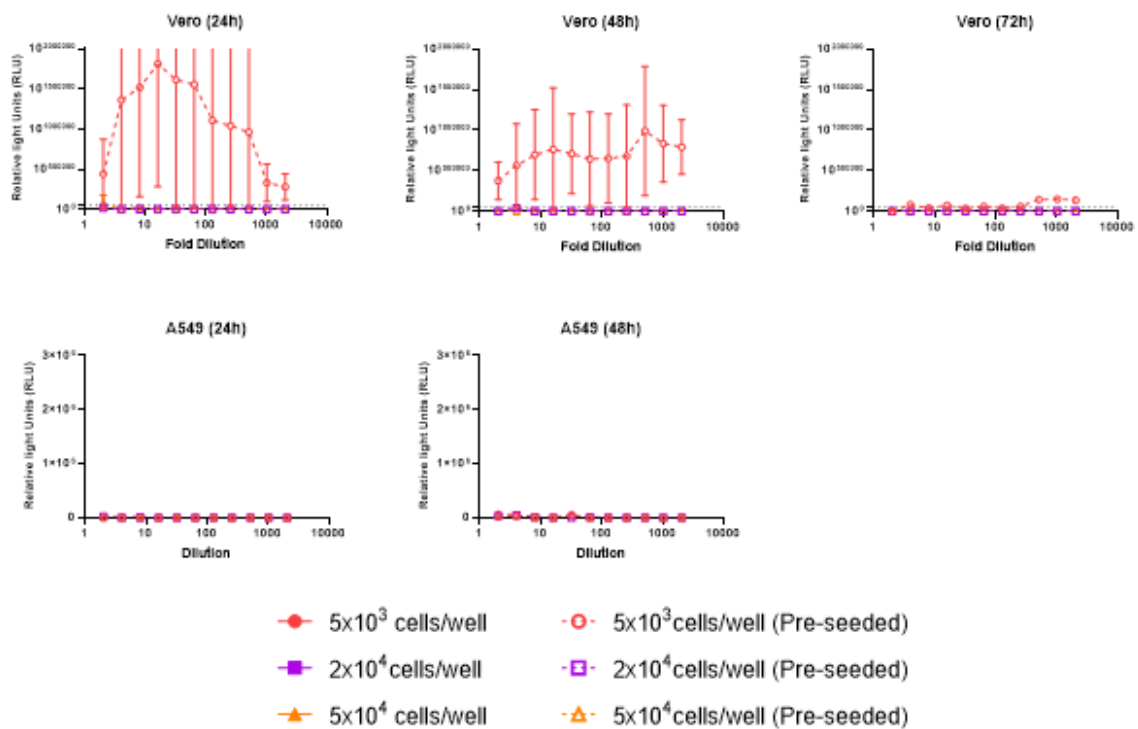


Figure A2.17. RSV-A VSV-G CT pseudoparticle infectivity evaluated in different cell lines and infection periods. RSV-A VSV-G CT pseudoparticle infectivity was evaluated in Vero (top panel), and A549 (bottom panel) at varying cell concentrations (5 000 (red) 20 000 (purple), and 50 000 (pink) cells/well) under pre-seeding conditions (dotted-line). In each case, infectivity was evaluated following a 24-, 48-, and 72- hour incubation (shown in the respective graph titles). Infectivity per virus dilution (x-axis) was reported as a function of light detected and is measured in RLU (y-axis).

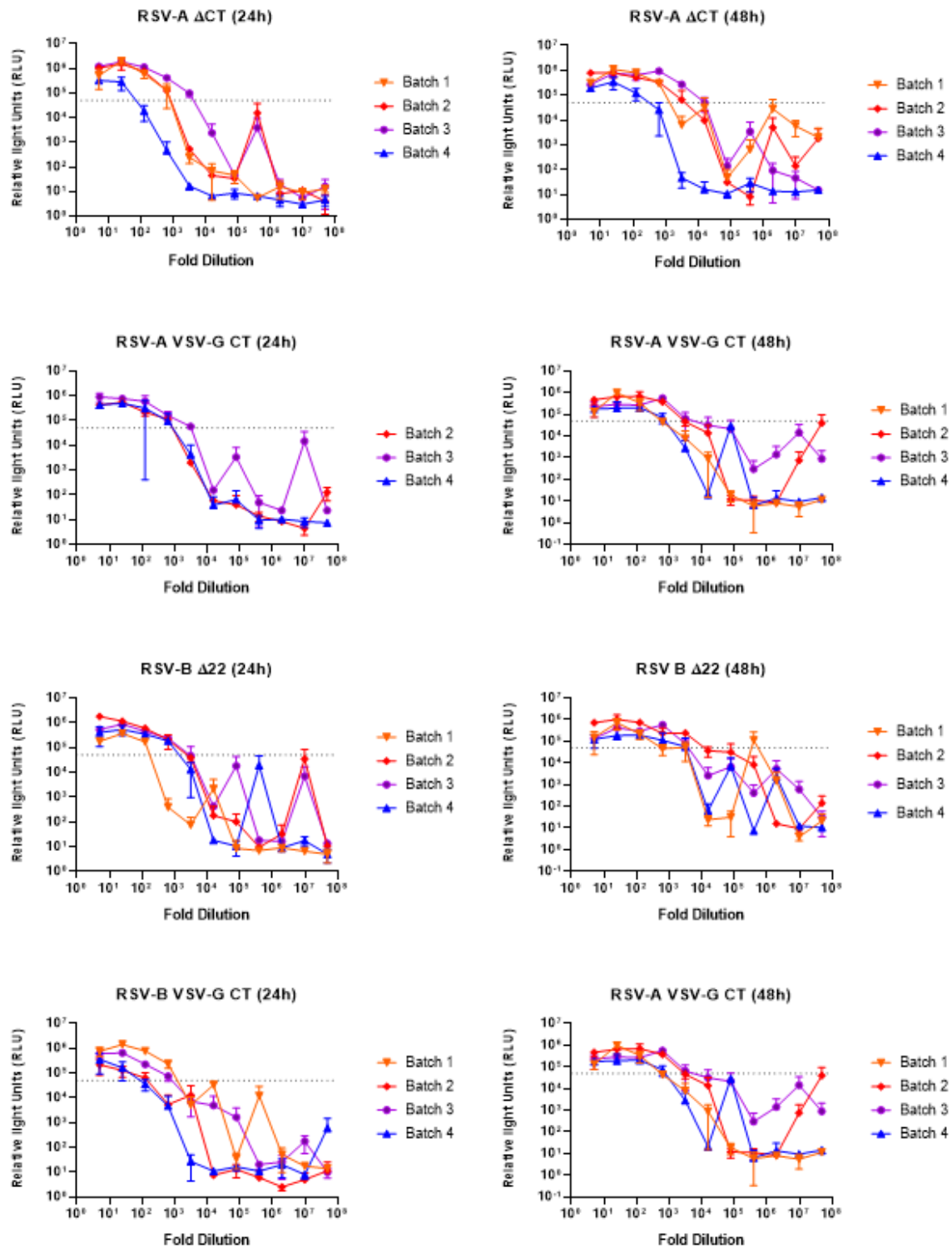


Figure A2.18. Reproducibility of modified RSV infection. RSV-A and RSV-B $\Delta 21$, and RSV-A and -B VSV-G CT were evaluated for infectivity in Vero cells (5 000 cells/well) after 24 and 48 hour incubations. Virus infectivity was evaluated across four batches (orange, red, purple and blue, respectively) for batch-batch reducibility assessment. Error bars indicate the standard deviation between replicate runs (n=2). Infectivity was measured in RLU (y-axis) for

each virus dilution (x-axis). The 50 000 RLU threshold for positive infection is indicated by the dotted line.

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