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Characterisation of RSV fusion proteins from South African patients with RSV disease, 2019 to 2020

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CHARACTERISATION OF RSV FUSION PROTEINS FROM SOUTH AFRICAN PATIENTS WITH RSV DISEASE, 2019 TO 2020

by

Prince Mabilo



A Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Sciences (MSc) in Medicine majoring in Medical Virology

Johannesburg, 2023

DECLARATION

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, **Prince Mabilo**, student number **[1455413]**, declare that this Thesis/Dissertation/Research Report is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis/Dissertation/Research Report.

Signature of Student ..... **Date** 10/03/2023

Name of Primary Supervisor Dr Florette Treurnicht

Signature of Primary Supervisor ..... **Date** 10/03/2023

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his/her Thesis/Dissertation/Research Report. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

Article 1: Title: Characterisation of RSV Fusion Proteins from South African Patients with RSV Disease, 2019 to 2020

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DEDICATION

This work is dedicated to my mother, for the great amount of love and support that she has always given me to pursue a career in research.

PUBLICATIONS AND CONFERENCE PROCEEDINGS

Publications

1. **Mabilo, P**.; Mthiyane, H.; Simane, A.; Subramoney, K.; Treurnicht, F.K. Characterisation of RSV Fusion Proteins from South African Patients with RSV Disease, 2019 to 2020. Viruses 2022, 14, 2321.https://doi.org/10.3390/v14112321. Impact factor: 5.048.

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ABSTRACT

Respiratory syncytial virus (RSV) is classified into subtypes A (RSV-A) and B (RSV-B), which are classified into different genotypes based on genetic variability of the G surface glycoprotein gene. The F surface protein gene is more conserved however variability in signal peptide, transmembrane domain, and antigenic sites have been reported. The study was conducted in the Virology laboratory, Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), South Africa. Study participants included patients of all ages from whom respiratory samples were submitted for respiratory viruses diagnoses from 2019 to 2020. The complete RSV F genes were amplified, library prep was preformed using the Nextera DNA prep kits and sequenced using amplicon-based next generation sequencing on the Illumina MiSeq sequencing platform. The Genome Detective Virus tool v2.27 was used to assemble sequencing reads and MEGA X was used for phylogenetic analysis. N-linked glycosylation and amino acid sequence variation was assessed. The overall prevalence of RSV was 5.8% (101/1 734). Seventy (69.3%; 70/101) RSV-positive samples were available for genetic characterisation of the F protein gene and thirty-one (30.7%; 31/101) were excluded due to insufficient sample volume. Only RSV-A strains were identified (91.2%; 31/34). Twenty three of thirty-one (74.2%) of the RSV F gene sequences from 2019 to 2020 clustered together with bootstrap values ranging from 64% to 99% and were NA1-like. A N-glycosylation site at position 120 gained by South African strains from 2018 is retained in strains from this study. This N-glycosylation site is present in approximately 25.8% of RSV strains from this study. The diversity of RSV-A F proteins was low, with amino acid variations observed at 30/571 (5.3%) sites. Ten mutations were detected in 4/6 antigenic sites (I, II, IV and V), with frequencies ranging from 0.3 to 100%. Antigenic changes seen exclusively among South African strains are: Y33H (0.3%) and V384T (7.3%) at site I and S275F (0.3%) at site II. Seven mutations associated with escape of human leukocyte antigen (HLA)-restricted cytotoxic T lymphocyte (CTL) were predicted in seven epitopes. Overall amino acid mutation frequency for 2019 to 2020 RSV-A F genes is similar to that reported for South African strains from 2018 (3.3% to 6.7%). For the first time in South Africa we detected the S275F mutation which causes palivizumab resistance.

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LIST OF ABBREVIATIONS

ADCC- Antibody Dependent Cellular Cytotoxicity

AEC- Alveolar epithelial cell

ARG- Argentina

ARIs- Acute respiratory infections

BEL- Belgium

BLT- Bead-like transposomes

CCA- Chimpanzee coryza agent

CCD- Central conserved domain

CHN- China

CMJAH- Charlotte Maxeke Johannesburg Academic Hospital

COVID-19- Coronavirus disease-2019

CPE- Cytopathic effects

CTL- Cytotoxic T lymphocyte

CX3CR1- CX3 chemokine receptor 1

DC-Dendritic cells

DEU- Germany

DNA- Deoxyribonucleic acid

EGF- Epidermal growth factor

ELISA- Enzyme-Linked Immunosorbent Assay

EPM- Enhaced PCR mix

ERD- Enhanced respiratory disease

ER-Endoplasmic reticulum

F- Fusion protein

FDA- Food and Drug Administration

FI-RSV- Formalin-inactivated RSV

FP- Fusion peptide

FTD-21- Fast Track Diagnostics Respiratory pathogens 21

G- Attachment protein

GISAID- Global Initiative on Sharing Avian Influenza Data

Gm- Membrane-bound G protein

Gs- Secreted G protein

GSK- GlaxoSmithKline

hAdV- Human adenovirus

HKY- Hasegawa–Kishino–Yano

HLA- Human histocompatibility leukocyte antigen

hMPV-Human metapneumovirus

HR-A- Heptad-repeat region A

HR-B- Heptad-repeat region B

hRV- Human rhinovirus

HRV2- Hypervariable region 2

HSPGs- Heparan sulfate proteoglycans

ICAM-1- Intercellular adhesion molecule 1

ICU- Intensive care unit

IFA- Immunofluorescence assay

IFN- Interferon

IFN- α/β- Alpha/beta interferon

IgG- Immunoglobulin G

IL- Interleukin

IRF-3- Interferon regulatory factor 3

KEN- Kenya

L- Large RNA polymerase

LAV- Live-attenuated vaccine

LB- Luria-Bertani

LFIA- Lateral flow immunoassay

LRTI- Lower respiratory tract illness

M- Matrix protein

M2- Matrix 2 protein

mAb- Monoclonal antibody

mAbs- Monoclonal antibodies

mRNA- Messenger RNA

N- Nucleoprotein

N residue- Asparagine residue

NAAT- Nucleic acid amplification tests

NaOH- Sodium hydroxide

NETs- Neutrophil extracellular traps

NF-κB- Nuclear factor κB

NHLS- National Health Sciences Laboratory Service

NICD- National Institute for Communicable Diseases

NLD- The Netherlands

NS- Non-structural proteins

ORF-Open-reading frame

P- Phosphoprotein

PCR- Polymerase chain reaction

PHL- Philippines

PIV- Parainfluenza virus

POCT- Point-of-care tests

Post-fusion- Post-F

Pre-fusion- Pre-F

R0- Basic reproduction number

RNA- Ribonucleic acid

RNPs- Ribonucleocapsid proteins

RSB- Resuspension buffer

RSA- The Republic of South Africa

RSV- Respiratory Syncytial Virus

RSV-A- Respiratory Syncytial Virus subtype A

RSV-B- Respiratory Syncytial Virus subtype B

RT-PCR- Reverse transcriptase polymerase chain reaction

RT-qPCR- Real-time quantitative PCR

RT-SIBA- Reverse transcription strand invasion-based amplification

SARS-CoV2- Severe acute respiratory syndrome coronavirus 2

SH- Small Hydrophobic protein

SP- Signal peptide

SPB- Sample purification beads

-ssRNA- Negative-sense single-stranded enveloped RNA

TAE- Tris-Acetate-EDTA

TB1- Tagmentation buffer 1

Th- Helper T lymphocytes

TLR4- Toll-like receptor 4

TSB- Tagmenation stop buffer

TSLP- Thymic stromal lymphopoietin

TWB- Tagmentation wash buffer

URTI- Upper respiratory tract illness

USA- United States of America

VNM- Vietnam

vRNA- genomic RNA

WHO- The World Health Organisation

CHAPTER 1

LITERATURE REVIEW

1.1. Background

Respiratory syncytial virus (RSV) is responsible for over 3 million severe acute respiratory tract infection (SARI)-associated hospitalisations and over 190 000 deaths occur each year [1]. Over 60% of children are infected with RSV before the age of 2 years [2]. More than 90% of mortalities associated with RSV occur in low to middle-income countries [1]. Children born with co-morbidities such as preterm infants are at a very high risk of severe RSV infections [3]. No vaccine has been approved to prevent RSV infections, however, palivizumab (Synagis®) is available only for high-risk infants and its use is very limited due to its cost [1]

RSV consists of two major surface glycoproteins namely attachment (G) and fusion (F) glycoproteins which can induce highly neutralising antibodies. The F protein of RSV is the mainstay of current vaccine development strategies because it is highly conserved [1]. RSV belongs to a single serotype and is grouped into 2 subtypes A and B and different genotypes based on a genetic variation on the G protein [1]. Despite the conservancy of the F protein, variations have been detected on the signal peptide (SP), transmembrane domain, and antigenic site Ø [4]. A South African study detected 7 and 11 variations on the antigenic sites of the F proteins of RSV-A and RSV-B at frequencies of 3.3% to 6.7% and 3.0% to 97.%, respectively [1]. Therefore, data on the variations among RSV F proteins is significant for vaccine or therapeutic development strategies, especially for South Africa.

1.2. RSV discovery

The discovery of RSV dates to 1956 from chimpanzees with acute respiratory disease and later in children during the bronchiolitis epidemic in the beginning of the 1960s [5]. During the investigation of the outbreak, an agent that caused a cytopathic effect (CPE) was isolated from one

of the chimpanzees who presented with upper respiratory tract illness (URTI). The agent was named "chimpanzee coryza agent" (CCA) after investigations [6]. After a thorough observation of the entire chimpanzee population, a high morbidity rate was discovered, and almost all the chimpanzees had been exposed to and infected by the CCA. Humans working on the chimpanzees were infected, and most presented with mild URTI and acute respiratory disease, somewhat less severe relative to the chimpanzees [6]. Subsequent studies recovered an agent from patients who had URTI, resembling CCA. The agent was named "respiratory syncytial virus" based on syncytial formation in tissue culture and its similarity to the agent from chimpanzees [6].

1.3. RSV classification and molecular biology

RSV is an enveloped, non-segmented, negative sense, single-stranded RNA (-ssRNA) virus classified under the family *Paramyxoviridae*, genus *Orthopneumovirus*, and subfamily *Pneumovirinae* [7;8]. RSV is a spherical virus ranging from, 150 to 300 nm in diameter [9].

The genome size is 15,2 kb and consists of a total of 10 genes encoding 11 distinct proteins (Figure 1.1) [10; 8]. The RSV proteins include two non-structural (NS) proteins, NS1 and NS2, and nine structural proteins, which include matrix (M); three envelope proteins, namely the G, F, and small hydrophobic (SH) proteins; five ribonucleocapsid proteins (RNPs); large RNA polymerase (L); nucleoprotein (N); phosphoprotein (P); and matrix 2 (M2), which has 2 open reading frames (ORF) [11; 12]. The G protein mediates virus-host cell attachment, while the F protein mediates fusion, viral entry, and transmission of the virus between cells. Both F and G proteins can induce neutralising antibodies against RSV infections. Hence, they are major targets for vaccine development [13]. The SH protein facilitates the formation of ion channels and acts as an apoptosis inhibitor [14]. The M protein acts as an inner envelope protein and provides further protection for the virus' genome. A total of 4 nucleocapsid-associated proteins, namely N, P, L, and M2-1, serve as viral transcription factors. M2-2 protein is involved in ribonucleic acid (RNA) synthesis during virion assembly [12]. It is assumed that NS1 and NS2 are involved in interferon (IFN)-release inhibition from infected cells [12]. These are described below in detail.

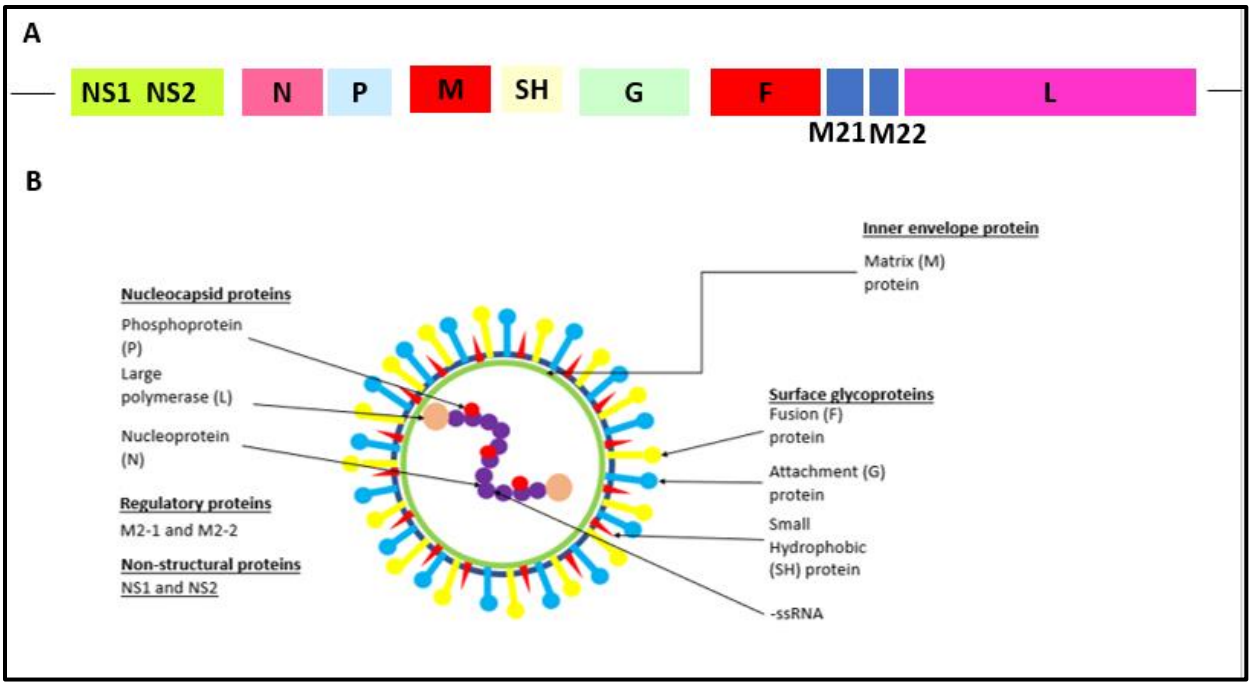


Figure 1.1. (A) RSV gene arrangement. (B) RSV virion structure. The virus consists of three envelope proteins, the G, F and SH proteins. The M protein is located below the envelope as the inner envelope protein. The nucleocapsid is made of -ssRNA that is encapsulated by the N protein. Associated with the nucleocapsid are the L protein and the P protein,adapted from [12].

1.3.1. NS1 and NS2 proteins

The NS1 and NS2 proteins are made-up of 139 and 124 amino acids, respectively, and are exclusive to RSV. NS1 and NS2 are not involved in viral assembly. However, the removal of these genes affects viral replication [15]. These proteins are involved in the evasion of the immune system [15; 16]. By mediating activation of nuclear factor B (NF-B), NS1 and NS2 inhibit activation of interferon regulatory factor 3 (IRF-3) and nuclear translocation, resulting in inhibition of induction of alpha- or beta-interferon (IFN) [16]. These proteins are also involved in apoptosis inhibition, resulting in a prolonged viral life cycle. In addition, these proteins interfere with viral RNA transcription and replication [16].

1.3.2. N proteins

The N protein is the major nucleocapsid protein, made up of 391 amino acids. It is the shortest of N proteins of all members of *the paramyxovirus* family. N protein is involved in multiple roles, including protection of the viral RNA from nucleases, RNA synthesis, replication, and virus assembly [15; 16].

1.3.3. P proteins

Likewise, the P protein of RSV is made up of 241 amino acids and is smaller than the P proteins of all other *paramyxoviruses* [15]. It is an essential polymerase cofactor [16]. It also forms a homotetramer and interacts with L, N, and M2-1. The P protein is extremely phosphorylated, and phosphorylation is essential for protein oligomerisation [15; 16].

1.3.4. M2-1 and M2-2 proteins

As previously mentioned, the M2 gene consists of two ORFs. The first ORF encodes the M2-1 protein, which is 194 amino acids long [15]. The M2-1 functions as an RNA polymerase complex co-factor [16] and is involved in the RNA synthesis process for the maintenance of polymerase association with the viral genome during RNA synthesis [15]. It is a zinc-binding phosphoprotein with two conformations (phosphorylated and dephosphorylated) and mostly helix-shaped secondary structures. In the phosphorylated conformation of M2-1, the phosphorylation sites are located at serine 58 and serine 6. These sites prevent M2-1 termination [16].

The M2-2 protein is 90 amino acids long. It is required for virus infection and pathogenesis but is not required for viral replication [16]. It is reported that extremely high levels of M2-2 expression interfere with RSV replication and are essential for the regulation of RNA transcription and replication [16].

1.3.5. L protein

The L protein is 2165 amino acids long and is the longest protein of all RSV proteins. The L protein forms part of the RNA-dependent RNA polymerase complex and is an important factor for primary enzyme activity in transcription and replication [16]. The L proteins have several functions, including catalysing the viral RNA transcription, capping and polyadenylating each mRNA. It consists of six conserved regions and two variable regions [16].

1.3.6. M protein

The M protein consists of 256 amino acids and is involved in the development of the overall virion shape and initiates virus assembly and budding of the host cell membrane [16]. It helps to recruit the RNPs to the plasma membrane for inclusion through the mediating M-to-M2-1 interaction. It is involved in interactions with the cytoplasmic tails of the G and F proteins and plays a role in the maturation of viral filaments. In the early stages, M is localised to the nucleus of the cell, interfering with host cell transcription. However, in later stages of infection, it is detected in cytoplasmic viral inclusion bodies for virus assembly and budding [16].

1.3.7. SH protein

The SH protein is a 64 amino acids long type II transmembrane protein. It neither plays virus replication nor entry. RSV without this gene is as infectious as wild-type RSV. Other studies have reported that it affects apoptosis inhibition. Its primary function is to form ion channels on the host cell membrane [16]. In contrast to F and G proteins, SH does not induce RSV neutralising, but facilitates antibody-dependent Dependent Cellular Cytotoxicity (ADCC) and other Fc receptor functions [17].

1.3.8. G protein

The G protein is a 298 amino acid-long type II integral membrane protein [18; 12]. The G protein is glycosylated, and it can be expressed as secreted (Gs) or membrane-bound (Gm), both containing a central fractalkine-like CX3C motif, but each having a specific role. The Gs is involved in neutralisation inhibition, while the Gm is involved in viral-host cell attachment [19 ;12]. The antigenic variation of the G protein is found at the C and N-termini. N and O-linked-glycosylation result in protein maturation and enhance immune response evasion mechanisms [12].

Only 53% of the amino acids on the G proteins are conserved across RSV strains. However, it has a 100% conserved and less glycosylated domain, which is essential for infectivity, namely the central conserved domain (CCD) [20]. The conserved nature of the CCD region has attracted most RSV vaccine development studies, but the role of adjacent regions should also be assessed [20]. In infants and young children, high titers of the G protein serum neutralising antibodies result in reduced RSV disease severity. As a result, the RSV-G protein induces clinically significant immune protection [21; 22].

1.3.9. F protein

The F protein is a 574 amino acid long class I fusion protein that has two fragments, F1 and F2 (Figure 2) [12; 17]. Furin cleavage occurs at amino acid positions 109 and 137 and results in the release of a p27 peptide, revealing the hydrophobic site at the F_1 fragment [23; 12]. Partial cleavage results in inactivated F proteins [24; 23]. The F_1 and F_2 fragments are linked by a cysteine-rich region that is found in two positions: between amino acid 70 and amino acid 212, and between 37 and 439. This region links together the F_1 and F_2 fragments (Figure 1.2). Furthermore, N-glycosylation occurs in the following amino acid positions: 27, 70, 116, 120, 126, and 500 [10]. The diversity of the F protein is extremely low, with only 25 (10%) amino acid differences and multiple antigenic sites [23; 12]. Since the F protein is the major focus of this study, more information related to the F protein will be discussed further in the next sections.

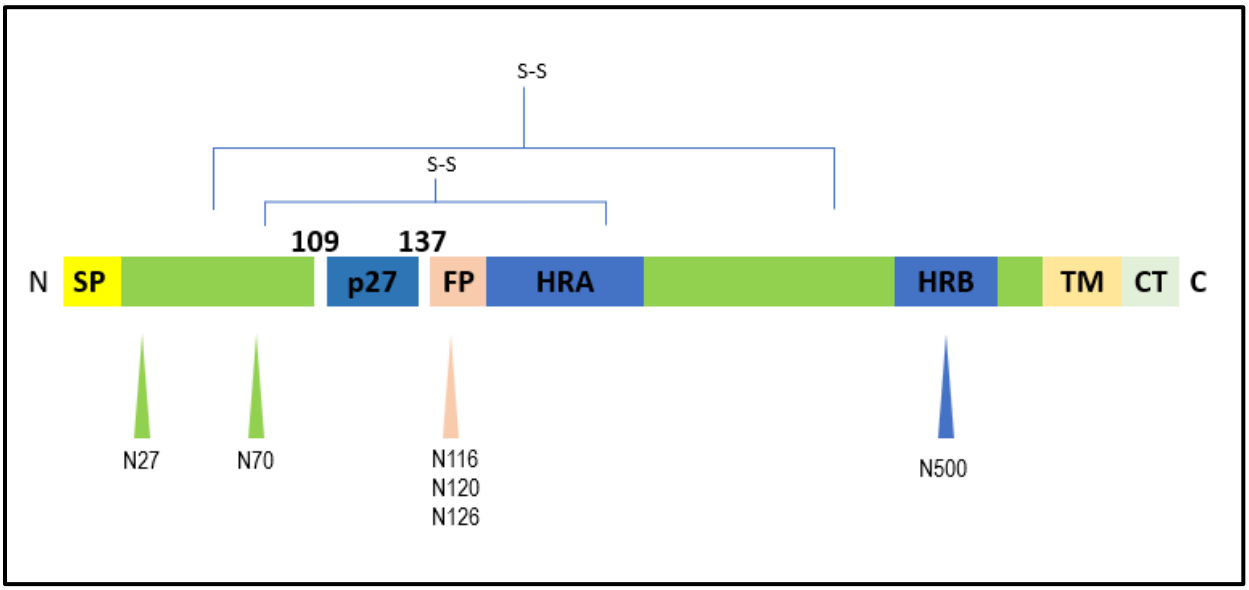


Figure 1.2. Structure of RSV F protein. The F protein is made of a cytoplasmic tail, the transmembrane domain, the two heptad-repeat regions (HR-A and HR-B), a fusion peptide (FP), and the SP. The furin cleavage sites (109 and 137) are indicated. Approximate positions of the N-glycosylation sites are shown by arrows, adapted from [16].

1.3.9.1. RSV F protein N-glycosylation

N-glycosylation occurs at the asparagine (N) residue. The F protein of RSV consists of up to 6 N-linked glycosylation sites (sequons) [16], with the F2 subunit consisting of two sequons at N27 and N70, which have slight impacts on the activity of the F protein. N500 occupies the F1 subunit in HR-B adjacent to the transmembrane domain, which is the only essential glycan for F protein functionality (Figure 1.2). The FP is occupied by 2 or 3 sequons at N116, N120, and N126 (Figure 1.2), depending on the RSV subtype [16]. The formation of N-glycan happens in the endoplasmic reticulum (ER), where a mannose core attaches to the N residues. Glycan maturation occurs in the Golgi apparatus, where these residues are replaced by more complex glycans such as N-acetyl-glucosamine and fucose. This process is essential for cell fusion [16]. Furthermore, it protects the protein against proteases. [25]. RSV genetic diversity affects the immunogenicity of surface

glycoproteins, which are defined by the profile of N-linked sequons in each strain [25]. It is assumed that N-linked glycans might shield up to 52% of the F protein surface, and the removal of specific glycans may enhance the induction of more potent neutralizing antibody responses [25].

1.3.9.2. RSV F protein conformations and antigenic sites

A vaccine must be able to induce the production of neutralising antibodies that target the F protein, which exists in 2 distinct conformations, namely, pre-fusion (pre-F) and post-fusion (post-F). Six F protein antigenic sites (Ø-V) have been identified, through monoclonal antibody (mAb) competition, structural studies of F protein-mAb complexes, and division of the surface of pre-F into six non-overlapping structural domains [26]. On the post-F surface, there are a total of 4 (sites I–IV). Meanwhile, antigenic sites found on the upper part of pre-F (sites Ø and V), have a significantly better antibody neutralisation potency than palivizumab, which targets site II [26].

The method of site-mAb isolation was critical because more mAbs were later isolated based on neutralization rather than binding. Therefore, facilitating the isolation of mAbs without existing pre-F constructs [27]. This subsequently led to the use of niservimab for stabilisation of pre-F conformation to produce stabilised pre-F candidate vaccines [27]. Furthermore, the site binds to other mAbs such as 5C4, AM22, and AM14, which are 100 times more neutralizing than palivizumab [12]. The antigenic site I was identified by the mouse mAb 131-2a. It is reported that mAbs binding to the site I are non-neutralising [27]. The MPE8, which neutralises several viruses of the *Pneumoviridae* family, mAb binds to antigenic site III. Additionally, mAbs specific to site III are elicited during the primary RSV infection in infants. This is supported by the isolation of ADI19425, from RSV infected infants. ADI19525 elicits potent neutralizing antibodies despite insufficient somatic hypermutation; it was co-crystallized with RSV pre-F protein. A mAb 101F binds to antigenic site IV and is found in both RSV F protein conformations. However, 101F cross-bind to the human metapneumovirus (hMPV) F protein. Additionally, a mouse mAb known as R4.C6, incorporating sites IV and II, has been isolated [27; 28].

Cryo-electron microscopic studies on the structure of the R4.C6 Fab-post-fusion F protein complex have shown that the antibody binds between sites II and IV [27]. Antigenic site V was described based on the isolation of a new stabilised pre-F construct [27]. The hRSV90 mAb binds to site V and was found to be competitive with mAbs that bind to site II and next to site Ø . Several site V-binding mAbs have been isolated from infants and adults. Therefore, highlighting the prevalence of these mAbs in the human anti-RSV repertoire. Other site V-specific mAbs include CR9501 and MC17, which cross-react with hMPV [27; 28].

Additionally, human histocompatibility leukocyte antigen (HLA)-restricted cytotoxic T lymphocyte (CTL) epitopes, including HLA-A*01, HLA-B*57, and HLA-Cw*12, have been identified on the RSV F protein [29]. The following evidence from pre-clinical studies that the stabilised pre-F protein has the ability to produce highly potent neutralising antibodies in animal models, the pre-F protein vaccine is in advanced clinical phases as a potential anti-RSV vaccine for use during pregnancy [12]. Structurally, the post-F is longer than the pre-F and can protect the pre-F protein from neutralising antibodies [30].

Metastable pre-F is essential for viral entry during infection and facilitates virus-host cell fusion, and stable post-F is adopted after cell membrane invasion [26]. Following the formation of pre-F, the hydrophobic site at the N-terminus of F1 anchors HR-A to the cell membrane [17]. When HR-A is attracted to HR-B neighboring the transmembrane domain, the virus combines with the host cell membrane, resulting in fusion and pore opening, which results in the incorporation of the viral genome into the host cell (Figure 1.3) [17].

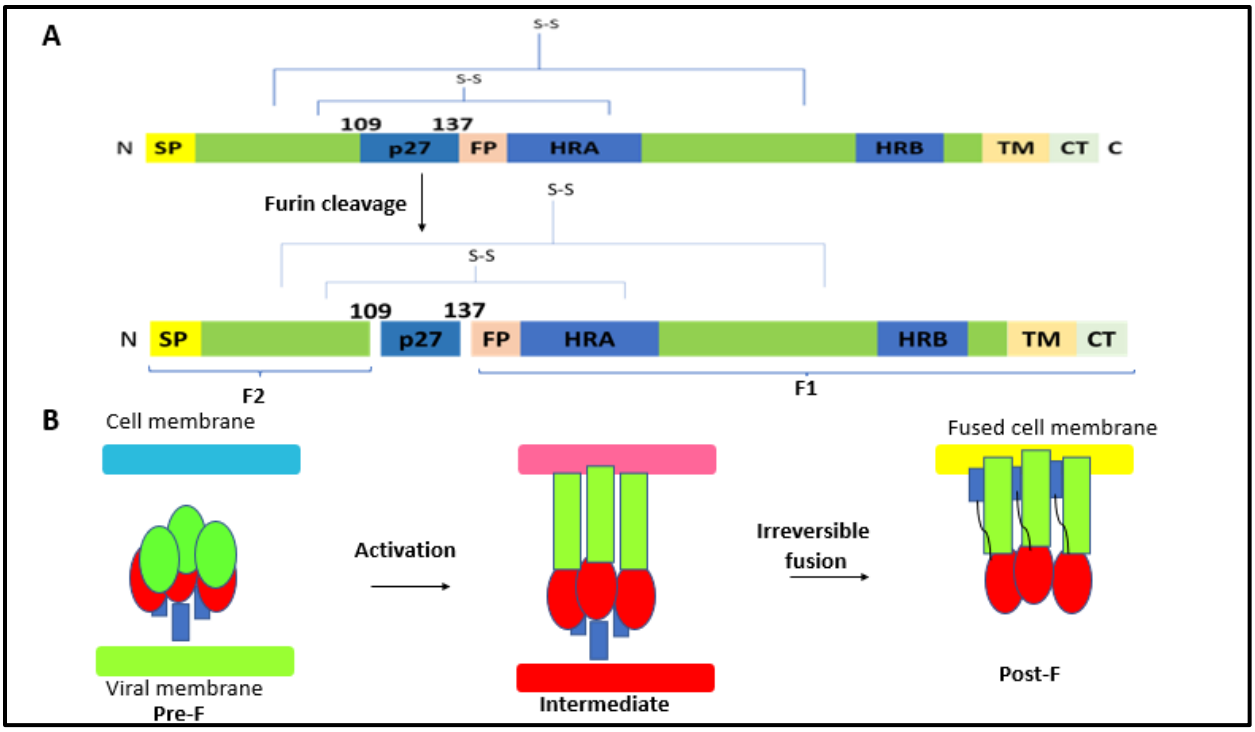


Figure 1.3. Activation and refolding of RSV pre-F into post-F conformation. (A) The biologically inactive F0 precursor is post-translationally cleaved by furin proteases at amino acids 109 and 137, to form two fragments (F2 and F1), resulting in the release of p27 peptide that separates C-terminus F1 and N-terminus F2. (B) Upon triggering, pre-F partially refolds and integrates its hydrophobic F peptide into the host cell membrane to form a pre-hairpin intermediate. The refolding of the pre-hairpin intermediate into the post-F conformation facilitates further viral F protein-host cell membrane fusion, adapted from [31].

Previously, most recent vaccine candidates and the historic FI-RSV vaccine contained the post-F conformation F protein [32]. Fortunately, understanding the atomic level structure of pre-F protein resulted in pre-F protein stabilization, allowing for the expression of a stable recombinant pre-F protein [33; 32]. This immunogenic subunit vaccine candidate, known as DS-Cav1, was tested in clinical trials (phase I) and provided a proof of concept [33; 32]. DS-Cav1 was tested in healthy adults, and a single dose induced a more than 10-fold boost in pre-existing neutralisation titers. This vaccine was well tolerated, and findings suggest commercial development of this vaccine development [33; 32].

1.4. RSV epidemiology

There is only one RSV serotype that is categorized into 2 subtypes, namely, RSV-A and RSV-B. Commonly, both subtypes circulate simultaneously; however, circulation may differ according to the geographic region [7; 34]. However, the RSV-A subtype is more frequent and transmissible than the RSV-B subtype [34]. Additionally, the subtypes are grouped into distinct genotypes based on the nucleotide sequence diversity in the G gene hypervariable region 2 (HRV2) [8]. Novel genotypes continue to emerge, resulting in a low prevalence and the extinction of other genotypes. Twenty RSV-A genotypes have been identified, GA1-7, SAA1-2, NA1, NA1-4, ON1-4, CB-A, and TN1-2 [8; 35]. More than 37 RSV-B genotypes have also been identified, including GB1-13, THB, BA1-14, BA-CCA, BA-CCB, SAB1-4, and URU1-2 [8; 36]. In 2017, the ON1 and BA9 genotypes were most prevalent in South Africa and globally [37; 21]. The global spread of these genotypes is the result of the immunologically naïve population [38].

The prevalence of the ON1 genotype varies geographically, and it may become a sole genotype of RSV-A in the next decade. The ON1 genotype is characterised by 72 nucleotide insertions in the C-terminus region of the G protein gene [21]. On the other hand, the BA9 genotype possesses 60 nucleotide duplications on the C-terminus of the G protein gene [38]. To better characterise prevalent and emerging RSV genotypes, constant surveillance of the clinical and molecular epidemiology of RSV genotypes is necessary.

1.4.1. RSV seasonality

The seasonality of RSV circulation varies across the world. In temperate regions, RSV circulates during winter and peaks between December and January, with the season offset being observed in late spring. On the one hand, in tropical regions, RSV circulation is observed during summer [12; 39]. In South Africa, RSV circulation differs across the provinces, with the onset usually in

KwaZulu-Natal in summer, Gauteng in autumn, and the Western Cape in March [40]. However, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) pandemic in 2020 has shifted the RSV circulation paradigm in South Africa. In October 2020, the National Institute for Communicable Diseases (NICD) of South Africa declared a resurgence of RSV infections [41]. In 2020, the detection of RSV during the RSV season declined significantly [41; 42]. The detection rate once again crossed the seasonal threshold in the middle of July of the same year. Furthermore, beginning in the third week of September, the detection rate increased to a moderate level, exceeding the 10-year average [41].

1.4.2. RSV disease burden

Yearly, approximately 34 million RSV-associated acute respiratory infections (ARIs) in children younger than 5 years cause 3 million hospitalisations worldwide. More than 90% hospitalisations and 99% of RSV related deaths occur in low to middle- income countries [37; 39]. The incidence of RSV-associated illnesses is predominant among infants aged below 5 months [39]. Despite high rates of ARIs on the African continent, there are limited studies of ARIs in the African population [39]. Hospitalization for RSV is common among very young infants in South Africa, suggesting that maternal antenatal vaccination or a birth dose of mAb may be beneficial in reducing disease severity [42]. HIV-exposed infants younger than 20 weeks are at higher risk of RSV-associated hospitalisation [43]. Buchwald et al., (2020) reported that acute LRTIs cause 8% of all deaths among South African children below the age of 5 years. RSV is the most common cause of acute LRTI in pregnant women with co-morbidities [39]. Nevertheless, there is insufficient information on the rate and severity of the disease, suggesting a need for more studies on RSV epidemiology. Most RSV studies that include pregnant women rely on case reports or secondary analyses of influenza vaccine trials. A recent study conducted in Houston, Texas, has found that over 33% of pregnant women presenting acute respiratory symptoms showed symptoms such as breathing difficulties and wheezing, and morbidity was linked to viral coinfections, including human rhinovirus (hRV), human coronaviruses (229E, NL63, OC43, and HKU1). Notably, the overall RSV infection rate according to polymerase chain reaction (PCR) tests among outpatient pregnant women was 10%, and 13% was confirmed through serologic tests [44].

Data from influenza vaccine trials are commonly used to investigate RSV epidemiology during pregnancy [43]. Two large maternal influenza vaccine trials that were conducted in Nepal and South Africa explored the incidence of RSV-associated maternal respiratory disease in pregnant women. The prevalence of RSV in the Nepal trial was 0.2% with an incidence of 3.9/1000 individuals. RSV prevalence was 2% in the South African trial, with an incidence of 14.4–48.0/1000 people [43]. No South African cases presented fever, and infection was not associated with an elevated risk of reduced birth weight or preterm birth in either Nepal or South Africa [43].

1.5. RSV transmission, replication, pathogenesis, and clinical manifestation

1.5.1. Transmission

Respiratory viruses differ in transmissibility and transmission modes. Therefore, it is crucial to understand different viral transmission modes to inform the effectiveness of non-pharmaceutical interventions in the population to mitigate transmission [44]. The basic reproduction number (R0) for RSV can be as low as 1, however, could rise from 8 to more than 10 [44]. RSV can be transmitted via direct contact when an infected person coughs or sneezes, indirect contact with nasopharyngeal secretions, fomites, and food from infected persons, or by contact with RSV contaminated surfaces [13; 44]. Disinfection with alcohol sanitisers and frequent washing of hands reduce the circulation of RSV infections, and hospital-acquired infections [13]. Additionally, Wong S-C et al., (2021) suggest that during this unprecedented coronavirus disease 2019 (COVID-19) era, universal masking is effective in reducing the shedding of nosocomial RSV and influenza A and B. This suggests that the masks may be effective in preventing RSV transmission between persons. Nonetheless, further investigation of universal masking in hospital settings is required [45].

1.5.2. RSV replication

The life cycle of RSV resembles that of all other paramyxoviruses [46]. Over more than the past decade, there has been progress in understanding the life cycle of RSV (Figure 1.4) [9]. This provides an opportunity for the development of robust diagnostics and effective vaccines, as well as therapeutics [9]. A detailed summary of the RSV replication life cycle is discussed below.

The development of therapeutics is strictly targeted toward receptors that interact with the virus [9]. Several host cell receptors for RSV attachment have been identified, namely annexin II, CX3 chemokine receptor 1 (CX3CR1), epidermal growth factor (EGF) receptor, calcium-dependent lectin, Toll-like receptor 4 (TLR4), intercellular adhesion molecule 1 (ICAM-1), nucleolin, and heparan sulfate proteoglycans (HSPGs) [9]. However, other receptors such as EGF are RSV strain specific [9]. Some of these receptors, such as annexin II, HSPGs, and C-type lectins, bind to the carbohydrate-rich regions of the F and G proteins [9]. The interaction of viral G protein with host cell receptors facilitates attachment and subsequently fusion of the viral envelope and host cell membrane via the RSV F protein, where RSV F switches from metastable pre-F to stable post-F conformation [47]. After viral fusion and entry, the viral nucleocapsids are released into the cytoplasm of the infected cell and used as templates for mRNA and genomic RNA (vRNA) synthesis by the viral RNA-dependent RNA polymerase [48]. Researchers suggest that these processes occur inside inclusion bodies, which are composed of nucleoprotein (N), phosphoprotein (P), and vRNA [48; 49]. Mutants lacking G and SH glycoproteins but containing them can mediate entry and infection. This feature shows that the F protein plays a critical role in viral attachment and entry, therefore highlighting the need for developing therapeutics targeting fusion protein [49]. After the assembly of newly manufactured nucleocapsids inside inclusion bodies, they are transported to assembly sites at the plasma membrane by cytoskeletons and eventually coalesce with other viral proteins [49]. This allows the transfer of infectious particles by forming syncytia to allow the transmission of multiple genomes between adjacent cells, thereby facilitating viral spread [49].

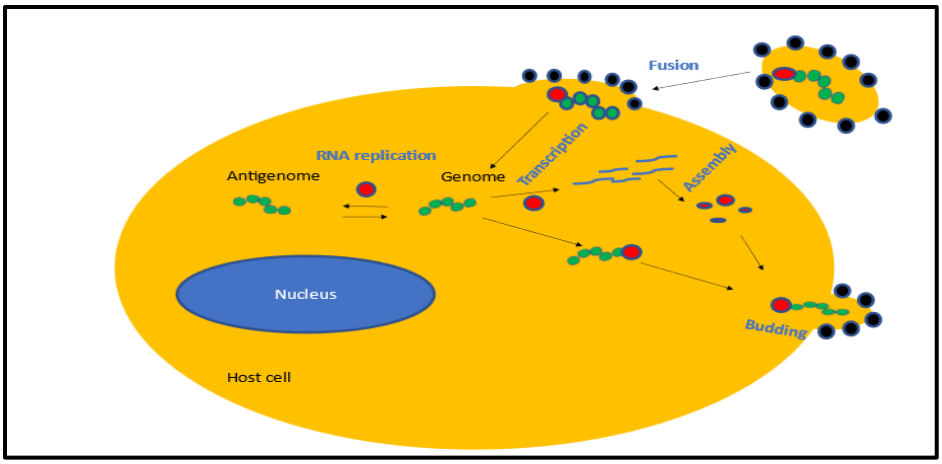


Figure 1.4. RSV life cycle. Firstly, viral-host cell attachment and fusion occurs when the virus enters the cell . The viral genome is released to start . Transcription occurs and it is followed by assembly and budding to generate new virions,adapted from [50].

1.5.3. RSV pathogenesis

RSV pathogenesis begins when RSV infects ciliated epithelial cells in the upper respiratory tract and eventually infects the bronchial epithelial cells [51]. *In vitro* culture studies have shown that RSV has a variety of cellular reservoirs that aid the virus in maintaining its pathogenicity, including the ability to infect intraepithelial dendritic cells (DCs) and basal epithelial cells [51]. One *in vitro* alveolar epithelial cell (AEC) model demonstrated that infection by RSV occurs more frequently in non-continuous cells. The following events occur during the progression of an RSV-induced infection: shedding of apical AECs, loss of cilia, formation of syncytia, and over-secretion of mucus, resulting in the formation of thick plugs in the lumen of the bronchioles in vivo.In addition, RSV has demonstrated the capability to infect and cause mucus secretion in basal cells rather than goblet cells [51]. RSV indirectly causes mucus secretion in the lumen of the bronchioles by inducing goblet cell proliferation [51].

RSV infection leads to the production of cytokines such as thymic stromal lymphopoietin (TSLP) and interleukin (IL)-33, which are essential in asthma development [51; 52]. Secretion of TSLP and IL-33 occurs after inflammation has occurred, leading to hypersecretion of mucus and immune cells such as eosinophils and neutrophils, T helper (Th), IL-5, and IL-13. In some viral infections,

the presence of these cells is related to the presence of web-like networks of neutrophil DNA called neutrophil extracellular traps (NETs), which are significant for trapping and eliminating pathogens [51]. It is important to note that more research on RSV biology and pathogenesis is necessary to guide vaccine development strategies [53].

1.5.4. Clinical manifestations

RSV infections cause mild or cold-like symptoms such as rhinorrhea, dry cough, wheezing, sneezing, and fever, however, these symptoms vary depending on age, the strength of the immune system, and medical history of infection with RSV [54-56]. These symptoms usually appear in stages, rather than simultaneously [7;57]. The incubation period is 3 to 5 days [45]. In new-borns, symptoms include reduced activity, irritability, and difficulties in breathing [57]. In some cases, severe symptoms such as bronchiolitis and pneumonia may be present. Infection of the lower respiratory tract occurs in up to 50% of children encountering RSV for the first time. In about 5–10% of infants admitted to hospitals, the disease is usually very severe and may require admission to the intensive care unit (ICU) [7].

1.6. RSV laboratory diagnosis

It is almost impossible to differentiate respiratory viruses clinically, as most demonstrate similar clinical signs and symptoms [44]. A correct and rapid diagnosis is necessary for the correct treatment and to prevent unnecessary antibiotic usage, prevent mortalities, and curb the spread of viruses to prevent epidemics or pandemics [58]. The laboratory diagnosis of RSV is achieved through tissue culture, serology, and molecular technology [59]. Very less sensitive, time-consuming, and labor-intensive methods such as tissue culture and immunofluorescence assay (IFA) have been reliable over the past decades. However, novel diagnostic methods with improved sensitivity, including nucleic acid amplification tests (NAAT) and point-of-care tests (POCT), have emerged [59]. The different RSV laboratory diagnostic techniques are outlined in more detail in the next page:

1.6.1. Tissue culture

The isolation of viruses in tissue culture on HeLa cells is a gold standard due to its specificity and the fact that the experiments occur in mammalian cell lines [59]. This technique relies on the detection of a syncytium in cells inoculated with RSV, which usually manifests within 3 to 7 days [59; 60]. However, because of its lower sensitivity and longer turnaround time, which results in missed RSV patients, it has been surpassed by other faster tests, such as reverse transcriptase polymerase chain reaction (RT-PCR) and some serology tests [55].

1.6.2. Serology

1.7.2.1. Enzyme-Linked Immunosorbent Assay and Immunofluorescence Assay

Enzyme-Linked Immunosorbent Assay (ELISA) and IFA are traditionally reliable serologic assays for RSV detection [59]. A modified, rapid, and inexpensive ELISA method directed against RSV F protein has been developed [59]. The IFA can be direct or indirect and use fluorescently-tagged primary or secondary antibodies to detect RSV antigens [59]. The direct IFA has a sensitivity and specificity of 94% and 97%, respectively, and it is widely used because it is simple and rapid [59]. Hence, it is used in low-income countries because it provides results within a short period and can help prevent a prolonged hospital stay due to delays in diagnostic test results [59].

1.6.2.2. Lateral flow immunoassay

Lateral flow immunoassay (LFIA) is a method that is used for the rapid detection of RSV from nasal washes and aspirates through immunochromatography [59; 61]. LFIA is performed over a strip, with different parts including the sample application pad, conjugate pad, nitrocellulose pad, and adsorption pad, all of which are assembled on plastic backing [62]. The positivity of the test is determined when the antigen-antibody complex forms a band showing the presence of an antigen in the sample [61].

1.6.3. Nucleic acid-based amplification tests

1.6.3.1. Reverse transcriptase polymerase chain reaction

RT-PCR has emerged as the new gold standard diagnostic technique owing to its robust sensitivity, specificity, and short turnaround time, resulting in faster treatment of patients [55; 61]. RT-PCR has been developed for both circle time and operability in adult infections and can detect the presence of viral infection in low viral load samples [59].

1.6.3.2. Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) is a rapid, specific, and sensitive TaqMan PCR method used primarily for detecting, subgrouping, and quantifying pathogens. RT-qPCR is more sensitive than conventional PCR and requires two primer-probe pairs, for the N gene and the F gene, targeting both RSV subtypes [59]. The TaqMan PCR assay is more accurate and sensitive than other techniques such as cell-culture-based assays and IFA [59].

On the other hand, locked nucleic acid-based one-tube nested real-time PCR is a highly sensitive and low cross-contamination incidence assay for RSV detection [59]. After sequencing the one-tube nested real-time PCR products, 143 nasopharyngeal aspirates that tested RSVnegative by RTqPCR tested RSV-positive [59; 63], indicating the importance of one-tube nested real-time PCR in clinical samples [59].

1.6.3.3. Rapid reverse-transcription recombinase-aided amplification

A rapid reverse-transcription recombinase-aided amplification test has been developed for the detection of RSV in clinical samples [59]. This assay uses an enzyme mixture, including ssDNA

binding protein, recombinase, and DNA polymerase, for the detection of RNA amplicons. It has high specificity and provides results in 30 minutes or less [59].

1.6.3.4. Reverse transcription strand invasion-based amplification

Furthermore, reverse transcription strand invasion-based amplification (RT-SIBA) has been developed (55). RT-SIBA has excellent sensitivity and can detect RSV rapidly. It can detect ten copies of RSV RNA in less than 20 minutes [59; 65]. Moreover, highly purified RNA is not necessary, which makes sample preparation easy and does not require highly purified RNA, for this reason, it increases the simplicity of sample preparation and shortens the detection period [59].

1.6.3.5. The Multiplex PCR

Multiplex real-time PCR allows for the detection of multiple viral infections at the same time from a single tube and 96 samples can be run simultaneously at a short turnaround time [59]. Several multiplex real-time PCR systems have been developed and are discussed below.

One of the novel multiplex real-time PCR systems is the Luminex NxTAG Respiratory Pathogen Panel. It has a high sample throughput and was upgraded from the xTAG Respiratory Viral Panel Fastv2 assay [55]. Its sensitivity and specificity are 98.9% and 99%, respectively, for the detection of adenovirus (hAdV) and hRV [59].

The Panther Fusion respiratory assay was also developed [59]. The Panther Fusion respiratory assay consists of 3 PCR panels, namely the influenza A and B/RSV panel, the parainfluenza virus (PIV) panel, and the hAdV/hMPV/RV panel [59; 66].

Fast Track Diagnostics Respiratory pathogens 21 (FTD-21) kit has been developed for 21 respiratory viruses [59]. It demonstrated a sensitivity and specificity of 90.7% and 100%, respectively, in a total of 665 samples [59].

A GeXP-based multiplex RT-PCR assay was developed for the detection of 16 different respiratory viruses [59]. It uses 17 sets of chimeric primers that can initiate amplification and two universal primers [59]. This test can reach a sensitivity of up to 200 copies for a single virus, and 1000 copies in the presence of all 16 viral targets [59].

The FilmArray multiplex PCR system can detect a total of 17 viruses, including RSV, and 2 bacterial pathogens [59]. This assay is rapid and generates results in 1 hour [59]. The test has a sensitivity of 86.4% and 100% for RSV-A and RSV-B, respectively, with 100% specificity for both subtypes [59].

1.7. RSV prevention and therapy

1.7.1. History of formalin-inactivated RSV vaccine

After the successful formalin-inactivated vaccine development against poliovirus, measles, and parainfluenza virus in the 1950s, studies on formalin-inactivated RSV (FI-RSV) were conducted within 10 years after RSV discovery [11]. From the preliminary studies of the FI-RSV vaccine, it was demonstrated that vaccinees developed antibodies with poor neutralising potency and had no FI-RSV-associated adverse effects for 10 days [11]. Although it appeared to be safe and effective, it resulted in deviant immunity to the natural virus, which caused more life-threatening pulmonary disease [11;68). Another different formulation was developed subsequently. However, it failed to enhance robust neutralising antibodies [11]. Some studies claim that the vaccine was not effective because it caused an allergic-like Th2 cellular immunity against RSV in the naïve vaccinees, which accelerated the infiltration of eosinophils and neutrophils and caused lung damage [67-69].

The RSV F protein is a major component of most recent vaccines in clinical development due to its conserved nature among RSV isolates [14]. Despite their conserved nature, recent studies have shown noticeable diversity across F proteins when historic and recent RSV strains were compared and suggested that these mutations affect the tertiary structure of the F protein, which may in turn affect antigenicity [29]. This may impact the effectiveness of vaccine-induced neutralizing antibody responses against recent RSV strains [29]. In 2015, the World Health Organisation (WHO) declared that several of those vaccines and mAbs are in advanced clinical phases and will possibly be on the market [70].

1.7.2. Challenges in RSV vaccine development

RSV prevention remains a global health priority after over 60 years of existence without a vaccine [71]. Failure to develop a safe and effective RSV vaccine is due to the following factors: young age, the peak of disease ability of a non-live virus vaccine to enhance RSV disease in young children aged 2-4 months who were immunized with FI-RSV vaccine plus alum adjuvant vaccine, failure to provide a robust and long-lasting immune response resulting in reinfections, and

expensive vaccine trials [11; 54]. In addition, severe RSV disease occurs early in life in children as young as 2–3 months of age, resulting in limited time for the implementation of national immunisation programs [11]. Infants' immune response to vaccination, is affected by the presence of high titers of immunoglobulin G (IgG) and a weak humoral response to RSV. The humoral response to RSV is weak in newborns, and high titers of IgG, tend to affect the infant's response to vaccination. Furthermore, severe disease is more likely to occur in immunocompromised and elderly people with comorbid conditions and immune deterioration, which may impair vaccine response [11].

Immunisation of the elderly may have different protective correlates. To prevent infections, humoral immunity may be targeted, while T cell-mediated immune responses are targeted to prevent severe disease [72]. The candidate RSV vaccines are likely to not trigger sterilising immune responses but prevent severe disease. This is because neither the natural infection nor the experimental infection can induce strong immunity against RSV reinfection [11]. Re-infection by the same RSV strain is possible, although reinfections result in less severe illness [72]. According to WHO, there is a high likelihood that vaccines and therapeutics will be available for public use within the next decade [4]. Additionally, several strategies have been implemented for the development of vaccines and mAbs targeting different population groups (Figure 1.5).

1.7.3. Current and future prevention strategies

1.7.3.1. Live-attenuated vaccines

A vaccine that provides a protective immune response in infants is desperately needed [73]. Live-attenuated vaccine (LAV) remains a very attractive platform for the prevention of infantile RSV infections, particularly in seronegative infants [11]. However, effective immunization has proven difficult in very young infants as young as three months [17]. The benefit of the LAV is that it is administered intranasally and triggers mucosal and systemic immunity in the presence of maternal antibodies, allowing immunisation of high-risk infants [11;74]. Another advantageous feature is that LAV has a proven track record of safety in seronegative infants [11]. In addition, LAV is not

linked to enhanced respiratory disease (ERD) [11;74]. However, immune responses in infancy are not sufficient, and it is not clear whether a pediatric RSV vaccine could prevent RSV disease [74]. Nevertheless, RSV-LAV is sufficiently attenuated for safety and has adequate virulence to trigger a long-lasting immune response. Additionally, there have been new and promising developments in RSV LAVs [11]. The existence of reverse genetics has resulted in the capacity to create new attenuation virus backbone mutations that result in ample attenuation to produce multi-phenotypic LAVs [11]. These advancements present a high probability for approval of replicating the vaccine for seronegative infants in the future [11]. However, potential reversion to wild-type RSV is a threat [11].

1.7.3.2. Monoclonal antibodies for neonatal protection

MAbs to prevent severe RSV diseases have been used for passive immunisation [72]. Even after the licensure of RSV vaccines, mAbs may still be used for prophylaxis in population groups that do not tolerate active immunisation, such as immunosuppressed individuals and pre-term infants with severe RSV disease and insufficient antibodies from mothers [72]. The drawback of this strategy is the fact that passive antibodies can protect against infection for as long as they circulate in the blood stream [72]. Palivizumab, which neutralises the F protein, is administered monthly during the RSV season. It is the first mAb approved by the Food and Drug Administration (FDA) to prevent severe disease in infants with co-morbidities [7]. Palivizumab reduces RSV-related hospitalizations by 50% when compared to placebo [75]. Palivizumab, due to its high cost, should only be used in high-risk infants in the first 24 weeks after birth, according to South African guidelines [40].

MAbs with higher neutralising potency and longer duration of protection are under development [72]. D25 which targets site Ø, is one of the mAbs being developed to protect infants against RSV infections for the duration of the RSV season with an extended half-life and a single intramuscular dose [72; 76]. Suptavumab (REGN2222) developed by Regeneron, was accelerated from phase 1

clinical trial to phase 3 by the FDA in 2015. Unfortunately, the trial did not meet the primary objectives to prevent infection by RSV in children younger than 5 months [72].

1.7.3.3. Maternal immunisation

Active immunization of mothers is an alternative method to administering mAbs at birth to newborns [17]. The role of maternal antibodies in RSV disease in young infants has been studied for many years [40]. Active transfer of IgG from mothers to fetal circulation via placental FcRn receptors occurs in the third trimester [45]. There was concern about an increase in RSV disease with the presence of maternal IgG antibodies in naive infants because maternal antibodies suppress the infants' immune response to RSV [44; 68]. However, it has been shown that infants with RSV disease have 2-4 lower RSV-specific titers compared to those without RSV disease [44]. RSV-specific serum antibodies were transferred from mothers to neonates via the placenta and protected the infants against LRTI during the first few months after birth [44]. One advantage of maternal immunisation is that it provides the infant with polyclonal antibodies and may also contribute to antibodies in breast milk [21]. As a result, breastfeeding may provide an added benefit to maternal immunization [68].

This approach aims to provide adequate neutralizing activity levels with one dose during the 2nd or 3rd of pregnancy [17]. South Africa participated in the multi-country clinical trial to assess the effectiveness of a nanoparticle RSV F vaccine (Novavax) based on the historic RSV-A Long strain. Unfortunately, the outcome of the clinical trial was disappointing in that it did not achieve the predetermined success measures [76]. Despite the poor effectiveness of the RSV F vaccine, it gave hope that the future implementation of an RSV vaccine is real. Ineffective vaccine-induced neutralizing antibody responses against recent RSV strains could be attributed to F protein drift from recent strains compared to the vaccine's F protein, which dates back to the 1960s [76]. GlaxoSmithKline (GSK) has developed an RSVPreF3 candidate vaccine using an engineered version of stabilised pre-F protein for use in young and older adults. After vaccination during the first trimester, the unadjuvanted RSVPreF3 candidate vaccine can provide a passive immune

response in young children. To combat the issue of waning immune response in the elderly, the RSVPreF3 vaccine has been adjuvanted with AS01, a liposome-based adjuvant consisting of 3-o-desacyl-4'-monophosphoryl lipid A and sapoin QS-21 that help to elicit a robust and long-lasting immune response against RSV infection [77].

1.7.3.4. Toddlers, older children and older adults

Despite the high burden of RSV disease in very young children and the elderly, there are still major public health costs linked to RSV disease in the elderly [11]. Immunization of older adults may reduce RSV circulation and the impact it has on young children and the elderly by indirectly affecting infants and older adults by decreasing viral shedding [11]. In 2020, 10 RSV vaccines for toddlers and older adults were in early clinical trial stages, such as the adenovirus-vector RSV vaccine in phase 2 clinical trial (Clinicaltrials.gov Identifier: NCT03303625) [11]. There are some difficulties in developing an RSV vaccine for older adults, including inadequately sensitive endpoints, a lack of immune correlates specific to the elderly, comorbid conditions, and extremely low and variable infection rates [11]. Because there is a lack of data indicating that the serum antibody repertoire in the elderly differs from that of adults, and cellular immunity appears to be weakened with age, the goal of vaccination of older adults is to strengthen T cell-mediated immunity [11].

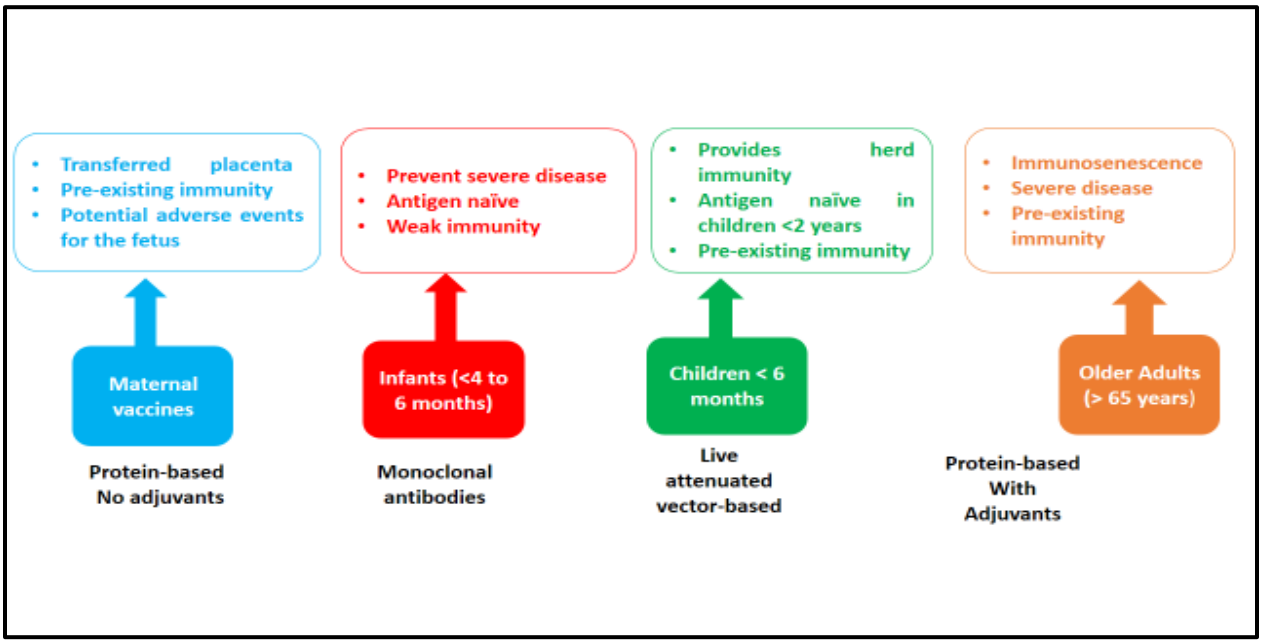


Figure 1.5. Summary of RSV vaccine types in development and clinical trials with target populations also indicated, adapted from [78].

1.7.4. RSV disease therapy

1.7.4.1. Antiviral therapy

Currently, there is no licensed antiviral drug to treat RSV disease; however, ribavirin, which has been previously used to treat other viral diseases such as hepatitis C and some hemorrhagic fevers, has been approved by the FDA for usage in RSV cases [7; 71]. Ribavirin is a guanosine analogue available in three formulations: oral, intravenous, and aerosol. Aerosolised ribavirin is recommended to treat severe RSV disease in children [11]. However, ribavirin is costly, and treatment is not assured [57; 71]. Furthermore, due to its toxicity, it is only used in severe cases in immunocompromised patients [70]. Currently, multiple antiviral agents are in trials, including presatovir (GS-5806), ziresovir (AK0529), lumicitabine (ALS-008176), JNJ-53718678, JNJ-64417184, and EDP-938, broad-spectrum antivirals such as favipiravir, VH244, remdesivir, and EIDD-1931/EIDD-2801; and host-directed strategies including nitazoxanide, eritoran, and

diltiazem [70; 74]. These antiviral drugs block various stages of the RSV life cycle, including entry, viral replication, polymerisation, and release [71].

1.7.4.2. Supportive therapy

Supportive therapy, including oxygenation, nasal suctioning, mechanical ventilation, and hydration, is the mainstay for bronchiolitis management [79; 80]. In nature, the disease is self-limiting (2-3 weeks). In cases of severe disease, hydration and feeding are recommended [79]. However, researchers discourage the use of nebulised hypertonic saline, nebulised epinephrine, and nasal suctioning in children. Inpatient and outpatient care both rely on nasal suctioning for bronchiolitis management [80]. One study revealed that the length of stay was long in infants who were subjected to deep suctioning or with long lapses of more than 4 hours between suctioning, which suggests that superficial suctioning may be the most effective and safest method [77]. Lack of oxygen is a distressing sign and requires hospitalisation [79; 81]. Therefore, oxygenation is a requisite for inpatient bronchiolitis management, despite the existing disagreements over when to implement and discontinue oxygen therapy [80]. Additionally, home oxygen therapy was reported to decrease the rate of hospitalisation and length of stay. Fluid support is fundamental in bronchiolitis management. Poor feeding is likely to result in worrying outcomes and hospitalisation. However, infants with adequate intake and milder symptoms only require close monitoring [79]. For infants who cannot sustain the required intake, parenteral fluid administration is recommended [79; 81].

1.8. Host immune response

1.8.1. Innate immunity

Infants without prior exposure solely rely on innate immune responses [82]. Early-life immune responses can be suppressive [12]. Because of the presence of maternal antibodies in the bloodstream and underdeveloped organs, infants' immune responses are known to be tolerant and

defective [12]. Although fetal and adult naïve Th cells are phenotypically similar, these cells differ in functionality, as fetal Th cells differentiate into T regulatory cells after induction, making an adaptive immune response a need in infants [12; 82]. This increases infants' susceptibility to infections in comparison to mature children [12]. Immunomodulation in early life is caused by both soluble and cellular mechanisms. Immunosuppressive plasma factors such as adenosine, and cytokines such as interleukin 1 receptor antagonist (IL-1RA), interleukin 4 (IL-4), IL-10, IL-11 and IL-13 weaken inflammation, as do T-regs which are found in higher numbers in newborns and preterm infants. T-regs secrete transforming growth factor beta molecules, which have modulatory effects on the function of natural killer cells [12; 82]. Failure of innate immunity causes activation of adaptive immunity following antigen presentation, usually by DCs to Th cells and B cells, resulting in viral clearance by cell-mediated and humoral immune responses [12].

1.8.2. Adaptive immunity

Likewise, the adaptive immune response has components that are fundamental for viral clearance or neutralisation such as CTL and Th cells and neutralising antibodies produced after infection [83]. However, this role is limited by the lower production of DCs in early life, which results in a weak immune response [12]. Following an RSV infection in early life, Th cells fail to differentiate into Th1 and Th2, indicating that immunomodulation can generate Th2-derived immunity [12; 82]. The CTLs are major participants in viral clearance following an infection. Factors such as severe combined immunodeficiency and transplantation of bone marrow and lungs result in mortality in RSV-infected individuals with reduced CTL levels [12]. Severe RSV infections occur in early life due to impaired CTL function, which could result in infantile deaths [12]. Like a cellular response, a humoral response is a challenge in early life. The significance of the humoral response to RSV in very young children manifests in some Fc-induced auxiliary processes, resulting in the production of neutralising antibodies that are essential for in reduction of infected cells, viral pathogenicity, and viral pathogenesis [12]. Therefore, decreased production of antibodies because of limited B cell maturation and differentiation into plasma cells contributes to the severity of the RSV disease [12].

1.9. Problem identification

For over 6 decades since RSV was discovered in 1956 and there is no vaccine to control infections by RSV and diseases, especially in vulnerable populations. For this reason, RSV remains a major public health burden. Acute respiratory infections, particularly pneumonia and bronchiolitis due to RSV, continue to be responsible for a considerable disease burden worldwide. Antibodies against RSV following a natural infection are only functional for a short period. Therefore, it is not sufficient to prevent re-infection. While palivizumab is used to prevent RSV infection in high-risk infants, the challenge remains that due to the high cost and need for well-timed administration, it may not be available to many who need it, particularly in developing countries, including South Africa. Failure of recent RSV vaccine candidates to induce protective immune responses may be linked to its inability to recognize the neutralizing epitopes in recent strains which had evolved to escape neutralizing antibodies induced by strains from the 1960s. Following the discovery that serum neutralising antibodies in RSV infected individuals target the F protein and maternal immunisation of MAbs that neutralise the F protein can reduce RSV-associated hospitalisations, the RSV F protein has in recent years become a subject in most of the RSV vaccine and therapeutics studies. Therefore, research aimed at discovering effective and safe vaccines and therapeutics are required. Despite the conserved nature of RSV strains, the use of F genes from more recent infections is advisable. This study was conducted to provide information on the characteristics of recent RSV F proteins.

CHAPTER 2

MATERIALS AND METHODS

2.1. Study design and sample size

The study included residual RSV-positive samples from patients who were admitted at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and their samples were submitted to the Department of Virology for respiratory virus diagnostic, National Health Sciences Laboratory Service (NHLS) from 01 July 2019 to 31 December 2020. The patients' clinical information, available on the NHLS TrakCare system and linked to virology laboratory test reports, was used. Sample size calculation was based on a 6% overall annual prevalence of RSV in South African and a minimum sample size of 87 per annum was estimated. A total of 70 residual RSV-positive samples with Ct values < 30, including nasopharyngeal swabs, nasal swabs, throat swabs, and sputum obtained from patients of all ages, were included for characterisation. The samples were stored in a -80ºC freezer for the preservation of viral RNA until further use**.**

2.2. Laboratory Methods

2.2.1. Viral RNA Extraction

The stored RSV-positive samples were retrieved from -80 storage and allowed to thaw at room temperature. Viral RNA was extracted using the Qiagen QiaAmp Viral RNA Mini Extraction Kit (Qiagen, Hilden, Germany) following our in-house protocol. In a 1.5 ml tube, 560µl of thawed lysis AVE buffer with lysis carrier RNA solution was added. Subsequently, a 200µl residual of the sample was added to the tube, followed by room temperature incubation for up to 10 minutes. The tube was filled with 560µl of absolute ethanol and vortexed thoroughly. A 600µl of the lysate was added into a column/assembly collection tube and followed by centrifugation for 1 minute at 8000

rpm. This step was repeated with the remaining amount of lysate. The column was transferred into a new collection tube and 500µl of AW1 buffer was added into the column, followed by centrifugation at 8000 rpm for 1 minute. A 500µl of AW2 was added to the column and centrifuged at 8000 rpm. Another 500µl of AW2 buffer was added and centrifuged at maximum speed for 3 minutes. The column was transferred into a new collection tube and centrifuged at maximum speed for 3 minutes. The column was added into 1.5ml tubes and elution was performed with 60µl of AVE buffer, followed by incubation for 2 minutes at room temperature. The tubes were centrifuged at 8000 rpm for 1 minute and caped. The Viral RNA extracts were stored at -80ºC for long-term storage.

2.2.2. One-Step RT-PCR

RSV F gene amplification was performed from 70 RNA extracts using the SuperScript™ III One-Step RT-PCR system (Invitrogen, Carlsbad, California) following our in-house protocol (Tables 2.1 and 2.2). All RT-PCR experiments were run on an Applied Biosystems 9700 (Life Technologies, California, USA) thermal cycler). RSV-A and RSV-B F gene-specific primers (Table 2.3) [84]. The cycling conditions for the RT-PCR for the RSV-A F gene are detailed below (Table 2.2). The cycling conditions for the RSV-B F gene were similar to those for the RSV-A F gene, except for the annealing temperature, which was set at 55˚C. Unsuccessful, RT-PCR amplification was repeated using a smaller volume (2µl) of the RNA template under the same cycling conditions to ensure that the RNA template concentration is low and that the *Thermus aquaticus* (Taq) polymerase when used first time is not inhibited due to carryover of inhibitors or insufficient denaturation.

2.2.3. Agarose gel electrophoresis

To assess the success of RT-PCR, a 1% agarose, Molecular Grade (Bioline, London, England), was prepared by dissolving 2g of agarose powder into 200 ml of 1×Tris-Acetate-EDTA (TAE)

buffer to examine the results of the RT-PCR. The agarose gel was stained with 1µl of a fluorescent dye called GelRed ® Nucleic Acid (Biotuim, Fremont, CA) before it was poured into a gel casting tray. The samples were prepared for loading into gel wells by mixing 5µl of the amplicons and 1µl of 6×loading dye (Thermo Fisher Scientific, USA). A 1kb DNA ladders were used (Promega Corporation, Madison, USA) as a reference. A 5µl of DNA ladder was added into the well, followed by 5µl of the amplicons/6× loading dye mix. The gel electrophoresis was run at 120V and 140 Amps for 60 minutes. The results were visualised under UV light using the Gel-doc system (Syngene, Frederick, USA).

Table 2.1. Reaction components and volumes used for the RT-PCR for RSV F gene amplification using SuperScript™ III One-Step RT-PCR system.

Component	**Volume (µl) per reaction**
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
2× reaction buffer	12.5
Nuclease free water	4.5
RT/Platinum Taq enzyme mix	1
Total reaction mix	**20**
RNA template	5
Total	**25**

Table 2.2. Cycling conditions for RSV-A F gene RT-PCR.

Step	Temperature (°C)	Time	No. of cycles
Reverse transcription	50	20 minutes	1
Initial denaturation	94	2 minutes	1
Denaturation Annealing Extension	94 48 68	20 seconds 30 seconds 3 minutes	40
Final extension	68	4 minutes	1
Hold	4	Hold	Infinite

Table 2.3. RSV-A and RSV-B F gene primer sequences used for RT-PCR RSV F gene amplification.

Primer	Target gene	*Position	Sequence	Size (bp)	Tm (°C)

Forward	RSV-A F	5648-5671	5'- GGG GCA AAT AAC AAT GGA GTT-3'	1771	50.45
Reverse		7394-7418	5'- CAT TGT AAG AAC ATG ATT AGG TGC T-3'		52.76
Forward	RSV-B F	5502-5524	5'- CGA AAA CAC ACC ACT CC ACA C-3'	1901	57.06
Reverse		7381-7402	5'- GTG GTT TTT TGT CTA TTT GCT G-3'		49.25

*Position refers to the region where the primer anneals on A2 strain (M74568.1) for RSV-A and wild-type B1 (AF013254.1) for RSV-B.

2.2.4. Next-generation sequencing

Next-generation sequencing was performed following the Nextera Flex protocol. PCR products were quantified using Qubit. Qubit working solution was prepared by diluting 0.25ml Qubit dsDNA HS reagent with 49.75ml Qubit dsDNA HS buffer in a 50ml tube. The mixture was vortexed to ensure a homogeneous working solution and stored in a dark environment until ready to use. The assay mix was prepared mixing 10µl standard (Standard 1 and 2) and 190µl working solution, 1µl of a sample, and 199µl working solution. The tubes were vortexed briefly and incubated at room temperature for 2 minutes prior to quantification. The standard 1 and 2 tubes were placed in the Qubit to automatically generate a standard curve from the readings. Readings for each sample tube were measured and recorded with the volume set at 1µl and units set at ng/µl. Using these Qubit readings, the concentrations for each PCR product were calculated and recorded. Fragment analysis was performed using the LabChip GX Touch- 384-well-plate. To perform tagmentation, 30µl DNA was added to each well of a 96-well plate. The bead-like transposomes (BLT) were vortexed for 10 seconds. The Tagmentation mastermix was prepared using details below (Table 2.4).

Table 2.4. Tagmentation mastermix.

Component	**Volume (µl) per sample**

BLT (bead-like transposomes)	11
Tagmentation buffer 1 (TB1)	11
Total	**22**

The mastermix was vortexed, and 20µl of the mastermix was added to each well that contained a sample. The mixture was resuspended by pipetting each sample 10 times. The plate was sealed, and the tagmentation was run on an Applied Biosystems 9700 (Life Technologies, California, USA) thermal cycler using the following conditions (Table 2.5).

Table 2.5. Tagmentation cycling conditions.

Temperature (°C)	**Time**	**No. of cycles**
55	15 min	1
10	Hold	Infinite

Ten microliters of tagmenation stop buffer (TSB) were added to the reaction. The beads were resuspended by pipetting each well 10 times. A post-tagmentation cleanup program was performed by running the tagmetation reaction for 15 minutes at 37°C followed by holding at 10°C on an Applied Biosystems 9700 (Life Technologies, Carlifonia, USA) thermal cycler. The plate was placed on a magnetic stand for 3 minutes until the liquid was clear and the supernatant was removed. The pellet was washed two times by removing the sample plate from the magnetic stand

and by slowly adding 100µl of tagmentation wash buffer (TWB) onto the beads until fully resuspended. A mastermix to amplify the tagemented DNA samples was prepared as follows (Table 2.6).

Table 2.6. Mastermix for amplification of tagmented DNA.

Component	**Volume (µl) per sample**
Enhanced PCR mix (EPM)	11
Nuclease-free water	11
Total	**22**

Forty microliters of PCR mastermix were pipetted into each well and mixed until the beads were completely resuspended. The plate was sealed and centrifuged at 280 × g for 3 seconds. Ten microliters of adapters were added to each well, mixed by pipetting 10 times, and centrifuged at 280 × g for 3 seconds. The plate was placed in an Applied Biosystems 9700 (Life Technologies, California, USA) thermal cycler using the following conditions (Table 2.7).

Table 2.7. BLT PCR amplificaton.

Temperature (°C)	Time	No. of cycles
68	3 minutes	1
98	3 minutes	1
98	45 seconds	8
62	30 seconds	
68	2 minutes	
68	1 minute	1
10	Hold	Infinite

Libraries were cleaned up using the following protocol: The plate was placed on a magnetic stand until the liquid was clear. A 45µl supernatant was transferred from each well to the corresponding

well on a new plate. The sample purification beads (SPB) were resuspended by vortexing and inverting several times, and 40µl nuclease-free water was added to each well. A 45µl SPB was added to each well and mixed by pipetting 10 times. Subsequently, the plate was incubated at room temperature for 5 minutes. The plate was placed on a magnetic stand for 5 minutes until the liquid was clear. A 15µl of thoroughly vortexed SPB (undiluted stock solution) was added to each well. A 125µl supernatant was transferred from each well of the initial plate into the corresponding wells of the new plate containing 15µl of undiluted SPB, mixed by pipetting 10 times, and incubated at room temperature for 5 minutes. The new plate was placed on the magnetic stand for 5 minutes until the liquid was clear, and the supernatant was carefully removed without disturbing the beads. The beads were washed twice by adding 200µl of freshly prepared 80% ethanol with the plate on the magnetic stand, followed by 30 seconds of incubation at room temperature. The supernatant was removed carefully without disturbing the beads. Residual ethanol was removed by using a 20µl pipette. The wells were air-dried on the magnetic stand for 5 minutes and then removed from the stand. Thirty-two microliters of resuspension buffer (RSB) were added to each well on the plate, resuspended by pipetting, and incubated for 2 minutes at room temperature. Subsequently, the plate was placed on a magnetic stand for 2 minutes, and the 30µl of supernatant was transferred onto a new plate. The DNA was quantified using the Qubit, and fragment lengths were determined using the LabChip as described previously. The libraries were normalised to 8 pM by diluting with an RSB buffer. A 5µl of the diluted sample library was transferred to an 8-strip tube and spun briefly using a minicentrifuge. The library samples were pooled from 8-strip tubes into a labelled Pool Amplicon Library 2ml tube. The libraries were denatured by preparing Sodium Hydroxide (NaOH) by combining 490µl nuclease-free water and 10µl stock 1.0N NaOH in a 1.5ml tube. The solution was mixed by inverting the tube several times. The library denaturation was performed by preparing 500µl of 0.2 NaOH by mixing 490µl of nuclease-free water and 10µl stock 1.0 N NaOH in a 1.5ml microcentrifuge tube. The tube was inverted several times to mix. A 5µl of 4nM sample DNA was mixed with 5µl of 0.2N NaOH. The remaining dilution of 0.2N NaOH was discarded, and the sample solution was mixed and centrifuged for 1 minute at a speed of 280×g, followed by incubation at room temperature for 5 minutes to denature the DNA. A 10µl of denatured DNA was mixed with 990µl of pre-chilled HT1. The denatured DNA was placed on ice until a final dilution was performed. The MiSeq run was prepared, and DNA samples were

sequenced. A post-run wash was performed as follows; a 10% wash solution was prepared by mixing 5ml 100% Tween 20 with 45ml nuclease-free water. To make a 0.5% Tween 20 wash solution, a 25ml solution of 10% Tween 20 was mixed with 475ml of nuclease-free water. The solution was mixed by inverting several times. The wash components were prepared by adding 6ml wash solution to each reservoir of the wash tray, and 350ml wash solution was added to the 500ml wash bottle. The FASTQ output data was stored for future analysis.

2.2.5. Sequence assembly and multiple sequence alignment

The open-source Genome Detective Virus Tool v2.27 was used to assemble sequencing reads generated in FASTQ format into contigs to extract the consensus sequence. Assembled sequence contigs were exported in FASTA format and imported into BioEdit v7.2 Sequence Alignment Editor to align the F gene sequences from this study, together with sequences retrieved from GenBank originating from various countries, including the United States of America (USA), Argentina (ARG), the Netherlands (NLD), Germany (DEU), Belgium (BEL), China (CHN), Vietnam (VNM), the Philippines (PHL), Kenya (KEN), and the Republic of South Africa (RSA). South African sequences from four 2021 strains, of which two were not deposited in any database, were also used (available on the Global Initiative on Sharing Avian Influenza Data (GISAID) database; EPI_ISL_14170477 and EPI_ISL_14170847). Reference sequences used for sequence analysis were the RSV-A2 long strain (accession numbers M74568.1 and KT992094.1) and the VR-26 strain (accession number AY911262.1).

2.2.6. Phylogenetic analysis

Multiple sequence alignments were submitted for phylogenetic tree construction using the MEGA X version 10.2 software [85]. The maximum likelihood method was used to construct a phylogenetic tree using the Hasegawa–Kishino–Yano (HKY) model [86]. The reliability of the phylogenetic tree was estimated using the bootstrap method with 500 replicates [87]. A cut-off of 60% was applied. This analysis involved 100 nucleotide sequences, each with 1716 positions in the final dataset, and all ambiguous positions were removed using the complete deletion method. As a root, the RSV-A2 long strain was used.

2.2.7. Amino Acid Variations at Antigenic Sites of the F Protein

The RSV F gene alignment in FASTA format was translated into amino acid sequences using BioEdit v7.2. To characterise the diversity of the F protein, amino acid variations on the F protein at antigenic sites Ø, and I-V were analysed. Sequences from this study were compared to South African sequences from 2018 and 2021 and global RSV F protein sequences. F protein sequence from the historical RSV-A2 long strain (accession number: M74568.1) was used as a reference.

2.2.8. N-Linked Glycosylation and Impact on Immune Epitopes

The HIV databases were used to determine the number and positions of N-linked glycosylation sites occurring at N residues. The consensus sequence to predict potential N-glycosylation sites was presented as the sequon NX(T/S)X, where N =asparagine, S = serine, T = threonine, and X represents any amino acid except proline [88]. Nine-mer cytotoxic T-lymphocytes (CTL) epitopes were predicted using NetTepi-1.0 software estimated from a combined score for binding affinity, peptide stability, and CTL propensity for the most prevalent HLA-A (HLA-A01:01, HLA-A02:01, HLA-A03:01, HLA-A11:01, HLA-A24:02, HLA-A26:01) and HLA-B (HLA- B07:02, HLA-B15:01, HLA-B27:05, HLA-B35:01, HLA-B39:01, HLA-B40:01, and HLA-B58:01) alleles [89; 90]. To obtain a global % rank score peptides were compared against 200 000 natural peptides

[89]. Strong binders were identified as peptides with a % rank of < 0.8% [91]. Similarly, the A2 Long strain was used as a reference against sequences from our study together with other South African sequences.

2.3. Cloning of RSV F gene

2.3.1. F gene Amplification

For cloning purposes, one-step RT-PCR was performed using primers with restriction sites for XbaI and XhoI (Table 2.8) [92] using SuperScript III One-Step RT-PCR system (Invitrogen, Carlsbad, California). The mastermix preparation procedure and PCR cycling conditions were similar to those in Table 2.1 and Table 2.2, except for the annealing temperature which was 58ºC. The amplicons were subjected to 1% agarose gel electrophoresis and visualised under UV transilluminator. A 1 kb DNA ladder (Promega Corporations, Madison, USA) was used as a reference for the expected positive 1.7 to 1.9 kb PCR products.

Table 2.8. Primers used for RT-PCR amplification of RSV F gene.

Primer	Sequence	Tm (°C)
Forward	5'-ATCTCCCCCGGGCTCGAG ACAATGGAGTTGCTAATCC-3'	70.0
Reverse	5'-GCATAAGAATGCGGCCGCTCTAGATTAGTTAGTAAATGCAATAT-3'	65.46

Highlighted in bold letters are XhoI and XbaI restriction sites. On forward primer the start of F coding region is underlined. On the reverse primer the end of F coding region is also underlined.

2.3.2. PCR product gel clean-up

Gel clean-up of the PCR product was done using the Wizard® SV Gel and PCR Clean-Up System (Promega Corporations, Madison, USA), following the manufacturer's instructions. A 1% agarose gel electrophoresis was performed and the DNA fragment corresponding to the amplified RSV F gene was excised using a sterile scalpel. Membrane binding solution was then added to the gel slice at a ratio of 10µl of solution per 10mg of agarose gel slice. The mixture was vortexed and incubated at 60°C until the gel slice was completely dissolved. The mixture was added to a SV minicolumn and centrifuged at 16,000 x g for 1 minute. The column was washed by adding 700µl of Membrane wash solution to the SV minicolumn and centrifuged for 1 minute at 16,000 x g). The column was rewashed with 500µl of Membrane Wash Solution and centrifuged for 5 minutes at 16,000 x g. DNA was eluted using 30µl of nuclease-free water applied directly to the centre of the column, incubated at room temperature for 1 minute and then centrifuged for 1 minute at 16,000 x g. The eluted gel purified product was stored at 4 °C. The DNA quality and concentration was determined using a nanophotometer (Implen, Munich, Germany).

2.3.3. A-Tailing

A-Tailing was performed in a 96-PCR plate to enable TA cloning of the plasmid vector and the purified PCR product (Table 2.9). The plate was incubated at 72°C for 30 minutes in a Applied Biosystems 9700 (Life Technologies, California, USA) thermal cycler.

Table 2.9. A-tailing of the PCR product.

Component	**Volume (µl)**
PCR product	15
10X PCR buffer	2
2mM dNTPs	2
Platinum™ Taq polymerase (3U/µl)	1
Total	**20**

2.3.4. Ligation of PCR product in pcDNA 3.4-TOPO vector (Linearized)

The purified RSV F gene was ligated into a pcDNA 3.4-TOPO TA- mammalian expression vector using the pcDNA 3.4-TOPO TA cloning kit (Invitrogen, Carlsbad, California) following manufacturer's instructions. To enable a 1:1 molar ratio of vector: insert in the ligation reaction, the reaction mixture was prepared (Table 2.10). The ligation mixture was mixed briefly and then incubated for 10 minutes at room temperature. After ligation the mixture was placed on ice for 10 minutes.

Table 2.10. Ligation reactions for F gene into pcDNA 3.4-TOPO vector.

Component	**Volume (µl) per reaction**
PCR product	3
Salt solution	1
Dilute Salt Solution	-
Nuclease-free water	1
TOPO vector	1
Total	**6**

2.3.5. Transformation

After the ligation process, One-Shot™ TOP10 chemically competent *E.coli* cells were transformed as described in the instruction manual. For each reaction, one vial of One-Shot™ TOP10 chemically competent *E.coli* cells were thawed per on ice. A 2µl of ligation mixture was added to the vial. For positive control purposes, 1µl of pUC19 control plasmid was added to another vial. The tubes were gently mixed by flicking the tubes. The tubes were incubated on ice for 30 minutes

and heat-shocked for 30 seconds in a water bath at 42°C without shaking. The tubes were immediately placed on ice and incubated for 2 minutes after which 250µl of room temperature super optimal broth (SOC) medium (Invitrogen, Carlsbad, California) was added to the tubes. The tubes were tightly capped and incubated for 1 hour at 37°C with shaking at 200 rpm. A 50µl of the transformation mixture was plated onto duplicate 100µg/ml ampicillin Luria Bertani (LB) plates. The plates were incubated overnight at 37°C. Single colonies were selected and inoculated into 5ml LB broth containing ampicillin. The mixture was incubated overnight at 37°C while shaking at the speed of 200 rpm.

2.3.6. Plasmid purification

Plasmid purification was performed using Qiagen Plasmid mini purification kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The bacterial cells were harvested by centrifugation at 6000 x g for 15 minutes at 4°C. The cells pellet was resuspended in 0,3ml of Buffer P1 that is mixed with RNase A. A 0.3ml of Buffer P2 was added and mixed thoroughly by inverting the tube 6 times. Subsequently, the tube was incubated at room temperature for 5 minutes. A 0.3 ml of chilled Buffer P3 was and mixed thoroughly by vigorously inverting 6 times followed by incubation on ice for 5 minutes to enhance precipitation. The tubes were centrifuged at maximum speed in a microcentrifuge for 10 minutes and the supernatants were removed in each tube. To equilibrate QIAGEN-tips 1ml Buffer QBT was applied, and the column was allowed to empty by gravity flow. The initially removed supernatant was applied to the QIAGEN-tip 20 and allowed to enter the resin by gravity flow. The tip was subsequently washed twice with 2ml Buffer QC and the Buffer QC was allowed to move through by gravity flow. To elute DNA 0.8ml Buffer QF was added directly to the center of the column using 1.5ml Eppendorf tube to collect the eluates. DNA was precipitated by adding 0.7 volumes (0.56ml per 0.8ml of elution volume) of room temperature isopropanol to the eluted DNA, with subsequent mixing and centrifugation at ≥15,000 x g rpm for 30 minutes. The DNA pellets were washed with 1ml of 70% ethanol and centrifuged at 15,000 x g for 10 minutes. the supernatants were carefully decanted without

disturbing the pellets. The pellets were air-dried for 10 minutes. DNA was dissolved in a 30µl of nuclease- free water. DNA quantification was performed using a nanophotometer (Implen, Munich, Germany).

2.3.7. Identification of positive clones

Double restriction enzyme digestion was performed to identify clones containing the RSV F gene using 1.5ml Eppendorf tubes. (Table 2.11). The tubes were incubated at 37°C for 1-4 hours. After restriction enzyme digestion, the digest was subjected to 0.6% agarose gel electrophoresis and visualised using a UV transilluminator. The purified plasmid DNA were subjected to PCR using RSV F gene primers designed for cloning, followed by 0.6% agarose gel electrophoresis.

Table 2.11. Double restriction enzyme digestion of purified plasmid DNA.

	Standard reaction	**Control reaction**
Component	**Amount**	**Amount**
Plasmid DNA	1µg	1µg
10X CutSmart buffer	5µl	5µl
XhoI	1µl	-
XbaI	1µl	-
Nuclease-free water	To 50µl	To 50µl
Total	**50**	**50**

2.3.8. Directional cloning

To optimise cloning, new RSV F gene primers without restriction sites were designed for directional cloning (Table 2.12). RT-PCR was performed as per previous protocol (Table 2.1 and Table 2.2); however, the annealing temperature was set at 55°C. A 1% agarose gel electrophoresis was performed to confirm successful amplification of the RSV F gene. The PCR product was cleaned-up as described previously. A-tailing, ligation, transformation, and plasmid purification

were performed as per previous protocols. Restriction enzyme digestion was performed using AgeI ad XbaI restriction enzymes that cut both ends of the pcDNA 3.4-TOPO vector**.** The purified plasmid DNA were subjected to PCR using the RSV F gene primers designed for directional cloning, followed by 0.6% agarose gel electrophoresis.

Table 12. Primers used for cloning optimisation.

Primer	**Sequence**	**Tm (°C)**
Forward	5'-CACCATGGAGTTGCYRATCCTYAAAHCAARTGC-3'	60.68
Reverse	5'-AAAHTTACTAAATGCAATATTATTTATACC-3'	49.32

2.3. Statistical analyses

Data analysis was performed using Statistica version 14.0.0.18. Descriptive statistics were presented as frequencies and proportions to summarise data distribution for demographic and clinical variables.

2.4. Ethics statement

The University of the Witwatersrand's Human Research Ethics Committee (Reference No. M201161) approved the study (Appendix IV).

CHAPTER 3

RESULTS

3.1. CHARACTERISATION OF RSV FUSION PROTEINS FROM SOUTH AFRICAN PATIENTS WITH RSV DISEASE, 2019 TO 2020

viruses

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Article

Characterisation of RSV Fusion Proteins from South African Patients with RSV Disease, 2019 to 2020

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Abstract: Respiratory syncytial virus (RSV) is classified into RSV-A and RSV-B, which are further classified into genotypes based on variability in the G gene. The fusion (F) protein is highly conserved; however, variability within antigenic sites has been reported. This study aimed to characterise F proteins from RSV strains detected in South Africa from 2019 to 2020. Patients of all ages, from whom respiratory samples were submitted to the National Health Laboratory Service at Charlotte Maxeke Johannesburg Academic Hospital, South Africa during 2019 to 2020, were included. Complete RSV F genes were amplified for next-generation sequencing. MEGA X software was used for phylogenetic analysis. The overall prevalence of RSV was 5.8% (101/1734). Among 101 RSV positive samples only 69.3% (70/101) were available for characterization of the RSV F protein gene. Among cases included for F gene characterisation, viral co-infections were observed in 50% (35/70) and 25.7% (18/70) were admitted to intensive care units (ICU). About 74.2% (23/31) of F gene sequences cluster with other African NA1/ON1 genotypes. At antigenic site I, the V384I mutation was replaced by V384T in South African strains. The S275F mutation was seen in a single South African strain. The N120 N-linked glycosylation site was present in 25.8% (8/31) of RSV-A F proteins described in this study. For the first time, we detected the rare S275F mutation that is associated with palivizumab resistance.

Keywords: RSV; respiratory; F protein; antigenic sites; palivizumab; South Africa

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1. Introduction

Respiratory syncytial virus (RSV) causes approximately 3.2 million severe acute respiratory tract infection (SARI)-associated hospitalisations, with overall mortality ranging between 55,000 and 200,000 annually [1,2]. Infection with RSV occurs in 60% of all children before they reach 1 year of age and nearly all children are infected by RSV at least once within 2 years after birth [3]. Over 90% of RSV-associated mortalities are exclusively reported in children less than 5 years of age from developing countries [1,2]. Premature infants and those born from HIV-positive mothers are at high risk of RSV infection, leading to high morbidity and mortality rates in these populations [4,5]. There is currently no licensed vaccine for RSV prevention [6]. Palivizumab (Synagis®) is the only prophylactic monoclonal antibody (mAb) treatment available for the prevention of RSV disease, but due to high cost its use is restricted to high-risk infants residing in low-, middle- and high-income countries [7–9].

The RSV genome encodes eleven proteins, including attachment (G) and fusion (F) glycoproteins which contain neutralising antibody epitopes capable of inducing a neutralising antibody response, and are targeted in RSV vaccine development strategies [1]. RSV is classified into subtypes A and B, which are characterised by different genotypes based on antigenic and genetic variability of the second hypervariable region (HVR2) of the G protein [1]. Among the two glycoproteins, F protein is the most conserved, with about

90% amino acid sequence identical in both RSV subtypes A and B [10]. Although the F protein is generally conserved, variability in some of the F domains has been observed in the signal peptide, transmembrane domain, and antigenic site Ø [10]. The F protein exists in two conformations (pre-F and post-F) and six antigenic sites (Ø, I to V) are defined. Sites Ø and V are found on the pre-F conformation and have significantly better antibody neutralisation potency than sites I–IV found on the post-F conformation [1]. Site II is the target for palivizumab-binding, site Ø is the target of MEDI8897; site IV is the target of MK-165412; and site V is the target of suptavumab [1]. A study conducted in China observed variability within the antigenic sites, with 56 amino acid differences between the RSV-A and RSV-B F protein [11]. A more recent study that included South African RSV strains from 2018 reported a total of 7 and 11 mutations, respectively, in antigenic sites of the RSV-A and RSV-B F protein sequences at frequencies ranging from 3.3% to 6.7% and 3.0% to 97.0% [1]. This highlights that variability among RSV strains is considerable, and that characterisation of local RSV strains' F proteins may contribute to a more strategic vaccine design or selection strategy for South Africa. The aim of the study was to characterise F proteins from South African RSV strains during 2019 to 2020.

2. Materials and Methods

2.1. Setting, Study Population and Sample Size

The study was conducted at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) in the Department of Virology, National Health Laboratory Services (NHLS) and the University of the Witwatersrand. The study population included patients of all ages, from whom respiratory samples were submitted for respiratory viral infection diagnosis from 16 July 2019 to 31 December 2020. For an expected 6% overall annual prevalence for RSV [12], a population size of about 11.5 million served by CMJAH and 95% confidence interval, a minimum sample size of 87 per annum was estimated [13].

2.2. Study Samples

Respiratory samples such as nasopharyngeal swabs, nasal swabs, throat swabs and sputum were tested for RSV and other respiratory viruses with the FastTrack Diagnostics (FTD, Junglinster, Luxembourg) 21-plex respiratory pathogen real-time multiplex PCR assay following total nucleic acid extraction from 200 µL of each sample on the automated MagNA Pure 96 extraction system (Roche Diagnostics, Meylan, France) using the MagNA Pure 96 DNA and Viral NA small volume kit [14]. Stored residual respiratory samples that tested positive for RSV were retrieved for genetic characterisation.

2.3. RSV F-Protein Gene Amplification

Viral RNA was extracted from 200µL of each respiratory sample using the QIAamp RNA mini kit (Qiagen, Hilden, Germany) and RNA was eluted in 60 µL of elution buffer. Five microliters of viral RNA from each sample were used to synthesise complementary DNA (cDNA) followed by amplification using the SuperScript III One-step RT-PCR System with Platinum *Taq* polymerase (Invitrogen, Carlsbad CA, USA). The following primers were used to amplify the RSV F protein gene, RSV-A Forward: A5648-S: 5′-GGG GCA AAT AAC AAT GGA GTT-3′(Tm 50.45) and reverse: A7418-AN: 5′-CAT TGT AAG AAC ATG ATT AGG TGC T-3 (Tm 52.76), RSV-B forward: B5502-S: 5′-CGA AAA CAC ACC ACT CCA CAC-3′ (Tm 57.06) and reverse: B7402-AN: 5′- GTG GTT TTT TGT CTA TTT GCT G-3′ (Tm 49.25) [15]. The cDNA synthesis was performed at 50 °C for 20 min. The initial denaturation was performed at 94 °C for 2 min. This was followed by 40 cycles PCR amplification at 94 °C for 20 s, 48 °C for 30 s, 68 °C for 3 min, followed by final extension at 68 °C for 4 min for RSV-A. The PCR amplification cycling conditions for RSV-B were similar to those for RSV-A, except for the annealing temperature, which was set at 55 °C.

Successfully amplified PCR products were visualised using 1% agarose gel electrophoresis (60 min at 120 V and 400 mA). A 1 kb DNA molecular weight marker (Promega,

Madison, USA) was used as a size marker to identify the F gene amplicons with the estimated sizes for RSV-A (1.7 kb) and RSV-B (1.9 kb) PCR products.

2.4. Next-Generation Sequencing

Library preparation was performed on F gene specific amplicons using the Nextera DNA prep kit (Illumina, San Diego, CA USA) as per manufacturer's protocol. Libraries were subsequently normalised to 8 pM and sequenced on the MiSeq system using the MiSeq V2 500 cycle sequencing reagents (Illumina, San Diego, CA USA).

2.5. Sequence Read Assemblies and Analysis

The open-source Genome Detective Virus Tool v2.27 [16] was used to assemble sequencing reads generated in FASTQ format to contigs to extract the consensus sequence. Assembled sequence contigs were exported in FASTA format and imported into BioEdit v7.2 Sequence Alignment Editor to align the F gene sequences from this study, together with sequences retrieved from GenBank [17] originating from various countries including the United States (USA), Argentina (ARG), the Netherlands (NLD), Germany (DEU), Belgium (BEL), China (CHN), Vietnam (VNM), Philippines (PHL), Kenya (KEN) and South Africa (RSA). South African sequences from four 2021 strains, of which two were not deposited on any database, were also used (available on the Global Initiative on Sharing Avian Influenza Data [GISAID] database; EPI_ISL_14170477 and EPI_ISL_14170847). Reference sequences used for sequence analysis were the RSV A2 Long strain (Accession number M74568.1 and KT992094.1) and VR-26 strain (Accession number AY911262.1).

2.6. Phylogenetic and Genetic Diversity Analysis

Multiple sequence alignments were submitted for phylogenetic tree construction using the MEGA X version 10.2 software [18]. The maximum likelihood method was used to construct a phylogenetic tree using the Hasegawa–Kishino–Yano (HKY) model [19]. The reliability of the phylogenetic tree was estimated by using the bootstrap method with 500 replicates [20]; a cut-off of 60% was applied. This analysis involved 100 nucleotide sequence, each sequence with 1716 positions in the final dataset, and all ambiguous positions were removed using complete deletion method. The RSV A2 Long strain was used as root.

2.7. Amino Acid Variation, N-Linked Glycosylation and Impact on Immune Epitopes

The BioEdit v7.2 Sequence Alignment editor was also used to translate nucleotide sequences to amino acids for variation analysis using A2 Long strain (Accession number: M74568.1) and sequences from various countries as references. The HIV Databases [21] were used to determine the number and positions of N-linked glycosylation sites occurring at Asn residues. The consensus sequence to predict potential N-glycosylation sites was presented as the sequon NX(T/S)X where N =Asn, S = Ser, T = Thr and X representing any amino acid except Pro. [22]. Nine-mer cytotoxic T-lymphocytes (CTL) epitopes were predicted using NetTepi-1.0 software [23] estimated from a combined score for binding affinity, peptide stability and CTL propensity for the most prevalent HLA-A (HLA-A01:01, HLA-A02:01, HLA-A03:01, HLA-A11:01, HLA-A24:02, HLA-A26:01) and HLA-B (HLA-B07:02, HLA-B15:01, HLA-B27:05, HLA-B35:01, HLA-B39:01, HLA-B40:01 and HLA-B58:01) alleles [24,25]. To obtain a global % rank score peptides were compared against 200 000 natural peptides [24]. Strong binders were identified as peptides with % rank of < 0.8% [26]. Similarly, the A2 Long strain was used as a reference against sequences from our study together with other South African sequences.

2.8. Statistical Analyses

Data analysis was performed using Statistica version 14.0.0.18. Descriptive statistics were presented as frequencies and proportions to summarise data distribution for demographic and clinical variables.

2.9. Ethics

The study was approved by the Human Research Ethics Committee (HREC) of the University of the Witwatersrand (M201161), Johannesburg, South Africa.

3. Results

3.1. Demographic Characteristics of Study Population

Between 16 July 2019 to 31 December 2020, 1734 respiratory samples were tested for respiratory viruses, of which 39.4% (684/1734) were from 2019 and 60.6% (1050/1734) from 2020. The overall prevalence of RSV was 5.8% (101/1734), of which 70 (69.3%) RSV positive cases with residual stored samples could be characterized genetically (Table 1). Males accounted for 48.6% (34/70) of cases, females for 42.9% (30/70) and data on gender classification was missing for 8.6% (6/70). Distribution of the patients by age group showed that most patients (75.7%; 53/70) were aged ≤2 years, (4/70; 5.7%) were >2 to 5 years, (2.9%; 2/70) were >5 years to <65 years old (Table 1).

Table 1. Demographic and clinical characteristics of the RSV-positive patients with available respiratory samples following diagnostic testing at the National Health Laboratory Service, Charlotte Maxeke Johannesburg Academic Hospital, Johannesburg, South Africa, 2019 to 2020.

Variable	2019 N (%)	2020 N (%)	Total N (%)
Age group			
≤2 years	13 (68.4)	40 (78.4)	53 (75.7)
>2 to 5 years	0 (0)	4 (7.8)	4 (5.7)
>5 to <65 years	2 (10.5)	0 (0)	2 (2.9)
≥65 years	0	0 (0)	0 (0)
Missing	4 (21.1)	7 (13.7)	11 (15.7)
Total	19 (100)	51 (100)	70 (100)
Gender			
Female	5 (26.3)	25 (49.0)	30 (42.9)
Male	11 (57.9)	23 (45.1)	34 (48.6)
Missing	3 (15.8)	3 (5.9)	6 (8.6)
Total	19 (100)	51 (100)	70 (100)
Ward			
[a]ICU	5 (26.3)	13 (25.5)	18 (25.7)
Non-ICU	12 (63.2)	36 (70.6)	48 (68.6)
Missing	2 (10.5)	2 (3.9)	4 (5.7)
Total	19 (100)	51 (100)	70 (100)
Viral co-infections			
0	11 (57.9)	24 (47.1)	35 (50)
1	5 (26.3)	15 (29.4)	20 (28.6)
2	2 (10.5)	8 (15.7)	10 (14.3))
≥3	1 (5.3)	4 (7.8)	5 (7.1)
Total	19 (100)	51 (100)	70 (100)

[a]ICU: Intensive care unit.

3.2. Other Respiratory Virus Infections

Among the 70 RSV-positive cases included for genetic characterisation, co-infection with other respiratory viruses were observed in 35 (50.0%) (Table 1). Most 28.6%; 20/70) were co-infected with one respiratory virus, 14.3% (10/70) were co-infected with two respiratory viruses and 7.1% (5/70) were co-infected with three or more respiratory viruses. Admission to intensive care units (ICU) were reported for 18/70 (25.7%) cases. Among ICU admissions, 38.9% (7/18) of cases were co-infected with another respiratory virus. Rhinovirus was the most frequently detected co-infecting virus (57.1%; 4/7), followed by human adenovirus (42,9%; 3/7), enterovirus (28,6%; 2/7), and bocavirus at (14.3%; 1/7).

3.3. Phylogenetic Analysis of RSV F Genes

F gene sequence data were generated for (91.2%; 31/34) samples and only RSV-A strains were identified in 2019–2020. Maximum likelihood tree analysis was conducted and the majority (74.2%; 23/31) of sequences from this study clustered together with bootstrap values ranging from 64% to 99% (Figure 1). Meanwhile, other sequences clustered with other South African sequences from 2018 and 2021 that were NA1-like. It should be noted the genotype assignment was based on G protein gene genotyping, and ON1 genotypes fall within the NA1 group (Figure 1). Strains previously genotyped as GA2 had F genes that formed two strongly supported sub-clusters within the main NA-1 cluster and these included strains from Kenya sampled in 2011 to 2015 (Figure 1). We did not see close clustering of South African RSV F protein genes with sequences from other countries. The RSV F gene sequences were uploaded on the GISAID database and accession numbers for download by registered users are provided (Supplementary Table S1).

3.4. Amino Acid Variations at Antigenic Sites of the F Protein

Ten amino acid changes were detected in four antigenic sites (I, II, IV and V) when compared with global reference sequences from 2001 to 2021, with frequencies ranging from 0.3 to 100% (Table 2). Five amino acid changes were detected for the majority of sequences at high frequencies at the following positions: I379V (100%; 329/329) and V384I (90.8%; 299/329) at antigenic site I, N276S (20.1%; 66/329) at antigenic site II, M447V (100%; 329/329) at antigenic site IV and V152I (93%; 307/329) and L178V (100%; 329/329) at antigenic site V. V152I amino acid change was prevalent in RSV-A strains from all countries. N276S was prevalent among South African sequences from this study at a frequency of (94.3%; 51/54). Amino acid change V384T (7.3%; 24/329) at antigenic site I and S275F (0.3%; 1/329) at antigenic site II were exclusive to South African sequences. No amino acid changes were detected at site Ø and III (Table 2).

Table 2. Frequency of mutations within the RSV F protein antigenic sites in South African and strains from other countries (N = 329).

Antigenic Site	Positions	Mutation	[a] Frequency of Detection, n (%)	* Countries Where Detected
I	27–45; 312–318; 378–389	***Y33H***	***1 (0.3)***	RSA
		I379V	329 (100)	RSA, USA, NLD, ARG, DEU, CHN, PHL, VNM, BEL, KEN
		V384I	299 (90.8)	RSA, USA, NLD, ARG, DEU, CHN, PHL, VNM, BEL, KEN
		V384T	***24 (7.3)***	RSA
II	254–277	***S275F***	***1 (0.3)***	RSA
		N276S	***66 (20.1)***	RSA, NLD, CHN, PHL, VNM, KEN
IV	422–471	M447V	329 (100)	RSA, USA, NLD, ARG, DEU, CHN, PHL, VNM, BEL, KEN
V	55–61; 146–194; 287–300	V152I	307 (93)	RSA, USA, NLD, ARG, DEU, CHN, PHL, VNM, BEL, KEN
		L178V	329 (100)	RSA, USA, NLD, ARG, DEU, CHN, PHL, VNM, BEL, KEN

[a] Amino acid changes detected at low frequency are indicated in bold-italics. * RSA: Republic of South Africa is underlined; USA: United States of America; ARG: Argentina; NLD: The Netherlands; DEU: Germany; PHL: Philippines; VNM: Vietnam; CHN: China; BEL: Belgium; KEN: Kenya.

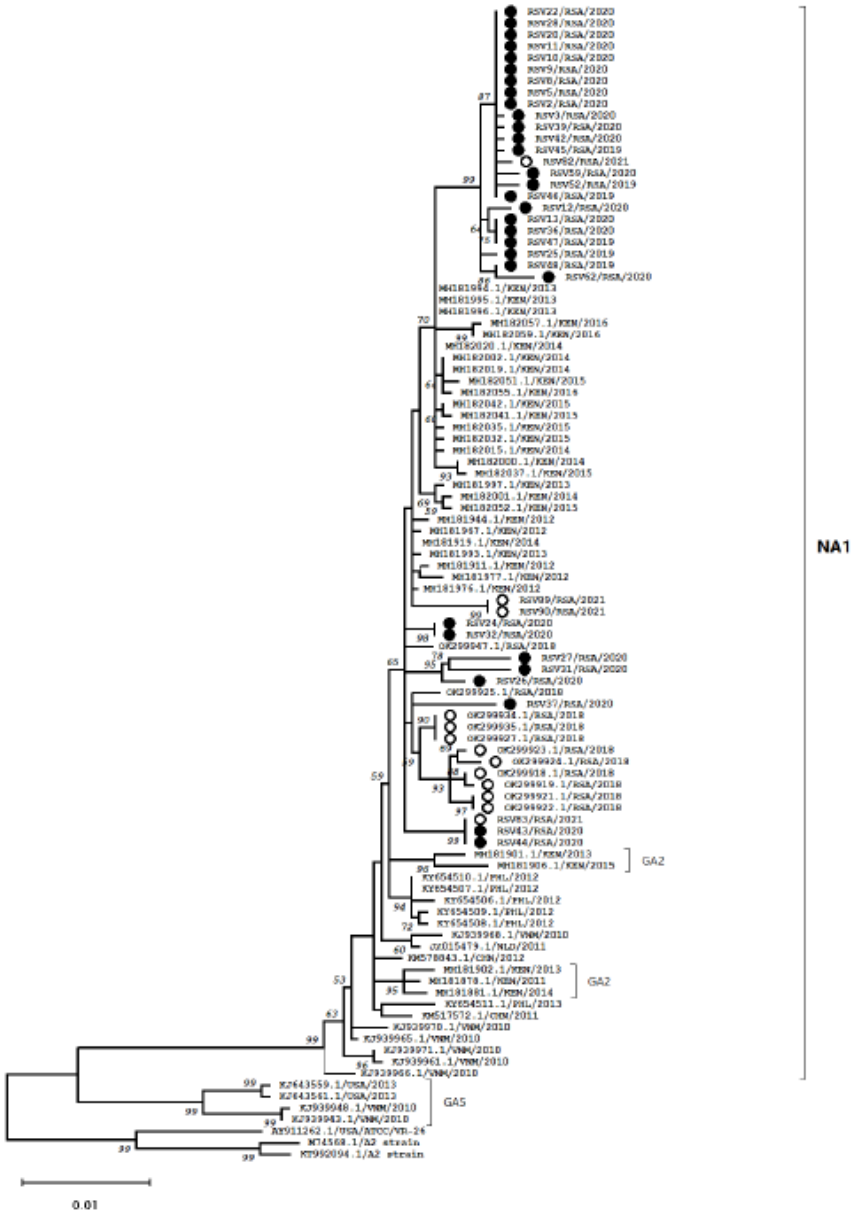


Figure 1. Maximum-likelihood phylogenetic tree based on the 1716 bp long F gene sequences of RSV-A strains from South Africa that circulated in 2019 and 2020 (filled black circles). Reference South African strains are indicated by open black circles. A2 Long strains were used as root. Genotype classification used are based on G protein gene sequence genotypes assigned to reference sequences.

3.5. N-Linked Glycosylation and CTL Epitope Predictions among Deduced F Proteins

A total of 6 N-glycosylation sites (N27, N70, N116, N120, N126 and N500) were predicted and similar between our sequences and reference sequences. Five N-glycosylation sites were predicted at high frequency at positions N27, N70, N116, N126 and N500 (Figure 2). Gain of the potential glycosylation site at position 120 was identified in 25.8% (8/31) of 2020 sequences from this study (Figure 2). This gain was also observed in South African reference sequences from 2018 and 2021 at frequencies of 100% (19/19) and 25.0% (1/4), respectively.

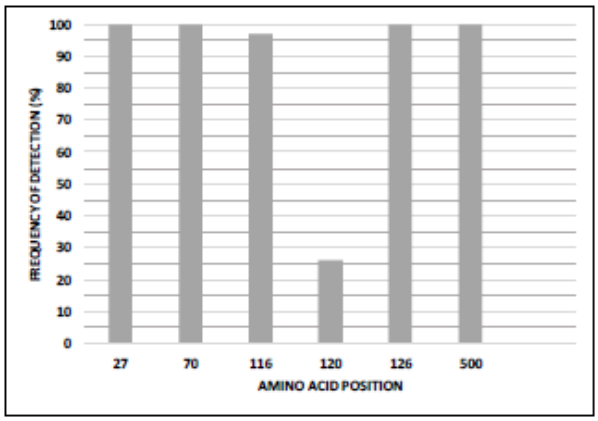


Figure 2. A graphic representation of N-glycosylation site positions across the South African RSV F protein sequences and the frequency of detection of NX(T/S)X sequons, 2019 to 2020.

The following mutations were observed in the RSV F protein signal peptide sequences: A8T, T16A/I, T12I/A and F20L. The V152I cytotoxic T lymphocyte (CTL) escape mutation in epitope SAIASGVAV restricted by HLA-B07:02 was observed in 2018 to 2021 strains from South Africa (Table 3). The Y33H escape mutations were seen in epitopes FYQSTCSA and GQNITEEFY restricted by HLA-A24:01 and HLA-B15:01, respectively. The S275F mutation was located in the HLA-A11:01 restricted epitope MSNNVQIVR at antigenic site II. CTL escape mutation K123Q in the MNYTLNNAK epitope (restricted by HLA-A03:01; HLA-A11:01) among strains from 2019 and 2020 was associated with a reversion (T > A) mutation at position 122 (Table 3). Among South African strains from 2018, an A23T mutation was seen in the LTAVTFCFA epitope; however, this mutation reverted in almost all South African strains from 2019 to 2020.

Table 3. CTL escape mutations in predicted RSV F epitopes.

Position	HLA Restriction	* Peptide (Amino Acid Change Position in Bold)	Amino Acid Change and Frequency (%)	Detected in South African Strains
25–33	HLA-B15:01	GQNITEEFY	G25S (100); Y33H (1.85)	2018
32–40	HLA-A24:01	FYQSTCSAV	Y33H (1.85)	2018
114–122	HLA-A02:01	FMNYTLNNA	A122T (51.9)	2018, 2020, 2021
115–123	HLA-A03:01; HLA-A11:01	MNYTLNNAK	K123Q (55.5)	2019, 2020, 2021
133–141	HLA-B27:05	RKRRFLVFL	V139G (100)	2018, 2019, 2020, 2021
146–154	HLA-B07:02	SAIASGVAV	V152I (100)	2018, 2019, 2020, 2021
274–282	HLA-A11:01	MSNNVQIVR	S275F (1.85)	2019

* A2 strain sequence was used as reference.

4. Discussion

The F protein of RSV is a key immune response determinant and target for antiviral and vaccine development, due to its conserved nature when compared to the G surface glycoprotein. Several mutations have been observed in the F gene between different RSV geographic strains [11,15]. This study was one of the few studies in South Africa to report on the molecular epidemiology of the RSV F protein. In this study, conducted from 16 July 2019 to 31 December 2020, we mainly identified F gene sequences characterised as belonging to the RSV-A subtype. This may be due to the predominance of RSV-A strains in South Africa during this period. Both RSV-A and RSV-B have been found to co-circulate within seasonal epidemics in South Africa [27]. Respiratory virus co-infections and ICU admissions were common among infants that were predominantly ≤2 years of age, accounting for 33.3% of co-infections. The influence of RSV on ICU admissions is inconclusive, as few studies found that co-infections increase ICU admissions, while others report a decrease in ICU admissions in the presence of co-infections [28–30].

Most candidate vaccines for RSV are based on the historical RSV A2 Long strain, which were used in this study as a reference [10]. Phylogenetic analysis showed that F gene sequences from this study are similar to South African strains from 2018 and Kenyan strains that were genotyped mainly as NA1 or ON1. The South African strains from 2018 were also genotyped as ON1 [27]. The characteristic 72-nucleotide duplication in the HVR2 of G protein of ON1 is suggested to increase viral replication, which thus contributed to its current widespread dominance [1].

Overall, the diversity of RSV F protein in South African strains was extremely low, with 5.3% positions with variations. Antigenic sites Ø and V are prone to mutations because they elicit high potency of neutralising antibodies [27]. However, no changes were observed at antigenic site Ø. Antigenic site V had two highly frequent amino acid changes, V152I and L178V. These changes were detected in strains from all featured countries. In contrast, antigenic site I elicited low potency neutralising antibodies and was previously reported as unlikely to be prone to mutations [31]. However, in this study the highest number of mutations were found in antigenic site I. In South African sequences, the wild-type amino acid change V384I in antigenic site I was replaced by the V384T mutation. Amino acid change N276S at antigenic site II has been reported to affect binding. The high frequency of N276S in antigenic site II was similar to what was observed among South African sequences from 2018 and, in addition, we also observed the S275F mutation among South African strains from this study. The N276S mutation at antigenic site II is located in the binding site for palivizumab. This highly prevalent N276S mutation is not associated with palivizumab resistance [15]. However, here we report for the first time the rare S275F mutation which is one of four single amino acid mutations that cause palivizumab resistance in vitro [32]. Therefore, there is a need to monitor the prevalence of these naturally resistant RSV strains through continued genomic surveillance to inform therapy and vaccine strategies.

We confirm that both the position and number of N-glycosylation sites across the F protein are highly conserved, suggestive of the importance of these glycosylation sites on the folding and biological activity of the F protein [33]. However, the importance of N-glycosylation for RSV F protein functionality is still unclear, although it was indicated that it may impact fusion activity [33,34]. The ability of a virus to change N-glycosylation patterns is one of the mechanisms for immune response evasion [5,35]. In this study, we show loss of the NXT site at position N120 in South African RSV-A strains detected after 2018, whereas the same was also reported for RSV strains from China sampled from 2003 to 2014 [36]. This may indicate that antibody-based immune selection pressure may be a key driver of evolution of F in this region, although the role of CTL pressure cannot be overruled.

Several amino acid mutations occurred in the signal peptide region of the F proteins. Mutations in almost all of these signal peptide positions were described previously, however, its impact on F protein expression and functionality were not discussed [10]. The concomitant reversion observed at position 122 with the escape mutation at position 123

may indicate the importance of these positions for maintaining functionality of the fusion peptide. These fusion peptide mutations are also thought to be associated with the helix structure which forms the antibody binding pocket for the pre-F D25 monoclonal antibody [37].

In conclusion, we show that despite the conserved nature of the RSV F protein among South African strains, amino acid changes potentially associated with CTL and antibody escape were identified. This confirms that continued immune adaptation is responsible for the evolution of the RSV F protein genes.

Supplementary Materials: The RSV F gene sequences were uploaded on the GISAID database and are available online at https://www.mdpi.com/article/10.3390/v14112321/s1, Table S1: Summary of F gene sequence data from this study that are available on the GISAID database.

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Informed Consent Statement: Participants were tested for RSV as part of clinical management and additional informed consent for this laboratory based study was not required. Approval to conduct the study was also obtained from the Charlotte Maxeke Johannesburg Academic Hospital, Gauteng Department of Health before ethical approval was granted by the University of the Witwatersrand HREC. Study samples and data (without patient identifiers) were retrieved in adherence to the National Health Laboratory Service's Confidentiality Agreement Policy which include registration of the project on their Academic Affairs and Research Management System.

Data Availability Statement: Sequence data are available on GISAID (https://gisaid.org/).

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3.2. CLONING OF RSV F GENE

3.2.1. One-Step RT-PCR of RSV F gene amplification

One-step RT-PCR was performed using RSV F gene specific primers with restriction sites for XbaI and XhoI as described previously. Samples (sample 2, 3, and 4) that were successfully amplified for genetic characterisation were selected for RT-PCR and cloning. A 1% agarose electrophoresis was performed to analyse the results of the RT-PCR amplification (Figure 3.1A). After successful RT-PCR amplification one PCR product (Sample 2) was cleaned-up from the gel and used cloning (Figure 3.1B).

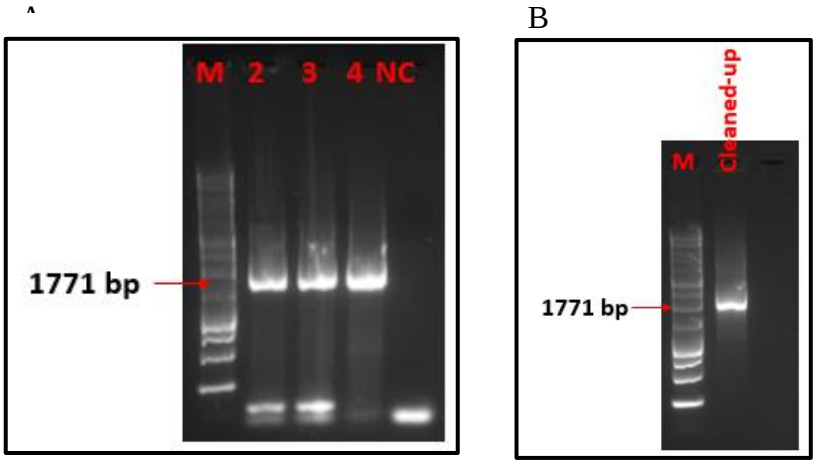


Figure 3.1. (A) A 1% agarose gel picture for RT-PCR using RSV F gene primers with restriction sites. (B) A 1% agarose gel picture for the cleaned-up PCR product.

3.2.2. Confirmation of positive clones

Only two colonies were observed on the ampicillin selective plate. Double restriction digestion of the plasmid DNA, PCR using RSV F gene primers with restriction sites and 0.6% agarose gel

electrophoresis of the digested and undigested plasmid DNA did not show any presence of the RSV F gene in the clones (Figure 3.2).

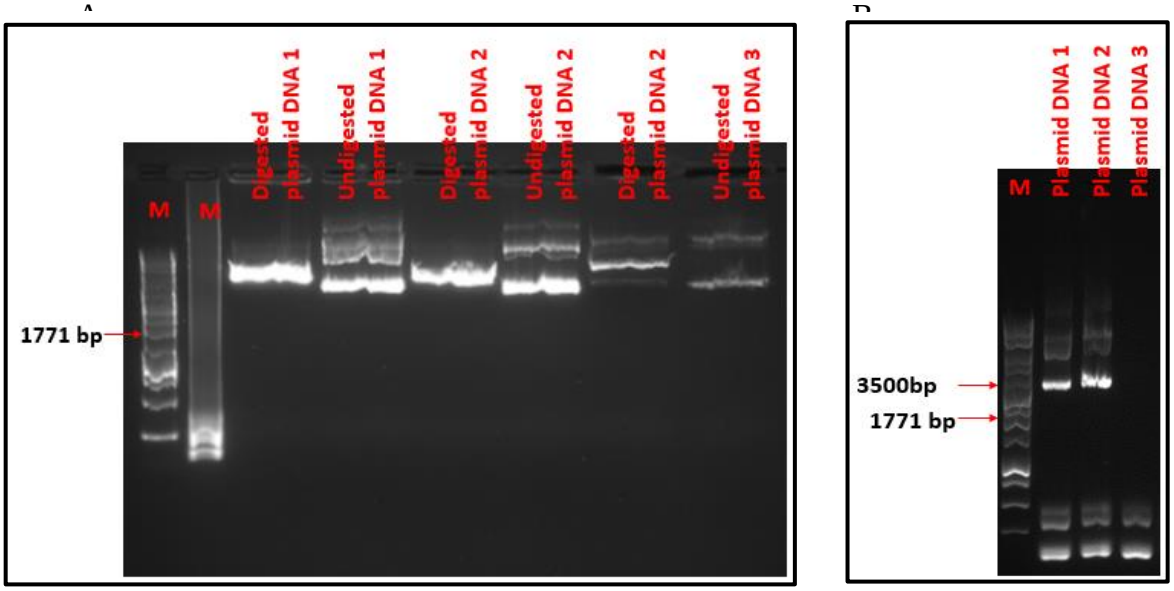


Figure 3.2. (A) A 0.6% agarose gel electrophoresis picture for double restriction digestion of the purified plasmid DNA. (B) A 0.6% agarose gel electrophoresis picture for PCR amplification of RSV F gene from the purified plasmid DNA.

Directional TA cloning

Thirteen colonies were picked from ampicillin selective plates. Likewise, double restriction digestion of the plasmid DNA, and 0.6% agarose gel electrophoresis of the digested and undigested plasmid DNA did not show any presence of the RSV F gene in the clones (Figure 3.3 and Figure 3.4).

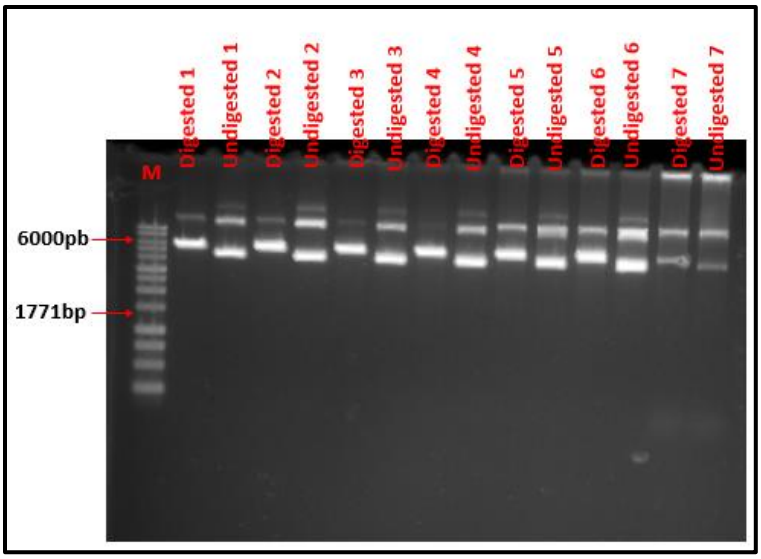


Figure 3.3 A 0.6% agarose gel electrophoresis picture for double restriction digestion of the purified plasmid DNA.

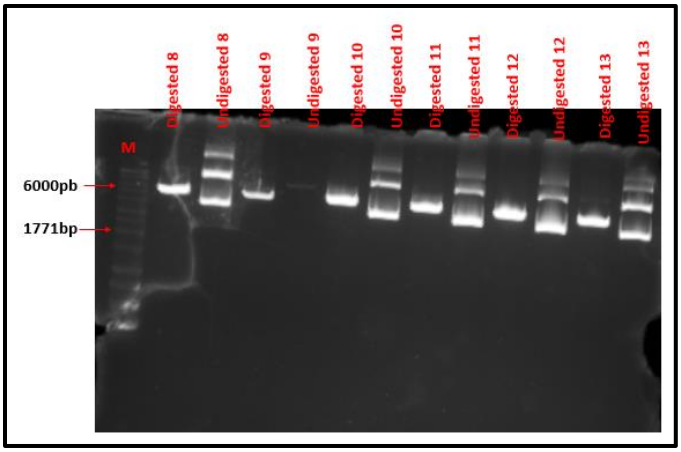


Figure 3.4. A 0.6% agarose gel electrophoresis picture for double restriction digestion of the purified plasmid DNA.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

RSV is responsible for a high morbidity and mortality rate, particularly in children and older adults, and a safe and effective vaccine is not yet approved [93]. New possibilities for passive treatment or prevention and the development of a vaccine have been opened up by recent developments in our understanding of the structure, function, and antigenicity of RSV F as well as the isolation and characterisation of novel RSV F-directed antibodies [93]. However, it is unclear whether the availability of newer therapeutics will make RSV treatment more affordable. This report describes the molecular evolution of the RSV F proteins and their implication in vaccine and therapeutic development.

Although, the diversity of RSV F proteins from South African strains in this study was extremely low. Amino acid variations at N-glycosylation sites, antigenic sites, and HLA-restricted CTL epitopes were detected, which can impact antibody neutralisation and viral clearance by the CTLs. Continued immune pressure and a lack of proofreading by the viral RNA-dependent polymerase of RSV are likely to be responsible for the evolution of the RSV F protein. Therefore, continual surveillance of these variations is necessary to monitor the emergence of new genotypes that could have a negative impact on the vaccines and antivirals under development. The characterisation of local RSV F proteins may contribute data to guide a more strategic vaccine design or selection strategy for South Africa.

Genetic information on recent RSV strains may also help to unravel the reasons for the poor protective immune responses induced by candidate vaccines evaluated in clinical trials. Several limitations were observed during of this study, the estimated sample size of 87 per annum was not reached because in 2019 RSV diagnosis was implemented in July 2019 which is outside the annual South African RSV season which usually start in February and ends in May. In 2020, there was decline of RSV circulation due to the COVID-19 pandemic [42]. The PCR amplification success rate was low due to long-term storage of RNA prior to RT-PCR or probably due to the presence

of inhibitors in the RNA samples. Hence, F sequence data was generated from only 31 cases and included in the bioinformatics analysis. Expression of natural RSV proteins in cell culture will contribute to our current understanding of what is needed to improve current vaccines. Unfortunately, we were unable to successfully generate full-length F protein expression clones even after lowering the transformation and bacterial culture temperatures. Successful generation and expression of F proteins to date required either the deletion of some of the N-terminal amino acids or the SP of the F protein [94]. Cell-free cloning could be another alternative method to overcome this problem [95]. In vitro studies are necessary to examine the effect of SP mutations on RSV F protein expression. Furthermore, to achieve the highest levels of expression and resolve limited expression issues, codon-optimised F molecular constructs with lower CpG content and site-directed mutagenesis should be considered [96].

In conclusion, the study shows that the South African RSV F genes this study and global RSV strains are fairly conserved. However, several genetic variations that are potentially associated with immune response pressure were observed. Thirty-one RSV F gene sequences were generated for 2019–2020, and data are available for future studies. In addition, for the first time, we identified the palivizumab-resistant mutation at position 275 of the F protein in a single RSV-A strain from South Africa, which also warrants further investigation into the frequency of this rare mutation. Genomic surveillance of local RSV F proteins may contribute to a more stratergic vaccine design or selection strategy for South Africa.

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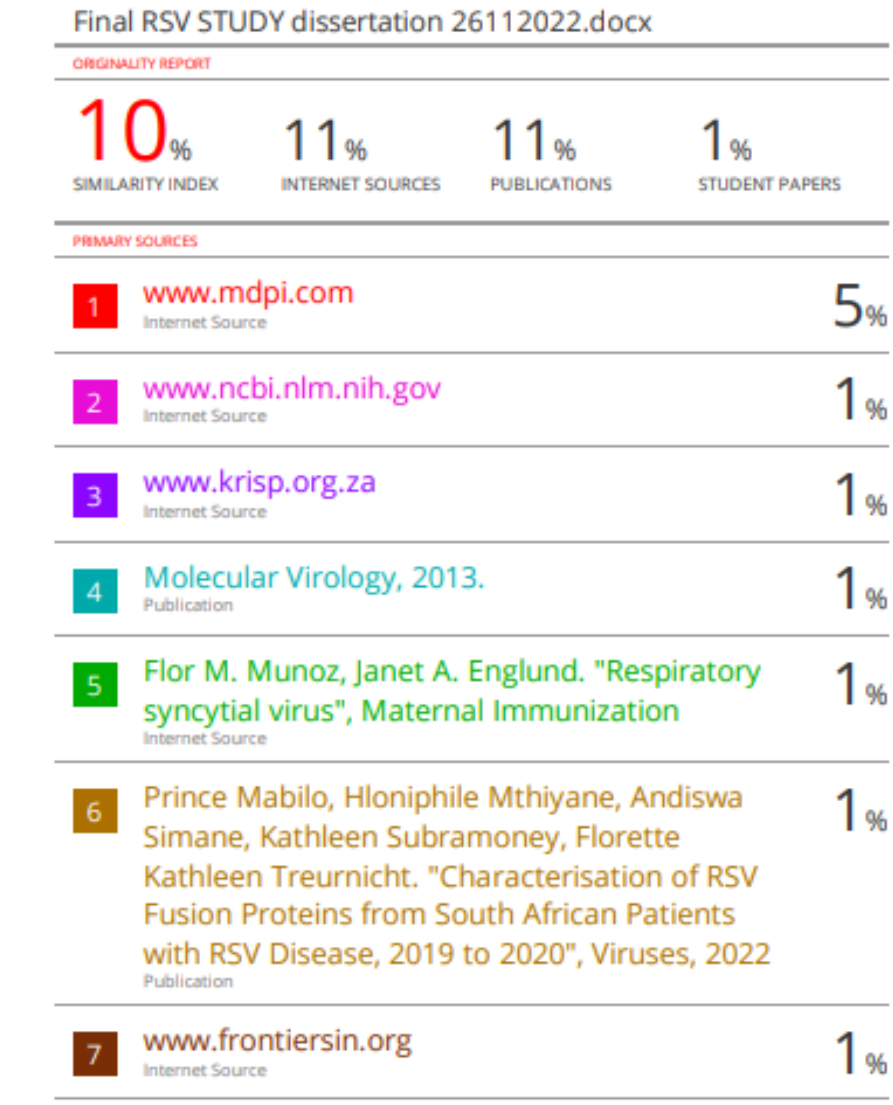
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APPENDICES

Appendix I: Turnitin Report



Final RSV STUDY dissertation 26112022.docx

ORIGINALITY REPORT

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Appendix II: Supplementary data

Supplementary table

Table S1. Summary of F gene sequence data from this study that are available on the GISAID database.

Shortened Sequence name (*Tree)	Full Sequence name (GISAID)	Accession number	Year of detection
RSV25/RSA/2019	hRSV/A/South_Africa/NCV280-25/2019	EPI_ISL_14204587	2019
RSV45/RSA/2019	hRSV/A/South_Africa/NCV496-45/2019	EPI_ISL_14204598	2019
RSV47/RSA/2019	hRSV/A/South_Africa/NCV498-47/2019	EPI_ISL_14204599	2019
RSV48/RSA/2019	hRSV/A/South_Africa/NCV499-48/2019	EPI_ISL_14204600	2019
RSV52/RSA/2019	hRSV/A/South_Africa/NCV500-52/2019	EPI_ISL_14204601	2019
RSV2/RSA/2020	hRSV/A/South_Africa/p5-2/2020	EPI_ISL_14171764	2020
RSV3/RSA/2020	hRSV/A/South_Africa/p6-3/2020	EPI_ISL_14172564	2020
RSV5/RSA/2020	hRSV/A/South_Africa/p7-5/2020	EPI_ISL_14173335	2020
RSV8/RSA/2020	hRSV/A/South_Africa/p8-8/2020	EPI_ISL_14174342	2020
RSV9/RSA/2020	hRSV/A/South_Africa/p9-9/2020	EPI_ISL_14204579	2020
RSV10/RSA/2020	hRSV/A/South_Africa/NCV273-10/2020	EPI_ISL_14204580	2020
RSV11RSA/2020	hRSV/A/South_Africa/NCV274-11/2020	EPI_ISL_14204581	2020
RSV12/RSA/2020	hRSV/A/South_Africa/NCV275-12/2020	EPI_ISL_14204582	2020
RSV13/RSA/2020	hRSV/A/South_Africa/NCV276-13/2020	EPI_ISL_14204583	2020
RSV20/RSA/2020	hRSV/A/South_Africa/NCV277-20/2020	EPI_ISL_14204584	2020
RSV22/RSA/2020	hRSV/A/South_Africa/NCV278-22/2020	EPI_ISL_14204585	2020
RSV24/RSA/2020	hRSV/A/South_Africa/NCV279-24/2020	EPI_ISL_14204586	2020
RSV26/RSA/2020	hRSV/A/South_Africa/NCV317-26/2020	EPI_ISL_14204588	2020
RSV27/RSA/2020	hRSV/A/South_Africa/NCV318-27/2020	EPI_ISL_14204589	2020
RSV31/RSA/2020	hRSV/A/South_Africa/NCV321-31/2020	EPI_ISL_14204590	2020
RSV32/RSA/2020	hRSV/A/South_Africa/NCV322-32/2020	EPI_ISL_14204591	2020
RSV36/RSA/2020	hRSV/A/South_Africa/NCV323-36/2020	EPI_ISL_14204592	2020
RSV37/RSA/2020	hRSV/A/South_Africa/NCV324-37/2020	EPI_ISL_14204593	2020
RSV39/RSA/2020	hRSV/A/South_Africa/NCV325-39/2020	EPI_ISL_14204594	2020
RSV42/RSA/2020	hRSV/A/South_Africa/NCV327-42/2020	EPI_ISL_14204595	2020
RSV43/RSA/2020	hRSV/A/South_Africa/NCV328-43/2021	EPI_ISL_14204596	2020
RSV44/RSA/2020	hRSV/A/South_Africa/NCV495-44/2020	EPI_ISL_14204597	2020
RSV59/RSA/2020	hRSV/A/South_Africa/NCV501-59/2020	EPI_ISL_14204602	2020

*Partial F gene sequences from 2 cases were not uploaded to GISAID

The supplementary table can be found at the following site: https://www.mdpi.com/1999-4915/14/11/2321#supplementary.

Appendix III: Buffers and solution preparation

Preparation of 1 × TAE (pH 8.0) buffer

1. Dilute 20ml of UltraPure™ DNA Typing Grade® 50× TAE stock (Life Technologies, California, USA into 980ml of distilled water and mix.

Preparation of 1% agarose gel

2. Weigh 2g of agarose powder and add in 200ml of 1× TAE buffer.
3. Dissolve the agarose powder by heating in a microwave.
4. Allow the gel to solidify at room temperature for 30 minutes.

Gel Red Nucleic acid precast staining

1. Add 1µl of Gel Red™ nucleic acid stain10 000X water (Merck, Johannesburg, South Africa) into a cool dissolved gel before it solidifies.

Preparation of Luria-Bertani (LB) broth and agar with ampicillin

1. Dissolve 25 grams of medium (Merck, Johannesburg, South Africa) in 1L of distilled water and mix thoroughly.
2. Sterilise by heating at 121˚C for 15 minutes in an autoclave and cool down. Add ampicillin to a final concentration 100µg/ml into the broth and store at 4 ˚C.
3. To prepare LB agar add 1.5% of LB agar powder (Merck, Johannesburg, South Africa) into the 1L broth and mix.
4. Sterilise by heating at 121˚C for 15 minutes in an autoclave and cool down.

Preparation of glycerol stocks

Add 150µl of 100% glycerol (Glentham Life Sciences, Wiltshire, United Kingdoms) into 850µl of bacterial culture and mix thoroughly. Store at -80˚C.

Preparation of Qubit working solution

1. Dilute Qubit dsDNA HS reagent with Qubit dsDNA HS buffer in 50ml falcon tube.

2. Vortex the to ensure a homogeneous working solution and stored in a dark environment until ready to use.

Preparation of 0.5% next-generation sequencing post-run wash solution

1. Mix 5ml of 100% Tween 20 with 45ml nuclease-free water.
2. Mix 25ml of 10% Tween 20 with 475ml nuclease-free water and mix the solution by inverting several times.

Appendix IV: Ethics approval certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R49 Mr P Mabilo

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M201161

NAME: (Principal Investigator)	Mr P Mabilo
DEPARTMENT:	School of Pathology Department of Virology Medical School University
PROJECT TITLE:	*Expression and characterisation of F proteins from South African respiratory syncytial virus (RSV) strains, 2019 to 2020*
DATE CONSIDERED:	2020/11/27
DECISION:	Aproved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr F Treurnicht and Miss H Mthiyane
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2021/03/10

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. I agree to submit a yearly progress report. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **«Missing mail merge field»** and therefore reports and re-certification will be due in the month of **«Missing mail merge field»** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

11/03/2021
Date