Investigation of the association of Rhinovirus and Respiratory Syncytial Virus with Severe Acute Respiratory Illness in South Africa

by

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I certify that the thesis hereby submitted to the University of Pretoria for the degree PhD (Medical Virology) has not been previously submitted by me in respect of a degree at any other university.

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Ethics

This protocol have been reviewed and approved by University of the Witwatersrand Human Research Ethics Committee (HREC), University of KwaZulu Natal Human Biomedical Research Ethics Committee (BREC) and Faculty of Health Sciences Research Ethics Committee university of Pretoria protocol number M081042, BF157/08 and 324/2013, respectively. Subsequent Ethical clearance was received for the cytokine expression patterns, by the HREC M110728, healthy controls and ILI by the BREC BF 080/12.

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Summary

Pneumonia is a major cause of morbidity and mortality in children worldwide and causes 18% of all deaths in children less than 5 years of age. In order to best understand the full burden of acute respiratory infection in South Africa, it is important to conduct surveillance and measure the burden of mild, outpatient disease as well as more severe, inpatient disease. By comparing the prevalence of these respiratory viruses detected in patients with influenza-like illness (ILI) and severe acute respiratory illness (SARI) to control patients the contribution of these respiratory viruses to SARI maybe better understood.

The aim of this study was to determine the contribution of the major respiratory viruses to severe acute respiratory illness in South Africa both during and after the pandemic and compare molecular epidemiology and host innate immune responses as factors that may influence the role of the two most frequently detected viruses, respiratory syncytial virus (RSV) and rhinovirus as pathogens in a population with a high HIV sero-prevalence.

To facilitate the detection of these viruses, a multiplex reverse transcription real-time polymerase chain reaction (rRT-PCR) assay that can detect the ten major respiratory viruses influenza A and B, RSV, enterovirus, human metapneumovirus, adenovirus, rhinovirus and parainfluenza 1, 2 and 3, was developed. This assay allowed us to determine the prevalence of these respiratory viruses in patients with SARI, ILI and controls, as well as to determine the attributable fraction of each of these viruses in SARI and ILI relative to controls. We were also able to determine the seasonality of each of these viruses in South Africa. While RSV was detected most frequently in children, rhinovirus was by far the most prevalent virus detected over all age groups in the study. We described the molecular epidemiology of rhinovirus in SARI, ILI and control cases to determine whether a specific rhinovirus species could be linked to severe disease. All 3 species of rhinovirus was identified in South Africa, although RV-A and RV-C were more common than RV-B. While rhinovirus was associated with symptomatic respiratory illness; there was no association between RV species and disease severity.

In order to determine if RSV genotypes could be identified in more severe cases and how the virus re-establish epidemics over time, we described the molecular epidemiology of RSV over 16 years in South Africa. Positive selection drove both RSV-A and B genotypes to evolve, resulting in replacement of all genotypes over the 16-year period in South Africa. We were unable to attribute more severe disease to either RSV-A or B; rather that it was linked to the predominant strain of the season, which was likely driven by herd immunity in previous seasons.

In a previous study in South Africa RSV-B deletion mutants were identified in HIV-infected patients in South Africa. To determine the frequency of this phenomena and the possible role in disease in these patients the surveillance program was used to

address these questions in a larger population group. Deletions in the G-protein of both RSV-A and RSV-B strains were identified in HIV-infected SARI patients. Whole genome analysis of the deletion mutants revealed that even though there were unique non-synonymous mutations for the deletions mutants and the cumulative effect of these substitutions might affect RSV fitness, no changes were observed that could result in altered virus-host attachment, changes in immune evasion strategies or distorted fusion abilities. The specimens obtained from the patients carrying the RSV strains containing the G-protein deletions contained significantly lower levels of pro-and anti-inflammatory cytokines, suggesting that the absence of the G-protein inhibited the virus's ability to activate the normal RSV anti-viral response.

The inflammatory responses of children infected by RSV, rhinovirus or both showed that rhinovirus infection elicits a strong Th1 response, while RSV infection skews the balance towards a Th2 response. Co-infections did not result in more severe disease and the effect that these 2 viruses have on the innate immune response may results in a further Th1/Th2 imbalance.

Understanding the contribution of RSV and RV to severe respiratory disease will allow for informed decision making when selecting and setting criteria for vaccine development and implementation as well as therapeutic interventions.

To conclude, this study provided a useful tool for defining the major respiratory viruses in SARI and confirmed the importance of Influenza, RSV, rhinovirus, and human metapneumovirus as pathogens. Changes in the circulating strains of RSV may overcome herd immunity and re-establish infections. RSV G-protein deletion mutants appeared only in HIV-infected patients and had a much reduced immune-modulatory effect relative to wild type strains. Results suggest that a co-infection

between RSV and rhinovirus will further imbalance the innate immune response and may affect the pathogenesis in HIV infected and young patients.

Publications and Communications

Publications:

1. Venter M, Naidoo D, **Pretorius M**, Buys A, McAnerney J, Blumberg L, Madhi SA, Cohen C, Schoub B. Evolutionary dynamics of 2009 pandemic influenza A virus subtype H1N1 in South Africa during 2009-2010. J Infect Dis. 2012 Dec 15;206 Suppl 1:S166-72.
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Anne von Gottberg, Nicole Wolter, Marietjie Venter, Shabir A. Madhi for the South African Severe Acute Respiratory Illness (SARI) Surveillance Group. Human metapneumovirus-associated severe acute respiratory illness hospitalisation in HIV-infected and HIV-uninfected South African children and adults. J Clinical Virology DOI: http://dx.doi.org/10.1016/j.jcv.2015.06.089.

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Pending publications:

1. Pretorius MA, Treurnicht FK, Tempia S, Walaza S, Cohen AL, Moyes J, Variava E, Dawood H, Madhi SA, Cohen C, Venter M. Naturally occurring respiratory syncytial virus A and B glycoprotein ecto-domain deletion mutants in HIV-infected patients hospitalized with severe acute respiratory illness in South Africa. *Under review with co-authors in preparation of submission.*
2. Pretorius MA, Tempia S, Walaza S, Cohen AL, Moyes J, Variava E, Dawood H, Cohen C, Venter M. T-helper 1 and 2 cytokine responses associated with severe acute respiratory illness in immune compromised infants infected with rhinovirus vs. respiratory syncytial virus.

International Communications:

1. Marietjie Venter**,** Dhamari Naidoo, **Marthi Nieuwoudt**, Ria Lassauniere, Amelia Buys, Lucille Blumberg, Cheryl Cohen, Adrian Puren, Barry Schoub. Molecular Epidemiology of the 2009 Pandemic Influenza A (H1N1) outbreak in South Africa [oral presentation] Influenza in Africa conference hosted by the CDC 7 - 9 December 2009 National Institute for Communicable Diseases, Sandringham, Johannesburg.
2. **M.A. Nieuwoudt[1]**, M. Venter, A. Buys[2] and A.J. Puren[1]: Validation and Development of an in-house Multiplex PCR for the Detection of Respiratory Viruses, [oral presentation] Influenza in Africa conference hosted by the CDC 7 - 9 December 2009 National Institute for Communicable Diseases, Sandringham, Johannesburg.
3. Dhamari Naidoo, **Marthi Nieuwoudt**, Amelia Buys, Cheryl Cohen, Lucille Blumberg, Marietjie Venter. Surveillance of Genetic Markers for Resistance and Virulence in Pandemic H1N1 Viruses from South Africa, [oral presentation] Influenza in Africa conference hosted by the CDC. 7 - 9 December 2009 National Institute for Communicable Diseases, Sandringham, Johannesburg.

4. **Marthi Nieuwoudt**, M. Venter, A. Buys[2] and A.J. Puren[1]: The application of Multiplex PCR platform for the detection of the Common Respiratory Virus Infections , [oral presentation] 1st Annual SARI and Rotavirus Principal Investigator and Surveillance Officers Meeting, NICD, PRF Auditorium, 28-30th October 2009.
5. **Naidoo D*, Nieuwoudt M**, Buys A, Mcanerney J, Cohen C, Blumberg L, Puren A, Schoub B, **Venter M.** Investigation of genetic drift in the Neuraminidase gene and its effect on antiviral resistance and pathogenic mutations in pandemic H1N1 influenza viruses from South Africa, 2009-2010 [Poster] Options for the Control of Influenza conference VII 3-7 September 2010, Hong Kong SAR, China(*presenter)
6. **Nieuwoudt M*, Naidoo D,** Madhi S, Groom M, Buys A, McAnerney J, Moyes J, Walaza S, Dawood H, Chagan M, Haffjee S, Twine R , Kahn K, Cohen C, Puren A, Schoub B, **Venter M.** Comparison of the prevalence and seasonal distribution of respiratory viruses in hospitalized patients presenting with severe acute respiratory illness vs. influenza like illness in South Africa, 2009-2010 [Poster] Options for the Control of Influenza conference VII 3-7 September 2010, Hong Kong SAR, China(*presenter)
7. Venter M*, Naidoo D, **Nieuwoudt M,** Buys A, McAnerney J, Cohen C, Blumberg l, Puren A, Schoub B. Evolution of Influenza A in South Africa since the emergence of pandemic H1N1[Poster] Options for the Control of Influenza conference VII 3-7 September 2010, Hong Kong SAR, China (*presenter)
8. **Marthi Pretorius** Venter M, Naidoo D, Buys A, McAnerney J, Cohen C, Blumberg l, Puren A, Schoub B**.** Comparison of the prevalence and distribution of respiratory viruses in patients presenting with SARI and ILI in South Africa, 2009-2010. The 2nd annual African Network for Influenza Surveillance and Epidemiology (ANISE) conference in Accra Ghana, 10th to the 14th of January
9. **Marthi Pretorius**, Dhamari Naidoo, Florette Treurnicht, Andronica Rakgantso, Sibongile Walaza, Cheryl Cohen, Marietjie Venter. Influenza Syndrome: Respiratory Viruses. [Oral Presentation] Influenza Symposium, 8th March 2011, NICD, Johannesburg
10. Dhamari Naidoo**,** Florette Treurnicht, Andronica Rakgantso, **Marthi Pretorius**, Amelia Buys, Marietjie Venter, Molecular Analysis of the 2010 South African novel H1 isolates [Oral presentation] Influenza Symposium, 8th March 2011, NICD, Johannesburg.
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the risk of elevated blood pneumococcal loads and associated mortality in hospitalised pneumonia patients. Oral presentation. 8th International Symposium On Pneumococci And Pneumococcal Diseases (ISPPD),Iguaçu Fall, Brazil 11-15 March 2012

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22. Cohen C, Moyes J, Walaza S, Groome M, Kgokong B, Tempia S, Dawood H, Kahn K, Variava E, Wolter N, von Gottberg A, **Nieuwoudt M**, Venter M, Madhi SA. Increased risk of death amongst HIV-infected persons hospitalized with influenza confirmed illness in South Africa. Oral presentation. Federation of Infectious Diseases Societies of Southern Africa (FIDSSA) conference, Durban, 8-11 September 2011:
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24. Walaza S, Moyes J, Kgokong B, Groome M, Tempia S, Dawood H, Cohen AL ,Kahn K, Variava E, **Pretorius M,** Venter M, Madhi SA, Cohen C. Risk of death amongst TB patients hospitalized with influenza in South Africa 2009-2010. Oral Presentation. 7th Public Health Association of South Africa (PHASA) conference, Sandton, Johannesburg,
25. **M. Pretorius**, Amelia. Buys, Shabir. A. Madhi, Marietjie. Venter: “Duplication mutation and positive evolution identified in the dominant Respiratory Syncytial Virus subtype A genotype GA2, previously persisting for 10 years in South Africa” (poster presentation). International 8th Annual Respiratory Syncytial virus Symposium, Santa Fe, New Mexico, USA, 26th to 30th September 2012
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29. **M Pretorius**, S van Niekerk; S Tempia; J Moyes, C Cohen, S Madhi, M Venter: "Replacement and positive evolution of subtype A and B Respiratory Syncytial Virus G-protein genotypes from 1997-2012 in South Africa". Options for the Control of Influenza VIII 5th-9th September 2013, Cape Town, South Africa (poster presentation).
30. Hellferscee, **M Pretorius**, C Cohen, S Walaza, J Moyes, E Variava, H Dawood, K Kahn, S Tempia, A Cohen, S Madhi, M Venter:" Association of cycle threshold values with disease severity in HIV-infected and HIV-uninfected patients with Influenza-associated respiratory illness; South Africa Options for the Control of Influenza VIII 5th-9th September 2013, Cape Town, South Africa (poster presentation).
31. Hellferscee, C von Mollendorf, F Treurnicht**, M Pretorius**, E Variava, H Dawood, S Walaza, S Tempia, A Cohen, C Cohen, M Venter: "Influenza viral shedding in HIV-infected and HIV-uninfected individuals, South Africa, 2012" Options for the Control of Influenza VIII 5th-9th September 2013, Cape Town, South Africa (poster presentation)
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Table of Contents

List of Abbreviations

%	Percentage
°C	degrees Celsius
µl	microliter
µM	micromolar
AdV	Adenoviruses
AF	attributable fraction
ALRI	acute lower respiratory tract infection
aRRR	adjusted RRR
BEAST	Bayesian Evolutionary Analysis by Sampling Trees
bp	base pair
CARD	caspase recruitment domain
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CDS	CoDing Sequence
COPD	chronic obstructive pulmonary diseases
CPE	cytopathic effect
Ct	crossing point
dN	non-synonymous
DNA	deoxyribonucleic acid
dS	synonymous
EIAs	enzyme immunoassays
ELISA	enzyme-linked immunosorbent assay
EV	Enteroviruses
FADD	Fas-associated death domain-containing protein

G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hCoV	human coronavirus(es)
HIV	human immunodeficiency virus
hMPV	human metapneumovirus
IFN	interferon
IFN-γ	interferon-γ
IFNs	interferons
IL	interleukin
ILI	influenza-like illness
INF	influenza virus
IP-10	interferon-gamma-inducible protein 10
IRAK-4	interleukin-1-receptor (IL-1R)-associated kinase-4
LRTI	lower respiratory tract infection
MAb	monoclonal antibodies
MCMC	Bayesian Markov chain Monte Carlo
MDA5	melanoma differentiation antigen 5
MDG	Millennium Development Goal
Min	Minute
ML	Maximum likelihood
MRCA	most recent common ancestor
mRNAs	messenger RNAs
MyD88	myeloid differentiation factor 88
NF- κB	Nuclear Factor-κB
NICD	National Institute for Communicable Diseases

OR	Odds Ratio
PCR	Polymerase chain reaction
p-distance	Pairwise amino acid distances
pg/ml	picograms per millilitre
PIV	parainfluenza viruses
$Prev_{Illness}$	prevalence of each virus attributable to mild or severe illness
PRRs	pattern recognition receptors
QCMD	Quality Control for Molecular Diagnostics
ra	receptor antagonist
RANTES	Regulated on Activation Normal T-cell Expressed and Secreted
RIG-I	RNA helicases retinoic acid inducible gene-I
RNA	Ribonucleic acid
RR	relative risk
RRR	Relative Risk Ratio
rRT-PCR	real-time reverse transcriptase polymerase chain reaction
RSV	respiratory syncytial virus
RV	rhinovirus
RVU	Respiratory Virus Unit
SA	South Africa
SADM	South African deletion mutants
SARI	Severe acute respiratory illness
SARS-CoV	Severe Acute Respiratory Syndrome coronavirus
Th	T helper cell
TLRs	toll-like receptors
TRAF3	tumor necrosis factor-receptor associated factor 3

TRIF	TIR-domain containing adapter inducing IFN-β
U	Units
UP	University of Pretoria
USA	United States of America
WHO	World Health Organization

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Chapter 1

Literature Review

1.1 Introduction

Pneumonia is a major cause of morbidity and mortality in children worldwide and causes 18% of all deaths in children less than 5 years of age (Black, Cousens *et al.* 2010). Respiratory viruses traditionally associated with acute respiratory tract infection include influenza (INF) A and B; respiratory syncytial virus (RSV); parainfluenza virus (PIV) types 1, 2 and 3; adenovirus (AdV); enterovirus (EV); human metapneumovirus (hMPV) and rhinovirus (RV) (Brittain-Long, Nord *et al.* 2008, Tiveljung-Lindel, Rotzen-Ostlund *et al.* 2009). While a few studies have determined the frequency of respiratory viruses in patients with acute lower respiratory tract illness in Africa, (Joosting, Harwin *et al.* 1979, Kristensen, Thiel *et al.* 2004, Madhi, Ludewick *et al.* 2007, Smuts, Workman *et al.* 2011) these studies have mainly been limited to single sites and a limited number of viruses, and little has been reported on viral co-infections

Comparative studies have shown that the detection of respiratory viruses using real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assays is substantially more sensitive than using conventional methods such as viral culture and immunofluorescence assays (IFA) (Hendrickson 2004, Paranhos-Baccala, Komuruan-Pradel *et al.* 2008, Lassauniere, Kresfelder *et al.* 2010). Furthermore, compared to conventional PCR and other real-time methods, multiplex rRT-PCR has a significant advantage as it permits simultaneous amplification of several viruses in a single reaction(Paranhos-Baccala, Komuruan-Pradel *et al.* 2008, Lassauniere, Kresfelder *et al.* 2010, Venter, Lassauniere *et al.* 2011). This facilitates cost-effective diagnosis, enabling the detection of multiple viruses in a single clinical specimen. In order to best understand the full burden of acute respiratory infection in South Africa, it is important to conduct surveillance and measure the burden of mild, outpatient

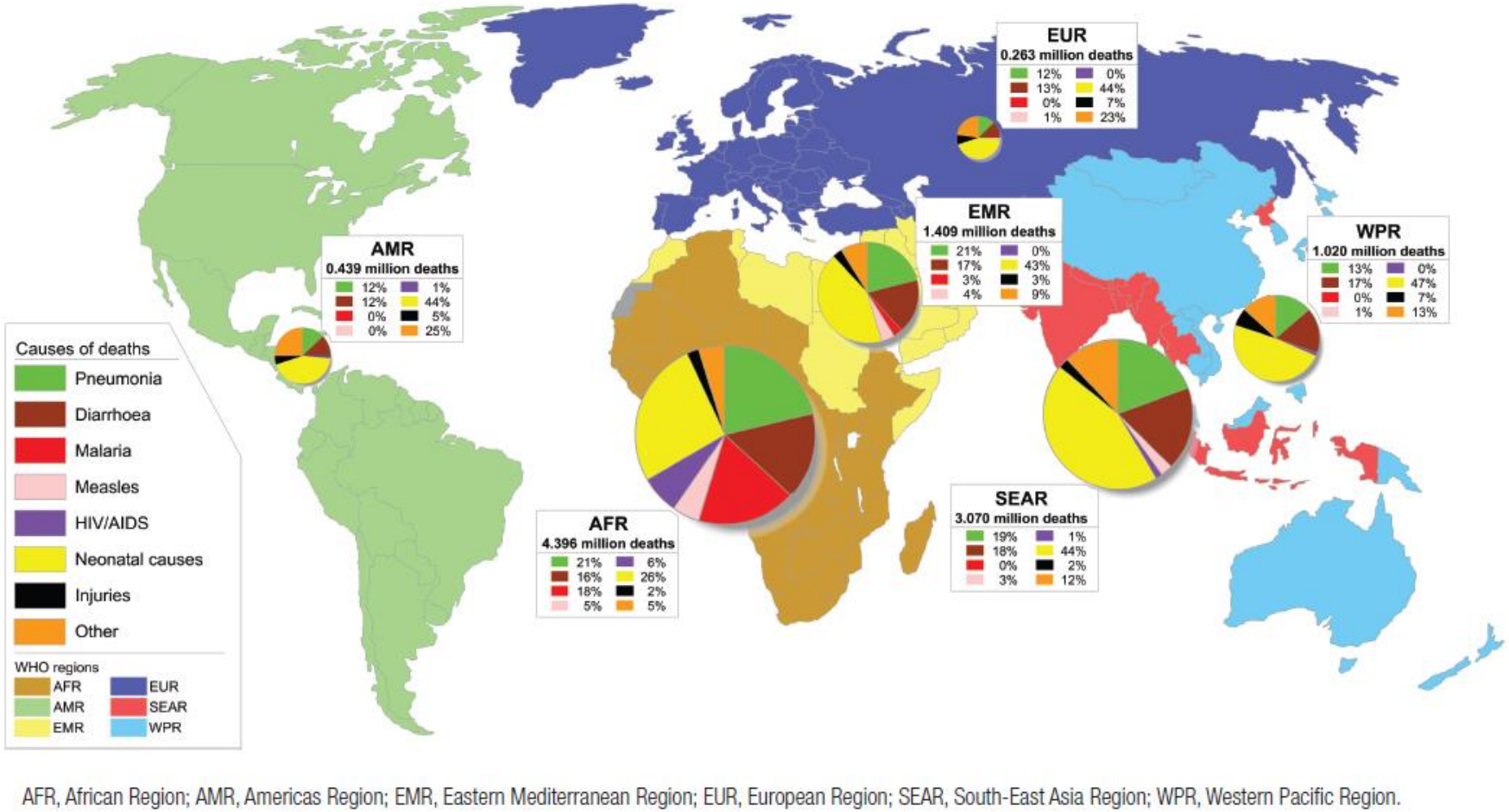
disease as well as more severe, inpatient disease. By comparing the prevalence of these respiratory viruses detected in patients with influenza-like illness (ILI) and severe acute respiratory illness (SARI) to control patients the contribution of these respiratory viruses to SARI maybe better understood.

1.2 Pneumonia

1.2.1 Burden and mortality of pneumonia

Previous estimates from the 1970's to early 1990's attributed up to a third of deaths in children aged less than 5 years were due to or associated with respiratory infection (Cockburn and Assaad 1973, Bulla and Hitze 1978, Leowski 1986, Garenne, Ronsmans *et al.* 1992). An increased focus on childhood mortality arising from the Millennium Declaration and the Millennium Development Goal (MDG) 4, which deals with the reduction of the mortality rate in children less than 5 years old with two-thirds by 2015. This focus made [16]monitoring the interventions to control these deaths crucial if these goals are going to be reached (Figure 1.1) (Rudan, Boschi-Pinto *et al.* 2008).

Several meta-analysis studies have since reported on childhood mortality in general and for specific syndromes like pneumonia and diarrhea (Rudan, Boschi-Pinto *et al.* 2008, Black, Cousens *et al.* 2010, Nair, Nokes *et al.* 2010, Nair, Brooks *et al.* 2011, Walker, Rudan *et al.* 2013). Rajaratnam *et al.,*2010 (Rajaratnam, Marcus *et al.* 2010) reported on the decrease in worldwide mortality in children less than 5 years of age from 11.9 million in 1990 to 7.7 million in 2010, however there was still a substantial amount of deaths occurring in the low-income countries: 33% in South Asia and 49% occur in sub-Saharan Africa, with less than 1% of deaths occurring in high-income countries. In their analysis which included data from 187 countries showed the



AFR, African Region; AMR, Americas Region; EMR, Eastern Mediterranean Region; EUR, European Region; SEAR, South-East Asia Region; WPR, Western Pacific Region.

Figure 1.1: Distribution of deaths from pneumonia and other causes in children aged less than 5 years, by WHO region (Rudan, Boschi-Pinto *et al.* 2008)

number of deaths in children younger than 5 years dropped from 16 million in 1970 to 7·7 million in 2010. (Rajaratnam, Marcus *et al.* 2010).

The set MDG 4 target for decrease in mortality in children younger than 5 years is 4.4% per year[16]. Taking into account the set target of 4.4% the 35% decline in childhood mortality reported in developing countries calculated to a yearly rate of 2.2%, which is lower that the MDG 4 target but still represents substantial progress across countries and economic status (Figure 1.2).

1.2.2 Incidence of clinical pneumonia

In 2004 Rudan *et al* (Rudan, Tomaskovic *et al.* 2004) published one of the first global estimates of clinical pneumonia in children from developing countries. In the meta analysis from 28 different studies published since 1961 The median incidence estimated from those studies was 0.28 episodes per child-year. This equates to an annual incidence of 150.7 million new cases of childhood pneumonia, 11-20 million (7-13%) of which are hospitalized. Together with large population-based studies published from Europe and USA (McConnochie, Hall *et al.* 1988, Jokinen, Heiskanen *et al.* 1993, Weigl, Bader *et al.* 2003) which reported that the incidence of community-acquired pneumonia among children less than five years old is approximately 0.026 episodes per child-year, still suggests that 95% of all episodes of clinical pneumonia in young children worldwide occur in developing countries.

A major public health to be considered is the distribution of the estimated 156 million new episodes by region and country to enable the role out of realistic preventative interventions and case management at community and facility levels which would include vaccination and antibiotic availability. Rudan *et al.,* 2008

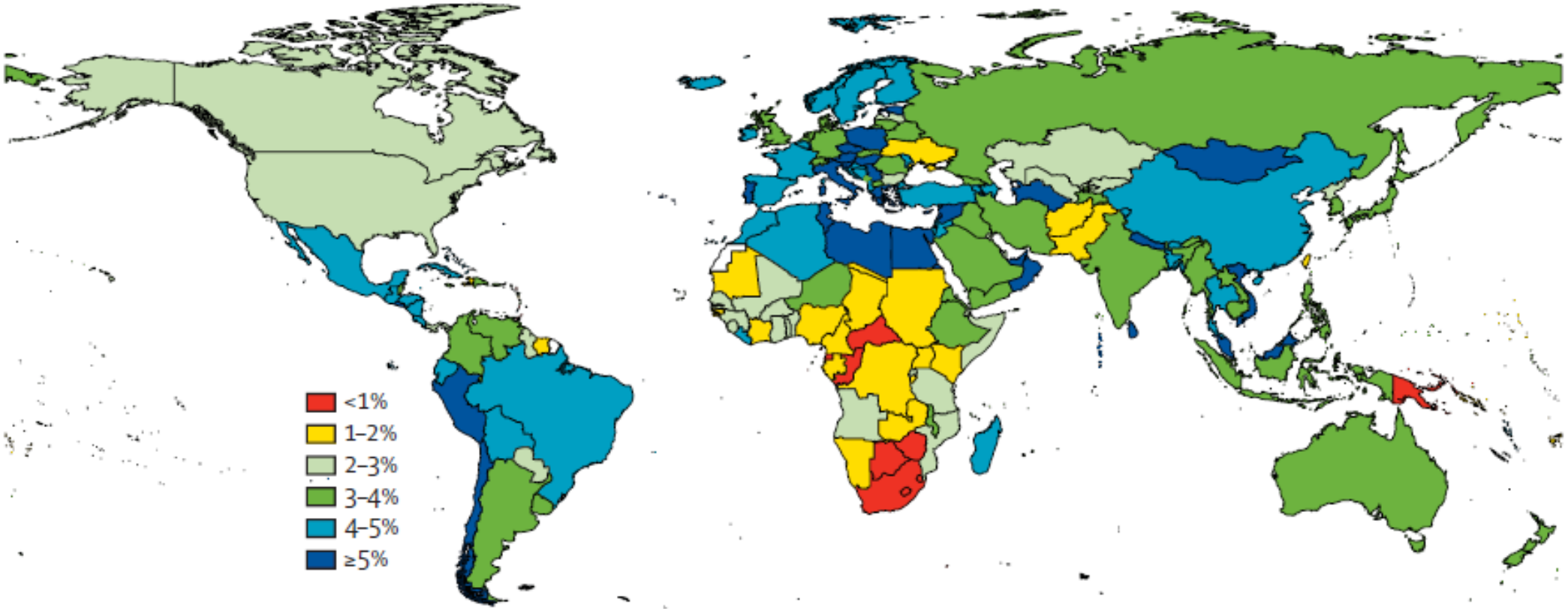


Figure 1.2: Yearly percentage decline in mortality in children younger than 5 years between 1990 and 2010 (Rajaratnam, Marcus *et al.* 2010)

(Rudan, Boschi-Pinto *et al.* 2008) identified the 15 countries with the highest predicted incidences of pneumonia. They calculated that these countries account for 74% of the estimated 156 million global episodes and that more than half of the new pneumonia cases are concentrated in five countries: India (43 million), China (21 million), Pakistan (10 million) and Bangladesh, Indonesia and Nigeria each with 6 million (Figure 1.3). The aggregated results together with the updated estimates of new episodes per child-year allowed Rudan *et al.,* 2008 (Rudan, Boschi-Pinto *et al.* 2008) to calculate the estimated incidence of pneumonia in the six WHO regions: on the higher spectrum South-East Asia (0.36 episodes per child-year), followed by Africa (0.33 episodes per child-year), the Eastern Mediterranean (0.28 episodes per child-year), and on the lower spectrum the Western Pacific (0.22 episodes per child-year), the Americas (0.10 episodes per child-year) and European regions (0.06 episodes per child-year).

1.2.3 Childhood pneumonia

Clinical pneumonia in childhood is caused by a combination of several factors which include the level of exposure to risk factors related to the host, the environment and infection.

1.2.3.1 Risk factors related to the host and the environment

Risk factors that affect incidence of childhood clinical pneumonia in the community in developing countries have been divided into three probable categories *i.e.* definite risk factors, likely risk factors and possible risk factors (Rudan, Boschi-Pinto *et al.* 2008). The first category include risk factors that could influence the child the first months postnatally *i.e.* malnutrition (weight-for-age z-score < –2), low birth weight

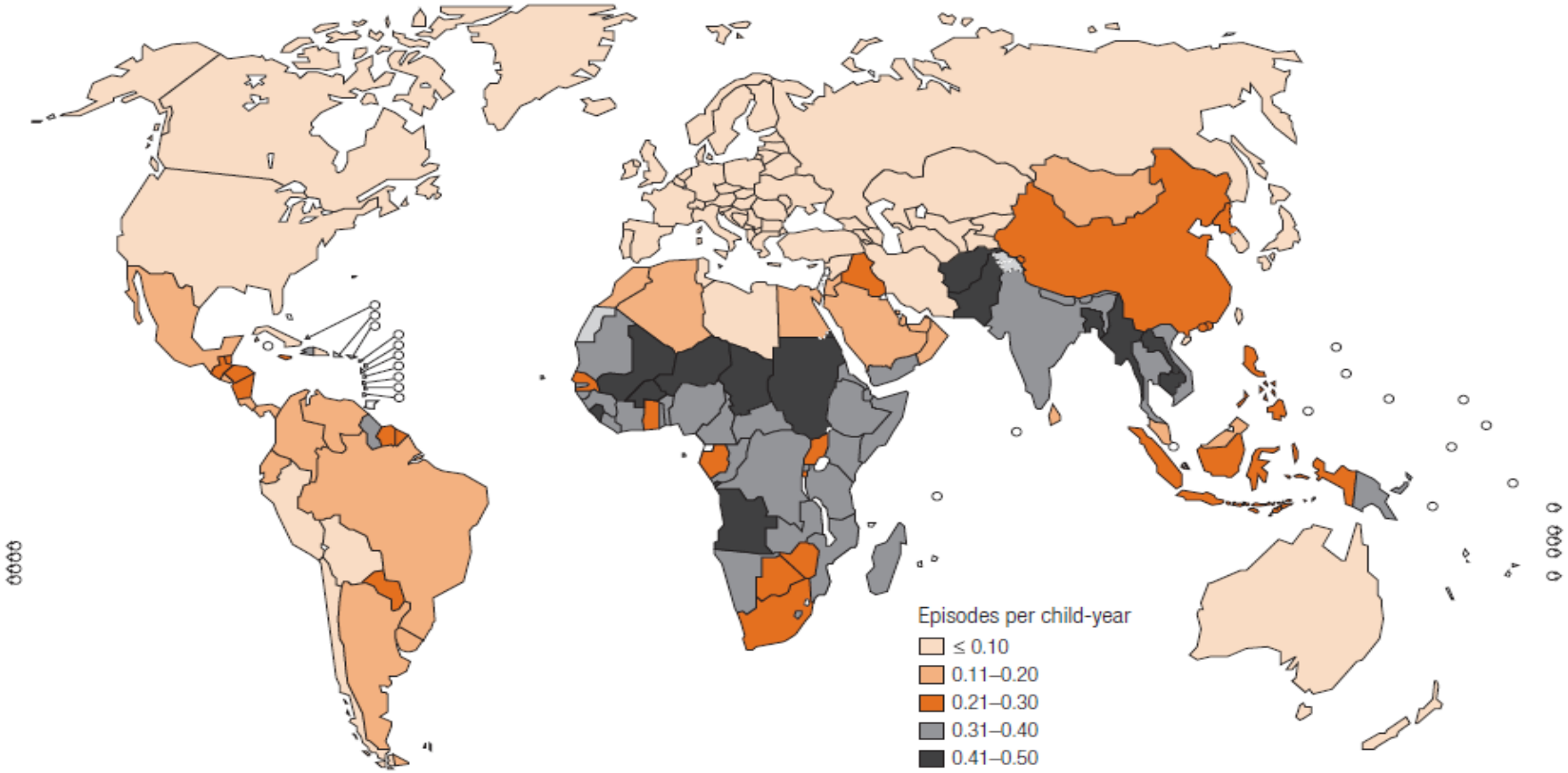


Figure 1.3: Incidence of childhood clinical pneumonia at the country level (Rudan, Boschi-Pinto *et al.* 2008)

(2500 g), non-exclusive breastfeeding (during the first 4 months of life), lack of measles immunization (within the first 12 months of life) and crowding where the person-to-person spread of virus-laden aerosol particle is greatly enhanced by having a dense population of susceptible individuals surrounding each infected subject, thereby maximizing the potential of the spread if infection (Souza, Ramos *et al.* 2003). The second category includes parental smoking, Zinc deficiency, the mother's experience as a caregiver, co-morbidities (e.g. diarrhoea, heart disease, asthma). The last category includes factor such as the mother's education which would influence her health care seeking behaviour, day-care attendance and the importance of the substantial difference in frequency of respiratory viruses in all climatic and geographical areas (Rudan, Boschi-Pinto *et al.* 2008). Respiratory virus infections often have seasonal distributions, especially in temperate climates and while the peak incidence varies year to year there is often a predominant seasonal occurrence (Kesson 2007b).

1.2.3.2 Aetiology of childhood pneumonia

Before vaccines were readily available the etiological cause of childhood pneumonia was of great interest as specific therapy was available for pneumococcal pneumonia of certain serotypes (Rudan, Boschi-Pinto *et al.* 2008, Walker, Rudan *et al.* 2013). Studies have identified *Streptococcus pneumoniae* (pneumococcus) and *Haemophilus influenzae* as the main bacterial causes of pneumonia, especially in the developing world that had limited access to treatment (Shann 1986).

Since the development of PCR detection techniques pneumonia aetiology studies that include viral causes have shown that in children especially RSV is the leading viral cause of severe pneumonia. It is estimated that 33·8 million new episodes of

RSV-associated acute lower respiratory tract infection (ALRI) occur worldwide in children under 5 years of age. With an estimated 66 000–199000 deaths in the same age group, 99% of these deaths occurring in developing countries (Nair, Nokes *et al.* 2010).This statistic is closely followed by 20 million cases of Influenza associated ALRI in the same age group with an estimated 28 000–111 500 deaths. Of these deaths 99% occurs in developing countries (Nair, Brooks *et al.* 2011). The other respiratory viruses like, parainfluenza, human metapneumovirus and adenovirus have also been shown to play a role (Rudan, Boschi-Pinto *et al.* 2008, Lassauniere, Kresfelder *et al.* 2010, Venter, Lassauniere *et al.* 2011). Several studies have reported on the interaction between viruses and bacteria, especially influenza and pneumococcus and that primary viral infection can lead to secondary bacterial infection or that viral or bacterial co-infection is very common (Ghafoor, Nomani *et al.* 1990, Forgie, O'Neill *et al.* 1991, O'Brien, Walters *et al.* 2000, McCullers 2006, Wolter, Cohen *et al.* 2014).

In recent years the emergence of the HIV epidemic has had a considerable impact not only on the incidence and mortality but also on the aetiology of childhood pneumonia (Rudan, Boschi-Pinto *et al.* 2008). In children with HIV, bacterial causes of pneumonia remains the major contributor, but additional pathogens such as *Pneumocystis jiroveci* also play a role (Klugman, Madhi *et al.* 2007). Available vaccines have been shown to have lower efficacy in children infected with HIV, however a significant proportion of children would still be protected against disease (Zar and Madhi 2006).

1.2.4 Diagnosis of respiratory viruses

1.2.4.1 Cell culture methods

Viral Tissue culture was traditionally the “gold standard” used for diagnosing respiratory virus infections(Fenner and White 1994), however in the last 10 year it has been replaced with molecular techniques. Virus isolation needs to remain an important part of respiratory virus diagnostics to maintain a source for analyzing not only genotypic changes but phenotypic changes as well in the virus populations of which the annual influenza vaccine is the best example(Hendrickson 2004). Respiratory samples that is suitable for virus isolation include nasopharyngeal aspirates, nasopharyngeal and/ or oropharyngeal swabs, nasopharyngeal washes, bronchi alveolar lavages, sputum and lung biopsies (Forman and Valsamakis 2011). Quality of the specimen as determined by: adequate sample collection, correct transport of specimens and processing methods also play a big role in successful viral isolation. Detection of viruses in cell culture requires a considerable expertise and is performed by microscope examination looking for degenerative morphological changes in the cell monolayer called cytopathic effect (CPE). Not all viruses grow in all cell types as such clinical specimens are usually inoculated into several cell culture types to provide an environment for the isolation of several viruses (Kesson 2007b).

More recently newer viral culture formats have been developed which allow for more rapid detection of viruses, especially for viruses which are known to grow slowly in conventional cell culture by centrifugation of the culture after the sample has been added. The enhanced detection rate may result from better contact between cells in the specimen and the cell culture thus allowing for earlier and more extensive

infection of the cell culture. Furthermore the time it takes to identity viral proteins have also decreased, with fluorescent-labeled (e.g. FITC) monoclonal antibodies, that are labour intensive but could detect the most common viruses within 24 – 48 hours (Kesson 2007b). Nevertheless, most culture methods have a severe disadvantage relative to PCR due to lack of sensitivity and specificity, especially during times of low prevalence (off season) or in special populations, such as the immune-compromised or the elderly (Hendrickson 2004).

1.2.4.2 Antigen detection methods

The ability to detect viruses was significantly enhanced by the development if monoclonal antibodies (MAb) linked with the fluorescent molecule FITC, directed against specific viral proteins for the viruses herpes simplex virus (HSV)-1, HSV-2, varicella zoster virus (VZV), cytomegalovirus (CMV), Influenza A and B, parainfluenza 1-3 and adenoviruses. These staining procedures are rapid and results are available within 3 hours and have an excellent specificity and sensitivity (Schutzbank and McGuire 2000). The performance characteristics of the rest depend on the type of specimen, age of the patient, duration of the illness, the MAb reagent and most importantly the level of expertise of the staff. However, this method has a distinct disadvantage that the availability of the MAb is limited and not available for the newer respiratory viruses like the Coronaviruses.

Membrane based enzyme immunoassays (EIAs), optical immunoassays and immunochromographic or lateral flow systems have been introduced for less technically demanding detection of respiratory viruses (Mackie, Joannidis *et al.* 2001). These techniques have the advantage of decreasing turn-around time for test results and have a considerably simplified technical component, but these tests are

not as sensitive or specific as the viral culture or Immunofluorescence tests and confirmatory testing is required (Cazacu, Greer *et al.* 2003).

1.2.4.3 Nucleic acid detection methods

Viruses can be detected in clinical samples using highly specific nucleic acid probes that are complementary in sequence to viral RNAs or viral DNAs and over the past ten years, nucleic acids amplification tests have been developed for a number of respiratory viruses and have become the new benchmark for respiratory virus detection. The published sensitivities and specificities are usually nearly 100% when compared with cell culture or antigen assay (Fan and Henrickson 1996, Woo, Chiu *et al.* 1997, Fan, Hendrickson *et al.* 1998, Hendrickson 2004). In fact studies that have compared molecular assays with tissue culture assays have demonstrated significantly increased sensitivity of up to 30% (Fan and Henrickson 1996, Fan, Hendrickson *et al.* 1998, Falsey, Formica *et al.* 2002, Falsey, Formica *et al.* 2003, Hendrickson 2004). Multiplex PCR assays have been used to detect the presence of one or more respiratory viruses e.g. Influenza virus (INF) A and B; (Fan, Hendrickson *et al.* 1998, Bellau-Pujol, Vabret *et al.* 2005, Kaye, Skidmore *et al.* 2006, Lam, Yeung *et al.* 2007, Mahony, Chong *et al.* 2007), Parainfluenza viruses (PIV) types 1, 2, 3 and 4 (Fan, Hendrickson *et al.* 1998, Osiowy 1998, Coiras, Aguilar *et al.* 2004b, Bellau-Pujol, Vabret *et al.* 2005, Lam, Yeung *et al.* 2007, Mahony, Chong *et al.* 2007); human Respiratory Syncytial Virus (RSV) (Osiowy 1998, Bellau-Pujol, Vabret *et al.* 2005, Kaye, Skidmore *et al.* 2006, Lam, Yeung *et al.* 2007, Mahony, Chong *et al.* 2007), human Metapneumovirus (hMPV) (Bellau-Pujol, Vabret *et al.* 2005, Lam, Yeung *et al.* 2007, Mahony, Chong *et al.* 2007), human Rhinoviruses (RV) (Coiras, Aguilar *et al.* 2004b, Bellau-Pujol, Vabret *et al.* 2005,

Lam, Yeung *et al.* 2007, Mahony, Chong *et al.* 2007), human Coronaviruses (hCoV-229E, hCoV-OC43) and Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) (Lam, Yeung *et al.* 2007, Mahony, Chong *et al.* 2007), human enteroviruses (EV) (Coiras, Aguilar *et al.* 2004b, Mahony, Chong *et al.* 2007) and Adenoviruses (AdV) (Osiowy 1998). Disadvantages, however, include higher start-up costs, higher reagent costs, and extensive and specific training is required (Hendrickson 2004).

In the next sections the two major respiratory viruses which were characterized further in this study will be described in more detail.

1.3 Respiratory Syncytial Virus

1.3.1 Classification

RSV falls into the Paramyxoviridae family of viruses includes some of the most successful respiratory pathogens causing epidemics of major medical and veterinary importance. The genome consists of a single strand of negative (-) polarity RNA in a linear arrangement of around six genes. These viruses must carry RNA polymerase in the viral particle because the incoming viral RNA can neither be translated nor copied by the cellular machinery. All these viruses have an envelope with surface projections surrounding the nucleocapsid. The projections are thought to be major targets for a protective immune response and induce neutralizing antibodies and resistance to infection (Mackie 2003).

Pneumovirinae sub-family is constituted of Metapneumovirus genus, together with the Pneumovirus genus. The genome of Respiratory Syncytial Virus (RSV) encodes 10 sub-genomic messenger RNAs (mRNAs). These mRNAs are translated into 11 known proteins: four nucleocapsid proteins – nucleocapsid N protein,

phosphoprotein P, large polymerase subunit L, and transcription elongation factor M2-1; three transmembrane envelope glycoproteins – fusion F protein, attachment G protein and a small hydrophobic SH protein; two non-structural proteins – NS1 and NS2; a matrix M protein; and a RNA regulatory factor M2-2 (Figure 1.4).

The G and F glycoproteins are the major protective antigens and induce RSV-neutralizing antibodies and resistance to infection. Two major subgroups have been identified RSV-A and RSV-B and described based on antigenic and genetic variability, subsequently the strains have been divided into separate genotypes based on distinct genetic patterns (Peret, Hall *et al.* 1998, Peret, Hall *et al.* 2000). Most studies for RSV have been based on phylogenetic changes observed in the G protein. To date, eleven genotypes for RSV-A have been identified internationally including GA1-7 (Peret, Hall *et al.* 1998, Peret, Hall *et al.* 2000); SAA1 (Venter, Madhi *et al.* 2001); NA1 and NA2 and ON1 with a 72bp duplication insertion(Eshaghi, Duvvuri *et al.* 2012). For RSV-B the genotypes are GB1-4 (Peret, Hall *et al.* 1998, Peret, Hall *et al.* 2000), SAB1-3 (Venter, Madhi *et al.* 2001, Venter, Collinson *et al.* 2002) and the BA genotype with the a 60bp insertion (Trento, Galiano *et al.* 2003) which has now evolved into sub-genotypes of BA1-10 (Dapat, Shobugawa *et al.* 2010, Trento, Casas *et al.* 2010).

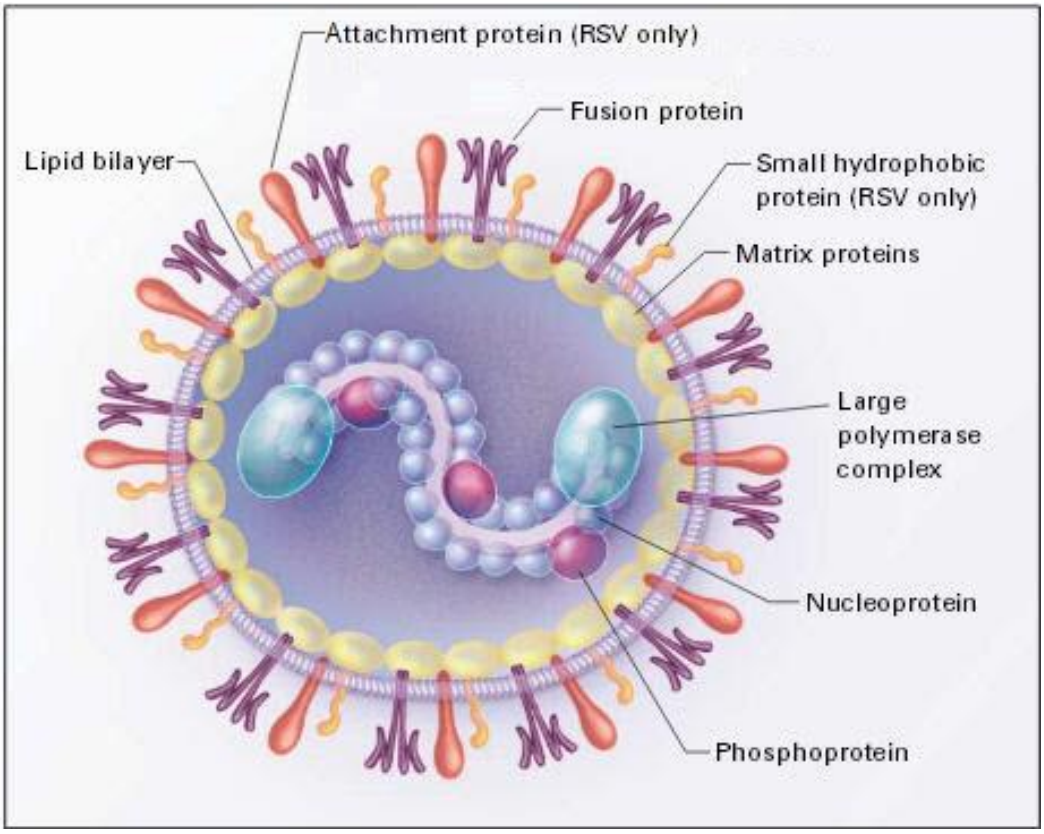


Figure 1.4: Structure of Respiratory Syncytial Virus (RSV). The genome of RSV is translated into 11 known proteins: nucleocapsid N protein, phosphoprotein P, large polymerase subunit L, and transcription elongation factor M2-1; three transmembrane envelope glycoproteins: fusion F protein, attachment G protein and a small hydrophobic SH protein; two non-structural proteins – NS1 and NS2; a matrix M protein; and RNA regulatory factor M2-2 (modified from http://www.kuleuven.be/rega/mvr/images/RSV-1.jpg).

1.3.2 Epidemiology

1.3.2.1 RSV in children

RSV is a common cause of childhood respiratory illness and several studies are underway to develop a vaccine (Nair, Verma *et al.* 2011). In 2005, Nair *et al* estimated that 33.8 million hospitalizations occurred globally in children younger than 5 years of age due to RSV, of which 66 000–199000 died and most of these deaths occurred in developing countries (Nair, Nokes *et al.* 2010). In the United States annual hospitalization rates due to RSV have been estimated to be 17/1000 children in the age group < 6months and 3/1000 children aged <5 years (Hall, Weinberg *et al.* 2009). Moyes *et al.,*(Moyes, Cohen *et al.* 2013) reported on the case fatality ratio (CFR) as well as the incidence of RSV-associated hospitalization in both HIV infected and uninfected children younger than 5 years of age in Soweto, South Africa. The observed CFR of 1% in South Africa was very similar to the CFR of 2.1% observed in other low-income countries. The estimated RSV-associated hospitalizations among HIV-uninfected children were 24/1000, which was similar to the reported estimations in Kenya (20/1000) (Nokes, Okiro *et al.* 2004) and other low-income countries (22.3/1000) (Nair, Nokes *et al.* 2010).The estimated incidence was the highest amongst HIV-infected children between 15/1000 (children younger than 5 years of age) up to 128/1000 (children younger than 1 year of age) (Moyes, Cohen *et al.* 2013).

1.3.2.2 RSV in Adults

Several studies (Falsey 1998, Falsey and Walsh 2000, Falsey, Hennessey *et al.* 2005) have shown that RSV infection is an important illness in elderly and high-risk adults, with a disease burden similar to that of non-pandemic influenza A. In the

United States annual hospitalization rates due to RSV have been estimated to be 23.4/10,000 person periods in adults older than 65 years of age. No significantly elevated hospitalization rate in low-risk adults aged between 50-64 years of age were identified (Mullooly, Bridges *et al.* 2007). Moyes *et al.,* reported on the CFR as well as the incidence of RSV-associated hospitalization in both HIV infected and uninfected adults in Soweto, South Arica. They reported that even though 50% of the observed of the deaths were in the age group 25 to 44 years, the CFR was highest in the elderly aged more than 65 (CFR 27%). The odds of an elderly (≥65) individual dying was nearly 4 times that of those aged 25- 44 OR 3.8 (Moyes, Walaza *et al.* 2013). The incidence of RSV-associated hospitalization in individuals older than 65 years of age infected with HIV was estimated to be 383/100 000 and for HIV-uninfected individual it was estimated to be 19/100 000.

1.4 Rhinovirus

1.4.1 Classification

The picornavirus family is a diverse group of human pathogens, including enteroviruses and rhinoviruses that together constitute the most common causes of infections of humans in the developed world. The genome is a single strand of (+) sense RNA that encodes a single ‘‘polyprotein’’ of between 2100 and 2400 amino acids (Minor 2000).

This polyprotein is cleaved in a highly specific manner by proteases to produce both structural molecules and proteins involved in viral replication. The non-enveloped capsid consists of a densely packed icosahedral arrangement of promoters, each consisting of four polypeptides; VP1, 2, 3 and 4, all derived from cleavage of the original promoter VP0. The viral particle is small (hence pico-RNA), 27–30 nm in

diameter, while the length of the genome is ~ 2500 nm and consequently tightly packed into the capsid (Minor 2000) (Figure 1.5). After it was first identifiedin 1956, more than 100 serotypes have been identified and comprise of 3 species, RV-A, RV-B and RV-C (Briese, Renwick *et al.* 2008).

Although the majority of infections are associated with mild disease, their impact on overall morbidity and their economic cost worldwide is considerable (Mahony 2008). Even though RV infection has been well established as the cause for the common cold and upper respiratory tract illness inducing symptoms such as rhinorrhoea, sneezing, nasal obstruction and sore throat, more recent reports have shown that RV is associated with up to about 50% of complications such as asthma (Miller, Edwards *et al.* 2009, Peltola, Jartti *et al.* 2009, Piotrowska, Vazquez *et al.* 2009), chronic obstructive pulmonary diseases (COPD) exacerbations (Smuts, Workman *et al.* 2011), pneumonia and bronchiolitis in young children (Miller, Edwards *et al.* 2009). However, some studies suggests that infection with RV-C may result in more severe illness compared to RV-A and RV-B (Miller, Edwards *et al.* 2009).

1.4.2 Epidemiology

Although rhinoviruses have frequently been linked to the cause of the common cold, the group of viruses have not been considered as an important cause of acute respiratory hospitalizations in children (Miller, Lu *et al.* 2007). Although there are reports available on the prevalence of rhinovirus since the introduction of more sensitive viral detection methods like polymerase chain reaction, especially with the emergence of large pneumonia aetiology studies that include multiple viral causes have shown that rhinovirus is not only associated with severe respiratory illness but also occur frequently among asymptomatic patients (Arden, McErlean *et al.* 2006,

Fry, Lu *et al.* 2011). Consequently the clinical relevance of detecting RV among hospitalized patients is difficult to interpret. A study performed in the United States by Miller *et al.,* (Miller, Lu *et al.* 2007) estimated that annual hospitalization rates due to RV were 17.6/1000 in children less than 5 months followed by 6/1000 for children between 6-23 months and 2/1000 for children between the ages of 24-59 months. However when comparing children with or without a history of wheezing or asthma an increase in rhinovirus hospitalizations were observed 25.3/1000 vs. 3.1/1000, respectively (Miller, Lu *et al.* 2007).

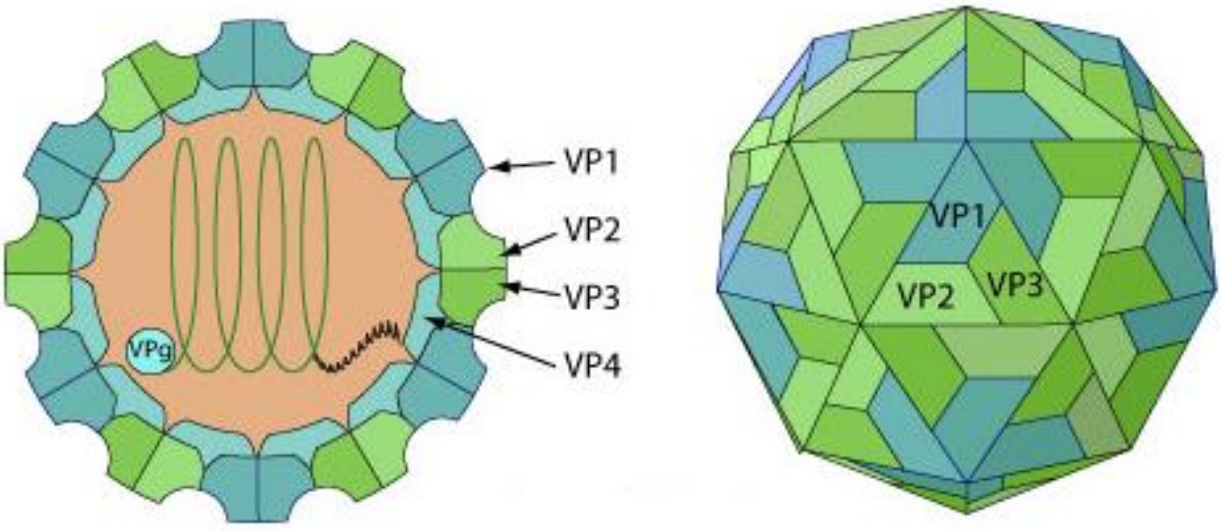


Figure 1.5: Structure of Rhinovirus, the genome is a single strand of (+) sense RNA that encodes a single ‘‘polyprotein’’ of between 2100 and 2400 amino acids. This polyprotein is cleaved to produce both structural and non-structural molecules. The non-enveloped capsid consists of a densely packed icosahedral arrangement of promoters, each consisting of four polypeptides; VP1, 2, 3 and 4, all derived from cleavage of the original promoter VP0.

1.5 Innate Immune Response

The pathogenesis of respiratory virus infections has been associated with the level of inflammatory cytokines and chemokines released during infections (Cheung, Poon *et al.* 2002, Koyama, Ishii *et al.* 2008). Cytokines are a group of immune regulatory protein secreted by a broad range of cells, in response to immune stimuli, which serve to moderate and modulate the immune response during infection. Numerous cytokines are produced by a number of different host cells during the course of a viral infection. Type I interferons (IFN) are the dominant cytokines introduces in the antiviral response (Koyama, Ishii *et al.* 2008). They are divided into 3 main groups *i.e.* IFN-α, IFN-β and IFN-ɣ. IFN-α and IFN-β are mainly responsible to inhibit viral protein synthesis in host cells, whilst IFN-ɣ not only has a direct antiviral activity, but also as an immune regulator. It is responsible for upregulating class II MHC antigens and antigen presentation to lymphocytes, promotes cytotoxic activity of natural killer (Nk) cells and virus-specific T cells as well as activating macrophages (Aberle, Aberle *et al.* 2004). It is therefore not surprising, that invading viruses have developed multiple mechanisms targeting cytokines and their downstream effects, to evade the hosts immune response (Peters 1996). Mechanisms used or activated include molecular mimicking (either at the cytokine receptor or the cytokine itself), cytokine signalling inhibition, transcriptional inhibition of IFN-induced cells and impaired gene expression (Peters 1996).

Virus infection is detected through the interaction of toll-like receptors (TLRs); (Koyama, Ishii *et al.* 2008) and several pattern recognition receptors (PRRs) which are activated by different components of viral infection such as viral RNA, DNA or viral intermediate products to activate Nuclear Factor-κB (NF- κB) and the release of pro-inflammatory cytokines, namely tumour necrosis factor (TNF) -α, interleukin (IL) -

6, IL-12, monocyte chemotactic protein (MCP)-1, Regulated on activation, normal T cell expressed and secreted (RANTES) and the IFNs (Schlender, Bossert *et al.* 2000, Bossert, Marozin *et al.* 2003, Spann, Tran *et al.* 2004, Spann, Tran *et al.* 2005, Oshansky, Zhang *et al.* 2009). This milieu of cytokines is responsible for dendritic cells (DC) maturation, Nk cell and macrophage activation, resulting in the release of further pro-inflammatory cytokines and the expression of stimulatory molecules (Bueno, González *et al.* 2008, Oshansky, Zhang *et al.* 2009). This initial innate immune response already influences the subsequent adaptive immune response and determines whether the T-cell response will be polarized towards Th1 (pro-inflammatory) or Th2 (anti-inflammatory) (de Jong, Smits *et al.* 2005). Adequate virus clearance entails a Th1 response, defined by the presence of IFN-ɣ, IL-2, and IL-12, whilst a Th2 response, defined by the presence of IL-4, IL-5, IL-10 and IL-13, is not capable of effectively clearing the virus and can results in hypersensitivity, an allergy-like condition and an extended disease period (Openshaw 1995, Graham, Johnson *et al.* 2000, van Schaik, Welliver *et al.* 2000, Martinez 2003, Durbin and Durbin 2004, Becker 2006b).

1.5.1 RSV

It has been shown in multiple studies that RSV causes an aberrant host immune response by altering the expressed cytokine profile during infection, thereby skewing the Th1/ Th2 response, which in turn contributed to immune deregulation and disease pathogenesis (Bueno, González *et al.* 2008). RSV achieves this through a number of mechanisms, namely:

- Inhibiting transcriptional activation

RSV interferes with the hosts antiviral cytokine response through the non-structural proteins NS1 and NS2, which act as a type I IFN antagonist and supressed DC maturation (Schlender, Bossert *et al.* 2000, Bossert, Marozin *et al.* 2003, Valarcher, Furze *et al.* 2003, Spann, Tran *et al.* 2004, Lo, Brazas *et al.* 2005, Spann, Tran *et al.* 2005, Munir, Le Nouen *et al.* 2008, Oshansky, Zhang *et al.* 2009, Munir, Hillyer *et al.* 2011)

- Inhibition of cytokine signalling

  The RSV G-protein inhibits IFN-β by inducing suppression of cytokine signalling (SOCS) 1 and SOCS 3 expression. SOCS proteins are negative regulators of type I IFN resulting in a decreased antiviral response within a cell (Yoshimura, Naka *et al.* 2007, Croker, Kiu *et al.* 2008)

- Molecular mimicking

  The RSV G-protein contains a CX3C chemokine motif which interacts with CX3 CRI blocking both the chemokine expression and CX3CRI mediated leukocyte chemotosis (Peters 1996, Tripp, Jones *et al.* 2001). RSV infected DC cells lose their ability to activate RSV-specific T cells and may in fact also inhibit T-cells activation by inadequate synapsing the T-cell receptor (González, Prado *et al.* 2008). The RSV G-protein is also able to prime CD_4^+ T-cells towards a Th2 response (Oshansky, Zhang *et al.* 2009). In the absence of IFN-ɣ, the RSV Fusion protein induces CD_4^+ T-cells to secrete IL-5, which acts as a modulator of pulmonary eosinophilia (Oshansky, Zhang *et al.* 2009).

1.5.2 Rhinovirus

Rhinovirus on the other hand has been shown to circumvent the host immune system by creating a large number of serotypes, which together with a high mutation rate, might be part of the success of RV infections (Kirchberger, Majdic *et al.* 2007). It has been shown that RV infections alter the expression of many of the genes associated with the host immune response (Proud, Turner *et al.* 2008). It has also been shown that through the engagement of ICAM-I on DC cells by Rhinovirus triggers inhibiting signals that disables the T-cell stimulating function of DC cells (Kirchberger, Majdic *et al.* 2005). Another mechanism involves the cytokine profile which is releases by monocytes upon rhinovirus infection, which is dominated by IL-10, a Th2 cytokine with immunosuppressive properties, resulting in decrease local immunecompetence, which leads to secondary infections commonly seen with rhinovirus infected patients. It also enables inhibition of antigen-specific T-cell responses, mediated by the down regulation of the MHC class II molecule expression (de Waal Malefyt, Haanen *et al.* 1991). The immunosuppressive and immune regulatory properties of IL-10 are commonly achieved by other viruses, such as the members of the herpes virus group, to evade the immune response, even having evolved their own IL-10 homologs (Alcami and Koszinowski 2000).

1.5.3 Host factors

The strength of a Th1 or Th2 response to viral infection depends on multiple factors, not least of which is the host immune response and the factors which influences it such as

- Age

The immaturity of infant's immune systems can result in a deficient Th1 response. It has been shown that RSV infection in neonates results in more severe disease, characterized by a Th2 cytokine response, whereas RSV infection later in life is characterized by less severe disease and an enhanced IFN-ɣ production (Culley, Pollott *et al.* 2002, Aberle, Aberle *et al.* 2004). However, infection in infants with other respiratory viruses like influenza virus, results in a strong IFN-ɣ response, indicating that the immune system of neonates and infants are capable of competent IFN-ɣ production to respiratory viral infection (Kurt-Jones, Popova *et al.* 2000, Aberle, Aberle *et al.* 2004). Thus, it appears that a diminished IFN response maybe unique to RSV infections and the mechanisms the virus has evolved to evade the immune system (Aberle, Aberle *et al.* 2004).

- Immune status – HIV

  The HIV glycoprotein gp120 molecule contains super antigen motifs, which interfere with IgE V_H3 regions bound to mast cells, basophiles and monocytes, resulting in the release of large amounts of Th2 cytokines, IL-4, IL-5, IL-6, IL-10 and IL-13, which skew the Th1/ Th2 cytokine profile. Thus in HIV positive patients, the immune system is already primed towards a Th2 response and further invading viruses such as RSV (Welliver, Wong *et al.* 1981, Karray and Zouali 1997, Becker 2006b)

2. Aim and objectives

This PhD formed part of a bigger active surveillance program for Severe Acute Respiratory Illness, which was initiated by the National Institute for Communicable Diseases in 2009. The PhD was designed to answer the specific questions listed in

the objectives. While the PhD candidate was involved in the writing of the protocols, the candidate had no input in the actual setting up of the sentinel sites, training of the surveillance officers and enrolment of the patients. The PhD candidate focused more on the virological aspects of the study in designing the assay used for diagnosis and the subsequent molecular epidemiology of RV and RSV.

2.1 Aim

The aim of this study was to determine the contribution of the major respiratory viruses to severe acute respiratory illness in South Africa both during and after the pandemic and compare molecular epidemiology and host innate immune responses as factors that may influence the role of the two most frequently detected viruses, respiratory syncytial virus (RSV) and rhinovirus as pathogens in a population with a high HIV sero-prevalence.

2.2 Secondary objectives

1. Develop a real-time multiplex reverse transcriptase PCR (rRT-PCR) assay that could detect ten major respiratory viruses (Influenza A and B, respiratory syncytial virus, enterovirus, human metapneumovirus, adenovirus, rhinovirus, parainfluenza1, 2 and 3).
2. Define the disease association of the ten viruses by comparing the incidence detected in ILI (mild), SARI (moderate to severe) and control patients as single and co-infections.
3. Define the molecular epidemiology of rhinovirus in patients hospitalised with Severe Acute Respiratory Illness and investigate the disease association of rhinovirus genotypes detected in ILI (mild), SARI (moderate to severe) and control patients.

4. Define the molecular epidemiology of RSV in order to determine the role of genetic variation as a possible mechanism to re-establish annual RSV epidemics and determine if disease severity can be attributed to RSV-A or B.
5. Determine if there is an association between deletions in G-protein of RSV and HIV infection, by screening HIV positive and negative patients with confirmed RSV infection and sequencing the full G-protein.
6. Characterize and compare the cytokine profiles in ILI (mild), SARI (moderate to severe) and control patients in response to single and co-infections with RV and RSV during one season where the circulating genotypes are defined.

Chapter 2

Development and application of a 10-plex real-time polymerase chain reaction assay to identify the common respiratory viruses in patients hospitalised with Severe Acute Respiratory Illness—South Africa, 2009-2010

2.1 Introduction

Viral infections have been shown to play a major role in acute respiratory infections in the developed world, but apart from a few papers on specific viruses such as influenza and RSV in selected regions, data remain limited from sub-Saharan Africa (Berkley, Munywoki *et al.* 2010, Gessner, Shindo *et al.* 2011, Venter, Lassauniere *et al.* 2011). In April 2009, Influenza A (H1N1) pdm09 emerged as a new pathogen. South Africa reported 12640 cases and 93 deaths during the first wave from June-October 2009, the most of any country in Africa (Archer, Cohen *et al.* 2009).

Respiratory viruses traditionally associated with acute respiratory tract infection include influenza (INF) A and B; respiratory syncytial virus ; parainfluenza virus (PIV) types 1, 2 and 3; adenovirus (AdV); enterovirus (EV); human metapneumovirus (hMPV) and rhinovirus (RV) (Brittain-Long, Nord *et al.* 2008, Tiveljung-Lindel, Rotzen-Ostlund *et al.* 2009). While a few studies have determined the frequency of respiratory viruses in patients with acute lower respiratory tract illness in Africa, (Joosting, Harwin *et al.* 1979, Kristensen, Thiel *et al.* 2004, Madhi, Ludewick *et al.* 2007, Smuts, Workman *et al.* 2011) these studies have mainly been limited to single sites and a limited number of viruses, and little has been reported on viral co-infections. Few data are available about the contribution of other respiratory viruses to respiratory tract infections during the pandemic or their role in (H1N1)pdm09 infections in Africa, and limited data is available from elsewhere (Renois, Talmud *et al.* 2010, Razanajatovo, Richard *et al.* 2011).

Comparative studies have shown that the detection of respiratory viruses using real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assays is substantially more sensitive than using conventional methods such as viral culture and immunofluorescence assays (IFA) (Hendrickson 2004, Paranhos-Baccala,

Komuruan-Pradel *et al.* 2008, Lassauniere, Kresfelder *et al.* 2010). Furthermore, compared to conventional PCR and other real-time methods, multiplex rRT-PCR has a significant advantage as it permits simultaneous amplification of several viruses in a single reaction(Paranhos-Baccala, Komuruan-Pradel *et al.* 2008, Lassauniere, Kresfelder *et al.* 2010, Venter, Lassauniere *et al.* 2011). This facilitates cost-effective diagnosis, enabling the detection of multiple viruses in a single clinical specimen.

As part of a severe acute respiratory illness (SARI) surveillance programme which commenced in February 2009in South Africa, we developed a two-step real-time multiplex reverse transcriptase PCR (rRT-PCR) assay that could detect ten different viruses (Influenza A and B, RSV, EV, hMPV, AdV, RV, PIV 1, 2 and 3) in order to investigate the role of the most common viral agents as aetiological agents in patients hospitalised with SARI in South Africa.

2.2 Materials and Methods

2.2.1 Setting

Specimens used in this study were obtained through routine surveillance for hospitalized SARI in six government hospitals around the country, including Chris Hani Baragwanath (2009-2010),an urban hospital in Gauteng province; Edendale (2009-2010), a semi-urban hospital in KwaZulu-Natal province; Matikwana and Mapulaneng (2009-2010), two rural hospitals in the Bushbuckridge district in Mpumalanga province; and Tshepong and Klerksdorp hospital complex (2010), semi-urban hospitals in the North-West province (Figure 1). Nasopharyngeal aspirates were collected from children <5 years old and nasopharyngeal as well as oral pharyngeal swabs were collected from patients >5 years old. Specimens were

sent to the Respiratory Virus Unit (RVU) at the National Institute for Communicable Diseases (NICD) in Johannesburg within 72 hours of collection for processing and storage at -70°C.

2.2.2 Case Definition

We defined a case of SARI according to a previously suggested WHO case definition (Ortiz, Sotomayor *et al.* 2009).

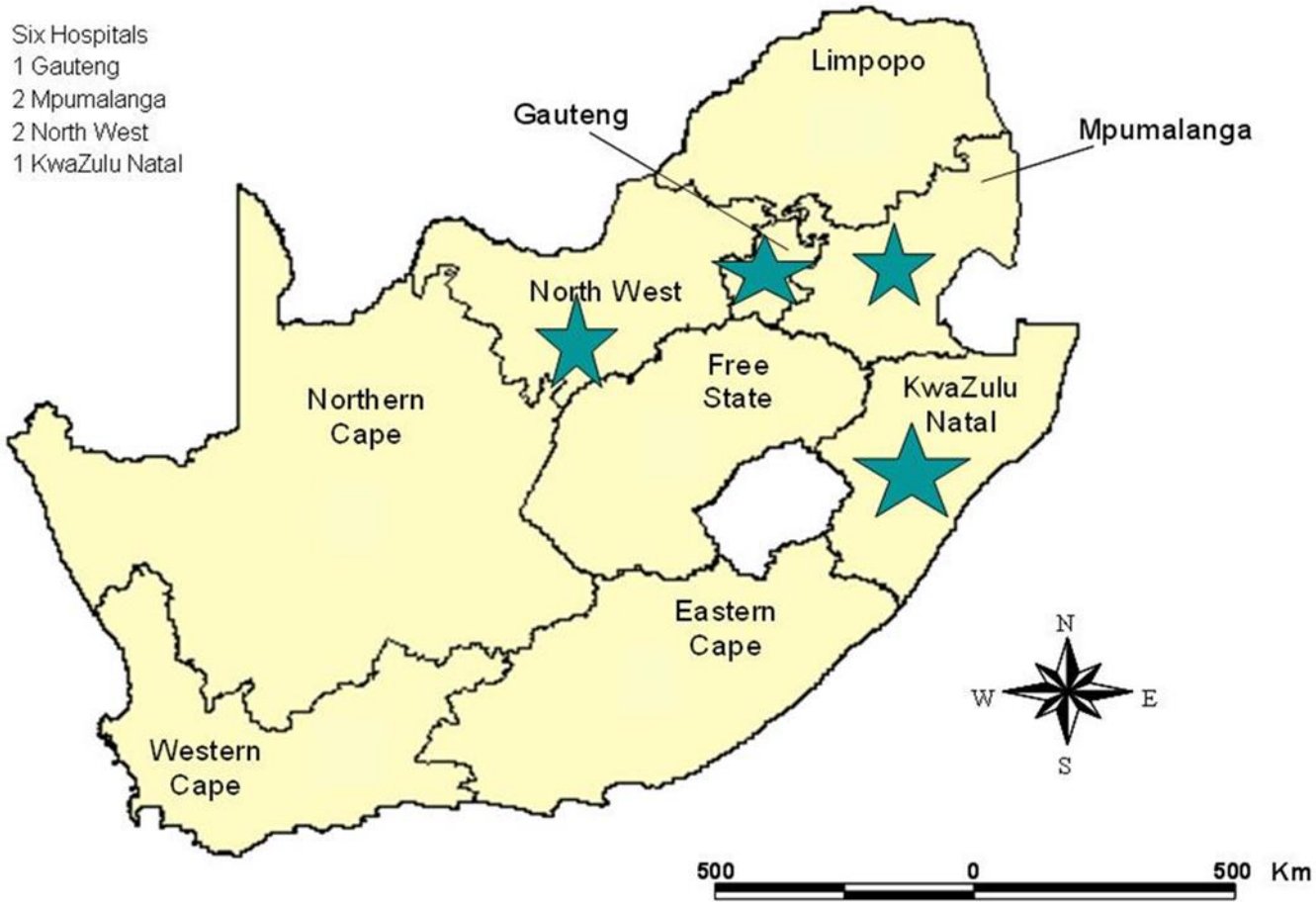


Figure 2.1: Geographical Map of South Africa indicating the locations of the SARI surveillance sites.

The methodology of this study has been previously by Cohen *et al.,* 2013 specfically by several others have also described the sampling methods (Cohen, Moyes *et al.* 2013, Moyes, Cohen *et al.* 2013, Cohen, Hellferscee *et al.* 2014, Cohen, Sahr *et al.* 2015, Cohen, Moyes *et al.* 2015, Cohen, Walaza *et al.* 2015a, Cohen, Walaza *et al.* 2015b, Groome, Moyes *et al.* 2015, Walaza, Tempia *et al.* 2015). For this study participants were enrolled at 3 public hospitals in 2 provinces of South Africa (Edendale Hospital in a peri-urban area of KwaZulu-Natal Province; and Klerksdorp and Tshepong Hospitals (the Klerksdorp-Tshepong Hospital Complex, KTHC) in a peri-urban area of North West Province). Patients were enrolled if they presented with symptom duration ≤7 days, provided written informed consent and met any of the following age-specific SARI case definitions: (i) children aged 2 days to <3 months with a diagnosis of suspected sepsis or physician-diagnosed lower respiratory tract infection irrespective of signs and symptoms; (ii) children aged 3 months to <5 years hospitalized with physician-diagnosed LRTI including bronchitis, bronchiolitis, pneumonia and pleural effusion; or (iii) individuals aged ≥5 years with sudden onset of fever (>38°C or history of fever) and cough or sore throat and shortness of breath or difficult breathing with or without clinical or radiographic findings of pneumonia. Surveillance officers administered a questionnaire with basic demographic and clinical information and examined medical records to collect data on admitting diagnoses. Specimens were collected on the day of admission.

All patients admitted during Monday through Friday were eligible, except for adult patients at CHBH, where enrolment was limited to 2 of every 5 working days (selected days varied systematically) per week because of large patient numbers and limited resources. The overall numbers of persons admissions, cases meeting study definitions, and persons enrolled were recorded. Study staff completed case

report forms until discharge and collected nasopharyngeal and throat swabs from patients >5 years of age or nasopharyngeal aspirates from patients <5 years of age and blood specimens from consenting patients. Hospital and intensive care unit admission and collection of specimens for bacterial culture, tuberculosis testing, and CD4+ T-cell counts were performed according to attending physician discretion.

2.2.3 Validation and Optimization of Real-Time rRT-PCR Multiplex

A real-time multiplex PCR assay detecting ten different viruses (Influenza A and B, RSV, EV, hMPV, AdV, RV, PIV 1, 2 and 3) was established as two-step rRT-PCR with 5 separate reactions (Table 1).The assay was validated using conserved regions of the target viruses, to minimize the effect of genetic changes within each of the viruses. External quality control panels which included isolates of PIV 1, 2, 3 and 4; RSVA and B; EV; hMPV I and II; AdV; RV; and INF A and B viruses and specimens for the bacterial species *Chlamydophila pneumoniae, Legionella pneumophila* and *Mycoplasma pneumoniae* from Quality Control for Molecular Diagnostics (QCMD, Glasgow, Scotland) were used to optimize and validate the multiplex assays. Optimal primer annealing temperatures and primer and probe concentrations were calculated by experimentation. The QCMD panels were used to test all primers and probes for possible competitive interactions. The cross reactivity of the assay was assessed in triplicate, to ensure repeatability, reproducibility, sensitivity and specificity. TaqMan technology was selected for the multiplex rRT-PCR assay to ensure adaptability to different real-time platforms.

2.2.4 Nucleic acid extraction

The MagNA Pure LC Total Nucleic acid Kit (Roche Diagnostics, Mannheim Germany) was used according to manufacturer's instructions using200µl sample and

a final elution volume of 50µl; excess extracted nucleic acids were stored at – 70°C. A negative and positive biological control was used in each extraction.

2.2.5 Primer and Probe multiplexing

Primers and probes for 10 respiratory viruses (PIV 1, 2 and 3; RSV; EV; hMPV; AdV; RV and INF A and B viruses) were identified for the qualitative studies. All primers and probes (Table 1) were optimized in different combinations for this assay. DNAMAN was used (Lynnon Corporation, Québec, Canada) to ensure primer complementary and primer dimers did not exist between different PCR groups and to select primer set candidates per multiplex. We used the influenza A primers recommended by the WHO Collaborating Center for Influenza, Centers for Disease Control and Prevention (CDC), USA for the universal detection of Influenza A strains, which were updated to include the(H1N1)pdm09, we also included the internal positive control Human sapiens ribonuclease RNP as recommended by the CDC to ensure the validity of the sample (The WHO Collaborating Centre for influenza at CDC Atlanta 2009).

2.2.6 Real-Time RT-PCR

cDNA was synthesized using the Transcriptor 1stStrand cDNA Kit (Roche Diagnostics, Mannheim Germany), according to manufacturer's instructions. Qualitative real-time polymerase chain reactions (PCR) using the LightCycler® 480 Probes Master kit (Roche Diagnostics, Mannheim Germany) and the LightCycler® 480 System (Roche Diagnostics, Mannheim Germany) were performed. Each real-time PCR reaction contained 15µl of 2X Master Mix, 1 µM of each primer and 0.5 µM of each probe and 10µl of cDNA reaction mixture as template for a final volume of 30µl. PCR cycles was initiated at 95°C for 15 minutes to activate *Taq* DNA

polymerase enzyme, followed by 45 cycles of 94°C for 15 seconds, 60°C for 20 seconds and 72°C for 10 seconds. Specimens were considered positive when the Ct value was equal or above the Ct value of the Lower limit of detection of the corresponding virus, which ranged between Ct=36 to Ct=40. The influenza positive specimens were subtyped using the CDC Real-time RTPCR (rRTPCR) Protocol for Detection and Characterization of Influenza, which was distributed to National Influenza Centres under a Material Transfer Agreement (The WHO Collaborating Centre for influenza at CDC Atlanta 2009)

Table 2.1: Primers and probes used in multiplex rRT-PCR run in five PCR mixtures as indicated.

Target	Gene	Oligonucleotide sequence (5'- 3')		REPORTER	PCR GROUP	Published
Parainfluenza PIV 1	HN gene	GTT GTC AAT GTC TTA ATT CGT ATC AAT AAT T	Forward			(Watzinger 2004)
		GTA GCC TMC CTT CGG CAC CTA A	Reverse			
		TAG GCC AAA GAT TGT TGT CGA GAC TAT TCC AA	Probe	Cy 5-BBQ		
PIV 2	HN gene	GCA TTT CCA ATC TTC AGG ACT ATG A	Forward		A§	(Watzinger 2004)
		ACC TCC TGG TAT AGC AGT GAC TGA AC	Reverse			
		CCA TTT ACC TAA GTG ATG GAA TCA ATC GCA AA	Probe	FAM-BHQ1		
PIV 3	HN gene	AGT CAT GTT CTC TAG CAC TCC TAA ATA CA	Forward			(Watzinger 2004)
		ATT GAG CCA TCA TAA TTG ACA ATA TCA A	Reverse			
		AAC TCC CAA AGT TGA TGA AAG ATC AGA TTA TGC A	Probe	Red 610-BHQ2		
Respiratory Syncytial virus (RSV)	Matrix protein	GCA AAT ATG GAA ACA TAC GTG AAC A	Forward			(Brittain-Long, Nord *et al.* 2008)
		GCA CCC ATA TTG TWA GTG ATG CA	Reverse			
		CTT CAC GAA GGC TCC ACA TAC ACA GCW G	Probe	FAM-BHQ1	B¥	
Influenza B (INF B)	HA	AAA TAC GGT GGA TTA AAT AAA AGC AA	Forward			(van Elden 2001)
		CCA GCA ATA GCT CCG AAG AAA	Reverse			
		CAC CCA TAT TGG GCA ATT TCC TAT GGC	Probe	Red 610-BHQ2		
Enteroviruses (EV)	5' UTR	TCC TCC GGC CCC TGA	Forward			(Nijhuis 2002)
		RAT TGT CAC CAT AAG CAG CCA	Reverse			
		CGG AAC CGA CTA CTT TGG GTG WCC GT	Probe	Cy 5-BBQ	C¥	
human Meta pneumovirus (hMPV)	N protein	GAA GAR ATA GAC AAA GAR GCA AG	Forward			(Tiveljung-Lindel, Rotzen-Ostlund *et al.* 2009)
		TCC CAC TTC TAT KGT TGA TGC TAG	Reverse			
		TCA GCA CCA GAC ACA CC	Probe	Red 610-LNA		
Adenoviruses (AdV)	Hexon	GCC ACG GTG GGG TTT CTA AAC TT	Forward			(Heim, Ebnet *et al.* 2003)
		GCC CCA GTG GTC TTA CAT GCA CAT C	Reverse			
		TGC ACC AGA CCC GGG CTC AGG TAC TCC GA	Probe	Red 610-BHQ2	D¥	
Influenza A (INF A)	M1	GAC CRA TCC TGT CAC CTC TGA C	Forward			*(The WHO Collaborating Centre for influenza at CDC Atlanta 2009)
		AGG GCA TTY TGG ACA AAK CGT CTA	Reverse			
		TGC AGT CCT CGC TCA CTG GGC ACG	Probe	FAM-BHQ1		
Rhinoviruses (RV)	5 ' UTR	GGT GTG AAG AGC CSC RTG TGC T	Forward			(Brittain-Long, Nord *et al.* 2008)
		GGT GTG AAG ACT CGC ATG TGC T	Reverse			
		GGG TGY GAA GAG YCT ANT GTG CT	Probe			
Human sapiens ribonuclease RNP (IQC)	30kDa subunit (RPP30)	GGA CAC CCA AAG TAG TYG GTY C	Forward		E¥	*(The WHO Collaborating Centre for influenza at CDC Atlanta 2009)
		CCG GCC CTG AAT GYG GCT AAY C	Reverse	FAM-BHQ1		
		AGA TTT GGA CCT GCG AGC G	Probe			
		GAG CGG CTG TCT CCA CAA GT	Forward			
		TTC TGA CCT GAA GGC TCT GCG CG	Reverse	Cyan 500-BHQ1		

* Primers and Probes were obtained from the CDC co-operative agreement after the first case of Pandemic H1N1 was detected. ¥Multiplex was combined from pre-existing published primer and probe sets. ¥Existing published multiplex

2.2.7 Statistical Analysis

We analysed the positive cases and seasonal patterns of the respiratory viruses included in the multiplex. Results were analysed according to virus proportion per month and subject age-group. The Chi-square test and Fischer's exact test were used for univariate analysis to compare the results of the different age groups to determine which viruses were most frequently detected in which age group and if compared to the 0-1 age group if the difference was significant, P-values <0.05 were considered to be statistically significant. Analysis was performed using STATA 11, (Stata Corporation, Texas USA). Data for Kappa and Bland-Altman analysis were analysed using Analyse-it® Method Evaluation Edition add-in software for Microsoft Excel 2007(Analyse-it Software Ltd., Leeds, UK). Stefano Tempia has assisted the student in running these statistical tests and assisted the student in the interpretation.

2.3 Results

2.3.1 Validation and Optimization of the Real-Time RT-PCR (rRT-PCR).

Using the external quality control panels provided by QCMD as the gold standard, the multiplex rRT-PCR assay had a high overall accuracy (98%) (*i.e.* the degree of closeness of measured or calculated quantity to its actual (true) value), negative predicative value (97%), positive predicative value (100%), sensitivity (91%) and specificity (100%)(Standards, Unit *et al.* 2008).The rRT-PCR assay was compared to Immunofluorescence assays (IFA) and the rRT-PCR assay was more sensitive in all cases (data not shown). Use of QCMD panels established reproducibility, repeatability, as well as lower detection limits ranging from Ct values of 36 to 40 depending on the different viruses. The coefficient of variation calculated from the

QCMD panels for the 5 reactions ranged from 0.2% to 0.7%. (Please see full validation report in Appendix A)

2.3.2 Study group demographics

From February 2009 up to December 2010, we collected and tested specimens from 8173 SARI patients. The median age of patients was 3 years (range 0-99 years).Half of patients (3974 (51.1%) were male. The largest age group was from children <1 year of age (3157 (38.6%), and 6098 (74.7%) were from patients admitted to Chris Hani Baragwanath Hospital in Soweto.

2.3.3 Application of the rRT-PCR for screening of surveillance specimens from patients with SARI

Of the 8173 patients tested with the rRT-PCR, 3240 (39.6%) had single infections, 1426 (17.4%) had co-infections with two or more viruses, and 3507 (42.9%) were negative for pathogens included in this assay. The most common respiratory viruses identified were RV (2034, 24.9%), RSV (1169, 14.3%), AdV (1083, 13.3%), and influenza (704, 8.7%; Table 2).Among the 3240 patients with single infections (Table 2.2, diagonal), RV was the most frequently detected virus, occurring in 1171 (36.1%) followed by RSV in 591 (18.2%) and AdV in 364 (11.2%). No difference was observed in the proportion of these viruses detected at each of the surveillance sites (data not shown).

The percent of specimens positive for influenza was similar in both years (393, 10.7% in 2009 vs. 324, 7.2% in 2010), but there were differences in influenza types and subtypes. In 2009 mainly Influenza A was detected (354, 9.7%), which comprised mostly of H3N2 (194, 5.3%) and (H1N1)pdm09 (160, 4.4%); Influenza B

Table 2.2: Distribution of Respiratory Virus infections and specimens received per age group, SARI surveillance, South Africa, 2009 and 2010

Age Groups		0-1	2-4	5-24	25-44	45-64	65+	Unknown
Specimens Received (n=)		3157	992	732	2086	961	227	18
RV	Total	985	399	186	318	119	20	7
	%	31.2%	40.2%	25.4%	15.2%	12.4%	8.8%	38.9%
	OR	-	1.48	0.76	0.39	0.31	0.21	
	p-values	-	0.001	0.003	0.001	0.001	0.001	
RSV	Total	845	189	33	73	26	2	1
	%	26.8%	19.1%	4.5%	3.5%	2.7%	0.9%	5.6%
	OR	-	0.64	0.13	0.09	0.07	0.02	
	p-values	-	0.001	0.001	0.001	0.001	0.001	
AdV	Total	513	315	88	110	47	6	4
	%	16.2%	31.8%	12.0%	5.3%	4.9%	2.6%	22.2%
	OR	-	2.41	0.71	0.28	0.26	0.14	
	p-values	-	0.001	0.001	0.001	0.001	0.001	
EV	Total	318	133	28	23	7	5	1
	%	10.1%	13.4%	3.8%	1.1%	0.7%	2.2%	5.6%
	OR	-	1.38	0.36	0.99	0.67	0.20	
	p-values	-	0.003	0.001	0.001	0.001	0.001	
PIV3	Total	214	44	17	59	15	5	0
	%	7%	4%	2%	3%	2%	2%	0%
	OR	-	0.64	0.33	0.40	0.22	0.31	
	p-values	-	0.008	0.001	0.001	0.001	0.010	
hMPV	Total	188	52	13	36	12	1	1
	%	6.0%	5.2%	1.8%	1.7%	1.2%	0.4%	5.6%
	OR	-	0.87	0.29	0.29	0.19	0.06	
	p-values	-	0.401	0.001	0.001	0.001	0.008	
H3N2	Total	112	60	28	48	19	7	2
	%	3.5%	6.0%	3.8%	2.3%	2.0%	3.1%	11.1%
	OR	-	1.75	1.08	0.64	0.54	0.86	
	p-values	-	0.001	0.717	0.011	0.017	0.714	
(H1N1)pdm09	Total	72	28	37	38	22	7	0
	%	2.3%	2.8%	5.1%	1.8%	2.3%	3.1%	0.0%
	OR	-	1.24	2.28	0.79	1.00	1.36	
	p-values	-	0.333	0.000	0.257	0.987	0.441	
INF B	Total	58	28	22	80	28	7	1
	%	1.8%	2.8%	3.0%	3.8%	2.9%	3.1%	5.6%
	OR	-	1.61	1.72	2.09	1.57	1.71	
	p-values	-	0.040	0.034	0.000	0.049	0.188	
PIV1	Total	47	30	4	4	3	1	1
	%	1.5%	3.0%	0.5%	0.2%	0.3%	0.4%	5.6%
	OR	-	2.06	0.37	0.13	0.21	0.29	
	p-values	-	0.002	0.055	0.000	0.008	0.225	
PIV2	Total	40	16	8	16	2	1	1
	%	1.3%	1.6%	1.1%	0.8%	0.2%	0.4%	5.6%
	OR	-	1.28	0.87	0.60	0.16	0.34	
	p-values	-	0.412	0.719	0.088	0.120	0.294	

The Chi-square test and Fischer's exact test were used for univariate analysis and Odds ratio calculated for each of the age group. Analysis was performed using STATA 11, (Stata Corporation, Texas USA). P-values <0.05 were considered to be statistically significant.

was much less common (25, 0.7%). However, in 2010 mainly influenza B viruses were detected (198, 4.4%), followed by Influenza A (126, 2.8%), comprised of H3N2 (82, 1.8%) and (H1N1)pdm09 (44, 0.9%). During 2009 a total of 14/494 (2.8%) Influenza A specimens could not be subtyped due to too low concentrations.

In the univariate analysis patients infected with (H1N1)pdm09 (OR=2.28, p=0.001), were more likely comprised of H3N2 (82, 1.8%) and (H1N1)pdm09 (44, 0.9%). During 2009 a total of 14/494 between 5-24 years, while patients infected with H3N2 (OR=1.75, p=0.001), were between 2-4 year old, however the patients infected with Influenza B (OR=2.09, p=0.001) were more likely between 25-44 years (Table 2.2). In addition no difference was observed between the two years with regards to the distribution and proportion of each of the other respiratory viruses.

The highest overall virus detection rate was in the 2-4 year old age group, where 833 (83.9%) of 992 specimens were positive for at least one virus, and lowest in persons ≥65 years old, where 55 (24.2%) of 227 specimens were positive for at least one virus. Compared to 2-4 year olds, the positivity rate for other age groups was significantly less [0-1 years (76.5% positive), 5-24 years (51.0% positive), 25-44 years (33.8% positive), and 45-64 years (28.7% positive; p<0.001)] for each compared with 2-4 year olds.In the 0-1 year old age group the most common virus was RV (985/3157 (31.2%)) followed by RSV, (845/3157 (26.7%)). In contrast, in other age groups, the more common pathogens were RV, and AdV (Table 2.2).

2.3.4 Respiratory viral co-infections

Among the 1426 patients with co-infections (Table 2.3, matrix), RV was detected most frequently [860 (60.3%)] followed by AdV in 719 (50.4%) and RSV in 578 (40.5%). Of the 51 patients with (H1N1)pdm09 co-infections, RV was detected in 19

(37.3%), followed by PIV-3 in 10 (19.6%). There were no co-infections with Influenza A (H1N1)pdm09 and H3N2viruses. However, there was 1 co-infection with Influenza B and H3N2 (Table 2.3). Of the 94 patients with Influenza A H3N2 co-infections, RV was detected in 38 (40.4%) followed by RSV in 19(20.2%) and PIV 3 in 6(6.4%).

Of the 1426 co-infections, the following combinations of viruses were detected most frequently in the same specimen: 356 cases of RV and AdV, 320 cases of RV and RSV, 212 cases of RSV and AdV and 142 cases of AdV and EV (Table 3, matrix).

2.3.5 Seasonality

Seasonal patterns were visible for RSV, Influenza A and B, EV, hMPV and PIV3 in both 2009 and 2010. RSV occurred from February to June before the influenza season, which typically falls between May to September(McAnerney, Cohen *et al.* 2011). EV was detected throughout the year with peak activity between February and April and again between November and December. Peak activity for hMPV was observed between July and August and for PIV3 between September and November. Adenovirus and RV were detected throughout the two years without seasonal variability. PIV 1 and PIV 2 were detected sporadically throughout the two years (Figure 2.2).

The seasonal patterns of both Influenza A and B viruses during 2009 and 2010 were different. In 2009, Influenza A H3N2 and (H1N1)pdm09occurred as two waves peaking between May to July and July to October, respectively, while Influenza B appeared briefly in August. During 2010 Influenza B season predominated from June to November, H3N2 circulated from June to September and (H1N1)pdm09 circulated between the last week of July and October.

Table 2.3: Contribution of respiratory viruses to SARI in hospitalized patients in South Africa as single (diagonal) or co-infection (matrix)

Total specimens received n=8173 Total Single infection n=3240(39.6%), Total co-infections n=1426 (17.4%)											
Total viruses detected	RV n=2034 (25%)	RSV n=1169 (14%)	AdV n=1083 (13%)	EV n=515 (6%)	PIV3 n=354 (4%)	hMPV n=303 (4%)	H3N2 n=276 (3%)	INF B n=223 (3%)	H1N1)pdm09 n=204 (3%)	PIV1 n=90 (1%)	PIV2 n=84 (1%)
RV	1171										
RSV	320	591									
AdV	356	212	364								
EV	0	107	142	219							
PIV3	87	20	31	27	198						
hMPV	73	18	52	9	14	155					
H3N2	38	19	53	12	6	3	182				
INFB	32	10	38	6	6	5	1	144			
(H1N1)pdm09	19	3	6	3	10	7	0	0	153		
PIV1	28	21	23	10	4	3	0	2	0	34	
PIV2	28	16	18	12	5	2	3	3	1	2	23

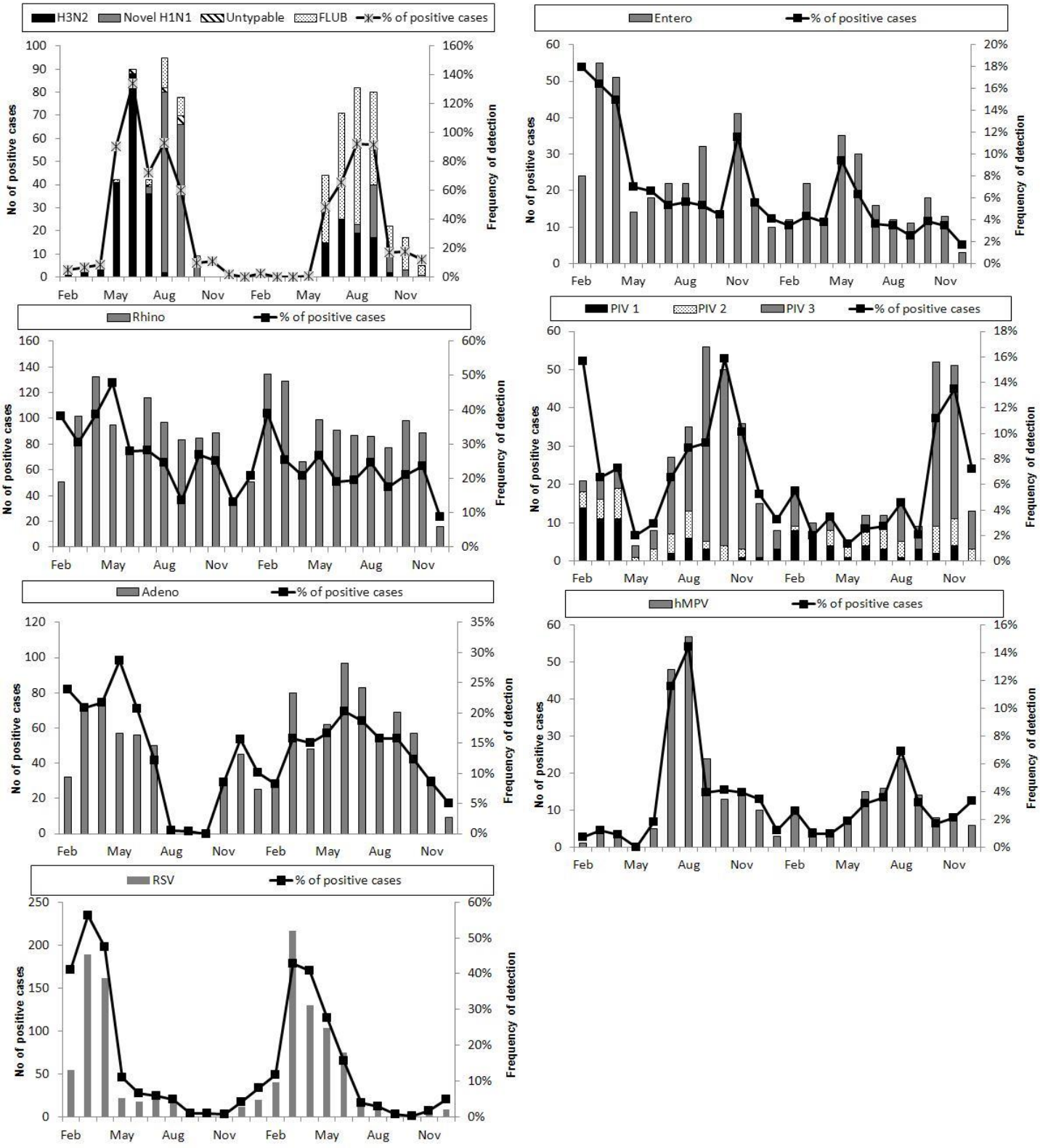


Figure 2.2: Distribution of Respiratory Viruses detected during 2009 and 2010, showing specific seasonal trends and peak activity. (Please note that the scales on the y-axis differ for each graph.)

2.4 Discussion

The validated assay was implemented for routine surveillance prior to the pandemic and enabled us to investigate the contribution of other respiratory viruses to SARI during the first two pandemic seasons. The assay also helped define the distribution and seasonality of these respiratory viruses in South Africa and the role of viral co-infections in hospitalized patients infected with (H1N1)pdm09 in South Africa during the 2009 and 2010 seasons. Using the validated rRT-PCR multiplex assay, viral agents were detected in 57% of cases identified through South Africa's SARI surveillance network, which is consistent with other studies using rRT-PCR multiplex assays for the detection of respiratory viruses (Berkley, Munywoki *et al.* 2010, Lassauniere, Kresfelder *et al.* 2010, Venter, Lassauniere *et al.* 2011). Validation of the rRT-PCR multiplex assay suggested that it is as specific but more sensitive than IFA. The assay does not give any false positive results while the lower detection limit determined may give false negatives with specimens with very low viral load.

The majority of patients enrolled were infants less than 1 year of age, and the most commonly identified pathogens within this group were RSV and RV. While RV was detected throughout the year, RSV was detected in a distinct seasonal pattern with the peak months of detection occurring from February to June. Seasonal peaks were also identified for PIV3, hMPV and enterovirus. Year-round detection of Rhinovirus and Adenovirus make these two viruses the more likely to co-infect with all other viruses. The seasonality of these viruses were assed again after a five year period by Cohen *et al.,* 2015 and there were no significant changes to the seasonality of the viruses with the exception of Influenza A where the distribution of the subtypes changed (Cohen, Walaza *et al.* 2015a).

Although rhinovirus was the most commonly identified pathogen in this study, further studies are needed to determine how much rhinovirus contributes to disease severity (Lau, Yip *et al.* 2007, Smuts, Workman *et al.* 2011, Venter, Lassauniere *et al.* 2011). In a study conducted two years prior to the 2009 pandemic in hospitals situated in South Africa, RSV was detected in a much higher rate in symptomatic infants with severe disease than in asymptomatic infants attending an immunization clinic in the same region (Lassauniere, Kresfelder *et al.* 2010). Nevertheless, in a study conducted during 2006 and 2007 in South Africa, RV was present in 18% of asymptomatic children and in >30% of children hospitalized with SARI, which suggests a possible role in disease severity (Venter, Lassauniere *et al.* 2011).

Although there are growing concerns for the potential of (H1N1)pdm09 reassorting with existing human influenza viruses giving rise to a highly transmissible or pathogenic virus (Liu, Li *et al.* 2010), no mixed infections were detected with either subtypes in patients with SARI in this study. The Influenza subtypes had co-circulated for overlapping periods both in 2009 and 2010, but peak months of detection was distinct for H3N2 and (H1N1)pdm09 in both years, while Influenza B was detected from June to November, overlapping with both H3N2 and pandemic H1N1 peaks. No seasonal H1N1 was observed during the 2009 and 2010 influenza season. In the present study we found pandemic cases mostly in older children and young adults, which is similar to surveillance reports of the 2009 pandemic in other parts of the world, which have shown that up to 57% of cases occurred among people between 5–24 years of agewith a detection rate of 5.1% (CDC 2009).

Our study has some limitations. First, rRT-PCR assays are more sensitive for detecting respiratory viruses compared to viral culture, and with the increased detection of mixed viral infections, the clinical interpretation of positive PCR results

have become more challenging. Although the viral nucleic acids detected here does not necessarily indicate the presence of viable virus, several studies have documented few persistent or recurrent PCR-positive respiratory specimens in patients after acute illness has resolved, suggesting a likely association with the diseased state (Falsey, Formica *et al.* 2003, Winther, Hayden *et al.* 2006). The relevance of the high frequency detected of respiratory viruses such as Rhinovirus in single and co-infections requires further investigation. Because of the low numbers of each specific co-infection combination, we did not report on clinical outcomes and how single and co-infections differed from each other clinically. This study also did not include bacterial testing and therefore gaps remain in our understanding of all the aetiologies of SARI in South Africa. Lastly, the study period of 2 years could also be a limitation since the circulation of viruses could change from year to year, however only two years of data is represented here, the surveillance study is on-going and changes in the seasonal circulation of the viruses will be detected. Understanding the contribution these viruses to severe respiratory disease will allow for informed decision making when selecting specific respiratory pathogens as part of sustainable respiratory disease surveillance. Currently the overall cost of the assay from extraction to detection is USD 63, with a panel (consisting of two to three viruses) costing USD 12 however, by selecting only the major contributors to SARI, the cost of running the assay could be reduced in future.

In conclusion this study indicates a contributing role for co-infecting viruses in patients presenting with SARI and highlights the important role of viral co-infection. Continued use of the rRT-PCR multiplex assay in conjunction with the SARI surveillance programme will enhance our ability to detect circulation of respiratory

viruses in patients hospitalised for SARI and help to clarify the contribution of these respiratory viruses among patients with SARI in South Africa.

Chapter 3

Investigation of the disease association of respiratory virus infection among patients with severe acute respiratory illness and influenza-like illness in South Africa, 2012-2015

3.1 Introduction

Pneumonia is a leading cause of childhood mortality globally, with about 1.6 million new cases per year, of which 1.2 million occur in the developing world (Rudan, Boschi-Pinto *et al.* 2008, Black, Cousens *et al.* 2010). Of these cases approximately 10% are severe enough to require hospitalization (Rudan, Boschi-Pinto *et al.* 2008). Before the worldwide availability of vaccines, *Streptococcus pneumoniae* (pneumococcus) and *Haemophilus influenzae* type b were identified as the main bacterial causes of pneumonia (Shann 1986, Rudan, Boschi-Pinto *et al.* 2008). Now, viruses are proportionally a much more common cause of pneumonia (Rudan, Boschi-Pinto *et al.* 2008).

Respiratory viruses infections have been detected through the use of polymerase chain reaction (PCR) among patients hospitalized with lower respiratory tract infection (LTRI) in several studies (Weber, Mulholland *et al.* 1998, Madhi, Venter *et al.* 2003, Berkley, Munywoki *et al.* 2010, Lassauniere, Kresfelder *et al.* 2010, Venter, Lassauniere *et al.* 2011, Hammitt, Kazungu *et al.* 2012, Pretorius, Madhi *et al.* 2012). While the use of sensitive PCR methods has significantly expanded the ability of laboratories to detect and identify pathogens, the clinical association between pathogen detection and disease remains difficult to interpret when considering viral shedding, replication and persistence of nucleic acids present during the pre- or post-syndromic phase of infection in the absence of comparison groups (Berkley, Munywoki *et al.* 2010, Hammitt, Kazungu *et al.* 2012, Pretorius, Tempia *et al.* 2014). Without comparing to control groups, the clinical relevance of identifying some respiratory pathogens by PCR testing remains difficult to determine (Weber, Kochs *et al.* 2004, Fry, Lu *et al.* 2011, Hammitt, Kazungu *et al.* 2012, Levine, O'Brien *et al.* 2012, Pretorius, Tempia *et al.* 2014).

Understanding the contribution of respiratory viruses to illness would allow the prioritization of respiratory pathogens for inclusion in diagnostic tests, disease surveillance, vaccine development and treatment. Using control subjects, we estimated the attributable fraction of 10 common respiratory viruses among patients hospitalized with severe acute respiratory illness (SARI) and outpatients with influenza-like illness (ILI).

3.2 Materials and Methods

3.2.1 Study design and population

SARI Surveillance: Study samples were obtained from participants enrolled in a prospective hospital-based surveillance program for SARI initiated in February 2009. The methodology of this study has been previously described (section 2.2.2) (Cohen, Moyes *et al.* 2013). For this study participants were enrolled at 3 public hospitals in 2 provinces of South Africa (Edendale Hospital in a peri-urban area of KwaZulu-Natal Province; and Klerksdorp and Tshepong Hospitals (the Klerksdorp-Tshepong Hospital Complex, KTHC) in a peri-urban area of North West Province) from May 2012 through April 2015. Patients were enrolled if they presented with symptom duration ≤7 days, provided written informed consent and met any of the following age-specific SARI case definitions: (i) children aged 2 days to <3 months with a diagnosis of suspected sepsis or physician-diagnosed lower respiratory tract infection irrespective of signs and symptoms; (ii) children aged 3 months to <5 years hospitalized with physician-diagnosed LRTI including bronchitis, bronchiolitis, pneumonia and pleural effusion; or (iii) individuals aged ≥5 years with sudden onset

of fever (>38°C or history of fever) and cough or sore throat and shortness of breath or difficult breathing with or without clinical or radiographic findings of pneumonia.

ILI and Control Surveillance: Study samples were obtained from participants enrolled in an active surveillance program for ILI and controls initiated in May 2012 and running through April 2015. Patients presenting with ILI and controls were enrolled at two outpatient clinics in the same catchment area to the above mentioned hospitals: Edendale Gateway Clinic, KwaZulu-Natal Province, and Jouberton Clinic, North West Province. An ILI case was defined as an outpatient of any age presenting with either temperature >38°C or history of fever, and cough of duration of ≤7 days. ILI cases that were referred for hospitalization subsequent to the visit were not eligible for enrolment.

A control was defined as an individual presenting at the same outpatient clinic with no history of fever, respiratory or gastrointestinal symptoms during the 14 days preceding the visit. The patients commonly presented to the clinic for visits such as dental procedures, family planning, well baby visits, voluntary HIV counselling and testing or acute care for non-febrile illnesses. We aimed to enrol one HIV-infected and one HIV-uninfected control every week in each clinic within each of the following age categories: 0-1, 2-4, 5-14, 15-54 and ≥55 years.

A standardized questionnaire was used to collect demographic and clinical information from enrolled SARI and ILI cases and controls. In addition, for SARI cases hospital records were reviewed to assess disease progression and outcome (i.e., discharge, transfer or in-hospital death). Medical and symptom history was systematically verified by a trained nurse using a structured checklist. This information was obtained through medical chart review and interview with the patient or legal guardian for children less than 15 years of age.

3.2.2 Respiratory Virus detection

Respiratory specimens (i.e., nasopharyngeal aspirates for children <5 years of age and nasopharyngeal and oropharyngeal swabs from individuals ≥5 years of age) were collected from all enrolled patients (SARI, ILI and Controls), placed in viral transport medium, stored at 4-8°C and transported to the National Institute for Communicable Diseases within 72 hours of collection for testing. All specimens collected over the study periods were tested for the presence of 10 respiratory viruses (influenza A and B viruses, parainfluenza virus (PIV) types 1, 2 and 3, RSV, adenovirus; rhinovirus; human metapneumovirus (hMPV), and enterovirus) using a real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay as previously described (section 2.2.6). Among consenting study patients, HIV status was established by enzyme-linked immunosorbent assay (ELISA) or PCR assay depending on the patients' age by using EDTA whole blood (Cohen, Moyes *et al.* 2013).

3.2.3 Statistical Analysis

We implemented a multivariable multinomial regression model to determine the association between specific respiratory viruses among patients with SARI or ILI compared to controls enrolled from May 2012 through April 2015. Multinomial regression allows modelling of outcome variables with more than 2 categories and relates the probability of being in category *j* to the probability of being in a baseline or reference category. A complete set of coefficients are estimated for each of the *j* levels (patients with ILI or SARI in this analysis) that are compared with the baseline category (controls for this analysis) and the effect of each predictor in the model is measured as relative risk ratio (RRR). The association of the 10 viruses with mild

(ILI) or severe (SARI) illness was assessed simultaneously irrespective of whether it was a single or co-infection using a multivariable model to adjust for the potential effect of co-infections.. In addition, all estimates were adjusted for age (<1, 1-4, 5-24, 25-44, 45-64 and ≥65 years of age), HIV serostatus and underlying medical conditions other than HIV.

In addition, we implemented an age-stratified analysis among individuals aged <5 and ≥5 years of age to evaluate potential differences in disease association in young children and older individuals. For both analyses we also adjusted the effect of the viral covariates by age within each age strata (<1 and 1-4 years of age for children aged <5 years and 5-24, 25-44, 45-64 and ≥65 years of age for persons aged ≥5 years), HIV serostatus and underlying medical conditions other than HIV.

Subsequently we estimated the attributable fraction (AF) from the relative risk (RR) obtained from the multinomial model for each virus using the following formula: $AF=(RR-1)/RR*100$. Lastly, we adjusted the observed detection rate ($Prev_{Obs}=n/N$) for each virus among ILI or SARI cases by the corresponding AF to obtain the prevalence of each virus attributable to mild (ILI) or severe (SARI) illness (adjusted prevalence, $Prev_{Illness}$) using the following formula: $Prev_{Illness}=Prev_{Obs}*AF/100$. The analysis was performed using STATA 13.1 (StataCorp®, Texas, and USA). Stefano Tempia has assisted the student in running these statistical tests and assisted the student in the interpretation.

3.3 Results

3.3.1 Characteristics of the Study Population and Detection of Respiratory Viruses

Over the study period, we enrolled 1959 SARI cases, 3784 ILI cases and 1793 controls. Children <5 years of age accounted for 73% (1431/1953); 28% (1075/3783) and 37% (658/1135) of SARI cases, ILI cases and controls, respectively. The HIV serostatus was known for 79% (1550/1959) of SARI cases, 87% (3280/3784) of ILI cases, and 92% (1643/1793) of controls. Among individuals with known HIV serostatus, the HIV prevalence was 26% (410/1550) among SARI cases, 30% (974/3280) among ILI cases, and 43% (702/1643; reflecting the enrolment criteria) among controls. Among SARI and ILI cases the HIV prevalence was lowest among infants <1 year of age [SARI: 10% (75/740), ILI: 2% (3/304)] and highest among individuals 25-44 years of age [SARI: 89% (174/196), ILI: 59% (608/1035)].

A virus was identified in 70% (1381/1959) of SARI cases, 59% (2230/3784) of ILI cases and 36% (645/1793) of controls. Among SARI cases the most commonly detected viruses were rhinovirus (34%; 667/1959), RSV (20%; 391/1959) and adenovirus (29%; 379/1959). Rhinovirus (28%; 1064/3784), influenza virus (15%; 577/3784) and adenovirus (12%; 434/3784) predominated among the ILI cases. Rhinovirus (21%; 374/1793) and adenovirus (12%; 207/1793) were the most prevalent among controls (Table 3.1).

3.3.2 Attributable fraction of respiratory virus infection to mild or severe illness

In the main unstratified analysis using multivariable multinomial regression, all viruses except adenovirus where significantly associated with mild illness (ILI) and all viruses except PIV2 were associated with severe illness (SARI) (Table

3.1 and 3.4). Nonetheless, the level of association (*i.e.,* magnitude of the AF) varied across pathogens. Among ILI cases the AF was highest for influenza (adjusted AF [aAF]: 93.3%; 95% confidence intervals [95%CI]: 89.6%-95.7%), PIV2 (aAF: 90.8%; 95%CI: 60.5%-97.9%) and hMPV (aAF: 86.6%; 95%CI: 74.9%-92.9%) (Table 3.4).

Table 3.1: Association of respiratory viruses among patients (all ages) with SARI and ILI compared to controls in South Africa, 2012 – 2014 (Control cases were used as the reference groups for the multinomial regression model, aRRR highlighted in bold indicate factors significant at p<0.05)

Factor	Control[a] n (%) N=1793	ILI n (%) N=3784	SARI n (%) N=1959	Multivariable Analysis ILI aRRR[b] (95% CI)	Multivariable Analysis SARI aRRR[b] (95% CI)
Influenza	25 (1.4)	577 (15.2)	111 (5.7)	**14.9 (9.6-23.2)**	**7.3 (4.5-11.9)**
Rhinovirus	374 (20.9)	1064 (28.1)	667 (34.1)	**2.1 (1.8-2.4)**	**1.9 (1.6-2.3)**
Adenovirus	207 (11.5)	434 (11.5)	379 (19.3)	1.1 (0.9-1.3)	**1.6 (1.3-1.9)**
Enterovirus	53 (3.0)	120 (3.2)	118 (6.0)	**1.7 (1.2-2.5)**	**1.9 (1.3-2.9)**
RSV	55 (3.1)	225 (5.9)	391 (19.9)	**2.7 (1.9-3.8)**	**6.1 (4.4-8.5)**
PIV 1	11 (0.6)	56 (1.5)	45 (2.3)	**4.0 (1.9-8.4)**	**4.3 (2.0-9.3)**
PIV 2	2 (0.1)	28 (0.7)	11 (0.6)	**10.9 (2.5-46.9)**	4.1 (0.8-21.6)
PIV 3	23 (1.3)	81 (2.1)	72 (3.7)	**2.9 (1.7-5.0)**	**2.6 (1.5-4.6)**
hMPV	13 (0.7)	141 (3.7)	86 (4.4)	**7.5 (4.0-14.1)**	**6.9 (3.6-13.4)**

[a] Reference group for the multinomial regression model. [b]Relative risk ratio (aRRR) adjusted by age, HIV serostatus and underlying medical conditions at multivariable analysis. Parainfluenza virus (PIV) 1, 2, 3; RSV; human metapneumovirus (hMPV).

Among SARI cases the AF was highest for influenza (aAF: 86.3%; 95%CI: 77.7%-91.6%), hMPV (aAF: 85.6%; 95%CI: 72.0%-92.6%), and RSV (aAF: 83.7%; 95%CI: 77.5%-88.2%) (Table 3.4).

In the age stratified analysis among children <5 years of age all viruses except adenovirus and enterovirus where significantly associated with mild illness (ILI) and all viruses except PIV2 were associated with severe illness (SARI) (Table 3.2 and 3.4). Among SARI cases <5 years of age, the highest significant AF (≥90%) were observed for influenza, hMPV and RSV, while the lowest significant AF was observed for enterovirus (38.2%) (Table 3.4). In this group among viruses with significant AF the estimated detection rate attributable to illness (adjusted prevalence) was 22.4% for RSV, 18.1% for rhinovirus, 10.1% for adenovirus, 5.2% for hMPV, 4.7% for influenza, 2.9% for PIV3, 2.8% for enterovirus and 2.4% for PIV1 (Table 3.4).

Among individuals ≥5 years of age adenovirus, RSV and PIV1 were not significantly associated with mild illness (ILI) and adenovirus, PIV1-3 and hMPV were not significantly associated with severe illness (SARI) (Table 3.3 and3.4). Among SARI cases ≥5 years of age the highest significant AF (>80%) were observed for enterovirus and influenza, while the lowest significant AF was observed for rhinovirus (42.7%). In this group among viruses with significant AF the estimated detection rate attributable to illness was 8.1% for rhinovirus, 5.9% for influenza, 3.4% for RSV, and 2.2% for enterovirus (Table 3.4).

Among ILI cases influenza had the highest AF and estimated prevalence associated with illness among children <5 years (AF: 95.8%; $Prev_{Illness}$: 13.9%) as well as individuals ≥5 years (AF: 91.9%; $Prev_{Illness}$: 14.5%) (Table 3.4).

Table 3.2: Association of respiratory viruses among children< 5 years of age with SARI and ILI compared to controls in South Africa, 2012 – 2014 (Control cases were used as the reference groups for the multinomial regression model, aRRR highlighted in bold indicate factors significant at p<0.05)

Variable	Control [a]	ILI	SARI	Multivariable Analysis[c]	
				ILI aRRR[b] (95% CI)	SARI aRRR[b] (95% CI)
	n (%) N=658	n (%) N=1075	n (%) N=1431		
Influenza	7 (1.1)	149 (13.9)	73 (5.1)	**24.0 (9.5-60.7)**	**12.7 (4.9-32.5)**
Rhinovirus	214 (32.5)	401 (37.3)	567 (39.6)	**1.6 (1.3-2.1)**	**1.8 (1.4-2.3)**
Adenovirus	122 (18.5)	212 (19.7)	323 (22.6)	1.1 (0.8-1.5)	**1.8 (1.4-2.4)**
Enterovirus	46 (6.7)	82 (7.6)	105 (7.3)	1.4 (0.9-2.2)	**1.6 (1.1-2.5)**
RSV	25 (3.8)	129 (12.0)	357 (24.9)	**4.1 (2.5-6.7)**	**9.9 (6.2-15.8)**
PIV 1	7 (1.1)	33 (3.1)	43 (3.0)	**4.1 (1.7-10.0)**	**4.8 (2.0-11.8)**
PIV 2	1 (0.1)	13 (1.2)	10 (0.7)	**12.1 (1.4-101.5)**	5.6 (0.6-49.4)
PIV 3	15 (2.3)	40 (3.7)	65 (4.5)	**3.2 (1.6-6.5)**	**2.8 (1.5-5.5)**
hMPV	4 (0.6)	53 (4.9)	79 (5.5)	**13.5 (4.1-44.9)**	**16.2 (4.9-53.4)**

[a]Reference group for the multinomial regression model. [b]Age and HIV adjusted relative risk ratio (aRRR) at multivariable analysis. [c]Only covariates significant at the multivariable analysis are reported. Parainfluenza virus (PIV) 1, 2, 3; RSV; human metapneumovirus (hMPV).

Table 3.3: Association of respiratory viruses among patients ≥ 5 years of age with SARI and ILI compared to controls in South Africa, 2012 – 2014 (Control cases were used as the reference groups for the multinomial regression model, aRRR highlighted in bold indicate factors significant at p<0.05)

Variable	Control [a]	ILI	SARI	Multivariable Analysis[c]	
				ILI aRRR[b] (95% CI)	SARI aRRR[b] (95% CI)
	n (%)N=1135	n (%)N=2708	n (%) N=522		
Influenza	18 (1.6)	428 (15.8)	38 (7.2)	**12.3 (7.5-20.3)**	**5.3 (2.9-9.9)**
Rhinovirus	160 (14.1)	663 (24.5)	100 (19.0)	**2.5 (2.0-3.0)**	**1.7 (1.3-2.4)**
Adenovirus	85 (7.5)	222 (8.2)	56 (10.6)	1.1 (0.8-1.4)	1.2 (0.8-1.8)
Enterovirus	7 (0.6)	38 (1.4)	13 (2.5)	**3.7 (1.4-9.7)**	**7.9 (2.7-23.5)**
RSV	30 (2.6)	96 (3.5)	34 (6.4)	1.4 (0.9-2.2)	**2.2 (1.2-3.9)**
PIV 1	4 (0.3)	23 (0.8)	2 (0.4)	2.4 (0.7-8.7)	1.7 (0.3-10.3)
PIV 2	1 (0.1)	15 (0.6)	1 (0.1)	**10.6 (1.3-82.9)**	Not estimated
PIV 3	8 (0.7)	41 (1.5)	7 (1.3)	**2.7 (1.1-6.3)**	2.5 (0.8-7.5)
hMPV	9 (0.8)	88 (3.25)	7 (1.3)	**4.8 (2.3-10.1)**	1.7 (0.6-5.3)

[a]Reference group for the multinomial regression model. [b]Age and HIV adjusted relative risk ratio (aRRR) at multivariable analysis. [c]Only covariates significant at the multivariable analysis are reported. Parainfluenza virus (PIV) 1, 2, 3; RSV; human metapneumovirus (hMPV).

Table 3.4: Attributable fraction, observed prevalence and adjusted prevalence of respiratory viruses among patients with SARI and ILI in South Africa, 2012 – 2014.

Viruses	Influenza-Like-Illness			Severe Acute Respiratory Illness		
	Attributable Fraction (%) (95% CI)	Observed Prevalence (%)	Adjusted Prevalence (%)[a]	Attributable Fraction (%) (95% CI)	Observed Prevalence (%)	Adjusted Prevalence (%)[a]
Individuals of any age						
Influenza	**93.3 (89.6-95.7)**	15.2	14.2	**86.3 (77.7-91.6)**	5.7	4.9
Rhinovirus	**52.0 (44.0-58.9)**	28.1	14.6	**46.9 (37.6-56.5)**	34.1	20.2
Adenovirus	5.9 (-15.1-23.1)	11.5	0.7	**36.4 (20.6-49.0)**	19.3	7.0
Enterovirus	**41.8 (15.5-59.9)**	3.2	1.3	**49.0 (24.9-65.4)**	6.0	2.9
RSV	**63.1 (48.6-73.5)**	5.9	3.7	**83.7 (77.5-88.2)**	19.9	16.7
PIV 1	**75.3 (48.8-88.1)**	1.5	1.1	**76.9 (50.6-89.2)**	2.3	1.8
PIV 2	**90.8 (60.5-97.9)**	0.7	0.6	75.9 (-25.9-95.4)	0.6	0.5
PIV 3	**66.1 942.5-80.0)**	2.1	1.4	**62.0 (34.1-78.1)**	3.7	2.3
hMPV	**86.6 (74.9-92.9)**	3.7	3.2	**85.6 (72.0-92.6)**	4.4	3.8
Children <5 years of age						
Influenza	**95.8 (89.5-98.3)**	13.9	13.3	**92.1 (79.7-96.9)**	5.1	4.7
Rhinovirus	**38.2 (21.1-51.6)**	37.3	12.7	**45.7 (31.0-57.2)**	39.6	18.1
Adenovirus	9.3 (-21.6-32.3)	19.7	1.8	**44.9 (27.0-58.4)**	22.6	10.1
Enterovirus	29.4 (-9.7-54.6)	7.6	2.2	**38.3 (5.0-59.9)**	7.3	2.8
RSV	**75.7 (60.3-85.1)**	12.0	9.1	**90.0 (84.0-93.7)**	24.9	22.4
PIV 1	**75.5 (39.8-90.0)**	3.1	2.3	**79.3 (49.3-91.5)**	3.0	2.4
PIV 2	**91.7 (30.6-99.0)**	1.2	1.1	82.1 (-57.7-97.9)	0.7	0.6
PIV 3	**69.2 (38.6-84.6)**	3.7	2.6	**64.8 (31.8-81.8)**	4.5	2.9
hMPV	**92.6 (75.4-97.8)**	4.9	4.6	**93.8 (79.6-98.1)**	5.5	5.2
Individuals ≥5 years of age						
Influenza	**91.9 (86.6-95.1)**	15.8	14.5	**81.3 (65.3-89.9)**	7.2	5.9
Rhinovirus	**59.6 (50.2-67.3)**	24.5	14.3	**42.7 (21.6-58.1)**	19.0	8.1
Adenovirus	8.1 (-23.2-31.4)	8.2	0.7	16.8 (-27.1-45.5)	10.6	1.8
Enterovirus	**72.8 (28.3-89.7)**	1.4	1.0	**87.4 (62.9-95.7)**	2.5	2.2
RSV	28.2 (-13.2-54.5)	3.5	1.0	**54.8 (19.9-74.5)**	6.4	3.4
PIV 1	58.7 (-48.4-88.5)	0.8	0.5	40.0 (-270.1-90.3)	0.4	0.1
PIV 2	**90.6 (26.2-98.1)**	0.6	0.5	Not estimated	0.1	Not estimated
PIV 3	**62.9 (13.5-84.1)**	1.5	0.9	59.4 (-24.3-86.7)	1.3	0.8
hMPV	**79.2 (56.1-90.1)**	3.25	2.6	41.7 (-81.5-81.3)	1.3	0.6

[a]Observed prevalence adjusted by the AF to obtain the prevalence attributable to illness. Parainfluenza virus (PIV) 1, 2, 3; Respiratory Syncytial Virus (RSV); human metapneumovirus (hMPV).

3.4 Discussion

We assessed the association between virus detection and mild or severe illness relative to controls. The estimated detection rate attributable to illness reported in this study reflects a more accurate description of the prevalence of viruses causing respiratory disease in both children and adults in South Africa than reporting viral detection rates alone. Most of the viral pathogens evaluated in this study were found to be associated with mild or severe disease. Nonetheless, the magnitude of this association varied between pathogens. The results of our study suggest that influenza, RSV and hMPV infections are highly associated with severe respiratory illness in South Africa relative to controls, especially in children <5 years of age. While rhinovirus and adenovirus had the lowest estimated AF the estimated detection rate attributable to illness remained high indicating that, while these viruses could act both as pathogen and bystander, they could also be responsible for a substantial proportion of severe disease. RSV, rhinovirus, adenovirus, hMPV and influenza were the most common pathogens causing disease among SARI cases, especially in children <5 years of age.

Our findings differ from those of other studies which have used multiplex PCR to detect a viral aetiology in non-invasive respiratory specimens and used a control group to interpret the findings. These studies found that fewer viruses were associated with disease. A study conducted among children ≤12 years of age hospitalized with pneumonia in the Kilifi District hospital in Kenya (Berkley, Munywoki *et al.* 2010) reported that RSV was the most common virus identified (34% of cases), and the only virus associated with disease. A case-control

study conducted among children ≤59 months in rural Kenya, also reported that only RSV was found to be significantly associated with severe pneumonia (Hammitt, Kazungu *et al.* 2012), while another study conducted in children reported that RSV and influenza were more commonly found among cases than controls (Feikin, Njenga *et al.* 2013). A study conducted among adults also found a disease association with RSV, influenza, and hMPV, with influenza being the most common pathogen causing disease (Feikin, Njenga *et al.* 2012). It should be noted that these studies had a limited number of controls potentially resulting in a lack of power to detect significant disease association for pathogens with low detection rates.

In our study RSV was found to be significantly associated with severe respiratory disease relative to controls across age groups. While RSV has been well documented as the leading cause of viral pneumonia in children <5 years of age not only in resource-limited setting but also in the developed world (Rudan, Boschi-Pinto *et al.* 2008, Nair, Nokes *et al.* 2010), RSV is an increasingly recognized cause of severe disease in adults (Falsey 1998, Falsey and Walsh 2000, Falsey, Formica *et al.* 2002, Falsey, Formica *et al.* 2003, Falsey, Hennessey *et al.* 2005). Several studies have shown that RSV infection is an important cause of illness in the elderly (≥65 years) and high-risk adults, with a disease burden similar to that of non-pandemic seasonal influenza (Falsey, Hennessey *et al.* 2005, Mullooly, Bridges *et al.* 2007). Although we found that RSV was associated with disease among both children and adults, other modelling studies conducted in South Africa have not found excess mortality or hospitalizations associated with RSV among persons aged ≥45 years of age (Kyeyagalire, Tempia *et al.* 2014, Tempia, Walaza *et al.* 2014).

This apparent contradiction may be related to the fact that the burden of RSV among older adults in South Africa may be too low to be detected in modelling studies.

While rhinovirus has been shown to be less associated with illness (low attributable fraction) in both children and adults, the estimated detection rate attributable to illness remains elevated when compared to other pathogens, suggesting that rhinovirus may still causes a substantial proportion of clinical disease that manifests either as ILI or SARI. The high prevalence of rhinovirus among controls indicates that rhinovirus may potentially have an extended shedding period and can be detected in patients without symptoms. Several studies have reported the high positivity rate of rhinovirus in asymptomatic individuals and none so far have been able to give a clear cut indication of the role that rhinovirus plays in severe respiratory infection, although several have suggested that rhinovirus can act as both a bystander and a pathogen (Fry, Chittaganpitch *et al.* 2010, Smuts, Workman *et al.* 2011, Onyango, Welch *et al.* 2012). Similar results were obtained for adenovirus in this study.

In our study, influenza and hMPV were found to be significantly associated with severe disease relative to controls among children less than 5 years of age. Influenza has been described as one of the leading causes of pneumonia in children, the elderly, and adults with HIV infection (Rudan, Boschi-Pinto *et al.* 2008, Cohen, Simonsen *et al.* 2010, Nair, Brooks *et al.* 2011, Cohen, Moyes *et al.* 2013). Since its initial description in 2001, hMPV has been reported worldwide. However, so far studies of hMPV have been limited; although it has been suggested that hMPV mirrors the epidemiology of RSV and influenza with more severe infections occurring in the very young, elderly and immune-

compromised individuals (Hamelin, Abed *et al.* 2004, Gaunt, McWilliam-Leitch *et al.* 2009).

Our study has limitations that warrant discussion. First, several viruses were detected at low prevalence in the control group which would account not only for the high adjusted relative risk ratios but also for the wide confidence intervals. Second, comparing detection rate of pathogens among symptomatic patients with controls doesn't prove or disprove disease association in individual patients. Other approaches such as viral load and host interactions are needed to determine what role some of these viruses play in severe respiratory disease, while taking into account factors such as replication or persistence of nucleic acids present during the pre- or post syndromic phase of infection. Last, we did not adjust for the potential role of bacterial infections as this information was not available. The role of bacterial super-infection on severe illness following a viral infection cannot be excluded.

In conclusion, influenza, RSV and hMPV can be considered likely pathogens if detected in South African patients with ILI or SARI; whereas rhinovirus and adenovirus were commonly identified also among controls suggesting that they may cause only a proportion of clinical disease observed in positive patients. Nonetheless, given their high estimated detection rate attributable to illness, they may be important contributors to disease. The pathogens listed above had the highest AF or estimated detection rates attributable to illness and they may be considered for routine surveillance. This data together with other matched case-control studies like PERCH (Pneumonia Etiology Research for Child Health) (Levine, O'Brien *et al.* 2012) will provide useful information on how each pathogen impacts disease severity and may assist to better interpret

surveillance data, to prioritize pathogens to be included in surveillance programs and to guide prevention interventions.

Chapter 4

Genetic Diversity and Molecular Epidemiology of Human Rhinoviruses in South Africa

4.1 Introduction

Currently more than 100 different serotypes of rhinovirus and three genetically characterised species (RV-A, RV-B and RV-C) have been described (Briese, Renwick *et al.* 2008). Although the majority of RV infections are associated with mild disease, their impact on overall morbidity and economic cost worldwide is thought to be considerable (Mahony 2008). Some studies have suggested that infection with RV-C may result in more severe illness compared to RV-A and RV-B (Miller, Edwards *et al.* 2009).

Using real-time PCR methods, we previously reported that rhinovirus was identified in 25% of patients that were hospitalised with severe acute respiratory illness (SARI) in South Africa (Pretorius, Madhi *et al.* 2012). Rhinovirus has also been identified among asymptomatic patients (Fry, Chittaganpitch *et al.* 2010) with a reported prevalence of 12-22% among children and 13% among immune-compromised patients (Smuts, Workman *et al.* 2011). Rhinovirus has a relatively short shedding period in otherwise healthy persons; however, prolonged shedding of over and above 28 days have been reported for immune-compromised patients (Peltola, Waris *et al.* 2013). Consequently the clinical relevance of detecting rhinovirus among hospitalized patients is difficult to interpret, especially in a population with a high HIV sero-prevalence. According to Statistics South Africa, 10% of the South African population is HIV-positive. This suggests a high percentage of potentially vulnerable individuals (Statistics South Africa 2013), and highlights the need to determine the association of respiratory viruses like rhinovirus with SARI relative to patients with milder illness or without respiratory symptoms.

We investigated the prevalence, epidemiological characteristics, genetic diversity and disease association of rhinovirus, including type, among patients with SARI, influenza-like illness (ILI) and asymptomatic controls in South Africa.

4.2 Materials and Methods

4.2.1 Study design and population

SARI Surveillance: Study samples were obtained from participants enrolled in a prospective hospital-based surveillance program for SARI initiated in February 2009 which aimed to describe the aetiology and risk factors for acute lower respiratory tract infection (ALRI) in all age groups in South Africa. The methodology and case definitions of this study have been previously described (section 2.2.2) (Cohen, Moyes *et al.* 2013). All patients were enrolled only once and followed through until discharged from the hospital.

ILI and Asymptomatic Control Surveillance: Study samples were obtained from participants enrolled in an active surveillance program for ILI and asymptomatic controls initiated in May 2012 through 2013. Patients presenting with ILI and asymptomatic controls were enrolled at two outpatient clinics serving the population surveyed at two of the SARI sentinel sites: the Gateway Clinic, KwaZulu Natal Province and Jouberton Clinic, Northwest Province. An ILI case was defined as an outpatient of any age presenting with cough duration of ≤7 days with either temperature >38°C or history of fever. ILI cases that were referred for hospitalization subsequent to the visit were not eligible for enrolment.

An asymptomatic control was defined as an individual presenting at the same outpatient clinic with no history of fever, respiratory or gastro-intestinal symptoms during the 14 days preceding the visit. The patients commonly presented to the clinic

for visits such as dental procedures, family planning, well baby clinics, voluntary HIV counselling and testing or acute care for non-febrile illnesses. Medical and symptoms history was systematically verified by a trained nurse using a structured checklist. This information was obtained through medical chart review and interview with the patient or legal guardian for children less than 15 years of age. One HIV-infected and one HIV-uninfected control were enrolled every week in each ILI clinic within each of the following age categories: 0-1, 2-4, 5-14, 15-54 and ≥55 years.

A standardized questionnaire was used to collect demographic and clinical information from each enrolled SARI and ILI case and control. In addition, for SARI cases hospital records were reviewed to assess disease progression and outcome (i.e., discharge, transfer or in-hospital death).

4.2.2 Sample Selection of Two Groups for Molecular Characterization

2009-2010 Cohort: SARI cases: was randomly selected from single positive rhinovirus SARI patients, Specimens were sorted according to randomly assigned numbers and the first 381 were selected (37%, 381/1039) for molecular characterization.

2012-2013 Cohort: SARI, ILI, and controls: for the disease association analysis we assumed a 25% rhinovirus prevalence among cases and a 15% rhinovirus prevalence among controls, which resulted in a needed sample size of 214 rhinovirus positive cases in each group to statistically assess significance using a 95% confidence interval and 80% power and a random selection (as described above) of single rhinovirus-positive specimens was characterized further.

4.2.3 Laboratory testing

4.2.3.1 Rhinovirus detection

Respiratory specimens (i.e., nasopharyngeal aspirates for children <5 years of age and nasopharyngeal and oropharyngeal swabs from individuals ≥5 years of age) were collected placed in viral transport medium, stored at 4-8°C and transported to the National Institute for Communicable Diseases within 72 hours of collection for testing. All specimens were tested for the presence of 10 respiratory viruses using the real- assay as previously (section 2.2.6). Among consenting study patients, HIV status was established by enzyme-linked immunosorbent assay (ELISA) or PCR depending on the patients' age.

4.2.3.2 Sequencing of the Rhinovirus VP4/VP2 genomic fragment

A 440 base pair region of VP4 and VP2 was amplified and sequenced for 595 randomly selected rhinovirus-positive specimens (single infection) consisting of: 381 SARI specimens from 2009-2010, and 214 SARI, ILI and control specimens from 2012-2013. Briefly, the first round of RT-PCR was performed using primers PR-1 (Forward) and PR-2 (Reverse) (Coiras, Aguilar *et al.* 2004a). Nested PCR was performed, using primers hRV 01.3 (Arden, McErlean *et al.* 2006) and RV2n with an expected band size of 550bp (Coiras, Aguilar *et al.* 2004a). Amplicons were purified using the ExoSAP-IT enzyme system (USB Corporation, Cleveland OH) and sequenced using the Big Dye terminator version 3.1 cycle Sequencing Ready Reaction kit (Life Technologies, Foster City, USA) using nested primers. Sequences were assembled using Sequencher® version 5 (Gene Codes Corporation, Michigan, USA) and alignments were performed using MAFFT multiple sequence alignment program (Katoh and Standley 2013). The nucleotide substitution model used in the

Maximum likelihood (ML) analysis was determined using jModelTest (Guindon and Gascuel 2003, Darriba, Taboada *et al.* 2012) and the ML trees were generated using PhyML 3.0 (Guindon and Gascuel 2003, Guindon, Lethiec *et al.* 2005).

4.2.4 Statistical Analysis

We implemented three multivariable multinomial regression models. First, using the 2009-2010 cohort of SARI cases we evaluated factors associated with each rhinovirus type. For this analysis the RV-A type was defined as the baseline category as it was the most common type detected. Second, using the 2012-2013 cohort of SARI, ILI, and control cases, we evaluated disease severity associated with rhinovirus infection comparing the rhinovirus prevalence among SARI and ILI cases to controls (reference group). Third, also using the 2012-2013 cohort, we evaluated disease severity associated with rhinovirus type by comparing the proportion of rhinovirus type among SARI and ILI cases to controls. Statistical significance was defined as $p<0.05$. The analysis was performed using STATA 12, (Stata Corporation, Texas, USA). Stefano Tempia has assisted the student in running these statistical tests and assisted the student in the interpretation.

4.3 Results

4.3.1 Phylogenetic comparison of rhinovirus strains identified in 2009-2010 and 2012-2013 cohorts

Maximum likelihood phylogenetic comparison of clinical specimens from 2009-2010 and 2012-2013 to international reference sequences indicated that RV-A (285,48%) and RV-C (247,41%) were more commonly identified than RV-B (67,11%) and that the South African sequences for each of the type formed numerous subclusters

within each type, with statistically significant bootstrap support (Figure 4.1). Several distinct bootstrap supported clusters of South African viruses were identified in type C (Figure 4.1).

4.3.2 Epidemiology of rhinovirus infection and factors associated with rhinovirus type in 2009-2010 cohort

The 2009-2010 study cohort data was used for this analysis. From February 2009 through December 2010, we obtained laboratory results from 7641 SARI patients. Of these 3171 (41%) were negative for the viral pathogens in our assay. Rhinovirus was detected in 1949 (25%) subjects, of which it was the only virus identified in 1039 (53%) cases. In multivariable analysis adjusting for age and year of circulation infection, RV-C compared with RV-A type was associated with asthma or having a history of asthma (adjusted Relative Risk Ratio (aRRR) =3.4 95% CI 1.1; 11.1) (Table 4.1). No difference between RV-B and RV-A type were detected in the multivariable analysis.

Rhinovirus was detected throughout the year with no evident seasonality. RV-A and RV-C co-circulated in 2009-2010, while RV-B was detected sporadically mainly in 2010 (Figure 4.2A).

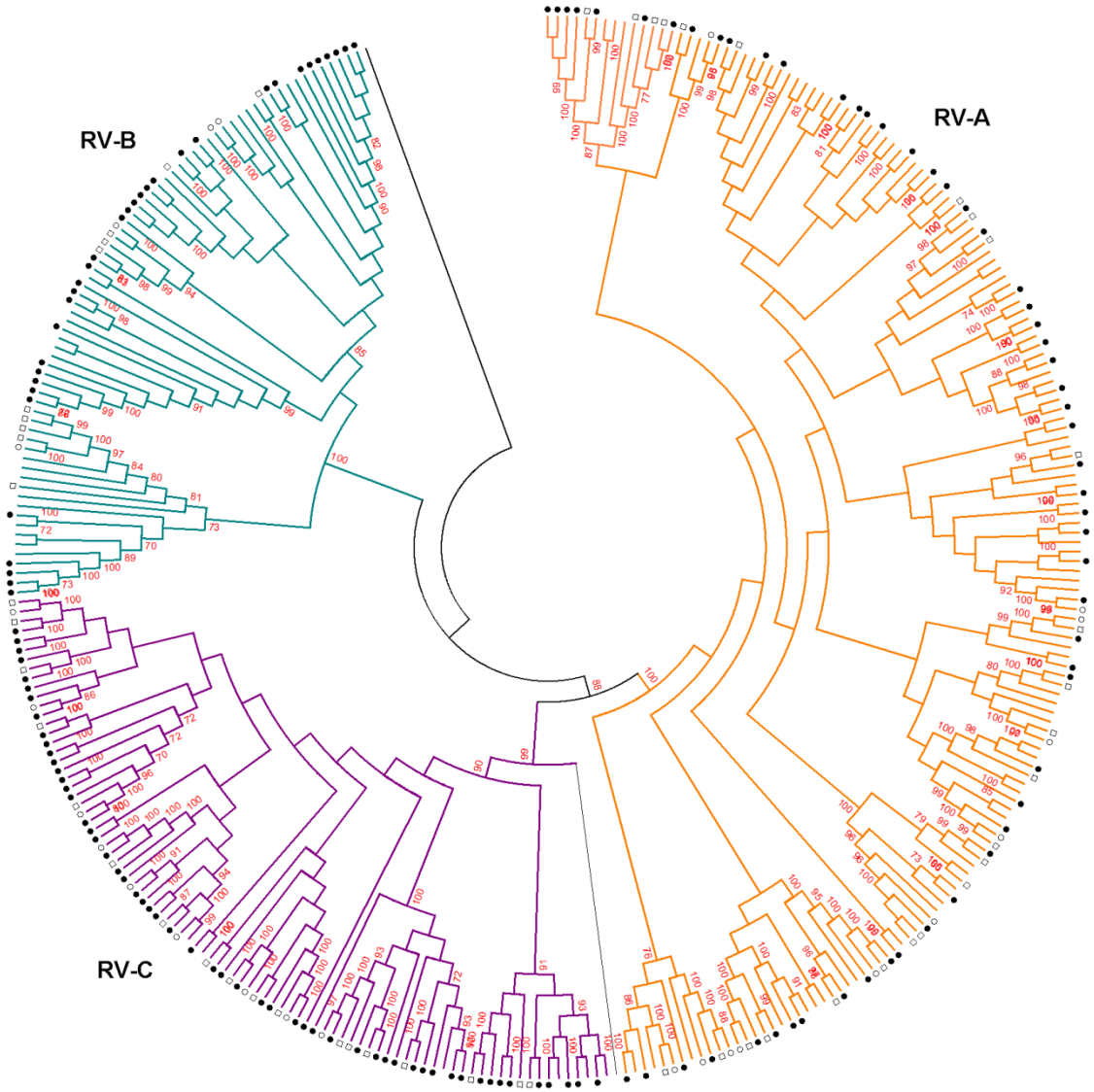


Figure 4.1: Phylogenetic analysis of rhinovirus type by Maximum Likelihood method of the VP4/VP2 region, South Africa, 2009-2010 and 2012-2013. Phylogenetic analysis of Rhinovirus sequences from South Africa and reference sequences from Genbank using Maximum Likelihood method of the VP4/VP2 region. Sequences with closed circle denotes type identified in SARI patients, those with open squares denotes type identified in ILI patients, while those with open circles denotes type identified in control patients, those without denotation are the reference sequences. Bootstrap values (100 replicates) shown on the branches, with values < 70% omitted from the tree.

Table 4.1: Factors associated with rhinovirus type among patients hospitalized with severe acute respiratory illness, South Africa, 2009-2010

Factor	Univariable analysis					Multivariable analysis[d]	
	RV-A[a]	RV-B		RV-C		RV-B	RV-C
	n/N (%)	n/N (%)	RRR[b] (95% CI)	n/N (%)	RRR[b] (95% CI)	aRRR[c] (95% CI)	aRRR[c] (95% CI)
Age group, yrs							
<5	78/162 (48)	9/39 (23)	1	90/156 (58)	1	1	1
5-14	9/162 (6)	1/39 (3)	0.9 (0.1-8.5)	10/156 (6)	0.9 (0.4-2.5)	1.1 (0.1-9.3)	0.9 (0.3-2.2)
15-24	9/162 (6)	2/39 (5)	1.9 (0.3-10.3)	6/156 (4)	0.6 (0.2-1.7)	1.8 (0.3-9.5)	0.6 (0.2-1.9)
25-44	42/162 (26)	19/39 (49)	**3.9 (1.6-9.4)**	38/156 (24)	0.8 (0.5-1.3)	**3.5 (1.4-8.5)**	0.9 (0.5-1.6)
45+	24/162 (15)	8/39 (20)	**2.9 (1.1-8.3)**	12/156 (8)	**0.4 (0.2-0.9)**	2.6 (0.9-7.8)	**0.4 (0.2-0.9)**
Sex (male)	82/162 (51)	13/39 (33)	0.5 (0.2-1.1)	75/156 (48)	0.9 (0.6-1.4)		
Year							
2009	52/162 (33)	6/39 (15)	1	85/156 (54)	1	1	
2010	109/162 (66)	33/39 (85)	**2.7 (1.1-6.7)**	71/156 (46)	**0.4 (0.2-0.6)**	2.0 (0.7-5.2)	**0.4 (0.3-0.7)**
Duration of symptoms >2 days	115/162 (71)	33/39 (85)	2.4 (0.9-5.7)	101/156 (65)	0.7 (0.5-1.2)		
Length of hospitalization >5 days	68/161 (42)	25/39 (64)	**2.4 (1.2-5.0)**	58/155 (37)	0.8 (0.5-1.3)		
HIV infection	71/153 (46)	26/37 (70)	**2.7 (1.2-5.9)**	60/135 (44)	0.8 (0.5-1.3)		
Asthma[e]	5/162 (3)	1/39 (2)	0.8 (0.1-7.2)	10/156 (6)	2.1 (0.7-6.4)	0.6 (0.1-5.5)	**3.4 (1.1-11.1)**
Underlying illness[f]	15/162 (9)	2/39 (5)	0.5 (0.1-2.4)	16/156 (10)	1.1 (0.5-2.3)		
Oxygen therapy	56/161 (35)	20/39 (51)	2.0 (0.9-4.0)	63/156 (40)	1.3 (0.8-2.0)		
Patient died	8/162 (5)	1/39 (3)	0.5 (0.1-4.2)	8/156 (5)	1.0 (0.4-2.8		

[a.]Reference group for the multinomial regression model. [b] Unadjusted relative risk ratio (RRR) at univariable analysis. [c] Adjusted relative risk ratio (aRRR) at multivariable analysis. [d] Only covariates significant at the multivariable analysis are reported. [e] Asthma was defined in our database as a history of asthma no distinction was made if they were undergoing an exacerbation of their asthma. [f] Underlying illness includes: chronic lung diseases, cirrhosis/liver failure, chronic renal failure, heart failure, valvular hearth disease, coronary heart disease, immunosuppressive therapy, splenectomy, diabetes, burns, kwashiorkor/marasmus, nephritic syndrome, spinal cord injury, seizure disorder or emphysema. RRR highlighted in bold indicate factors significant at p<0.05

4.3.3 Association of rhinovirus infection and rhinovirus-type with respiratory disease severity in 2012-2013 cohort

The 2012-2013 study cohort data was used for this analysis. From May 2012 through April 2013 we obtained laboratory results from 3907 patients, of which 2125 (54%) had SARI, 1325 (23%) had ILI and 457 (11%) were controls. Children <5 years of age accounted for 35% (743), 23% (299) and 26% (119) of the SARI, ILI and controls, respectively (p=0.226). Rhinovirus was identified in 24% (515/2125), 27% (356/1325) and 20% (91/457) of SARI, ILI and controls, respectively (Table 3). On multivariable analysis adjusting for age (<5, 5-14, 15-44 and ≥45 years age groups; p=0.003) and HIV status (p=0.012), Rhinovirus infection was associated with both ILI (aRRR: 1.9; 95% CI 1.4-2.6) and SARI cases (aRRR: 1.6; 95% CI 1.2-2.2) compared to controls.

No significant difference was observed between the different rhinovirus type and disease severity among the characterized cases (results not shown).

4.4 Discussion

We describe the rhinovirus type circulating among patients from all age groups with acute upper and lower respiratory tract infections and controls in South Africa. While we detected a statistically significant difference in the prevalence of rhinovirus among SARI and ILI cases compared to controls, the elevated positivity rate of rhinovirus among controls indicates that rhinovirus may act as pathogen but could also be present in asymptomatic infections. This suggests that only a proportion of rhinovirus infections (along with other factors such as viral load and host interactions) may be responsible for the clinical disease that manifests as ILI or SARI. Rhinovirus was detected with no evident seasonality.

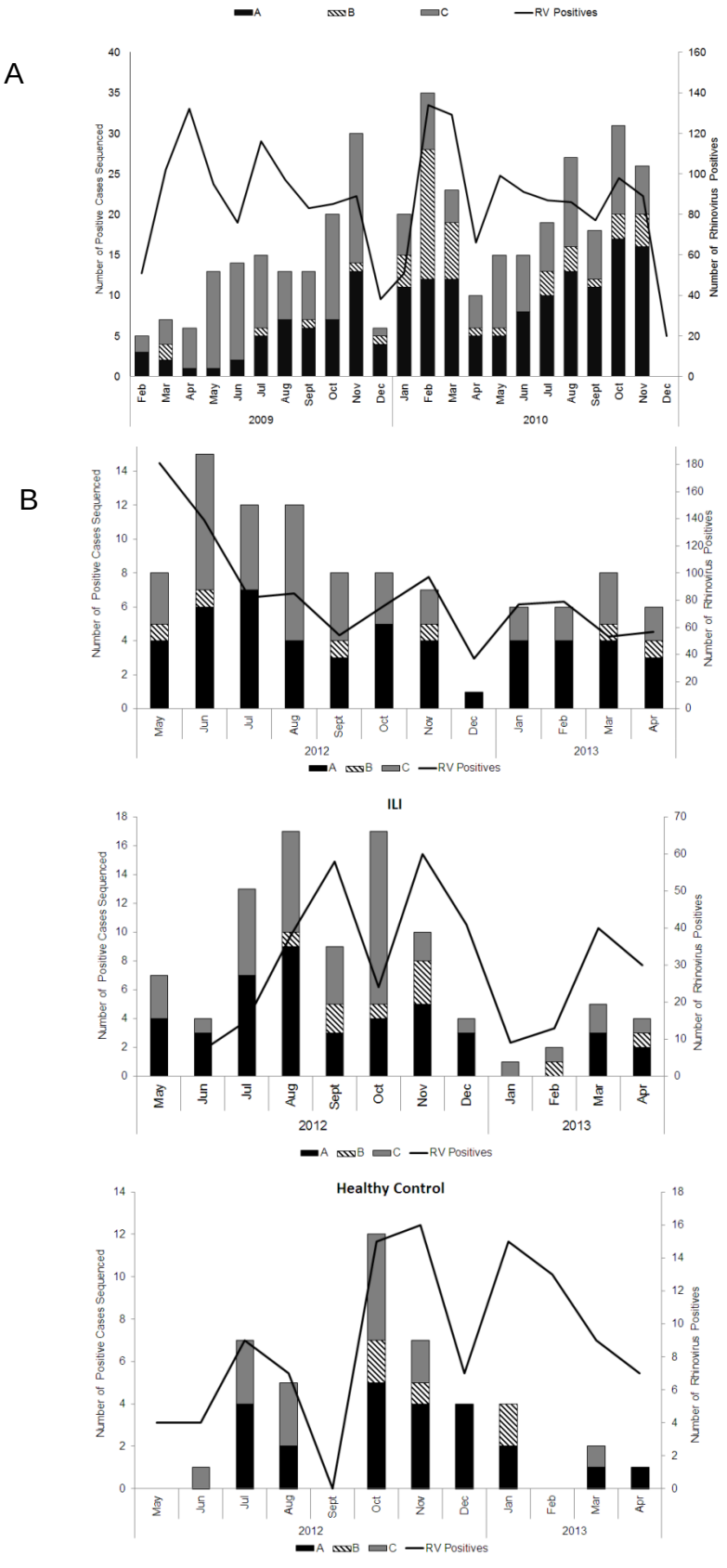


Figure 4.2: Number of positive cases and detection rate of Rhinovirus by month in South Africa, 2009-2010 SARI (A) and 2012-2013 SARI, ILI and control cases (B).

RV-A and RV-C co-circulated in 2012-2013, while RV-B was detected sporadically throughout (Figure 4.2B).

We did not identify any difference in disease severity due to different rhinovirus type. Similar results have been reported by a study in Thailand (Fry, Lu *et al.* 2011) whereby a rhinovirus prevalence of 19% in outpatients with ILI and 9% in controls was observed and no difference in disease severity by rhinovirus type was identified. The study in Thailand (Fry, Lu *et al.* 2011) and a more recent study conducted in Kenya (Onyango, Welch *et al.* 2012) showed similar distributions of rhinovirus type that we observed in South Africa: RV-A and RV-C co-circulated with no clear seasonality and RV-B was observed sporadically.

We found that HRV-C is not associated with more severe disease, but does appear to be associated with a history of asthma. This suggests that HRV-C induces asthma, or alternatively, that asthmatic children are vulnerable to HRV-C infections. Studies have shown HRV-C was not only related to wheezing illnesses and asthma, but was also associated with an increased risk of prior and subsequent hospital respiratory admissions (Kotaniemi-Syrjanen, Vainionpaa *et al.* 2003, Lau, Yip *et al.* 2007, Bizzintino, Lee *et al.* 2011, Smuts, Workman *et al.* 2011, Cox, Bizzintino *et al.* 2013).

Our study has several limitations. We only recorded in the initial interview if participants had a history of asthma. We did not follow-up on participants over the course of the study to determine whether the rhinovirus infection led to a diagnosis of wheezing or asthma or whether any viral nucleic acids detected using RT-PCR may represent the pre-syndromic phase of a viral infection. Also, the case definition for SARI and ILI was restricted to patients with duration of symptoms ≤ 7 days so we could have missed some cases that might have more prolonged illness. The

collection of different types of samples may potentially affect the sensitivity of rhinovirus detection. However, when comparing the detection rate of respiratory viruses in different samples Blaschke et al., (Blaschke, Allison *et al.* 2011) have shown that non-invasive methods for collecting respiratory samples can be used to identify respiratory viruses with multiplex PCR testing. Finally, while adjusting for age and HIV status in our analyses on association with disease severity, we were not powered to implement age and HIV stratified analysis, hindering the ability to detect differences among different groups.

In conclusion, we showed that there was a high diversity in the sequences of the rhinovirus type that circulated in South Africa. Rhinovirus is detected in a proportion of outpatient and hospitalized respiratory disease but is also detected in individuals with no history within the past 14 days of respiratory illness, which suggests that rhinovirus can act as a disease causing agent and be found in asymptomatic infection. Further studies are necessary to determine if other factors such as viral load or host interactions play a role in rhinovirus-associated disease.

Chapter 5

Positive evolution and molecular epidemiology of subtype A and B Respiratory Syncytial Virus G-protein genotypes from 1997-2013 in South Africa.

5.1 Introduction

While affecting all age groups, RSV infection can be particularly severe in infants, immune-compromised patients and the elderly (Nair, Nokes *et al.* 2010). RSV causes infection and re-infection during infancy and throughout life, despite maternal antibodies in young infants and prior infections in adults, without the need for significant antigenic changes(Bukreyev, Yang *et al.* 2008).Antigenic variation may, however, contribute to the susceptibility of some individuals to repeated infection (Agoti, Mbisa *et al.* 2010).

The two major RSV subgroups are A (RSV-A) and B (RSV-B) and they are further characterized into several genotypes, based on antigenic and genetic variability of the second hypervariable region of the G-protein, which provides a reliable proxy for the entire G-protein variation (Peret, Hall *et al.* 1998, Peret, Hall *et al.* 2000). Several genotypes can co-circulate in a single epidemic season, and different genotypes can dominate in consecutive seasons (Venter, Madhi *et al.* 2001, Zlateva, Lemey *et al.* 2004a, Zlateva, Lemey *et al.* 2005, Matheson, Rich *et al.* 2006, van Niekerk and Venter 2011). To date, eleven genotypes for RSV-A have been identified and include GA1-7 (Peret, Hall *et al.* 1998, Peret, Hall *et al.* 2000); SAA1 (Venter, Madhi *et al.* 2001); NA1; NA2; and ON1 with a 72-base pair(bp) duplication insertion(Papenburg, Carbonneau *et al.* 2012). For RSV-B, the genotypes are GB1-4 (Peret, Hall *et al.* 1998, Peret, Hall *et al.* 2000); SAB1-3 (Venter, Madhi *et al.* 2001, Venter, Collinson *et al.* 2002); and the BA genotype with a 60-bp insertion (Trento, Galiano *et al.* 2003) which has subsequently been classified into sub-genotypes BA1-10(Dapat, Shobugawa *et al.* 2010, Trento, Casas *et al.* 2010).

From previous studies conducted in South Africa during 1997-2001, we reported the circulation of GA2, GA5, GA7, GB3 and GA4, as well as novel genotypes such as SAA1, SAB1, SAB2 and SAB3 (Venter, Madhi *et al.* 2001, Venter, Collinson *et al.* 2002, Madhi, Venter *et al.* 2003). These novel genotypes have subsequently been identified worldwide (Galiano, Palomo *et al.* 2005, Matheson, Rich *et al.* 2006). The BA genotype was first detected in South Africa during the investigation of a nosocomial outbreak, in 2006 (Visser, Delport *et al.* 2008), and we subsequently showed that BA had replaced all previously described RSV-B genotypes in South Africa (van Niekerk and Venter 2011). Also, until 2009, GA2 and GA5 were the only subtype A genotypes still circulating in South Africa, suggesting that these RSV-A genotypes have been stable over the past 9 years. However, poor bootstrap and pairwise distance analysis suggested that drift may be occurring. The objective of this study was to compare the genotypic characteristics of RSV identified through a surveillance program investigating the prevalence of respiratory viruses in individuals hospitalized for severe acute respiratory illness (SARI) conducted between 2009-2013; to compare these to the genotypes of RSV identified from 1997-2001, 2006-2009; and to RSV identified elsewhere globally.

5.2 Materials and Methods

5.2.1 Study design

SARI Surveillance program (2009-2013): Specimens were collected as part of a prospective hospital-based sentinel surveillance program from February 2009-May 2013, which aimed to determine respiratory viruses and risk factors associated with hospitalization for severe acute respiratory illness (SARI) in South Africans of all age groups. The methodology of this study has been previously described (section

2.2.2). The participating SARI surveillance sites are Chris Hani Baragwanath Academic hospital (CHBAH, Gauteng Province); Edendale hospital (KwaZulu-Natal Province), Matikwana and Mapulaneng hospitals (Mpumalanga Province); and Tshepong and Klerksdorp hospitals (North-West Province).

RSV previously identified in South Africa: During 1997-2000 samples were obtained from children <5 years from CHBAH(Soweto) (Venter, Madhi *et al.* 2001); during 1998-2001from children <5 years from community clinics in Agincourt (Venter, Collinson *et al.* 2002); additional samples during 2000-2001fromhigh-risk children from across the country (Madhi, Venter *et al.* 2003) and during 2006-2009 from primarily from hospitalized patients, mostly children, in Steve Biko and Kalafong Academic Hospitals (Pretoria, South Africa) (van Niekerk and Venter 2011).

5.2.2 Sample Selection for Screening and Molecular Characterization

The specimens from all age groups were selected out of RSV single positive cases with available HIV status. Specimens were sorted according to randomly assigned numbers and the first 851/1459 (58%) were selected for molecular characterization.

5.2.3 Detection of Respiratory Viruses

All SARI specimens were tested using the real-time reverse transcription polymerase chain reaction (rRT-PCR) as previously described (section 2.2.6) and screened for parainfluenza 1-3, RSV, influenza A and B, RSV, adenovirus, enterovirus, human metapneumovirus and rhinovirus.

5.2.4 Full RSV G-protein gene amplification

The complete or near complete RSV G genes were amplified by nested PCR using the ABI Gene Amp 2700. Briefly, cDNA was synthesized with random primers using SuperScript™ reverse transcriptase (Invitrogen, Life Technologies, USA) according to

manufacturer's instructions. Full-length RSV G gene were amplified using Platinum®*Pfx* DNA polymerase (Invitrogen, Life Technologies, USA) with primers G1-21 (Trento, Viegas *et al.* 2006) and F164 (Sullender, Sun *et al.* 1993). In brief, 5µl cDNA was added to 7.5µl 10X reaction buffer, 10mM each deoxynucleoside triphosphate (dNTP), 10 pmol each primer (G1-21 and F164), 50mM $MgSO_4$1.25 U of Platinum®*Pfx* DNA polymerase up to 50µl final volume. The following cycling conditions were used: 94°C for 2 min; 40 cycles of 94°C for 15 s, 55°C for 30 s, 68°C for 2 min; and 68°C for 10 min. This was followed by nested PCR with forward primers G32A(RSV-A), G32B(RSV-B), G598A(RSV-A) and G604B(RSV-B) (Venter, Collinson *et al.* 2002) and reverse primers G665R (Venter, Collinson *et al.* 2002) and F1 (Peret, Hall *et al.* 1998).The nested PCR was conducted in a 50µl reaction mixture volume. In brief, 1µl of the first round product was added to 7.5µl10X reaction buffer, 10mM each dNTP, 10 pmol each primer, 50mM $MgSO_4$1.25 U of Platinum®*Pfx* DNA polymerase using the same cycling conditions as the first round PCR cycler used ABI Gene Amp 2700. PCR products were analyzed on a 1% agarose gel against a 100-bp ladder as molecular weight marker (DNA molecular marker XIV; Roche Diagnostics, Mannheim, Germany).

5.2.5 Subgrouping:

A Subgroup was defined based a region spanning 270 nucleotides in RSV-A and 330 nucleotides in RSV-B, representing the second hypervariable region of the G-protein gene (Peret, Hall *et al.* 1998). Subgroup-specific primers were used for subgrouping as described before on full-length G-protein gene by nested PCR with forward primers G52B, G598A, (Venter, Madhi *et al.* 2001, Venter, Collinson *et al.*

2002) and reverse primer F1 (Peret, Hall *et al.* 1998), or directly by sequencing using a combination of the primers listed (Table 5.1).

Table 5.1: Primers selected for amplifying and sequencing the G-protein gene.

Primer	Orientation	Subtype	Purpose[a]	Sequence (5'-3')
G1-21	Forward	A & B	1st round	GGGGCAAATGCAACCATGTCC
G32A	Forward	A	S	GCA AAC ATG TCC AAA ACC AAG
G32B	Forward	B	S	GCAACCATGTCCAAACACAG
G598A	Forward	A	G/ S	GGAAAGAAAACCACCACCAA
G604B	Forward	B	G/ S	AAACCAACCATCAAACCCACA
G665R	Reverse	A & B	G/ S	TTTTGGGGCTCTTTTGTTTG
F1	Reverse	A & B	G/ S	CAACTCCATTGTTATTTGCC
F164	Reverse	A & B	1st round	GTTATGACACTGGTATACCAACC

[a]1st round= 1st round amplification of G-protein, S= complete or near complete G-protein sequencing, G= Genotyping of RSV-A and RSV-B

5.2.6 Nucleotide sequencing

PCR products were purified using the ExoSAP-IT enzyme system (USB Corporation, Cleveland OH). Cycle sequencing was performed with a BigDye terminator 3.1 cycle sequencing kit as recommended by the manufacturer (Applied Biosystems, Foster City, CA). Nucleotide sequencing was carried out on both strands, and the editing was performed with Sequencher® version 5 (Gene Codes Corporation, Michigan, USA).

5.2.7 Phylogenetic analysis

A region spanning 270 nucleotides in RSV-A and 330 nucleotides in RSV-B), representing the second hypervariable region of the G-protein gene, was used for phylogenetic analysis (Peret, Hall *et al.* 1998). The South African RSV sequences were compared against reference sequences of each genotype in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html). All South African strains present in the database from our previous investigations in Pretoria (2006-2009) (Visser, Delport *et al.* 2008, van Niekerk and Venter 2011), Soweto (1997-2001), Agincourt, Pretoria, Bloemfontein, Cape Town and Durban(2000) (Venter, Madhi *et al.* 2001, Venter, Collinson *et al.* 2002) were also included. Nucleotide sequences of RSV-A and RSV-B viruses were aligned separately with ClustalX 1.81(Thompson, Gibson *et al.* 1997)embedded in BioEdit Sequence Alignment Editor version 7.0.4.1(Hall 1999). Because most previously published sequences were based only on the second variable region, phylogenetic analysis was based on this region for all strains.

5.2.6 Nucleotide and amino acid sequence analysis

Pairwise distance (p-distance) was calculated between individual genotypes, as well as within each genotype, using Mega version 5 (Tamura, Dudley *et al.* 2007b). Estimates of evolution were based on the ratio of non-synonymous substitution per non-synonymous site (*Ka*) to synonymous substitutions per synonymous site (*Ks*) calculated with the Modified Nei-Gojobori method in MEGA version 5 (Kumar, Nei *et al.* 2008), where $Ka/Ks>1$ indicates positive selection. Amino acid analysis for individual genotypes were calculated for the full G-protein ecto-domain, of

representative strains of each genotype using Genedoc v 2.6.003 (*www.nrbsc.org/gfx/genedoc/*).

5.2.8 Evolutionary rate and most recent common ancestor (MRCA).

The evolutionary analyses over time were conducted using a coalescent Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in the BEAST (Bayesian Evolutionary Analysis by Sampling Trees) version 1.4.6(Drummond and Rambaut 2007), to estimate rate of evolution. MCMC chains were run for sufficient time to achieve convergence (as assessed using TRACER). Dates were introduced according to the year of isolation. Maximum clade credibility trees were generated using Tree Annotator and all trees were plotted using FigTree v1.1.2 (http://tree.bio.ed.ac.uk/). Using the root-to-tip regression plot as performed with Path-o-Gen version 1.3 (http://tree.bio.ed.ac.uk/software /pathogen); this displays the correlation between phylogenetic branch length and the time of sampling of the viral strains to calculate the most recent common ancestor (MRCA) for all the sequences under analysis.

5.2.9 Selective pressure analysis

Selective pressure analysis was conducted making use of the Datamonkey webserver which estimated the rates of non-synonymous and synonymous changes at each site in a sequence alignment in order to identify sites under positive or negative selection (Kosakovsky Pond and Frost 2005, Pond and Frost 2005, Delport, Poon *et al.* 2010)(www.datamonkey.org).

5.2.10 Statistical analysis

The frequency of RSV subgroups was calculated with Fisher's exact test using STATA 10 (College Station, TX). P values of <0.05 were considered significant. We

implemented a multivariable multinomial regression model. Using the 2009-2013 SARI cases we evaluated factors associated with each RSV type, while adjusting for year of circulation. For this analysis the RSV-A type was defined as the baseline category. Statistical significance was defined as $p<0.05$. The analysis was performed using STATA 12, (Stata Corporation, Texas, USA). Stefano Tempia has assisted the student in running these statistical tests and assisted the student in the interpretation.

5.2.11 Nucleotide sequence accession numbers

Sequences of partial and full G-protein genes were submitted to GenBank under accession numbersKC476656 - KC477097.

5.3 Results

5.3.1 Detection of respiratory viruses and characteristics of RSV-positive patients identified in the SARI surveillance program during 2009-2013

During February 2009-May 2013, we enrolled 20,287 patients meeting the SARI case definition and tested for respiratory viruses. RSV was detected in 2989 (15%) individuals, including 1457 (49%) in which RSV was the sole pathogen identified. The proportion testing positive for RSV varied by age group: 30% (2119/7166) for the 0-1 years age group, 19% (404/2158) for 2-4 years, 7% (56/768) for 5-14 years, 4% (31/804) for 15-24 years, 4% (242/5559) for 25-44 years, 4% (114/2718) for 45-64 years and 3% (22/632) forage >65 years ($p<0.001$).

5.3.2 Evolution of RSV subgroups

Among the 670 RSV positive specimens that could be amplified and investigated further from the SARI program between 2009-2013, RSV-A was detected in 426

(64%),and RSV-B in 244 (36%), p=0.001. RSV-A predominated in three of the five seasons (Table 5.2). Subgroup switching and replacement between genotypes during 1997-2013 are indicated in Figure 5.4. This indicated that all genotypes initially identified in South Africa during 1997 had been replaced by 2013, for both subgroups A and B.

5.3.3 Phylogenetic comparison of strains identified in 2006-2013 relative to earlier periods

5.3.3.1 Diversity:

Maximum likelihood (Figure 5.1) and Bayesian (Figure 5.2) phylogenetic comparison of 670 RSV strains identified in clinical specimens from 2006-2013 to strains identified in earlier studies from 1997-2002 in South Africa, as well as international reference sequences, indicated that 441 (66%) strains detected since 2006 clustered with strains that were formerly assigned to the GA2 genotype. However, none of them clustered with significant bootstrap values to the older strains, while 67 (10.2%) clustered with GA5 (Figure 5.4). Several clusters seemed to have evolved from the GA2 genotype and grouped together with statistically significant bootstrap values to form new genotypes (Figure 5.1A). Some of these clusters were similar to genotype NA1 (421, 43%) and NA2 (20, 2%) previously identified in Japan and ON1 (13, 1.3%) previously identified in Canada with bootstrap support of 72, 82 and 98 respectively, while one cluster was unique to South Africa and putatively named SAA2 (12, 1.8%) (Bootstrap support of 83) ;(Figure 5.1A). Although strong bootstrap values could be obtained for all strains clustering with GA5, 3 GA5 subclusters were identified since its emergence 19 years ago and were named GA5I-GA5III (Fig 5.1A, 5.2A). All of the RSV-B isolates since 2006 clustered within the BA genotype and

had the characteristic 60-nucleotide duplication (Fig 5.1B), all previously identified RSV-B genotypes in South Africa were replaced by the BA genotype. Analysis showed that of 244 RSV-B strains selected since 2006, 139 (14%) clustered with BA9, 106 (11%) with BA10 and 4 (0.4%) with BA8 (Fig 5.3).

Table 5.2: Frequencies of RSV subgroups A and B in South Africa from 1997-2013.

Subgroups	Number (%) of Specimens positive for subtype in:																					
	1997		1998		1999		2000		2006		2007		2008		2009		2010		2011		2012		2013	
A	31	(62)	30	(43)	41	(75)	51	(88)	48	(63)	69	(81)	29	(91)	8	(8)	127	(84)	71	(43)	154	(88)	66	(89)
B	19	(38)	40	(57)	14	(26)	7	(12)	28	(37)	16	(19)	3	(9)	95	(92)	24	(16)	95	(57)	22	(13)	8	(11)
Total	50		70		55		58		76		85		32		103		151		166		176		74	

a) Samples obtained primarily from children aged <5 years from CHBAH during 1997-2000 (Venter, Madhi *et al.* 2001)
b) Samples obtained from children < 5 years from community clinics in Agincourt obtained from 2000-2001, (Venter, Collinson *et al.* 2002) as well as other samples from high-risk children from across the country (Madhi, Venter *et al.* 2003)
c) Samples obtained from hospitalized children at Steve Biko and Kalafong Academic Hospitals (Pretoria South Africa) 2006-2009 (Visser, Delport *et al.* 2008, van Niekerk and Venter 2011)
d) Samples obtained from SARI surveillance program 2009-2013 (in 4 provinces).

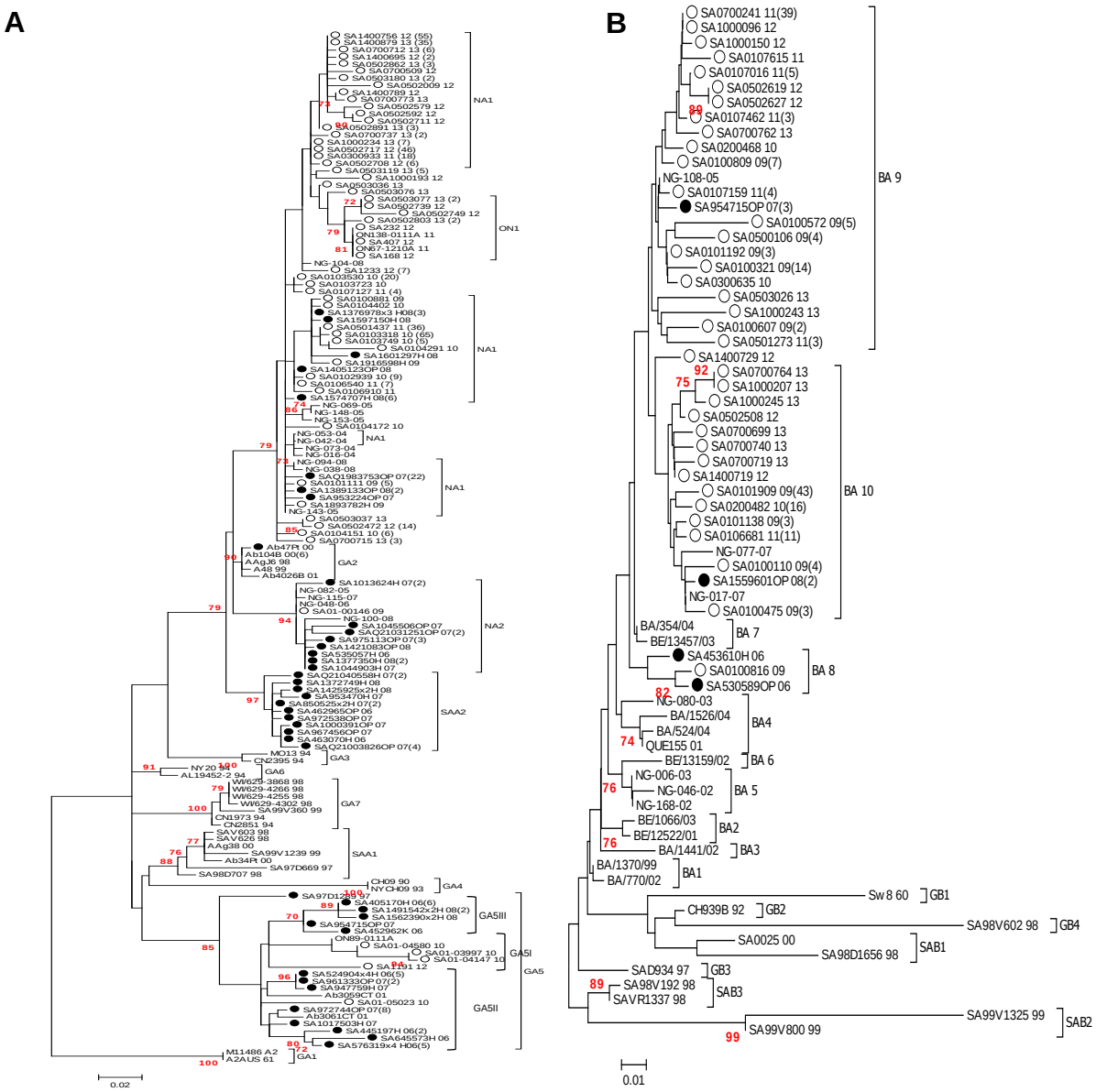
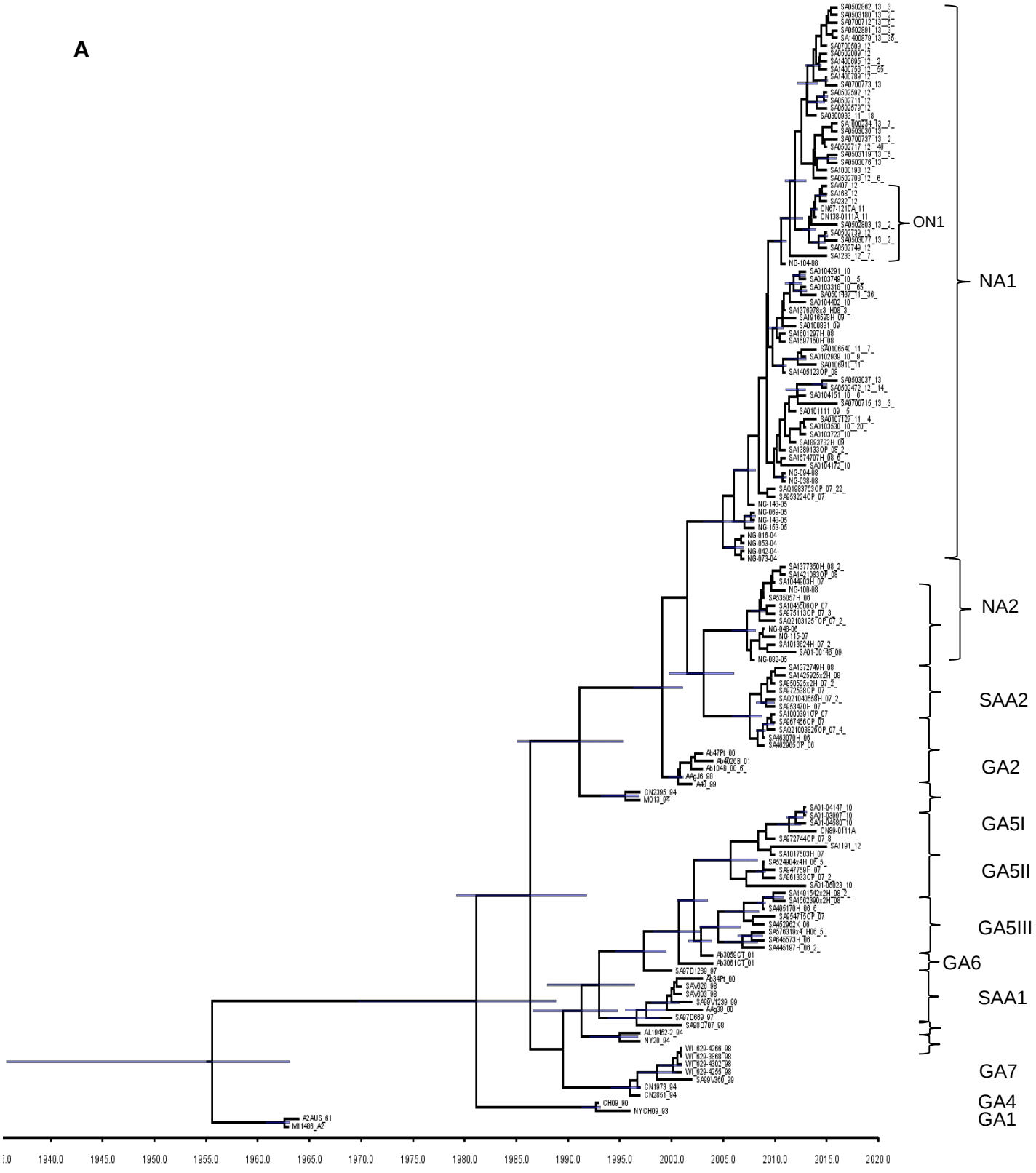


Figure 5.1: Maximum likelihood tree RSV genotypes in South Africa (1997 – 2013).

Midpoint-rooted maximum-likelihood trees for subtype A (A) and subtype B (B) constructed under the HKY codon position substitution model using MEGA version 5 (Tamura, Dudley *et al.* 2007a), drawn to scale with the bars indicating 0.02 nucleotide substitutions. Estimates were based on bootstrap resampling carried out with 1,000 replicates. Only bootstrap values of 70 are shown. The virus designations refer to the place of isolation (SA, South Africa)/isolate number/year of isolation. Sequences with closed circles denote South African sequences from patients from 2006-2008 and open circles from patients from 2009-2013. The number of identical sequences is indicated as (n). The genotypes assigned are indicated at the right by brackets. The prototype strain for subtype A is strain A2 (A2AUS61, Australia), and prototype strains for subtype B are Sw8-60 (Sweden) and 18537 (CH18537_62, United States).



B

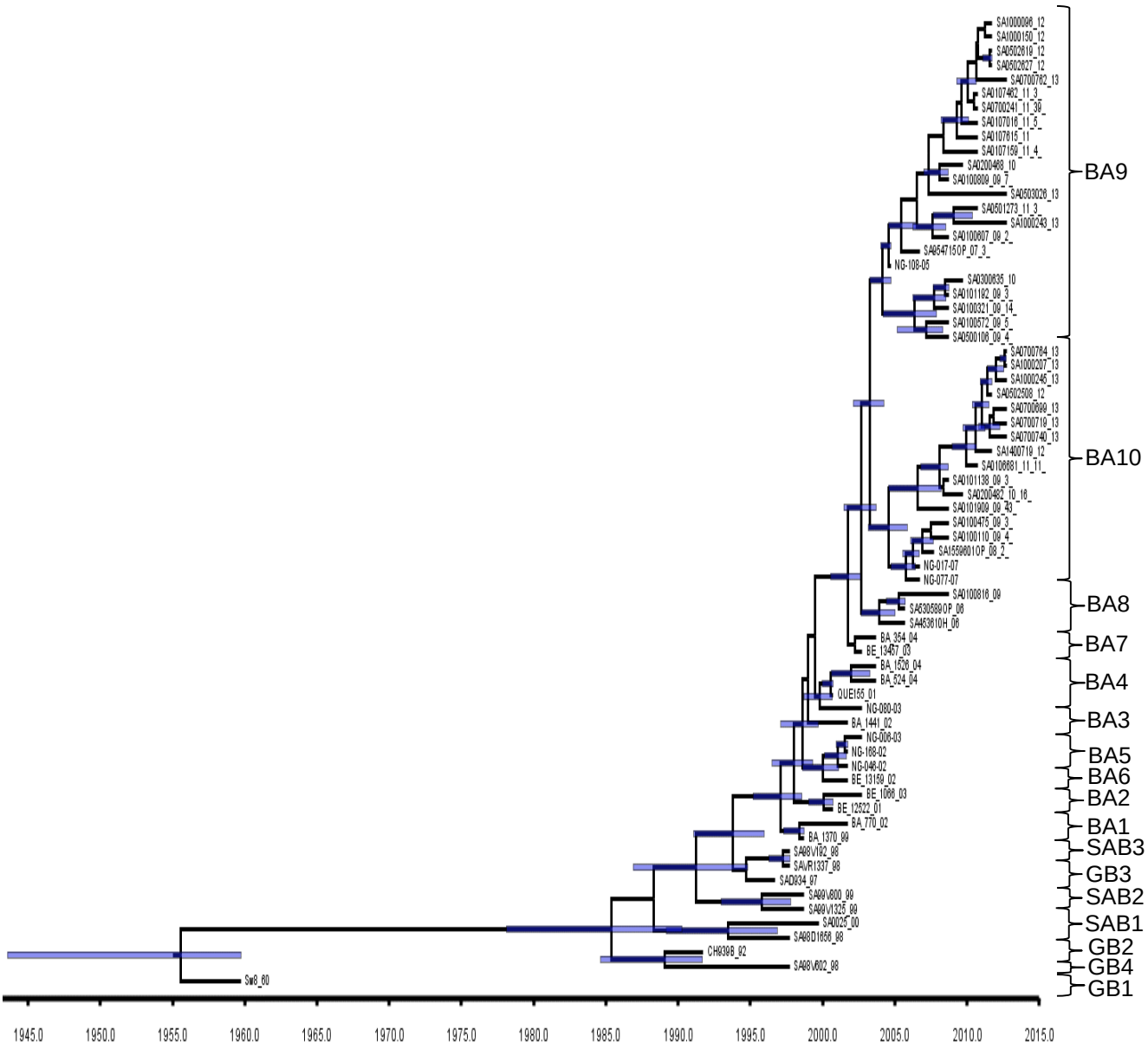


Figure 5.2: Bayesian Analysis RSV genotypes in South Africa (1997 – 2013).

A midpoint-rooted BEAST phylogenetic tree of RSV-A (A) and RSV-B (B) genotypes isolated worldwide since 1960 together with all the South African genotypes isolated during this study (2006 to 2013) and other South African sequences from 1997-2000. The lengths of the horizontal lines are proportional to the genetic distance between viruses. The bar represents 7 substitutions per site. The virus designations refer to the place of isolation (SA, South Africa)/isolate number/ year of isolation. South African strains were compared to strains from different continents, the sources of the reference sequences obtained from Genbank were as follows: NY, New York; AL, Alabama; MO, Missouri; TX, Texas; CH, Rochester; WI, Wisconsin in the United States; ON, Ontario; Que, Quebec, CN, in Canada; MON, Montevideo, Uruguay; MAD, Madrid, Spain, NG, Nagasaki, Japan, BE, Belgium, BA, Buenos Aires and Cam, Cambodia. The prototype strain for subtype A is strain A2 (A2AUS61, Australia).

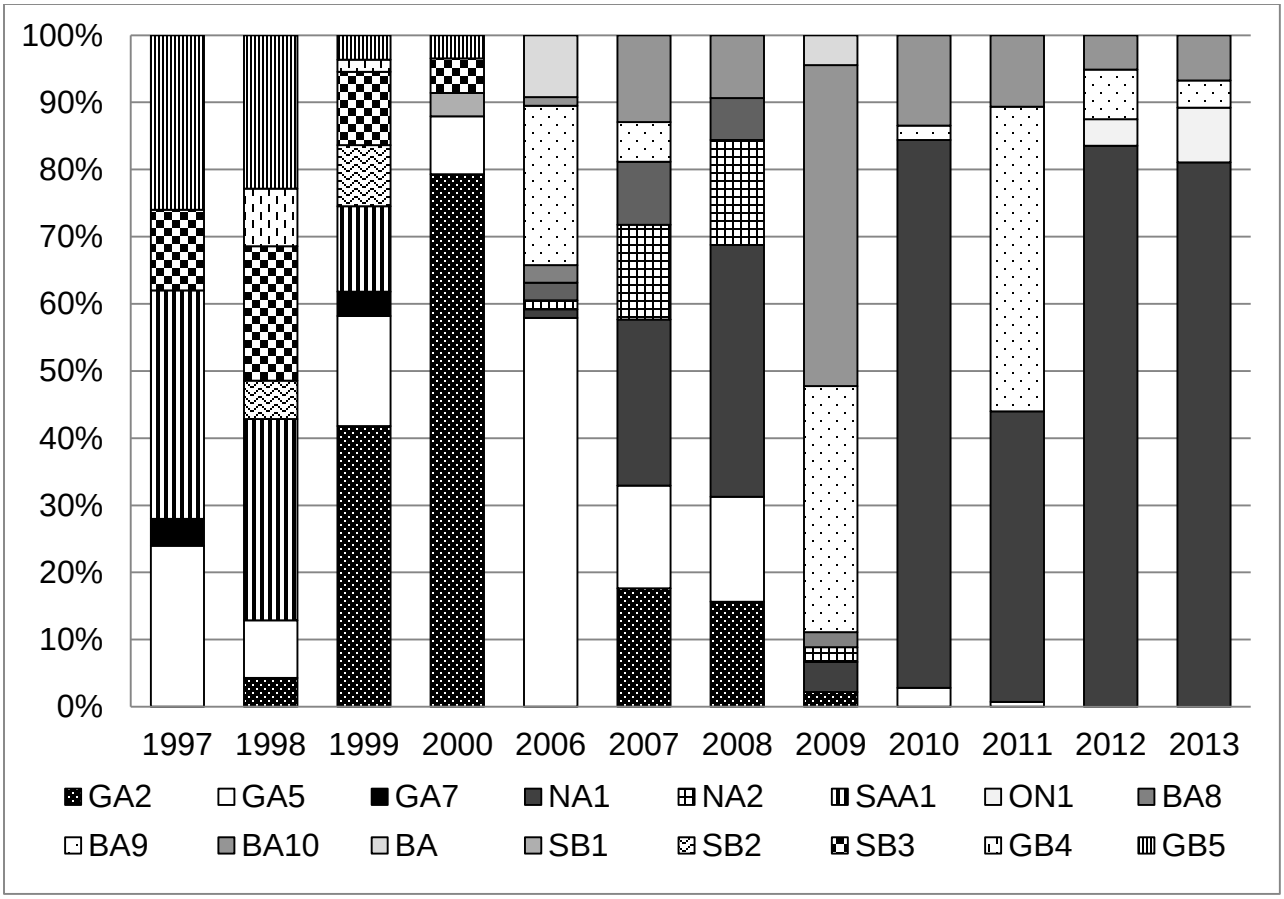


Figure 5.3: Frequencies of RSV genotypes in South Africa (1997 – 2013).

The genotypic profile of RSV obtained from the SARI program (2009-2013) was compared to that of RSV identified previously from 2006-2009 in South Africa. These samples were primarily obtained from hospitalized children at Steve Biko and Kalafong Academic Hospitals (Pretoria South Africa) (van Niekerk and Venter 2011), from children aged <5 years from CHBAH during 1997-2000, (Venter, Madhi *et al.* 2001) and from children < 5 years from community clinics in Agincourt obtained from 2000-2001 (Venter, Collinson *et al.* 2002), as well as other samples from high-risk children from across the country (Madhi, Venter *et al.* 2003).

5.3.3.2 Pairwise distances:

SAA2 strains were distinct from strains belonging to genotypes GA2, NA1 and NA2, (average p-distances of 4.0%, 6.2% and 6.1% respectively).GA5 also displayed the largest intragenotypic range of P-distances (0-12%). The intragenotypic p-distances for the different genotypes are shown on the diagonal of Table 5.3A.The average intergenotypic p-distance between the different genotypes within RSV-A ranged from 4to 18%; including 13.2% difference between the two dominant genotypes NA1 and GA5. Among the 426 RSV-A isolates from 2009-2013, five termination codons were used, 20 (5%) used the UAG stop codon, 346 (81%) used UGA, and 2 (0.5%) used UAA at position 894, while 55 (13%) used stop codon UAG at position 895 and 3 (0.7%) used UGA in position 964.

For the RSV-B strains, the intragenotypic p-distances for the different genotypes detected are shown on the diagonal of Table 5.3B. The average intergenotypic p-distance ranged from 2-18%, with 4.3% difference between the two dominant genotypes, BA9 and BA10. Among the 244 RSV-B isolates identified from 2009-2013, two termination codons were used: 223 (91%) used UAA at position 931, and 21 (9%) used UAG at position 952. Figure 5.4 indicates the amino acid alignment of the full G-protein of genotypes identified in South Africa from 1997-2013 relative to the reference strains.

Table 5.3: Average percentage nucleotide p-distance between and within RSV-A (A) and RSV-B (B) South African strains used in Figure 5.1. P-distances calculated between the individual genotypes are shown below the diagonal in each column of the table, and the range of p-distances within each genotype is highlighted on the diagonal.

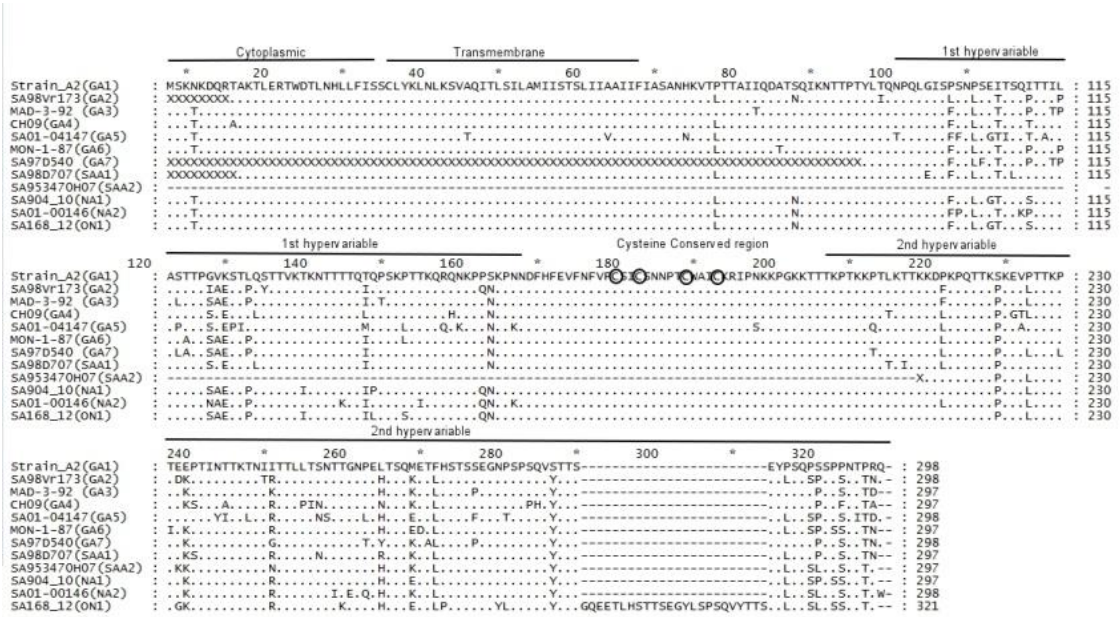
A

	GA1	GA2	SAA1	GA5	GA6	GA4	GA7	GA3	NA1	NA2	ON1	SAA2	
GA1	**0.0**	n/c											
GA2	12.6	**2.7**	(0-6)										
SAA1	14.3	7.6	**2.7**	(0.4-7)									
GA5	17.2	11.4	10.8	**5.4**	(0-12)								
GA6	12.9	6.0	5.9	9.9	**1.9**	(0-2)							
GA4	18.6	13.5	12.5	15.9	12.2	**0.0**	n/c						
GA7	14.0	7.8	8.3	11.5	6.5	12.6	**1.0**	(0-2)					
GA3	14.2	5.8	7.8	11.6	5.4	12.9	7.4	**3.5**	(0-6)				
NA1	14.7	4.9	10.2	13.2	8.6	15.6	10.5	8.2	**2.6**	(0-7)			
NA2	14.3	5.0	10.3	13.8	8.7	15.0	10.4	8.3	5.8	**3.0**	(0-10)		
ON1	17.7	6.2	12.6	16.1	10.6	18.1	13.1	10.4	4.6	7.6	**0.2**	(0-0.6)	
SAA2	13.3	4.0	9.3	12.5	7.8	15.1	9.0	7.4	6.2	6.1	7.5	**1.3**	(0-3)

B

	GB1	GB2	GB3	GB4	SAB1	SAB2	SAB3	BA1	BA2	BA3	BA4	BA5	BA6	BA7	BA8	BA9	BA10
GB1	**2.59**	n/c															
GB2	8.10	**n/c**	n/c														
GB3	11.47	6.30	**n/c**	n/c													
GB4	15.06	8.42	11.47	**3.16**	(0.3-5)												
SAB1	10.72	5.79	7.75	11.50	**5.27**	n/c											
SAB2	14.47	9.02	7.94	11.77	11.01	**5.33**	n/c										
SAB3	11.80	5.90	3.96	10.13	7.80	6.66	**0.53**	n/c									
BA1	10.86	5.14	3.03	10.50	7.12	6.78	2.78	**0.66**	(0-1)								
BA2	11.51	4.74	4.73	11.42	7.78	8.50	4.50	2.54	**0.59**	(0.6-1)							
BA3	12.26	6.04	4.98	10.68	9.07	8.74	4.75	2.75	3.74	**0.44**	n/c						
BA4	12.80	5.98	4.91	12.45	8.45	8.71	4.71	2.72	3.20	3.04	**1.85**	(0.3-4)					
BA5	11.82	5.07	4.13	11.50	8.10	7.88	3.89	2.09	3.11	2.41	2.38	**0.84**	(0-2)				
BA6	10.47	4.75	3.68	11.16	7.78	7.44	3.44	1.65	2.65	2.86	2.80	2.20	**n/c**	n/c			
BA7	11.86	6.15	5.09	12.57	9.19	8.86	4.85	3.20	3.60	3.52	2.72	2.86	3.31	**1.50**	(0.3-5)		
BA8	11.75	5.92	5.33	12.51	9.02	8.98	5.09	3.03	3.15	3.35	2.69	2.70	3.14	2.33	**1.81**	(0.3-5)	
BA9	11.45	5.85	4.80	12.15	8.46	8.58	4.58	3.34	3.76	3.83	3.03	3.16	3.35	2.54	2.65	**2.06**	0-7)
BA10	12.39	6.67	5.61	13.05	9.72	9.32	5.37	3.47	3.71	3.94	3.26	3.26	3.42	2.70	2.84	3.07	**2.23**	(0-8)

A



B

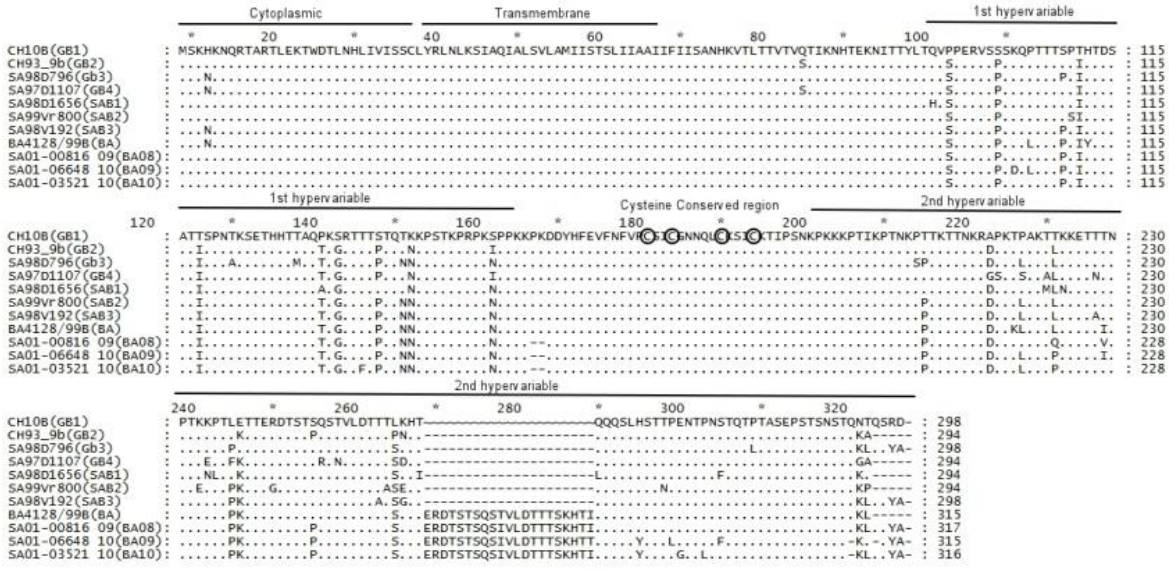


Figure 5.4: The amino acid alignment of RSV-A (A) and RSV-B (B) indicates the genotypes identified in South Africa from 1997-2013 relative to the reference strains.

5.3.3.3 Analysis of selective pressure:

Full RSV G-protein bioinformatics calculations were based on the complete RSV G-proteins of strains sequenced from the SARI samples and representative full RSV G-protein sequences for each of the genotypes previously identified in South Africa and elsewhere available on GenBank. The genetic changes observed due to nucleotide substitution were as follows: 196 substitutions in RSV-A strains, with 75synonymous and 120 non-synonymous; with 203 substitutions in RSV-B strains, with 94 synonymous and 109 non-synonymous. The Nei-Gojobori method identified positive selection within both RSV-A and RSV-B. Genotypes SAA1, GA6, GA3, SAB1, GB4 and BA5 had *Ka*/*Ks* ratios of 1.304, 1.177, 1.051, 1.07, 1.26, and 1.42 respectively; where a ratio >1 indicates positive selection. Investigation of *Ka*/*Ks* ratios between genotypes that emerged from the GA2 genotype indicated intergenotypic positive selection between NA1, SAA2 andON1. Intergenotypic positive selection was also identified within the BA genotype between sub-genotypes BA1-BA5 and BA7(Nei and Gojobori 1986, Kosakovsky Pond and Frost 2005, Pond and Frost 2005).Calculations for positive selective sites identified 16 sites in the G glycoprotein gene that had a posterior probability >0.5, which indicates positive selection in both RSV-A and B (Kosakovsky Pond and Frost 2005, Pond and Frost 2005, Delport, Poon *et al.* 2010). For RSV-A, 2 positively selected sites were located in the first hypervariable region (amino acid positions 94 and 136), and six positively selected sites were located in the second hypervariable region (amino acid positions 233, 237, 250, 262, 274 and 290). Four amino acids (positions 237, 262, 274 and 290) had posterior probabilities for positive selection above 90%: one (position 233) was>80%, two (positions 136 and 250) were

>70% and one (position 94) was >60%. For RSV-B, one positively-selected site was located in the first hypervariable region (amino acid position 103) and seven positively selected sites were located in the second hypervariable region (amino acid positions 209, 245, 248, 251, 268, 269 and 285). Four amino acids (positions 251, 268 269 and 285) were identified to be under positive selection above the 90% level: one (position 248) was >80%, two (positions 103 and 209) were >70%, and one (position 245) was >50%.

5.3.3.4 Evolutionary rates:

The evolutionary rate was estimated as $4.68X10^{-3}$(95% HPD, $3.5499X10^{-3}$ to$5.8881X10^{-3}$) substitutions/site/year for RSV-A and $5.8907X10^{-3}$(95% HPD, $14.2816X10^{-3}$ to$7.6298X10^{-3}$) substitutions/site/year for RSV-B. A root-to-tip transgression plot estimation indicated the MRCA to date back to 1945 for RSV-A and to 1957 for RSV-B.

5.3.4 Disease association of RSV-A and B.

On multivariable analysis adjusting for year of circulation RSV-B infection was associated with both a symptom duration of more than 3 days (aRRR: 2.8; 95% CI 1.1-7.6) and a hospitalization stay of more than 7 days (aRRR: 2.9; 95% CI 1.2-7.1) compared to RSV-A.

No significant difference was observed between RSV-A and RSV-B over the different year, however it was related to which ever subtype was dominant that year (Table 5.4).

Table 5.4: Factors associated with RSV type among patients hospitalized with severe acute respiratory illness, South Africa, 2009-2013

Factor	Univariable analysis			Multivariable analysis[d]
	RSV-A[a]	RSV-B		RSV-B
	n/N (%)	n/N (%)	RRR[b] (95% CI)	aRRR[c] (95% CI)
Year				
2009	4/354 (1)	74/215 (34)		
2010	126/354 (36)	24/215 (11)	**0.01 (0.003-0.03)**	**0.01 (0.003-0.03)**
2011	65/354 (18)	94/215 (44)	**0.08 (0.02-0.2)**	**0.07 (0.02-0.2)**
2012	103/354 (29)	17/215 (8)	**0.01 (0.002-0.03)**	**0.01 (0.001-0.02)**
2013	56/354 (16)	6/215 (3)	**0.01 (0.001-0.02)**	**0.003 (0.001-0.02)**
Age (years)				
<1	254/354 (71)	154/214 (72)		
1-4	53/354 (15)	33/214 (15)	1.02 (0.6-1.7)	**1.9 (1-3.7)**
5-24	7/354 (2)	8/214 (4)	1.8 (0.6-5.3)	2.5 (0.6-10.8)
25-44	30/354 (8)	13/214 (6)	0.7 (0.4-1.4)	0.5 (0.2-1.2)
45 +	11/354 (3)	6/214 (3)	0.9 (0.3-2.5)	0.6 (0.2-2.1)
Duration of symptoms >3 days	80/352 (23)	48/213 (23)	0.9 (0.6-1.5)	**1.8 (1-3.2)**
Length of hospitalization > 7 days	73/345 (21)	61/211 (29)	**1.5 (1-2.2)**	**3.0 (1.7-5.4)**
HIV infection	53/327 (16)	29/192 (15)	0.9 (0.5-1.5)	
Oxygen therapy	165/348 (47)	98/214 (46)	0.9 (0.6-1.3)	**0.6 (0.4-0.9)**
ICU	0/348 (0)	2/214 (0.9)		
Patient died	7/346 (2)	4/212 (2)	0.9 (0.2-3.2)	

[a.]Reference group for the multinomial regression model. [b] Unadjusted relative risk ratio (RRR) at univariable analysis. [c] Adjusted relative risk ratio (aRRR) at multivariable analysis. [d] Only covariates significant at the multivariable analysis are reported. RRR highlighted in bold indicate factors significant at p<0.05

5.4 Discussion

The implementation of SARI surveillance in four South African provinces with distinct geographic and climatic conditions provided an opportunity to analyse the molecular epidemiology of RSV at a national level, expanding on previous work undertaken in South Africa (Venter, Madhi *et al.* 2001, Venter, Collinson *et al.* 2002, Madhi, Venter *et al.* 2003). Phylogenetic analysis in this study confirmed previous observations that similar genotypes dominated in different locations in South Africa and globally during one season (Venter, Collinson *et al.* 2002, Zlateva, Lemey *et al.* 2004a, Zlateva, Lemey *et al.* 2005, Matheson, Rich *et al.* 2006). We also identified strains that were identical to a new genotype (ON1, identified in Canada in 2011 with a 72-bp insertion) not previously seen in South Africa, confirming the global spread of RSV strains (Eshaghi, Duvvuri *et al.* 2012). The SARI surveillance data confirmed that although the RSV season peaked between February-May each year, on-going low-level circulation outside of the epidemic season was detected and may contribute toward the persistence of strains between seasons. Although drift within genotypes was also visible over the seasons, strains from later seasons were further from the root of each genotype across the tree. Figure 5.3 illustrates genotype distribution over 16 years, and indicates that different genotypes co-circulate within a season, with certain genotypes dominating and then declining before being replaced with a different genotype; all genotypes originally described for both subtype A and B were replaced by 2012 (Venter, Madhi *et al.* 2001, Matheson, Rich *et al.* 2006, Zlateva, Vijgen *et al.* 2007). Although several studies have suggested that RSV-A produce more severe disease than RSV-B, we like others have

been unable to attribute any disease severity to RSV-A or B specifically (Devincenzo 2004, Oliveira, Freitas *et al.* 2008) and results suggest that genotype switching re-establishes annual epidemics and reinfection throughout life supporting the recent findings that genomic differences within the G-protein has been associated with disease severity (Fletcher, Smyth *et al.* 1997, Martinello, Chen *et al.* 2002).

The GA2 genotype was first detected in 1998 and was continuously detected up to 2009 (van Niekerk and Venter 2011). In 2007, new clusters emerged closely related to GA2, but with insignificant bootstrap values and p-distances greater than 4%. One of these clusters was unique to South Africa and we named it SAA2, to follow on the previous genotype identified here in 1997, SAA1. The other clusters resembled genotypes identified in Japan, before NA1, the dominant genotype from 2010-2012. The ON1 genotype identified in 2011 was the first instance where a duplication insertion was described for RSV-A (Eshaghi, Duvvuri *et al.* 2012). It will be interesting to follow this genotype over the next seasons to see if it will have the same impact on RSV-A evolution as the BA genotype had for RSV-B, and if the 72-nucleotide duplication will provide an evolutionary advantage to the virus. Drift also occurred in the GA5 genotype, with p-distance analysis suggesting that 3 subclusters, GA5I-III, could be identified.

Investigation of RSV-B evolution over the 16 years showed that the BA genotype had not only replaced all the previously identified RSV-B genotypes in South Africa (van Niekerk and Venter 2011), but is also undergoing positive selection and evolving into new genotypes. The BA genotype has become dominant since it was first described in 1999 across most parts of the world (Venter, Madhi *et al.* 2001, Visser, Delport *et al.* 2008, Shobugawa, Saito *et al.* 2009, Rebuffo-Scheer, Bose *et al.* 2011, van Niekerk and

Venter 2011, Yamaguchi, Sano *et al.* 2011, Papenburg, Carbonneau *et al.* 2012). In the early investigation of the natural history of the BA genotype, it was divided into six sub-genotypes, BA1-BA6 (Trento, Viegas *et al.* 2006). Further genetic drift since its emergence 13 years ago resulted in four new sub-genotypes, BA7-BA10 (Dapat, Shobugawa *et al.* 2010). The South African RSV-B strains identified since 2006 belong to two main genotypes, BA9 and BA10, and none of the South African-specific RSV-A and RSV-B genotypes identified in earlier studies (SAA1, SAA2, and SAB1-3), were identified to be circulating during 2009-2012 (van Niekerk and Venter 2011).This may be due to community-wide immunological pressure induced by previous exposure.

We identified seven novel positively-selected sites in RSV-A (2 sites) and RSV-B (5 sites) located mainly in the second hypervariable region for the G-protein gene, confirming the value of this region in phylogenetic analysis (Zlateva, Lemey *et al.* 2004b, Zlateva, Lemey *et al.* 2005, Botosso, Zanotto *et al.* 2009). This suggests that evolution of both RSV-A and B is driven by the selection of new variants due to positive selective pressure at certain codon positions. Unlike other respiratory viruses, such as influenza, it would be impossible to make predictions about future evolutionary changes to the G-protein, since the mechanism that drives changes in the neutralization site is not completely understood; however immune pressure seems to be a likely explanation because positive selection has been identified. Although, random changes may also occur probably due to mistakes introduced by the polymerase and mutations retained could have an evolutionary advantage for the virus. Use of alternative stop codons and tolerance to large insertion mutations contribute to the higher evolutionary rate of RSV-B (Zlateva, Lemey *et al.* 2004a, Zlateva, Lemey *et al.* 2005, Matheson, Rich *et al.*

2006). Emergence of a subgroup A strain with a 72bp insertion could also provide evolutionary advantage to subgroup A. Our study is subject to a number of limitations: we report on several different studies, where differences in design and study population enrolled (in terms of age and severity of disease) made it difficult to assess the potential association of different genotypes with disease severity among the different studies. There is also a 4-year gap (2002-2005) in the RSV molecular data, where we could not report on the molecular changes in RSV.

We have been investigating RSV genetic and antigenic variation in patients over the past 15 years. Data from these studies reflect findings from other parts of the world but also identified differences unique to our population, and may provide some insight about RSV molecular evolution in the rest of Africa. We identified several genotypes for the first time in South Africa that have been identified across the world, suggesting that emergence of new RSV strains in distant areas could contribute to RSV strains circulating globally, and to the reoccurrence of annual epidemics.

Chapter 6

Naturally occurring Respiratory Syncytial Virus A and B Glycoprotein ecto-domain deletion mutants- association with severe acute respiratory illness in HIV-infected South Africans

6.1 Introduction

RSV is a negative-sense, single-stranded RNA virus of the family *Paramyxoviridae*, with genome length of ~15Kbp. It readily infects and re-infects throughout life, despite maternal antibodies or antibodies induced from prior infections, without the need for significant antigenic change (Bukreyev, Yang *et al.* 2008).Two major groups, RSV-A and RSV-B, have been identified based on antigenic and genetic variability (Peret, Hall *et al.* 1998, Peret, Hall *et al.* 2000). Most studies have been based on the changes observed in the RSV G-protein, which consists of an cytoplasmic region, a transmembrane region, an ecto-domain consisting of a central region of four conserved cysteines and a putative receptor binding site, flanked by two hypervariable regions (Garcia, Martin *et al.* 1994).

Previous studies have shown that the G-protein have the ability to accommodate not only large frameshift mutations leading to truncations and shorter proteins (Melero, Garcia-Barreno *et al.* 1997, Martinez, Valdes *et al.* 1999), but also the deletion of the central conserved domain and cysteine noose, which suggested that the G-protein is not required for infection(Teng and Collins 2002). The complete deletion of the G-protein inhibits the replication of the virus in the respiratory tract of mice, suggesting that the G-protein is important for the replication of virus *in vivo*(Teng, Whitehead *et al.* 2001). Venter *et al.* previously identified subtype B strains lacking nearly the entire G-protein ecto-domain in one HIV-infected and one HIV-exposed-uninfected child hospitalized with pneumonia (Venter, van Niekerk *et al.* 2011). Implications of infection with RSV strains carrying mutations in the G-protein on clinical disease presentation are not clear. We aimed to investigate the prevalence of these mutants, its association with

HIV infection, severity of infection, and subtype-specificity in a larger cohort of HIV-infected and -uninfected individuals of all ages with severe acute respiratory illness (SARI) and to compare the prevalence of these mutations to RSV strains from individuals with non-hospitalized influenza-like illness (ILI) and asymptomatic control persons enrolled though a prospective surveillance program in South Africa.

6.2 Materials and Methods

6.2.1 Study design and population

SARI Surveillance: Study samples were obtained from participants enrolled in a prospective hospital-based surveillance program for SARI initiated in February 2009 which aimed to describe the aetiology and risk factors for acute lower respiratory tract infection (ALRI) in all age groups in South Africa. The methodology and case definitions of this study have been previously described (section 2.2.2) (Cohen, Moyes *et al.* 2013). All patients were enrolled only once and followed through until discharged from the hospital.

ILI and Control Surveillance: Study samples were obtained from participants enrolled in an active surveillance program for ILI and asymptomatic controls initiated in May 2012 through June 2013. The methodology and case definitions of this study have been previously described (section 4.2.1).

6.2.2 Sample Selection for Screening and Molecular Characterization

A total of 1012 samples were selected from all surveillance cohorts as outlined below.

2009-2013 SARI cases: The specimens from all age groups were selected out of RSV single positive cases with available HIV status. Specimens were sorted according to

randomly assigned numbers and the first 851/1459 (58%) were selected for molecular characterization.

2012-2013 ILI and controls cases: All single positive RSV specimens with available HIV status from individuals of all age groups among those enrolled as ILI and control patients were selected. In total 2067 ILI cases were enrolled and 544 control cases out of these (127/2067; 6%) ILI and (34/544; 6%) controls fit the selection criteria and were also evaluated.

6.2.3 RSV detection

Respiratory specimens (i.e., nasopharyngeal aspirates for children <5 years of age and nasopharyngeal and oropharyngeal swabs from individuals ≥5 years of age) were collected and placed in viral transport medium, stored at 4-8°C and transported to the National Institute for Communicable Diseases within 72 hours of collection for testing. All specimens were tested for the presence of 10 respiratory viruses using the real-time assay as described (section 2.2.6). Among consenting study patients, HIV status was established by enzyme-linked immunosorbent assay (ELISA) or PCR depending on age (Cohen, Moyes *et al.* 2013).

6.2.4 Screening for G-protein deletion mutants:

The complete or near complete RSV G genes were amplified by nested PCR as previously described (section 5.2.4). PCR products were analyzed on a 1% agarose gel against a 100-bp ladder as molecular weight marker (Hyperladder I; Bioline, London, UK) and the specimens with significantly smaller product were selected for further screening. Limiting dilution of the cDNA was made as described by Rousseau *et al.,*

2006 (Rousseau, Birditt *et al.* 2006) and the G-protein nested PCR repeated. PCR Product was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Cycle sequencing was performed with a BigDye terminator 3.1 cycle sequencing kit as recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). Sequences were assembled using Sequencher® version 5 (Gene Codes Corporation, Michigan, USA) and alignments were performed using MAFFT multiple sequence alignment program (Katoh and Standley 2013). A region spanning 897 nucleotides representing the G-protein gene was used for phylogenetic analysis.

6.2.5 RSV genome sequencing

6.2.5.1 Nucleic acid extraction

The remaining aliquots of each of the confirmed deletion mutants were pooled and the viral RNA manually extracted. Briefly, after the samples were pooled and vortexed the samples were centrifuged for 20 min at 4000*g* to collect all cell debris. The supernatants were collected and filtered through a 0.45µm filter to remove bacterial contamination. After which the supernatant was concentrated using the Amicon Ultra-15 filter (Merck Millipore, Billerica, MA USA) for 25 min at 4000*g*. The concentrate was collected and TRIzol® (Invitrogen, Life Technologies, USA) was added in a 1:3 ratio and incubated at room temperature for 5 min after which chloroform was added in a 1:5 ratio, shaken incubated at room temperature for 5 min and centrifuged for 15 min at 12000*g*, the RNA aqueous phase was removed and the viral RNA extracted using the QIAamp® Viral RNA Mini (Qiagen, Valencia, CA USA) according to the manufacturer's instructions. The extracted RNA was treated with 500 U of DNase for 1 h at 37°C. The extracted RNA

was treated with Ribo-Zero™ Magnetic Gold Kit* (Epidemiology) (epicentre® Madison, WI USA) according to manufacturer's instructions to remove all rRNA contamination and the resulting RNA elute was cleaned up using the RNeasy® MinElute® Cleanup (Qiagen, Valencia, CA USA).

6.2.5.2 Illumina MiSeq Sequencing

The library preparation and next generation sequencing was done by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria Gauteng, South Africa). Briefly, the RNA obtained from the manual extraction was converted into double stranded cDNA using the Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Fisher Scientific Inc. Waltham, MA USA) according to manufacturer's instructions and 1ng of double stranded cDNA was used as input material for the Nextera DNA Sample Preparation Kits (Illumina San Diego, CA USA) according to manufacturer's instructions. Sequences were assembled using CLC Genomics Workbench (CLC Bio, Cambridge MA USA) and contig sequences were obtained.

6.2.6 Phylogenetic analysis of full genome sequences

6.2.6.1 Maximum likelihood analysis

Alignments were performed using MAFFT multiple sequence alignment program (Katoh and Standley 2013). The nucleotide substitution model; general time reversal model (GTR) +I+G (where the rate variation among sites in the sequences was described using gamma distribution (G) and proportion of invariable sites (I) in the dataset) used in the Maximum likelihood (ML) analysis was determined using jModelTest(Guindon and

Gascuel 2003, Darriba, Taboada *et al.* 2012) and the RSV full genome ML trees were generated using PhyML 3.0 (Guindon and Gascuel 2003, Guindon, Lethiec *et al.* 2005).

6.2.6.2 Synonymous and Non-synonymous Mutations

Mutations in each CDS (CoDing Sequence) were analyzed by running codon aligned sequences for each gene were through the Highlighter program (http:// http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/HIGHLIGHT_XYPLOT/highlighter .html) (Keele, Giorgi *et al.* 2008) and SNAP (http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html) (Korber 2000) was used to calculate the variability for each CDS. Alignments included the CDS sequences from the available whole genomes for RSV A genotype NA1 (JF920046-54, JX015479-98, KC731483 and KF530261). The Non-Synonymous differences were plotted using excel and the unique non-synonymous changes in the truncated sequences were indicated.

6.2.6.3 Sequence Variability and Selective pressure.

Pairwise amino acid distances (p-distance) were calculated between individual genotypes, as well as within each genotype, using Mega version 5(Tamura, Dudley *et al.* 2007b) for each of the gene segments. The evolutionary pressures for each CDS regions was quantified as an average ratio of substitution rates at non-synonymous (dN) and synonymous (dS) sites for the two RSV A specimens with whole genome sequences, the SNAP program (Korber 2000) was used for these calculations. The calculated dN/dS ratios was used to estimate selective pressure where dN/dS>1 indicates positive selection, dN/dS<1 purifying selection and dN/dS=1 neutral selection.

6.2.6.4 Protein Conformation analysis

The CDSs of each region of the SAT strains were analyzed using DeepView - Swiss-PdbViewer (http://www.expasy.org/spdbv/) (Guex and Peitsch 1997) to determine if any of the unique non-synonymous changes identified in the SAT strains resulted in a theoretical change in protein conformation.

6.2.7 Accession Numbers for reference sequences

The nucleotide sequences from the Dutch (JQ901447-JQ901458), Belgian (JX015479-JX015499) (Tan, Lemey *et al.* 2012), Milwaukee (JF920046-JF920070; JN032115-JN032123) (Rebuffo-Scheer, Bose *et al.* 2011), Singapore (GU591758-GU591771) (Kumaria, Iyer *et al.* 2011) studies and the prototype reference strains A2 (M74568.1), Long (AY911262.1), Line19 (FJ614813.1) were included in protein analysis studies.

6.2.8 Inflammatory cytokines and chemokines

6.2.8.1 Sample Selection for Cytokine assay

To further characterize the pro- and anti-inflammatory cytokine and chemokine response a total of 81 samples were selected from all surveillance cohorts as outlined below

2009-2013: SARI cases

The 2009-2013 SARI cases was comprised of randomly selected SARI patients ≥ 1 year old with single positive RSV infection (n= 470), with available HIV data enrolled from February 2009 through June 2013 at all 6 sentinel sites in four provinces. The specimens for each individual selection was sorted according to the randomly assigned numbers and for RSV the first 66 were selected (14%, 66/470) for cytokine analysis.

2012-2013: ILI and controls

The 2012-2013 ILI and control cases enrolled at the outpatient clinics situated in KwaZulu Natal and North-West Provinces during the period of May 2012 through June 2013. All available samples collected from patients ≥ 1 year old with available HIV data with single positive RSV infection (ILI n=7); (Controls n=3) enrolled during this time period at the sites were selected.

South African Deletion Mutants

All 5 deletion mutants were included in the cytokine selection.

6.2.8.2 Cytokine assay

The level of inflammatory cytokines and chemokines released during infection was measured by using the Bio-PlexPro™ Human Cytokine Group I 15-plex assay (BioRad Laboratories Inc. USA) targeting Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferon (IFN)-γ, Interleukin (IL)-6, IL-8, IL-10, IL-7, IL-12, IL-13, Monocyte chemoattractant protein (MCP)-1, Granulocyte-colony stimulating factor (G-CSF), Eotaxin, IL-1ra, IL-15, Interferon gamma-induced protein (IP)-10 and Regulated on activation, normal T cell expressed and secreted (RANTES). The Bioplex system contained dyed beads conjugated with monoclonal antibodies targeting specific cytokines and chemokines. The assays were performed as per manufacturer's instructions and cytokine levels were expressed in picograms per milliliter. Cytokine responses in negative healthy controls were used to normalize cytokine responses in study samples.

6.2.8.3 Statistical analysis

All values obtained were expressed as an absolute concentration and normalized using the mean of the negative control samples. The distribution of the data was significantly different from a normal distribution (skewness and kurtosis); consequently unpaired non-parametric statistical methods were applied. Differences in the levels of cytokines and chemokines were assessed between the various clinical groups using the Kruskal-Wallis test and correcting for multiple comparisons using the Dunn's test. P-values < 0.05 were considered significant. All data was analyzed using GraphPad Prism 6®, GraphPad Software Incorporated (La Jolla, USA).

6.3 Results

6.3.1 Initial identification of South African deletion mutants

The full length G-protein were successfully amplified and sequenced in 61% (622/1021). Of these, 570 (92%) were SARI cases, 47 (8%) were ILI cases and 5 (0.8%) were controls. The HIV prevalence was 36% (206/570) among SARI cases, 15% (7/47) among ILI cases, however due to our selection criteria for the controls the HIV prevalence was higher at 60% (3/5). Following limiting dilution PCR, PCR amplicons of 400bp, which was smaller than the expected size for full-length (980bp) G-protein sequence were detected in 5/622 (0.8%) specimens (Figure 6.1). An additional four cases that consisted of two species of G-protein were detected, a full length G-protein amplicon present at low levels as well as a smaller deletion mutant species (Figure 6.2). Phylogenetic analysis and investigation of deduced amino acid alignments showed that

4 of these strains were identified as RSV-A with G deletions corresponded to the following amino acid positions: 128 to 317, 64 to 267,109 to 281 and 103 to 317 (Figure 6.2A). The remaining case was identified as harbouring a subtype B strain with deletions corresponding to amino acids 112 to 307 (Figure 6.2B). All 5 cases (5/206; 2.4%) were hospitalized with SARI and were HIV positive. Three (60%) were children less than 2 years of age. All 5 cases were discharged from the hospital and survived the infection (Table 6.1).

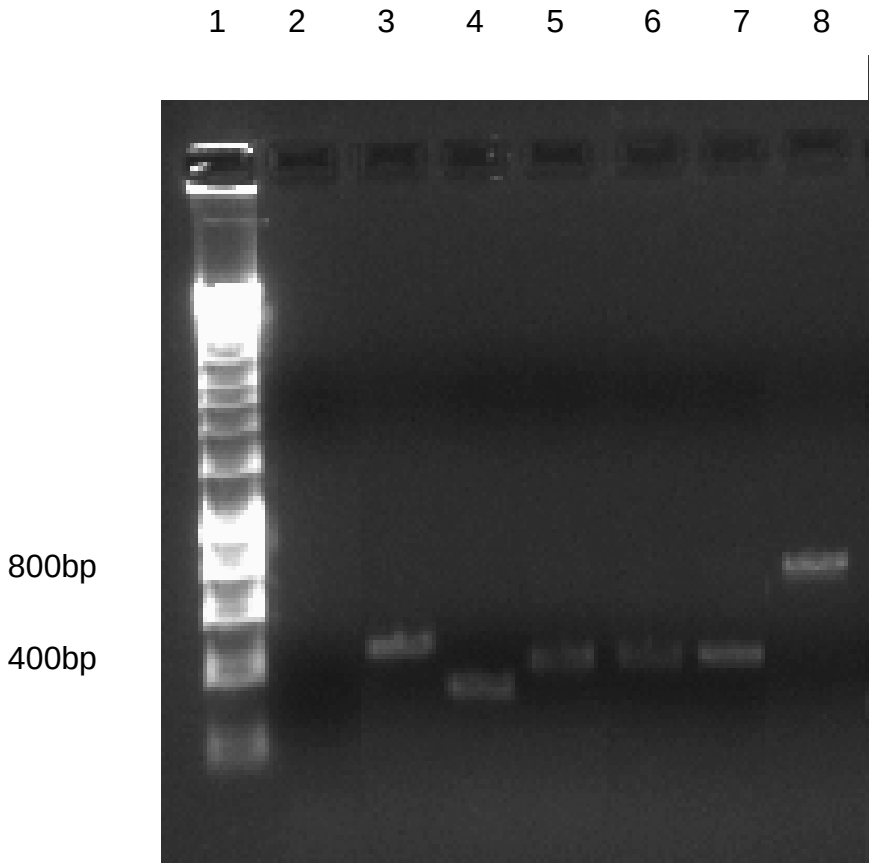
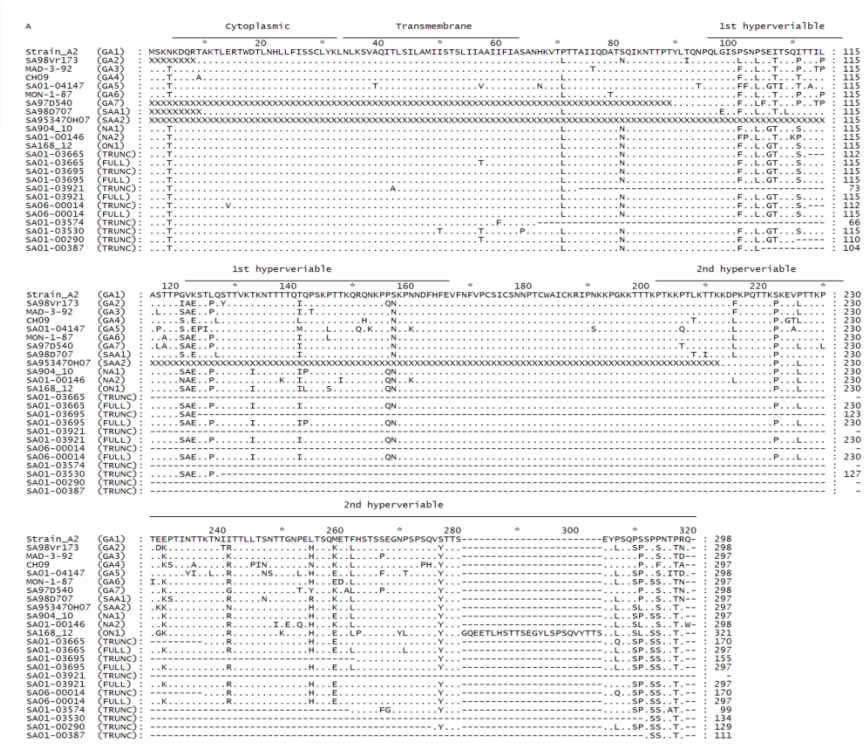


Figure 6.1 Full G protein gene nested PCR products for the detection of deletion mutants (400bp) relative to a full length G protein RSV A strain: lane 1, negative control; lane 2 to 7, clinical specimens; lane 8, Full length RSV A positive-control (800bp).

A



B

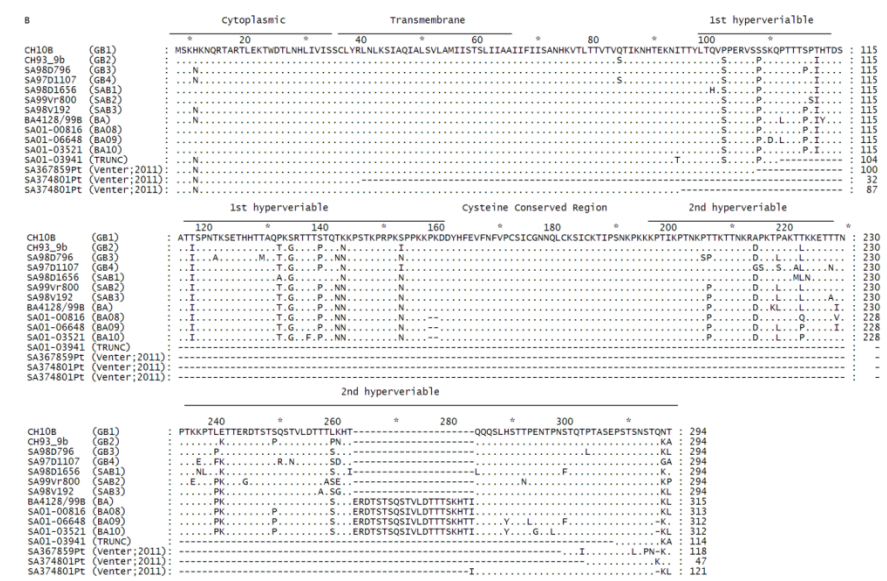


Figure 6.2: The amino acid alignment of RSV-A (A) and RSV-B (B) G-protein ecto domain deletion mutants identified in South Africa from 2009-2013 relative to the reference strains.

Table 6.1: Demographic and clinical characteristics of sever acute respiratory illness (SARI) and influenza-like-illness (ILI) patients and controls as well as SARI patients harbouring RSV G-protein deletion-mutant strains selected for the cytokine screening.

Factor	SARI		ILI	Controls	p-value
	Non-mutants n/N (%)	Deletion mutants n/N (%)	n/N (%)	n/N (%)	
Age group, months 0-5 6-11 12	 39/66 (59) 15/66 (23) 12/66 (18)	 2/5 (40) - 1/5 (20)	 3/7 (43) 1/7 (14) 3/7 (43)	 2/3 (67) 1/3 (33) -	0.252
≥5 years	0/66 (0)	2/5 (40)	0/7 (0)	0/3 (0)	-
Sex (male)	33/66 (50)	2/5 (40)	1/7 (14)	1/3 (33)	0.317
Duration of symptoms 3-7 days	30/66 (45)	5/5 (100)	3/7 (43)	0/3 (0)	0.038
HIV infection	24/66 (36)	5/5 (100)	0/7 (0)	1/3 (33)	0.005
Oxygen therapy	24/65 (37)	2/5 (40)	0/7 (0)	0/3 (0)	0.143
Patient died	1/65 (2)	0/5 (0)	0/7 (0)	0/3 (0)	0.973

6.3.2 Full genome analysis of South African G-protein deletion mutant strains

Of the two samples on which we were able to perform next generation sequencing to recover the full-length genomes, 1 sample harboured evidence of both wild type and a deletion mutant version of the G-protein. While, in the second sample only a truncated version of the G-protein was recovered, with open reading frames for all other proteins intact. Both recovered genomes clustered within the NA1 genotype of RSV-A (Figure 6.3). The longer branch length observed is the result of the truncated G-protein in the genome sequence, and does not reflect strain diversity.

Comparing the complete coding sequence (CDS) of the two South African deletion mutants, one of which did contain a full length copy of the G-protein, against the consensus NA1 CDS showed that the highest numbers of non-synonymous substitutions were in the G, L and F, with G also being the most variable at protein level with 17% amino acid variability relative to the consensus NA1 sequence (Figure 6.4, Table 6.2). However, the G-protein deletion mutants showed that the M2-2 protein (0.8095) displayed the highest selective pressure ratio, followed by G (0.3654), SH (0.1415) and NS2 (0.1096); all the other ratios were <0.05 (Table 6.2). The unique amino acid changes for each protein in the G-protein deletion mutants strains are also indicated (Table 6.2). No protein conformational changes are predicted for the G-protein deletion mutants strains due to non-synonymous changes observed.

Within the F protein, the F1 domain contained the most substitution sites; however there were no substitutions within the fusion peptide region on the F1 domain. The signal peptide and the F2 domain had 4 and 3 substitution sites respectively, whereas the rest of the F protein was conserved. Within the F2 domain all predicted N-glycosylation sites

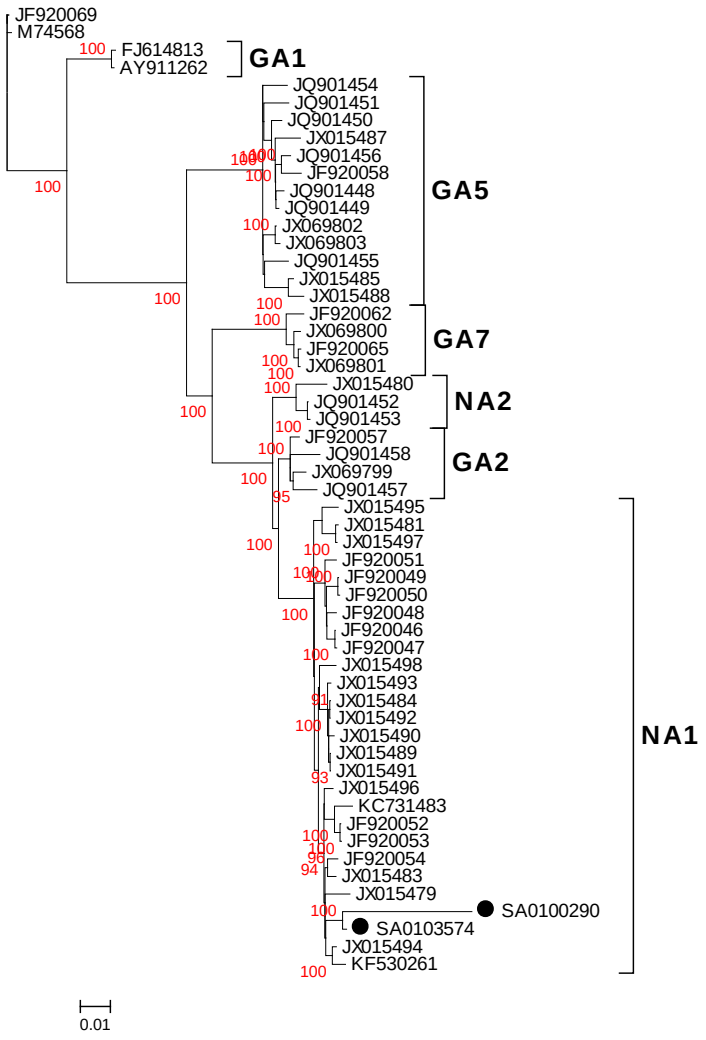


Figure 6.3: Maximum likelihood tree of RSV A South African deletion mutants complete genome

Phylogenetic analysis of RSV A complete genome sequences from South Africa indicated by the closed circles and reference sequences using Maximum Likelihood method. The nucleotide substitution model; general time reversal model (GTR) +I+G (where the rate variation among sites in the sequences was described using gamma distribution (G) and proportion of invariable sites (I) in the dataset) used in the Maximum likelihood (ML) analysis was determined using jModelTest (22, 23) and ML trees were generated using PhyML 3.0 (23, 24). Bootstrap values (100 replicates) shown on the branches, with values < 70% omitted from the tree.

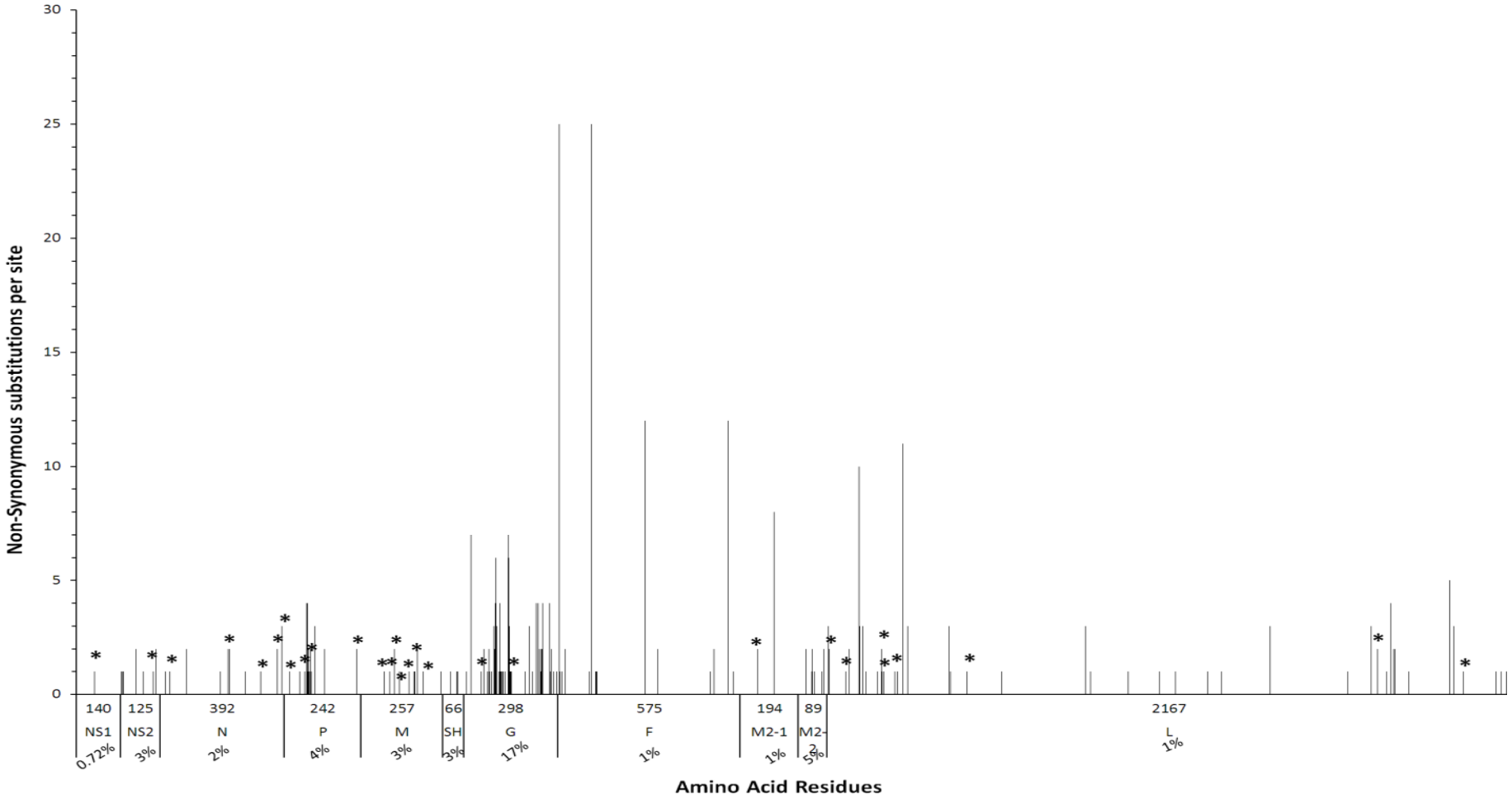


Figure 6.4: Sequence variability in the coding sequence (CDS) of the two South African deletion mutants against the consensus NA1 CDS. The number of non-synonymous substitutions per site (black bars) and the intragenotypic amino acid variability (%) in each of the proteins calculated within the NA1 genotype. * Indicates a unique non-synonymous amino acid change in the South African deletion mutants as indicated in Table 6.2.

Table 6.2 Sequence Variability and Selective pressure of each coding sequence of the two South African deletion mutants against the consensus NA1 CDS indicating the unique non-synonymous amino acid changes in the South African deletion mutant sequences.

Gene	Average dS	Average dN	dN/dS	Total Sites (CDS)	Number of Non-synonymous sites	Unique Non-synonymous deletion mutant changes relative to RSV-A NA1
NS1	0.0205	0.0002	0.0098	140	1	V59D
NS2	0.0228	0.0025	0.1096	125	9	H289Q
N	0.0546	0.0010	0.0183	392	16	S379N, F566L, H666N, A719T, D733E
P	0.0457	0.0028	0.0547	242	28	R765K, I815T, P826T, Q829K, P832T, P846Q, G978D
M	0.0342	0.0016	0.0468	257	12	V1109I, M1127T, C1145S, S1176A, V1207I, R1224K, N1233H, S1252A
SH	0.0106	0.0015	0.1415	65	3	
G	0.0260	0.0095	0.3654	297	101	A1586T, Q1680R
F	0.0275	0.0019	0.0691	575	88	
M2-1	0.0272	0.0012	0.0441	195	10	T2558A
M2-2	0.0042	0.0034	0.8095	89	10	
L	0.0244	0.0013	0.0538	2167	95	S2805N, N2807S, I2858M, L2961S, Q2976H, S3015N, V3254I, S4544L, F4816I

for amino acid residues 27, 70, 116, 120, 126 and 500 were highly conserved within the G-protein deletion mutants.

6.3.3 Inflammatory Cytokines and Chemokines

To further characterize the G-protein deletion mutants the profiles of the cytokines present were determined, together with RSV single positives of SARI, ILI and control samples. The patient characteristics were similar between the different subgroups of SARI, ILI, controls and deletion mutants (Table 6.1).

As can be observed the measured levels for the G-protein deletion mutants were either very low or below detectable thresholds (Figure 6.5). In the multiple comparison analysis samples from the G-protein deletion mutants contained significantly lower levels of the pro-inflammatory cytokines IL-8, IL-13, Eotaxin and G-CSF compared to those of HIV uninfected SARI patients (Figure 6.5A). The same applied to the levels of the anti-inflammatory cytokine IL-1ra and IL-10 (Figure 6.5B). Similarly the G-protein deletion mutant contained significantly lower levels of pro-inflammatory cytokines IL-12, IL-13, Eotaxin, IFN-ɣ, IP-10 and RANTES when compared to HIV infected SARI patients (Figure 6.5A). The G-protein deletion mutant also contained significantly lower levels of pro-inflammatory cytokines IL-7, IL-12, Eotaxin and IFN-ɣ when compared to ILI patients (Figure 6.5A). There were also no significant differences between the measured cytokine levels for both the pro- and anti-inflammatory cytokines of SARI HIV infected and uninfected.

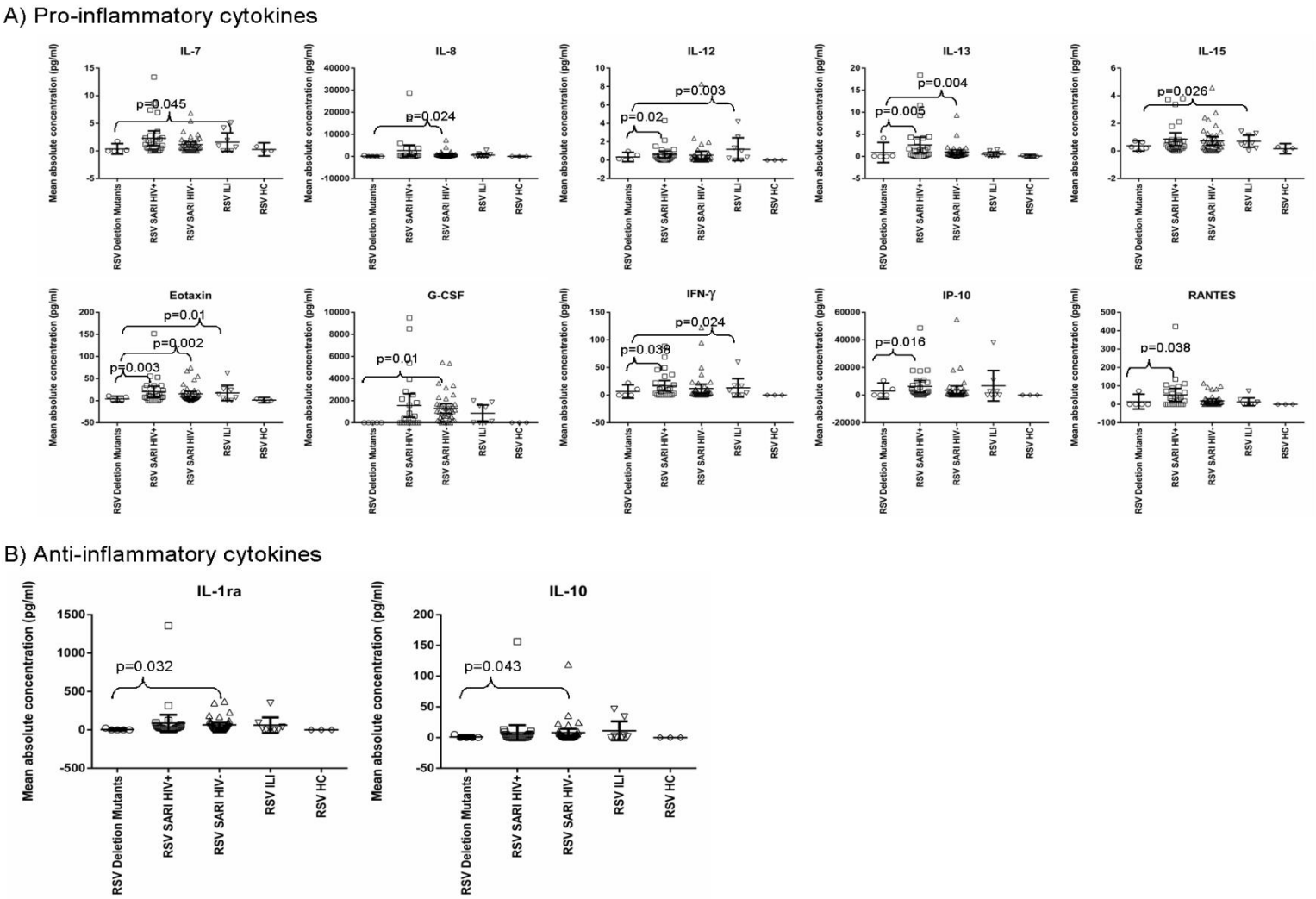


Figure 6.5 A and B: Scatter dot plots showing the mean absolute concentration of each cytokine in pg/ml, the error bars indicate 95% CI. The RSV deletion mutant specimens were compared against patients enrolled in SARI with single RSV infection HIV infected (RSV SARI HIV+) and HIV uninfected (RSV SARI HIV-), patients enrolled in ILI with single RSV infection (RSV ILI) and patients enrolled as controls with single RSV infection (RSV HC). The Kruskal-Wallis test and correcting for multiple comparisons using the Dunn's test was used to determine if the differences observed were significant, p-values < 0.05 were considered significant.

6.4 Discussion

Screening of specimens from HIV-infected and -uninfected patients infected with RSV identified G-protein deletion mutants from both subtype A and B strains that lacked a large portion of the ecto-domain. We observed a prevalence of 2.4% of the deletion mutants in HIV-infected patients with SARI.

Sequence analysis showed that most of the 1st and 2nd hypervariable regions and the entire conserved cysteine region were deleted. Full gene and full genome analysis have shown evidence that the G-protein deletion quasi-species exist even when the full-length protein is still present. We hypothesize that the G-protein might be necessary for initial infection, but is not necessary for replication in HIV-infected individuals. These G-protein deletion mutants may only be using the F-protein to facilitate replication. The F-protein fuses the cell walls and causes syncytia which enables RSV to move from cell to cell and spread the infection (Sun, Pan *et al.* 2013).This may suggest that the deletion mutant viruses cannot be transmitted to other people as it lacks the G-protein to establish initial infection. Although some studies have suggested the formation of defective virus particles is a way to evade the immune system, the precise mechanism is still not well understood (Mlera, Melik *et al.* 2014). In our study as well as others all the other RSV genes showed no positive selective pressure, suggesting that the impact of immune pressures on the evolution of these genes is minimal (Zlateva, Lemey *et al.* 2004a, Rebuffo-Scheer, Bose *et al.* 2011, Pretorius, van Niekerk *et al.* 2013). However, the cumulative effect of the substitutions observed in these genes and the deletion of

the ecto-domain of the G-protein might very well affect RSV fitness and its transmission dynamics.

No changes in potential N-glycosylation of the F protein that could result in altered virus-host attachment, changes in immune evasion strategies or distorted fusion abilities were observed (Zimmer, Trotz *et al.* 2001, Tan, Lemey *et al.* 2012). Position N500, which is known to be required for efficient syncytia formation, was present in both deletion mutants. As previously described, acute RSV infection seems to be predominately characterized by the pro-inflammatory immune response which contribute to disease pathogenesis (Bueno, González *et al.* 2008, Bueno, González *et al.* 2011, Breindahl, Rieneck *et al.* 2012). We demonstrated that patients infected with the G-protein deletion mutants had significantly lower levels of pro- and anti-inflammatory cytokines than patients infected with full length RSV strains with SARI that were HIV-uninfected.

RSV attachment to epithelial cells leads to the activation of pattern recognition receptors *i.e.* troll-like receptors (TLRs), specifically TLR 3 and 4 expressed in respiratory epithelial cells (Liu, Jamaluddin *et al.* 2007). TLR3 promotes nuclear factor-ƙβ activation followed by secretion of IFN-β. Simultaneously the engagement of surface TLR4 by RSV induces the secretion among other things of IP-10, Eotaxin, MCP-1, RANTES, IL-6 and IL-8 (Bueno, González *et al.* 2011). This would suggest that the absence of the G-protein, which stimulates the pro-inflammatory cytokine response, would inhibit the virus' ability to activate these pathways. Patients with the deletion mutants present were all hospitalized with severe respiratory illness and no other viral co-infections were detected using the real-time PCR assay (Pretorius, Madhi *et al.* 2012).

Our study has limitations that merit discussion. We did not take sequential specimens from these patients as the screening was retrospective, so we are unable to determine how these G-protein ecto-domain deletions evolved in these patients. Because of the incomplete data and few deletion mutants detected we are underpowered to determine the effect of the level of immunosuppression of our HIV-infected individuals. We were unable to culture these specimens and subsequently further characterize the deletion mutants. However, due to the fact that we were able to detect the deletions mutants using both limiting dilution PCR and next generation sequencing, we are confident that this is a real phenomenon and not due to poor fidelity of the Taq polymerase during PCR.

In conclusion, Both RSV-A and RSV-B G-protein deletion mutants were identified in HIV-infected SARI patients. These results suggest that G-protein deletion mutants are associated with an attenuated inflammatory response as measured by cytokine-induction in individuals who have been hospitalized with severe illness, have ILI or no recorded symptoms at all.

Chapter 7

T-helper 1 and 2 cytokine responses associated with severe acute respiratory illness in immune compromised infants infected with rhinovirus vs. respiratory syncytial virus

7.1 Introduction

Viruses have developed several different strategies to proliferate within host cells and evade host's immune response, the main target for evasion being type 1 interferon's (IFN) (Weber, Kochs *et al.* 2004, Koyama, Ishii *et al.* 2008). The pathogenesis of respiratory virus infections have been associated with the level of inflammatory cytokines and chemokines released during infections (Cheung, Poon *et al.* 2002, Koyama, Ishii *et al.* 2008) and depending on the viral pathogen, the subsequent respiratory infection in infants differ significantly with regards to the quantity of interferon production and this will contribute to the clinical course and outcome of the disease (Aberle, Aberle *et al.* 2004).

As a rule cytokines can be divided into two groups pro- and anti-inflammatory, with T lymphocytes being a major source of cytokines (Berger 2000). The two main subsets of T lymphocytes are CD4 and CD8, with CD4 cells being responsible for T helper (Th) cells, which can be further subdivided in to Th1 and Th2 cells expressing Th1 type cytokine and Th2 type cytokines, respectively (Berger 2000, Becker 2006b). Th1 cytokines of which IFN-ɣ is the main cytokine is mainly responsible for pro-inflammatory responses and to counter act this Th2 cytokines, which include IL-4, IL-5, IL-13, which promotes IgE and eosinophillic responses (Berger 2000, Mejías, Chávez-Bueno *et al.* 2004, Becker 2006b) have an anti-inflammatory affect (Becker 2006b, Bueno, González *et al.* 2011). The ideal scenario would be that humans would simulate a well-balanced Th1 and Th2 response.

During RSV infection an allergy-like condition develops due to increased levels of Th2 cytokines, especially in young children (Becker 2006a, Becker 2006b). It is

hypothesized that the viral-soluble G-protein contains a T cell super antigen that is capable of binding to the hematopoietic cells, basophils, mast cells and monocytes, similar to the case of allergens, and that this aggregation causes these innate system cells to degranulate and release large amounts of Th2 cytokines (interleukin (IL)-4, IL-5, IL-10, IL-13) into the blood. This is thought to be the mechanism RSV utilizes to skew the Th1/Th2 balance toward Th2 (Becker 2004, 2006a, Becker 2006b), which has an inhibitory effect of the innate immune system.

The immune evasion mechanism of small RNA viruses like rhinovirus creates the large number of serotypes, which together with a high mutation rate, might be part of the success of rhinovirus infections (Kirchberger, Majdic *et al.* 2007). Previous studies have observed rhinovirus infection induced an up regulation of inflammatory cytokines IL-8 and IP-10 (Wark, Bucchieri *et al.* 2007, Cakebread, Haitchi *et al.* 2014). Most of these studies were performed on bronchial epithelial monolayers from adults and to our knowledge a similar comparison in a paediatric population has not been performed. Studies have shown that children infected with rhinovirus mounts a predominant Th1 response (Wimalasundera, Katz *et al.* 1997), in the form of IFN-ɣ, which has been linked to rhinovirus virus loads.

The aim of our study was to compare systemic cytokine responses between immune-compromised infants infected with RSV, rhinovirus only and RSV and rhinovirus co-infections during the first year of life to determine if differences in the cytokine profiles induced by these viruses may influence the clinical outcome in HIV-infected and uninfected patients.

7.2 Materials and Methods

7.2.1 Study design and population

SARI Surveillance: Study samples were obtained from participants enrolled in a prospective hospital-based surveillance program for SARI initiated in February 2009 which aimed to describe the aetiology and risk factors for acute lower respiratory tract infection (ALRI) in all age groups in South Africa. The methodology and case definitions of this study have been previously described (section 2.2.2) (Cohen, Moyes *et al.* 2013). All patients were enrolled only once and followed through until discharged from the hospital.

ILI and Control Surveillance: Study samples were obtained from participants enrolled in an active surveillance program for ILI and asymptomatic controls initiated in May 2012 through June 2013. The methodology and case definitions of this study have been previously described (section 4.2.1).

7.2.2 Laboratory testing

Respiratory specimens (i.e., nasopharyngeal aspirates for children <5 years of age and nasopharyngeal and oropharyngeal swabs from individuals ≥5 years of age) were collected from SARI, ILI and control patients placed in viral transport medium, stored at 4-8°C and transported to the National Institute for Communicable Diseases within 72 hours of collection for testing. All specimens were tested for the presence of 10 respiratory viruses using the real-time multiplex pcr assay as described previously (section 2.2.6). *Streptococcus pneumoniae* was also tested for using the *lytA* PCR as described previously on blood samples collected from SARI patients (Wolter, Cohen *et*

al. 2014). Among consenting study patients, HIV status was established by enzyme-linked immunosorbent assay (ELISA) or PCR depending on the patients' age.

7.2.3 Sample Selection for Cytokine screening

7.2.3.1 2009-2013: SARI cases

The 2009-2013 SARI cases were comprised of randomly selected SARI patients ≥ 1 year old where *Streptococcus pneumonia* infection was excluded. There were n=470 RSV single positive, n=588 single positive RV and n=173 co-infections of RSV and RV only cases, with available HIV data enrolled from February 2009 through June 2013 at all 6 sentinel sites in four provinces available for selection. The specimens for each individual selection was sorted according to randomly assigned numbers and for RSV the first 66 were selected (14%, 66/470), for RV the first 71 were selected (12%, 71/588) and for RSV and RV co-infection the first 44 (25%, 44/173) for cytokine analysis.

7.2.3.2 2012-2013: ILI and controls

The 2012-2013 ILI and control cases enrolled at the SARI sentinel sites and outpatient clinics situated in KwaZulu Natal and North-West Provinces during the period of May 2012 through June 2013. All available samples collected from patients ≥ 1 year old with available HIV data that had single positive RSV (ILI n=7); (Controls n=3), single positive RV (ILI n=14), (Controls n=8) and co-infections of RSV and RV only (ILI n= 8), (Controls n=1) enrolled during this time period at the sites were selected. Approximately 222 specimens were selected for further cytokine characterization (Figure 1 Sample selection flow chart).

7.2.4 Cytokine assay

The level of inflammatory cytokines and chemokines released during infection was measured by using the Bio-PlexPro™ Human Cytokine Group I 15-plex assay (BioRad Laboratories Inc. USA) targeting Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interferon (IFN)-γ, Interleukin (IL)-6, IL-8, IL-10, IL-7, IL-12, IL-13, Monocyte chemoattractant protein (MCP)-1, Granulocyte-colony stimulating factor (G-CSF), Eotaxin, IL-1 receptor antagonist (ra), IL-15, Interferon gamma-induced protein (IP)-10 and Regulated on activation, normal T cell expressed and secreted (RANTES). The Bioplex system contained dyed beads conjugated with monoclonal antibodies targeting specific cytokines and chemokines. The assays were performed as per manufacturer's instructions and cytokine levels were expressed in picograms per milliliter. Cytokine responses in negative controls were used to normalize cytokine responses in study samples.

7.2.5 Statistical analysis

All values obtained were expressed as an absolute concentration and normalized using the mean of the negative healthy control patients. Differences in the levels between the various clinical groups were assessed using regression analysis (generalized linear, model with binomial distribution and log-link). The multivariate analysis of the differences in cytokine levels between RSV, RV infection and RSV/RV co-infection was adjusted to HIV cases and the outcome was not dependant. The cytokine data is presented as a fold-difference between RSV, RV infection and RSV/RV co-infection affected children

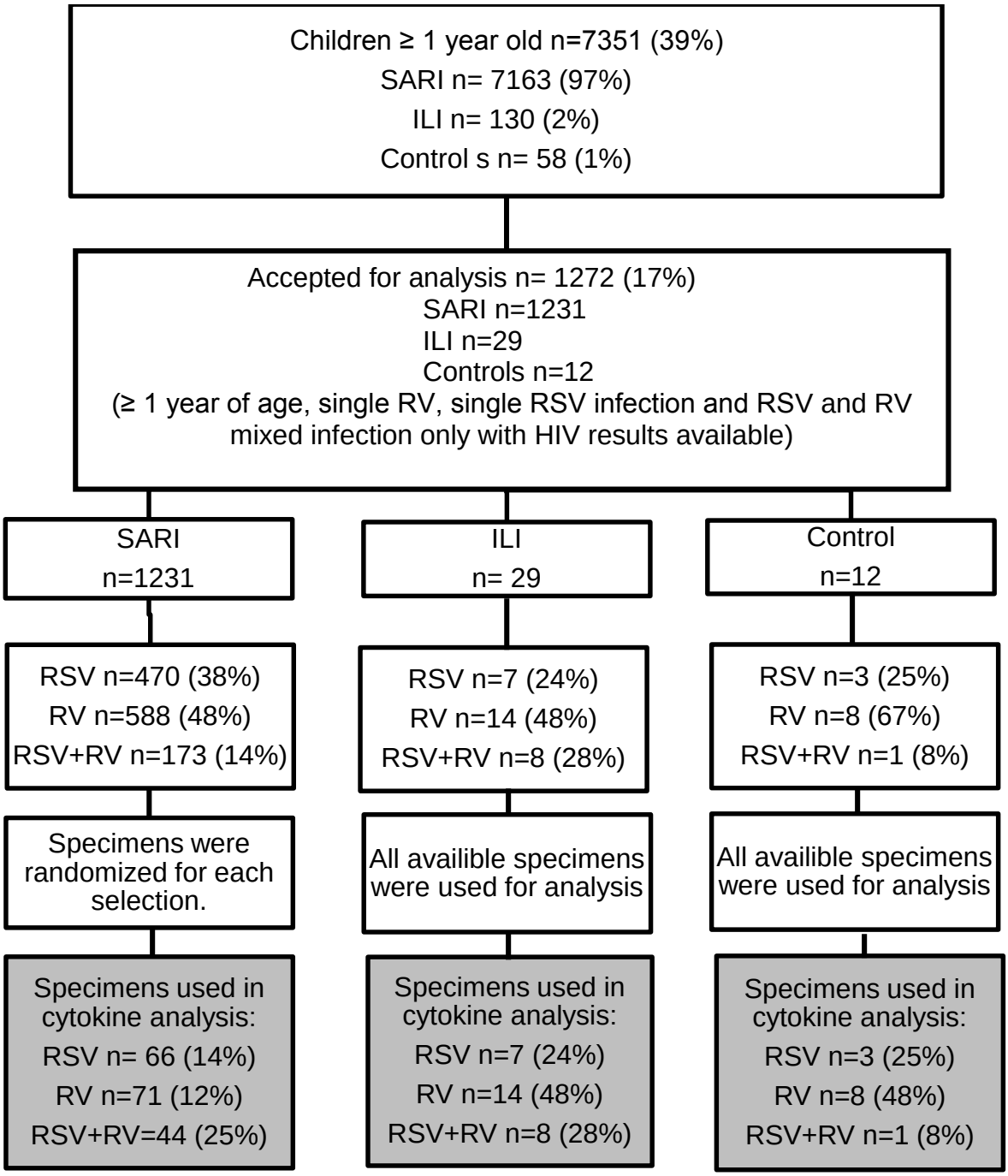


Figure 7.1: Specimen selection flow chart. Flowchart of patients enrolled and specimens tested in the severe acute respiratory illness study, South Africa, 2009-2013. The study group (n = 222) is highlighted in grey.

(Jartti, Paul-Anttila *et al.* 2009). The analysis was performed using STATA 12, (Stata Corporation, Texas, USA). Due to small number of specimens available in some of the selections no statistical power calculation was done for the cytokine analyses. Stefano Tempia has assisted the student in running these statistical tests and assisted the student in the interpretation.

7.3 Results

7.3.1 Characteristics of study children

During the study period from 2009 for the SARI surveillance and 2012 for the ILI and control surveillance, 16824 patients were hospitalized with severe acute respiratory illness and 1915 patients were enrolled as ILI or controls. 7351 were children ≥ 1year of age, of these 1272 children, fulfilled the study criteria which included single RV, single RSV infection as well as RSV and RV co-infection (Influenza, Parainfluenza type 1, 2 and 3, Enterovirus, Adenovirus, human Metapneumovirus and Streptococcus pneumoniae (for SARI patients only were ruled out by real-time PCR as part of the laboratory testing procedure) (Figure 7.1).

Of the 222 children included in the final analysis, 76/222 (34%) had confirmed single RSV infection, 93/222 (42%) had single RV infection and 53/222 (24%) had only RSV and RV co-infections (Figure7.1). The patient characteristics did not differ when compared between the different sub groups of SARI, ILI and controls or when the subgroups of RSV, RV and RSV/RV were compared (Table 7.1).

The children that were enrolled in SARI and tested positive for RV were more likely to have symptom duration of less than 2 days when compared to RSV and RSV/RV. In

contrast children that tested positive for RSV were more likely to have symptom duration of between 3-7 days. Children enrolled in the ILI study and tested positive for RSV were more likely to have symptom duration of less than 2 days and symptom duration of between 3-7 days when co-infected with RSV and RV (Table 1), however none of the differences detected were statistically significant.

7.3.2 Cytokine levels

The multivariable analysis comparing HIV infected to HIV uninfected revealed that the HIV infected patients had significant fold increases in: IL-7 (Coefficient: 1.4; 95% CI 0.23-2.05), Eotaxin (Coefficient: 8.23; 95%CI 1.12-15.34) and RANTES (Coefficient: 17.41; 95% CI 3.12-31.69) (Table 7.2) (Appendix A: Mean cytokine concentration (pg/ml) present in NPAs of children ≥1 year of age associated with single RSV, single RV and RSV and RV co-infection.

When comparing the NPAs of children hospitalized with SARI to ILI using the control group as a reference, it showed that SARI had significant fold increases in: IL-12 (Coefficient: 5.6; 95% CI 1.70-9.41) and IFN-γ (Coefficient: 13.9; 95% CI 3.12-24.68) (Table 7.2).

Comparing the NPAs of from RV children with the NPAs from children with RSV and RV co-infections, using RSV infections as a reference group, showed that RV was associated with significant fold increases in: IL-1ra (Coefficient: 102.99; 95% CI 43.162.51) and IL-12 (Coefficient: 6.45; 95% CI 4.44-8.46) and significant fold decreases in IL-10 (Coefficient: -5.25; 95% CI -9.85;-0.65) and Eotaxin (Coefficient: -8.91; 95% CI -16.19;-1.63) (Table 7.2).

RSV and RV co-infections were associated with significant fold increases in: IL-7 (Coefficient: 1.69; 95% CI 0.61-2.77) and G-CSF (Coefficient: 1040.5; 95% CI 278.60-1802.43) (Table 7.2) and significant fold decreases in IL-15 (Coefficient: -0.4; 95% CI -0.59; -0.11) (Table 7.3).

7.4 Discussion

In general infants tend to be born with a Th2 biased immune response, geared towards the production of IL-4, 5, 10 and 13, away from a Th1 response and the production of IFN-γ in response to viral infections (Berger 2000, Copenhaver, Gern *et al.* 2004, Jartti, Paul-Anttila *et al.* 2009). It has been hypothesized that the nature of this imbalance, especially a weak Th1 response has been linked to the development of allergies (Berger 2000, Copenhaver, Gern *et al.* 2004), which could explain why children less than 6 months of age are more severely affected by RSV infection.

In our study when comparing the Th1 and Th2 responses of young children with RV single infection and RV and RSV co-infection to that of young children with RSV single infection, a definite bias can be observed. Significant increases in the Th1 cytokines for the RV and RV/RSV group when compared to the RSV group.

The principal Th1 cytokine IFN-γ produced by Th lymphocytes as an nonspecific defence mechanism and an inflammatory cytokine in reaction to the activation of nuclear factor ƙβ (NF- ƙβ) (Jartti, Paul-Anttila *et al.* 2009, Bueno, González *et al.* 2011). In our study the Th1 cytokines were overall found to be significantly associated with patients who were infected with RV or a combination of RSV/RV, suggesting that co-infection with RV and RSV suppress RSV's ability to activate the Th2 cytokines.

Table 7.1: Patient characteristics in respiratory syncytial virus, rhinovirus and respiratory syncytial virus and rhinovirus co-infected affected children.

Factor	SARI				ILI				Controls			
	RSV n/N (%)	RV n/N (%)	RSV/RV n/N (%)	p-value	RSV n/N (%)	RV n/N (%)	RSV/RV n/N (%)	p-value	RSV n/N (%)	RV n/N (%)	RSV/RV n/N (%)	p-value
Age group, months												
0-5	39/66 (59)	37/71 (52)	30/44 (68)	0.317	3/7 (43)	3/14 (21)	1/8 (13)	0.232	2/3 (67)	1/8 (13)	1/1 (100)	0.219
6-11	15/66 (23)	22/71 (31)	11/44 (25)		1/7 (14)	9/14 (64)	5/8 (63)		1/3 (33)	3/8 (38)	-	
12	12/66 (18)	12/71 (17)	3/44 (7)		3/7 (43)	2/14 (14)	2/8 (25)		-	4/8 (50)	-	
Sex (male)	33/66 (50)	35/71 (49)	31/44 (70)	0.054	1/7 (14)	7/14 (50)	4/8 (50)	0.248	1/3 (33)	5/8 (63)	-	0.400
Duration of symptoms 3-7 days	30/66 (45)	24/69 (35)	18/44 (41)	0.447	3/7 (43)	7/13 (54)	5/8 (63)	0.748	-	-	-	
HIV infection	24/66 (36)	35/71 (49)	8/44 (18)	0.004	-	-	-		1/3 (33)	4/8 (50)	-	0.598
Underlying illness[a]	-	2/71 (3)	2/44 (5)	0.256	-	-	-		-	-	-	
Oxygen therapy	24/65 (37)	27/71 (38)	17/44 (39)	0.982	-	-	-		-	-	-	
Patient died	1/65 (2)	2/71 (3)	-	0.515	-	-	-		-	-	-	

[a]Underlying illness includes: chronic lung diseases, cirrhosis/liver failure, chronic renal failure, heart failure, valvular hearth disease, coronary heart disease, immunosuppressive therapy, splenectomy, diabetes, burns, kwashiorkor/marasmus, nephritic syndrome, spinal cord injury, seizure disorder or emphysema. None of the cases were admitted to ICU.

Table 7.2: Multivariable analysis of the difference in (a) Th1 cytokines, (b) Th2 cytokine and (c) Chemokines levels among patients ≥ 1year of age with single RSV, RV infections and RSV and RV co-infections compared to ILI and controls, South Africa. (Controls cases and RSV single infections were used as the reference groups for the multivariate analysis, values highlighted in bold indicate factors significant at p<0.05)

(a)

Variables	Th1 cytokine cell mediated immunity Coefficient (95% CI)													
	IL-1ra		IL-7		IL-12		IL-15		G-CSF		GM-CSF		IFN-γ	
HIV infection	-26.45	(-84.55;31.65)	**1.14**	**(0.23;2.05)**	1.24	(-0.72;3.2)	0.12	(-0.08;0.32)	-4.34	(-648.55;639.87)	0.38	(-0.02;0.78)	0.88	(-4.61;6.37)
Case:														
HC[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ILI	76.56	(-56.06;209.18)	0.74	(-1.35;2.82)	2.71	(-1.77;7.18)	0.34	(-0.12;0.81)	617.11	(-853.38;2087.60)	0.2	(-0.71;1.11)	6.16	(-6.36;18.69)
SARI	109.96	(-4.15;224.08)	1.44	(-0.35;3.23)	**5.56**	**(1.70;9.41)**	0.27	(-0.12;0.67)	714.99	(-550.32;1980.3)	0.43	(-0.35;1.21)	**13.9**	**(3.12;24.68)**
Virus infection:														
RSV	**-**	**-**	-	-	**-**	**-**	**-**	**-**	-	-	-	-	-	-
RV	**102.99**	**(43.48;162.51)**	0.61	(-0.32;1.55)	**6.45**	**(4.44;8.46)**	**-0.7**	**(-0.91; -0.49)**	-166	(-825.9;493.87)	0.05	(-0.36;0.46)	5.17	(-0.45;10.79)
RSV/RV	50.31	(-18.40;119.03)	**1.69**	**(0.61;2.77)**	0.49	(-1.83;2.81)	**-0.4**	**(-0.59;-0.11)**	**1040.5**	**(278.60;1802.43)**	0.071	(-0.4;0.54)	-5.54	(-12.03;0.95)

(b)

Variables	Th2 cytokines humoral immunity, Coefficient (95% CI)					
	IL-6		IL-10		IL-13	
HIV infection	189.9	(-41.82;421.67)	-1.03	(-5.52;3.46)	0.56	(-0.70;1.81)
Case:						
HC	-	-	-	-	-	-
ILI	93.73	(-435.27; 622.72)	2.07	(-8.17;12.32)	0.86	(-1.99;3.72)
SARI	90.65	(-364.53; 545.83)	0.16	(-8.66;8.98)	2.29	(-0.16;4.75)
Virus infection:						
RSV	-	-	**-**	**-**	-	-
RV	-208.5	(-445.93; 28.85)	**-5.25**	**(-9.85;-0.65)**	1.1	(-0.18;2.38)
RSV/RV	-157.1	(-431.18; 117.00)	-2.9	(-8.21;2.41)	-0.2	(-1.72;1.24)

(c)

Variables	Chemokines, Coefficient (95% CI)									
	IL-8		Eotaxin		IP-10		MCP-1		RANTES	
HIV infection	623.83	(-165.76;1413.42)	8.23	(1.12;15.34)	1205.01	(-2868.14;5278.15)	8.42	(-20.80;37.64)	17.41	(3.12;31.69)
Case:										
HCa	-	-	-	-	-	-	-	-	-	-
ILI	767.2	(-1035.15; 2569.55)	11.39	(-4.84;27.62)	4494.5	(-4803.03; 13792.03)	5.7	(-61.00;72.4)	8.28	(-24.34;40.89)
SARI	1327.8	(-223.12; 2878.61)	9.26	(-4.69;23.23)	5857.18	(-2143.03; 13857.39)	21.23	(-36.16;78.63)	15.27	(-12.8;43.33)
Virus infection:										
RSV	-	-	-	-	-	-	-	-	-	-
RV	-3.68	(-812.48; 805.13)	-8.91	(-16.19;-1.63)	1297.76	(-2874.51; 5470.03)	-22.22	(-52.15;7.71)	-11.52	(-26.15;3.12)
RSV/RV	-89.31	(-1023.16; 844.55)	6.98	(-1.43;15.38)	3677.02	(-1140.34; 8494.39)	-14.03	(-48.59;20.53)	1.77	(-15.13;18.66)

RSV: respiratory syncytial virus single infections, RV: rhinovirus single infections, RSV/RV: respiratory syncytial virus and rhinovirus co-infections; CI: confidence interval; Coefficient: fold-difference reference group and affected children

The data were analysed using regression analysis. In multivariate analysis, adjustments to HIV cases and the outcome was not dependant

Only significant adjustments ($P < 0.05$) were kept in the model.

1i.e. how many folds higher the cytokine levels is in the RV-group compared to the RSV-group or in the control group.

A previous study where T cells obtained from tonsils, showed that children have a predominated Th1 response when infected with RV (Wimalasundera, Katz *et al.* 1997). Papadopoulos *et al.,* (Papadopoulos, Stanciu *et al.* 2002) and Message *et al.,* (Message, Laza-Stanca *et al.* 2008) have shown that IFN-gamma response could be linked to RV virus loads and coupled with less severe symptoms this suggested a protective role in RV infection. As expected the IFN-ɣ response although not significant were low in RSV infected children (Tripp, Oshansky *et al.* 2005, Bueno, González *et al.* 2011).

Previous studies as well as our findings support the link between IFN-ɣ and IL-12 where during virus infection the induction of IL-12 elicits natural killer cell production of IFN-ɣ (Orange and Biron 1996) and is seen as the primary inducer of the Th1 response (Peters 1996), even though the interaction between IL-12 and IFN-ɣ seems to be less apparent in RV infections as presented by us and by others previously (Wimalasundera, Katz *et al.* 1997, Papadopoulos, Stanciu *et al.* 2002, Jartti, Paul-Anttila *et al.* 2009). Previous studies have shown that the ability of RV to elicit a Th2 response in less pronounced when compared to the IFN-ɣ response (Grunstein, Hakonarson *et al.* 2001, Hosoda, Yamaya *et al.* 2002). Yoon et al. (Yoon, Kim *et al.* 2007) showed that during RV infection IL-1ra inhibits IL-1 and in doing so appears to balance both the beneficial and detrimental effects of IL-1 as well as mediating symptom resolution.

In our study, increases in the Th2 cytokines and chemokine responses could be observed for RSV when compared to RV and RV/RSV. The acute phase RSV infection seems to be dominated by a pro-inflammatory response through the activation of NF- ƙβ releasing Th2 cytokines IL-4, -5, -6, -10 and -13 and skewing the balance of Th1/Th2

towards Th2 (Becker 2004, Becker 2006b, Breindahl, Rieneck *et al.* 2012). The increase IL-4 serum levels especially will inhibit the adaptive immune response by blocking the Th1 cytokine synthesis (Becker 2006b). Roman et al (Roman, Calhoun *et al.* 1997) found that RSV infected infants exhibit a suppressed IFN-ɣ production and increase IL-4 production, resulting in an increased IL-4/ IFN-ɣ ratio that will favour a Th2-type response. Simultaneously the binding of RSV to the troll-like receptor (TLR-4) in the airway epithelium cells results in the secretion of several chemokines including IL-8, Eotaxin, IP-10, MCP-1 and RANTES (Becker 2006b, Bueno, González *et al.* 2011).

Of the studied cytokines, IL-10 tends to have an anti-inflammatory function and is produced in an attempt to reduce airway inflammation (Jartti, Paul-Anttila *et al.* 2009, Bueno, González *et al.* 2011). Moreover, in combination with other Th2 cytokines like IL-4 promotes the failure of the immune system to clear RSV infection (Connors, Giese *et al.* 1994). The up regulation of IL-15 is likely to be an important mechanism in activating the host's immune response to RSV by pulmonary epithelial cells through the up regulation of MICA (MHC class I chain related protein) and the CTL response (Oshansky, Zhang *et al.* 2009).

Our study has several limitations. We could only exclude *Streptococcus pneumonia* as possible bacterial infections from the SARI patients not in ILI or controls as we were only able to test the SARI patients' blood for bacterial load. This is a cross sectional look at the cytokine responses at that point in time when the patient was enrolled, and may not reflect the true cascade of events of how the cytokines are activated, in fact the immaturity of the infants immune response may also alter the outcome of the viral infection, but at the same time the viral infection could shape the development of the

infants' immune response. The use of non-steroidal anti-inflammatory drugs was not recorded, however their effect on cytokine levels when recommended dosages are used is considered negligible (Sirota, Shacham *et al.* 2001). Despite finding increased levels of cytokines in patients enrolled with ILI and SARI when compared to control patients, we were underpowered to determine if there was any direct correlation between the level of Th1/Th2 cytokines and disease severity.

In conclusion, the manipulation of cytokine expression by RSV results in an allergy like cytokine profile, which in turn may contribute to longer periods of infection. The cytokine profile of children infected by RV and RSV differ greatly and our results support previous studies that RV infection elicits a strong Th1 response, while RSV infection skews the balance towards a Th2 response. Co-infection with RSV and RV resulted in strong Th1 response suggesting that the co-infection with RV manipulated the immune response of these viruses and the resulting immune competence is exacerbated during viral co-infection further skewing the Th1/Th2 response.

Chapter 8

Concluding remarks

Respiratory viruses have been well established as one of the major causes of pneumonia as well as one of the primary causes of childhood mortality especially in continents such as Africa (Rudan, Tomaskovic *et al.* 2004, Rudan, Boschi-Pinto *et al.* 2008, Black, Cousens *et al.* 2010). Well recognised viral agents traditionally associated with acute respiratory tract infection include influenza A and B, RSV, parainfluenza virus types 1, 2 and 3, adenovirus, enterovirus, human metapneumovirus and rhinovirus (Brittain-Long, Nord *et al.* 2008, Tiveljung-Lindel, Rotzen-Ostlund *et al.* 2009).

Traditionally viral cultures were the most common method for detection of respiratory viruses in clinical specimens, however the difficulties encountered with culturing certain viruses, meant that the aetiology and proportion of respiratory viruses responsible for pneumonia was severely under estimated. Over the last decade, nucleic acid detection methods have become increasingly popular for the detection of respiratory pathogens (Hendrickson 2004, Paranhos-Baccala, Komuruan-Pradel *et al.* 2008, Lassauniere, Kresfelder *et al.* 2010). Furthermore, compared to conventional PCR and other real-time methods, multiplex rRT-PCR has a significant advantage as it permits simultaneous amplification of several viruses in a single reaction. This facilitates cost-effective diagnosis, enabling the detection of multiple viruses in a single clinical specimen.

This was the objective for developing a two-step real-time multiplex reverse transcriptase PCR (rRT-PCR) assay that could detect ten major respiratory viruses (Influenza A and B, RSV, enterovirus, hMPV, adenovirus, rhinovirus, PIV 1, 2 and 3). This enabled us to screen the SARI and later ILI and control patients to determine the contribution of each of these viruses to SARI in South Africa.

The assay helped define the distribution and seasonality of these respiratory viruses in South Africa especially the role of viral co-infections during (H1N1)pdm09 pandemic in 2009 and 2010. We were able to assign a viral aetiology in 57% of the SARI cases. The most commonly identified pathogens were RSV and rhinovirus especially in the infants less than 1 year of age. As expected RSV displayed a very distinct seasonal pattern between February and June, with rhinovirus detected throughout the year. After which we hypothesized the primary objective of investigating the importance of RSV and rhinovirus as pathogens in SARI and the effect they and their genetic variation have on the innate immune response and disease severity.

While the use of sensitive PCR methods have significantly expanded the ability of laboratories to detect infections, the clinical association between pathogen detection and disease remains difficult to interpret in the absence of comparison groups, we calculated the adjusted prevalence of viruses using the attributable fraction. The obtained results indicated that influenza, RSV, hMPV and to a lesser extent rhinovirus infection were associated with both mild and severe respiratory disease relative to controls, which is a more accurate description of the prevalence of viruses.

We investigated rhinovirus as a pathogen by comparing patients with mild, severe or asymptomatic infection. The detection of rhinovirus by PCR is difficult to interpret; this may be due asymptomatic infections or the time it takes to clear rhinovirus RNA from the respiratory tract. The results obtained suggested that while we observed a significant difference in the prevalence of RV among SARI and ILI cases compared to controls, only a proportion of RV infections may be responsible for the clinical disease that manifests as ILI or SARI and that rhinovirus may act as a pathogen and a bystander.

We also tried to determine whether the rhinovirus species causing the infection may have an effect on the disease outcome. All three of the recognized rhinovirus species circulate in South Africa with RV-A and C more common than B. We were unable to identify any difference in disease severity due to rhinovirus type.

To examine RSV's ability to re-establish annual epidemics, we investigated RSV's genetic and antigenic variation in patients over the past 16 years. We identified several genotypes not previously described in South Africa that have been identified across the world, including a new genotype (ON1, identified in Canada in 2011 with a 72-bp insertion). None of the genotypes specifically identified in South Africa are still circulating and NA1 being the main genotype circulating. Several new positively-selected sites located mainly in the second hypervariable region for the G-protein gene were identified, confirming the value of this region in phylogenetic analysis. We were unable to attribute more severe disease to a specific RSV subtype, however our results suggests that genotype switching re-establishes annual epidemics and reinfection throughout life.

Screening of specimens from HIV infected and uninfected patients infected with RSV identified G protein deletion mutants in both subtype A and B strains. Patients with the deletion mutants present were all HIV-infected and hospitalized with severe respiratory illness and no other viral co-infections were detected using the real-time PCR assay. Subsequent sequence analysis showed that these strains lacked most of the 1st and 2nd hypervariable region and the entire conserved cysteine region. We observed 2.3% prevalence in the population studied. Patients with the deletion mutants had significantly lower pro- and anti-inflammatory cytokines responses than patients infected with full length RSV strains with SARI; both for HIV infected and uninfected cases. This would

suggest that the absence of the G-protein, which stimulates the pro-inflammatory cytokine response, would inhibit the virus' ability to activate these pathways.

To address the question on the effect that RSV relative to rhinovirus would have on the host's response, we characterized and compared the cytokine profiles in ILI (mild), SARI (moderate to severe) and control patients in response to single and co-infections with rhinovirus and RSV during one season where the circulating genotypes are defined. Our results suggested that the cytokine profile of children infected by RV and RSV differ greatly and our results support previous studies that RV infection elicited a strong Th1 response, while RSV infection skewed the balance towards a Th2 response. Whereas co-infection with RSV/RV together could not be linked to more sever disease and the cytokine profile is suggestive of an exacerbated effect further skewing the Th1/Th2 response.

To conclude, our study showed an important role for multiplex PCR in respiratory disease diagnosis and suggested a positive result for influenza, RSV, and hMPV infection to be significantly associated with disease. RSV genotypes were replaced over the 16 years suggesting a role for genetic change in re-establishing epidemics. Deletion mutants occurred in 2.3% of RSV patients and these strains had a much reduced inflammatory cytokine response relative to wild type strains. No difference in disease severity was associated with specific subgroups of RSV and rhinovirus. Although, rhinovirus have been shown to be associated with both patients with SARI and ILI, the frequency with which it is detected in asymptomatic patients made it difficult to assign a specific role to rhinovirus in respiratory infections although it still came up as significantly higher in patients with disease.

Cytokine studies did show that in patients with severe disease rhinovirus may have an effect on the immune induced pathogenesis.

Appendices

Appendix A: Validation for the implementation of the Respiratory Virus Two Step Multiplex PCR assay.

1. Motivation for implementation of Assay

Respiratory virus infections are a major public health problem, because of their worldwide occurrence, ease of spread and morbidity and mortality. Mortality from respiratory viral infections are rare in developed countries, but the mortality rate in developing countries could be high with up to 5 million children globally under 5 years of age dying annually (Kesson 2007a). Several meta-analysis studies have since reported on childhood mortality in general and for specific syndromes like pneumonia and diarrhea (Rudan, Boschi-Pinto *et al.* 2008, Black, Cousens *et al.* 2010, Nair, Nokes *et al.* 2010, Nair, Brooks *et al.* 2011, Walker, Rudan *et al.* 2013). Rajaratnam *et al.,*2010 (Rajaratnam, Marcus *et al.* 2010) reported on the decrease in worldwide mortality in children less than 5 years of age from 11.9 million in 1990 to 7.7 million in 2010, however there was still a substantial amount of deaths occurring in the low-income countries: 33% in South Asia and 49% occur in sub-Saharan Africa, with less than 1% of deaths occurring in high-income countries. Acute respiratory tract infections are a significant cause of morbidity particularly in the very young and elderly and in immune-compromised patients (Billings, Hertz *et al.* 2001), and is a major cause for hospitalization. However, on average, children are infected two to three times more frequently that adults, with acute respiratory virus infections being the most common infections experienced by children, resulting in loss of school time and a significant socioeconomic cost in medical visits, medication and parent loss of work time (Kesson,

2007). For diagnosing viral respiratory tract infections clinical virology laboratories historically have used traditional methods such as direct fluorescent-antibody assay (DFA) and culture for detection of six or seven conventional respiratory viruses. DFA offers a rapid turnaround time for results but is labour-intensive, subjective, and require trained technologists as well as specific monoclonal antibodies. With traditional methods, such as DFA and culture, that use microscopy, turnaround time times for results are slow, especially in laboratories handling large volumes of specimens. These methods are also limited by the availability of monoclonal antibodies for newly discovered viruses (Mahony, Chong *et al.* 2007).

Most of the viruses are transmitted by direct contact or droplets, although some are transmitted by aerosols. Viruses that primarily infect the respiratory tract include: Influenza (INF), Adenovirus (AV), Parainfluenza virus (PIV), Respiratory Syncytial virus (RSV), Coronavirus (CV), human Metapneumovirus (hMPV), rhinovirus and Enterovirus (EV). The INF, PIV, hMPV and RSV occur in epidemics while the AV, CV and RV occur endemically.

2. Procedure

2.1 Intended use of the test assay

The Respiratory Virus Two Step Multiplex PCR test is a real-time nucleic acid assay for the qualitative detection of Respiratory Virus RNA and DNA. The assay can detect Respiratory Virus RNA and DNA in respiratory specimens collected in VTM. Specimen preparation is automated using the MagNA Pure LC instrument with the Total Nucleic

Acid (TNA) small volume kit. The primers for this Multiplex approach have been adapted from literature.

2.2 Validation of equipment

Please refer to individual validations of the MagNA Pure 96 and LightCycler 480. All equipment used was validated for this assay during the validation and optimization of the assay

2.3 Published articles in support of validation

All of the primers and probes used in this assay have been published in literature, although they have been optimized to function as different groups

3. Materials and Methods

3.1 Kits and reagents used

Reagents will be used according to manufacturer's instructions:

Roche MagNA Pure LC Total Isolation Kit (Cat # 03038505001)

Roche MagNA Pure 96 DNA and Viral NA small volume (Cat # 5467454001)

Transcriptor 1st strand cDNA synthesis kit (Cat # 4897030001)

LightCycler® 480 Probes Master Kit (Cat # 04887301001)

3.2 Specimens/ samples used for validation

The specimens used for the validation was the QCMD Respiratory panels of 2008 (INFRNA 2008, MPV.RSV 2008, ADV 2008, EV 2008, PINF 2008, CV.RV 2008), including the GRACE panel from QCMD as well as specific panels made for us on request by QCMD. Due to the amount of specimen needed and the cost involved it was

decided to use the QCMD panel. The results obtained from the panel could then also be compared to that of the QCMD final report so that we could determine how our assay correlated to other laboratories using the same technology. A comparison with a 150 patient specimens were also performed using a commercial kit for method comparisons. Comparisons with culture-based detection were also made by calculating a Kappa value. Table 1 shows the relationship between accuracy and precision as well as the essential elements for calculating negative and positive predictive values.

4. Criteria and Expected Results

The RVPCR assay used real-time detection on LightCycler 480 as a final detection method, for the sample to be deemed positive, it has to comply with several conditions:

- The analysis done in the high confidence setting
- Have a crossing point (ct) value
- Have fluorescent intensity of at least 2
- A uniform amplification slope.

From the final QCMD report for 2008 we can determine that we fell within the expected range of 2SD, and that we correlate well with the other laboratories using the same technology. The data obtained was used to calculate the overall Accuracy (97.8%), Negative Predicative Value (97.2%), Positive Predicative Value (100%), Sensitivity (91%) and Specificity (100%) (See Table 1 for individual groups. For raw data used for calculation please consult section 8).

From the QCMD Panel A-E output reproducibility (Fig 1-5, Tables 3-7), repeatability (Fig 6A-E), as well as lower detection limits (Table 8) was established. For Reproducibility the panel was repeated five times and the results correlated with the expected results

(Fig 1-5) and from the same results (Tables 3-7) the repeatability could be established by correlating the obtained results with what was expected (Fig 6A – E). Using the QCMD panels during five independent rRT-PCR runs, the coefficient of variation was calculated and ranged from 0.2% to 0.7%.

The cross reactivity of the assay was assessed using the GRACE panel that included a mixture of all the respiratory viruses tested for in this panel as well as Coronaviruses which are not included in the assay and bacteria (*M. pneumoniae, L. pneumophila, C. pneumoniae*). This panel was repeated 3 times to ensure repeatability and reproducibility. This panel was also used as an inter-variability measurement as two other laboratories had run the same panel with similar results (Table 8). However we found that with the exception of the rhinovirus and Enterovirus PCR none of the other PCR groups showed any cross reactivity. Rhinovirus and Enterovirus are closely related in the Picornaviridae family and to accommodate all the subgroups make use of conserved areas and as such RV cross reacts with EV. An EV PCR which does not cross react with RV is used to distinguish the EV positive from RV positives.

Few studies are available where symptomatic screening has been conducted of all the most common respiratory viruses known to cause SARI, in several geographic locations across South Africa. Over the last decade, nucleic acid detection methods have become increasingly popular for the detection of respiratory pathogens. Comparative studies have shown that the detection of respiratory viruses using real-time reverse transcriptase polymerase chain reaction (rRT-PCR) is substantially more sensitive than conventional methods like viral culture and IFA (Hendrickson 2004, Paranhos-Baccala, Komuruan-Pradel *et al.* 2008, R.Lassauniere, T.Kresfelder *et al.* 2010). Furthermore,

compared to conventional methods and mono-specific PCR, rRT-PCR multiplex PCR for clinical diagnosis has a significant advantage as it permits simultaneous amplification of several viruses in a single reaction, facilitating more cost-effective diagnosis and enabling the detection of multiple viruses in a single clinical specimen.

Thus to compare culture based methods for virus detection with the Respiratory Virus Two Step Multiplex PCR assay the inter-rater agreement or concordance was determined by calculating a Cohen's Kappa coefficient for qualitative values (Cohen 1960). According to the interpretation that Landis and Koch (Landis and Koch 1977) used for the κ-value < 0 equals no agreement, 0.0 – 0.2 slight agreement; 0.21 – 0.40 fair agreement; 0.41 – 0.60 moderate agreement; 0.61 – 0.80 substantial agreement and 0.81 – 1.00 an almost perfect agreement. Here the calculated kappa statistics for some respiratory viruses (Table 10) were 0.93 for RSV, 0.83 for INF A, 0.62 for PIV, and 0.24 for AdV.

To conclude the method validation for the rRT-PCR Multiplex the degree of agreement with the Seeplex® RV/PB18 ASE Detection kit (Seegene, Seoul, South Korea) was calculated by Bland- Altman analysis (Bland and Altman 1995) (Table 11) . The mean bias and limits of agreement were as follows: AdV: 0.135 [0.073, 0.343(]; EV: 0.091 [0.02, 0.203]; PIV: 0.062 [0.046, 0.17]; RSV: 0.047 [0.035, 0.13]; RV: 0.039 [0.01, 0.088] and INF: 0.053 [0.033, 0.074]. Bland-Altman analysis showed that the mean bias close to 0 shows equality and the limits of agreement show variability (Bland and Altman 1995). Thus the mean bias of the individual tests are close to 0 with a narrow limits of agreement on both side of the bias, showed that the RVPCR assay results compared very well with that of the Seeplex commercial kit..

5. Results

5.1 Accuracy, Sensitivity, specificity, NPV, PPV using 2008 QCMD, GRACE Respiratory virus Panels

Table 1 Relationship between accuracy and precision

Test results	**Positive**	**True Positive = 35**	**False Positive = 0** →	Positive Predictive Value
	Negative	**False Negative = 2** ↓	**True Negative = 5** ↓ ↘	Negative Predictive Value
		Sensitivity	Specificity	Accuracy

Table 2: Breakdown of Accuracy, NPV, PPV, Sensitivity, and Specificity for each multiplex group

PCR Group	Accuracy	NPV	Sensitivity	PPV	Specificity
A	98.3%	97.7%	93%	100%	100%
B	98.3%	98%	91.3%	100%	100%
C	97.2%	97%	87%	100%	100%
D	96%	94.3%	89%	100%	100%
E	99%	99%	94%	100%	100%

5.1.1 Accuracy

The calculated accuracy for this assay, which represents the degree of closeness of a measured or calculated quantity to its actual value, accuracy of this assay = 97.8%.

5.1.2 Negative Predictive value (NPV)

The negative predictive value which indicates the proportion of samples with negative results that are correctly identified, NPV For this assay = 97.2 %.

5.1.3 Positive Predictive value (PPV)

The positive predictive value which indicates the proportion of samples with positive results that are correctly identified, PPV for this assay = 100 %.

5.1.4 Sensitivity

Sensitivity measures the proportion of actual positive which are correctly identified, the sensitivity for this assay = 91 %.

5.1.5 Specificity

Specificity, measures the proportion of negatives which are correctly identifies, the specificity of this assay = 100 %.

5.2 Reproducibility using the QCMD Respiratory Panels A-E

The QCMD EQA panel was repeated five times to ensure reproducibility (Table 3-7, Figure 1-5) and the Log_{10} differences between each panel and the expected results were calculated to show that result fell within the calculated 1 SD range, showing that statistically there was no difference between the panel results. These results show that the Respiratory Virus Two Step Multiplex PCR assay would be a suitable assay for testing for respiratory viruses in clinical specimens, and was implanted as of February 2009 and final validation was signed off October 2010.

Table 3: Group A Reproducibility

RA panel							
Expected values	Log Run 1	Log Run 2	Log Run 3	Log Run 4	Log Run 5	Mean Log	STD Log

RA-01	PIV 1	31	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RA-02	PIV 1	1 X 10-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RA-03	PIV 1	1 X 10-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RA-04	PIV 1	1 X 10-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RA-05	PIV 2	33	1.47	1.47	1.47	1.48	1.47	1.47	0.00
RA-06	PIV 2	1 X 10-1	1.51	1.51	1.51	1.51	1.51	1.51	0.00
RA-07	PIV 2	1 X 10-2	0.00	0.00	0.00	0.00	1.55	1.55	0.69
RA-08	PIV 2	1 X 10-3	1.53	1.53	1.53	1.52	1.53	1.53	0.00
RA-09	PIV 3	34	1.41	1.41	1.41	1.42	1.42	1.41	0.00
RA-10	PIV 3	1 X 10-1	1.46	1.46	1.46	1.46	1.46	1.46	0.00
RA-11	PIV 3	1 X 10-2	1.51	1.50	1.50	1.51	1.50	1.51	0.01
RA-12	PIV 3	1 X 10-3	1.54	1.53	1.55	1.53	1.53	1.53	0.01
RA-13	NEG	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00

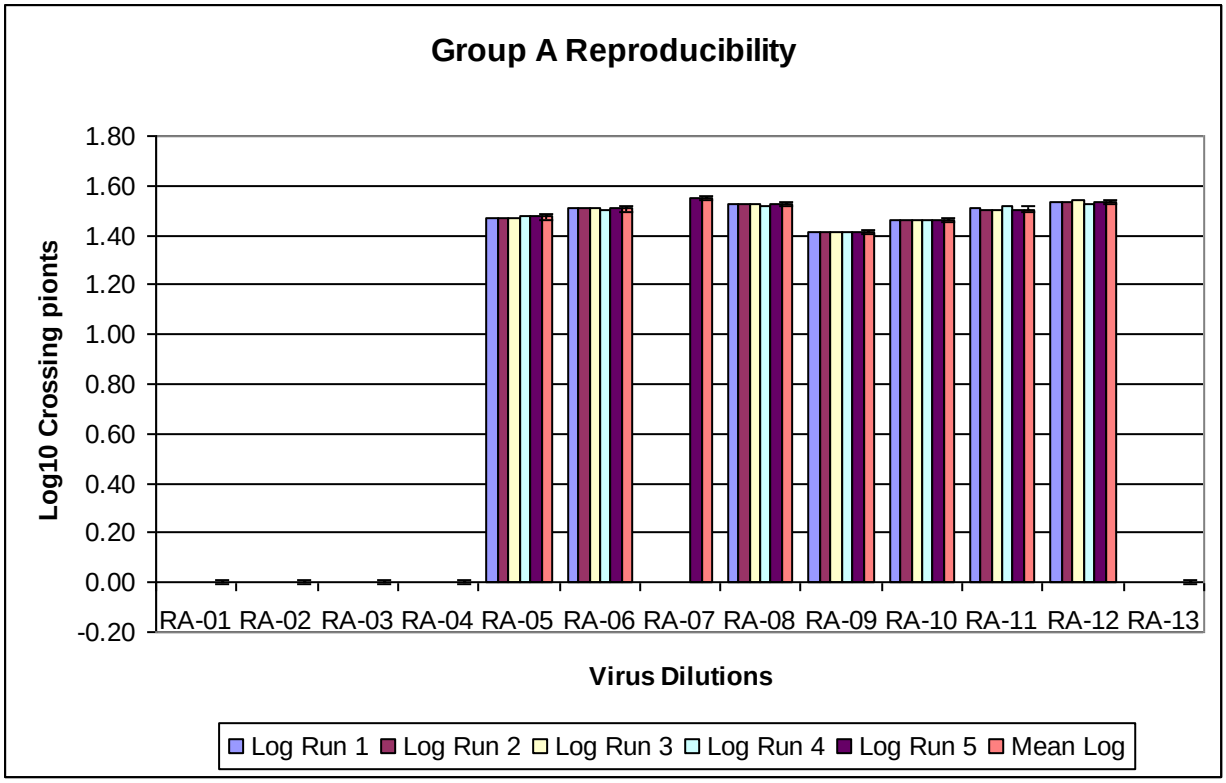


Figure 1: The graph represents the calculated Log_{10} values of the QCMD panel and the repeats of the panel. The error bars denotes the calculated 1 SD range

Table 4 Group B Reproducibility

RB panel									
Expected values			Log Run 1	Log Run 2	Log Run 3	Log Run 4	Log Run 5	Mean Log	STD Log
RB-01	RSV A	30.7	1.46	1.46	1.45	1.46	1.45	1.46	0.00
RB-02	RSV A	1 X 10-1	1.50	1.50	1.50	1.51	1.50	1.50	0.00
RB-03	RSV A	1 X 10-2	1.55	0.00	1.55	1.53	1.55	1.55	0.69
RB-04	RSV A	1 X 10-3	1.56	0.00	0.00	1.00	1.55	1.56	0.78
RB-05	RSV B	31.9	1.44	1.44	1.44	1.44	1.44	1.44	0.00
RB-06	RSV B	1 X 10-1	1.49	1.49	1.48	1.49	1.49	1.49	0.00

RB-07	RSV B	1 X 10-2	1.52	1.52	1.54	1.52	1.53	1.53	0.01
RB-08	RSV B	1 X 10-3	1.00	1.00	1.00	1.00	1.00	1.00	0.00
RB-09	INF B	33	1.47	1.47	1.47	1.47	1.47	1.47	0.00
RB-10	INF B	1 X 10-1	1.53	1.52	1.51	1.52	1.52	1.52	0.01
RB-11	INF B	1 X 10-2	1.55	0.00	0.00	0.00	1.55	1.55	0.85
RB-12	INF B	1 X 10-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RB-13	NEG	N/A	0.00	0.00	0.00	0.00	0.00	0.00	0.00

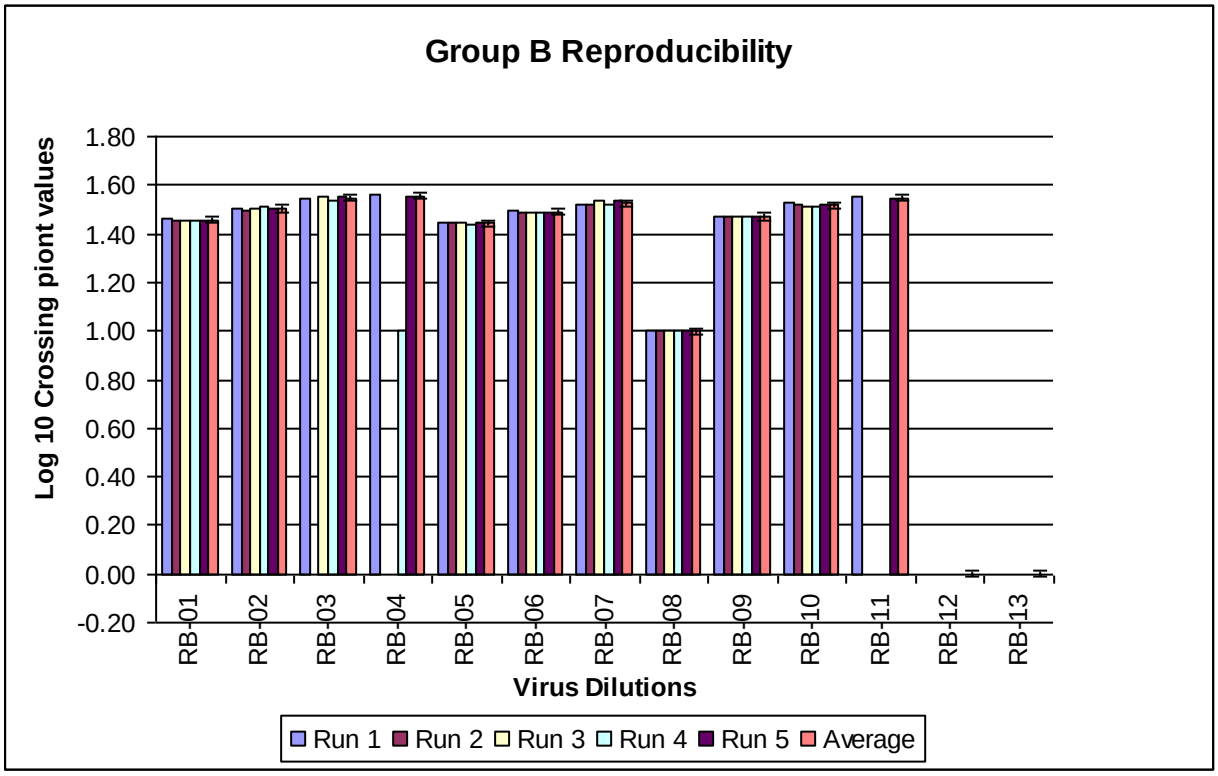


Figure 2: The graph represents the calculated Log_{10} values of the QCMD panel and the repeats of the panel. The error bars denotes the calculated 1 SD range.

Table 5 Group C Reproducibility

RC Panel

Expected values			Log Run 1	Log Run 2	Log Run 3	Log Run 4	Log Run 5	Mean Log	STD Log
RC-01	HMPV I	1 X 10	1.41	1.43	1.45	1.46	1.42	1.43	0.02
RC-02	HMPV I	1 X 10	1.45	0.00	1.48	0.00	0.00	1.46	0.80
RC-03	HMPV I	1 X 10 3	1.44	0.00	0.00	0.00	0.00	1.44	0.65
RC-04	EV71	1 X 10	1.32	1.33	1.36	1.36	1.32	1.34	0.02
RC-05	EV71	1 X 10	1.38	1.39	1.42	1.42	1.38	1.40	0.02
RC-06	EV71	1 X 10 3	1.43	1.43	1.46	1.45	1.42	1.44	0.01
RC-07	EV71	1 X 10 2	1.45	0.00	1.49	1.48	0.00	1.47	0.81
RC-08	COX B3	1 X 10	1.28	1.28	1.33	1.33	1.27	1.30	0.03
RC-09	COX B4	1 X 10	1.36	1.36	1.40	1.39	1.34	1.37	0.02
RC-10	COX B5	1 X 10 3	1.42	1.41	1.45	1.44	1.41	1.42	0.02
RC-11	COX B6	1 X 10 2	1.44	1.44	1.47	1.47	0.00	1.45	0.65
RC-12	COX B3	1 X 10 1	1.47	1.45	0.00	0.00	0.00	1.46	0.80
RC-13	COX A16	1 X 10	1.44	1.43	1.47	1.46	1.41	1.44	0.02
RC-14	COX A16	1 X 10 3	1.46	0.00	0.00	0.00	0.00	1.46	0.65
RC-15	COX A9	1 X 10	0.97	0.97	1.06	1.06	0.98	1.01	0.05
RC-16	COX A9	1 X 10	1.13	1.13	1.19	1.19	1.12	1.15	0.03
RC-17	COX A9	1 X 10 3	1.23	1.24	1.28	1.28	1.23	1.25	0.03
RC-18	COX A9	1 X 10 2	1.31	1.32	1.35	1.36	1.31	1.33	0.02
RC-19	COX A9	1 X 10 1	1.37	1.37	1.40	1.40	1.37	1.38	0.02
RC-20	POLIO 3	1 X 10	1.32	1.32	1.36	1.36	1.33	1.34	0.02
RC-21	POLIO 3	1 X 10	1.39	1.39	1.42	1.42	1.38	1.40	0.02
RC-22	POLIO 3	1 X 10 3	1.43	1.41	1.45	1.45	1.42	1.43	0.02

RC-23	POLIO 3	1 X 10 2	1.45	0.00	1.46	1.46	0.00	1.46	0.80
RC-24	POLIO 3	1 X 10 1	1.46	1.46	1.49	0.00	0.00	1.46	0.81
RC-25	NEG	N/A	0.00	0.00	0.00	0.00	0.00	0.00	0.00

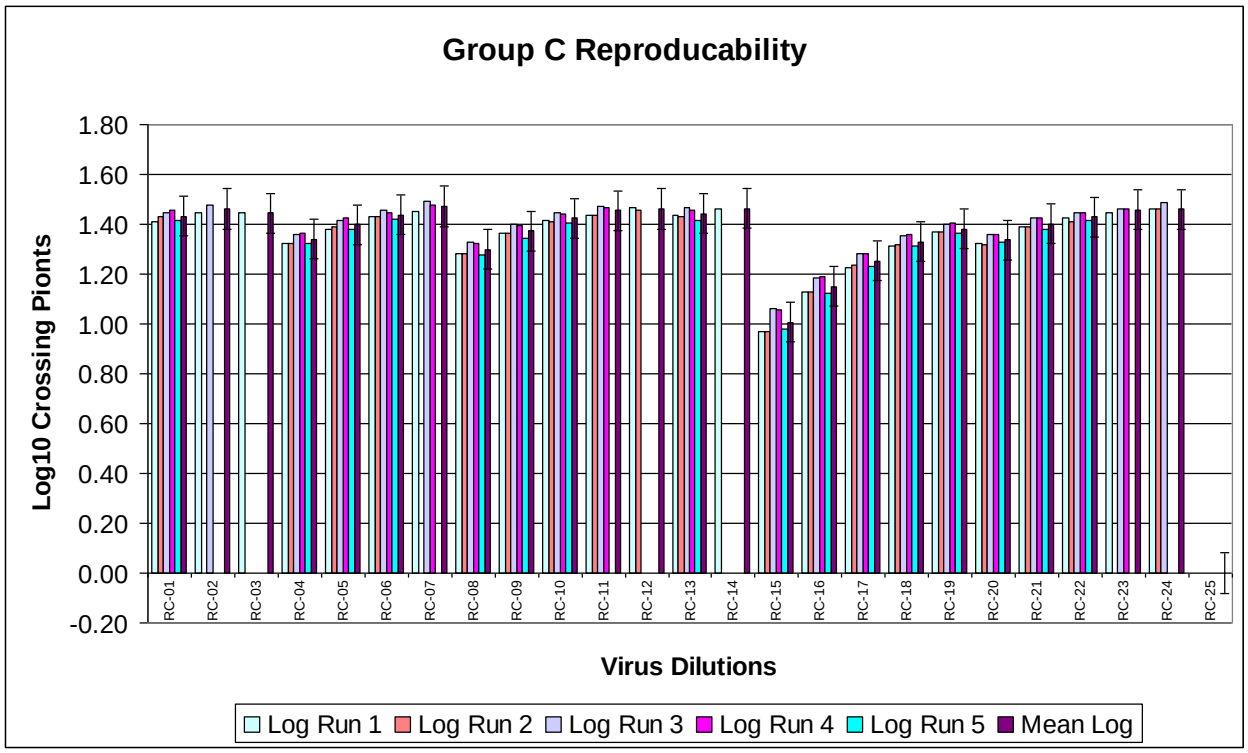


Figure 3: The graph represents the calculated Log_{10} values of the QCMD panel and the repeats of the panel. The error bars denotes the calculated 1 SD range.

Table 6 Groups D Reproducibility

RD panel									
Expected values			Log Run 1	Log Run 2	Log Run 3	Log Run 4	Log Run 5	Mean Log	STD Log
RD-01	ADV 1	1 X 10	1.49	1.50	1.49	1.50	1.48	1.49	0.01
RD-02	ADV 1	1 X 10	1.51	1.51	1.51	1.52	1.50	1.51	0.01

RD-03	ADV 1	1 X 10 3	1.52	1.51	0.00	0.00	1.52	1.52	0.83
RD-04	ADV 1	1 X 10 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RD-05	ADV 1	1 X 10 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RD-06	ADV 3	5 X 10 3	1.50	1.49	1.51	1.50	1.51	1.50	0.01
RD-07	ADV 3	5 X 10 2	1.52	0.00	1.53	1.52	1.52	1.52	0.68
RD-08	ADV 3	5 X 10 1	1.53	1.53	1.52	1.49	0.00	1.51	0.68
RD-09	ADV 4	1 X 10	1.49	1.49	1.49	1.53	1.48	1.50	0.02
RD-10	ADV 4	1 X 10 3	1.51	1.54	1.51	1.52	1.51	1.52	0.01
RD-11	ADV 4	1 X 10 2	1.52	0.00	1.52	0.00	0.00	1.52	0.83
RD-12	ADV 4	1 X 10 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RD-13	NEG		0.00	0.00	0.00	0.00	0.00	0.00	0.00
RD-14	H1	1X10-1	1.21	1.21	1.21	1.21	1.21	1.21	0.00
RD-15	H1	1X10-2	1.30	1.30	1.31	1.31	1.31	1.31	0.01
RD-16	H1	1X10-3	1.33	1.33	1.34	1.34	1.34	1.33	0.00
RD-17	H1	1X10-4	1.43	1.43	1.43	1.43	1.43	1.43	0.00
RD-18	H1	1X10-5	1.49	1.49	1.49	1.49	1.49	1.49	0.00
RD-19	H1	1X10-6	1.53	1.51	1.52	1.52	1.53	1.52	0.01
RD-20	H1	1X10-7	1.53	1.53	0.00	0.00	0.00	1.53	0.84
RD-21	H3	1X10-1	1.24	1.24	1.25	1.25	1.25	1.24	0.01
RD-22	H3	1X10-2	1.32	1.32	1.32	1.32	1.33	1.32	0.00
RD-23	H3	1X10-3	1.39	1.39	1.39	1.39	1.39	1.39	0.00
RD-24	H3	1X10-4	1.45	1.45	1.45	1.45	1.44	1.45	0.00
RD-25	H3	1X10-5	1.49	1.49	1.49	1.49	1.49	1.49	0.00
RD-26	H3	1X10-6	1.50	1.51	1.51	1.51	1.52	1.51	0.01
RD-27	H3	1X10-7	1.53	1.53	1.52	1.53	0.00	1.53	0.68

RD-28	H3	1X10-8	1.53	1.53	0.00	1.53	0.00	1.53	0.84

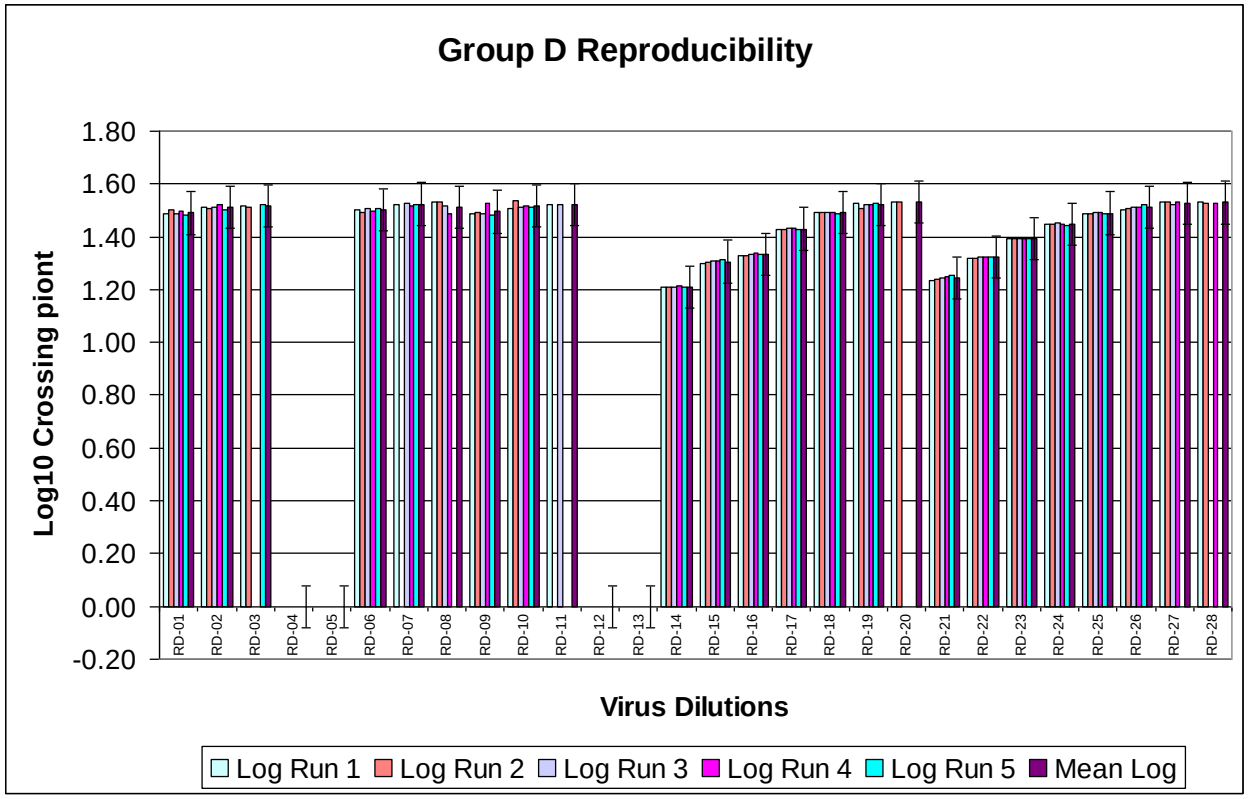


Figure 4: The graph represents the calculated Log_{10} values of the QCMD panel and the repeats of the panel. The error bars denotes the calculated 1 SD range.

Table 7: Group E Reproducibility

RE Panel								
Expected values			Log Run 1	Log Run 2	Log Run 3	Log Run 4	Log Run 5	Mean Log	STD Log
RE-01	RV	1X10-3	1.39	1.40	1.40	1.46	1.40	1.41	0.03
RE-02	RV	1X10-4	1.44	1.44	1.44	1.43	1.43	1.43	0.00
RE-03	RV	1X10-5	1.46	1.46	1.46	1.46	1.45	1.46	0.00
RE-04	RV	1X10-6	1.48	1.47	0.00	0.00	1.46	1.47	0.80

RE-05	RV	1X10-7	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RE-06	RV	NEG	0.00	0.00	0.00	0.00	0.00	0.00	0.00

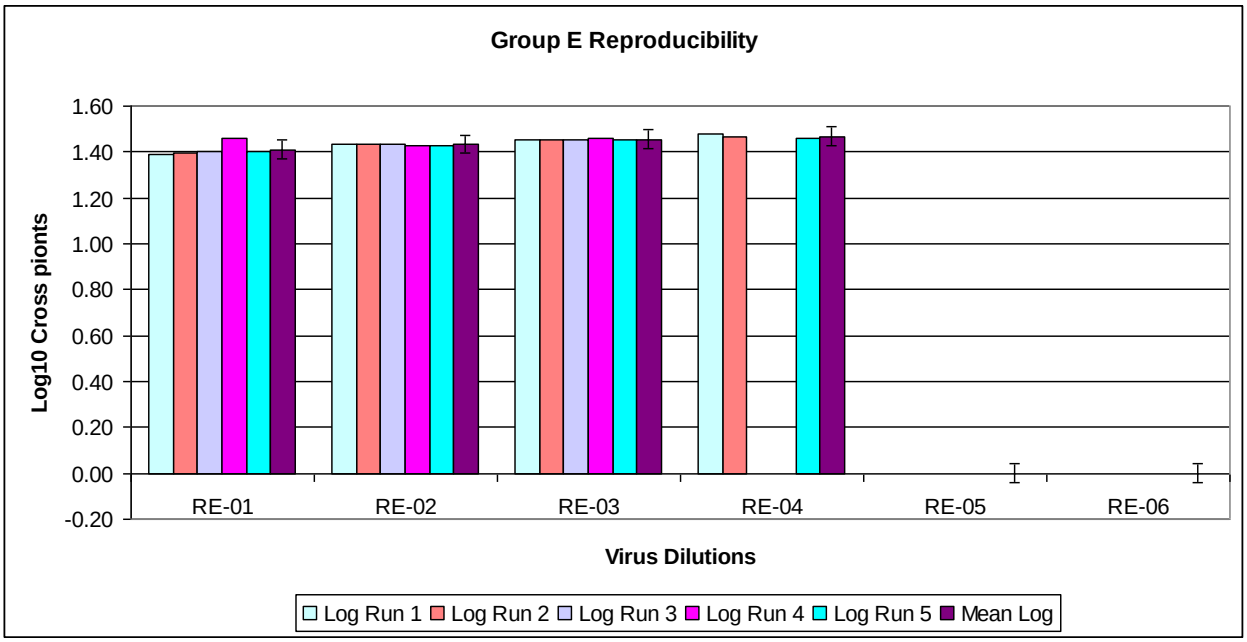


Figure 5: The graph represents the calculated Log_{10} values of the QCMD panel and the repeats of the panel. The error bars denotes the calculated 1 SD range.

Table 8: Inter variability with Grace Panel QCMD 2009

GRACE	Sample Content	Conc. / Dilution	QCMD EQA	Expected Result	UMC Utrecht	UMC Utrecht	UMC Leiden	UMC Leiden	RVU					
Sample Number		(Ct)	Panel Code		Results	CT Values	Results	CT Values	Run 1		run 2		run 3	
GRACE-01	HMPV-I	$1x10^{-6}$ (36)	RS.MV07-01	**hMPV** Positive	**hMPV** Positive	**37.93**	**hMPV** Positive	**45.1**	HMPV	33.56	NEG	NEG	NEG	NEG
GRACE-02	Influenza A virus Type H3	$1x10^{-7}$	INF06-08	**INF A** Positive	**PIV 2/4** Positive	**44.17**	Negative		INF A	>40.00	INF A	>40.00	INF A	39.69
GRACE-03	Parainfluenza virus 2	-34	N/A	**PIV** Positive	**PIV 2/4** Positive	**32.7**	**PIV 2** Positive	**33.1**	PIV 2	33.24	PIV 2	34.24	PIV 2	34.34
GRACE-04	Negative Transport Medium	0	N/A	**Negative**	**Negative**		Negative		NEG	NEG	NEG	NEG	NEG	NEG
GRACE-05	Negative Transport Medium	0	N/A	**Negative**	**Negative**		Negative		NEG	NEG	NEG	NEG	NEG	NEG
GRACE-06	Influenza B virus	$1x10^{-6}$	INF06-03	**INF B** Positive	**INF** Positive	**38.9**	**INF B** Positive	**39.3**	NEG	NEG	NEG	NEG	NEG	NEG
GRACE-07	HMPV-II	$1x10^{-5}$ (35)	RS.MV07-07	**hMPV** Positive	**hMPV** Positive	**37.85**	**hMPV** Positive	**41,5**	HMPV	32.88	HMPV	32.9	NEG	NEG
GRACE-08	Influenza A virus Type H3	$1x10^{-7}$	INF06-12	**INF A** Positive	**Negative**		**INF A** Positive	**39.5**	INF A	>40.00	INF A	>40.00	INF A	38.76
GRACE-	HMPV-II	$1x10^{-4}$	RS.MV07-06	**hMPV**	**hMPV** Positive	**34.02**	**hMPV**	**35.3**	HMPV	31.34	HMPV	31.05	HMPV	29.26

09		(33)		Positive			Positive							
GRACE-10	Parainfluenza virus 1	-30	N/A	**PIV** Positive	**PIV 1/3** Positive	**30.53**	**PIV 1** Positive	**34**	PIV 1	35.99	NEG	NEG	PIV 1	34.81
GRACE-11	Negative Transport Medium	0	N/A	**Negative**	**Negative**		Negative		NEG	NEG	NEG	NEG	NEG	NEG
GRACE-12	Influenza A virus Type H1	$1x10^{-6}$	INF06-02	**INF A** Positive	**INF** Positive	**40.39**	Negative		NEG	NEG	INF A	>40.00	INF A	37.81
GRACE-13	Parainfluenza virus 1	-34	N/A	**PIV** Positive	**PIV 1/3** Positive	**33.71**	**PIV 1** Positive	**38.1**	PIV 1	37.99	PIV 1	38.49	NEG	NEG
GRACE-14	HMPV-I	$1x10^{-4}$ (31)	RS.MV07-03	**hMPV** Positive	**hMPV** Positive	**31.38**	**INF A +hMPV** Pos	**40.3 + 32.2**	HMPV	31.12	HMPV	31.12	HMPV	29.86
GRACE-15	Influenza A virus Type H3	$1x10^{-6}$	INF06-04	**INF A** Positive	**INF** Positive	**37.75**	**INF A** Positive	**36.3**	INF A	39.08	INF A	39.54	INF A	37.69
GRACE-16	RSVB	$5x10^{-4}$ (32)	RS.MV07-10	**RSV** Positive	**RSV** Positive	**28.88**	**RSV** Positive	**33.4**	RSV	30.74	RSV	30.38	RSV	31.05
GRACE-17	Influenza A virus Type H1	$1x10^{-5}$	INF06-07	**INF A** Positive	**INF** Positive	**35.38**	**INF A** Positive	**34.4**	INF A	37.18	INF A	36.42	INF A	35.16
GRACE-18	Parainfluenza virus 3	-32	N/A	**PIV** Positive	**PIV 1/3** Positive	**34.7**	**PIV 3** Positive	**31.3**	PIV 3	31.9	PIV 3	30.92	PIV 3	30.49
GRACE-19	RSVA	$1x10^{-5}$ (30)	RS.MV07-04	**RSV** Positive	**RSV** Positive	**32.26**	**RSV** Positive	**40.7**	RSV	34.5	RSV	34.09	RSV	33.72
GRACE-	Parainfluenza virus 1	-27	N/A	**PIV** Positive	**PIV 1/3** Positive	**27.85**	**PIV 1**	**31.1**	PIV 1	31.84	PIV 1	31.89	PIV 1	32.73

20							Positive							
GRACE-21	RSVA	$1x10^{-3}$ (24)	RS.MV07-11	**RSV** Positive	**RSV** Positive	**24.24**	**RSV** Positive	**29.5**	RSV	25.88	RSV	25.48	RSV	26.91
GRACE-22	Coronavirus NL63($2x10^{-6}$)	(35-37)	RV.CV06-07	**CV** Positive	**CV** Positive	**32.79**	Negative		NEG	NEG	NEG	NEG	NEG	NEG
GRACE-23	Rhinovirus 90 (10^{-3})	(29-30)	RV.CV06-13	**RhV** Positive	**RhV** Positive	**29.58**	**RhV+ ADV4** Pos	**31.4 + 44.6**	RV	31.76	RV	31.78	RV	30.19
GRACE-24	Human Adenovirus 31	1.0x 10^{2}	ADV07-05	**ADV** Positive	**ADV** Positive	**38.89**	**ADV** Positive	**39.8**	ADV	39.46	ADV	>40.00	ADV	37.82
GRACE-25	Rhinovirus 16 (10^{-6})	(38-40)	RV.CV06-02	**RhV** Positive	**RhV** Positive	**39.7**	**RhV** Positive	**42.3**	NEG	NEG	NEG	NEG	NEG	NEG
GRACE-26	Negative Transport Medium	0	N/A	**Negative**	**Negative**		Negative		NEG	NEG	NEG	NEG	NEG	NEG
GRACE-27	Human Adenovirus 3	5.0x 10^{2}	ADV07-07	**ADV** Positive	**ADV** Positive	**34.06**	**ADV** Positive	**35.4**	ADV	>40	ADV	>40	ADV	36.85
GRACE-28	Rhinovirus 16 (10^{-4})	(29-30)	RV.CV06-09	**RhV** Positive	**RhV** Positive	**31.04**	**RhV+ ADV4** Pos	**34.6 + 40.0**	RV	33.15	RV	34.05	RV	31.47
GRACE-29	Human Adenovirus 4	2.5x 10^{2}	ADV07-12	**ADV** Positive	**ADV** Positive	**35.35**	**ADV** Positive	**37.1**	ADV	39.18	ADV	>40.00	ADV	37.31
GRACE-30	Coronavirus OC43 (10^{-4})	(34-36)	RV.CV06-08	**CV** Positive	**CV** Positive	**31.75**	**CV** Positive	**42.4**	NEG	NEG	NEG	NEG	NEG	NEG
GRACE-	Coronavirus 229E ($2x10^{-4}$)	(30-32)	RV.CV06-12	**CV** Positive	**CV** Positive	**31.83**	**CV+ ADV**	**37 + 45**	NEG	NEG	NEG	NEG	NEG	NEG

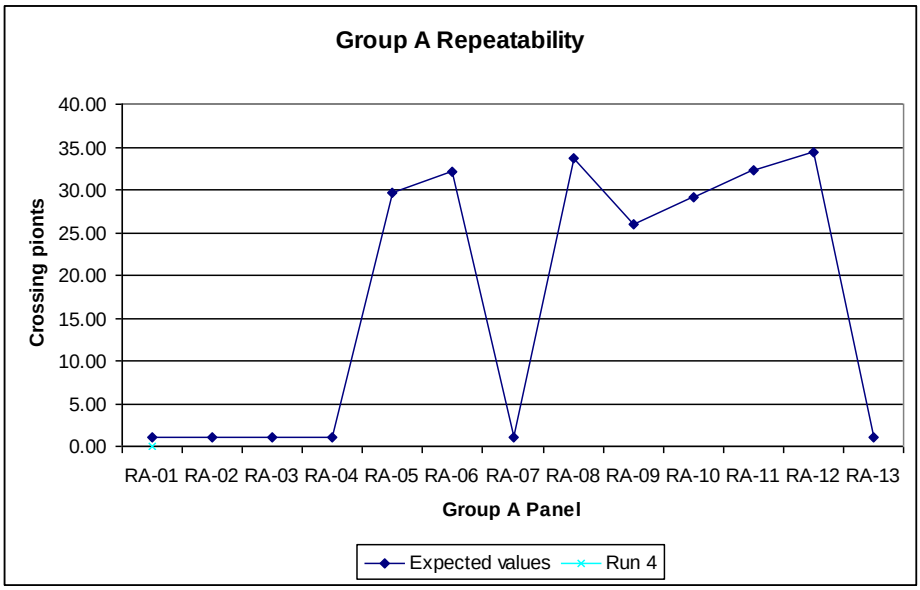
31							Positive							
GRACE-32	Human Adenovirus 1	1.0x 10^2	ADV07-11	**ADV** Positive	**ADV** Positive	**35.05**	**ADV** Positive	**38.4**	NEG	NEG	NEG	NEG	NEG	NEG
GRACE-33	Rhinovirus 90 (10^{-6})	(37-38)	RV.CV06-10	**RhV** Positive	**Negative**		Negative		NEG	NEG	NEG	NEG	NEG	NEG
GRACE-34	Rhinovirus 72 (10^{-5})	(33-34)	RV.CV06-06	**RhV** Positive	**RhV** Positive	**35.43**	**RhV** Positive	**39.1**	RV	35.83	RV	35.74	NEG	NEG
GRACE-35	*M. pneumoniae*	5000 CCU/ml	N/A	***Mp*** Positive	***Mp*** Positive	**29.15**	***Mp*** Positive	**36.3**	NEG	NEG	NEG	NEG	NEG	NEG
GRACE-36	Negative Transport Medium	0	N/A	Negative	Negative		Negative		NEG	NEG	NEG	NEG	NEG	NEG
GRACE-37	*L. pneumophila*	1800 CFU/ml	N/A	***Lp*** Positive	***Lp / Mp*** Positive	**33.25 / 32.7**	***Lp+ Mp*** Positive	**41.2 + 34.5**	NEG	NEG	NEG	NEG	NEG	NEG
GRACE-38	*M. pneumoniae*	50 CCU/ml	N/A	***Mp*** Positive	***Mp*** Positive	**34.49**	Negative		NEG	NEG	NEG	NEG	NEG	NEG
GRACE-39	*L. pneumophila*	180 CFU/ml	N/A	***Lp*** Positive	***Lp*** Positive	**36.73**	***Lp*** Positive	**36.4**	NEG	NEG	NEG	NEG	NEG	NEG
GRACE-40	*C. pneumoniae*	49 IFU/ml	N/A	***Cp*** Positive	***Cp*** Positive	**26.27**	***Cp*** Positive	**31.2**	NEG	NEG	NEG	NEG	NEG	NEG
GRACE-41	Negative Transport Medium	0	N/A	Negative	Negative		Negative		NEG	NEG	NEG	NEG	NEG	NEG
GRACE-	*M. pneumoniae*	500	N/A	***Mp*** Positive	***Mp*** Positive	**32.6**	***Mp*** Positive	**39.1**	NEG	NEG	NEG	NEG	NEG	NEG

42		CCU/ml												
GRACE-43	*C. pneumoniae*	490 IFU/ml	N/A	***Cp*** Positive	***Cp*** Positive	**22.88**	***Cp*** Positive	**28.5**	NEG	NEG	NEG	NEG	NEG	NEG
GRACE-44	*C. pneumoniae*	4.9 IFU/ml	N/A	***Cp*** Positive	***Cp*** Positive	**30.86**	***Cp*** Positive	**33.1**	NEG	NEG	NEG	NEG	NEG	NEG
GRACE-45	*L. pneumophila*	18 CFU/ml	N/A	***Lp*** Positive	***Lp*** Positive	**40.08**	Negative		NEG	NEG	NEG	NEG	NEG	NEG
GRACE-46	*C. pneumoniae*	4.9 IFU/ml	N/A	***Cp*** Positive	***Cp*** Positive	**29.91**	***Cp*** Positive	**34.8**	NEG	NEG	NEG	NEG	NEG	NEG
GRACE-47	*L. pneumophila*	60 CFU/ml	N/A	***Lp*** Positive	***Lp / Mp*** Positive	**38.07 / 35.9**	Negative		NEG	NEG	NEG	NEG	NEG	NEG
GRACE-48	*M. pneumoniae*	500 CCU/ml	N/A	***Mp*** Positive	***Mp*** Positive	**33.34**	***Mp*** Positive	**39.5**	NEG	NEG	NEG	NEG	NEG	NEG

5.3 Repeatability using the QCMD Respiratory Panels A-E

Using the data obtained from table 3 through 7, figure 6 illustrates the correlation between the expected and obtained results, and this in turn would be an indication of the repeatability of each specimen. As can be observed from the figures the specimens run parallel with each other and as seen from the previous correlating figures the log value of the obtained results fall within the SD1 specified range.

A



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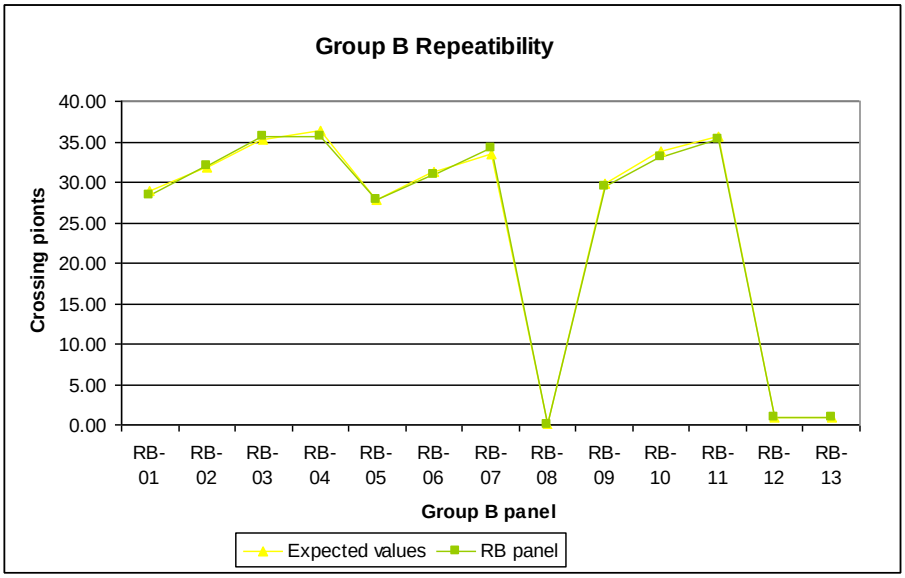

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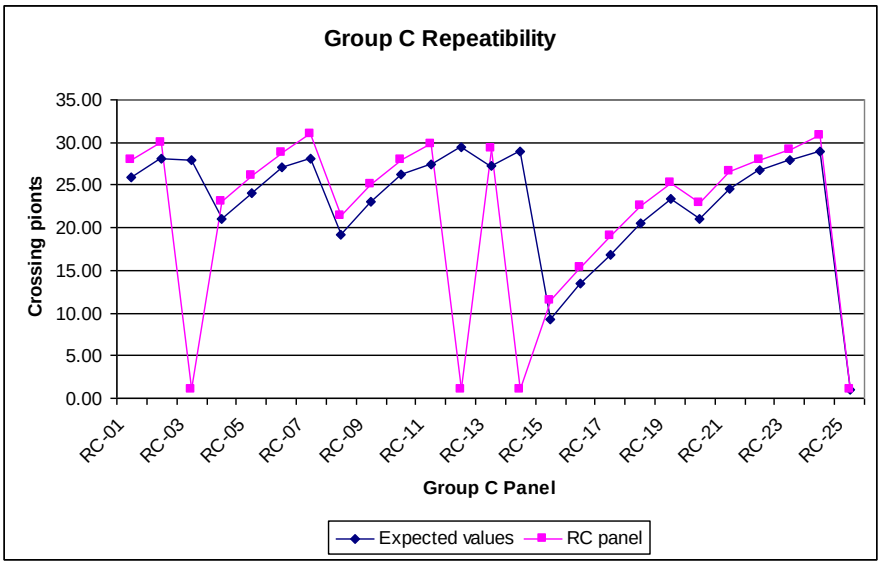

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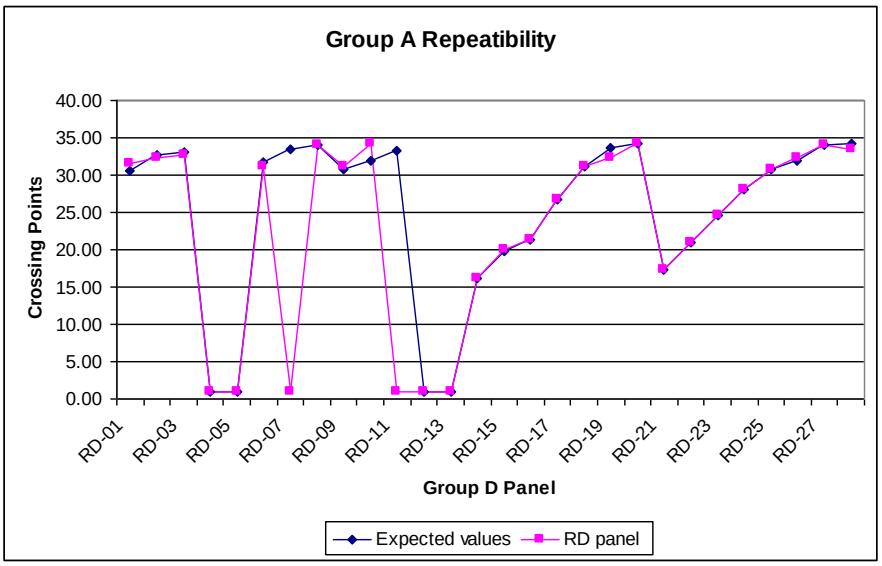

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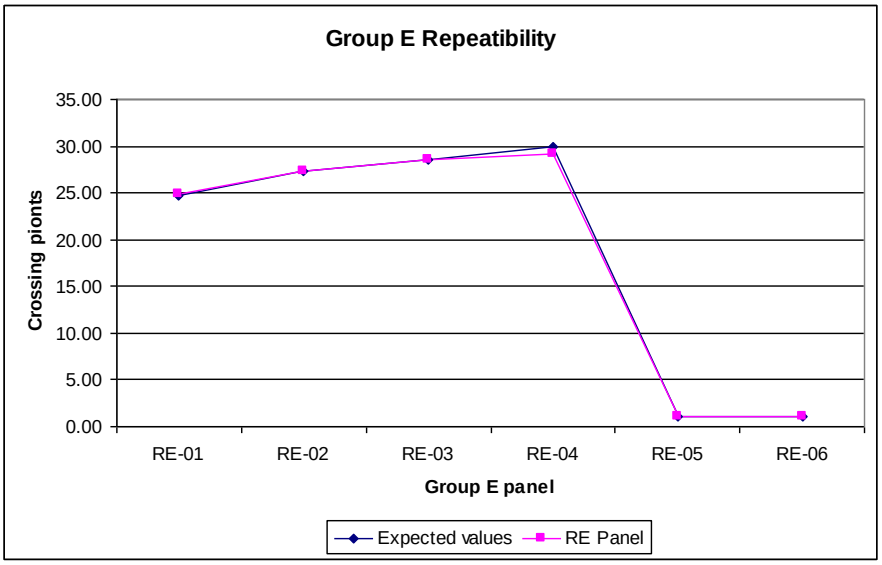


Figure 6: The graph A - E represents the comparison of the calculated Log_{10} values of the QCMD 2005 expected results versus the Obtained results in copies/ ml to establish the repeatability of the assay.

As can be observed from figure 6 it illustrates the correlation between the expected and obtained results for the QCMD, the specimens run parallel with each other. In figure 6 C and D which represents the correlation between the expected and obtained results for the RC panel and RD panel, some discrepancies can be observed. These discrepancies can be explained by the lower detection limits (Table8), where these specimens were outside our limit of detection. The panel performance for RVU was accepted as per the QCMD report and continuous monitoring will take place though QCMD EQA panels see Section 4.

5.4 Detection limits of the RVPCR assay

Table 9 Lower Detection Limits of the RVPCR assay.

Virus	Ct value	2 SD	Percentage Detection
PIV 1	34	0.34	100
	37	0	10
PIV 2	34	0.27	100
	38	0.57	100
	40	0	10
PIV 3	37	0.8	100
	39	1.15	100
RSV	36	0.58	100
	40	1.49	80
	41	1.02	40
INF B	38	0.96	100
	40	0.55	40
hMPV	32	2.42	100
	34	2.7	40
EV	32	1.88	100
	33	2.51	80
	34	1.25	40
INF A	38	1.11	100
	39	0.1	40
AV	37	0.99	100
	38	0.17	40
RV	33	0.31	100
	34	1.05	60

5.5 Method Comparisons

5.5.1 Kappa Values

Table 10: Kappa values for PCR Culture comparisons

Kappa values for Culture	
RSV	0.93
Av.	0.24
PIV	0.62
Inf.	0.83

As can be observed AV has a Kappa Value of 0.2, but if you look at the BA plot the Bias between the PCR technologies is close to 0 which indicates accuracy and the small limits of agreement show precision. According to the interpretation that Landis and Koch(Landis and Koch 1977) used for the κ-value where < 0 equals no agreement, 0.0 – 0.2 slight agreement; 0.21 – 0.40 fair agreement; 0.41 – 0.60 moderate agreement; 0.61 – 0.80 substantial agreement and 0.81 – 1.00 Almost perfect agreement. Most of the values calculated show a substantial to almost perfect agreement.

5.5.2 Bland Altman Plots

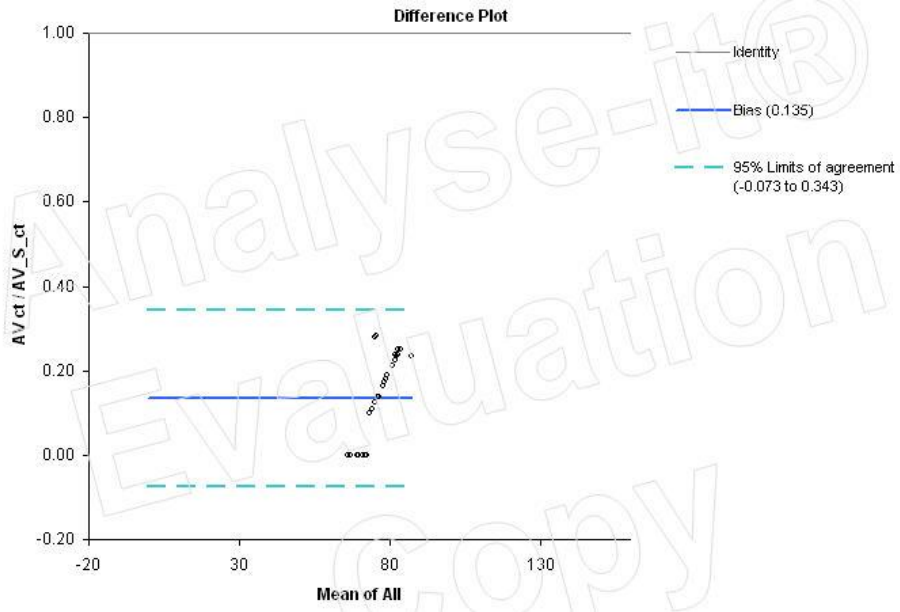
Ideally two methods compare favourably when the mean paired differences is close to zero (a zero means implies equality), limits of agreement are narrow and a few outliers are present.

Table 11 Bland Altman plots for comparison between assay and Seeplex kit.

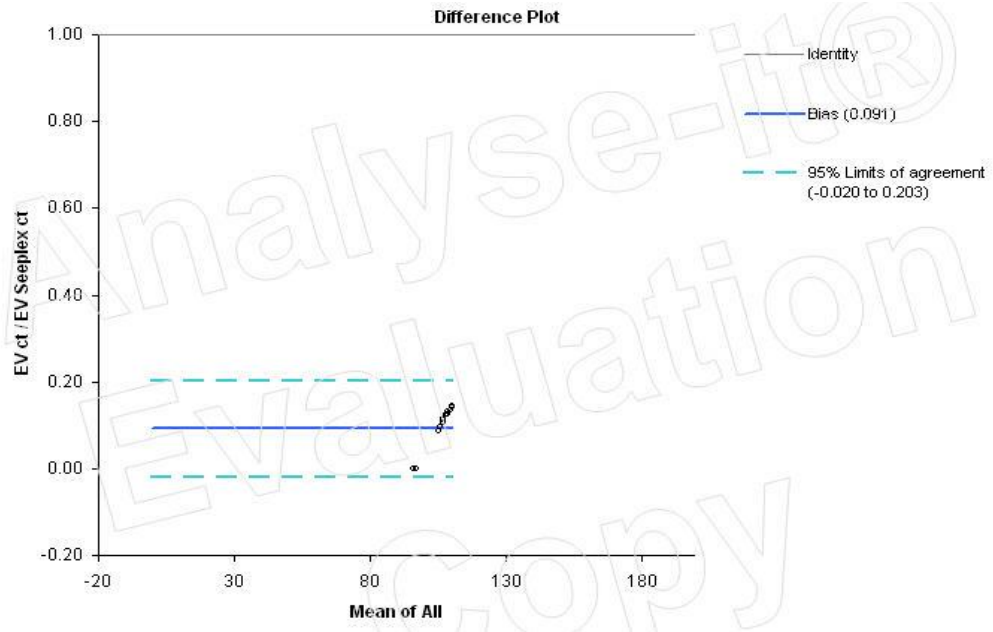
Virus	Bias	Limits of agreement		SD
AV	0.135	-0.073	0.343	0.016

EV	0.091	-0.02	0.203	0.057
PIV	0.062	-0.046	0.17	0.055
RSV	0.047	-0.035	0.13	0.042
RV	0.039	-0.01	0.088	0.025
INF	0.053	0.033	0.074	0.011

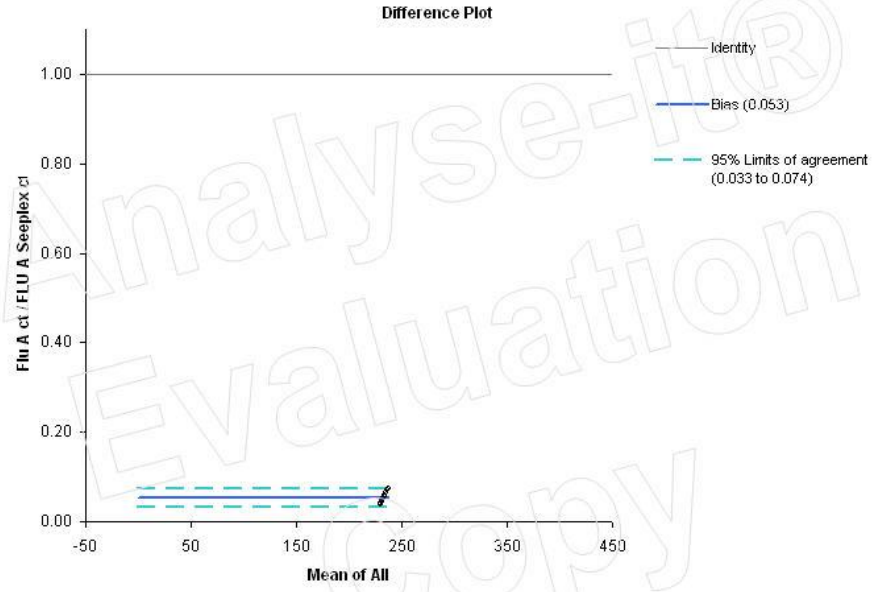
i Adenovirus



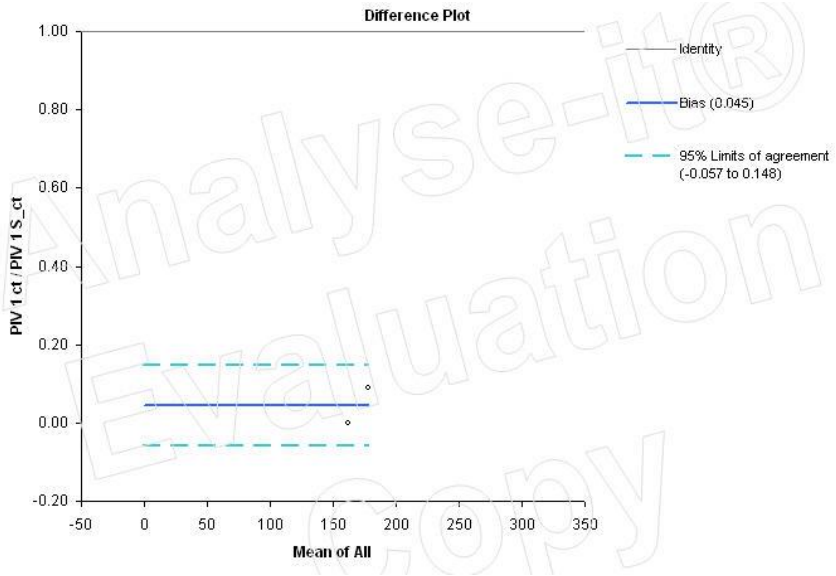
ii Enterovirus



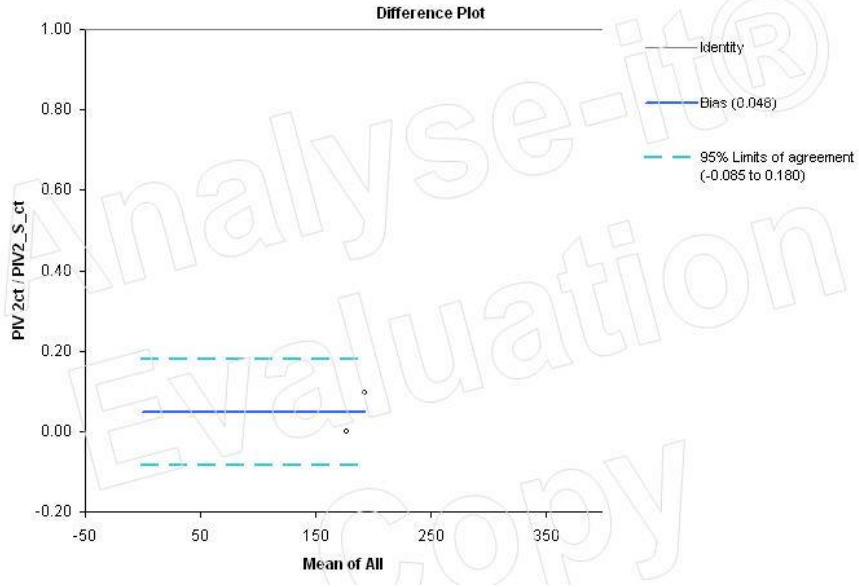
iii Influenza A



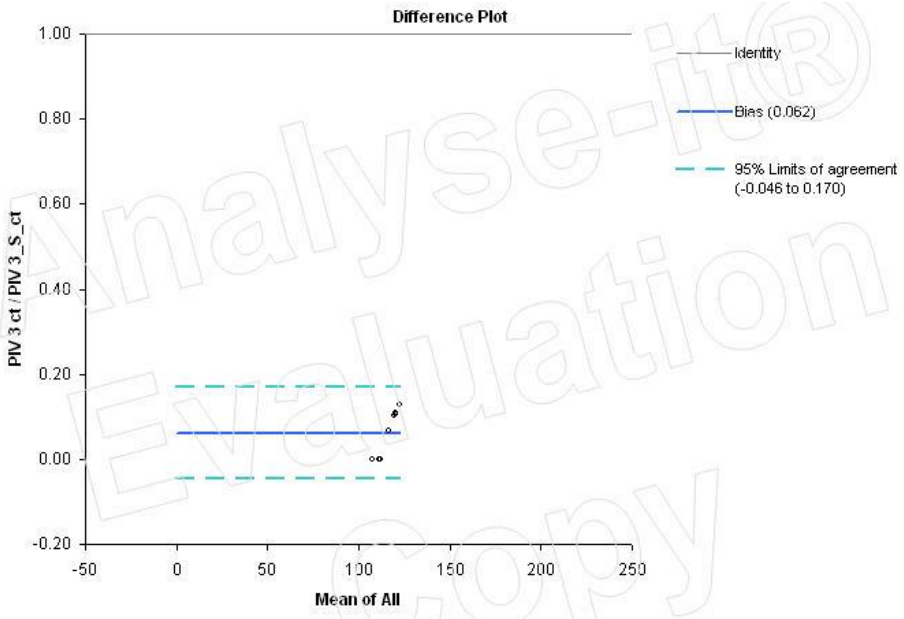
iv PIV 1



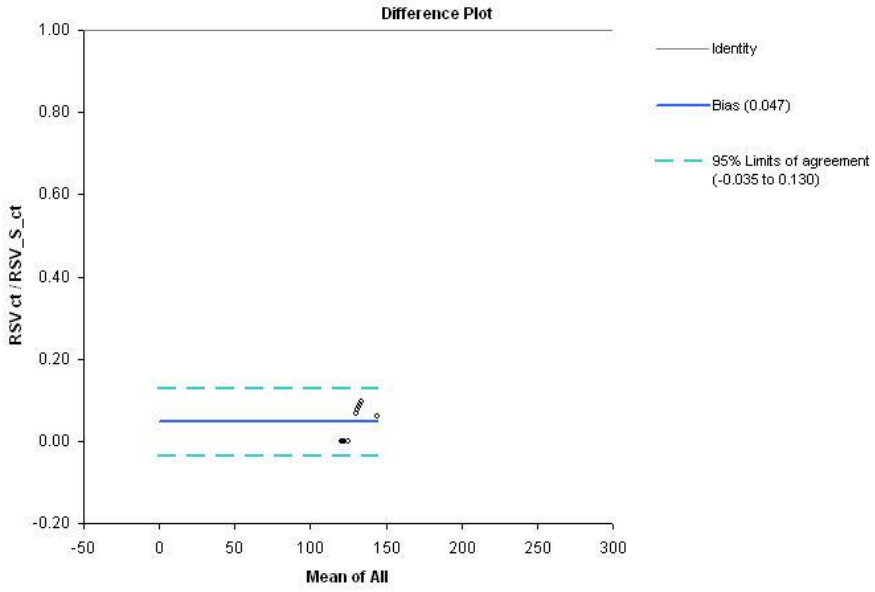
v PIV 2



vi PIV 3



vii RSV



viii RV

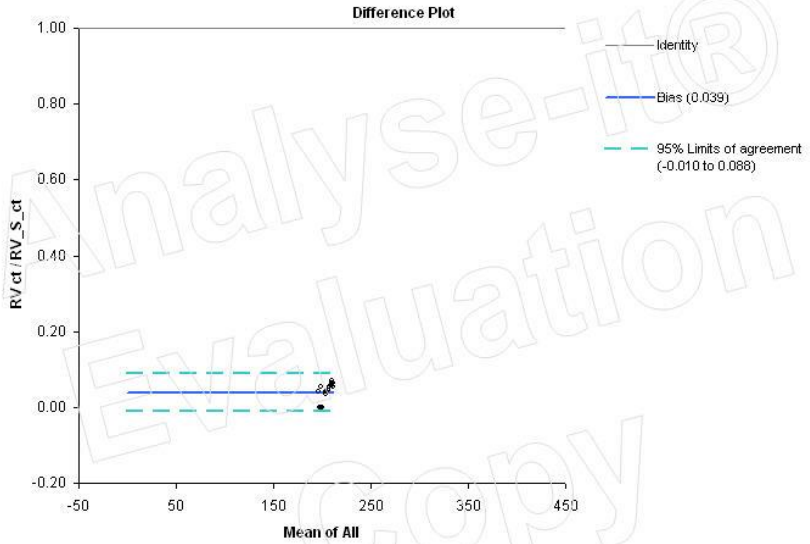


Figure 7: (i-viii) Bland Altman Plots of the RVPCR assay compared to the Seeplex® RV/PB18 ASE Detection kit (Seegene, Seoul, South Korea)

6. Uncertainty of measurement

PCR efficiencies are always important when doing Real-Time assays, no traceable WHO standards available that could be used to calculate this. As with any test procedure and assay, good laboratory practice is essential to the proper performance of this assay. The test has been validated for the use with respiratory specimens collected in VTM, accuracy of other specimens not. As most of the respiratory viruses are RNA viruses; cold chain, collection, transport of specimen, storage and processing procedures are of utmost importance. Amplicon contamination, contamination from positive controls and clinical specimens can be avoided only by good laboratory practices. The use of the assay should be limited to personnel that have been trained and deemed competent by set standards

7. Conclusion

Validation of molecular assays is complicated and there is continuous evaluation of the method with EQA but to date comparable data has been observed when comparing to cell culture methods and other established PCR methods with exceptions. The PPV and specificity at a 100 % indicates that the assay is adequate in that it does not give any false positive results. The NPV (97.2%) and sensitivity (91%) is determined by the lower detection limit of this assay, this assay gives false negatives with specimens with low viral load. The sensitivity and specificity together with NPV and PPV indicates that this assay is more than suitable for the detection of Respiratory Viruses in clinical specimens. This method has been validated and found suitable to use in the Respiratory Virus Unit, NICD. The assay was implemented from February 2009 with final validation signed off in October 2010. Although the test has been implemented evaluation is on going (dependant on cost and test sample availability) and new data points will be added as they become available.

Appendix B: Mean cytokine concentration (pg/ml) present in NPAs of children ≥1 year of age associated with single RSV, single RV and RSV and RV co-infection.

	Cytokine	SARI						ILI			Controls		
		RSV HIV+	RSV HIV-	RV HIV+	RV HIV-	RSV/RV HIV+	RSV/RV HIV-	RSV	RV	RSV/RV	RSV	RV	RSV/RV
		mean (95% CI)						mean (95% CI)			mean (95% CI)		
		n= 24	n= 42	n=35	n=36	n=8	n=36	n=7	n=14	n=8	n=3	n=8	n=1
Th1 cytokine cell mediated immunity	IL-1ra	96.97 (-19.76; 213.7093)	62.95 (35.77; 90.13)	143.8 (81.47; 206.13)	210.19 (120.49; 299.91)	108.34 (10.24; 206.43)	146.09 (95.73; 196.46)	71.19 (-46.16; 188.54)	165.68 (-5.77; 337.13)	76.56 (-25.86; 178.97)	Not detected	58.98 (-12.02; 129.99)	7.58
	IL-7	2.37 (0.94; 3.81)	1.166 (0.71; 1.62)	2.66 (1.32; 3.99)	2.34 (1.27; 3.42)	6.25 (0.07; 12.44)	2.72 (1.85; 3.59)	1.83 (-0.07; 3.72)	1.04 (0.33; 1.75)	1.53 (0.05; 3.01)	Not detected	1.23 (0.35; 2.11)	0.88
	IL-12	0.62 (0.22; 1.02)	0.55 (0.13; 0.97)	9.77 (5.30; 14.24)	7.65 (4.77; 10.52)	1.31 (0.03; 2.59)	0.64 (0.28; 1.00)	1.36 (-0.04; 2.76)	0.81 (-0.13; 1.76)	1.36 (-1.02; 3.74)	Not detected	Not detected	Not detected
	IL-15	0.89 (0.39; 1.39)	0.75 (0.44; 1.01)	0.06 (-0.02; 0.13)	0.01 (-0.01; 0.02)	0.71 (-0.21; 1.62)	0.38 (0.16; 0.59)	0.81 (0.37; 1.24)	0.22 (-0.01; 0.45)	0.19 (-0.01; 0.41)	Not detected	Not detected	Not detected
	G-CSF	1701.17 (572.63; 2829.72)	1229.49 (810.61; 1648.38)	945.33 (464.823; 1425.83)	1188.36 (465.94; 1910.77)	2036.46 (584.99; 3487.93)	2574.23 (1608.41; 3540.05)	982.38 (150.03; 1814.75)	1381.03 (-500.76; 3262.83)	1997.37 (-139.87; 4134.61)	Not detected	907.58 (-94.02; 1909.18)	Not detected
	GM-CSF	0.86 (-0.13; 1.85)	0.07 (-0.03; 0.17)	0.34 (-0.07; 0.75)	0.59 (0.04; 1.15)	1.45 (-1.33; 4.24)	0.14 (-0.01; 0.29)	0.07 (-0.10; 0.24)	0.06 (-0.05; 0.18)	0.004 (-0.01; 0.02)	Not detected	Not detected	Not detected

Table 2 continued

	IFN-γ	15.7 (5.79; 25.62)	12.89 (5.04; 20.74)	20.72 (14.65; 26.79)	22.11 (16.98; 27.23)	10.95 (-3.48; 25.38)	7.47 (3.61; 11.34)	15.07 (-4.27; 34.38)	5.05 (0.58; 9.53)	5.78 (-1.70; 13.25)	Not detected	5.83 (-7.52; 19.19)	Not detected
Th2 cytokine humoral mediated immunity	IL-6	626.83 (-347.42; 1601.09)	49.99 (16.17; 83.80)	23.61 (9.46; 37.76)	52.29 (18.28; 86.32)	22.39 (5.49; 39.28)	50.75 (25.54; 75.96)	44.08 (-15.05; 103.23)	28.74 (-20.26; 77.75)	18.12 (-16.24; 52.48)	Not detected	8.89 (-6.73; 24.51)	10.67
	IL-10	8.67 (-4.70; 22.05)	7.68 (1.69; 13.67)	1.62 (0.60; 2.63)	2.07 (0.94; 3.19)	3.42 (-1.32; 8.16)	6.87 (3.89; 9.84)	12.67 (-5.03; 30.37)	6.67 (-0.38; 13.73)	2.24 (-0.38; 4.86)	Not detected	5.78 (-4.54; 16.11)	1.59
Chemokines	IL-13	2.77 (0.89; 4.65)	0.98 (0.50; 1.47)	2.65 (0.61; 4.69)	3.46 (1.05; 5.87)	2.25 (-0.23; 4.73)	0.98 (0.66; 1.29)	0.58 (0.02; 1.14)	0.48 (0.22; 0.75)	0.62 (-0.01; 1.26)	Not detected	0.22 (0.17; 0.28)	0.17
	IL-8	2823.17 (40.89; 5605.46)	805.7 (397.85; 1213.55)	1449.21 (940.95; 1957.47)	1744.98 (1111.59; 2378.36)	1331.53 (-356.26; 3019.32)	1326.78 (871.52; 1782.04)	737.84 (-227.46; 1703.15)	771.49 (-506.37; 2049.35)	601.99 (25.82; 1178.17)	Not detected	300.98 (-79.58; 681.54)	302.56
	Eotaxin	21.23 (7.58; 34.88)	14.97 (9.54; 20.41)	12.45 (4.82; 20.07)	4.5 (1.85; 7.16)	38.86 (-7.42; 85.14)	21.14 (11.06; 31.23)	19.82 (0.44; 39.21)	9.99 (-0.67; 20.6624	15.97 (-0.16; 32.10)	Not detected	4.61 (-0.98; 10.19)	Not detected
	IP-10	6350.13 (1797.91; 10902.34)	3782.79 (912; 6653.59)	7034.11 (2877.48; 11190.75)	6828.92 (2580.02; 11077.81)	9538.85 (-3506.614; 22584.31)	8847.2 (1134.23; 16560.17)	7756.05 (-5117.183; 20629.28)	3309.23 (-1506.45; 8124.92)	5723.74 (-1417.99; 12865.48)	Not detected	880.49 (-268.63; 2029.61)	Not detected
	MCP-1	87.44 (-20.99; 195.87)	29.27 (13.54; 44.98)	12.99 (5.63; 20.34)	36.85 (12.93; 60.78)	19.4 (2.07; 36.73)	31.48 (11.17; 51.80)	11.63 (-0.78; 24.04)	12.77 (0.71; 24.83)	19.03 (-7.79; 45.87)	Not detected	4.92 (-4.51; 14.36)	88.36
	RANTES	55.1	18.66	22.97	17.35	43.89	27.89	15.51	10.74	13.31	Not	12.79	7.05

	(17.51; 92.69)	(9.07; 28.25)	(6.19; 39.74)	(1.77; 32.93)	(0.88; 86.91)	(13.64; 42.14)	(-9.19; 40.22)	(-1.06; 22.51)	(-4.08; 30.68)	detected (-6.59; 32.18)

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