Genomic Analysis of Respiratory Syncytial Virus Circulating in the Free State during the COVID-19 Pandemic

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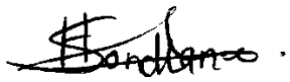
DECLARATION

"I, Hlengiwe Sondlane, declare that the Master's Degree research dissertation or interrelated, publishable manuscripts/published articles, or coursework Master's Degree mini-dissertation that I herewith submit for the Master's Degree qualification (Medical Virology), at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education."



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DEDICATION

I dedicate this thesis to my lovely family and everyone else who believed in me!

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LIST OF ABBREVIATIONS

AAP:	American academy of paediatrics
AARMS:	Academic affairs research management system
ALRTI:	Acute lower respiratory tract infection
AMPV:	Avian Metapneumovirus
ARTI:	Acute respiratory tract infection
Bp:	Base pairs
BRSV:	Bovine respiratory syncytial virus
BV-BRC:	Bacterial and Viral Bioinformatics Resource Center
CCA:	Chimpanzee coryza agent
CDC:	Centres for disease control and prevention
cDNA:	Complementary DNA
CHD:	Congenital heart disease
COVID-19:	Coronavirus disease 2019
CXC3CR1:	C-X-3 chemokine receptor 1
DFA:	Direct fluorescent antibody
dNTP:	Deoxynucleotide
EBREC:	Environmental and Biosafety research ethics committee
EGF:	Epidermal growth factor
ERD:	Enhanced respiratory distress
F:	Fusion protein
FDA:	Food and drug administration
FSDoH:	Department of Health
G:	Attachment glycoprotein
GISAID:	Global initiative on sharing all influenza data
HMPV:	Human Metapneumovirus
HPD:	High probability density
HSPGs:	Heparan sulphate proteoglycans
HSREC:	Health science research ethics committee
HVR2:	Second hypervariable region
IBS:	Inclusion bodies
ICAM-I:	Intercellular adhesion molecule 1
ICTV:	International committee on taxonomy of viruses
ICU:	Intensive care unit

L:	Large protein
LMICs:	Low- and middle-income countries
LRTI:	Lower respiratory tract infection
M:	Matrix protein
M2:	M2-1 protein and M2-2 protein
mAb:	Monoclonal antibody
N:	Nucleoprotein
NGS:	Next generation sequencing
NHLS:	Nation Health laboratory services
NPIs:	Non-pharmaceutical interventions
NPS:	Nasopharyngeal swab
NS:	Nasal swab
NS1:	Non-structural protein 1
NS2:	Non-structural protein 2
NW:	Nasal wash
OPS:	Oropharyngeal swab
ORSV:	Ovine respiratory syncytial virus
P:	Phosphoprotein
RADT:	Rapid antigen detection test
RdRp:	RNA dependent RNA polymerase
RNP:	Ribonucleoprotein
RSV:	Respiratory syncytial virus
RTI:	Respiratory tract infection
RT-PCR:	Reverse transcription polymerase chain reaction
SARI:	Severe acute respiratory infection
SARS-CoV-2:	Severe acute respiratory syndrome coronavirus 2
SH:	Small hydrophobic protein
TLR4:	Toll-like receptor 4
UFS:	University of the Free State
URTI:	Upper respiratory tract infection
VTM:	Viral transport media
WGS:	Whole genome sequencing
WHO:	World Health Organization

LIST OF SYMBOLS

-	Hyphen
&	And
()	Bracket
,	Comma
.	Full stop
:	Colon
;	Semicolon
≤	Less than or equal to

PROJECTED PUBLICATION FROM THIS STUDY

1. Whole genome molecular analysis of respiratory syncytial virus pre and during the COVID-19 pandemic in Free State Province, South Africa (Virus Research) -Under Review (Manuscript number-: **VIRUS-D-24-00044**)

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2. **Hlengiwe Sondlane.** RSV infection: A Persistent Ravaging Virus Masked and Exacerbated by the COVID-19 Pandemic. University of the Free State Institutional Three Minute Thesis (3MT) 13 October 2023
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AWARDS

1. The Faculty of Health Sciences Three Minute Thesis (3MT) competition, University of the Free State, Bloemfontein, South Africa, 18 September 2023. 1st -Runner up (MSc category) and cash award.
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OTHER PUBLICATIONS DURING THE STUDY

1. Ogunbayo AE, Mogotsi MT, **Sondlane H,** Sabiu S, Nyaga MM. Metagenomics characterization of respiratory viral RNA pathogens in children under five years with severe acute respiratory infection in the Free State, South Africa. J Med Virol. 2023 May;95(5): e28753. doi: 10.1002/jmv.28753. PMID: 37212321

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2. Ayodeji Emmanuel Ogunbayo, Milton Mogotsi, **Hlengiwe Sondlane**, Kelebogile Nkwadipo, Martin Nyaga. Application of next-generation sequencing and metagenomics in deciphering the human microbiome (with a focus on respiratory virome). Second Global Microbiome Network – GloMiNe – Symposium for Africa, 2022.

OTHER POSTER PRESENTATION EMANATED FROM THIS STUDY

1. Ayodeji Emmanuel Ogunbayo, Milton Tshidiso Mogotsi, **Hlengiwe Sondlane**, Saheed Sabiu, Martin Munene Nyaga. Increased Utility of Metagenomics Next-generation sequencing in diagnosing severe acute respiratory infection in paediatric patients. University of the Free State Faculty Research Forum, 2022.

SUMMARY

Respiratory syncytial virus (RSV) is a highly contagious virus that is responsible for most infant hospitalisations in developed nations and accounts for a significant childhood mortality in low- and middle-income countries (LMICs). The RSV has two main subtypes, namely A and B, which co-circulate and exhibit a predominance during different RSV epidemic seasons. This virus exhibits a high degree of genetic diversity, resulting in the emergence of various genotypes. The regulation of viral replication is primarily governed by two major RSV glycoproteins, namely the attachment (G) protein and the fusion (F) protein. These proteins possess potential glycosylation sites, although the variability is more pronounced in the G protein, while the F protein remains relatively conserved across different virus isolates.

Following the advent of severe acute respiratory syndrome coronavirus-2 and the onset of the coronavirus disease 2019 (COVID-19) era, a significant decline in RSV activity was observed, aligning with the enforcement of non-pharmaceutical interventions (NPI). Consequently, the COVID-19 pandemic brought about a significant shift in the seasonality and epidemiology of RSV, resulting in an out of season RSV outbreak. Therefore, this study aimed to investigate the genetic diversity of the circulating strains in South Africa before and during the COVID-19 pandemic.

In this study, 69 nasopharyngeal swabs were collected from children who presented with respiratory distress (n=50) and severe acute respiratory infection (SARI) (n=19) and required hospital admission in different participating hospitals in the Free State province, Bloemfontein, South Africa. The samples were then subjected to multiplex panels to detect RSV. The viral RNA was extracted from RSV positive samples and overlapping fragments of the RSVA/B genomes were amplified using the Superscript IV One-Step RT-PCR kit. Libraries were prepared with QIAseq-FX single-cell RNA library preparation kit. The RSV whole genome was sequenced using the Illumina MiSeq, platform, and the obtained data was then analysed using Genome Detective and Geneious software. The virus genotypes were identified through phylogenetic analysis utilising a Nextclade typing tool. The phylodynamics of RSV, genetic diversity, and evolutionary patterns were analysed using IQ Tree and BEAST software.

The sequence phylogenetic analysis revealed a notable level of genetic diversity among the RSV strains in South Africa. In this study, the analysis showed that all study strains belonged to the GA2.3.5 and GB5.0.5a genotypes, which were predominant during the whole study period. These genotypes have been characterised previously and are circulating globally as the predominating

genotypes. The phylogenetic analysis of the G-gene showed that strains responsible for the South African epidemic observed during the COVID-19 resurgence in 2020-2021 formed distinct clusters with contemporary strains from various geographic origins, suggesting continuous minor introduction within South Africa. Furthermore, the estimated mean evolutionary rates for RSV-A and RSV-B were found to be 1.48×10^{-3} and 1.92×10^{-3} nucleotide substitutions/site/year, respectively. The genetic diversity patterns in GA2.3.5 and GB5.0.5a genotypes were similar according to the Bayesian Skyride plot analysis. Our results suggest that the COVID-19 lockdown in South Africa caused a minor bottleneck suggesting other strains ceased to circulate, then followed by increased diversity as restrictions were lifted, indicating increased circulation of this variants.

The relaxation of COVID-19 restrictions in South Africa led to a surge in the off-season RSV epidemic among children. This increase in cases can be attributed to the presence of pre-existing strains, which may have been able to spread in the paediatric population that is immunologically naïve or have waned immunity. Therefore, it is crucial to conduct genomic sequencing to gain insights into the circulation patterns of RSV and the diversity of RSV strains. Given the need to comprehensively understand the RSV evolutionary patterns and its impact on vaccine effectiveness, it is imperative for genomic surveillance strategies to focus on and prioritize the use of whole genome sequencing.

Keywords: Off-season RSV epidemic, COVID-19, Whole genome sequencing, non-pharmaceutical interventions, paediatric, viruses, nasopharyngeal swabs, respiratory infection, respiratory distress.

Preface

The work presented here in chapter one provides a detailed background and rationale/significance of the study with a comprehensive description of the problem statement.

Detailed authors contributions are here-in described.

Authors detailed Contributions:

- **Proposal:** Hlengiwe Sondlane
- **Writing—review and editing**: Martin Nyaga, Dominique Goedhals, Hlengiwe Sondlane.

CHAPTER ONE: INTRODUCTION

1.1. Background

Respiratory syncytial virus (RSV) is a major contributor to acute respiratory tract infection (ARTI) globally in children ≤5 years, the elderly, and the immunocompromised population (Borchers et al., 2013; Malik et al., 2023; Raghunandan et al., 2021). Infants under six months are more vulnerable and at higher risk of severe consequences from RSV infection, including death (Gill et al., 2022; Nair et al., 2010). According to global estimates, in 2019 RSV was implicated in about 33 million episodes associated with lower respiratory tract infection (LRTI) in children below 5 years, resulting in 1.4 million hospital admissions and around 45,700 hospital fatalities worldwide (Li et al., 2022).

According to the recent classification by the International Committee on Taxonomy of Viruses (ICTV), RSV belongs to the family *Pneumoviridae* and the genus *Orthopneumovirus* (Rima et al., 2017). This virus is an enveloped, negative-sense, and single-stranded RNA virus (Kenmoe et al., 2018). Its genome is non-segmented and is composed of approximately 15,200 nucleotides (Zheng et al., 2017). The RSV genome is categorized into two main antigenically distinct subtypes, RSV-A and RSV-B (Mufson et al., 1985), with at least 15 and 30 genotypes existing for subtypes A & B, respectively (Chen et al., 2022). Subtype and genotype differentiation is based on the region encoding the attachment (G) protein, part of the gene containing the highest degree of genetic divergence (Fodha et al., 2008; Ramaekers et al., 2020). Generally, one subtype predominates in a particular season, with a dominant subtype with varying occurrence every one to three years (Borchers et al., 2013; Nam and Ison, 2019). Although multiple RSV genotypes can coexist in a population at once, in most cases a single genotype will predominate in a local epidemic, with new genotypes gradually replacing the original as they emerge (Otieno et al., 2016; Pangesti et al., 2018).

Generally, RSV has a distinct seasonal epidemic pattern with a high prevalence rate occurring in winter and throughout the summer season with few or no outbreaks (Yassine et al., 2020). In early childhood, RSV is the causative agent that leads to acute bronchiolitis and viral pneumonia, ranking second to malaria, as the leading cause of infant mortality following the neonatal period (Shang et al., 2021). Reinfections with RSV are common throughout a lifetime, due to short-lived and partial protective immunity following infection (Hacking and Hull, 2002; Ouyang et al., 2022; Russell et al., 2017). Most of the children frequently infected and hospitalised are previously healthy infants with no predisposing risk factors for severe disease (Bont et al., 2016; Collins and Melero, 2011; Robledo-Aceves et al., 2018; Shi et al., 2015). Despite RSV being deemed a global health threat, to date there

is no Food and drug Association (FDA) licensed vaccine for children or specific therapies are available (Broadbent et al., 2015; Chatterjee et al., 2021; Feng et al., 2022; Griffiths et al., 2017). The Palivizumab, a humanised monoclonal antibody (mAb) serves as a prophylaxis and is reserved for paediatric patient groups at high risk of developing severe RSV disease (Anderson et al., 2017) However, it is associated with a high cost and questionable efficacy (Mac et al., 2019; Simoes et al., 2018; Turner et al., 2014). Additionally, Nirsevimab, has recently received FDA approval for its use in preventing infections in infants. However, U.S. FDA has approved the first RSV vaccine known as Abrysvo, for administration to pregnant individuals, aimed at protecting new-borns against RSV severe disease during the initial 6 months following birth (Ernst, 2023).

Whilst childhood viral respiratory infections with RSV induce partial primary immunity; repeated infections occur throughout a lifetime and may lead to severe disease, which may be exacerbated with waned immunity (Glezen et al., 1986; Bont et al., 2002). This phenomenon mirrors the unexpected surge and sporadic RSV cases that were documented in children during the recent COVID-19 pandemic because of adopted non-pharmaceutical interventions (NPIs), such as mandatory face masks, social and physical distancing measures, and hand hygiene to curb the transmission of the virus (Yeoh et al., 2021). Currently, little is known about the genomics of the strains responsible for this outbreak. Besides, a dearth of information generally exists on the whole genome sequencing of RSV in South Africa and regionally in other African countries.

With next generation sequencing (NGS) becoming more rapidly available, globally, it is now possible to sequence whole genomes of RSV and acquire massive genomic data suitable for analysis and drawing inferences. However, there is limited RSV whole genome data in public databases such as national center for biotechnology information (NCBI), due to the limited availability of studies on whole genome sequencing of RSV (Langedijk et al., 2022). The attachment glycoprotein has been widely used in phylogenetic analysis and genotyping due to its high degree of genetic variability (Goya et al., 2023). In addition, advanced technologies have enabled the sequencing of bigger fragments and even whole genomes (Ramaekers et al., 2020). The availability of such genomic data will be valuable in approaches required for viral vaccine development and management and would simultaneously benefit epidemiological surveillance and tracking of emerging RSV strains in different epidemic seasons. Therefore, this study performed whole genome sequencing of RSV in South African children admitted with respiratory distress and SARI during the COVID-19 pandemic in the Free State Province, South Africa.

1.2. Problem Statement

The RSV is a major etiological agent of severe acute lower respiratory tract infections in the paediatric population, globally (Kini et al., 2019; Nair et al., 2010; Russell et al., 2017). It has a seasonal distribution pattern with typical wintertime peaks and causes seasonal outbreaks (Moore et al., 2009; Obando-Pacheco et al., 2018). Diversion of an immune response against an RSV type during a single season may render the population vulnerable to infection triggered by another RSV or similar strain in subsequent seasons (Rajendran et al., 2020). Moreover, reinfections with RSV are likely to re-occur multiple times in individuals during a lifetime even though an immune response against RSV has been developed post primary and secondary RSV infections experienced during infancy (Agoti et al., 2015; Walsh and Hall, 2015). Children may experience recurrent infections as RSV immunity wanes in a short period. However, secondary infections do not present severe disease and rarely necessitate hospitalisation (Openshaw et al., 2017; Sommer et al., 2011).

The coronavirus disease 2019 (COVID-19) pandemic, was implacable in 2020, and the NPIs implemented to reduce severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) had an enormous impact on the circulation of respiratory pathogens with few cases of RSV recorded (Tempia et al., 2021). The easing of COVID-19 imposed NPIs caused a delay in the RSV outbreak, resulting in a sudden surge in the number of RTI cases associated with RSV outside of its previously established seasonality (Binns et al., 2022; Mondal et al., 2022; Williams et al., 2021; Yeoh et al., 2021). This could have potentially resulted in the emergence of new RSV strains due to selective pressure or a matter of previous strains causing increased infections due to waned or absent immunity in the population, following a lack of exposure. The execution of new RSV vaccine studies may be hampered by this seasonal irregularity and the unpredictability of these infection peaks (Williams et al., 2021).

Currently, there is limited information regarding the circulating subtypes of RSV strains. Characterisation of the RSV subtypes circulating during an epidemic season using advanced sequencing techniques, such as NGS is crucial in elucidating the role of this virus in respiratory disease and may address changes in genetic diversity. In addition, there is still little knowledge about RSV at the entire genome level, particularly in many underdeveloped and developing nations. For research and intervention purposes, the characterisation of this viral agent through whole genome analysis may be crucial for infection control and knowledge on the possible existence and circulation of novel strains with the potential to cause interseason outbreaks.

1.3. Rationale and Significance of the Study

The RSV has emerged as a significant public health concern that has resulted in high hospitalization and fatality in children (Baviskar et al., 2013; Shi et al., 2017). Although RSV is recognised as a significant respiratory pathogen, there is very limited data regarding the circulating RSV strains available. This study provides an opportunity to advance our knowledge of the genotype distribution of RSV currently circulating in the community, during a global pandemic season. Besides, adequate knowledge on the communal introduction of RSV is required, which is still poorly understood, owing to the global scarcity of genomic sequence and complementary epidemiological data (Langedijk et al., 2020). Achieving sequencing and analysis of the whole genome of RSV will broaden our understanding of the phylogenetics and diversity of these strains. With the surge in RSV infection, there was a high need to conduct surveillance using genomics in preparation for its resurgence. The detection of this virus could ignite an urgency in vaccine development with a focus on the most vulnerable population.

1.4. Research Questions

1. Are we experiencing a new wave of RSV due to the emergence of new strains of the virus?
2. Are there any genetic similarities and differences between the currently circulating strains during COVID-19 and those prior to the pandemic?

1.5. Research Aim

To perform whole genome sequencing and genomic analysis of RSV strains during the COVID-19 pandemic.

1.6. Research Objectives

1. To perform whole genome sequencing of RSV positive clinical samples detected during the COVID-19 pandemic.
2. To perform genomic analysis of circulating RSV subtypes before and during the COVID-19 pandemic and compile a repository for RSV sequences.
3. To perform genetic analysis of RSV positive samples coinfected with other respiratory pathogens during the COVID-19 pandemic.

1.7. Thesis Organisation

This thesis is divided into five chapters, which are preceded by a preface.

- **Chapter one** includes background information on RSV and highlights the aims and objectives, the problem statement, and the significance of the study.
- **Chapter two** presents a detailed literature review on RSV, covering the epidemiology, genomic aspects, prevention, and treatment options, and highlights the existing gaps in the current research based on publications.
- **Chapter three** covers the detailed methodology and laboratory techniques employed in the study. In addition, optimized and validated techniques and protocols adopted in this study are presented herewith in this chapter.
- **Chapter four** is presented in a manuscript format that features the introduction, methodology, results, and discussion. It addresses objective one, two, and three, which is based on whole genome sequencing of RSV and explores the phylodynamics, evolutionary, and genetic diversity of RSV during the COVID-19 pandemic.
- **Chapter five** is presented in a manuscript format of a review paper. This review paper highlights the burden and the impact of the COVID-19 pandemic on RSV circulation, and seasonality including the clinical aspect.
- **Chapter six** covers the integrated summary, recommendations, and conclusion of the study.

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Preface

The work presented here in chapter two provides a general discussion on the genomics, biology, and epidemiology of RSV with a highlight on the existing gaps in the current research based on publications.

Detailed authors contributions are here-in described.

Authors detailed Contributions:

- **Proposal:** Hlengiwe Sondlane
- **Writing—review and editing**: Martin Nyaga, Dominique Goedhals, Hlengiwe Sondlane.

CHAPTER TWO: LITERATURE REVIEW

2.1. Historical Background of RSV

In 1956, researchers first discovered RSV in chimpanzees who had an upper respiratory illness at the Walter Reed Army Institute of Research in the United States (Morris et al., 1956; Wright and Piedimonte, 2011). The coryza-like symptoms that were present during the respiratory outbreak in the chimpanzee colony were characterised by coughing, sneezing, and nasal discharge (Carvajal et al., 2019). Morris and colleagues identified the causative agent as a virus and named it Chimpanzee Coryza Agent (CCA) (Kapikian et al., 2011; Morris et al., 1956). Subsequently, Charnock and colleagues were able to establish conclusively that the pathogen caused respiratory disease in humans by collecting isolates from two children suffering from laryngotracheobronchitis and one with bronchopneumonia that were identical to CCA (Chanock et al., 1961; Chanock and Finberg, 1957). The virus was named RSV, based on the distinctive cytopathic observation in tissue culture, in which the infected epithelial cells form characteristic giant multinucleated cells indicative of almost complete syncytium formation (Chanock et al., 1961; Gagliardi et al., 2017). Nearly six decades later, RSV is still acknowledged as the primary viral agent cause of infantile bronchiolitis and the principal global agent responsible for severe respiratory disease in children under five (Binns et al., 2022; Coultas et al., 2019; Douros and Everard, 2020; Ghazaly and Nadel, 2018).

2.2. Burden and Epidemiology of RSV

2.2.1. Disease Burden Worldwide and in South Africa

The RSV remains a major contributor to paediatric LRTI and childhood mortality worldwide, especially in low- middle-income countries (LMICs) (Carbonell-Estrany et al., 2022; O'Brien et al., 2019; Thomas et al., 2021). In all age groups, fatalities related to RSV are predominantly observed in LMICs, accounting for over 97% of the total deaths (Li et al., 2022). Most RSV deaths occur in the community and are sometimes unaccounted for in hospital based surveillance and are therefore not reflective of the true incidence of RSV attributable death in LMICs (Cohen and Zar, 2022; Kazi et al., 2021; Mazur et al., 2021; Simões et al., 2021; Srikantiah et al., 2021). Due to inadequate surveillance in LMICs, it is difficult to estimate the actual burden of RSV mortality, particularly to determine the RSV disease burden in communities (Arriola et al., 2020; Gill et al., 2022; Zanone et al., 2016). In 2015, RSV was estimated globally, to cause approximately 33.8 million RSV related ALRTI episodes, and 3.2 million hospital admissions, with about 110,000 fatalities in infants less than five years, with a large proportion occurring in developing countries (Shi et al., 2017). In a previous systematic review by Li and colleagues, RSV associated ALRTI was reported to be responsible for 3.3 million hospital

admission cases in children worldwide, and over 100,000 annual fatalities in children below five years (Li et al., 2022). Most of the documented fatalities in this study (95%) occurred in LMICs, with approximately 45% occurring within the first six months of life in infants (Li et al., 2022). In another study conducted by Lively and colleagues, RSV-related visits to the paediatric emergency room among infants under two years of age are estimated to be between 59.6-205.7 per 1000 children, respectively (Lively et al., 2019). Similarly, Rha and colleagues reported an RSV-related hospitalization rate of 2.9 per 1000 children under the age of five years in the United States (Rha et al., 2020).

Noteworthy, RSV infection in the adult population follows a comparable pattern to seasonal influenza infection especially in the elderly over 65 years (Ackerson et al., 2019; Korsten et al., 2021). Even throughout the influenza season, a significant health burden of RSV and high severity has been reported (Amini et al., 2019). In 2019, RSV was estimated to be responsible for 5.2 million cases of ARI, about 470,000 hospitalizations, and approximately 33,000 in-hospital mortality in elderly over 65 years of age in industrialised countries (Savic et al., 2023). According to a recent meta-analysis, the annual occurrence of RSV related LRTI among individuals aged 65 and above was approximated to be 6.7 cases per 1000 individuals (Shi et al., 2020). Consequently, the disease burden of RSV could be largely underappreciated at all stages of life.

In South Africa, the prevalence of RSV is substantially higher at 2%, with an incidence that ranges from 14.4 to 48.0 cases/1000 person-years (Madhi et al., 2018). In young children, RSV is a frequent cause of moderate to severe bronchiolitis and is 2-3 times higher in infancy compared to children over five years (White et al., 2016). In a cohort study conducted in South Africa, hospitalised RSV-associated LRTI in infants under six months had an incidence rate of 0.07 cases per child year (95% CI 0.05-0.10) in the first two years of life (Moyes et al., 2023). Additionally, the burden of RSV associated illness in South African children younger than five years was estimated by month and RSV associated ARI was the highest in 2-month-old (18,361/100,000 population) and SARI less than one month old (14,674/100,000), with a high mortality rate in the first and second month of life in this population (Moyes et al., 2023). Besides the health-related burden of RSV, severe RSV infection in South Africa has been associated with exorbitant out-of-pocket expenses, surpassing 100% of the average monthly income (Srikantiah and Klugman, 2023).

2.2.2. Seasonality and Transmission of RSV

The epidemiology of RSV varies significantly and is highly dependent on climate and weather patterns. (Janet et al., 2018; Li et al., 2019; Obando-Pacheco et al., 2018; Suryadevara and Domachowske, 2021). Viral stability, host predisposition, and dormant viral reactivation are all enhanced in low temperatures (Henry et al., 2019; Piedimonte and Perez, 2014). Seasonal or periodic outbreaks of RSV in different hemispheres present a substantial disease burden in paediatric patients that is clinically significant (Li et al., 2019). In each hemisphere, the seasonal onset of RSV activity differs and exhibits a latitudinal gradient; for instance, the tropics experience the onset of RSV activity in late summer, while the temperate zones experience it in late fall or early winter (Borchers et al., 2013; Chadha et al., 2020; Chatterjee et al., 2021; Li et al., 2019; Rose et al., 2018). Also, there is a correlation between an increase in cases of RSV and a decrease in temperature, which can be attributed to increased indoor crowding, which may facilitate the rapid transmission of the virus (Kaler et al., 2023; Wagatsuma et al., 2023).

Prior to the year 2020, seasonal trends in the circulation of RSV have been consistent and conform to the established norm of increased transmission in winter seasons. However, the unprecedented global COVID-19 pandemic caused by SARS-CoV-2, resulted in the adoption of several NPIs (Oh et al., 2021). These NPIs include hand sanitization, wearing of a mask, social distancing, and lockdown measure; all of which halted the transmission and circulation of SARS-CoV-2 and other respiratory viruses including RSV and influenza. (Bermúdez Barrezueta et al., 2022; Li et al., 2019; Tang and Loh, 2014). During this period, limited cases of RSV infections were reported with few to zero detections of influenza virus. Consequently, a delayed RSV epidemic was observed in the winter season followed by an offseason outbreak in the summer of 2020.

Like other respiratory viruses, the transmission of RSV is via direct or indirect contact with infectious respiratory droplets. Direct transmission involves inhalation of the infectious droplet while indirect transmission involves contact with contaminated surfaces like floors or skin, or contaminated fomites (Figure 2.1) (Leung, 2021). Comparatively, RSV has a reproductive ratio (R_0) of 3.0, which is higher than majority of other respiratory viruses including influenza with a (R_0) of 1.0-1.6. In essence, a single infection of RSV will lead, on average, to three other infections (Dare and Talbot, 2016; Grayson et al., 2017; Haber, 2018; Kulkarni et al., 2016).

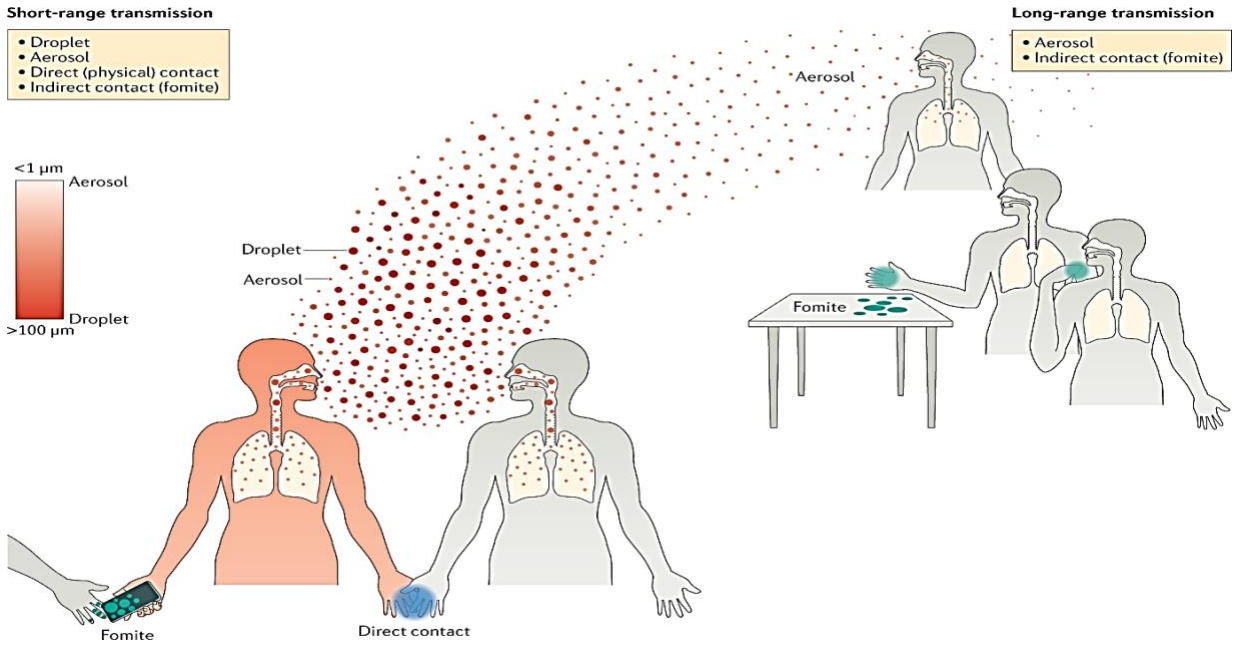

Figure 2.1: A graphical representation of different modes of viral transmission (Leung, 2021).

2.2.3. Clinical Manifestations of RSV in Children

In children, symptoms associated with RSV infection range from a moderate self-limiting upper respiratory tract infection (URTI) to severe respiratory infection that is primarily manifested by pneumonia or bronchiolitis (Smith et al., 2017). Bronchiolitis is a major clinical presentation of RSV infection and is responsible for significant hospitalisation as well as life threatening repercussions or complications such as respiratory failure, especially in paediatric intensive care unit (ICU) admissions (Barr et al., 2019; Blanken et al., 2013; Jha et al., 2016; Piedimonte and Perez, 2014). Bronchiolitis is a LRTI, characterized by symptoms such as coughing and wheezing (Paul, 2013; Pickles et al., 2015). In addition, RSV infection is often characterized by symptoms including rhinorrhoea, sneezing, nasal congestion, cough, and occasionally fever (Nam and Ison, 2019).

Most recurrent infections are limited or contained to the upper respiratory tract and have a milder course, manifesting as a prolonged cold that gradually gets well on its own and is seldom accompanied by serious clinical consequences (Meissner, 2016). However, the condition may proceed and develop into LRTI, particularly in immunocompromised individuals, which is characterised by more severe symptoms and, in rare cases, considerable morbidity and death (Anderson et al., 2022; Jain et al., 2017; Pierangeli et al., 2018). Moreover, RSV recurrence in older children is prevalent, but often less severe, although it can aggravate other pulmonary function-related diseases (Fauroux et al., 2017). In addition to severe disease, evidence also indicates or points to a higher risk of subsequent wheezing in infancy, hyper-reactive airways in childhood (Smith et al., 2017), and asthma in later life in children who had severe RSV infections or bronchiolitis (Baraldi

et al., 2020; Fauroux et al., 2017). Lethargy and difficulty ingesting food may be evident in very young children, and this might result in hospitalisation owing to dehydration and most likely malnutrition (Kaler et al., 2023).

2.2.4. Risk Factors Associated with RSV Infections

The involvement of the lower respiratory tract in the progression of RSV is frequently observed in individuals with compromised immune systems (Carvajal et al., 2019; Welliver et al., 2007). Preterm infants, as well as those with chronic lung illnesses such as bronchopulmonary dysplasia (Chaw et al., 2020), cystic fibrosis, interstitial lung disorders, down syndrome (Beckhaus and Castro-rodriguez, 2018), or haemodynamically severe congenital heart disease, have a greater morbidity and fatality rate from RSV disease (Anderson et al., 2021). Infants of all gestational ages (GAs) are more prone or at increased risk than healthy adults to have severe RSV illness that necessitates hospitalisation (Smithgall et al., 2020). Amongst other factors, premature delivery, immunodeficiency, gender, malnutrition, congenital heart disease (CHD), and underlying lung illness, are host variables in children that may contribute to hospitalisation from RSV infection (Bont et al., 2016; Carvajal et al., 2019). Other predisposing environmental factors in children include exposure to passive smoking, especially in the same household, crèche attendance, and overcrowding amongst others. (Coultas et al., 2019). Although the highest rates of RSV-related hospitalization are seen within high-risk groups, it is important to recognize that most of infections occur in otherwise healthy infants born at term (Murray et al., 2014; Driscoll et al., 2020).

2.2.5. Immunity against RSV

There are knowledge gaps pertaining to the immunologic mechanisms linked to immune protection against RSV (Koivisto et al., 2022). While several infections with either the same or distinct strains of RSV can occur, the virus fails to induce robust and enduring immunity (Openshaw et al., 2017). Although infants who exhibit the capability to produce robust RSV-specific humoral and cellular immune responses during their initial RSV infections often experience expedited recovery and milder symptoms (Schmidt et al., 2018), RSV naïve infants typically exhibit insufficient and temporary innate and adaptive immune reactions towards the virus (Graham, 2011). Nonetheless, RSV naïve infants may still receive higher levels of neutralising antibodies from their mothers through transplacental transfer, possibly supplemented through breastfeeding, and may exhibit reduced susceptibility to contracting the virus (Taleb et al., 2018).

2.3. Genomic and Virology of RSV

2.3.1. Taxonomy of RSV

The RSV has been separated from the original *Paramyxoviridae* family and reclassified into the *Pneumoviridae* family, found within the *Orthopneumovirus* genus in the order Mononegavirales (Afonso et al., 2016; Rima et al., 2017). The virus is categorised into two major subtypes, namely, subgroups A and B (Borchers et al., 2013). Each group is distinguished based on antigenic and genetic analyses of the G-protein second hypervariable region (HVR2) (Vandini et al., 2017). The presence of additional antigenic variability that exists within RSVA and RSVB contributes significantly to the pathogenicity of RSV and its ability to evade the immune system (Di Giallonardo et al., 2018; Kaler et al., 2023; Martinelli et al., 2014). This is primarily attributed to the extensive diversity observed in the attachment glycoprotein, both in terms of its antigenic and genetic characteristics (Jha et al., 2016). More so, each subtype is further characterised into genotypes consisting of multiple genotypes that coexist and circulate with one subtype predominant in any given year (Zlateva et al., 2004). During periods of epidemic outbreaks, it is common for subtypes A and B to coexist, with RSV-A being the dominant strain on a global scale. Additional members of the genus *Orthopneumovirus* of the RSV genus are bovine RSV (BRSV), ovine RSV (ORSV), and pneumonia virus of mice (murine pneumonia virus) (Collins et al., 2013). The family *Pneumoviridae* also includes the genus *Metapneumovirus* which encompasses viruses such as human and avian metapneumoviruses (HMPV and AMPV), which are also pathogens of clinical significance (Rima et al., 2017).

2.3.2. Genetic Diversity of RSV

As initially described, RSV is classified into 2 distinct subgroups: RSV-A and RSV-B, based on their antigenic and genetic sequence variability (Anderson et al., 1985; De-Paris et al., 2014), which may vary, even though antigenic regions of the F protein are widely thought to be well-conserved (Langedijk and Bont, 2023). Both groups are further divided into numerous genotypes, RSV-A contains approximately 20 genotypes, including GA1 to GA7, ON1, SAA1 to SAA2 NA1 to NA14 and CBA, compared to RSV-B which consist of more than 30 genotypes, such as GB1 to GB4, BAc, SAB1 to SAB4, URU1 to URU2, CB1 (GB5), CBB, BA to CCA, BA to CCB BA1 to 14, and THB (Ábrego et al., 2017; Bashir et al., 2017; Chen et al., 2022). Noteworthy, pre-existing genotypes can be replaced by emerging ones over time and predominate (Kamau et al., 2020; Otieno et al., 2018).

In the past 20 years, the G-Gene has been characterised by nucleotide (nt) duplication events that occur in G ectodomain that resulted in an emergence of RSV genotypes worldwide, which are Buenos Aires genotype (BA) in 1999 (Goya et al., 2023) and Ontario genotype 1 (ON1) in 2010 (Battles and McLellan, 2019; Eshaghi et al., 2012). The ON1 genotype has spread throughout the world, albeit, at a faster rate than the BA genotype (Duvvuri et al., 2015). Since their emergence, the BA and ON1 strains have supplanted and predominated every genotype demonstrating the G protein duplication's role of selective advantage (Otieno et al., 2018; Pretorius et al., 2013; van Niekerk and Venter, 2011). The prevalence of emergent genotypes on a global scale, facilitated by duplication events, may result in a competitive edge through selection advantage (Schobel et al., 2016). This advantage may be attributed to an augmented viral attachment. A potential consequence of this phenomenon is the increased spread and transmission of the emergent genotypes. In addition to the current genotypes, novel genotypes sporadically arise and intermittently supplant the prevailing strains, thereby becoming the predominant circulating variants on a global scale (Yu et al., 2021).

The G gene's genetic drift is widely acknowledged as the primary catalyst for the development of regional variations, subsequently leading to the seasonal resurgence of the pathogen and the occurrence of new epidemics (Kombe et al., 2021; Piedimonte and Perez, 2014). Currently, a definitive consensus regarding the criteria for genotyping has not been established (Goya et al., 2023; Ramaekers et al., 2020; Schobel et al., 2016). According to the recently suggested classification of genotypes by Goya and colleagues was to define clades through G ectodomains and the classification system which reduced the known genotypes three-fold and designated the genotypes into sub genotypes and lineages (Goya et al., 2020).

2.3.3. Genomic Structure and Characteristics of RSV

The RSV virion is a spherically shaped enveloped virus with a non-segmented negative-sense single-stranded RNA genome of approximately 15,200 nucleotides in length (Figure 2.2) (Agoti et al., 2017; Azzari et al., 2021; Collins and Melero, 2011; Rios Guzman and Hultquist, 2022; Schobel et al., 2016). The virus has particles with pleomorphic properties with either the spherical particles ranging from 100-350 nm in diameter or the long filaments, which are the dominant form, measuring approximately 60-200 nm in diameter and 1-10 um in length (Cao et al., 2021; Jeffree et al., 2003; Ke et al., 2018; Liljeroos et al., 2013). Like other RNA viruses, RSV possesses a genome that is highly mutable due to its reliance on an RNA polymerase that is incapable of proofreading and editing RNA (Falsey and Walsh, 2000).

Moreover, it is characterised by ten genes, encoding 11 proteins with 11 open reading frames (ORF), and these are sequentially arranged and transcribed in the following order Ns1, Ns2, N, P, M, SH, G, F, M2 (−1, −2), and L (Figure 2.2) (Mastrangelo et al., 2021; Rameix-Welti et al., 2014; Thornhill and Verhoeven, 2020).

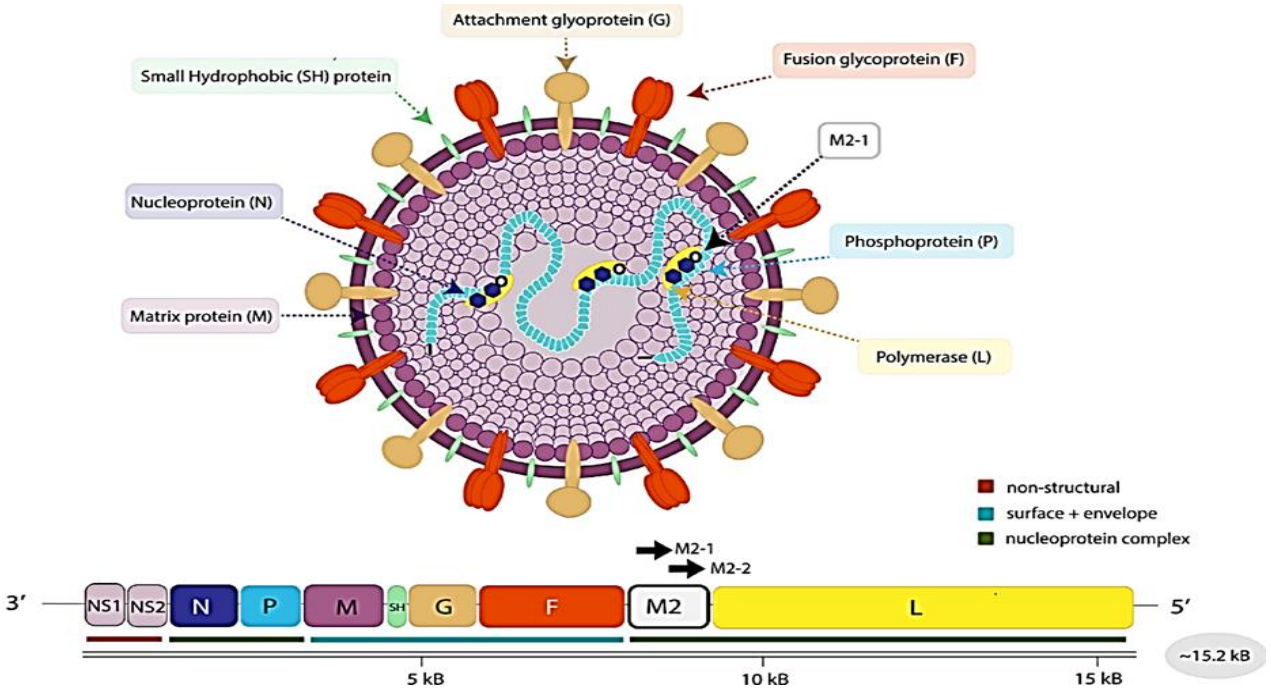

Figure 2.2: Schematic diagram of the RSV genome structure and organisation. The gene sequence of the virus exhibits a 3′-5′ orientation, with noncoding terminals and intergenic sections interspersed between each gene. Every gene, except for the M2 gene, translates into a single matching protein, with the M2 gene being the only exception due to its two overlapping open reading frames (ORFs) that encode two proteins. Specifically, the arrangement of the genes is as follows: NS1-NS2-N-P-M-SH-G-F-M2-L. Adapted from (Rios Guzman and Hultquist, 2022).

2.3.3.1 Structural and Non-structural Proteins of RSV

The RNA genome encodes non-structural and structural proteins. The non-structural proteins (NS1 and NS2) inhibit or interfere with interferon responses and allow the virus to escape innate immune responses (Mejias et al., 2019; Polak, 2004). In addition, these proteins aid in promoting viral replication by prolonging the process of apoptosis of infected cells (Bitko et al., 2007). There are structural proteins embedded internally, nucleoprotein (N), phosphoprotein (P), large RNA polymerase (L), and transcription factor (M2-1) (Liljeroos et al., 2013), as shown in figure 2.2. The M and N proteins are functional and necessary in the polymerase complex. The P and L proteins are primarily required, essential, and sufficient for RNA replication (Bouillier et al., 2019). Also, the

envelope is surrounded by three distinct viral transmembrane proteins, exposed surface glycoprotein (G), fusion protein (F), and the small hydrophobic (SH) protein (Shown in fig 2.2) (Divarathne et al., 2019; Jung et al., 2020). Furthermore, the SH protein was recently proposed to be an ion channel similar to viroporin, disrupting the host cell's regular functioning and making infection feasible (Thornhill and Verhoeven, 2020). The attachment G protein consists of a short cytoplasmic domain, transmembrane domain, and two hypervariable mucin-like domains linked by a conserved sequence involved in cell binding (Schobel et al., 2016). The RSV-F promotes virion attachment, but to a much lesser extent than G, its key function is to mediate the fusion of the virus and host cell membrane (Shown in fig 2.2) (Divarathne et al., 2019). The F glycoprotein is the major viral neutralization and protective antigen, followed by the G glycoprotein (Collins and Melero, 2011). The M protein functions as a non-glycosylated structural protein lining the inner leaflet of the viral envelope, and it associates with the cytoplasmic domain of the F protein and assembly of viral particles (Baviskar et al., 2013; Gomez et al., 2014; Shaikh et al., 2012). Each gene encodes a unique single protein except for the M2 protein which has two open reading frames (M2-1 and M2-2), that slightly overlap (Shown in figure 2.2) (Shook and Lin, 2017).

2.3.3.2. The Attachment and Fusion Glycoproteins of RSV

The attachment glycoprotein (G) and fusion (F) proteins are the primary RSV neutralizing antigens and play a crucial role in providing protection against the virus (Ha et al., 2020). The glycoprotein (G) is an integral protein II that is essential for viral fusion and consists of 298 amino acids (Bohmwald et al., 2016). On the other hand, the F protein is responsible for viral penetration and syncytium formation (Gagliardi et al., 2017). Among RSV strains, the G protein exhibits the most variation in its structure, which in turn determines the antigenic differences between RSV-A and RSV-B (Battles and McLellan, 2019). The extracellular portion of the G protein is composed of a central conserved domain and two hypervariable regions that contain numerous N- and O-glycosylation sites (Griffiths et al., 2017; McLellan et al., 2013; Pangesti et al., 2018). These glycosylation sites contribute to the antigenicity of the G protein (Griffiths et al., 2017).

In contrast, the F protein demonstrates a notable degree of genetic and antigenic stability, remaining conserved among RSV-A and B strains (Hause et al., 2017). This characteristic renders it a prime candidate for the development of vaccines and monoclonal antibodies (Andreano et al., 2021). The synthesis of the 574-amino-acid polypeptide, known as F0, which gives rise to the RSV F protein, is accompanied by, or undergoes post-translational modifications involving the addition of five or six

N-linked glycans (Kimura et al., 2017; Leemans et al., 2018). The F protein further plays a crucial role in facilitating the fusion of viral and host cell membranes, as well as the formation of syncytia (Busack and Shorr, 2022). This mature F protein exists in both prefusion and post fusion conformations (Mastrangelo et al., 2021).

2.3.4 Replication Cycle of RSV

2.3.4.1. Attachment

The viral attachment process and receptors utilised in RSV binding are not fully understood for members of the *Pneumoviridae* family (Rima et al., 2017). The important process through which the host cell entry is initiated is through the attachment of the G protein to host cell surface molecules (Figure 2.3) (Battles and McLellan, 2019), and this binding triggers the fusion of the virus with its host cell membranes (King et al., 2021).

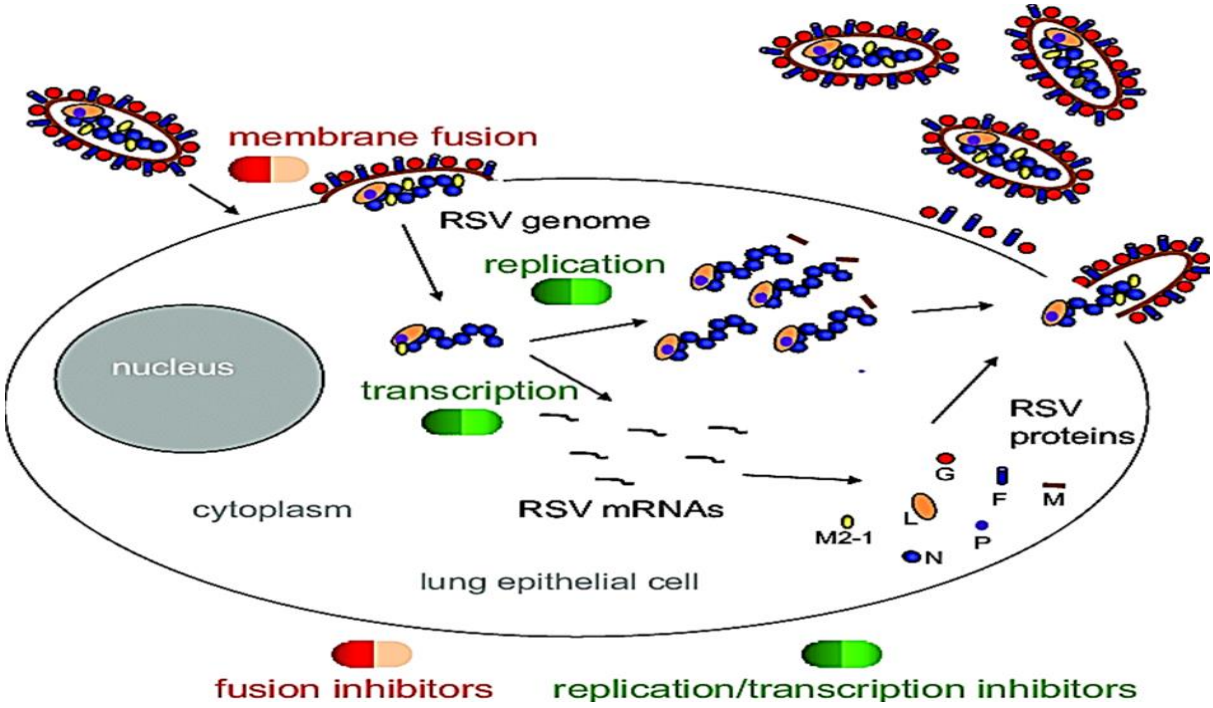

Figure 2.3: Replication cycle of RSV. The attachment glycoprotein attaches to the CX3CR1 chemokine receptor located on the apical surface of ciliated epithelial cells. The virion fuses with the cell membrane and enters the cell, one of the last events of virus entry that must take place for successful replication of RSV in the host cell. Transcription and replication occur simultaneously (Battles and McLellan, 2019).

Numerous potential cellular receptors involved in RSV entry have been described, including annexin II, epidermal growth factor (EGF) receptor, Toll-like receptor 4 (TLR4) (Kurt-Jones et al., 2000), intercellular adhesion molecule 1 (ICAM-1) (Behera et al., 2001), nucleolin and heparan

sulphate proteoglycans (HSPGs) (Johnson et al., 2015). The glycoprotein and fusion protein facilitate the virus attachment and cellular entry by primarily binding to viral cell receptors comprising heparin-binding domains and glycosaminoglycans as well as other types of cellular receptors such as CX3CR1 (C-X-3 chemokine receptor 1), and nucleolin (Griffiths et al., 2020). The G protein contains a CX3C motif that can bind the CX3CR1 receptor on host cells; mutation of this motif or inhibition of the G-CX3CR1 interaction with a blocking anti-CX3CR1 antibody is reported to reduce RSV infection (Boyoglu-Barnum et al., 2017).

2.3.4.2. Viral Fusion

After the attachment process, the obligatory step is the fusion of the viral and cellular membrane to accommodate the entry of the ribonucleoprotein (RNP) into the cytoplasm (Battles and McLellan, 2019; Hu et al., 2020). The viral fusion is triggered by the F-protein which promotes viral and host cell membranes to fuse on the cellular surface. This fusion process is essential for an enveloped virus as it results in entry and infection (Cullen et al., 2017). Virus cell membrane fusion is triggered by receptor binding in a pH-independent manner (Griffiths et al., 2017). Following fusion, the helical RNP is released into the cytoplasm of the host cell through membrane fusion (Shown in fig 2.3) (Battles and McLellan, 2019). The viral envelope fuses with the host cell at or near the plasma membrane in extremely dynamic lipid raft domains and forms part of the cell membrane (San-Juan-Vergara et al., 2012). Due to the development of a fusion pore, membrane fusion allows the virion capsid to be delivered into the host cells (Griffiths et al., 2017). Since evidence exists that RSV uses different mechanisms to enter the cell, the location of RSV fusion must be clarified (Van Der Gucht et al., 2017).

2.3.4.3. Viral Transcription, Translation, and Genome Replication

Immediately after viral entry, the viral replication complex is formed on intracellular membranes and is composed of the virus and specific host cell proteins (Munday et al., 2015). Replication is carried out through the viral RNA encoded RNA polymerase L and its cofactor phosphoprotein (Gilman et al., 2019; Shook and Lin, 2017). The interaction between the N protein and the RNA genome binding hinders the formation of a double-stranded structure (Bohmwald et al., 2016). Viral replication and transcription take place in the host cell cytoplasm, and infection within the cells results in virally prompted cytoplasmic inclusions known as inclusion bodies (IBs) (Cervantes-Ortiz et al., 2016; Rincheval et al., 2017). The IBs serve as the sites for viral transcription and replication, while also housing most of the viral proteins within the replication complex (Van Royen et al., 2022).

An RNA synthesis ribonucleoprotein (RNP) complex, comprising the N, L, P, and M2-1 proteins, is employed in viral production (As shown in figure 2.3) (Cao et al., 2021).

Both transcription and replication processes take place simultaneously (Collins et al., 2013). The virus may develop in enucleated cells in the presence of actinomycin D, demonstrating that nuclear involvement is not required for genome transcription and replication of RSV (Collins et al., 2013). The RSV nucleocapsid protein facilitates the reverse transcription of the virus single strand negative sense RNA (-RNA) into complementary DNA (cDNA) within the cytoplasm of the infected cell. This cDNA then replicates into positive double strand DNA (dsDNA). Once the positive dsDNA is transcribed into mRNA, the host cell translation machinery, which includes transfer RNA (tRNA) and ribosomal RNA (rRNA), along with free amino acids, are utilised for the synthesis (translation) of virus proteins (Oshansky et al., 2009). It contains noncoding sections at the 3' end and 5' end of its genome, known as the leader and tailer regions, respectively (Shang et al., 2021). The RNA-dependent RNA polymerase (RdRp) transcribes viral sub-genomic mRNA in addition to replicating and synthesizing full-length and positive sense antigenome intermediates that are required for replication and the formation of new negative-sense genomes packaged into virions (Noton and Fearns, 2015; Van Royen et al., 2022). Notably, the method by which the polymerase complex transitions from replication to transcription is still unknown (Bermingham and Collins, 1999; Shahriari et al., 2016; Shang et al., 2021).

The presence of newly generated soluble viral N and P proteins stimulates the elongation of RNA replication products, resulting in full-length encapsulated antigenomes and genomes (Fearns and Deval, 2016). Although the non-structural proteins NS1 and NS2 are not directly involved in RNA replication, they do aid RSV replication, these proteins mediate RSV replication by altering type I IFN production and signalling in the host (Van Royen et al., 2022). The N protein binds to the genome and antigenome independently throughout their full length to produce stable nucleocapsids (Collins et al., 2013). Additionally, the M protein plays a crucial role in facilitating viral assembly, thereby being indispensable for the replication of the RSV genome (Förster et al., 2015).

The F, M, N, and P proteins are the minimal viral proteins required to form virus-like particles able to transport the viral genomes to their respective target cells (Shahriari et al., 2016), and their expression stimulates the development of viral filaments. Moreover, the RNP complex associated with the RNA genome is assembled into viral filaments and transported to the plasma membrane assembly sites to develop new viral RSV particles (As shown in figure 2.3) (Fearns and Deval, 2016;

Vanover et al., 2017). In the absence of any other viral component, the RSV F protein located on the surface of the cell is sufficient to initiate fusion, leading to the formation of syncytia (Langedijk and Bont, 2023; Walsh and Hall, 2015). RSV is filamentous when it is first released from infected cells, even across several viral strains, irrespective of the cell line, or host cell polarization state (Shang et al., 2021).

2.4. Diagnosis, Therapeutic Options, and Prevention of RSV

2.4.1. Applicable Samples and Techniques used in the Diagnosis of RSV Infections

Accurate diagnosis of RSV infection depends on the identification of the virus, virus antigens, or virus-specific nucleic acid in respiratory secretions (Popow-kraupp and Aberle, 2011). RSV testing is not routinely recommended and often not performed; therefore, RSV is likely underdiagnosed and attributed to the infrequent administration of RSV testing and the lack of universal recommendations for its use (Binder et al., 2017). In clinical paediatric practice, obtaining prompt laboratory confirmation of RSV infection leads to a decrease in the number of ancillary tests, reduced antibiotic usage, and shorter hospitalization periods (Byington et al., 2002; van Houten et al., 2019). To date, no clinical symptoms have been reported to practically differentiate RSV infection from other respiratory infections except for diagnosis (Korsten et al., 2021). Diagnostic testing has become standardized and prevalent in recent years, encompassing the collection of specimens from the upper respiratory tract. These specimens include nasopharyngeal aspirates (NPA), nasopharyngeal swabs (NPS), nasal swabs (NS), nasal washes (NW), or oropharyngeal swabs (OPS) (Onwuchekwa et al., 2023). Although NW or tracheal secretions are more reliable for confirming RSV than NS (Eiland, 2009; Lambert et al., 2008), NS are more frequently used because they are a convenient method due to their ease of use. In intubated patients, it is recommended to collect lower respiratory samples as they tend to have a higher concentration of viral replication in the later stages of the disease (Nam and Ison, 2019).

Although nucleic acid amplification methods have revolutionised RSV diagnosis and are increasingly sensitive and specific, which provide rapid results, they have gradually replaced cell culture isolation, which was once considered the gold standard for RSV identification (Chartrand et al., 2015; Henrickson and Hall, 2007). Reverse transcription polymerase chain reaction (RT-PCR) was the first and is currently widely employed in nucleic acid assays and as the gold standard. Rapid antigen detection tests (RADT) and Direct fluorescent antibody (DFA) are molecular methodologies that have recently been used due to their high sensitivity (Prendergast and Papenburg, 2013; Shafik

et al., 2011). The lack of standardised clinical case definitions, due to the nonspecific nature and presentation of RSV symptoms makes the diagnosis of RSV infections even more difficult (Sáez-López et al., 2019).

2.4.2. Therapeutic Options for RSV

Like other numerous viral respiratory illnesses, RSV is inherently self-limiting in nature. The recommended measures are supportive care which includes supplemental oxygen, mechanical ventilation, and hydration, when necessary, until the patient's condition improves (Baraldi et al., 2022; Ralston et al., 2014; Simoes et al., 2018; Turnham et al., 2017). Currently, the only approved treatment for RSV disease is aerosolized ribavirin, an antiviral agent to treat RSV infection in high-risk groups such as preterm infants who are susceptible to getting life threatening complications (Behzadi and Leyva-Grado, 2019; Domachowske et al., 2021). Ribavirin is the only currently licensed therapeutic that was recommended for the treatment of RSV RTI and a nucleoside analogue with broad-spectrum antiviral activity against pathogens causing RTI (Cunningham et al., 2021; Elawar et al., 2021). Ribavirin can be administered in three different forms, aerosolised, oral, and intravenous, of which the latter is of limited availability, therefore is not FDA approved (Foolad et al., 2019). Aerosolized ribavirin has been licensed by the FDA for use in new-borns and children, but not in adults (Foolad et al., 2019; Stein et al., 2017; Tejada et al., 2022). Notably, ribavirin has limited and questionable efficacy in treating RSV, therefore available clinical guidelines advise against its routine use (Cunningham et al., 2021; Simoes et al., 2018). In addition, ribavirin is associated with significant side effects such as bronchospasm, nausea, and anaemia which can be adverse in individuals suffering from RSV infection (Hayden and Whitley, 2020; Trang et al., 2018; Turner et al., 2014). For early infection management, administration of antivirals within 24 to 48 hours after the onset of symptoms is advised to limit the progression of RSV LRTI in infants (Löwensteyn and Bont, 2020).

2.4.3. Prevention of RSV Infection

Reducing the disease burden caused by RSV remains a high priority, and it is crucial to prioritise prevention strategies, despite the lack of effective treatment options for RSV in newborns (Giersing et al., 2019; Gunatilaka and Giles, 2021; Messina et al., 2022). The Centers for Disease Control and Prevention (CDC) and the American Academy of paediatrics (AAP) recommend that caregivers practice good hand hygiene, limit tobacco smoke exposure, and avoid overcrowded settings, including day care, when possible, to prevent transmission of RSV among high-risk infants (Forbes

et al., 2008; Simoes et al 2018; Goldstein et al., 2017). Palivizumab (Synagis, AstraZeneca), a licensed prophylaxis that has been in existence for a considerable period, is an anti-RSV monoclonal antibody. It is administered to infants for a duration of 5 months, starting from the commencement of the RSV season. In 1998, palivizumab was licensed by the FDA, then used in numerous countries worldwide as a prophylactic prevention of RSV LRTI in specific paediatric population at risk of developing RSV severe disease (Andabaka et al., 2013; Cadena-Cruz et al., 2023; Elawar et al., 2021; Ginsburg and Srikantiah, 2021). This intramuscular recombinant antibody mainly targets the surface of the RSV-F protein (Chatterjee et al., 2021). In addition, palivizumab is exclusively administered to high-risk new-borns because of its high cost, poor efficacy, and not accessible for widespread use in LMICs, with a monthly dosing requirement in infants (Busack and Shorr, 2022; Garegnani et al., 2021; Homaira et al., 2014; Saravanos et al., 2019; Simoes et al., 2018). Interestingly, the use of palivizumab has had a significant decrease in poor health outcomes including a lower incidence of ICU admissions for RSV disease, reduced duration of RSV related hospitalization, the need for oxygen supplementation and hospitalisation days attributable to moderate or severe RSV infection (Garegnani et al., 2021). Several studies have described the cost effectiveness relative to the RSV medication, that the more risk groups are target, the more cost-effective immunoprevention is (Kim et al., 1969; Wang et al., 2011).

In this regard, a new monoclonal antibody, known as Nirsevimab under the trade name Beyfortus, been FDA approved to prevent the RSV LRT disease in neonates and infants during their initial RSV season. Nirsevimab, a mAb that confers passive immunity by specifically targeting prefusion conformation RSV F-protein (Levien and Baker, 2023) which offers a single dose of protection against hospitalization and infection caused by RSV in infants for the duration of the entire RSV season (Hammitt et al., 2022; Sun et al., 2023). The currently available evidence regarding Nirsevimab demonstrates that this drug has indeed the potential to impact significantly on the prevention of RSV bronchiolitis not only in preterm infants but also in healthy, term infants, born both during the epidemic season for RSV and outside the epidemic season (Esposito et al., 2021). The passive immunisation programmes targeting the first six months of life could have a substantial effect on reducing RSV morbidity and mortality burden (Li et al., 2022). New RSV prevention technologies in the pipeline include maternal vaccination and long-acting Mab (Vekemans et al., 2019).

2.4.4. Approaches in RSV Vaccine Development

Despite over 50 decades of global tremendous effort in the development of an effective vaccine, to date, no licensed vaccine is commercially available for the prevention of RSV infection (Langedijk et al., 2020; Tang et al., 2019). Approximately 33 candidate vaccines are currently under clinical development and divided into six categories which are recombinant vector, live attenuated, chimeric, subunit, particle-based, and nucleic acid vaccines, and monoclonal antibodies (Figure 2.4.) (Andabaka et al., 2013; Mazur et al., 2018).

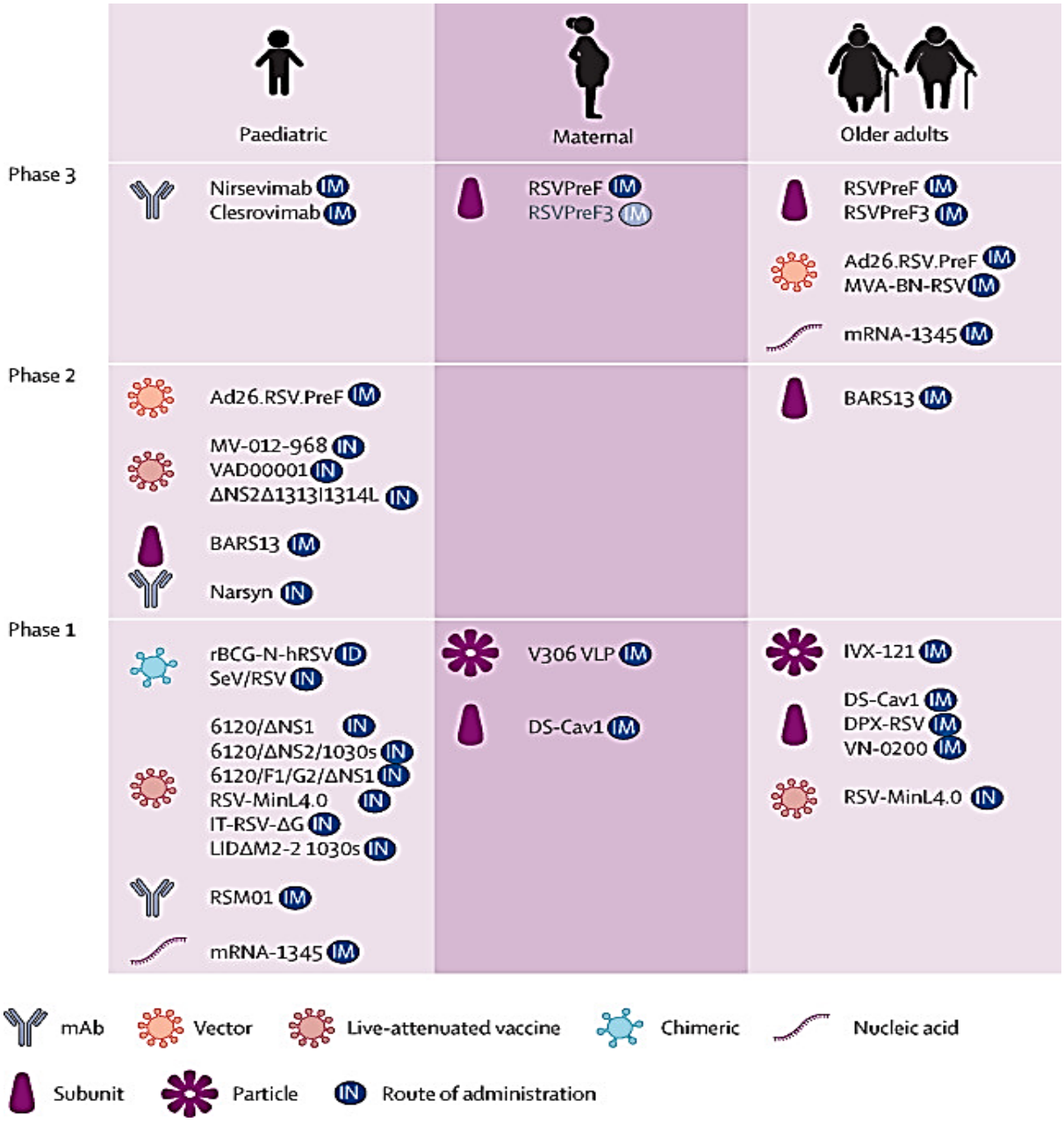

Figure 2.4: RSV vaccination and monoclonal antibody agents tailored to the target population. The ongoing research and development activities in the field of RSV vaccines hold great promise for the future, offering hope for improved prevention and control of RSV-related illnesses (Mazur et al., 2023).

The challenges associated with RSV vaccine development, include concerns regarding live virus-free vaccine in young children leading to enhanced RSV disease (ERD) with more severe illness (Muralidharan et al., 2017), difficulty in inducing and evaluating protective immunity and the cost of required clinical trials (Boyoglu-barnum et al., 2019). While many of present vaccinations are designed to protect a single major demographic, RSV vaccines will almost certainly need to protect three different groups: young infants (those born between the ages of three and six months), older infants and toddlers, pregnant women and the elderly (Figure 2.4) (Mazur et al., 2018), a situation which further complicates the development of vaccines suitable for three groups.

2.4.4.1. Paediatric Population

The use of monoclonal antibodies (mAbs) for passive immunoprophylaxis in new-borns under six months of age and live-attenuated vaccines (LAVs) for active immunisation in older infants (over six months) are leading approaches in the paediatric population (Mejias et al., 2019). Live-attenuated and chimeric virus vaccinations are largely targeted for the paediatric group, because of RSV-naive new-borns' shows incapacity to prime for vaccine-enhanced illness (Karron et al., 2013). LAVs are designed to mimic natural infections but with reduced virulence, aiming to stimulate a robust immune response that encompasses both local mucosal antibody and cellular reactions (Kerron et al., 2021). Chimeric live viral vaccine candidates, possessing satisfactory safety attributes, demonstrate the expression of RSV proteins within attenuated viruses of a similar nature (Clark and Guerrero et al., 2017). In the pursuit of global accessibility, the affordability aspect remains a pivotal consideration in the advancement of molecularly imprinted bags (Verwey et al., 2023). In most LMICs, monthly intranasal mAb administration or the use of a palivizumab biosimilar may be subject to programmatic limitations (Ananworanich and Heaton, 2021; Gieber et al., 2023). The epitopes for monoclonal antibodies (mAbs) currently under development exhibit a high degree of conservation, with minimal occurrence of naturally resistant strains (Ananworanich and Heaton, 202). These resistant strains have demonstrated comparable or lower viral fitness *in vitro* when compared to non-resistant viral strains (Mason et al., 2018).

2.4.4.2. Maternal and the Elderly

Recent studies and advancements have primarily concentrated on two approaches to combat RSV infection in infants. The first approach involves maternal immunization, wherein the levels of anti-RSV maternal antibody transferred through placental transfer are enhanced (Etti et al., 2022). The second approach, known as passive immune prophylaxis, entails the administration of a RSV

neutralizing antibody to the new-borns shortly after delivery (Gunatilaka and Giles, 2021). These strategies aim to bolster the infant's immune response and provide protection against RSV infection. (Tang et al., 2019). Due to the potential for vaccine-enhanced illness in RSV-naive children, it is recommended that particle-based and subunit-based vaccinations be reserved for the elderly or maternal immunization (Battles et al., 2019). The vaccine currently undergoing clinical development is the particle-based vaccine, which consists of a recombinant RSV post-F nanoparticle and an adjuvant (Mejias et al., 2019). Subunit vaccines demonstrate efficacy by employing the pre-F stabilised configuration as the primary antigen, alongside adjuvant proteins and a refined iteration of the pathogen (Killikelly et a., 2020). This amalgamation has exhibited encouraging outcomes in terms of immunogenicity and protection against the targeted pathogen (Qamar et al., 2020). In conclusion, ongoing late-stage studies are exploring three immunisation strategies that employ the pre-F antigen, namely nucleic acid, subunit, and vector-based vaccines, for the elderly population (Killikelly et a., 2020). Additionally, particle-based vaccines utilize particle assembly to display multiple antigens, thereby exhibiting promising immunogenic potential (Mazur et al., 2023). Furthermore, apart from candidate vaccines that would be made available to confer immunity to individuals during RSV season and prevent respiratory syncytial virus infection, the FDA approved a novel RSV vaccine known as ABRYSVO (Ernst, 2023; Ruckwardt, 2023). This vaccine is intended for immunising all healthy individuals aged 60 years and above, to induce immune response for prevention of RSV infections (Walsh et al., 2023). Furthermore, a second vaccine for RSV known as AREXVY, which is a recombinant fusion (F) glycoprotein, this vaccine has been recently approved by the FDA for individuals aged 60 years and above (Venkatesan, 2023; Ruckwardt, 2023). Another, RSV vaccine called ABRYSVO has been approved for pregnant women who are in their 32 to 36 weeks of pregnancy, as it helps protect new-born babies and infants from RSV infection (Kampmann et al., 2023). The main rationale to administer the ABRYSVO vaccine to expectant mothers during their third trimester is to enhance their immunity against RSV and subsequently transfer this protective immunity in the form of antibodies to the unborn child before delivery (Ruckwardt, 2023).

2.5. Whole Genome Sequencing of RSV and Sequencing Technologies

The comprehension and decoding of the code that encodes all forms of life on earth, along with the ability to detect and treat genetic diseases, is made possible by the breakthrough of DNA sequencing. These sequences, referred to as reads, consist of strings composed of the five letters (A, T, C, G) (Kchouk et al., 2017). The letter N is used where an element of uncertainty within the sequence

happens (Kchouk et al., 2017). Currently, the scarcity of whole genome sequences virtually limits our knowledge of the circulation and impact of RSV (Di Giallonardo et al., 2018; Eden et al., 2022; Trovão et al., 2019). Whole genome sequencing gives detailed sequence data that can inform, and describe molecular epidemiology and transmission dynamics of viruses, which collectively provide new strategic approaches for virus control and vaccine development (Ferdinand et al., 2021). Besides dearth of information exists on WGS of RSV in South Africa with few genomes available in public databases. Currently, there are only approximately 2200 whole-genome sequences of RSV available, even though RSV was identified in 1955 and these sequences can be accessed through the Global Initiative on Sharing All Influenza Data (GISAID) (Rios Guzman and Hultquist, 2022), as opposed to other respiratory pathogens such as SARS-CoV-2. The dearth of RSV sequences impedes our understanding of global RSV diversity, describing genotypes, and evolutionary dynamics of RSV (Patil et al., 2017).

The WGS provides a useful tool for global surveillance to better understand the evolution and epidemiology of RSV and provide essential information that may impact antibody treatments, antiviral drug sensitivity, and vaccine effectiveness (Pangesti et al., 2018). Analysis of genomic sequences is crucial for the identification of novel circulating strains and tracking viral transmission patterns including monitoring crucial strains with important antigenic sites in the G-gene (Ludlow, 2023; Otieno et al., 2018). Previous studies have applied whole genome sequencing to determine epidemiological, molecular, and evolutionary dynamics and distinct patterns of RSV (Bose et al., 2015; Otieno et al., 2018; Tulloch et al., 2021). The need for RSV sequencing and capacity in developing countries has been unmet. Previous sequencing efforts for RSV have predominantly concentrated on the comprehensive or partial sequencing of the G gene, which spans a range of 200 to 300 nucleotides (bp) (Muñoz-Escalante et al., 2019; Schobel et al., 2016). Although a vast wealth of sequence data is available to examine the evolutionary and phylodynamic of the RSV G gene, whole RSV genome sequencing has been less common as compared to other RNA respiratory viruses such as SARS-CoV-2 (Goya et al., 2023; Rios Guzman and Hultquist, 2022). Additionally, it is crucial to consider the potential influence of evolutionary signals originating from alternative gene regions when examining the phylogeny inferred solely from the complete or partial G gene (Chen et al., 2022). This is because the inferred phylogeny from other genes may potentially contradict the phylogeny inferred from the G gene alone (Chen et al., 2022).

Analysis of the RSV full genome sequences performed by Elawar and colleagues identified circulating serotypes and genotypes, which could have implications for vaccine development (Elawar et al., 2017). Similarly, an analysis of RSV genomes by Madi et al (2018) found high genetic variability of RSV type A as compared to RSV type B and co-circulating multiple clades (Madi et al., 2018). Overall, these studies highlight the need for advanced genome sequencing techniques to efficiently identify and detect circulating serotypes (Agoti et al., 2015; Tulloch et al., 2021). NGS is highly applicable for WGS, which yields faster and more accurate results (Kchouk et al., 2017). To appropriately characterise strains circulating in different or specific regions requires powerful tools such as NGS, which would generate data suitable for diversity interrogation.

2.5.1. First Generation Sequencing Technologies

First generation sequencing was initiated and developed by Frederick Sanger and colleagues in 1977. The Sanger sequencing method is also known as chain-terminating dideoxynucleotides (ddNTP) or sequencing by synthesis technique and has been widely employed for many years to sequence low-throughput or individual DNA samples (Kchouk et al., 2017; Sanger et al., 1977; Straiton et al., 2019). This method involved the incorporation of termination specific bases T, C, G, or A chain inhibitors that block DNA polymerization (Garrido-cardenas et al., 2017). The dNTPs are distinctly labelled with ddG, ddA, ddT, and ddC to correspond to each DNA base, with no hydroxyl group. The ddNTPs are like regular deoxynucleotide (dNTP), which the DNA polymerase uses to synthesize or elongate a new DNA strand from the template strand (Garrido-cardenas et al., 2017).

Alternatively, a method developed by Maxam and Gilbert is the modification of DNA and subsequent base specific chemical cleavage of DNA backbone at sites adjacent to the modified nucleotides (Maxam and Gilbert, 1977; Slatko et al., 2018). The DNA fragments obtained are separated based on the size and analysed using gel electrophoresis to determine the actual sequence (França et al., 2002). The visualization of fragments is based on radioactive labelling, followed by the introduction of dyes with fluorescence into the reaction to allow proper imaging (El-Metwally et al., 2014; Mcginn and Gut, 2013). Sanger sequencing technology is still applied in various laboratory settings, due to its high efficiency and low radioactivity (Kchouk et al., 2017). The Sanger sequencing technique, despite not being suitable for high throughput applications, remains immensely advantageous (Saini et al., 2023).

2.5.2. Second Generation Sequencing Technologies

The emergence of advanced NGS technologies has contributed to increased efficiency with massive sequencing data output (Straiton et al., 2019). With its high-throughput system, NGS allows the generation of millions of short reads per sequencing run from multiple samples in parallel (Hu et al., 2021; Kchouk et al., 2017). Herein, is an overview of the most used NGS technologies with a brief description of their performance.

The Illumina platform, a renowned biotechnology company based in San Diego, California, provides a widely used range of sequencing platforms, including iSeq, MiSeq, MiniSeq, NextSeq, HiSeq, and NovaSeq. These platforms have gained immense popularity and are extensively utilized in various published series (Gu et al., 2019). The Illumina sequencing technology holds a prominent position in the field of genomics due to its exceptional flexibility and widespread utilization. In the initial stage, the DNA samples undergo a process of random fragmentation into sequences, followed by the ligation of adapters to both ends of each sequence (Caruccio, 2011). In the subsequent phase, the amplification of every sequence linked to the solid plate is conducted through the utilization of "PCR bridge amplification (Uhlen and Quake, 2023). This process generates numerous replicas of each sequence, thereby enhancing their abundance (Uhlen and Quake, 2023). Consequently, a cluster is formed, comprising a compilation of sequences originating from a common source sequence. (Gu et al., 2019; Hu et al., 2021; Vincent et al., 2017). It employs a sequence by synthesis approach and reversible terminators whereby the nucleotides, primers, and DNA polymerase are part of the sequencing reagents, thus the primers eventually hybridize into the sequences (Gu et al., 2019). It allows DNA generated fragments to be independently sequenced as fluorescently labelled nucleotides incorporated on the growing strand on a slide. Lasers are employed to excite clusters, inducing the emission of distinct light signals corresponding to individual nucleotides (Rajesh and Jaya, 2017). Subsequently, a coupled-charge device (CCD) camera captures these signals, which are subsequently translated into a nucleotide sequence through computer programs (Kchouk et al., 2017).

The Ion Torrent technology by Thermo Fisher performs sequences by synthesis, while detection is performed during DNA polymerisation by using solid-state pH meters to measure released hydrogen ions (Rothberg and Myers, 2011). With a throughput of up to 10 Gb for ion proton sequencers, the Ion Torrent sequencers can generate reads with lengths of 200 bp, 400 bp, and 600 bp (Kchouk et al., 2017). The main benefits of Ion Torrent are its faster sequencing times of 2 to 8

hours and greater read lengths compared to other SGS sequencers. However, its drawback includes the inherent challenges of deletion and insertion errors that arise due to homopolymer sequences (Kchouk et al., 2017; Reuter et al., 2015). The ABI/SOLiD sequencing technology adopts sequencing by ligation (Levy and Myers, 2016). The ABI/SOLiD platform draws its advantage from double base reading which results in high accuracy, however, its lengthy run time and the relatively short reads it generates are its major drawbacks. This technology is also known to have base identification errors, which is due to the noise generated during the ligation cycle (Goodwin et al., 2016).

Next generation sequencing is invaluable and has allowed the identification of novel variant strains and detection of genes that cause diseases (Straiton et al., 2019). Although, SGS has its limitations such as introductions of nucleotide substitutions during sequencing (Goodwin et al., 2016). It is still the most widely used platform compared to first generation sequencing and has significant throughput and accuracy in DNA sequencing, especially in genomics and clinical settings (Pervez et al., 2022; Satam et al., 2023).

2.5.3. Third Generation Sequencing Technologies

Third generation sequencing (TGS) is creating a new resurgence and producing long reads of unprecedented quality to circumvent limitations experienced with SGS (Satam et al., 2023; Van Dijk et al., 2018). This technology allows sequencing without using the PCR amplification step and generates results efficiently and faster than other second-generation technologies (Giani et al., 2020). The TGS can generate longer reads that can go beyond kilobases, allow improved assembly of the reads, and resolve complex genomes with repetitive distinct regions (Van Dijk et al., 2018). The commonly employed sequencing platforms in the field of biosciences are Oxford Nanopore and Pacific Biosciences SMRT.

Pacific Biosciences released a single molecule real-time platform in 2011; an LRT that incorporates different nucleotides into DNA strands while monitoring polymerase activity (Rhoads and Au, 2015). While this technology comes with several third generation sequencing advantages, including speed (Liu et al., 2012), the platform often has randomly distributed deletions and insertion errors across the long read (Koren et al., 2013).

Oxford Nanopore Technologies (ONT) also introduced nanopore sequencing called MinIONs. In this technology, DNA bases are detected by measuring generated electrical conductivity as DNA flows through the biological pore (Lu et al., 2016). Low cost and sample size are among the

advantages offered by this sequencer: While MinION can provide very long reads with the ability to resolve contiguity of *de-novo* assembly; its drawback includes a high error rate that includes deletions, insertions, and mismatches (Ip et al., 2015). The ONT has continued to evolve with the introduction of sequencers such as PromethION, GridION X5, and SmidgION, which can be operated with smartphones (van Dijk et al., 2018). With longer-read technologies, genome regions can be fully resolved, and complex transcriptomes can be explored in detail (van Dijk et al., 2018).

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Preface

The work presented here in chapter 3 is a detailed description of the optimization and validation of the laboratory technique adopted in this thesis and is presented in three different sections.

Detailed authors contributions are here-in described.

Authors detailed Contributions:

- **Conceptualisation:** Martin M. Nyaga, Hlengiwe Sondlane
- **Optimization and Validation:** Hlengiwe Sondlane
- **Initial writing:** Hlengiwe Sondlane
- **Review and editing**: Martin M. Nyaga, Hlengiwe Sondlane, Dominique Goedhals, Armand Bester.

CHAPTER THREE: DETAILED METHODS OF OPTIMIZATION, VALIDATION AND ADOPTED METHODOLOGY

Section A: Study Design, Patient Recruitment and Ethical Considerations

3.1. Study Design and Patient Enrolment

This project was an experimental descriptive study, which served as a sub-study of a pre-approved ongoing project by the University of the Free State Health Science Research Ethics Committee (HSREC: UFS-HSD2020/0952/2807). The main study project commenced in 2020 wherein, it involved the collection of nasopharyngeal swabs from children aged 0-12 years presenting with respiratory distress requiring hospitalization at Pelonomi Hospital, Universitas Academic Hospital, or National District Hospital. Prior to enrolment, parents or legal guardians provided signed written informed consent on behalf of the children, after being fully informed of the procedures, in the three languages which predominate in the area namely, English, Afrikaans, or Sesotho. A trained clinician recruited the participants and collected samples using a sterile dry flexible flocked swab and placed in a sterile container. Following sample collection, samples were placed in a container at 4 °C, then transported on ice to the NHLS laboratory Division of Virology, National Health Laboratory Services (NHLS), Universitas, Bloemfontein, for routine viral diagnostic testing. On receipt in the diagnostic laboratory, each respiratory sample swab was suspended and vortexed in 2 mL of sterile saline for further processing. The swabs were tested for common respiratory viruses including RSV and the newly identified virus (SARS-CoV-2) using a respiratory panel. Viral-positive clinical samples were then labelled with a unique barcode provided and immediately stored at -80 °C until further processing. At the time of enrolment demographic and clinical data including, age, gender, and clinical presentation of each patient was collected and recorded for the main study.

Additional clinical samples were collected from a viral respiratory metagenomics cross-sectional study with HSREC approval number (UFS-HSD2019/1129/2910). At enrolment researchers collected NP/oropharyngeal swabs in Viral Transport Media (BD Diagnostics, Franklin Lakes, NJ, USA) (VTM) from patients between 0-5 years, requiring hospital admission in May 2020-August 2021 and fulfilled the World Health Organization (WHO) case definition for SARI. The WHO defines a case of SARI based on the following criteria: a patient with acute respiratory infection (ARI), requiring hospitalization, has a history of fever or a measured fever of ≥38 °C, and has experienced a cough with onset within the last ten days. These samples were collected at Pelonomi Hospital, Botshabelo Hospital, and National District Hospital. Samples collected were placed in 1 mL VTM and stored at -80 °C. Samples were tested for several respiratory pathogens using a QIAstat-Dx respiratory SARS-

CoV-2 panel (Qiagen, Hilden, Germany). The study provided the demographic information collected on the admission of patients and pathogens detected with their Ct values.

3.2. Eligibility Criteria

The inclusion criteria for sample selection were participant's ≤5 years, with the provision of sample collection date, where samples should be collected during the COVID-19 pandemic period. The clinical data and written informed consent should have been obtained from parents or legal guardians.

The exclusion criteria: Samples collected in the pre-pandemic period were excluded, as well as those from children above 5 years.

3.3. Ethical Consideration

The study protocol was approved by the UFS HSREC, (approval number: UFS-HSD2021/1616/2501). The use of biological infectious samples as part of this study was approved by the UFS Environmental and Biosafety Research Ethics Committee (EBREC), approval number UFS-ESD2021/0256/21. In compliance with relevant laws and institutional guidelines, further approval was sought. The Academic Affairs Research Management System (AARMS), NHLS, approved the use of clinical samples and access to the facility, in which available study samples that met the inclusion criteria were identified and selected. Moreover, additional approval was granted by the Free State Department of Health (FSDoH), and the initial study with the viral metagenomics cross-sectional study. For the study, further approval was granted by the UFS HSREC (UFS-HSD2021/1616/2501-0002). Nineteen (n=19) supplementary nucleic acid samples were incorporated into the project, from the study which had been approved earlier. The specimens were obtained from paediatric patients suffering from an acute respiratory infection and requiring hospital admission. The selection criteria for these samples involved identifying children who had been screened with multiple respiratory pathogens using the QIAstat-Dx SARS-CoV-2 respiratory panel for detection. Patient identifiers from the initial study, such as personal information, for instance, names, address, and hospital numbers were delinked from the sample sheet to protect patient confidentiality and do not appear in any part of the documentation in this study.

Section B: Optimization and Troubleshooting Procedures

The set of protocols outlined section B delineates the attempted techniques that were optimised with various troubleshooting (Nucleic material) steps but were not adopted due to inadequate results.

3.4. Viral RNA Extraction

3.4.1. Viral Extraction Using a Partially Automated Platform

The automated NUCLISENS® EASYMAG® instrument (Biomerieux, Marcy I'Etoile, France) was employed to directly extract viral genomic RNA from 200 µL of samples, following the manufacturer's instructions. The methodology utilized a silica bead-based extraction principle that facilitated the binding of RNA to the beads. The cleaning process was initiated automatically using cleaning buffers, leading to the purification of nucleic acid material during the washing steps. Subsequently, a heating step was performed to allow the separation of nucleic acid from silica. Afterward, the magnetic silica was pulled by the magnetic device, causing it to separate from the eluted nucleic material in a volume of 50 µL. Then the RNA that was extracted was subsequently stored at a temperature of -20 °C until it could be further processed.

3.4.2. PureLink Viral RNA/DNA Mini kit

The process of extracting total nucleic material from nasopharyngeal swabs was done using the PureLink Viral RNA/DNA Mini kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Briefly, in a sterile 1.5 mL microcentrifuge tube, 25 µL of Proteinase K was added together with a 200 µL cell-free sample. Subsequently, 200 µL of the Lysis buffer, except for carrier RNA was added into the lysate and briefly vortexed for 15 seconds. The solution was incubated at 56 °C for 15 minutes. After this step, a binding and washing step was carried out, involving the addition of 100% ethanol to the lysate tube, and mixing by vortexing for 15 seconds. The lysate containing ethanol was incubated for 5 minutes at room temperature. Precisely, 674 µL of the lysate was transferred into the Viral Spin Column and subjected to centrifugation at 68000 $x\ g$ for 1 minute. Subsequently, the collection tube containing the flow-through was discarded. The spin column was positioned in a clean collecting tube. The 500 µL of wash buffer II with ethanol was added to the column. The column was centrifuged once more at 6800 $x\ g$ for 1 min and the flowthrough was discarded. The preceding step was repeated. The spin column was then transferred to a clean sterile wash tube and centrifuged at maximum speed for 1 minute. Subsequently, the viral spin column was placed and carefully transferred to a 1.5 mL recovery tube, and 50 µL of the sterile nuclease-free water was added onto the column and incubated for 1 minute at room temperature.

Following this, the column was centrifuged at maximum speed for 1 minute and the resulting purified DNA was stored at -20 °C for further analysis.

NOTE: Following the RNA extractions, the concentration was assessed using Qubit 3.0 Fluorometric assay. During the quality check, most of the samples had low-quality RNA concentrations. Therefore, samples were repeated for extraction with the same protocol by increasing the volume of the cell-free sample and the subsequent buffers and reagents. Following these, better concentrations were achieved, albeit still not across all samples.

3.5. Reverse Transcription, Amplification, and Library Preparation

The optimisation steps were adapted from previously published protocols.

3.5.1. RSV Complementary DNA Synthesis through One-Step RT PCR

Reverse Transcription (RT) was performed on the extracted RNA to synthesise a complementary DNA (cDNA). Followed by the amplification of the cDNA using specific primers (Goya et al., 2018) (Table 3.1), with 5 overlapping RSV fragments of around 2300 to 4500 in length. The specific primers annealed to the conserved site on the RNA template. Each fragment was amplified in an independent reaction using the OneStep RT-PCR kit (Qiagen, Hilden, Germany). The initial reaction mixture contained 0.6 µL of the 25 µM forward and reverse primer and 6 µL of RNA template and incubation at 65 °C for 5 minutes, then placed the plate on ice for 2 minutes. Following that was the 25 µL PCR reaction contained 5 µL of 5x Buffer, 10.55 µL of H_2O DNase/RNase free, 0.25 µL RNase inhibitor, 1 µL of dNTP mix, and 1 µL of enzyme mix. The 25 µL PCR mixture was subjected under the following conditions: 45 °C for 30 minutes and 95 °C for 15 minutes, followed by 40 cycles of 94 °C for 10 sec, 55 °C for 30 sec, and 68 °C for 4.5 minutes and the final extension of 68 °C for 10 minutes and hold at 4 °C. This procedure was repeated using concentrated RNA and inconsistent amplification of the RSV fragments was observed.

Table 3.1: Primer set for RT PCR for amplifying full RSV genome.

Primer fragment	Primer sequence (5′-3′)	Primer position of RSV	Expected amplicon length (bp)
Fragment 1 Fw	ACGCGAAAAAATGCGTACwAC	1-21	2385
Fragment 1 Rev	GCrTCTTCTCCATGrAATTC	2366-2385	
Fragment 2 Fw	GCwGGyCTAGGCATAATG	2124-2141	2753
Fragment 2 Rev	GTTGTTrGTGTrACTTTGT	4858-4876	
Fragment 3 For	AyCCyGCATCACTwACAAT	3315-3333	3359
Fragment 3 Rev	TAACTCTCTAryACTCCAACTAyACC	7823-7798	
Fragment 4 Fw	TGATGCATCAATATCTCAAGTC	7095-7116	4158
Fragment 4 Rev	TAAATATTAAACTGCATAAT	11233-11252	
Fragment 5 For	AGTyTkACAAGATATGGTGATCT	10439-10461	4743
Fragment 5 Rev	AAGTGTCAAAAACTAATrTCTCGT	15158-15181	

Key: Degenerate code to be incorporated in the primer sequence

Symbol	Mixed bases
K	G, T
R	A, G
Y	C, T
W	A, T

NOTE: Following PCR amplification, amplification of all the required fragments could only be achieved in some of the samples. It was proposed that genetic changes in the viral genomes could be a contributory factor resulting in failure of the primers to achieve target amplification.

3.6. Library Preparation

3.6.1. Nextera XT DNA Library Preparation Kit

3.6.1.1. Fragmentation of the DNA

The Nextera XT DNA Library Preparation kit (Illumina, San Diego, California, United States), was utilized to successfully construct DNA libraries for high-throughput sequencing. The expected fragment size and the PCR steps were considered in accordance with the protocol instructions. This protocol was only employed to troubleshoot at least two samples. Therefore, the starting material was normalised to 0.2 ng/μL to generate small fragments. Firstly, all required reagents were equilibrated on ice. The workflow for library construction began with the tagmentation of the synthesized complementary DNA. This step involved random fragmentation of the cDNA using Nextera transposase enzyme while simultaneously tagging with platform-specific adapter sequences. In the 96 well plate, 10 μL of tagment DNA buffer (TD) and 5 μL of 1 ng of DNA were combined and thoroughly mixed by pipetting for a duration of 15 seconds. Subsequently, 5 μL of amplicon tagment mix (ATM) was pipetted into each well of the same PCR plate. The PCR plate was covered with a Microseal A film and centrifuged at 280 × g for 1 minute. Following this, the PCR plate containing the tagmentation mix was immediately placed in a pre-programmed thermocycler. The thermocycler was set to preheat the lid at 60 °C and incubate the plate at 55 °C for 4 minutes, followed by a 10 °C hold. Immediately after the fragmentation reaction was completed, the process was halted by adding 5 μL of NT to each well. This was achieved by pipette mixing 10 times, followed by another centrifugation step at 280 × *g* for 1 minute. The plate was then incubated at room temperature for 5 minutes. This step played a crucial role in neutralizing the activity of the transposase enzyme and its fragmentation process.

3.6.1.2. Library Amplification

After the tagmentation process, the libraries undergo a brief PCR step to introduce barcodes using the Nextera XT Index kit (Illumina, San Diego, California, United States). These barcodes, known as unique dual indexes, consist of DNA oligonucleotides that are used in distinguishing each sample during sequencing. To achieve this, 15 μL of the NPM, along with 5 μL of index primer 1 and 5 μL of index primer 2, were added to the PCR plate containing the tagmented DNA. The mixture was then gently mixed by pipetting 10 times, sealed, and subjected to centrifugation at 280 *x g* for a minute.

3.6.1.3. PCR Clean Up

After the process of indexing, it became necessary to perform a post-PCR clean-up procedure to eliminate any unwanted substances or impurities that were present in the samples. Residual enzymes and unincorporated index primers, which require elimination, are among the substances that may be present. Following that, the indexed libraries were subjected to size selection and PCR clean-up utilizing AMPure XP beads (Beckman Coulter, Indiana, United States) and 80% ethanol to eliminate fragments of both low and high molecular weight, as well as PCR impurities. Before use, the AMPure XP beads (Beckman Coulter, Indiana, United States) were allowed to equilibrate to room temperature. Subsequently, the 50 μL of the indexed libraries in a PCR plate were centrifuged at 280 *x g* for 1 minute at room temperature, to allow settling of all the constituents. The beads were thoroughly vortexed for 30 seconds until uniformly distributed and subsequently added to a trough. From there 30 μL of the beads were pipetted using a multichannel pipette P200 and added into each well on the PCR plate. The PCR plate was sealed and shaken for 2 minutes at 1800 rpm, followed by incubation for 5 minutes. The plate was then placed on the magnetic stand for 2 minutes until the liquid was clear and the supernatant was removed and discarded. In a two-step washing process, 200 μL of 80% ethanol was added to the beads while they were positioned on the magnetic stand and incubated for 30 seconds. Subsequently, the supernatant was carefully removed and discarded without disturbing the beads. The excess ethanol was then eliminated, and the beads were left to dry for 15 minutes.

3.6.1.4. Elution

The plate was removed from the magnetic stand. The beads were mixed with 52.5 μL of the resuspension buffer was added to beads and the plate was sealed before being subjected to a plate shaker for 2 minutes at r1800 rpm. The plate was sealed and positioned on a plate shaker for 2 minutes at a rotational speed of 1800 rpm. Subsequently, it was subjected to incubation at room temperature for an additional 2 minutes. Afterward, the plate was placed on the magnetic stand again for 2 minutes until the supernatant was clear. Lastly, the 50 μL supernatant containing the eluted DNA was transferred into a new PCR plate and stored at -20 °C.

NOTE: The libraries that were prepared for these samples exhibited commendable quality. Nevertheless, due to certain samples possessing a comparatively low concentration, it was not feasible to prepare libraries using this protocol. Consequently, the use of this protocol could increase the likelihood of the samples with low concentration being unsuccessful during the library procedure.

SECTION C: Optimization and Validation of Adopted Method

The set of protocols outlined here in section C delineates the optimised, validated and adopted techniques.

3.7. QIAamp Viral RNA Mini Extraction Kit

Viral RNA extraction was performed using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany), following the manufactures protocol. The clinical sample obtained from the nasopharyngeal swab was allowed to reach room temperature. Subsequently, 50 µL of Buffer AVL was introduced into a microcentrifuge tube, except for the use of a carrier RNA addition. A 140 µL cell-free respiratory sample was added to the AVL buffer and mixed through pulse vortexing for 15 seconds, to obtain a homogeneous solution. The viral particle lysis mixture was incubated at room temperature for 10 minutes. After, incubation the tube was centrifuged to remove any droplets from inside a lid. Then 560 µL of 96-100% ethanol was added to the sample and mixed by pulse vortexing for 15 seconds. Subsequently, the mixture in the tube was briefly centrifuged to remove any droplets on the lid. The 630 µL solution was carefully transferred into a QIAamp Mini column and placed in a 2 mL collection tube. The column was centrifuged at 6000 *x g* (8000 rpm) for 1 minute. Afterwards, the QIAamp Mini column was inserted into a clean 2 mL collection tube and the filtrate was disposed of. Subsequently, 500 µL of Buffer AW1 was added into the QIAamp Mini column and subjected to centrifugation at 6000 *x g* (8000 rpm) for 1 minute. After this step, the QIAamp Mini column was transferred to another 2 mL collection tube while discarding the tube containing the filtrate. The 500 µL Buffer AW2 was added into the QIAamp Mini column and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 minutes. The QIAamp Mini column was positioned in a clean 2 mL collection tube and spun at maximum speed for 1 minute, leading to the disposal of the previous collection tube containing the filtrate. Subsequently, the QIAamp Mini column was transferred to a sterile 1.5 mL microcentrifuge tube, where it was combined with 60 µL of Buffer AVE that had been equilibrated to room temperature. The mixture was incubated at room temperature for 1 minute and subsequently centrifuged at 6000 x g (8000 rpm) for 1 minute. The extracted viral RNA was stored at -20 °C.

NOTE: The samples with low RNA quality yield were repeated. The sample volume was increased to more than 140 µL, and the amount of ethanol proportionally increased (e.g., a 280 µL sample requires 1120 µL ethanol).

NOTE: The samples that were deemed unsatisfactory during the quality control evaluation of the extraction were subjected to the Chemagic™ automated extraction instrument.

3.8. Chemagic™ 360 Automated Viral Extraction

The Chemagic™ 360 (PerkinElmer, Waltham, MA, USA), system was prepared for the extraction process by thoroughly inspecting all manifolds and ensuring their proper functionality. In this process, the preloaded buffers were dispensed into the machine's channels sequentially. After the machine initialised, a priming protocol was implemented to prevent blockage during buffer loading. To process the samples, nasopharyngeal swabs were equilibrated to room temperature, and 10 µL Proteinase K was added to a 300 µL sample. This was followed by the addition of 300 µL of lysis buffer I into the wells containing the sample. The extraction process was set up on the Chemagic™ (PerkinElmer, Waltham, MA, USA), by ensuring the functionality of all manifolds and channels for the sequential dispensing of preloaded buffers into the machine. The QA software guided the placement of the sample plate onto the tracking system. Following the instructions, 150 µL of magnetic beads and 75 µL of elution buffer were loaded onto distinct deep well plates, which were subsequently placed on the tracking system. The machine prompted the placement of the disposable tips and tip racks into their respective positions on the tracking system. This was done in a clockwise orientation for each position. The software integrated into the machine recorded the sample IDS and provided instructions based on the required step in the protocol. Throughout the process, the plate was carefully oriented and fitted to ensure accuracy. The addition of Binding Buffer 2, Wash Buffer 4, and Wash Buffer 5 was automated for consistency. Finally, the plate containing the eluent DNA of 67 µL was positioned on a magnetic stand and the supernatant was carefully transferred to a new plate and stored appropriately.

3.9. Quantification of RNA Using Qubit 3.0 Fluorimeter

The quantification procedure is an essential step in evaluating the viral extract's quality prior to moving forward with downstream applications. Quantification of the RNA extracted from the 69 samples was performed using the RNA high sensitivity assay on the Qubit™ 3.0 Fluorimeter (Thermo Fisher Scientific, Waltham, USA). The Qubit™ 3.0 Fluorimeter is a benchtop laboratory equipment that employs fluorescence to quantify the amount of nucleic acid present in each sample. It consists of a series of applications and assays that are implemented within, and function on the use of sensitive dyes when combined with either DNA, RNA, or proteins and measured. In an RNA HS assay, the fluorescence dye binds specifically to the RNA, and loaded within the machine and

the amount of light exerted excites the dye to highly become fluorescence. This gives a signal that is converted and measures the amount of RNA concentration in that sample with the range pre-determined by using the Qubit™ standards. Using the RNA HS assay, 1:200 dilutions were prepared by adding 13930 µL of the Qubit™ buffer and 70 µL of the Qubit™ Reagent with a fluorescent dye into a falcon tube. Subsequently, 190 µL of the working solution and 10 µL of Qubit™ Standards were added into each qubit tube. The RNA HS sensitivity assay was opted for on the Qubit™ Fluorometer device, and standards were calibrated beforehand using the standard prepared. Furthermore, sterile qubit tubes were utilized to add 1 µL of the RSV sample with 199 µL of the qubit working solution. After a brief vortexing, the Qubit™ tubes were incubated at room temperature for a duration of 2 minutes. Subsequently, the RNA samples were measured for their concentration using the instrument, and the readings were recorded as output results in ng/µL. Samples exhibiting low concentrations were subjected to repetition, and if no improvement was observed, they were excluded from further downstream analysis.

3.10. Reverse Transcription and PCR Amplification

The viral RNA was subjected to reverse transcription (RT) to generate a complementary DNA (cDNA) from the extracted RNA. Subsequently, the cDNA was amplified using specific primers and a protocol that had been previously established and described (Langendik et al., 2020) (Table 3.2). The four overlapping fragments spanning the RSV-A and RSV-B genome, were around 4000 to 4500 bp in length. Our clinical specimen was not subjected to an initial reaction to ascertain the RSV subtype that was present. Consequently, each of the RSV fragments were subjected to both RSV-A and RSV-B primer sets and amplified in an independent reaction using the SuperScript IV One-Step RT-PCR System (Thermo Fisher, Waltham, MA, USA)**.** The 10 µL PCR reaction mixture placed on ice contained, 0.5 µL of the 10 µM forward and reverse primer and 2 µL of RNA template, followed by 5 µL of 2X Platinum™ SuperFi™ RT-PCR Master Mix, 0.1 µL of SuperScript™ IV RT Mix, 1.9 µL Nuclease-free water. The thermocycling conditions of the PCR mixture at 55 °C 10 minutes for the cDNA synthesis step, 98 °C for 2 minutes to inactivate the enzyme, followed by 40 cycles at 98 °C for 10 seconds, 61 °C for 30 seconds, and 72 °C for 3 minutes and the final extension of 72 °C for 5 minutes and hold for ∞. The RSV amplicons were stored at -20 °C until use.

Table 3.2: The primer sequence set employed for the amplification of RSV-A and B genomes.

Primer Fragment	**Primer sequence (5′-3′)**
RSVA-fragment 1-Fw	AAAAATGCGTACWACAAACTTGC
RSVA-fragment 1-Rev	GTTGGTCCTTGGTTTTGGAC
RSVA-fragment 2-Fw	CACAGTGACTGACAACAAAGGAG
RSVA-fragment 2-Rev	GCTCATGGCAACACATGC
RSVA-fragment 3-Fw	CGAGGTCATTGCTTGAATGG
RSVA-fragment 3-Rev	CACCACCACCAAATAACATGG
RSVA-fragment 4-Fw	AGGGTGGTGTCAAAAACTATGG
RSVA-fragment 4-Rev	ACGAGAAAAAAAGTGTCAAAAACT
RSVB-fragment 1-Fw	AAAAATGCGTACTACAAACTTGC
RSVB-fragment 1-Rev	TTGTGCTTGGCTTGTTGTTC
RSVB-fragment 2-Fw	AAGGGTTAGCCCATCCAAMC
RSVB-fragment 2-Rev	TGCTAAGGCTGATGTCTTTCC
RSVB-fragment 3-Fw	GTCCTCGTCTGARCAAATTGC
RSVB-fragment 3-Rev	TAGGTCCTCTTTCACCACGAG
RSVB-fragment 4-Fw	GAGGGATCCACAGGCTTTAGG
RSVB-fragment 4-Rev	ACGAGAAAAAAGTGTCAAAAACT

3.11. Agarose Gel Electrophoresis

The gel electrophoresis technique was employed to analyse the four PCR products obtained from each sample, serving as a confirmatory method to validate the positive amplification. A 2% agarose gel and 2 grams of agarose powder were dissolved in 50 mL of TBE buffer (Sigma-Aldrich, Missouri, USA)). The mixture was then heated in a microwave until it reached a uniform consistency (homogenous solution). The molten agarose was permitted to cool down and then stained using 2 μL of Pronasafe (Condalab, Torrejon de Ardoz, Madrid). Afterward, the mixture is poured into a gel tray containing combs and left undisturbed until it solidifies. The PCR product and negative controls, along with a loading dye, were loaded onto the gel in their respective wells, with a volume of 1 μL. Followed by a 2 μL DNA molecular weight marker (Thermo Fisher, Waltham, MA, USA) loaded in a separate lane. The agarose gel was resolved in a gel electrophoresis chamber with TBE buffer at 140 volts for 45 minutes. The results were visualized under a UV transilluminator.

3.11.1. Quantification of PCR Products

The cDNA amplicon concentration was determined using DNA quantification on the Qubit 3.0 Fluorimeter (Thermo Fisher, Waltham, MA, USA) by utilizing the 1x dsDNA High Sensitivity assay kit (Thermo Fisher, Waltham, MA, USA). The amplicons from each sample were then normalized and pooled into equal volumes and concentrations.

3.12. Purification of PCR Products

The PCR amplicons, which showed distinct single bands of the correct size compared to the DNA ladder observed in the resolved gels, and demonstrated sufficient concentration, were prepared using the Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA), following the guidelines provided by the manufacturer. The objective of the purification process was to eliminate undesired impurities, primer dimers, and other reagents. Briefly, Purification by centrifugation was done for each sample, and the PCR amplicons were processed by adding an equal volume of the sample and the membrane binding solution in a 1.5 mL single tube. The prepared mixture was transferred into a mini column and placed in a collection tube. Followed by incubation at room temperature for 1 minute. Subsequently, the mini column was subjected to centrifugation at 16,000 × *g* for 1 minute and then transferred to a fresh collection tube. Afterwards, a washing step was carried out by adding 700 µL of membrane wash solution containing ethanol onto the mini column, which was then centrifuged at 16 000 x *g* for 1 minute. The flow through was discarded, and the collecting tube was replaced. The preceding step was repeated, however, 500 µL of the membrane wash solution was added and followed by centrifugation for a duration of 5 minutes. To allow for the evaporation of any surplus ethanol, the centrifugation step was repeated for 1 minute with the microcentrifuge lid left open. Upon completion, the mini column was placed onto a new sterile 1.5 mL microcentrifuge tube. Subsequently, 50 µL of nuclease-free water was transferred to the mini column and allowed to incubate for a minute at room temperature. Afterward, a centrifugation step at 16,000 x g for another 1 minute was performed. The purified cDNA was then stored at -20 °C for further analysis.

3.13. QIAseq FX Single Cell RNA Library Preparation

3.13.1. Fragmentation, End Repair and A-Addition

The libraries were prepared using the QIAseq FX Single cell library preparation kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The 10 µL purified cDNA was transferred into a PCR tube and kept on ice. The reaction set was prepared for insert fragment size of 300 bp.

The prepared reaction mixture consisted of 40 µL, comprising 5 µL of FX Buffer 10x, 20 µL of H2O, 5 µL of FX Enhancer, and 10 µL of FX Enzyme Mix. The purified cDNA sample was carefully transferred onto a PCR plate and subsequently chilled on ice. Following this, 40 µL of the FX Reaction Mix, which had been previously prepared, was added to the sample and mixed gently. The PCR plate was momentarily shaken and centrifuged before being immediately transferred to a pre-chilled (4 °C) thermocycler. The programmed procedure was then initiated immediately. Upon completion of the fragmentation process, the sample plate was placed onto a bed of ice.

3.13.2. Adapter Ligation

To ensure optimal performance, it is recommended to allow the Agencourt AMPure XP beads (Beckman Coulter, Indiana, United States), to equilibrate at room temperature for 20-30 minutes prior to usage. The adapter plate was vortexed and centrifuged, and the protective cover of the plate was removed. The aluminium foil seal was carefully perforated and 5 µL of the DNA adapter was transferred into each of the samples used. Each of the unique barcodes used for each sample was tracked and documented meticulously. The aspect library preparation procedure incorporates unique indexes called barcodes with 8 bases of DNA oligonucleotides to identify each sample before multiplexing. A sample sheet was generated for all 36 samples using the Illumina Experiment Manager software version 1.15 (Illumina, Inc., California, United States). The sample sheet comprised the identification details of the samples, the corresponding indexes assigned to each sample, the sequencing instrument workflow, and the expected sequence read length.

Afterward, a separate tube was used to prepare a ligation master mix of 45 µL, consisting of 10 µL of DNA Ligase Buffer 5x, 15 µL of H^2O, and 10 µL of DNA Ligase. After the addition of the 45 µL ligation master mix to each sample, a 15 minutes incubation at 20°C was carried out. Subsequently, the adapter ligation clean-up step was performed, which involved the addition of 80 µL of re-suspended Agencourt AMPure XP beads (Beckman Coulter) and slurry to each ligated sample through pipette mixing. Thereafter, the mixture was incubated at room temperature for 5 minutes. The beads were then subjected to a magnetic stand for 2 minutes to pellet them, and the supernatant was eliminated. Following this, the beads were washed with 200 µL of freshly prepared ethanol for each pellet on a magnetic stand for 2 minutes, and the supernatant was discarded. The preceding procedure was repeated for two consecutive washes of the beads using ethanol. Subsequently, the plate was placed on the magnetic stand for 5-10 minutes to allow the beads to completely dry. Careful observation was conducted to prevent excessive drying of the beads, as it may result in diminished DNA recovery. The bead plate was then detached from the magnetic stand. The elution

step was performed by resuspending the DNA in 52.5 µL 10 mM Tris-HCl, pH 8.0. Afterwards, the beads were pelleted on a magnetic stand, and 50 µL of the resulting DNA was transferred to a separate PCR plate. Subsequently, the second purification process was carried out by adding 50 µL of the 1x Agencourt AMPure XP beads to the sample plate and thoroughly mixing them. The mixture was then incubated at room temperature for 5 minutes, and another incubation step of 50-10 minutes was repeated allowing the beads to dry. Following this, the beads were eluted by resuspending them in 26 µL of 10 mM Tris-HCl, pH 8.0. The DNA/supernatant was then transferred to a clean PCR plate, and the libraries were temporarily stored at -20 °C.

3.13.3. Quantification of the Library

The quality of the libraries was assessed by DNA quantification on Qubit 3.0 Fluorimeter (Thermo Fisher, Waltham, MA, USA) using the 1x dsDNA High Sensitivity (HS) assay kit (Thermo Fisher, Waltham, MA, USA).

3.14. Quality Validation

3.14.1. Library Validation and Quantification

Library validation is an essential stage in the pre-sequencing process, aimed at evaluating the presence or absence of adapter or adapter dimers. This was achieved by using the 2100 Bioanalyzer (Agilent Technologies, California, United States) instrument, which employs a chip-based capillary electrophoresis. This instrument evaluates the quality of the prepared library to provide the size fragment distribution and integrity of the prepared library (Panaro et al., 2000). This method functions as a means of validation to prevent the production of low-quality runs and the introduction of bias in our sequences. The Bioanalyzer utilizes microfluidic electrophoretic separation on microfabricated chips, providing a visual depiction of the various sizes of DNA fragments comprising the library. The library analysis was conducted using the Agilent HS DNA kit. Prior to use, the High sensitivity dye concentrate, and DNA gel matrix were allowed to equilibrate at room temperature for 30 minutes. Once completely thawed, they were vortexed and spun down for 10 seconds. The DNA gel matrix was supplemented with 15 µL of gel dye concentrate using a pipette to create the gel dye mix. Afterward, the mixture was vortexed for a duration of 10 seconds. Subsequently, it was transferred into a sterile spin filter and subjected to pulse centrifugation in a prism microcentrifuge at a speed of 2240 $x\ g$ for approximately 10 minutes at room temperature. Finally, the spin filter was discarded. The 1.2 mL microcentrifuge tube was vortexed with a foil to protect it from light. All the reagent, including the HS DNA chip, was placed on the chip priming station, wherein 9 µL of the gel dye matrix was pipetted into a specific well

labelled G and allowed to disperse. A fresh, sterile syringe was positioned on the priming station, and pressure was exerted on the plunger positioned at 1 mL. The priming station was then closed for 60 seconds. Subsequently, the plunger was pressed downwards until it was securely held by the clip. Following this, the plunger was released back to its original position using the clip release mechanism for another 60 seconds. The priming station was then opened, and a volume of 9 µL of the gel dye mix was carefully added into each well. Following this, 5 µL of the HS DNA marker was added into the well marked with a ladder symbol and into the other 11 wells. Subsequently, 1 µL of HS DNA ladder was pipetted into the well with a ladder symbol and then 1 µL of the sample was added into the 11 unused wells. The chip was placed in a horizontal position on the adapter of the vortex mixture and vortexed at 2400 rpm for 60 seconds. The chip was inserted into the Bioanalyzer instrument, and a run was initiated. Each cartridge can only accommodate 11 samples and for each run, a comprehensive report containing the electropherogram with fragment size distribution, gels with amplicon sizes or smears, and other significant findings was produced.

3.14.2. Library Normalisation

The libraries were normalised to an equimolar concentration using the average fragment sizes obtained from the bioanalyzer and DNA concentrations from Qubit™. This step was crucial to ensure that each library had equal representation during the MiSeq sequencing. To determine the library concentration in nanomolar (nM) units, a formula (as shown below) was employed. Subsequently, the purified libraries were normalized to either 4nM or 2nM, depending on the initial library concentrations, before pooling them together.

$$\boldsymbol{Concentration\ in\ ng/\mu l / (660 g/mol\ x\ Average\ library\ size)\ X\ 106 = Concentration\ in\ nM}$$

3.14.3. Library Pooling, Denaturation and Dilution

The inclusion of this procedure was crucial to achieve optimal cluster density and facilitate Illumina MiSeq sequencing. The libraries, which had been normalized to equal concentrations, were pooled in a 1.5 mL microcentrifuge tube by adding 5 µL of each library to the tube. The pooled library tube was briefly vortexed before undergoing chemical denaturation and dilution. To prepare for this, a freshly diluted 0.2 N NaOH solution was made. Then, 5 µL of the 4 nM pooled library and 5 µL of the freshly made 0.2 N NaOH were combined in a clean 1.5 mL microcentrifuge tube, resulting in a total volume of 10 µL. The tube was vortexed and centrifuged at 280 *x g* for 1 minute, followed by incubation for 5 minutes at room temperature to allow denaturation of the DNA library and dilution to 2 nM. After the incubation, 990 µL of the pre-chilled hybridization buffer HT1 was added to the

2 nM pool which has 10 μL, to further dilute the library to 20 pM. The 20 pM library was further diluted to a final loading 8 pM concentration.

3.14.4. Phix Control

Phix, a bacteriophage genome, is utilized as a positive control during Illumina sequencing. To achieve an optimal cluster density, the Phix control is first diluted and denatured. Following this, 3 μL of a 10 nM PhiX library is added to 2 μL of Elution buffer, which is equivalent to 10 nM Tris-HCl, pH 8.5. Consequently, 5 μL of the PhiX of the 4 nM is denatured using 5 μL of 0.2 N NaOH. The mixture is then thoroughly mixed by pipette and centrifuged at 280 x g for 1 minute, followed by incubation at room temperature for 5 minutes. To further dilute the 4nm Library to 2nM at 20 pM, 990 μL of pre-chilled HT1 buffer is added. The PhiX control serves creating diversity within the library. Therefore, a 1.5% spike-in is performed by combining 9 μL of the 20 pM denatured PhiX with 591 μL of the 10 pM denatured and diluted library.

3.15. Raw Data Retrieval and Analysis

The sequence data was demultiplexed on the instrument using barcodes. The raw paired end reads in Fastq file data sequence was analysed with the aid of genome detective bioinformatics software. The web-based tool interrogates high throughput next generation sequencing data and generates an accurate, quick, and automated system for virus identification. The programme examines the NGS data by pre-processing the reads iteratively, through quality assessment. The reads are divided into, utilised, allocated, and unallocated groups, and a thorough report is provided which includes the input sample's length distribution. The quality of sequencing reads was evaluated using FastQC programme. The FastQC generates graphs and tables to easily evaluate the quality of the raw Fastq reads. Filtering and trimming of low-quality read sequences done using Trimmomatic, implemented in Genome Detective, prior to downstream analysis (Bolger et al., 2014). The forward and reverse reads were trimmed at extreme lengths and quality reads below Q20 removed. The trimmed paired-end reads were de novo assembled using metaSPAdes to generate contigs (Bankevich et al., 2012). The DIAMOND tool embedded in Genome Detective was then used to align candidate viral reads against a complete viral genome on the NCBI RefSeq virus database (Vilsker et al., 2019).

3.16. Analysis on Geneious Prime Software

The preceding sequence reads with good genome coverage were loaded on the software as paired end raw reads on Geneious v 2022.1.2 and trimmed the sequences using the Trim ends tool to remove unwanted bases (Kearse et al., 2012). The RSVA/B reference sequences previously noted

were retrieved from GenBank and uploaded to the software. RSV-A or RSV-B subgroups reference sequences (GenBank accession numbers NC_001803 and NC_001781), were used in the mapping assembly respective of the sequence assigned subtype. All study sequences were mapped and assembled to the RSVA/B reference sequence on Geneious and generated a full-length consensus sequence. The consensus sequences were interrogated, and ambiguities were corrected against the reference sequence to accurate the base quality call in that position. From the whole genome, the complete coding region of the G-gene was extracted and the final output from Geneious as a nucleotide sequence consisting of at least 399 nucleotides, thus exported, and subsequently used for phylogeny.

3.17. Alignments and Data Curation

The study strains were named as per the classification system. Available RSVA and B sequences were mined from the Global Initiative on Sharing All Influenza Data (GISAID) and GenBank (https://www.ncbi.nlm.nih.gov/) databases. GenBank entries for each of these sequences were mined, such as the collection date, isolation country, and previously known genotype omitted strains with ambiguous sequences and sequences that were not accompanied by information on the detection year and region. Reference genomes from the African continent available in the Bacterial and Viral Bioinformatics Resource Center (BV-BRC, https://www.bv-brc.org/) were retrieved (Olson et al., 2023). Strains with 100% nucleotide sequence similarity (homologous strains) in the same detection year and country were omitted. The global RSVA/B sequences were retrieved and then aligned with the study G-Gene sequences using the MAFTT algorithm. After the alignment, the sequences were trimmed to the G-gene open reading frame. The alignment was analysed on MEGA 7.0 Software (Kumar et al., 2016), to inspect the alignment using the MAFTT plugin. The alignment was trimmed using the motif of the G-gene start sequence ATGTCCAAAACCAAGG and the end G-gene end start codon AAAATGATAG. The upstream and downstream nucleotides were deleted accordingly. The global retrieved RSVA sequences were groups based on regions, aligned independently on MAFTT. The same was done for RSV-B reference sequences, South African G-gene, and South African BA genotype. The large alignments were examined for the following errors/insufficiencies duplicate sequences were therefore eliminated. All alignments were checked and edited as appropriate. Identical sequences occurring multiple times, with various names, and yet precisely the same were removed. The difficult to align regions were also eliminated from the alignment.

3.18. Recombination Analysis

Simplot, a widely recognized recombination tool, was utilised to determine the potential RSV breakpoints. This tool utilizes a graphical user interface, enabling the loading of various file formats such as FASTA, Nexus, or other alignment formats. The loaded sequences were segregated into two distinct groups: RSVA and B, with RSV-B strains serving as the consensus. The resultant consensus sequence formed the basis for the Simplot and recombination analysis. Following calculation, each consensus sequence was carefully inspected to establish an appropriate confidence threshold. Potential signals for genomic inter-subgroup recombination events were investigated in samples sequenced. A Jukes-Cantor distance model was applied with a window length of 200 bp and step size of 20 bp applied within Simplot 3.5 (Samson et al., 2022).

3.19. Phylogenetic Analysis

3.19.1. RSV-A and RSV-B genotype Assignment

The genotypes in this study were assigned based on the recently proposed classification system by Goya et al., 2020. Clade determination was performed using an online clade genotyping tool available in Nextclade (https://clades.nextstrain.org) (Aksamentov et al., 2021). Generated phylogenetic trees comprising study strains and reference strains were exported and subsequently visualised using Auspice (https://auspice.us) (Hadfield et al., 2018).

3.19.2. Maximum Likelihood Phylogenetic Tree for RSV-A and RSV-B

Maximum likelihood (ML) is a commonly employed method in molecular systematics for inferring phylogenetic relationships (Felsenstein et al., 2008). The most optimal nucleotide substitution model for each dataset was determined using ModelFinder (Kalyaanamoorthy et al., 2017). IQ-TREE is a highly efficient program for reconstructing ML trees, offering both time and search efficiency. It serves as a valuable addition to the existing pool of ML-programs and outperforms RAxML or PhyML in terms of ML search (Nguyen et al., 2015). Then, ML trees were inferred with IQ-TREE v1.6.12 software, and node support values were evaluated by bootstrapping to 1000 replicates (Nguyen et al., 2018).

3.20. Evolutionary Analysis

Subsequently, the dataset was explored on BEAUTI, and the tip dates were set, sites with substitution models were specified, the clock model was defined, and prior tree parameters were set. Phylogenetic relationships were deduced by employing an uncorrelated relaxed molecular clock, which was defined with branch rates derived from a lognormal distribution. This approach

was adopted to accommodate the diverse evolutionary rates observed among different lineages. The GMRF Bayesian Skyride tree prior was implemented, and an evolutionary analysis was conducted. Gene-specific phylogenies were estimated using Markov chain Monte Carlo sampling chains consisting of 100 million to 200 million generations. Parameters and trees were recorded to ensure a total of 10,000 sampled trees per run. Default priors were used for each analysis except for the ucld.mean procedure for which rate reference prior was used. The calculation of the estimated sample size (ESS) and along with the highest posterior probability density (HPD) interval, was performed based on the specified run parameters. The output file on BEAUTi was saved as .Xml file format and prepared to run on BEAST in triplicates. All analyses were evaluated with Tracer v1.6 (available at http://tree.bio.ed.ac.uk/software/tracer/) to determine the success of the chain sampling based on effective sample size values for each parameter. The BEAST run generated three output files, namely .log, .ops, and .tree, which were subsequently utilized downstream. The .log file inspected scrutiny in tracer to determine an ESS exceeding 200, following a 10% burn-in, and under sampling explore and reach convergence. The posterior probability was evaluated and deemed to be statistically significant, were visualized with 95% highest probability density (HPD) using Tracer v1.7 (Suchard et al., 2018). The data generated from the dataset from Beast, was condensed using TreeAnnotator and compiled to a maximum credibility clade tree. The tree was annotated using Figtree v1. The mean estimates of the times to most recent common ancestor (TMRCA) was estimated for both RSV-A and RSV-B, GA2.3.5 and GB5.0.5a lineages, respectively. In addition, the rates of molecular evolution were also calculated by suitable models selected for each dataset as described.

3.21. Selection Pressure Analysis

The full G-gene and F-gene sequence alignment was analysed to calculate the rates of synonymous (dS) and non-synonymous (dN) substitutions as well as total substitution rates. These are single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), mixed effects model of evolution (MEME) and fast, unconstrained Bayesian Approximation for inferring selection (FUBAR). The SLAC, FEL, and MEME method was performed with a posterior probability of 0.9 while FUBAR determined positively selected sites (dN/dS >1) and negatively selected sites (dN/dS <1). The SLAC method was used to estimate the overall mean substitution ratio (dN/ dS). The MEME method aims to detect sites evolving under positive selection in a proportion of branches, in contrast to the FUBAR method which uses a Bayesian approach and assumes that selection pressure is constant along the entire phylogeny.

3.22. N-glycosylation

The F and G genes of the RSV-A and B datasets comprised of African strains and South Africa strains were interrogated to identify putative N-glycosylation sites utilising NetNGlyc 1.0. Server (http://www.cbs.dtu.dk/services/NetNGlyc/)

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Preface

The work presented here in chapter four has been submitted in *Virus Research._Under Review*

Detailed authors contributions are here-in described.

Authors detailed Contributions:

- **Project conceptualisation:** Martin M. Nyaga, Hlengiwe Sondlane.
- **Study development**: Hlengiwe Sondlane.
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- **Data capturing:** Hlengiwe Sondlane.
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- **Visualization and original draft**: Hlengiwe Sondlane
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CHAPTER FOUR: WHOLE GENOME MOLECULAR ANALYSIS OF RESPIRATORY SYNCYTIAL VIRUS PRE AND DURING THE COVID-19 PANDEMIC IN FREE STATE PROVINCE, SOUTH AFRICA

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Abstract:

Respiratory syncytial virus (RSV) is the most predominant viral pathogen worldwide in children with lower respiratory tract infections. The Coronavirus disease 2019 (COVID-19) pandemic and resulting nonpharmaceutical interventions perturbed the transmission pattern of respiratory pathogens in South Africa. A seasonality shift and RSV resurgence was observed in 2020 and 2021, with several infected children observed. Conventional RSV-positive nasopharyngeal swabs were collected from various hospitals in the Free State province, Bloemfontein, South Africa, from children suffering from respiratory distress and severe acute respiratory infection between 2020 to 2021. Overlapping genome fragments were amplified and complete genomes were sequenced using the Illumina MiSeq platform. Maximum likelihood phylogenetic and evolutionary analysis were performed on both RSV-A/-B G-genes with published reference sequences from GISAID and GenBank. Our study strains belonged to the RSV-A GA2.3.2 and RSV-B GB5.0.5a clades. The upsurge of RSV was due to pre-existing strains that predominated in South Africa and circulating globally also driving these off-season RSV outbreaks during the COVID-19 pandemic. The variants responsible for the resurgence were phylogenetically related to pre-pandemic strains and could have contributed to the immune debt resulting from pandemic imposed restrictions. The deviation of the RSV season from the usual pattern affected by the COVID-19 pandemic highlights the need for ongoing genomic surveillance and the identification of genetic variants to prevent unforeseen outbreaks in the future.

Keywords: Respiratory syncytial virus, COVID-19 pandemic, whole genome sequencing

4.1. Introduction

Respiratory syncytial virus (RSV) is recognised as the most frequent cause of viral lower respiratory tract infection (LRTI) in infants and children ≤5 years worldwide, and a leading cause of hospitalisation, mortality, and morbidity (Demont et al., 2021; Shi et al., 2017; Weinberg, 2017). The RSV infects nearly 90% of children within the first two years of life, with nearly 40% of whom develop LRTI, the first infection episode (Brady et al., 2014; Meissner, 2016). Also, RSV offers partial immunity due to waning immunological memory, characterized by recurrent infections that may continue into adulthood (Griffiths et al., 2017; Nam and Ison, 2019). Almost 90% of RSV-associated fatalities are recorded in children ≤ 5 years from low income (or low resourced) countries (Liu et al., 2020).

In temperate regions, RSV has a distinct seasonal circulation in the late autumn or early winter and in tropical regions, RSV infections predominate during the rainy season (Khor et al., 2012). Despite the global impact of RSV on public health, there are currently no licensed RSV vaccines or effective antivirals for acute infection available (Feng et al., 2022; Mazur et al., 2018). Palivizumab prophylactic monoclonal antibody (mAb) has been widely used in the prevention of RSV and has proven to be effective against RSV infections in high-risk children (Cadena-Cruz et al., 2023; Elawar et al., 2021). In addition, novel mAb Nirsevimab, a newly approved by the FDA for the prevention of RSV infection infants has been made available (Simões et al., 2023). Another vaccine, known as ABRYSVO, administered in pregnant women, to protect infants from RSV associated LRTI after birth and individuals who are 60 years old and above has been made available (Ernst, 2023).

Recently RSV has been reclassified under the genus human *orthopneumovirus*, belonging to the family *Pneumoviridae* and order monogavirales (Rima et al., 2017). The genome has approximately 15, 200 nucleotides of single stranded, negative sense RNA encoding 11 viral proteins (Azzari et al., 2021). The virus is classified into RSV-A and RSV-B, the two primary antigenic groups that have been identified as the prevalent strains within the human population (Vandini et al., 2017). The attachment glycoprotein (G) has been extensively employed for RSV genotyping due to its high degree of diversity (Goya et al., 2020; Hibino et al., 2018; Muñoz-Escalante et al., 2021). Numerous studies have proposed a unified manner of designating genotypes. Adoption and the implementation of this methodology facilitated the categorization of lineages and sub-genotypes or clades, while simultaneously decreasing the number of identifiable genotypes by almost half in both groups (Chen et al., 2022; Goya et al., 2020). Based on the newly proposed genotype nomenclature,

three genotypes were identified and classified within RSV-A, namely GA1, GA2, and GA3, similarly, within RSV-B, seven genotypes were designated as GB1, GB2, GB3, GB4, GB5, GB6 and GB7 (Goya et al., 2020).

Before the COVID-19 pandemic, an estimated 6,000 RSV-associated deaths occurred annually in South Africa (Cohen et al., 2018). The unprecedented COVID-19 pandemic prompted the South African government and other nations to implement nonpharmaceutical interventions (NPIs) to contain the spread of SARS-CoV-2. These NPIs included social distancing, frequent hand washing, travel restrictions, school closures, curfews, and mask wearing (Alanezi et al., 2020; Bents et al., 2022; Ijaz et al., 2022). Although the implemented NPIs were successful in controlling SARS-CoV-2, they have indirectly disrupted the circulation of other respiratory viruses with a known seasonal pattern, such as RSV, in many countries worldwide (Baker et al., 2020; Tempia et al., 2021; Yeoh et al., 2021; Zheng et al., 2021). Surveillance data from South Africa suggested a significant decline in RSV circulation from the 2020-2021 winter season (Olsen et al., 2021; Tempia et al., 2021). The impact of the NPIs was not uniform amongst the respiratory pathogens, but there was a simultaneous reduction of influenza, and RSV during the COVID-19 pandemic compared to Rhinoviruses and respiratory Adenoviruses (Abo et al., 2021; Tempia et al., 2021). A delayed RSV season was observed in late 2020 and early 2021 and its resurgence was accompanied by offseason outbreaks following the gradual relaxation of lockdown restrictions (Agha and Avner, 2021; van Summeren et al., 2021). The altered epidemiology and resurgence of RSV was probably due to waned immunity from reduced or lack of exposure to the virus, (Reicherz et al., 2022). This highlights the importance of molecular surveillance to predict potential outbreaks that could have an impact on vulnerable populations.

There is limited data on characterisation of strains driving the RSV epidemic in South Africa, and none in the Free State province. Currently, there is a dearth of whole genome data on RSV strains in public databases, particularly during the COVID-19 era in South Africa. Next generation sequencing technology and its applicability, allow the broader characterisation of circulating RSV strains through whole genome sequencing (WGS), and this information could aid in antiviral medication and vaccine development using epidemiologic surveillance and characterisation of RSV diversity (Wang et al., 2022). The progress made in the field of WGS presents a significant opportunity to tackle unresolved concerns related to the viral evolution of RSV and reduce dearth of information

in underrepresented communities and less described sequenced genomic regions of RSV (Rios Guzman and Hultquist, 2022).

To the best of our knowledge, this is the first study to describe RSV complete genome sequences collected in South Africa during the COVID-19 pandemic. However, it is unclear whether the RSV outbreaks observed in this period were due to the emergence of novel strains with an enhanced selective advantage or influenced by selective pressure resulting from the low RSV transmission in 2020. The objective of this study was to perform whole genome sequencing of RSV in children ≤5 years of age with respiratory distress and severe acute respiratory illness (SARI) and investigate circulation patterns of RSV during the COVID-19 pandemic in South Africa.

4.2. Methodology

4.2.1. Study design and patient enrolment

This study utilised samples collected from two existing studies that previously received ethical clearance. Samples were utilised from a paediatric clinical study enrolling children (0-12 years) with respiratory distress requiring hospital admission at Pelonomi Regional Hospital, Universitas Academic Hospital, or National District Hospital in the Free State Province, South Africa. Nasopharyngeal swabs (NPs) were collected and transported for routine testing of RSV, SARS-CoV-2, and *Bordetella pertussis* at the National Health Laboratory Services (NHLS), Bloemfontein, Free State Province, South Africa. Secondly, the cross-sectional study recruited children who were presenting with severe acute respiratory tract infection (SARI) and required hospitalisation. Patients were recruited in Botshabelo District Hospital, Pelonomi Regional Hospital, and National District Hospital in the Free State Province, South Africa. The World Health Organisation (WHO) definition of SARI was used as criteria for sample collection as described previously (Ogunbayo et al., 2022). Nasopharyngeal swabs (BD Diagnostics, Franklin Lakes, NJ, USA) were collected from the children and inserted into a viral transport media (VTM) and placed on ice (4 ℃) then transported to the University of the Free State Next Generation Sequencing Unit (UFS-NGS Unit), and were stored at -80°C until processed. Subsequently, the NPs were tested for several respiratory viruses including RSV as described previously (Ogunbayo et al., 2022). Upon admission, clinicians and or nurses collected relevant clinical and demographic information. Study numbers were used instead of patient identification information.

4.2.3. Ethical consideration

The study protocol was approved by the Health Science Research Ethics Committee (HSREC) of the University of the Free State (HSREC initial approval number: UFS-HSD2021/1616/2501). The Environmental and Biosafety Ethics Research Committee also granted clearance for the use of biological samples in this study (EBREC number: UFS-ESD2021/0256/21). Furthermore, permission to acquire/retrieve samples at NHLS was granted by the Academic Affairs Research Management System (AARMS). Additional HSREC approval was sought, for the additional samples used in this study (HSREC number: (UFS-HSD2021/1616/2501-0002). All procedures were carried out in compliance with the established institutional guidelines.

4.2.4. Sample collection

The NPs that were identified as positive for RSV were selected in children ≤5 years at the time of sampling and samples collected during the COVID-19 pandemic. These samples were retrieved at NHLs (n=50) and UFS-NGS unit (n=19) archives along with the provision of cycle threshold (Ct) values and the list of detected viruses on respiratory panels.

4.2.5. RNA extraction and cDNA synthesis

Viral RNA was extracted directly from 250 µL of RSV-positive clinical sample using the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations, except for the use of a carrier RNA. RNA eluted in 50 µL of elution buffer was stored at -20 °C until use. Additionally, samples with low-quality RNA were further extracted using an automated extraction machine Chemagic™ 360 (PerkinElmer, Waltham, MA, USA), according to the manufacturer's recommendations.

Subsequently, the RNA was reverse transcribed to cDNA and four overlapping RSV fragments were simultaneously amplified in an independent reaction using the SuperScript IV One-Step RT-PCR System (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (Langedijk et al., 2020). The PCR fragments were resolved by agarose gel electrophoresis (2% agarose gel) and visualised using a gel imaging system, then quantified using Qubit™ 1X dsDNA High sensitivity assay kit (Thermo Fisher, Waltham, MA, USA), normalised and pooled in equimolar amounts per amplicon for each sample. Finally, the amplified cDNA products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA), according to the manufacturer's instructions.

4.2.6. Whole genome sequencing of RSV

The purified amplicons were used as a template for Illumina NGS library preparation using the QIAseq FX single cell RNA library preparation kit (Qiagen, Hilden, Germany), following the manufacturers instruction. Prior to sequencing, a Bioanalyzer Agilent 2100 instrument (Agilent Technologies, Santa Clara, CA, USA), was used to analyse the library fragment size distribution. The libraries were then normalized using Qubit 2.0 (Thermo Fisher, Waltham, MA, USA), pooled, diluted, and sequenced using 300-cycles MiSeq reagent v2 standard kit (2x150 bp paired-end) (Illumina, San Diego, CA, USA).

4.3. Data Analysis

4.3.1. Genome assembly

Raw pair-end sequence reads were analysed using Genome Detective, an online based bioinformatics tool (https://www.genomedetective.com/, Accessed 22 November 2022) (Vilsker et al., 2019). The RSV subtypes were assigned, and genome coverage was generated for each sample during the assembly process. Sequences with over 99% genome coverage were further inspected and analysed using Geneious Prime® version 2022.0.1 (Kearse et al., 2012). The reads were assembled/mapped to a reference sequence for both RSVA and B (Accession number NC_013235 and NC_ 001781), respectively using Geneious Prime®, and generated a consensus sequence for each sample.

4.3.2. Generation of global datasets for phylogenetic analysis

All global RSV samples available in the GISAID database (Shu and McCauley, 2017), were downloaded (accessed 24 June 2023). Additionally, all RSV strains from the African continent available in the Bacterial and Viral Bioinformatics Resource Center (BV-BRC, https://www.bv-brc.org/) (Olson et al., 2023), were also downloaded. The Kenyan strains from BV-BRC were excluded due to the overrepresentation of Kenyan strains in the dataset and the GISAID Kenyan dataset was stratified by year and identical sequences removed. Identical sequences representing the repeated sequencing of the same isolate (determined by the corresponding isolate name and date of collection) were removed where identified.

Separate RSV A and B datasets were generated, and multiple sequence alignments were performed using MAFFT v7.490 (Katoh et al., 2002; Katoh and Standley, 2013), applying the FFT-NS-2 algorithm in Geneious. Alignments were visually inspected, and RSV A and B alignments were trimmed to the open reading frame of the G and F genes. Strains with poor sequencing quality, shorter than 300 bp or lacking sampling date or isolation country were excluded.

Prior to phylogenetic analysis, the RSV A and B alignments were tested for recombination by applying multiple models (RDP, GENECONV, Bootscan, Chimaera, SiScan, MaxChi, and 3Seq) using Recombination Detection Program version 5 (RDP5) and any strains with recombination events detected by three or more models were removed from the dataset (Martin et al., 2021).

4.3.3. Recombination

Potential signals for genomic inter-subgroup recombination events were investigated in samples sequenced in this study except for sample hRSV/A/ZAF/UFS-NGS-UNIT/RD-3|2021-02-24 which was excluded due to poor sequence quality. The consensus of the RSV/A samples was used as the comparison reference genome in a similarity plot analysis. A Jukes-Cantor distance model was applied with a window length of 200 bp and step size of 20 bp applied within Simplot 3.5 (Samson et al., 2022).

4.3.4 Phylogenetic analysis of RSVA/B strains

4.3.4.1. Clade assignment

All strains from this study were analysed for clade determination using the online RSV clade typing tool available in Nextclade (https://clades.nextstrain.org) (Aksamentov et al., 2021). Generated phylogenetic trees comprising study strains and reference strains were exported and subsequently visualised using Auspice (https://auspice.us) (Hadfield et al., 2018).

4.3.4.2. Maximum likelihood phylogenetic tree analysis

All global strains were classified into G-clade using the RSV clade typing tool available in Nextclade (https://clades.nextstrain.org) and randomly subsampled, clade-specific alignments were generated for G5.0.5a and GA2.3.5 The optimal nucleotide substitution model for each dataset was determined using ModelFinder (Kalyaanamoorthy et al., 2017). Maximum likelihood (ML) phylogenetic trees were inferred using IQTree version 1.6.12 and node support values were evaluated by bootstrapping to 1000 replicates (Nguyen et al., 2018).

4.3.4.3. Bayesian evolutionary analysis

G-clade datasets were examined for adequate temporal signal using TempEst and divergent samples were removed (Rambaut et al., 2016). Phylogenetic relationships and viral demographic histories were inferred using BEAST 1.10 (Suchard et al., 2018). The optimal nucleotide substitution model was determined using ModelFinder in IQTree. An uncorrelated relaxed molecular clock was specified with branch rates drawn from a lognormal distribution to account for varied evolutionary rates among lineages. A Gaussian Markov Random Fields (GMRF) Bayesian Skyride demographic prior was specified (Suchard et al., 2018). The chain length of MCMC sampling was 100 million generations with sampling every 10,000 generations. Convergence and mixing were examined using Tracer 1.7 and maximum clade credibility (MCC) trees were summarised using TreeAnnotator.

4.3.4.4. Selection pressure analysis

Selection pressures acting on the G and F genes were investigated using the Single-Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL), (Mixed Effects Model of Evolution (MEME), and Fast, Unconstrained Bayesian AppRoximation (FUBAR) methods ($P < .05$ or posterior threshold 0.9 to minimize false positives) (Weaver et al., 2018), implemented in HyPhy (Hypothesis Testing using Phylogenies) (version 2.5.33) (Kosakovsky Pond et al., 2020). As results can differ depending on the chosen method, sites selected by three or more methods were considered robust.

4.3.4.5. N-linked glycosylation

The F and G genes of the RSV A and B datasets comprised of African strains were interrogated to identify putative N-glycosylation sites utilising the N-Glycosite web tool (https://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html) (Zhang et al., 2004).

4.4. Results

4.4.1. Patient Demographics and Clinical Characteristics

Participants were enrolled in 2020 to 2021 and a total of 69 samples were collected. The demographic profiles among the participants were similar, comparable, and unbiased in both groups with respiratory distress and SARI. Among the RSV positive patients, 47/69 (68%) had LRTI, and 11/69 (16%) had URTI. The remaining 16% had no clinical data available. The clinical characteristics of children are shown in Table 4.1. Detailed demographics of the participants of which samples were subsequently used in the analysis are represented in the supplementary material Table 4S1-4S2.

Table 4.1: Clinical Characteristics of Patients infected with RSV during the COVID-19 Pandemic

Patient demographics	Paediatric clinical study	Viral metagenomic cross sectional study
RSV infection cases	66% ≤1 year	37% ≤ 1year
Gender		
Males	48% (24/50)	63% (12/19)
Females	36% (18/50)	37% (7/19)
Unspecified	16% (8/50)	-
Suspected diagnosis		
LRTI	78% (39/50)	37% (7/19)
URTI	2% (1/50)	47% (9/19)
Unspecified	16% (8/50)	16% (3/19)
PICU admission	8% (4/50)	None
Unspecified	16% (8/50)	5 % (1/19)
Feeding difficulty	-	32% (6/19)
HIV infected	4% (2/50)	11% (2/19)
Uninfected	58% (29/50)	89% (17/19)
Exposed	22% (11/50)	-
Unspecified	16% (8/50)	-
Chest indrawing	-	47% (9/19)
Household member smoking	16% (8/50)	16% (/19)
Unspecified	16% (8/50)	-
Unknown	2% (1/50)	-

4.4.2. Genome Sequencing and Assembly

During the study period, only 50 patients with respiratory distress and 19 with SARI were RSV positive. Of these, 72% (50/69) samples had quantifiable viral RNA extracted, of which 76% (38/50) were amplified, and 95% (36/38) were sequenced. In this group 58% (21/36) of the samples had RSV whole genomes successfully sequenced [17 ≥ 99% genome coverage, while four were near-complete (approx. 80% genome coverage)]. Of the total sequenced samples, 25/36 were identified as subtype

RSV-A and 11 as subtype RSV-B with genome coverage ranging from 80-100% (Supplementary Table 4S3-4S4)

4.4.3. Recombination Analysis on Sequenced Study Strains

Four RSV-A study strains were identified as potential inter-subtype recombinants with multiple breakpoints across the genome and regions derived from RSV-B (Figure 4.1, Supplementary Table 4S5). Additional figures are provided in appendix figure A6-A7.

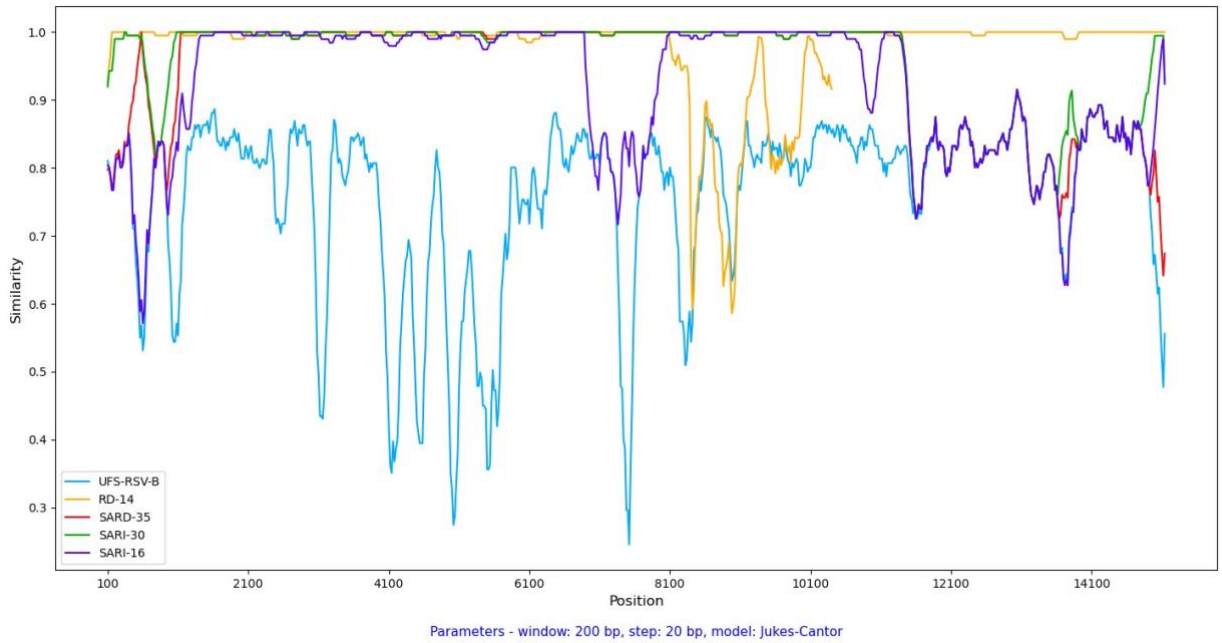

Figure 4.1. Recombination analysis of the complete genome of the sequenced study strains. The consensus of non-recombinant RSV-A study strains was used as the reference whilst the consensus of RSV/B study strains is presented by "UFS-RSV-B".

4.4.4. Clade Assignment

The RSV-A strains from this study were classified as clade GA2, corresponding to the previously recognised ON1 genotype. Six lineages have been identified within the GA2.3 sub-genotype (GA2.3.1-GA2.3.6) with strains from this study clustering within GA2.3.5 (Figure 4.2). The RSV B strains were classified within clade GB5.5 corresponding to the previously recognised BA type. Five lineages have been identified within GB5.0 (GB5.0.1-GB5.0.5). The samples from this study belong to GB5.0.5a (Figure 4.3).

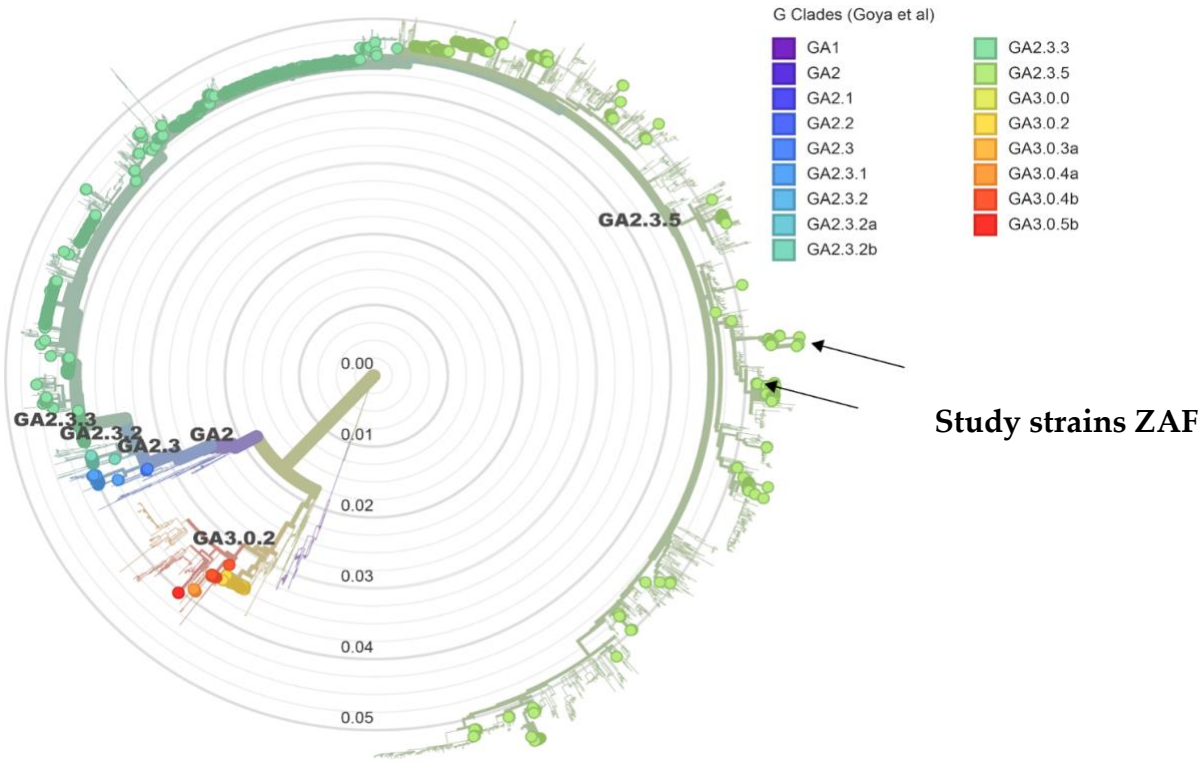

Figure 4.2: Phylogenetic tree of major RSV A clades generated using NextClade. Previously characterised South African taxa are represented by circles coloured based on clade. The strains from this study formed two separate clusters (indicated with black arrows) within GA2 clade GA2.3.5 sub-genotype.

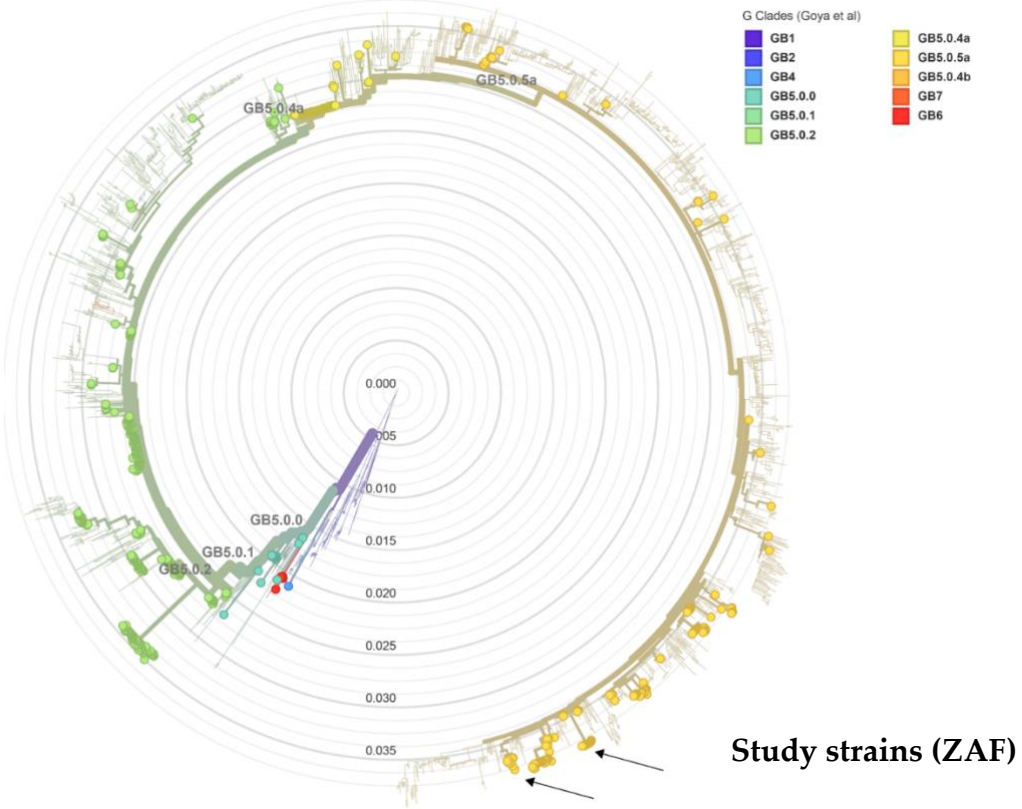

Figure 4.3: Phylogenetic tree of major RSV B clades generated using NextClade. Previously characterised South African taxa are represented by circles coloured based on clade. The strains from this study formed two separate clusters (indicated with black arrows) within GB5 clade GB5.0.5a sub-genotype.

4.4.5. Maximum Likelihood Phylogenetic Tree of RSV-A

In the pre-COVID-19 era, specifically, from 2015 to 2018, there were several co-circulating variants of GA2.3.5 in South Africa, showing high genetic similarity to global strains, primarily from Europe, North America, and other countries within the African continent (Figure 4.4). The phylogenetic tree showed minor endemic circulation of strains within Africa reflecting the continued introduction of variants from outside the continent. Several variants that circulated in the pre-COVID-19 era were not detected from 2018 onwards including the dominant South African variant that circulated between 2015-2018 which was also detected in Mozambique.

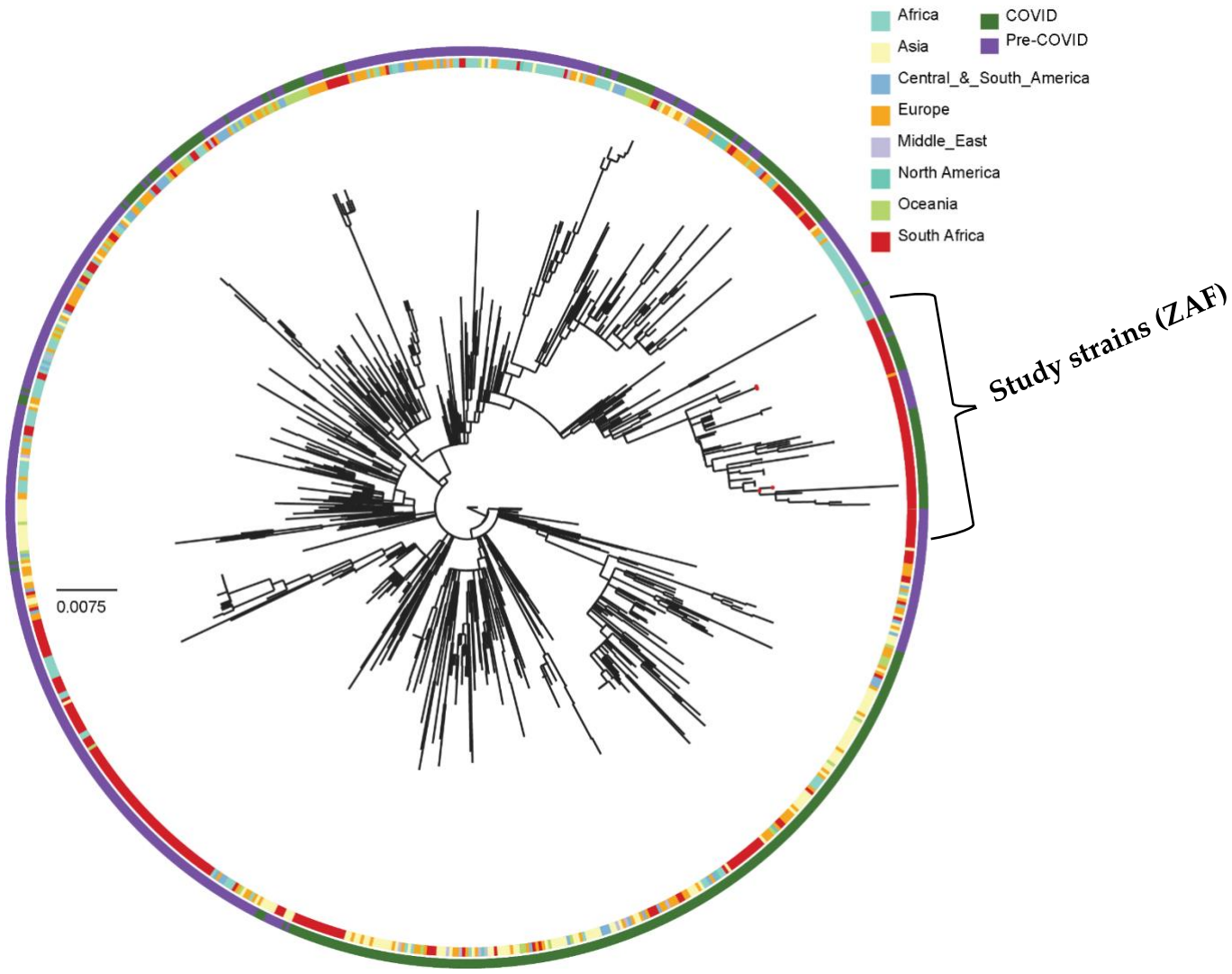

Figure 4.4: Phylogenetic tree of global representative GA2.3.5 strains constructed by maximum likelihood method at 10000 bootstrap replicates. Study strains are indicated by a red circle. The bootstrap values are not displayed at the branch nodes of the tree. A detailed phylogenetic tree with bootstrap support shown is presented in Supplementary Figure 4S1. The phylogenetic tree is drawn to scale; the scale bar represents the number of nucleotide substitutions per site.

The strains detected in South Africa between 2020-2021 were genetically diverse forming a number of small clusters and most of these small clusters were closely related to South African strains detected in 2018, suggesting that the increase in RSV-A detected was not due to a dominant new variant recently introduced into South Africa, rather the population was largely derived of strains that had likely evolved within the country over the intervening years. A small number of variants appeared to be recent introductions following the re-opening of borders showing a high degree of genetic similarity to globally circulating strains. No unique clustering patterns were observed with strains from other regions that could suggest sustained introductions of novel variants into the population. The samples from the Free State formed two discrete clusters, with the largest cluster forming a monophyletic cluster with contemporary South African strains from other provinces and most closely related to samples from the Gauteng province. Based on the phylogeny this variant was likely derived from viruses that circulated in Kenya between 2015-2018. There was a high degree of circulation of all variants across the country indicating the widespread nationwide transmission of variants.

4.4.6. Maximum Likelihood Phylogenetic Tree of RSV-B

The sequencing data for South African RSV-B strains indicated that GB5.0.5a circulated as the dominant variant from the mid-2000s to 2015 with no sequencing data from 2016 and 2017. The GB5.0.5a was a minor variant in 2015 and emerged in 2018 to become the dominant clade (Figure 4.5). Between 2018 and 2019, multiple GB5.0.5a variants co-circulated within South Africa (Figure 4.5). These strains exhibited a high degree of genetic diversity, forming numerous clusters with contemporary strains of diverse geographic origins reflecting the repeated seeding of global variants into South Africa rather than the endemic circulation of African variants. The dominant lineage of South African strain that circulated prior to the COVID-era was largely undetected after 2020. Between 2020 and 2022, multiple GB5.0.5a variants co-circulated with contemporaneous strains from various geographic regions with the strains probably introduced recently into South Africa rather than evolving from variants which circulated in the country prior to the COVID-19 era. The samples from the Free State formed two discrete clusters, with the largest cluster forming a monophyletic cluster with contemporary South African strains from other provinces and most closely related to samples from the Western Cape and North West provinces. The smaller Free State cluster was not closely related to contemporary South African strains and were most closely related to strains from various geographic regions. There was a high degree of circulation of all variants

across the country with limited geographic clustering indicating the widespread nation-wide transmission of variants.

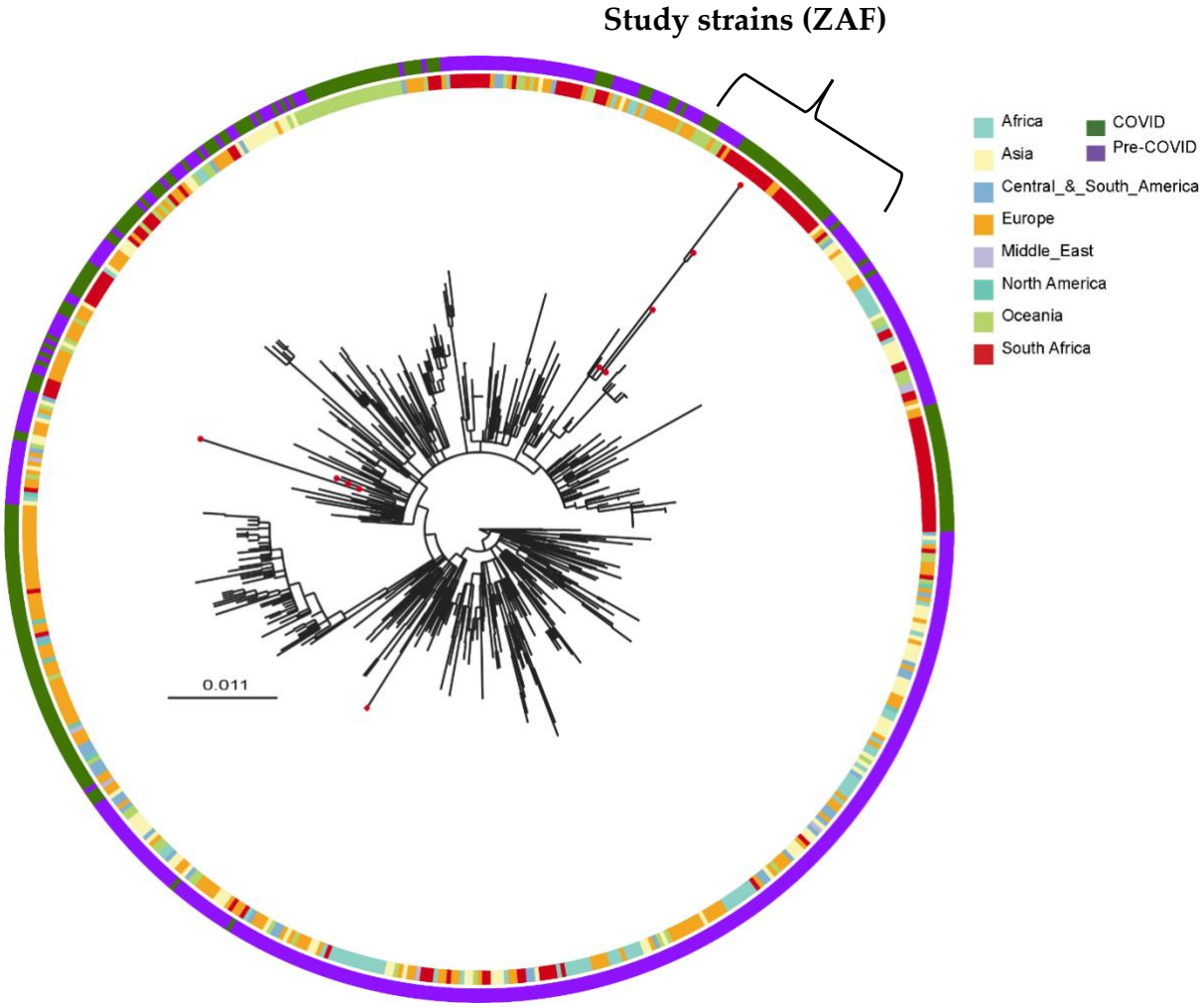

Figure 4.5: Maximum likelihood phylogenetic tree Global G-Gene GB5.0.5a strains. Study strains sequenced in this study are shown in a red circle. A detailed phylogenetic tree with bootstrap support shown is presented in Supplementary Figure 4S2. The scale number indicates the number of nucleotide substitutions per site.

4.4.7. Bayesian Reconstruction of Evolutionary Histories

The time to most recent common ancestor (TMRCA) of the South African GA2.3.5 strains from the most recent tip (22nd of June 2022) (Figure 4S3), was 11.64 years (95% HPD interval [11.0216, 12.2639]) resulting in an estimated emergence of this clade in late 2010 (2010.833, 95% HPD interval [2010.2074, 2011.4497]). The nucleotide substitution rate for the South African GA2.3.5 strains was estimated at 2.033×10^{-3} (95% HPD interval [1.6708×10^{-3}, 2.428×10^{-3}]. The TMRCA of the South Africa GB5.0.5a strains from the most recent tip (19th of July 2022) was 8.51 years (95% HPD interval [7.8568, 9.2842]) resulting in an estimated emergence of this clade in early 2014 (2014.035, 95% HPD

interval [2013.261, 2014.6884]). The nucleotide substitution rate for the South African GB5.0.5a (Figure 4S4), strains was estimated at 4.081×10^{-3} (95% HPD interval [2.5524×10^{-3}, 5.801×10^{-3}]).

4.4.8. The Relative Genetic Diversity of the GA2.3.5 and GB5.0.5a Strains

Between 2017 and 2022 the South African GA2.3.5 and GB5.0.5a strains showed very similar patterns of genetic diversity with a slight bottleneck observed between 2019 and 2020 which represented multiple variants ceasing to circulate in the population during the early years of the COVID-19 era coinciding with lockdowns and extensive NPIs. This was followed by an increase in diversity between 2021 and 2022 as multiple variants co-circulated with the introduction of new variants, with diversity decreasing throughout 2022 due to reduced sampling (Figure 4.6).

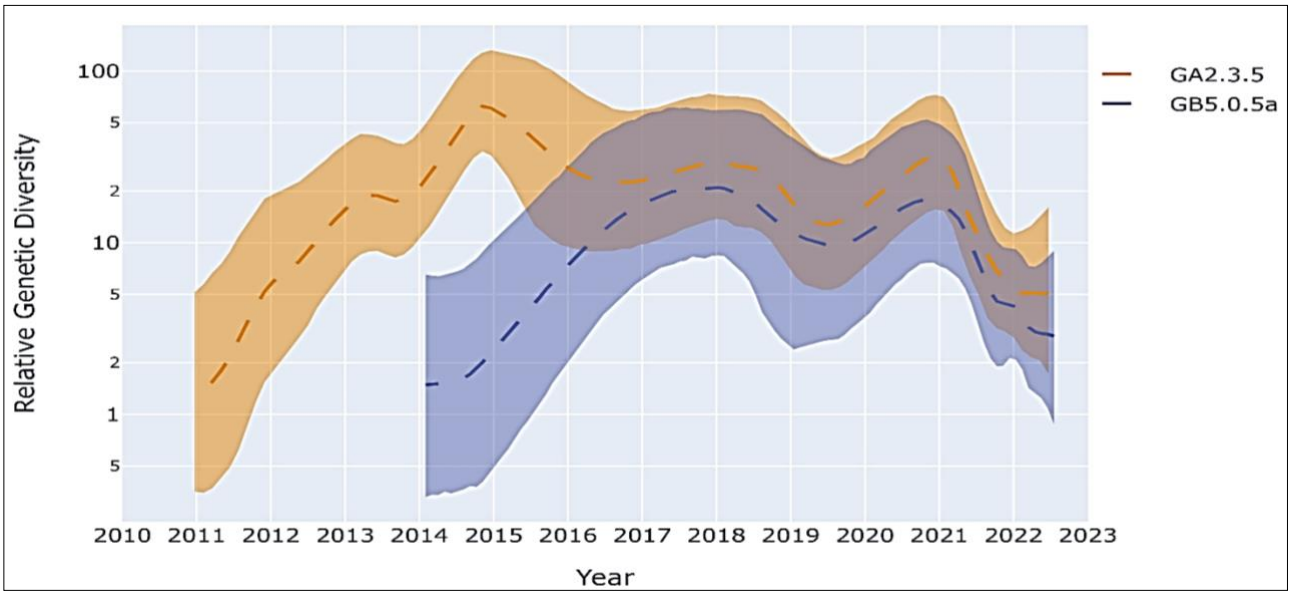

Figure 4.6. The relative genetic diversity of South African GA2.3.5 and GB5.0.5a strains. A measure of relative genetic diversity is given on the y-axis with the 95% highest posterior density shown in solid colour and the median as a dashed line.

4.5. Selection Pressure Analysis

4.5.1. Selection on RSVA/B strains

The entire coding region of the F gene of all African strains (regardless of clade due to the conserved nature of the F gene) were analysed for sites under positive selection. In addition, the South African strains were analysed separately to infer any country-specific selection pressure. In the African RSV-A F African dataset (Table A3), the amino acid position 554 was found to be under positive selection by all models. The country-specific analysis conducted on the RSV-A South African dataset did not identify any selection sites that exhibited positive selection.

For the G gene, multiple sites within the RSV-A African GA2.3.5 dataset (Table A1), were detected under positive selection, specifically, amino acid positions 273, 290, 297, and 314 were consistently identified as undergoing positive selection across all the models used in the analysis. Furthermore, a separate analysis was conducted on the GA2.3.5 South African strains to determine if there were any selection pressures specific to the country. Notably, sites 298, 308, and 314 were identified as being subjected to positive selection, indicating potential evolutionary pressures unique to South Africa (Table A2).

Positive selection acting on the fusion protein was detected at amino acid position 125 in the RSV-B F African dataset (Table A6), as indicated by the four selection models employed in the study. A consensus among all the models indicates that amino acid position 4 in the RSV-B F South African dataset was subject to positive selection (Table A7). The amino acid positions 217, 226, 229, 245, 252, 268, 285, 303, and 310 within the RSV-B GB5.0.5a G gene African dataset (Table A4), exhibited positive selection across all models. For the South African GB5.0.5a G gene dataset, amino acid positions 217 and 285 were identified under positive selection (Table A5).

4.6. N-Linked Glycosylation

In the G gene of South African GA2.3.5 strains, residues 85, 103, 135, 237 and 318 were identified as N-glycosylation sites. Residues 85, 103, 135 were highly conserved in COVID-19 era strains and some pre COVID-19 era strains but absent in strains in South Africa in 2018. This may be evidence of selection of strains with these glycosylation sites. The absence of an N-glycosylation site at residue 237 was infrequently detected in strains both prior to and during the COVID-19 era, of note, 8/11 strains sequenced in this study lacked this N-glycosylation site. Residue 318 varied in strains both prior to and during the COVID-19 era.

In the G gene of South African GB5.0.5a strains residues 81, 86, 228, and 294 were identified as frequent N-glycosylation sites. Residues 81 and 86 were more frequently identified as potential N-glycosylation sites in strains that circulated from the COVID-19 era. Residue 228 was identified as a potential N-glycosylation site in a minority of samples mostly from the COVID-19 era including all samples sequencing in this study. In most South African strains, a proline amino acid was observed at this site which prohibits the formation of *N*-linked glycans.

In both the RSV-A and RSV-B F gene African datasets five potential N-glycosylation sites were noted located at residues 27, 70, 116, 126, and 500. These sites were found to be highly conserved in all

South African strains regardless of year of detection. The presence of a sixth N-glycosylation site at residue 120 showed more variability. In contrast to the other strains, the study strain SARI-18 displayed an additional distinctive N-glycosylation site at position N437.

4.7. Discussion

To curb the transmission of SARS-CoV-2 during the peak of the COVID-19 pandemic, the government of South Africa declared a national state of disaster on 15 March 2020 with a national lockdown implemented from 27 March to 30 April 2020 with international and local travel restrictions, closure of all non-essential businesses and schools, and citizens were confined to their residences (Bents et al., 2022). Additional non-pharmaceutical interventions including social distancing, travel bans, school closures, and mask wearing were in place between March and November of 2020. These measures impacted the transmission of RSV within South Africa with decreased RSV disease reported in 2020 and the previously established seasonality not observed. Coinciding with the relaxation of NPIs, an out-of-season outbreak of RSV was reported that continued into 2021 (Bents et al., 2022; Perofsky et al., 2022). Disease was reduced between March and November of 2021 as stricter NPIs were implemented to control the third wave of COVID-19 (Bents et al., 2022). This phenomenon was not restricted to South Africa. The global rate of RSV infection decreased dramatically during the peak of the COVID-19 pandemic (Abu-Raya et al., 2023). As NPI measures were relaxed an off-season resurgence of RSV was reported. An example, is the atypical surge in cases reported during the Australian summer of December 2020 to February 2021 (Britton et al., 2020). Another atypical resurgence of RSV in Senegal characterised by increased RSV detections with a temporal shift was observed between September and October 2022 (Jallow et al., 2022).

This study undertook a whole genome sequencing of both RSV-A and -B strains detected in 2021 and 2022 in the Free State province to understand specific changes in the circulating clades or the emergence of novel strains, if any, associated with the increased disease following the peak of the COVID-19 pandemic. The phylogenetic analysis revealed that over the study period, the circulating strains in South Africa were lineages GA2.3.5 (previously ON1) and GB5.0.5a (previously BA9). Recent studies have corroborated these findings, indicating that GA2.3.5 and GB5.0.5a were the prevailing types circulating prior to and following the COVID-19 pandemic in several countries (Goya et al., 2023; Jallow et al., 2022; Redlberger-Fritz et al., 2023). The RSV-A ON1 lineage has been globally dominant worldwide since its initial detection in Ontario, Canada, in 2010 (Eshaghi et al.,

2012; Lu et al., 2019), and during the COVID-19 pandemic (Eden et al., 2022; Jia et al., 2022; Lee et al., 2023). The BA genotype has gained global prevalence since its emergence and is currently the dominant RSV-B genotype in circulation across numerous countries (Gaymard et al., 2018; Trento et al., 2010).

As previously described, in the years immediately prior to the COVID-19 pandemic (2015-2017), ON1 genotype strains were dominant in South Africa (Liu et al., 2020). These variants were frequently introduced into South Africa from diverse geographic origins. During 2020, there was a reduction in the number of GA2.3.5a variants co-circulating in the country evident in both the phylogenetic analysis and in the reconstruction of the relative genetic diversity of GA2.3.5a strains. The strains circulating during the upsurge of disease were largely derived from strains that circulated prior to the COVID-19 era with a small number of variants introduced into the country. The phenomenon of diversification occurring at the local level has been documented and is believed to contribute to the persistence of ON1 variants within a population across various seasons (Agoti et al., 2017; Duvvuri et al., 2015; Lu et al., 2019). This is also in line with what has been observed in other countries where strains that circulated prior to the COVID-19 era were associated with the upsurge in cases once NPIs ceased (Jia et al., 2022; Lee et al., 2022). This contrast with the resurgence of RSV in Argentina in 2021 which was attributed to the introduction of new viral strains from other countries as potential drivers of the outbreak (Dolores et al., 2022).

Of note, the GA2.3.5a strains that persisted from the pre-COVID-19 era in South Africa appear to have given rise to the dominant variant detected in 2021 and 2022 which lacked N-glycosylation residues 85, 103, 135 which were a feature of COVID-19 era strains suggesting a pressure acted on this variant during 2019-2020 selecting strains with these N-glycosylation residues. Interestingly these N-glycosylation residues were detected frequently in strains that circulated in 2013-2015. Residues 298, 308, and 314 were determined to be under positive selection in the South African dataset with residues 273, 290, 297, and 314 in African GA2.3.5. The sites under positive selection were not unique to strains circulating in the COVID-19 era.

In contrast to RSV-A, where the same G-clade had predominated for many years prior to the COVID-19 era, GB5.0.2 circulated as the dominant variant from the mid-2000s to 2015 and GB5.0.5a emerged as dominant from 2018 onwards. The dominant lineage of South African GB5.0.5a strains that circulated prior to the COVID-19 era was largely undetected after 2020. Unlike the GA2.3.5 strains that were largely derived from pre-existing variants, multiple GB5.0.5a variants co-circulated in

2020-2022 with global contemporaneous strains introduced recently into South Africa rather than evolving from endemic variants. For the South African GB5.0.5a G gene dataset, amino acid positions 217 and 285 were identified under positive selection but were not associated with strains emerging in the COVID- 19 era. Residues 81 and 86 were more frequently identified as potential N-glycosylation sites in strains that circulated from the COVID-19 era. This is consisted with a previous study (Kamau et al., 2020).

The period of emergence of the GA2.3.5 clade in South Africa was late 2010 and early 2014 for GB5.0.5a. This corresponds with the global emergence of these clades and suggests they were seeding into South Africa soon after their global emergence. The evolutionary estimates in our study differ slightly from previous estimates, 2.033×10^{-3} and 4.081×10^{-3} nucleotide substitutions/site/year for GA2.3.5 and GB5.0.5a respectively. The previously described rates of evolution have been for the ON1 lineage rather than the GA2.3.5 clade, with estimates reported of 3.06×10^{-3} nucleotide substitutions/site/year for global strains and country-specific analysis of Kenyan isolates reported a rate of 2.89×10^{-3} nucleotide substitutions/site/year (Otieno et al., 2016). Similarly, a prior South African study that estimated the rate of evolution for the entire BA lineage reported at 5.8907×10^{-3} nucleotide substitutions/site/year (Pretorius et al., 2013).

A decline in the relative genetic diversity was observed in both South African GA2.3.5 and GB5.0.5a viral populations. This bottleneck can be attributed to the reduced circulation of RSV during the peak of the COVID-19 pandemic when NPIs reduced the transmission of respiratory pathogens in the community and numerous variants died out. A subsequent increase in relative diversity could be attributed to increased off-season cases reported as public health measures were eased suggesting the resurgence of RSV variants circulating in the population.

The pandemic-era NPIs created a unique environment where infants lacked exposure to RSV, creating an immunity debt. The increased cohort of immunologically naïve children may have been a contributing factor in selecting which variants flourished during the off-season outbreaks. The glycosylation of RSV is a significant characteristic that determines the antigenicity of the virus and exhibits considerable variation among different strains of RSV (Gimferrer et al., 2015). Notably, the virus can modify the potential N-linked glycosylation sites, resulting in changes to the antigenic properties of the viral protein (Feng et al., 2022). This modification masks specific epitopes from the host's immune system, thereby affecting the virus's interaction with host immunity (Collins et al., 2013). In both the RSV-A and RVS-B F gene datasets the same potential N-glycosylation sites were

observed and were highly conserved regardless of the year of detection suggestive of the significance of the sites in the protein function and biological activity (Feng et al., 2022; Leemans et al., 2019; Schobel et al., 2016). The present research extends the previous finding of an absence of an N-glycosylation site at residue 120 in the RSV-A Fusion protein from strains in South Africa that were identified after 2018 (Mabilo et al., 2022). The N-glycosylation for the G protein showed more variability in strains that emerged in the post-COVID-19 era. For GA2.3.5 strains, N-glycosylation of residues 85, 103, and 135, was associated with the emergence of COVID-19 era strains. N-glycosylation residues 237 and 318 were inconsistently detected in both pre- and COVID-19 era samples. For South African GB5.0.5a strains residues 81 and 86 were more frequently identified as potential N-glycosylation sites in strains that circulated from the COVID-19 era. The strains from the Free State province exhibited an additional N-glycosylation site at residue 228 that was rarely seen in other strains.

Based on our clinical data, the risk factors for severe RSV infection identified and recorded in this study were few. Therefore, in our study, during the 2020–2021 epidemic season, the clinical data collected and observed was gender, age, exposure to smoke, length of hospital stay, or ICU admissions. Moreover, there was a slight variation observed in the prevalence of subtypes noted during the 2020-2021 outbreak, and RSV-A being the predominant subtype in our study samples. Additionally, coinfections have the potential to heighten the severity of RSV disease, however, a direct correlation between coinfection and disease severity remains inconclusive, as others found no definitive link between disease severity and coinfection (Goka et al., 2014; Luo et al., 2020; Scotta et al., 2016; Tan et al., 2021). In this study, due to the multiplex PCR system employed in the diagnosis of children in the SARI group, co-infection involving other respiratory viruses such as rhinovirus, SARS-CoV-2, and adenovirus were detected. However, children in the respiratory distress arm had no co-infection documented and may be due to the use of a multiplex PCR with limited viral pathogens targeted. In essence, the adoption of a multiplex system in the diagnosis of respiratory infection in children could aid early identification of co-infection which could guide clinical decisions and ultimately result in better clinical outcomes (Lamrani Hanchi et al., 2021).

This study underscores the continued importance of surveillance efforts in detecting and characterizing new variants of RSV to understand the circulating patterns of these strains. The primary limitation of this research was the sample size (samples collected during the pandemic), limited clinical information, and quantity of RSV genomes sequenced in this study. Nevertheless, to

the best of our knowledge, this is the first report on the diversity of RSV-A and -B circulating strains during the COVID-19 period characterised by off-season outbreaks of RSV in South Africa.

In conclusion, this study demonstrated that 2020-2021 RSV surge in South Africa during the COVID-19 pandemic was primarily caused by pre-existing lineages rather than any unique or novel genetic variant. The implementation of NPIs for COVID-19 exerted pressure on the transmission dynamics of RSV, resulting in the delay of epidemic seasons in 2020 and an atypical rise in RSV-positive cases during the spring-summer period. Further investigation is necessary to explore the impact of the shift in RSV epidemiology and on potential future outbreaks. Gaining a deeper understanding of the molecular diversity and evolution of RSV through continuous monitoring during pandemic periods is important, it also can offer valuable insights and assist in making informed public health decisions as well as advancing vaccine development.

Supplementary Data

Additional materials have been provided to supplement the main content.

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Supplementary Table 4S1: Clinical information for children under the age of ≤5 years recruited for the Paediatric clinical study.

Sample ID	Age months	Gender	Symptoms	Suspected diagnosis	Adm_PICU	HIV_Status	Pas_Smoking_Ex	Duration of stay (days)	Ct Values
RD-1	72	Male	Cough Shortness of breath Sore throat	URTI croup	No	Uninfected	Yes		24,33
RD-2	7	Female	Cough Fever >38 °C Shortness of breath Nausea/Vomiting General weakness	LRTI	No	Uninfected	No	12	30,84
RD-3	NA	Male	Cough Fever >38 °C Shortness of breath Nausea/Vomiting	LRTI	No	Exposed uninfected	Yes	5	20,91
RD-4	2	Female	Cough Fever >38 °C Shortness of breath	LRTI	No	Exposed uninfected	No	4	28,5
RD-5	46	Male	Cough Fever >38 °C Nausea/Vomiting	LRTI	No	Uninfected	No	3	23,46
RD-6	18	Male	Cough Shortness of breath Nausea/Vomiting Diarrhoea	LRTI	No	Uninfected	Yes	2	25,61
RD-7	<1	Male	Cough Fever >38 °C Shortness of breath Other	Other	Yes	Uninfected	No	16	36,37
RD-8	30	Male	Cough Sore throat Nausea/Vomiting Irritability/confusion	LRTI	No	Uninfected	Yes	2	19,49
RD-9	6	Male	Cough Shortness of breath	LRTI	No	Unknown	No	4	18,09
RD-10	12	Male	Cough Fever >38 °C Shortness of breath Diarrhoea Irritability/confusion	LRTI	No	Uninfected	No	5	21,23
RD-11	4	Male	Cough Fever >38 °C Shortness of breath Nausea/Vomiting	LRTI	No	Uninfected	Yes	3	27,91
RD-12	1	Female	Cough	LRTI	No	Uninfected	No	0	15,15
RD-13	2	Female	Cough Shortness of breath Irritability/confusion	LRTI	No	Uninfected	No	2	19,92
RD-14	9	Male	Cough Fever >38 °C Nausea/Vomiting	LRTI	No	Infected on ART	No	1	29,49
RD-15	11	Female	Cough Fever >38 °C	LRTI	No	Exposed uninfected	No	12	21,52

			Shortness of breath						
RD-16	1 4	Female	Cough Fever >38 °C Shortness of breath	LRTI	No	Uninfected	No		32,6
RD-17	1	Male	Cough Fever >38 °C Shortness of breath Irritability/confusion General weakness	LRTI	No	Uninfected	No	4	30,18
RD-18	1	Male	Cough Fever >38 °C Shortness of breath	LRTI	No	Uninfected	No	13	25,08
RD-19	3	Female	Cough Shortness of breath	LRTI	No	Uninfected	Yes	2	20,07
RD-20	1	Male	Cough Nausea/Vomiting	LRTI	No	Uninfected	No	3	21,62
RD-21	1	Male	Cough Shortness of breath Irritability/confusion General weakness	-	No	Uninfected	Yes	5	26,38
RD-22	2	Male	Cough Shortness of breath Diarrhoea Nausea/Vomiting	LRTI	No	Uninfected	No	2	28,88
RD-23	4	Female	Cough Shortness of breath	LRTI	No	Exposed uninfected	No	5	-
RD-24	1	Male	Shortness of breath Irritability/confusion	LRTI	No	Uninfected	No	7	-
RD-25	NA	Male	Cough	LRTI	Yes	Infected on ART	No	30	-
RD-26	3	Female	Cough	LRTI	Yes	Exposed uninfected	No	10	-
RD-27	12	Male	Cough	LRTI	No	Exposed uninfected	No		-
RD-28	3	Male	Cough Fever >38 °C Shortness of breath Nausea/Vomiting Diarrhoea Irritability/confusion	LRTI	No	Uninfected	No	7	-
RD-29	11	Female	Cough Fever >38 °C Shortness of breath	LRTI	No	Uninfected	No	5	-
RD-30	5	Male	Cough Shortness of breath	LRTI	No	Uninfected	No	12	-
RD-31	37	Female	Cough Fever >38 °C	LRTI	No	Exposed uninfected	Unknown	3	-

RD-32	12	Female	Cough Fever >38 °C Shortness of breath Nausea/Vomiting Diarrhoea General weakness	LRTI	No	Exposed uninfected	No		25,11
RD-33	<1	Female	Cough Fever >38 °C	LRTI	No	Exposed uninfected	No	5	31,12
RD-34	30	Female	Cough Fever >38 °C Myalgia Nausea/Vomiting Irritability/confusion General weakness	LRTI	No	Uninfected	No	1	22,81
RD-35	1	Male	Cough Shortness of breath Nausea/Vomiting	LRTI	No	Uninfected	No	3	-
RD-36	44	Male	Cough Shortness of breath	LRTI	No	Uninfected	Yes	3	-
RD-37	4	Female	Cough Shortness of breath	LRTI	No	Uninfected	No	6	-
RD-38	3	Female	Cough Shortness of breath Nausea/Vomiting	LRTI	Yes	Uninfected	No	13	-
RD-39	2	Female	Cough Shortness of breath Irritability/confusion	LRTI	No	Exposed uninfected	No	4	24,7
RD-40	11	Male	Cough Fever >38 °C Shortness of breath Myalgia Diarrhoea Irritability/confusion	LRTI	No	Uninfected	No	4	24,1
RD-41	3	Female	Cough Fever >38 °C Shortness of breath Other	LRTI	No	Exposed uninfected	No	4	31,84
RD-42	47	Male	Cough Fever >38 °C Diarrhoea General weakness	LRTI	No	Uninfected	No	7	31,61

Supplementary Table 4S2: Clinical information for children under the age of ≤5 years recruited for the Metagenomics cross sectional study

Sample synonymous no	Age	Gender	Symptoms	Suspected diagnoses	ICU admission	HIV infected	Household member smoking	Feeding difficulty	Chest indrawing	Need for oxygen	Creche attendance	Viruses detected (coinfections)	RSV ct values
SARI-6	4 months	Male	Fever ≥ 38° C, Cough, Difficulty in breathing	URTI (Pneumonia)	No	Yes	No	No	Yes	Yes	No	-	26.2
SARI-15	6 Months	Male	Fever ≥ 38° C, Cough, Difficulty in breathing	URTI (Pneumonia)	No	Yes	No	No	Yes	Yes	No	-	25.6
SARI-16	3 Years	Female	Fever ≥ 38° C, Cough, Wheezing	LRTI (Pneumonia)	No	No	No	No	No	No	No	Adenovirus (33.4)	22.5
SARI-17	2 weeks	Female	Fever ≥ 38° C, Cough, Difficulty in breathing	URTI (Pneumonia)	No	No	No	Yes	Yes	Yes	No	-	25.9
SARI-18	3 years 2 months	Female	Fever ≥ 38° C, Cough, Severe Pneumonia	URTI (Pneumonia)	No	No	No	No	Yes	Yes	No	Rhinovirus/Enterovirus (30.9)	28.2
SARI-19	2 years 2 months	Female	Fever ≥ 38° C, Cough, Difficulty in breathing	LRTI (Pneumonia)	No	No	No	no	Yes	Yes	No	Human metapneumovirus (34.3)	26.5
SARI-20	1 year 2 months	Female	Fever ≥ 38° C, Cough, wheezing	URTI (Pneumonia)	No	No	No	Yes	No	Yes	No	Rhinovirus/Enterovirus (35.2)	26.3
SARI-21	10 months	Male	Fever ≥ 38° C, Cough, Difficulty in breathing, wheezing	LRTI (Bronchiolitis)	No	No	Yes	yes	No	Yes	Yes	Rhinovirs/Enterovirus (31.0)	22.8
SARI-22	8 Months	Male	Fever ≥ 38° C, Cough, Difficulty in breathing	LRTI (Pneumonia)	No	No	No	Yes	Yes	Yes	No	Rhinovirus (29.1)	20.8

SARI-23	2 Years	Male	Fever ≥ 38° C, wheezing	URTI (Pneumonia)	No	No	No	No	No	No	No	Coronavirus NL63 (32.0)	20.7
SARI-24	1 year 4 months	Male	Fever ≥ 38° C, Cough, Difficulty in breathing	URTI (Pneumonia)	No	No	Yes	No	Yes	Yes	Yes	-	21.3
SARI-25	2 years 3 Months	Male	Fever ≥ 38° C, Cough	URTI (Pneumonia)	No	No	No	No	No	No	No	Parainfluenza virus 3 (31.0), Rhinovirus (32.0)	22.5
SARI-26	1 year 9 months	Male	Fever ≥ 38° C, Cough, Difficulty in breathing	LRTI	No	No	Yes	No	No	No	No	-	26.2
SARI-27	1 year 1 months	Female	Fever ≥ 38° C, Cough, Difficulty in breathing	URTI (Pneumonia)	No	No	No	Yes	Yes	Yes	No	Parainfluenza virus 3(31.7), Rhinovirus/Entero virus (33.3),	34.9
SARI-30	5 months	Male	Fever ≥ 38° C, Cough, Difficulty in breathing, wheezing	Unspecified (Pneumonia)	No	No	No	No	Yes	Yes	No	-	20.3
SARI-31	8 months	Female	Fever ≥ 38° C, Cough, Difficulty in breathing	LRTI (Pneumonia)	unspecified	No	No	No	No	No	No	-	15.3
SARI-34	1 year 3 months	Male	Fever ≥ 38° C, Cough, Wheezing	LRTI (Broncholitis)	No	No	No	no	Yes	Yes	No	Rhinovirus/Entero virus (34.3), SARS-CoV-2 (35.5)	24.1
SARI-35	3 years 8 months	Male	Fever ≥ 38° C, Cough	Unspecified (Broncholitis)	No	No	No	No	No	No	No	Rhinovirus (32.9)	25.4
SARI-36	1 year 3 months	Male	Fever ≥ 38° C, Cough	Unspecified	No	No	No	Yes	No	No	Unspecified	Adenovirus (34.1)	26.6

Supplementary Table 4S3: Genome coverage assigned subtype and the length of RSV-A and RSV-B sequences analysed.

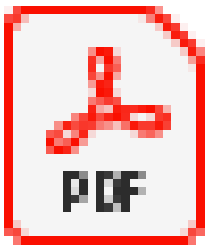
Sample	Subgroup	Reads Mapped to reference	Coverage	Depth	Length	Viruses detected	Coinfection detected by QIAstat panel	Cycle threshold (Ct)
Participants								
hRSV/A/ZAF/UFS-NGS UNIT/RD-3| 2021-02-24	A	210328	84.2	1716.1	12815	RSV	-	20,91
hRSV/A/ZAF/UFS-NGS UNIT/RD-4|2021-02-28	A	272038	100	1923.9	15218	RSV	-	28,5
hRSV/A/ZAF/UFS-NGS UNIT/RD-5| 2021-03-11	A	209278	100	1534.8	15220	RSV	-	23,46
hRSV/B/ZAF/UFS-NGS UNIT/SARI-6|2021-02-11	B	344108	100	2373.8	15219	RSV	-	26.2
hRSV/A/ZAF/UFS-NGS UNIT/RD-8| 2021-02-21	A	224844	100	1560.8	15217	RSV	-	19,49
hRSV/A/ZAF/UFS-NGS UNIT/RD-9| 2021-02-23	A	115064	99.7	797.3	15170	RSV	-	18,09
hRSV/A/ZAF/UFS-NGS UNIT/RD-10|2021-03-16	A	130005	99.8	1568.9	15180	RSV	-	21,23
hRSV/B/ZAF/UFS-NGS UNIT/RD-11|2021-05-04	B	174056	88.9	1361.3	13539	RSV	-	27,91
hRSV/B/ZAF/UFS-NGS UNIT/RD-12|2021-03-16	B	241883	87.5	1997.8	13319	RSV	-	15,15
hRSV/A/ZAF/UFS-NGS UNIT/RD-13| 2021-03-14	A	215118	100	1535.7	15216	RSV	-	19,92
hRSV/A/ZAF/UFS-NGS UNIT/RD-14| 2021-03-15	A	200327	85.2	1629.8	12976	RSV	-	29,49
hRSV/B/ZAF/UFS-NGS UNIT/SARI-15|2021-06-16	B	164869	97.7	1230.4	14876	RSV	-	25.6
hRSV/A/ZAF/UFS-NGS UNIT/SARI-16|2020-12-21	A	138328	100	1005.7	15216	RSV	Adenovirus (33.4)	22.5
hRSV/B/ZAF/UFS-NGS UNIT/SARI-17|2020-21-12	B	363351	99.9	2605.1	15204	RSV	RSV A+B (25.9)	25.9
hRSV/B/ZAF/UFS-NGS UNIT/SARI-18|2020-12-02	B	232861	98.7	1503.5	15025	RSV	Rhinovirus/Enterovirus (30.9)	28.2
hRSV/B/ZAF/UFS-NGS UNIT/SARI-19|2020-12-29	B	252485	99.9	1743.7	15204	RSV	Human metapneumovirus (34.3)	26.5
hRSV/A/ZAF/UFS-NGS UNIT/SARI-30| 2021-01-05	A	358164	100	2509.1	15221	RSV	-	20.3
hRSV/B/ZAF/UFS-NGS UNIT/SARI-31|2021-02-11	B	361250	99.5	2672	15142	RSV	-	15.3
hRSV/B/ZAF/UFS-NGS UNIT/SARI-34|2021-03-02	B	226421	99.9	1711.8	15212	RSV	Rhinovirus/Enterovirus (34.3), SARS-CoV-2 (35.5)	24.1
hRSV/A/ZAF/UFS-NGS UNIT/SARI-35|2021-03-02	A	447648	100	3458.3	15221	RSV	Rhinovirus (32.9)	25.4
hRSV/B/ZAF/UFS-NGS UNIT/SARI-36|2020-12-14	B	602006	99.9	4629.3	15217	RSV	RSV A+B (26.6), Adenovirus (34.1)	26.6

Supplementary table 4S4: Nucleotide coverage % for each of the RSV coding regions and protein against the reference strain

Sample	NS1	NS2	N	P	M	SH	G	F	M2-1	M2-2	L
hRSV/A/ZAF/UFS-NGS UNIT/RD-3| 2021-02-24	78.6%	-	49.0%	28.5%	52.9%	100%	100%	100%	100%	100%	100%
hRSV/A/ZAF/UFS-NGS UNIT/RD-4|2021-02-28	100	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
hRSV/A/ZAF/UFS-NGS UNIT/RD-5| 2021-03-11	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
hRSV/B/ZAF/UFS-NGS UNIT/SARI-6|2021-02-11	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
hRSV/A/ZAF/UFS-NGS UNIT/RD-8| 2021-02-21	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
hRSV/A/ZAF/UFS-NGS UNIT/RD-9| 2021-02-23	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
hRSV/A/ZAF/UFS-NGS UNIT/RD-10|2021-03-16	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
hRSV/B/ZAF/UFS-NGS UNIT/RD-11|2021-05-04	100%	100%	100%	100%	100%	100%	1.7	58.1	100%	100%	100%
hRSV/B/ZAF/UFS-NGS UNIT/RD-12|2021-03-16	69.3	59.2	62.5	60.3	72.4	27.3	85.7	99.8	100%	100%	100%
hRSV/A/ZAF/UFS-NGS UNIT/RD-13| 2021-03-14	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
hRSV/A/ZAF/UFS-NGS UNIT/RD-14| 2021-03-15	100%	100%	100%	100%	100%	100%	100%	100%	100%	73.3	72.6
hRSV/B/ZAF/UFS-NGS UNIT/SARI-15|2021-06-16	100%	100%	100%	100%	100%	100%	73.7	95.8	100%	100%	100%
hRSV/A/ZAF/UFS-NGS UNIT/SARI-16|2020-12-21	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
hRSV/B/ZAF/UFS-NGS UNIT/SARI-17|2020-21-12	100%	100%	100%	100%	100%	100%	98.3	99.3	100%	100%	100%
hRSV/B/ZAF/UFS-NGS UNIT/SARI-18|2020-12-02	100%	100%	100%	100%	100%	100%	78.3	100%	100%	100%	100%
hRSV/B/ZAF/UFS-NGS UNIT/SARI-19|2020-12-29	100%	100%	100%	100%	100%	100%	98.0	99.8	100%	100%	100%
hRSV/A/ZAF/UFS-NGS UNIT/SARI-30| 2021-01-05	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
hRSV/B/ZAF/UFS-NGS UNIT/SARI-31|2021-02-11	100%	100%	100%	100%	100%	100%	93.3	99.8	100%	100%	100%
hRSV/B/ZAF/UFS-NGS UNIT/SARI-34|2021-03-02	100%	100%	100%	100%	100%	100%	99.0	99.8	100%	100%	100%
hRSV/A/ZAF/UFS-NGS UNIT/SARI-35|2021-03-02	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
hRSV/B/ZAF/UFS-NGS UNIT/SARI-36|2020-12-14	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

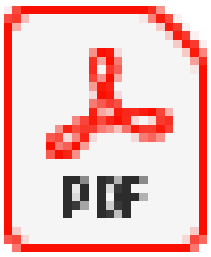
Supplementary table 4S5: Recombination breakpoints across the whole genome derived from RSV-B.

RSV-A Sample ID	Whole genome recombination events									
	Ns1	NS2	N	P	M	SH	G	F	M2	L
hRSV/A/ZAF/UFS-NGS-UNIT/SARI-16|2020-12-21	●	●	-	-	-	-	-	●	●	-
hRSV/A/ZAF/UFS-NGS-UNIT/SARI-30|2021-01-05	-	●	-	-	-	-	-	-	-	●
hRSV/A/ZAF/UFS-NGS-UNIT/SARI-35|2021-03-02	●	●	-	-	-	-	-	-	-	●
hRSV/A/ZAF/UFS-NGS-UNIT/RD-14|2021-03-15	-	-	-	-	-	-	-	-	-	●



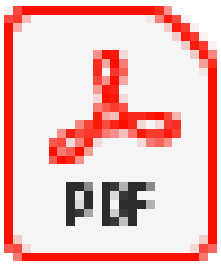
GA2.3.5_GLOBAL_AF
RICA_RSV_A_LINEAR

Supplementary figure 4S1: Maximum likelihood phylogenetic tree for RSV-A GA2.3.5 global strains. Each nation is assigned a specific colour. The tips of tree represent the clinical isolates of the infants investigated in this research and the location. Nodes with bootstrap values are represented by black dots.



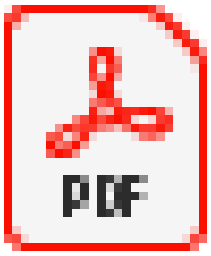
GB_5.0.5a_RSV_B_LI
NEAR_TREE_LOCATI

Supplementary figure 4S2: Maximum-likelihood phylogenetic tree for RSV-B GB.5.0.5a global strains. The colours and indications remain consistent with those in Figure S1



GA2.3.5_RSV_A_SO
UTH_AFRICA_G_MC(

Supplementary figure 4S3: Maximum credibility clade tree for RSV-A GA2.3.5



GB5.0.5a_RSV_B_SO
UTH_AFRICA_G_MC(

Supplementary figure 4S4: Maximum credibility clade tree for RSV-B GB5.0.5a

The work presented here in chapter five has been prepared for submission in *Virus Research.*

Detailed authors contributions are here-in described.

Authors detailed Contributions:

- **Project conceptualisation:** Martin M. Nyaga, Hlengiwe Sondlane.
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- **Study review**: Martin M. Nyaga,
- **Project supervision**: Martin M. Nyaga, Dominique Goedhals, Armand Bester.
- **Data capturing:** Hlengiwe Sondlane.
- **Original draft**: Hlengiwe Sondlane
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CHAPTER FIVE: INCIDENCE AND CLINICAL MANIFESTATION OF RSV IN CHILDREN BELOW FIVE YEARS, PRE, AND DURING COVID-19 PANDEMIC: A MINI REVIEW

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Abstract:

Respiratory syncytial virus (RSV) contributes significantly to the burden of respiratory tract infection in the paediatric population globally. This virus follows a distinct seasonal pattern in both temperate and tropical regions. However, the introduction of non-pharmaceutical interventions (NPIs) due to the COVID-19 pandemic disrupted the transmission dynamics of most respiratory viruses on a global scale, including RSV. As NPIs were gradually eased, a resurgence of RSV was experienced in several countries worldwide, following a period of reduced activity that lasted for over a year. Early data from the winter seasons in Europe, Australia, and South Africa indicated similar patterns. The clinical manifestations of RSV infection can vary greatly, exhibiting a broad spectrum of clinical features that can range from mild Upper respiratory tract infection (URTI) to severe and potentially life-threatening. This review paper aimed to delineate the clinical presentations of RSV in children in the pre-pandemic years and during the COVID-19 pandemic and shed light on the impact of COVID-19 on RSV infections in South Africa and other selected regions. While also comparing the clinical manifestations in children <5 five years before and during the COVID-19 pandemic. Cough, need for oxygen, and ICU admission were the common manifestations of RSV infection in children mostly during the COVID-19 pandemic. Of note, infants ≤1 year were the predominant group presenting particularly with RSV infection. The current study indicates a correlation between RSV outbreaks and shift in seasonality observed during the COVID-19 era due to reduced circulation caused by adopted NPIs. This shift has resulted in increased hospitalisation and reported cases of RSV in several parts of the world.

Keywords: RSV, COVID-19 pandemic, seasonality, clinical manifestation.

5.1. Introduction

RSV is a leading cause of lower respiratory tract infection (LRTI) in children ≤5 years of age, with a significant burden on healthcare systems worldwide (Li et al., 2022). Despite the established risks associated with RSV infection in children with underlying medical conditions, it is important to recognize that healthy, full-term infants are also vulnerable and account for a substantial number of RSV hospitalisations (Rha et al., 2020). The mini narrative review herein reports a review of the impact of the COVID-19 pandemic on the seasonality and clinical presentations of RSV in South Africa and in selected regions.

5.1.1. Overview of RSV Seasonality

The RSV season exhibits a consistent trend wherein outbreaks commence in November and cease in April in the Northern Hemisphere. However, in the Southern Hemisphere, the RSV season spans from April to August, while in the equatorial regions, it extends from August to December (Kuitunen and Renko, 2021). The onset of the RSV season in South Africa commences in February, as the virus tends to be highly prevalent during the autumn and early winter months (Green et al., 2010). A comprehensive study on the seasonality of RSV at the regional and global levels has been impeded by the limited availability of global surveillance data. Consequently, limited studies have utilized a multicounty dataset and a standardized methodology to investigate RSV seasonality (Staadegaard et al., 2021).

Previous studies have indicated that the seasonality of RSV in temperate regions remains consistent over time, with most countries witnessing a noticeable surge in RSV cases per season (Staadegaard et al., 2021). Furthermore, a significant factor that impedes comprehensive comparison between studies is the wide range of statistical techniques employed in defining seasonality within the existing literature. Reviews or meta-analyses that surpass the national level in their scope are primarily focused on and rely on published national seasonality estimates (Obando-Pacheco et al., 2018; Pangesti et al., 2018).

5.1.2. COVID-19 Public Health Measures and RSV

Restrictive measures imposed to combat the spread of SARS-CoV-2 were introduced in the Southern Hemisphere before the onset of winter and the duration of these measures varied depending on the extent of SARS-CoV-2 transmission. On 11 March 2020, the coronavirus disease 2019 (COVID-19) was officially declared a pandemic, leading to the implementation of various restrictive measures put in place across the globe to control its spread (Yang et al., 2020). In the initial wave of the

pandemic, nearly every country implemented a comprehensive lockdown strategy which entailed the complete closure of schools in March and April 2020 (Buonsenso et al., 2021).

The primary measures implemented globally were nonpharmaceutical interventions (NPIs), which included mandatory protective face masks, restricted gatherings and observed social distancing. However, these lockdown strategies varied across nations at different levels, depending on transmission intensity (Cowling et al., 2020; Vittucci et al., 2021). The implementation of a national lockdown in South Africa commenced on 27 March 2020, which included stringent restrictions imposed on both domestic and international travel (Tempia et al., 2021). These measures not only proved effective in curbing the spread of SARS-CoV-2, but they also played a role in a substantial decline in the transmission of respiratory infections caused by other respiratory viruses, including RSV (Di Mattia et al., 2021; Kim et al., 2021; Pelletier et al., 2021).

Following the implementation of NPIs, there was a substantial decrease in the spread and prevalence of influenza across most temperate regions and certain tropical regions across the globe (Olsen et al., 2021). One of the most striking outcomes is the apparent extinction of the influenza B Yamagata lineage (Koutsakos et al., 2021). The most apparent outcome is a notable decrease in RSV cases observed in both the Southern and Northern Hemispheres in March 2020 (Di Mattia et al., 2021; Edwards, 2021; Trenholme et al., 2021). The outbreak of SARS-CoV-2 in the Northern Hemisphere occurred simultaneously with the expected surge of respiratory viruses such as influenza and RSV (Di Mattia et al., 2021).

Based on reports from Italy, Finland, Belgium, the United Kingdom (UK), and the United States of America (USA), the RSV epidemic season ended abruptly and prematurely early in March 2020 in contrast to past years, with essentially no cases detected in the subsequent months (Sherman et al., 2021). In the southern hemisphere, reports from Chile and South Africa described a decreased incidence of paediatric RSV and influenza cases and hospital admissions (Olsen et al., 2021).

In Australia, Europe, and America, a diminished spread of RSV and a delayed re-emergence interseason were observed during the winter of 2020 (Haapanen et al., 2021; Lumley et al., 2022; Mondal et al., 2022; Yeoh et al., 2021). RSV infections significantly declined in 2020 during Australia's winter months, nonetheless, unexpected offseason RSV outbreaks occurred in the spring of the same year in Western Australia and New South Wales (Britton et al., 2020; Foley et al., 2021). The RSV cases remained minimal both in Europe and Australia, even after the most stringent restrictions

were lifted and basic preventive measures such as hand washing, social isolation, and the requirement to wear face masks remained in place (Curatola et al., 2021; Van Brusselen et al., 2021).

The unexpected outbreak of RSV and the strain on local healthcare systems may be attributed to social interaction, gradual relaxation of NPIs, and the accumulation of an immune debt in children who have not been exposed to RSV in the past year (Binns et al., 2022).

Surprisingly, the extended durations of limited exposure have paradoxically emerged as a threat to the healthcare system, as the overwhelming demand for supportive care systems was witnessed during the resurgence of RSV in the summer season (Bardsley et al., 2023; Billard et al., 2022). The resurgence of RSV would undoubtedly put further strain on health medical professionals who are already overwhelmed in response to the COVID-19 pandemic (Cong et al., 2022). It is crucial to comprehend the potential outcomes in future epidemic seasons after the transmission of SARS-CoV-2, considering the clinical impact and epidemiology of RSV.

5.1.3. Clinical Presentation of RSV

The typical signs and symptoms commence following a period of 4 to 6 days of incubation, characterized by nasal congestion, runny nose, fever, persistent cough, or elevated body temperature (Walsh, 2017). In some patients, the disease course may progress to the lower airways causing wheezing, tachypnoea, chest indrawing, hypoxemia, or respiratory distress that needs hospital admission (Smith et al., 2017). Children diagnosed with RSV bronchiolitis experience a significantly more intense clinical course in comparison to those with non-RSV bronchiolitis (García et al., 2010). In addition, RSV infection may lead to extended periods of hospitalisation, heightened requirements for oxygen supplementation, increased admissions to the paediatric intensive care unit, and a greater necessity for mechanical ventilation (Ghazaly and Nadel, 2018). Disproportionately, bronchiolitis-related deaths are more prevalent in low-income countries, whereas high-income countries witness a higher incidence of infant hospitalisations attributed to RSV respiratory illness (Dalziel et al., 2022).

In children, immunity acquired following natural infection appears to be short-lived, with reinfection occurring frequently, although subsequent infections tend to be mild (Bont et al., 2002). Severe cases require supportive therapy, such as oxygen supplementation and hydration, and less frequently ventilatory support (Barbati et al., 2020). The impact of age on disease severity cannot be overstated, particularly during the first few months of life. Research has shown that the risk of

developing serious illnesses is highest between 1 and 3 months when infants are no longer protected by maternal antibodies (Meissner, 2016). RSV not only contributes to the onset of acute illness, but it also assumes a pivotal function in the progression of wheezing in early childhood and the subsequent manifestation of asthma in later stages of life (Walsh, 2017). The unmet clinical need for RSV LRTI has been further complicated by the emergence of COVID-19.

5.2. Methodology

5.2.1. Selection Strategy and Criteria

Relevant data were retrieved from previously published work to give a snapshot of the RSV outbreak, resurgence, and clinical manifestation in a selected cohort age in different selected regions before and during the COVID-19 pandemic. A search was undertaken on the PubMed database. The key search words on PubMed including, 'RSV', respiratory syncytial virus', 'COVID-19', 'Pandemic'; 'seasonality', 'SARS-CoV-2', with no restrictions on the date of publication or journal type. This review provides an update and overview since the beginning of the COVID-19 pandemic by analysing publicly available data presented as results (Clinical characteristics). Information was selected based on recent literature on the COVID-19 pandemic, by relevance of the published data compared to data on RSV published before the pandemic.

5.2.2. Clinical Data on RSV Presentation and Demographics

The collection of clinical published data was chosen to capture the clinical manifestation and outcomes of suspected respiratory infection, pertaining to the hospitalisation of the patients and the age of the participants between 0-3 months, 1 year, and 0-5 years respectively. The status of the participant when hospitalised; ICU admission, length of stay, respiratory symptoms (cough, history of asthma), number of children hospitalised in the individual study cohorts. Clinical data retrieved from multiple studies was collected to compare the clinical manifestations and impact of COVID-19 prior to and during the COVID-19 pandemic.

5.2.3. Data Collection and Documentation

Subsequently, the clinical information was gathered and organized in a Microsoft Word document to accurately document the observed outcomes. Considering the variability of data emanating from sources or publications, the presentation of continuous variables such as medians and interquartile ranges were documented. Data with standard values, such as percentages, were presented as indicated in the publication. The compilation of the data was contingent upon the availability of clinical data, thus imposing no restrictions on the number of sources included in the study.

5.2.4. Article Inclusion and Exclusion Criteria

Selection of eligible articles required full-text articles during and before the COVID-19 pandemic. The exclusion criteria for this review involve the omission of publications that were not reported in English, as well as those involving adults and outpatients.

5.3. Results

During the pre-pandemic years, most studies have reported on the clinical presentations of RSV in patients participating in various studies. In South Africa, RSV infection cases vary in different age demographics, and most of the patients in the pre-pandemic period required oxygen therapy as compared to ICU admission. Characteristics of participants and their clinical manifestations in various selected countries are shown in Table 5.1. The RSV cases notably declined during COVID-19 as reported by many countries. In addition, off-season RSV outbreaks occurred when lockdown restriction were lifted in various countries. Seasonality of RSV in pre-selected countries during the COVID-19 are shown in Table 5.2.

Table 5.1: Clinical Characteristics of the Study Population in the Selected Published Articles.

Season	Study	Age (Months, %)	Cough	Bronchiolitis	Admission	Oxygen	Asthma/reactive airway	Wheeze	Hospital length of stay	ICU	Country	Publication
2011-2012	Hospital-based, sentinel surveillance program	n=1157 <6 637/1157 (55) 6–11 226/1157 (20) 12–23 195/1157 (17) 24–60 99/1157 (9)	1136/1157 (98)	-		404/1154 (35)	16/1157 (1)	300/1157 (26)	2 (1–5) Prolonged Hospital stay (More than 5 days) 231/1157 (20)	-	South Africa	(Moyes et al., 2013)
2012-2015	Hospital-based SRI surveillance program	n=2509 <3 months 229/601 (38.1) 3–5 months 134/601 (22.3) 6–11 months 106/601 (17.6) 12–23 months 78/601 (13.0) 24–59 months	581/596 (97.5)	-	-	409/599 (68.3)	7/600 (1.2)	-	302/601 (50.2)	24/598 (4.0)	South Africa	(Valley-omar et al., 2022)

		54/601 (9.0)										
2020-2021	cross-sectional study	n=149 ≤5 Median age 14.5	149 (100%	33%	149 (100%)	66 (44.2%)	5 (3.3%)	-	-	0 (0%)	South Africa	(Ogunbayo et al., 2022)
2014-2018	single-centre, retrospective, observational study	7.9 (2.5–18.3)	170 (85.0%)	-	-	111 (55.5)	-	(99, 49.5%	2.0 (1.0–4.0)	29 (14.5)	Australia	(Saravanos et al., 2021)
2019	Observational study	2.8 (1.4-6.4)	-	240 (64)	398	60 (25.4)	-	112 (47.5)	2.6 (1.5-3.9)	30 (12.7)	Western Australia	(Foley et al., 2021)
2020/2021		3.6 (1.7-8.2)	-	251 (82)	563	91 (37.6)	-	110 (45.5)	1.9 (1-3.7)	17 (7)		
2015-2016	Prospective Multi-centre Study	10 (4.1–21) n=191	187 (98%)	-	65 (34%)	40/65 (62%)	-	52 (27%)	2 (1–3)	11/65 (17%)	USA	(Haddadin et al., 2021)
2014-2018	-	< 3 months 222 (55.0) 3–6 months 90 (22.3) >6–24 months 92 (22.8)	380 (94.1)	-	-	-	199/404 (49.3)	208 (51.5)	4.2 [3.4-6.9]	n=99 59 (59.6) 23 (23.2) 17 (17.2)	USA	(Brenes-Chacon et al., 2022)

2022	Cross-sectional study	0-2 323 3-5 127 6-11 150	-	-	-	Low Flow- 51 (8.5) High flow- 253 (42.2)	-	-	5 (4-10)	-	USA	(Halasa et al., 2023)
2015-2020	Retrospective Cohort Study	0–5 74 (146) 6-11 26 (51)	-	65 (267) 35 (145)		-	-	-	4.7	69%	USA	(Movva et al., 2022)
2020-2021		0–5 67 (24) 6-11 33 (12)	-	56 (37) 44 (29)		-	-	-	3.5	60%		
2016 to 2019		n=298 6 months (range: 12 days to 9 years)	-		197 (66.7%)		-	-	4 days.	(160 of (197; 81%)	USA	(Agha and Avner, 2021)
2019–2020		17 months (range: 11 days to 18 years)	-				-	-	3 days.	45%		
2018-2019	retrospective review	≤12 months 58 (64.4%) 13-24 months	-	-	42 (46.7%)	25 (27.8%)	13 (14.4%)	-	-	-	USA =4d	(Halabi et al., 2022)

		14 (15.6%) 25-35 month 1 (1.1%) 13 (14.4%)										Season 2018-2019 143 cases
2020-2021		≤12 months 49 (54.4%) 13-24 months 17 (18.9%) 25-35 months 8 (8.9%) 3-6 years 11 (12.2%)	-		59 (65.6%)	44 (48.9%)	4 (4.4%)	-	-	-		Season 2019-2020 1162 RSV cases
2016-2019	retrospective study	n=186 <6 months, 115 (44.9) 6 months - 1 year 33 (12.9) 1–2 years 73 (28.5) 2–3 years 27 (10.5) 3–4 years,	-	171 (66.8)	-	147 (57.4%)	3 (1.2)	-	4.9 ± 5.7	34 (13.3)	Taiwan	(Lin et al., 2022)

		4 (1.6) 4–5 years 2 (0.8)										
2020		N=256 <6 months 19 (10.2) 6 months - 1 year 26 (14.0) 1–2 years 48 (25.8) 2–3 years 42 (22.6) 3–4 years 24 (12.9) 4–5 years 17 (9.1)	-	66 (35.5)	-	72 (38.7%)	3 (1.6)	-	3.4 ± 4.5a	14 (7.5)		
2018- 2022 Before		n=170 < 1 57 (33.5%) 2-3	133 (78.2)	-	-	-	-	26 (15.3)	10 (7-15)		China -	(Jia et al., 2022)

		38 (22.4) 4-5 16 (0.4) 6-11 17 (10) 12-23 17(10) 2-4y 23 (13.5)										
During		n=233 < 1 54 (23.2) 2-3 58 (24.9) 4-5 25 (10.7) 6-11 24 (10.3) 12-23 28 (12) 2-4y 35 (15)	143 (61.4)	-	-	-	-	29 (12.4)	10 (7-16)	-		

2012-2015		*6.1 (2.4–11.7) (n = 547)	534 (97.6%)	289 (52.8%)	-	-	14 (2.6%)	420 (76.8%)	-	26 (4.8%)	Mexico	(González-Ortiz et al., 2019)
2009-2015		n=749 <6 350 (46.7) 6 to <12 184 (24.6) 12 to <24 134 (17.9) 24 to <60 81 (10.8)	-	-	-	-	20 (2.7)	-	5.6 (5.6)	29 (3.9)	Mexico	(Esparza-Miranda et al., 2023)
2022-2023		n=51 <6 months 350 13 (25.5) 6 to <12 months 9 (17.6) 12 to <24 months 17 (33.3) 24 to <60 months 12 (23.5)	-	-	-	-	3 (5.9)	-	4.9 (4.1)	2 (3.9)		

2015-2018	Retrospective Medical Record Review Study	Age 0 to <6 (n = 108) 2.0 (1.0–3.0) Age 6 to <12 (n = 48) 8.0 (7.0–9.0) Age 1 to <2 Y (n = 78) 18.0 (14.0–20.0) Age 2–5 Y (n = 78) 34.5 (28.0–47.0	87 (80.6) 28 (58.3) 44 (56.4) 54 (69.2)	86 (79.6) 9 (81.3) 40 (51.3) 31 (39.7	-	62.0% (67/108) 56.3% (27/48) 51.3% (40/78) 59.0% (46/78		61 (56.5) 27 (56.3) 29 (37.2) 22 (28.2)	4 (3–6.5)	9.3 (10/108) 0.0 3.8 (3/78) 3.8 (3/78)	Germany	(Hartmann et al., 2022)
13 March 2020 and 30 November 2021		n-169 3 (0; 57)	88.8% (n = 150)	-	-	39.6% (n = 67)	-	-	4 (0; 21)	10.1% (n = 17)	Germany	(Meyer et al., 2022)

Table 5.2: Surge in RSV Cases Reported in Various Countries and Number of Cases.

Country	Hemisphere	Study	NPI introduction	Surge in Cases	Median Age Before	Median age during	Increase or decrease in the number of detections
South Africa	Southern	(Tempia et al., 2021)	27 March to 30 April (weeks 13–18)	Observed during Jul–Oct 2020	-	-	The detection of RSV in 2020 showed that 4.1% of outpatients and 10.5% of inpatients had RSV, which is lower than the percentage of RSV in the years between 2013 and 2019, where 6% of outpatients and 15.4% of inpatients were reported to be infected with RSV
South Africa	Southern	(Bents et al., 2022)	27 March to 30 April (level 5 restrictions; weeks 13–18)	August-December of 2020,	A total of 70% of hospitalisations are accounted for by infants who are 2 years old or younger.	-	-
Australia (Western Australia)	Southern	(Yeoh et al., 2021)	20 March (week 12)	-	-	-	In the winter of 2020, Western Australian children experienced a significant decline in RSV detections, with a remarkable reduction of 98% being reported.
Australia (Western Australia)	Southern	(Foley et al., 2021).	20 March (week 12)	Observed during Nov 2020	7.3–12.5 months for seasons between 2012 and 2019	18.4 months for the 2020	-
France	Northern	(van Summeren et al., 2021)	17 March 2020	The 2020-2021 season experienced a delay of at least 12 weeks and was shorter by 2-3 weeks compared to	2.2 to 3.1 months 2016/2017-2019/2020	4.8 months in 2020/21	-

				the seasons observed between 2016 and 2020.			
UK	Northern	(Lumley et al., 2022)	-	Noted among children aged 0 to 3 years old in July 2021	The duration of seasons from March 2016 to March 2020 is 0.3 years.	1.8 years of age from March 2020 to July 2021	-
USA	Northern	(Bardsley et al., 2023)	-	-	-	-	During the winter of 2020-2021, a significant decrease in RSV activity was witnessed, as indicated by a remarkable relative reduction of 99.6% in RSV test positivity. The recorded RSV test positivity rate stood at a mere 0.1%, with only 7 cases reported.

5.4. Discussion

The COVID-19 pandemic has emerged as the most significant health crisis in recent history, compelling numerous nations to implement stringent public health interventions to curb transmission (Bermúdez Barrezueta et al., 2022). Consequently, this has resulted in a notable alteration in the typical seasonal prevalence of other respiratory viruses. This study aimed to describe and compare changes in RSV clinical presentation in children ≤5 years and explore the seasonality observed during the COVID-19 pandemic in selected countries.

From the selected studies, the age distribution remained unchanged with children ≤1 year accounting for most clinical presentations. The occurrence of bronchiolitis and pneumonia is often associated with RSV infection, which has been found to be responsible for as much as 70% of hospitalisations for bronchiolitis (Mansbach et al., 2013). In the reviewed studies, the prevalence of bronchiolitis among patients varied across different countries, excluding South Africa (Valley-omar et al., 2022), and China (Jia et al., 2022), with no records. In Australia, USA, Taiwan, Mexico, and Germany, the percentage of RSV infected patients presenting with bronchiolitis ranged from 52% to 67% before the onset of the COVID-19 pandemic and a relatively lower percentage during the COVID-19 era. Notably, Australia experienced a significant increase of 82% in bronchiolitis cases compared to other countries during the COVID-19 period (Foley et al., 2021).

Notably, studies from the countries included in this review reported a marginal decline in the need for oxygen therapy and ICU admissions, except for Australia, with a significant rise in ICU admissions before the COVID-19 pandemic (Foley et al., 2021). During the pre-COVID-19 period, the rate of RSV related PICU admissions in the USA was higher compared to the COVID-19 period, with a percentage of 69% as opposed to 60% (Movva et al., 2022). In another study, the admission rates for LRTI in PICU in Latin America witnessed a marked decrease (Vásquez-Hoyos et al., 2021). In the specific context of New York City, the 2020-2021 RSV season, spanning from November to April, witnessed a significant decrease in the total number of RSV-positive cases reported in paediatric departments, with a drop from 1162 cases during the 2019-2020 season to 143 cases during the COVID era (Halabi et al., 2022).

A growing body of evidence suggests that RSV LRTI may be associated with the development of asthma, wheezing, or impaired lung function later in life (Shi et al., 2020). In this review, there were no notable differences observed among patients who had asthma and wheezing in South Africa, the USA, Taiwan, Australia, and Mexico before and during the COVID-19 pandemic. However, the

understanding of the factors that influence the development of asthma or wheezing, such as underlying mechanisms and genetic susceptibility, is currently inadequate.

During the pandemic, hospitalisation was a common outcome for most patients who presented with respiratory clinical symptoms associated with RSV. Nonetheless, some patients required shorter hospital stays than others, highlighting the variability in disease severity and outcomes among individuals. However, it is important to note that previously healthy children can have severe outcomes that may necessitate hospitalisation (Hall et al., 2009). Of note, the duration of hospital stay exhibited in the reviewed studies ranged from 2-5 days prior to the COVID-19 pandemic, which varied across different countries. However, during the pandemic, the range of hospitalisation duration narrowed down to 3-4 days in the selected countries. In this review, China had the longest hospitalisation period for patients, lasting between 7-15 days before the COVID-19 pandemic and 7-16 days during the pandemic, surpassing the hospitalisation durations observed in other countries (Jia et al., 2022). In essence, the duration of hospitalisation is an important factor that contributes to the burden of RSV disease (Simões, 2022).

Cough is a significant symptom associated with RSV infection, symptomatic cough cases were prevalent across all countries, with a significant rise in the number of patients presenting with cough. In the reviewed study, notably, South Africa witnessed a slight increase from approximately 98% before the onset of the COVID-19 outbreak to a complete 100% during the pandemic period. The primary focus of the COVID-19 study in South Africa was on children who were afflicted with SARI (Ogunbayo et al., 2022). The study utilized a multiplex detection panel to determine the Ct value and RSV in comparison to other respiratory viruses. The results indicated that RSV could be the pathogen responsible for the clinical manifestations observed in these children, due to its relatively lower Ct values, indicative of higher viral load (Ogunbayo et al., 2022).

A comprehensive study revealed that the occurrence of hospitalisation caused by respiratory infections related to RSV varied across different regions. The study found that in Asia and Africa, the incidence rates ranged from 7 to 13 cases per 10,000 individuals, whereas in the USA, the rates were significantly higher, ranging from 190 to 254 cases per 10,000 people (Tin Tin Htar et al., 2020). It is important to note in this review paper that the estimates provided are not comprehensive and may not accurately reflect the true burden of RSV in children. The limited focus on a small group of participants and the primary focus on detecting SARS-CoV-2 infections could lead to an

underestimation of the actual prevalence of these respiratory illnesses. Therefore, further research is needed to better understand the true burden of RSV in children across different countries.

The NPI implemented during the COVID-19 pandemic have resulted in an unintended consequence of altering the seasonality of respiratory viruses, such as RSV. Nevertheless, there were notable differences in the characteristics of the RSV resurgences. Across all the countries analysed, the onset of the first RSV epidemic was postponed amidst the COVID-19 pandemic. Consequently, these delayed outbreaks led to an increased rate of hospitalisations, despite the manifestation of comparatively milder clinical symptoms (Fourgeaud et al., 2021). Furthermore, most research investigations focused on the duration of lockdowns rather than analysing the impact of specific NPIs. A notable example is the case of South Africa, where the presence of RSV was identified in 4.1% of individuals seeking outpatient care and 10.5% of those admitted as inpatients during the year 2020. In contrast, during the period spanning from 2013 to 2019, RSV was detected in 6% of outpatients and 15.4% of inpatients in the same region (Tempia et al., 2021).

The 2020-2021 season in France experienced a significant delay, commencing at least 10-12 weeks later than usual, and was also shorter by approximately 2 to 3 weeks compared to the seasons observed between 2016 and 2020 (Delestrain et al., 2021; van Summeren et al., 2021). Despite experiencing a delay in its peak season, the overall number of RSV cases during the 2021-2022 season was higher compared to the previous year, 2020-2021 (Delestrain et al., 2021). Following the implementation of NPIs, the rate of RSV positivity experienced a significant decline, dropping to 0.03% between December 2020 and March 2021 (Chuang et al., 2023). In contrast to the preceding statement, in 2020, RSV LRTIs in South Africa followed the typical seasonal pattern, but with a lower peak intensity compared to previous years. Interestingly, there was also an unexpected RSV occurrence outside the usual season and with a smaller magnitude compared to the average RSV surge in the country (Tempia et al., 2021).

During the period of January 4 to April 4, 2020, the USA witnessed a decline in the weekly percentage of positive RSV results from 15.3% to 1.4%. Subsequently, for the following year, the levels remained consistently low, measuring below 1.0% per week, which can be considered historically low (Olsen et al., 2021). From December 2019 to March 2020, a research study conducted in the USA revealed that RSV accounted for 5.4% of the total positive specimens of respiratory pathogens detected prior to the onset of the COVID-19 pandemic (Chuang et al., 2023). During the period from March 2022 to May 2021, a notable decline of 86% in the overall occurrence of RSV was

documented in the USA, as indicated by a previous investigation conducted amidst the COVID-19 outbreak (Yuan et al., 2022).

The UK experienced an unforeseen resurgence of RSV during the summer of 2021 (Chuang et al., 2023; Hussain et al., 2021). The 2020-2021 season in France witnessed a delayed occurrence of the RSV outbreak, with the epidemic commencing and reaching its peak during the months of February and March 2021 (Chuang et al., 2023; Fourgeaud et al., 2021). In Western Australia, there was a noticeable decline in RSV activity after the implementation of local COVID-19 restrictions in week 14. This decline persisted even after the gradual easing of these restrictions, with RSV activity remaining at significantly lower levels compared to previous seasons (Yeoh et al., 2021). Children in Western Australia experienced a significant decline in the detection of RSV infections during the winter of 2020, with a decrease ranging from 98.0% to 99.4% compared to previous winter seasons between 2012 and 2019 (El-heneidy et al., 2022; Yeoh et al., 2021). Recent research conducted in a tertiary paediatric hospital revealed a significant decrease of 68% in the RSV positive rate during the COVID-19 pandemic in comparison to the rates observed previously (Abo et al., 2021). In New South Wales, the detection of RSV during April to June 2020 was significantly lower by 94.3% compared to the predicted values based on the data from 2015 to 2019 (Britton et al., 2020). It has been established that RSV infection resurged in Australia from January to March 2021 (Cooney et al., 2022).

In light of this, it is important to acknowledge that there are additional factors beside NPIs that may play a role in the variations observed among different countries. The winter of 2020-2021 in the UK observed a significant decrease in RSV activity, as compared to previous years. The estimated relative reduction in RSV test positivity was a staggering 99.6%, with the observed RSV test positivity rate being 0.1%. This is in stark contrast to other regions where RSV activity remained high during the same period (Bardsley et al., 2023). In Germany, similarly, the RSV positivity rate dropped from 10–21% to 0% in the 2020/21 season until the end of March 2021 (Engels et al., 2022). During the COVID-19 era, Taiwan observed a marked reduction in the incidence of RSV cases, even during the peak season for RSV infections (Lee et al., 2022). The implementation of NPIs is expected to contribute to a rise in the susceptible population, consequently leading to more extensive outbreaks in comparison to the years prior to the pandemic (Baker et al., 2020).

5.5. Conclusion

In this review, cough was a prevalent clinical symptom among most RSV patients, regardless of their age or risk group. Nonetheless, a true comparison of RSV prevalence in different geographic areas is difficult to perform considering the differences in the study design with varying seasonality. The current narrative review has certain limitations. Firstly, the comparison of clinical information is solely based on a few selected studies, rather than a comprehensive collection of studies mined from the database. As a result, the findings may not provide a complete reflection of COVID-19 impact and clinical characteristics of children before and during the COVID-19 pandemic. Hence, it is imperative to broaden the investigation's scope to precisely uncover the clinical manifestations and attributes of children affected by RSV infection. Additionally, the studies obtained encompassed a range of sources, including multicentre and retrospective studies. Consequently, a comprehensive analysis is indispensable to draw accurate conclusions. The existence of missing or incomplete data in certain cases could have potentially resulted in confounding factors.

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Preface

The work presented here in chapter six summarises the thesis covering the general discussion of preceding chapters, conclusion and future remarks.

Detailed authors contributions are here-in described.

Authors detailed Contributions:

- **Writing:** Hlengiwe Sondlane
- **Review and editing**: Martin Nyaga, Dominique Goedhals, Hlengiwe Sondlane.

CHAPTER SIX: SUMMARY

6.1. Discussion

Respiratory infection due to RSV has emerged as a prominent public health concern, leading to substantial rates of hospitalisation and mortality in children (Coutinho and Gava Chakr, 2022). The clinical presentation of RSV may exhibit symptoms that are indistinguishable from those caused by other prevalent respiratory pathogens, which may complicate treatment options when not specifically diagnosed (Bianchini et al., 2020).

The impact of the adopted NPIs on the transmission dynamics of other respiratory viruses during the Coronavirus disease 2019 (COVID-19) pandemic was documented globally (Garg et al., 2022; Homaira et al., 2014; Liu et al., 2021; Oh et al., 2021; Olsen et al., 2021; Park et al., 2021; Redlberger-Fritz et al., 2023), with several reports on out of season RSV outbreak and hospitalization in different countries due to the perturbed seasonality and possible immunological naivety in the children.

Although RSV is a well-studied ssRNA virus, and despite the approval of new RSV vaccines available for use by pregnant women, the lack of a licensed vaccine and its virulent potential, especially in primary infection in children, poses daunting questions. A potential consequence is emergence of a novel RSV strain that could threaten the development of a universal RSV vaccine. Besides, a dearth of information exists on the whole genome sequencing of RSV in South Africa and regionally in other African countries. More so, it holds great significance to give due consideration and adequately prepare for the potential resurgence of RSV that deviates from the typical season (Williams et al., 2021). The first objective of this study (addressed in chapter 3) was to conduct whole genome sequencing of RSV from clinical samples obtained during the pandemic. The current study employed next generation sequencing (NGS), to generate complete RSV genomes. Despite having good coverage, certain genomes were deemed unsuitable for downstream analysis due to their low quality. There are several potential factors that can contribute to inadequate genome coverage. One plausible explanation is the quality of the samples that were used, as poor sample quality or possible RNA degradation can lead to suboptimal results. Moreover, insufficient sample input may have played a role in compromising the sequencing quality, as the DNA obtained may not have been a true representation of the complete genome. This phenomenon is not unique to our study and has been previously reported (Agoti et al., 2019; Rebuffo-scheer et al., 2011; Rios Guzman and Hultquist, 2022). Despite the abundance of sequence data for studying the evolutionary and phylodynamic aspects of the RSV G gene, the complete sequencing of the RSV genome has been comparatively

limited when compared to other respiratory viruses like influenza and SARS-CoV-2 (Goya et al., 2020). To the best of our knowledge, there has been limited research conducted on RSV whole genome sequencing, with no studies that have been reported in South Africa. This study in this regard contributed to the body of available knowledge by generating whole genome sequences of RSV during the pandemic which will be made available in public repositories.

The second objective of this study (addressed in chapter 3) aimed to conduct a genomic analysis of the circulating RSV strains in South Africa and investigate their genetic similarity prior to and during the COVID-19 pandemic. Objective three was an integral part of this section wherein focused on the phylogenetic analysis of the RSV strains. The latter was carried out using the limited number of genomes that were sequenced in this study, which also included co-infected samples. Furthermore, this study analysis incorporated unpublished RSV South African sequences from GISAID, as supplementary to our analysis. Numerous studies have predominantly focused on sequencing the complete or partial G gene (Chen et al., 2022; Kang et al., 2020; Krivitskaya et al., 2021). The majority of previous studies have primarily focused on the second hypervariable portion of G, located at the C-terminal, which is both necessary and adequate for distinguishing between the two RSV groups and the genotypes within each group (Muñoz-Escalante et al., 2021; Ren et al., 2014). Currently, in this region, there exist proposed criteria for the unified designating of genotypes, which encompass three RSV-A and seven RSV-B genotypes that were recently characterized (Goya et al., 2020). In this study, all RSV sequences that were accessible from the databases were considered and used to construct phylogeny and assign genotypes to the study strains. This assignment was preferentially performed on the virus' G gene. To better understand the circulating strains in South Africa driving RSV outbreaks during the COVID-19 pandemic phylogeny of RSV was undertaken. Phylogenetic analysis results showed that the RSV-A and RSV-B, belonged to GA2.3.5 and GB5.0.5a lineage, respectively, and were the predominant genotype circulating during the COVID-19 pandemic, consistent with reports from other regions (Dolores et al., 2022; Goya et al., 2023; Jallow et al., 2022). The GA2.3.5 corresponds to the previously described ON1 genotype which is predominantly circulating globally (Lu et al., 2019). The GB5.0.5a strains of RSV-B correspond to the classified BA genotype, which has been identified in numerous countries across the globe (Haider et al., 2018).

In the comparison of South African strains to global strains, the phylogenetic tree of RSV-A GA2.3.5 indicated minor endemic circulation of strains in Africa, which suggests that variants are continuously introduced from other regions. The genetic similarity observed among the GB5.0.5a

South African strains was notable, forming multiple clusters with contemporary strains found in different geographic origins. This finding strongly suggests the repeated introduction of global variants into South Africa rather than solely circulating African variants. In essence, our results suggest that the upsurge of RSV outbreaks in the year 2020-2021 in South Africa was primarily driven by pre-existing strains that had already been circulating, rather than novel or emerging strains. In addition, the genome-scale analysis, of the G gene phylogeny revealed a decrease in diversity indicative of a genetic bottleneck in both the GA2.3.5 and GB5.0.5a where these previously established lineages have significantly declined or even disappeared during the COVID-19 outbreak period. The latter coincided with lockdown restrictions implemented to curb the SARS-CoV-2. Furthermore, a slight increase in genetic diversity in 2021 and 2022 was observed. During that period continuous co-circulation of multiple variants and dominance of these lineages were observed, resulting in significant RSV surges as lockdown measures were eased.

The third objective of the study (addressed in chapter 3), aimed to perform genomic analysis from South African RSV positive samples which are coinfected with other respiratory pathogens amidst the COVID-19 pandemic. Numerous studies have demonstrated a remarkable decline in the prevalence of RSV and other prevalent respiratory pathogens amidst the COVID-19 period (Agha and Avner, 2021; Chuang et al., 2023; Eden et al., 2022; Foley et al., 2021; Tempia et al., 2021). Research findings also indicate that the simultaneous presence of two pathogens commonly results in a heightened severity of the disease (Meissner et al., 2016; Florin et al., 2017). In our study, we observed the simultaneous detection of multiple pathogens, including SARS-CoV-2 and Influenza, as RSV commonly co-infects with other respiratory viruses. However, it is inconclusive from our research whether the concurrent detection of both subtypes worsens the illness in relation to its symptoms. In some countries where both COVID-19 and rhinovirus were present, it was observed that rhinovirus activity peaked before the resurgence of RSV during the onset of the pandemic (Liu et al., 2021; Thongpan et al., 2021). In the aftermath of the COVID-19 pandemic, it is crucial to acknowledge the clinical significance of RSV infection along with other prevalent upper respiratory viral infections.

In a concise review format, in chapter four, this thesis provides an expanded analysis of the occurrence and clinical presentation of RSV in children less than 5 years before and amidst the COVID-19 pandemic by comparing different published studies from selected countries. Furthermore, this review also presented the seasonal patterns of RSV in these nations before and

amidst the COVID-19 outbreak. The clinical manifestations presented by children varied amongst the children of different age groups. The impact of COVID-19 on the circulation of RSV is apparent, as it has caused a notable change in its anticipated seasonality. A resurgence of RSV has been reported in numerous countries worldwide, with a notable increase in cases and hospitalizations among the paediatric population (Abu-raya et al., 2023; Bardsley et al., 2023; Foley et al., 2021; Jia et al., 2022). However, it is challenging to conduct a comprehensive comparison of RSV prevalence across various geographical regions due to variations in study designs. In our study, the prevalence of RSV varied significantly depending on the season and geographic location.

6.2. Conclusion

It is apparent that RSV infection is a major factor in the development of respiratory tract infections in young children. The COVID-19 pandemic brought about unforeseen alterations in the seasonality and epidemiology of RSV, which could hold significance in the implementation of efficient strategies for preventing RSV. Based on the phylogenetic analysis, it revealed that the outbreak samples had sufficient genetic similarity to suggest that these viruses were already present in South Africa before the COVID-19 restrictions were put in place. The traditional patterns and expectations regarding RSV disease have been significantly disrupted by these dynamics. In the aftermath of the COVID-19 pandemic, it is imperative to prioritize a heightened focus on the potential resurgence of RSV. To gain comprehensive insights into the local and global transmission patterns of RSV, it is essential to conduct nationwide surveillance studies. These studies will play a crucial role in unravelling the underlying mechanisms that drive the circulation of RSV. Additionally, this study emphasizes the urgent need for ongoing surveillance of RSV, and other significant respiratory pathogens to analyse changes in their genetic diversity and inform vaccine development strategies.

6.3. Limitations and Future Directions

This study proposed to compile a repository of RSV sequences derived from South Africa, however, sample limitation was a contributing factor and only a few genomes were sequenced. Furthermore, the complete genome sequences produced in this study will be deposited in the GenBank database. Another important limitation to the study, is the small sample size and challenges associated with sequencing RSV whole genomes and the minimal success rate of obtaining a whole genome through sequencing due to RNA degradation, making it difficult to establish clear correlations between clinical and genetic data. The RSV possesses a genome that is composed of a single strand, making it highly sensitive to freezing and thawing, which could lead to RNA degradation before the

sequencing process. Additionally, the utilisation of samples obtained from archives that have been stored for extended periods contributes to the slow success rate. Although, in our case, samples were stored for a shorter period, relatively few whole genomes were obtained. Our research was limited by the number of sequences obtained from clinical samples. Hence, it is imperative to collect an extensive range of clinical samples and process them for sequencing. In that that way there will be more genomes generated and analysed to draw inferences.

The utilization of whole genome sequence data increases the likelihood of identifying variations in comparison to individual genes, thus making it imperative for establishing relatedness and phylogenetic construction. By expanding the scope of genomic surveillance for RSV, it becomes possible to conduct a more in-depth analysis of the epidemiological trends and trace origins of different lineages. One further limitation was that this review article focused on clinical data found in selected representative published papers, rather than considering the complete pool of papers available for RSV across all databases. The future remarks of this investigation indicate the necessity for enhanced monitoring and synchronization of sample and clinical data collection. Additionally, it is imperative to explore methods for effectively incorporating and comprehending complete genome sequences in conjunction with clinical variables.

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Williams, T.C., Sinha, I., Barr, I.G., Zambon, M., 2021. Transmission of paediatric respiratory syncytial virus and influenza in the wake of the COVID-19 pandemic. Eurosurveillance 26, 2100186.

Appendixes

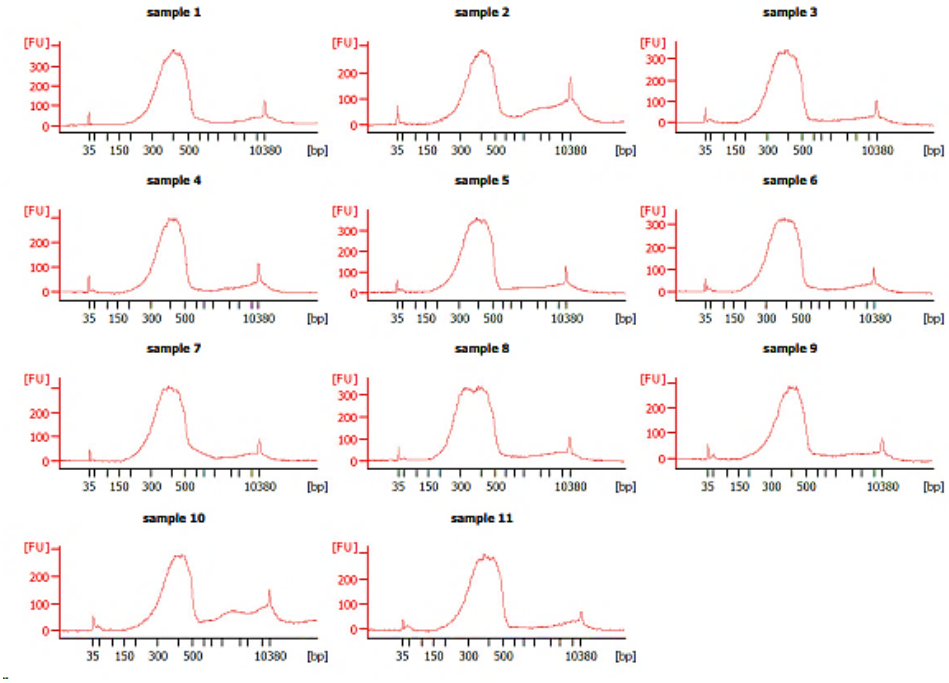


Figure A1: A high sensitivity dsDNA assay was utilized to determine the library size distribution, which is depicted in the Bioanalyzer electropherogram. All validated samples exhibit an average library size of approximately 350 bp, as indicated by the electropherogram.

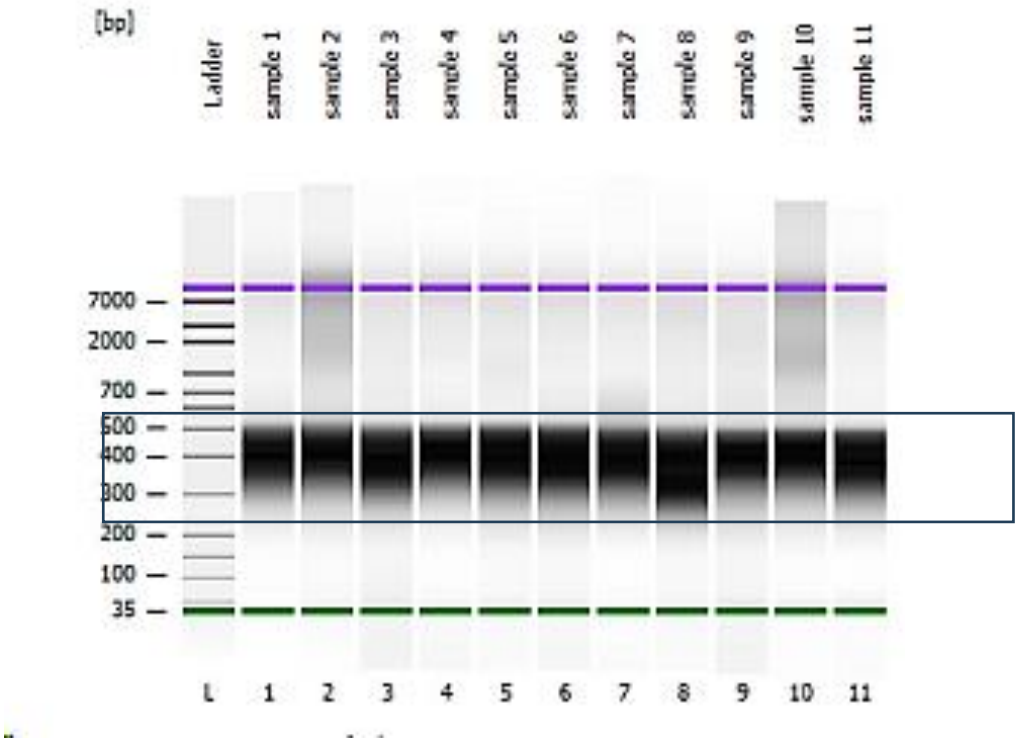
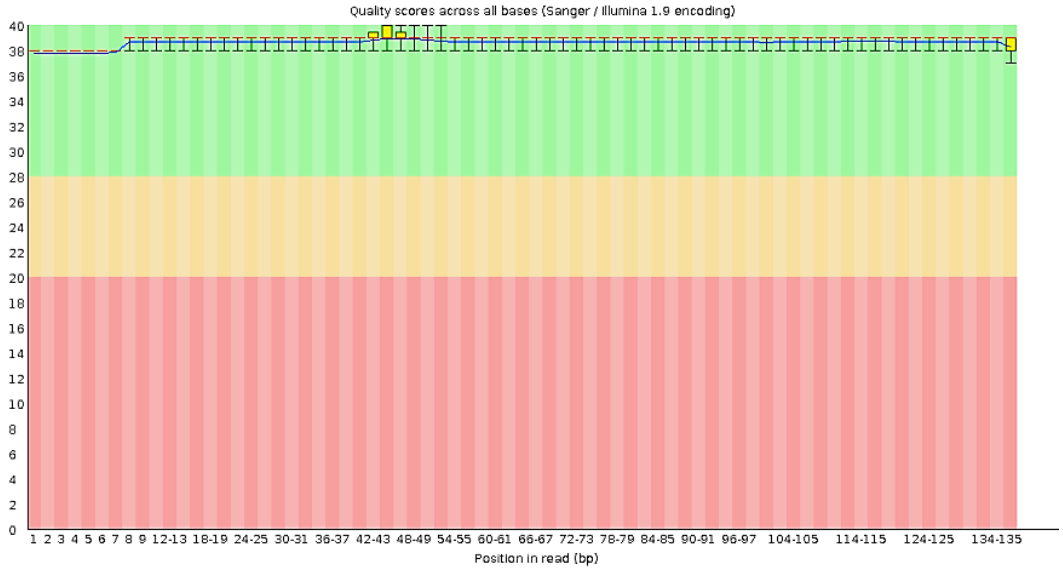


Figure A2: Depiction of the Bioanalyzer gel image showing the library size distribution of each of the samples.



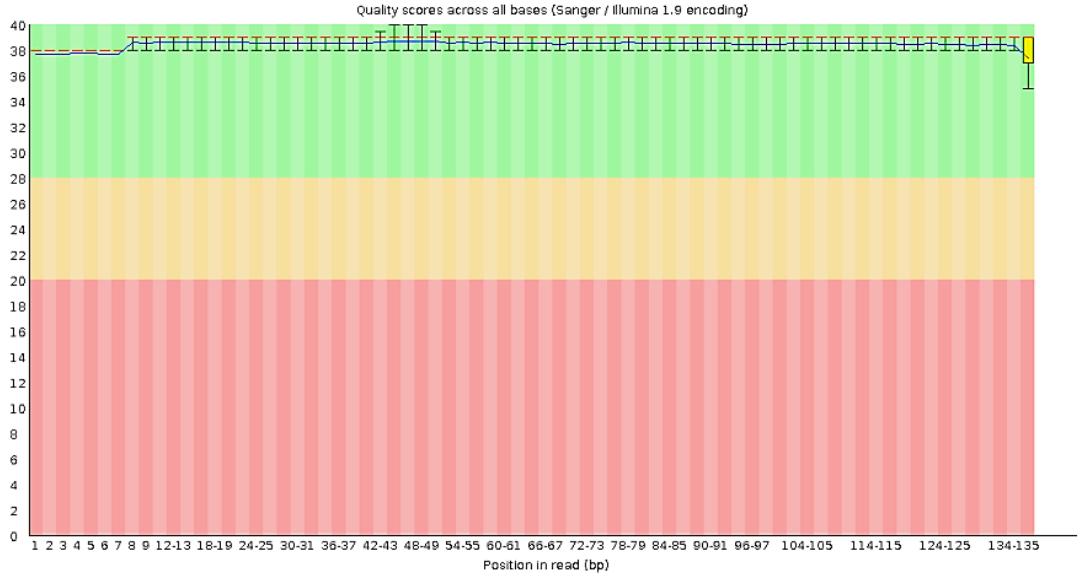
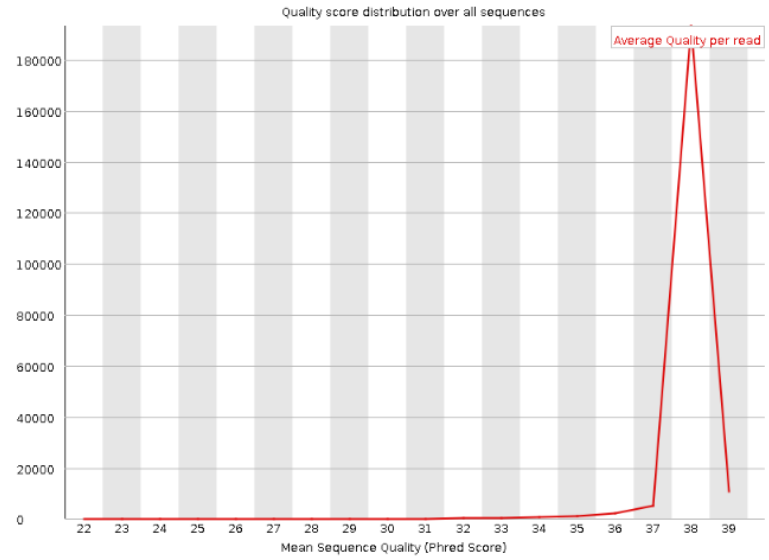


Figure A3 A graph illustrating the quality score of RSV sequences analysed in this study. The analysis of RSV whole genome sequences was conducted using the FastQC program on Genome detective. Focusing on the quality of the base sequence and the distribution of length of RSV sequences. The forward RSV sequence reads are depicted in A, while the reverse RSV sequence reads are shown in B. The sequences are represented by yellow bars, and their proximity to the orange region indicates the gradual decrease in quality of the reads. It is noteworthy that most of the sequences fulfilled the Quality score criteria, as they exceeded the Q30 quality score threshold.



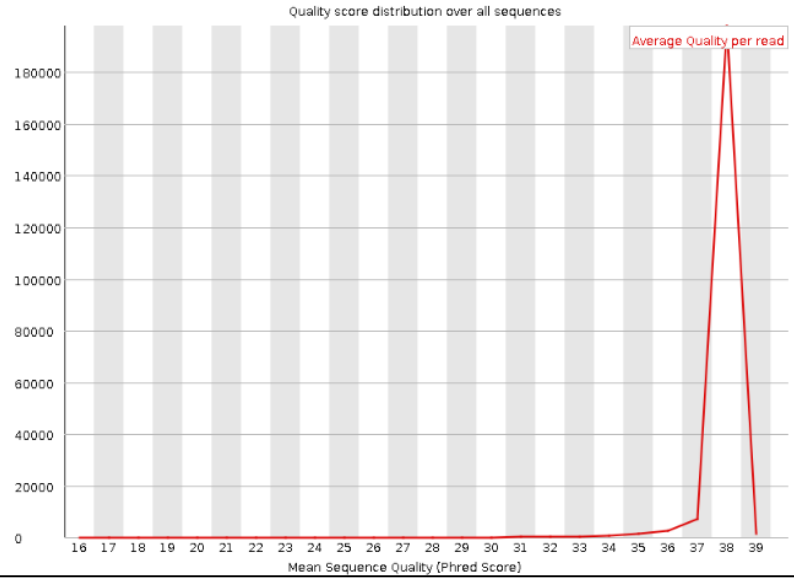


Figure A4: Depiction of RSV genome sequences length distribution. A and B image show the average quality per read and the quality score distribution over all sequences.

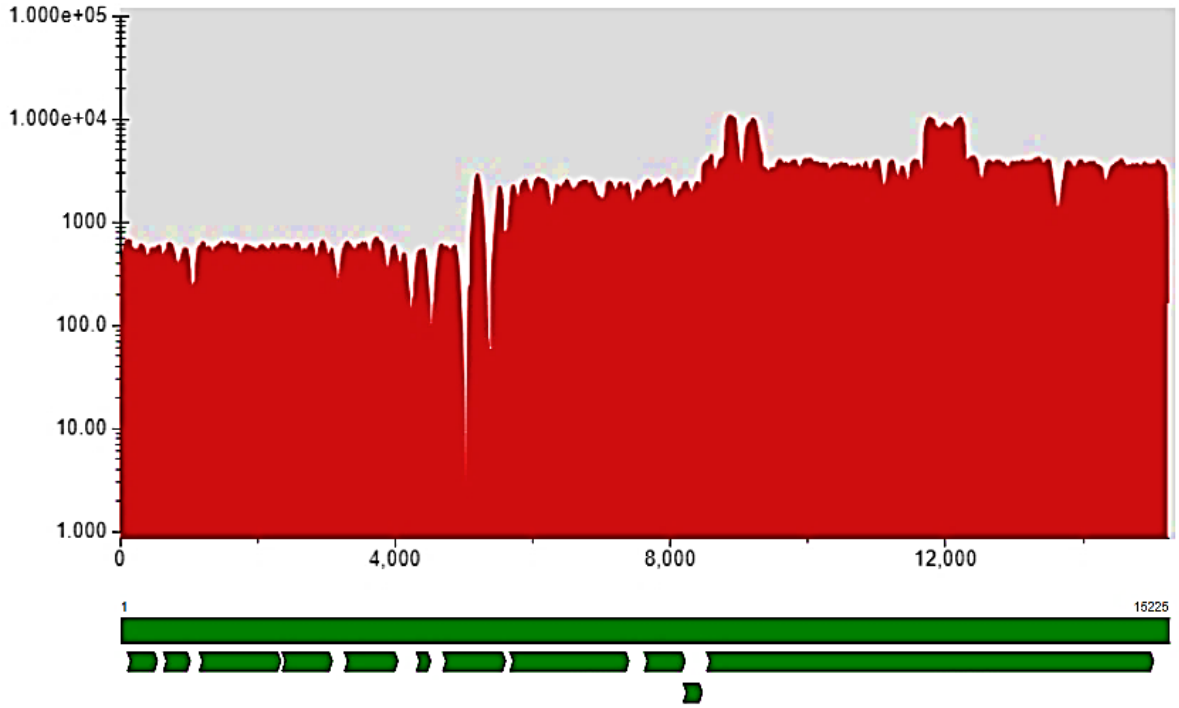


Figure A5: Depiction of RSV genome coverage map obtained from genome detective. The red colour indicates the regions covered on the whole genome. The green section represents the genome organisation of RSV and the specific genes and coverage across the reference genome.

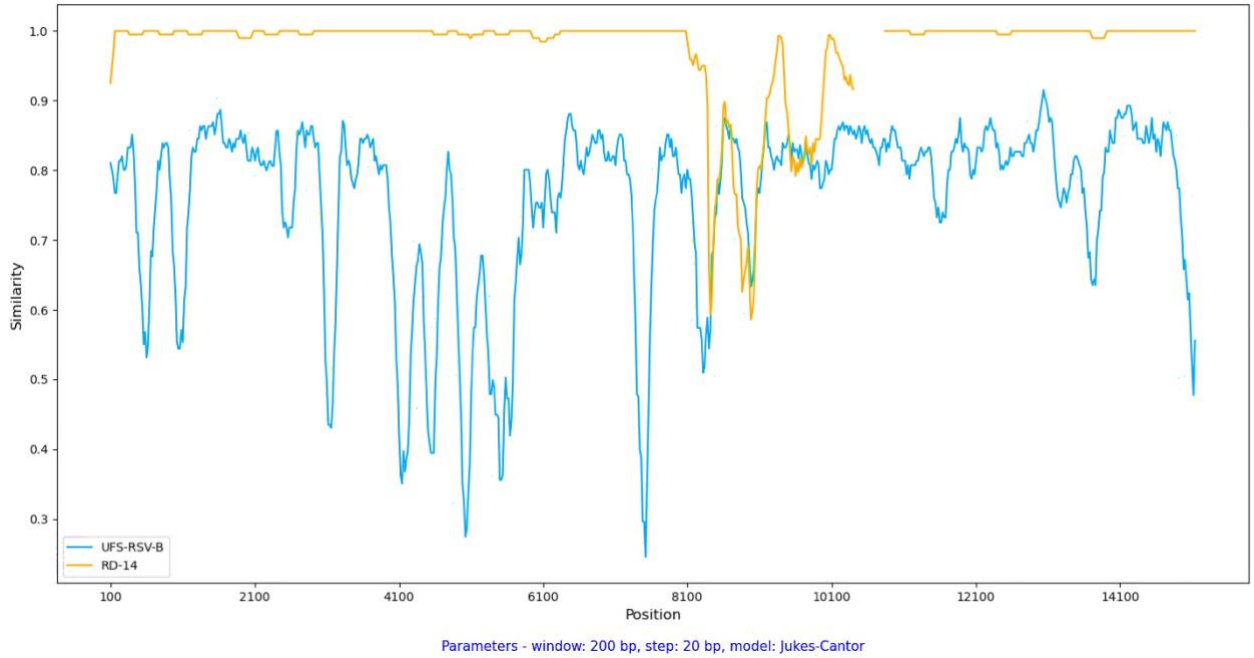


Figure A6: Recombination analysis in RSV-A RD-14 sample by using representative RSV-B consensus. Recombination defined as breakpoints across the RSV b genome. Positions of the specific RSV genes are annotated in letters.

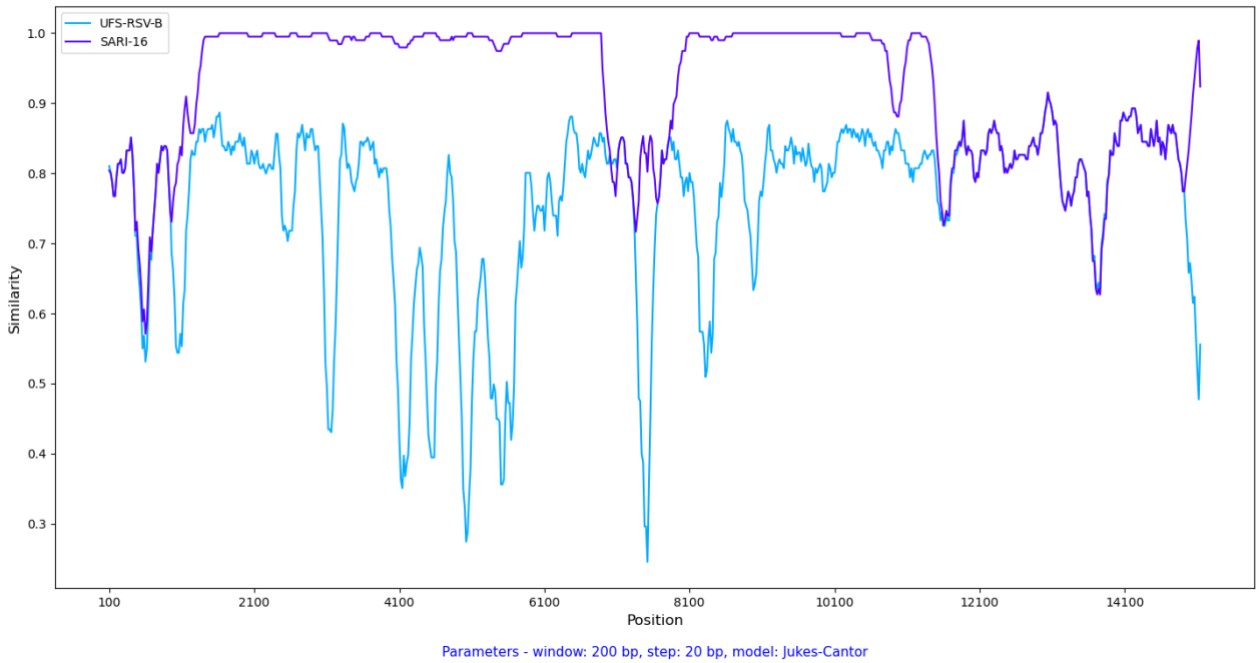


Figure A7: Recombination analysis in RSV-A SARI-16 sample by using representative RSV B consensus. Recombination defined as breakpoints across the RSV b genome. Positions of the specific RSV genes are annotated in letters.

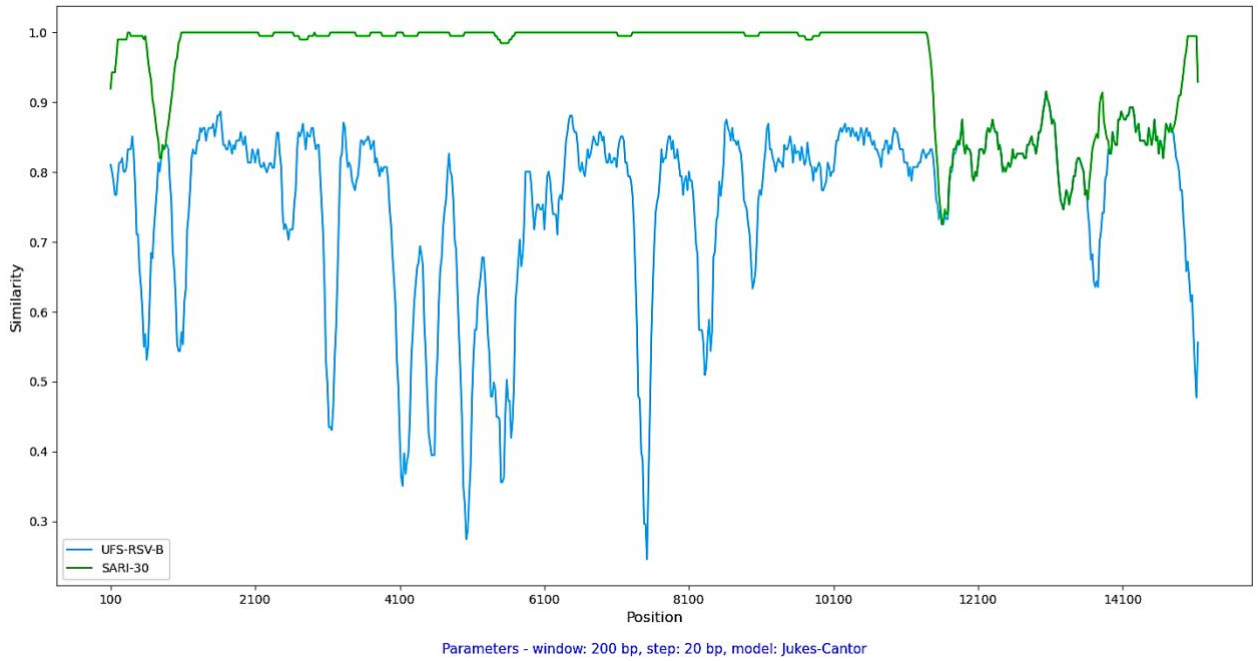


Figure A8: Recombination analysis in RSV-A SARI-30 sample by using representative RSV B consensus. Recombination defined as breakpoints across the RSV-B genome. Positions of the specific RSV genes are annotated in letters.

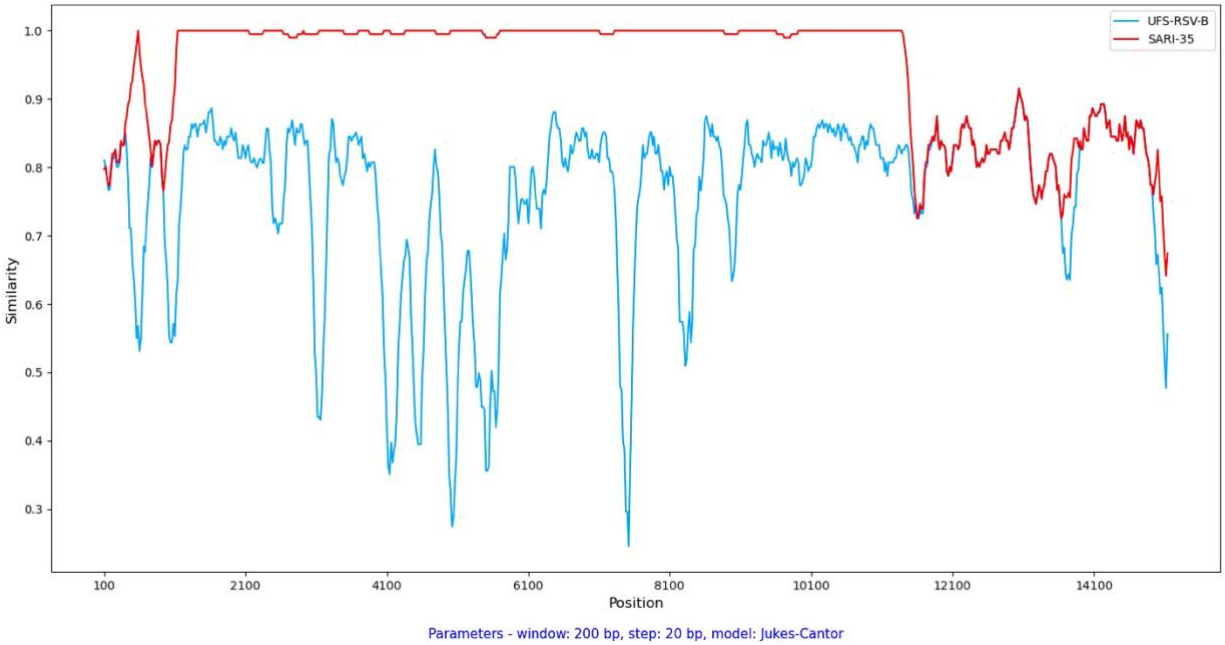


Figure A9: Recombination analysis in RSV-A SARI-35 sample by using representative RSV B consensus. Recombination defined as breakpoints across the RSV-A genome. Positions of the specific RSV genes are annotated in letters.

Selection Pressure Analysis

Table A1: Selection pressure analysis on GA2.3.5 African Dataset

Position	MEME	FEL	SLAC	FUBAR
38	0.0127			
47	0.0051			
111		0.0459		
115			0.00	
142	0.0014			
178	0.0368			
200	0.0339	0.0225		0.9270
225			0.04	
232	0.0495			
255	0.0026	0.0013		0.9845
273	0.0000	0.0000	0.00	1.0
274			0.00	
276			0.04	
284	0.0227	0.0143		0.9423
290	0.0289	0.0188	0.05	0.9571
297	0.0000	0.0000	0.01	0.9999
298		0.0469	0.00	0.9375
301			0.05	
308	0.0215	0.0136		0.9858
310			0.00	
314	0.0018	0.009	0.00	0.9970

Table A2: Selection pressure analysis on GA2.3.5 South African Dataset

Position	MEME	FEL	SLAC	FUBAR
178	0.0156			
207	0.0525	0.0393		0.921
255	0.0127	0.0079		0.968
273	0.0143	0.0082		0.972
297	0.0038	0.0021		0.991
298*	0.0337	0.0155	0.01	0.972
308*	0.0339	0.0227		0.944
314*	0.0277	0.0177	0.00	0.952

Table A3: Selection pressure analysis on RSVA F-gene African Dataset

Position	MEME	FEL	SLAC	FUBAR
23				0.9264
276				0.9936
553	0.0003			
554	0.0021	0.0061	0.03	0.9996
555	0.0005			

Table A4: Selection pressure analysis on GB5.0.5a African Dataset

Position	MEME	FEL	SLAC	FUBAR
46	0.0000			
54	0.0029			
78	0.0089			
79	0.0069			
80	0.0050			
81	0.0042			
103				0.9004
114	0.0071	0.0530		
141	0.0059			
142	0.0151			
143		0.0518		
144	0.0092	0.0536		

154		0.0054		0.9655
190	0.0211			
217	0.0004	0.0002	0.000	0.9982
225	0.0274			
226	0.0096	0.0094		0.9567
229	0.0017	0.0109	0.000	0.9725
244	0.0235			
245	0.0323		0.013	0.9249
252	0.0383	0.0388	0.003	0.9898
263	0.0078			
267	0.0006			
268	0.0011	0.0006	0.011	0.9993
272	0.0519			
273	0.0524	0.0369		
276	0.0489			
279	0.0008			
280	0.0073			
281	0.0027	0.0180		0.9187
282	0.0001			
285	0.0000		0.000	1.0000
294	0.0193	0.0124		0.9556
303	0.0070	0.0040	0.055	0.9854
310	0.0139	0.0087	0.040	0.9653

Table A5: Selection pressure analysis on GB5.0.5a South African Dataset

Position	MEME	FEL	SLAC	FUBAR
78	0.0186			
79	0.0122			
81	0.0072			
90				0.9286
97	0.0049			
114	0.0412			
137				0.9401
141	0.0115			
142	0.0075			
217	0.0243	0.0156	0.002	0.9952
229			0.053	0.9665
244	0.0054			
245	0.0046			0.9095
252				0.9346
267	0.0002			
268				0.9801
272				0.9349
279	0.0002			
281	0.0075			
282	0.0016			
285	0.0085	0.0049	0.050	0.9986

Table A6: Selection pressure analysis on RSV-B F-gene African Dataset

Position	MEME	FEL	SLAC	FUBAR
4		0.0997		
12		0.0955		
23		0.0872		
113	0.0530	0.0372		
125	0.0054	0.0092	0.01	0.9887
173	0.0059			
439	0.0003			
440	0.0033			

Table A7: Selection pressure analysis on RSV-B F-gene South African Dataset

Position	MEME	FEL	SLAC	FUBAR
4	0.0516	0.0361		0.9671
173	0.0116			
209				0.9198
440	0.0114			

Appendix

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Health Sciences Research Ethics Committee

06-Mar-2023

Dear **Hlengiwe Sondlane**

Ethics Number: UFS-HSD2021/1616/2501-0002

Ethics Clearance: **Genomic analysis of respiratory syncytial virus circulating in the Free State during the COVID-19 pandemic**

Principal Investigator: **Hlengiwe Sondlane**

Department: **School of Pathology Department (Bloemfontein Campus)**

Submission Page

SUBSEQUENT SUBMISSION APPROVED

With reference to your recent submission for ethical clearance from the Health Sciences Research Ethics Committee. I am pleased to inform you on behalf of the HSREC that you have been granted ethical clearance for your request as stipulated below:

In this study we request to use additional samples (19) which have been identified from similar patient groups and nucleic acid material has been extracted and stored at the UFS-NGS Unit. The study ethical clearance number is (UFS-HSD2019/1129/2910).

- Our initial proposed sample collection was 50 and were pre- selected based on availability. During the sample retrieval at NHLS we experienced challenges, the freezers where majority of the samples were stored had a fault, as such several samples were discarded including our potential study samples.
- Given that our sampling and sample size has already been limited and could not achieve the initial target of at least 50 genomes (samples), incorporating these recently identified group of samples would compensate for the samples that were discarded, also could result to meaningful and significant conclusions to our study.
- Changes has been updated in the study protocol and can be found in page 14, section 3.2 of the study protocol with the heading "collection of Samples".

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2020); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; International Council for Harmonisation (ICH) Harmonised Guideline, Integrated Addendum to ICH E6(R1), Guideline for Good Clinical Practice (GCP) E6(R2), 2016, SAHPRA Guidelines as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

The Principal Investigator (PI) bears final responsibility for the RIMS application. In the event of any misconduct or improper activities perpetuated by a third party, the PI will be held vicariously liable. The HSREC will bear no responsibility or liability for any actions of a PI and/or third party or breach of confidentiality caused by the PI and/or third party.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this request for ethical clearance and we wish you continued success with your research.

Yours Sincerely

Prof. A. Sherriff
Chairperson : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00011992; REC 230408-011; IORG 0010096; FWA 00027947
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
RESEARCH DEVELOPMENT
NAVORSINGSONTWIKKELING

Environment & Biosafety Research Ethics Committee

09-Dec-2021

Dear **Hlengiwe Sondlane**

Project Title: **Genomic analysis of respiratory syncytial virus circulating in the Free State during the COVID-19 pandemic.**

Department: **Medical Microbiology Department (Bloemfontein Campus)**

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Environment & Biosafety Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2021/0256/21**

Please note the following:

1. **This ethical clearance is valid for two years from the issuance of this letter.**
2. **If the research takes longer than two years to complete, please submit a Continuation Report to the Ethics Committee before ethical clearance expires.**
3. **If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an Amendment.**
4. **When the research is concluded, please submit a Final Report to the Ethics Committee.**

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Environment & Biosafety Research Ethics Committee
University of the Free State

Directorate: Research Development
T: +27 (0)51 401 9398 | +27 (0)51 401 2075 | E: smitham@ufs.ac.za
Johannes Brill Building, Room 106D, First Floor
205 Nelson Mandela Drive | Park West, Bloemfontein 9301 | South Africa

Appendix

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Health Sciences Research Ethics Committee

17-Dec-2021

Dear **Hlengiwe Sondlane**

Ethics Clearance: **Genomic analysis of respiratory syncytial virus circulating in the Free State during the COVID-19 pandemic**

Principal Investigator: **Hlengiwe Sondlane**

Department: **School of Pathology Department (Bloemfontein Campus)**

Submission Page

APPLICATION APPROVED

Please ensure that you read the whole document

With reference to your application for ethical clearance with the Faculty of Health Sciences, I am pleased to inform you on behalf of the Health Sciences Research Ethics Committee that you have been granted ethical clearance for your project.

Your ethical clearance number, to be used in all correspondence is:**UFS-HSD2021/1616/2501**

The ethical clearance number is valid for research conducted for one year from issuance. Should you require more time to complete this research, please apply for an extension.

We request that any changes that may take place during the course of your research project be submitted to the HSREC for approval to ensure we are kept up to date with your progress and any ethical implications that may arise. This includes any serious adverse events and/or termination of the study.

A progress report should be submitted within one year of approval, and annually for long term studies. A final report should be submitted at the completion of the study.

Research conducted in any Department of Health facility: Researchers are required to sign and return the HSREC approval letters to the provincial Department of Health where they applied. It is also a requirement for researchers to submit electronic copies of their final research findings, and/or make a presentation of their findings and recommendations at departmental research days when and where indicated.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; International Council for Harmonisation (ICH) Harmonised Guideline, Integrated Addendum to ICH E6(R1), Guideline for Good Clinical Practice (GCP) E6(R2), 2016, SAHPRA Guidelines as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this proposal for ethical clearance and we wish you every success with your research.

Yours Sincerely

Prof. A. Sherriff
Chairperson: Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00011992; REC 230408-011; IORG 0010096; FWA 00027947
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

Appendix

health
Department of Health
FREE STATE PROVINCE

02 February 2023

Ms. Hlengiwe Sondlane
School of Pathology Department
University of the Free State

Dear H Sondlane

Subject: Genomic analysis of respiratory syncytial virus circulating in the Free State during the COVID-19 pandemic.

- Please ensure that you read the whole document, Permission is hereby granted for the above–mentioned research on the following conditions:
- Participation in the study must be voluntary and written consent by each participant must be obtained.
- Serious adverse events to be reported to the Free State department of health and/ or termination of the study.
- Ascertain that your data collection exercise neither interferes with the day-to-day running of **Pelonomi, Botshabelo and National Hospitals** nor the performance of duties by the respondents or health care workers.
- The Department of Health expects that the researcher will be the responsible data manager according to the POPI Act. The responsibility thus lies with the researcher to ensure that the processing of all participants' personal information and research data is lawful according to the stipulations of the POPI Act (Protection of Personal Information Act 4 of 2013).
- Confidentiality of information will be ensured and please do not obtain information regarding the identity of the participants.
- Department of Health to be fully indemnified from any contravention of the POPI Act as you conduct this study.
- **Research results and a complete report should be made available to the Free State Department of Health upon completion of the study (a hard copy plus a soft copy).**
-
- Progress report must be presented not later than one year after approval of the project to the Ethics Committee of the University of Free State and to the Free State Department of Health.
- Any amendments, extensions, or other modifications to the protocol or investigators must be submitted to the Ethics Committee of the University of Free State and to the Free State Department of Health.
- **Conditions stated in your Ethical Approval letter should be adhered to and a final copy of the Ethics Clearance Certificate should be submitted to Sebeelats@fshealth.gov.za/Gwantshuws@fshealth.gov.za before you commence with the study**
- No financial liability will be placed on the Free State Department of Health.
- **Please discuss your study with the Institution Manager on commencement for logistical arrangements.**
- Department of Health is to be fully indemnified from any harm that participants and staff experience in the study.
- As part of the feedback, you will be required to present your study findings/results at the Free State Provincial Health Research Day.

Trust you find the above in order.

Kind Regards

MR. MNG MAHLATSI
HEAD: FREE STATE DEPARTMENT OF HEALTH

Date: 7/02/2023

Head: Health
PO Box 227, Bloemfontein, 9300
4th Floor, Executive Suite, Bophelo House, cnr Maitland and, Harvey Road, Bloemfontein
Tel: (051) 408 1646 Fax: (051) 408 1556 e-mail:khusemi@fshealth.gov.za / chikobvup@fshealth.gov.za
www.fs.gov.za

Appendix

NATIONAL HEALTH LABORATORY SERVICE

Academic Affairs and Research
Modderfontein Road, Sandringham, 2031
Tel: +27 (0)11 386 6155
Fax: +27 (0)11 386 6296
Email: babatyi.kgokong@nhls.ac.za
Web: www.nhls.ac.za

19 April 2022

Applicant: Hlengiwe Sondlane
Institution: University of Free Sate
E-mail Address: hlengiwesondlane@gmail.com
Tel: 051 401 9158 **Cell:** 082 354 6974

CC: Dominique Goedhals, Phillip Bester, Martin Munene Nyanga

Project Title: Genomic analysis of respiratory syncytial virus circulating in the Free State during the COVID-19 pandemic
Reference Number: PR2222645

Research Application Type(s):
1. Request for Samples

RE: APPROVAL LETTER: REQUEST TO ACCESS NHLS RESOURCES FOR RESEARCH PURPOSES

This letter serves to advise that the application requesting permission to conduct the above-mentioned research using the listed NHLS resources has been reviewed and **"Approved"**. Please note that the approval is granted on the condition that you comply with the NHLS Research Material and Data Access Policy and requirements stated below.

1. All material and data requested shall be used as per the research protocol submitted to the NHLS and as approved by the relevant Health Research Ethics Committee (HREC) in South Africa.
2. Access to the NHLS material and/or data shall be limited to the minimum required for successful completion of the approved study and shall be made available ***without patient names***.
3. Confidentiality shall be maintained at the participant and institutional level and there shall be no disclosure of personal information or confidential information.
4. The material and/or data obtained from the NHLS shall be anonymised and not, for any reason, be used to track or recruit patients as no pre-approval/consent is obtained from patients.
5. Processes shall be discussed with the relevant NHLS departments (i.e. Corporate Data Warehouse (CDW), NHLS Laboratory Management, Operations Office, etc.) and agreed upon.
6. Any amendments to the study requirements, including the use of the material and/or data for purposes not initially disclosed to the NHLS) shall be cleared by an approved HREC and submitted to the NHLS for approval via the AARMS system – https://aarms.nhls.ac.za.
7. The NHLS shall be acknowledged as a source of material and/or data in any output, such as abstracts and journal articles, emanating from the project.
8. A final report of the research study and any published output resulting from this study shall be submitted to the NHLS via AARMS

Please note that this letter constitutes approval by the NHLS Academic Affairs and Research Office. The NHLS entities tasked with providing the material and/data may have additional requirements for access. Data related queries may be directed to NHLS CDW, email: zarina.sabat@nhls.ac.za; contact number: 011 386 6074 and sample related queries (if applicable) shall be directed to the relevant business manager.

Dr Babatyi Malope-Kgokong
National Manager: Academic Affairs and Research