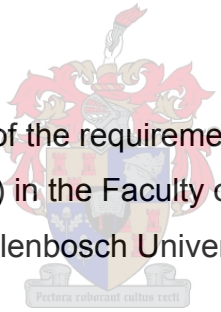
Prevalence and Risk Factors of Acute Respiratory Infection by Human Respiratory Syncytial Virus in Children at Provincial General Hospital of Bukavu, Democratic Republic of the Congo

By Landry Kabego Cihambanya



Thesis presented in fulfilment of the requirements for the Degree of Master of Medical Science (Medical Virology) in the Faculty of Medicine and Health Sciences at Stellenbosch University

Supervisor: Dr Corena de Beer

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part, submitted it at any university for a degree.

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ABSTRACT

Human Respiratory Syncytial Virus (HRSV) is the major cause of acute respiratory infection in children (ARI) and it is responsible for substantial morbidity and mortality, especially in younger children. The present study had two main objectives. The first one was to determine the prevalence of HRSV and non-HRSV ARI in children under the age of 5 years at the Provincial General Hospital of Bukavu (PGHB). The second objective was to analyse factors associated with the risk of ARI to be diagnosed as lower respiratory tract infection (LRTI).

A total of 146 children under 5 years visiting the PGHB for ARI between August and December 2016 were recruited. A clinical examination was made and a questionnaire was completed by the parent or the guardian after which a nasopharyngeal swab was performed to collect respiratory fluid. The sample was analysed by a multiplex reverse transcriptase polymerase chain reaction for the detection of 15 different viruses, among which HRSV A and B, Influenza A and B, human Rhinovirus (HRV) A/B/C, Parainfluenza (PIV) viruses 1, 2, 3 and 4, Adenovirus (ADV), Bocavirus, Coronavirus OC43 and 229E/NL63, Enterovirus and human Metapneumovirus.

Of 146 samples collected, 84 (57.5%) displayed a positive result of at least one of the 15 viruses. The overall prevalence of HRSV was 21.2%. HRSV A (30, 20.5%) was the virus the most detected, followed by HRV (24, 16.4%), PIV3 (20, 16.6) and ADV (7, 4.79%). The other viruses were detected in three or less cases. There were only 11 (7.5%) of co-infection. In bivariate analyses, HRSV infection, malnutrition, younger age, rural settings, low income and mother illiteracy were associated with the risk of ARI to be diagnosed as LRTI. However, in multivariate analyses, only HRSV infection and younger age predicted LRTI. Children with HRSV infection had 6.45 times higher odds to exhibit LRTI when compared to children without HRSV infection. Older children (by one month) had 6% lower odds of LRTI than younger children (adjusted odds ratio = 0.94, 95% CI: 0.90 – 0.97, p-value = 0.004).

OPSOMMING

Respiratoriese sinsitiale virus (RSV) is die hoofoorsaak van akute respiratoriese infeksie (ARI) by kinders en dit is verantwoordelik vir erge morbiditeit en mortaliteit, veral by jonger kinders. Die huidige studie het twee hoofdoelwitte gehad. Die eerste een was om die voorkoms van RSV en nie-RSV ARI in kinders onder die ouderdom van 5 jaar by die Provinsiale Algemene Hospitaal van Bukavu (PGHB) te bepaal. Die tweede doel was om faktore te analiseer wat verband hou met die risiko dat ARI gediagnoseer word as lae lugweginfeksie.

'n Totaal van 146 kinders onder 5 jaar wat die PGHB vir ARI besoek het tussen Augustus en Desember 2016 is gewerf vir die studie. 'n Kliniese ondersoek is gedoen en 'n vraelys is deur die ouer of voog voltooi, waarna respiratoriese vloeistof met behulp van 'n nasofaringeale depper versamel is. Die monster is geanaliseer met behulp van 'n multiplex-omgekeerde transkriptase-polimerase kettingreaksie vir die opsporing van 15 verskillende virusse, waaronder RSV A en B, Influenza A en B, menslike Rhinovirus (HRV) A / B / C, Parainfluenza virus (PIV) 2, 3 en 4, Adenovirus (ADV), Bocavirus, Coronavirus OC43 en 229E / NL63, Enterovirus en menslike Metapneumovirus.

Van 146 monsters wat versamel is, is minstens een van die 15 virusse bevestig in 84 (57.5%) gevalle. Die algehele voorkoms van RSV was 21.2%. RSV A (30, 20.5%) was die virus wat die meeste bespeur is, gevolg deur HRV (24, 16.4%), PIV3 (20,16.6) en ADV (7, 4.79%). Die ander virusse is slegs in drie of minder gevalle bevestig. Daar was slegs 11 (7.5%) ko-infeksie. In ’n twee-veranderlike analise is RSV-infeksie, wanvoeding, jonger ouderdom, landelike woongebiede, lae inkomste en ongeletterdheid in moeders geassosieer met die risiko dat ARI gediagnoseer word as lae lugweginfeksie. In ’n meer-veranderlike analise het slegs RSV-infeksie en jonger ouderdom lae lugweginfeksie voorspel. Kinders met RSV-infeksie het ’n 6.45 keer hoër kans gehad om lae lugweginfeksie te vertoon in vergelyking met kinders sonder RSV-infeksie. Ouer kinders (met een maand) het ’n 6% laer kans gehad op lae lugweginfeksie as jonger kinders (aangepaste kansverhouding = 0.94, 95% CI: 0.90 – 0.97, p-waarde = 0.004).

DEDICATION

To my parents

For their unconditional love

Vincent Kabego and Praxeda Mahongole

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“Much unhappiness has come into the world because of bewilderment and things left unsaid.”

Fyodor Dostoyevsky

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LIST OF ABBREVIATIONS

aa: Amino acid
AAP: American academy of paediatrics
ADV: Adenovirus
APC: Antigen-presenting cell
ARI: Acute respiratory infection
cDC: Conventional dendritic cell
cDNA: Complementary DNA
CI: Confidence interval
CoV: Coronavirus
CTL: Cytotoxic T lymphocyte
DC: Dendritic cell
DFA: Direct immunofluorescence assay
DRC: Democratic Republic of Congo
EV: Enterovirus
EIA: Enzyme immunoassay
GAG: glycosaminoglycans
GE: Gene end
GS: Gene start
HBoV: Human bocavirus
HMPV: Human metapneumovirus
HRV: Human rhinovirus
IFN: Interferon
IL: Interleukin
IP: Inducible protein
IPC: Infection prevention and control
IQR: Interquartile range
Le: Leader
LRTI: Lower respiratory tract infection
MCP: Monocyte chemotactic protein
MHC: Major histocompatibility complex
MIP: Macrophage inflammatory protein
mRNA: Messenger RNA
OR: Odds Ratio
ORF: Open reading frame
pDC: Plasmacytoid dendritic cell
PGHB: Provincial General Hospital of Bukavu

PIV: Parainfluenza virus

RNA: Ribonucleic acid

HRSV: Human Respiratory syncytial virus

RT-PCR: Reverse transcription polymerase chain reaction

Th: T helper

TLR: Toll-like receptor

Tr: Trailer

TrC: Complement of the trailer

URTI: Upper respiratory tract infection

USA: United States of America

UTM: Universal transport medium

CHAPTER 1: INTRODUCTION

1.1 Rationale

Human Respiratory syncytial virus (HRSV) is a major cause of acute respiratory infection (ARI) in children worldwide (Kim *et al.*, 2000; Tsolia *et al.*, 2003; Kwofie *et al.*, 2012). It is responsible for seasonal outbreaks (Madhi *et al.*, 2006; Bloom-Feshbach *et al.*, 2013) and is associated with substantial morbidity and mortality rates, especially in people with some underlying conditions (Anderson *et al.*, 2016). Every year, HRSV is a source of significant hospital admissions in young children with a considerable rate of mortality in high-risk groups (Szabo *et al.*, 2013; Reeves *et al.*, 2017). HH ARI has a major impact on healthcare resources and costs (Forbes *et al.*, 2010; Anderson *et al.*, 2017). Furthermore, there is growing evidence that HRSV ARI in childhood is linked to long-term impaired lung function that can manifest as recurrent wheezing or asthma (Fauroux *et al.*, 2017).

Various socio-demographic factors are associated with severe HRSV ARI. Children born before 37 weeks of gestational age tend to develop more severe HRSV ARI than those born at term (Shi *et al.*, 2015) and those under 1 year experience the highest incidence of HRSV-associated hospitalizations (Rowlinson *et al.*, 2013). Severe stunting, low birthweight, crowding, high numbers of siblings under 6 years in a household, lack of exclusive breast feeding and maternal smoking have been shown to be major factors associated with severe HRSV ARI (McNamara & Smyth, 2002; Okiro *et al.*, 2008; Jackson *et al.*, 2013).

Non-HRSV virus co-infection is common in HRSV-infected children (Yoshida *et al.*, 2013). The following non-HRSV viruses are known to be involved in co-infection during HRSV infection: Influenza virus, Human rhinovirus (HRV), Adenovirus (ADV), Coronavirus (CoV), Parainfluenza virus (PIV), Human metapneumovirus (HMPV), Human bocavirus (HBoV) and Enterovirus (EV). Non-HRSV virus co-infection may increase the severity of HRSV disease (Harada *et al.*, 2013).

Investigating the epidemiology of viral respiratory infections, understanding their interaction with the human body and increasing the knowledge on the disease progression can be a valuable tool to contain their spread. Infection prevention and control (IPC) measures, such as strict observance of hand washing, use of gloves

and gowns by caregivers, limiting visitors during HRSV season, active surveillance for HRSV infection and cohorting of infected patients and caregivers, are efficient in limiting the spread of the virus (Collins & Karron, 2013). Moreover, passive immunization with palivizumab (a monoclonal antibody) is used in the prevention of HRSV infection in children with a high risk of a severe disease.

To date, the burden of HRSV infection in children in the Democratic Republic of Congo (DRC), especially in the East part of the country, has not been studied. Moreover IPC measures are not strictly practised and high-risk children do not receive palivizumab for passive immunization against the virus due to its very high cost. This study will bring some insight on respiratory infections in this country and propose some recommendations on how to improve children's health.

1.2 **Hypotheses:**

Hypothesis 1: The prevalence of HRSV is higher in ARI in children under 5 years of age when compared to other respiratory viruses.

Hypothesis 2: HRSV, as well as some socio-demographic factors and medical features, is associated with the risk of ARI to be diagnosed as lower respiratory tract infections (LRTI) in children under the age of 5 years.

1.3 **Aim:**

To investigate the epidemiological features of respiratory infections in paediatric patients visiting the Provincial General Hospital of Bukavu (PGHB).

1.4 **Objectives:**

- To collect socio-demographic information, clinical data and nasal swabs from children under the age of 5 years
- To gather literature on HRSV infection
- To determine the prevalence of HRSV ARI and non-HRSV ARI in children under the age of 5 years.
- To identify socio-demographic and clinical characteristics associated with the risk of ARI to be diagnosed as LRTI in children under the age of 5 years.

CHAPTER 2: LITERATURE REVIEW

In this section, the HRSV structure and replication cycle, its interaction with the host immunity, epidemiology, diagnosis, as well as prevention and treatment, will be briefly reviewed.

2.1 The Infectious Agent:

2.1.1 The Virion:

HRSV belongs to the order of *Mononegavirales*, the family of *Pneumoviridae*, the genus of *Orthopneumovirus* and the species of Human orthopneumovirus (Rima *et al.*, 2017). Its virion consists of a nucleocapsid surrounded by a lipid membrane derived from the bi-layer plasma membrane of the host (Figure 2.1). Virions produced in cell culture consist of spherical particles of 100–350 nm in diameter and long filaments that usually predominate and are 60–200 nm in diameter and up to 10 µm in length (Jeffree *et al.*, 2003).

Electron tomography and calculation of subtomogram averages using Dynamo™ software have revealed that the HRSV nucleocapsid is helical left-handed with a pitch of 68 Angstrom (Bakker *et al.*, 2013).

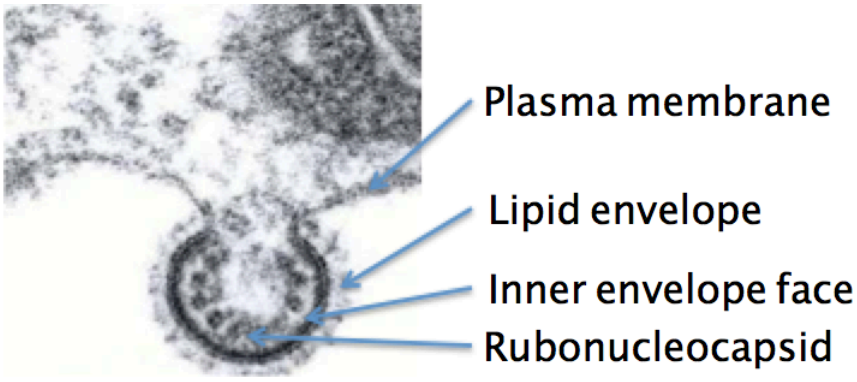

Figure 2.1 Electron micrograph image of an HRSV virion budding from an infected cell (Garofalo *et al.*, 2016).

2.1.2 The Genome

The HRSV genome is a non-segmented negative-sense single-stranded ribonucleic acid (RNA) of approximately 15 200 nucleotides. This genome contains 10 different

genes in the order 3'NS1-NS2-N-P-M-SH-G-F-M2-L. Each gene encodes for a corresponding messenger RNA (mRNA) (Figure 2.2).

The first 9 genes are separated by intergenic regions that vary in length from a single adenosine residue (N-P intergenic sequence) to 52 nucleotides (G-F intergenic sequence). The M2 and L genes, on the other hand, overlap by 68 nucleotides (Collins *et al.*, 1986).

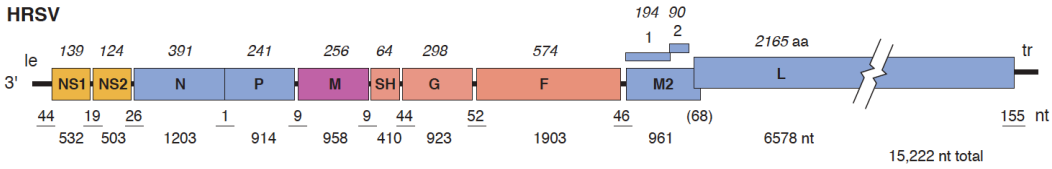

Figure 2.2. The 3' to 5' negative-sense genomes of HRSV strain A2. The overlapping open reading frames (ORFs) of the M2 mRNA are illustrated over the M2 genes. Numbers beneath each map indicate nucleotide (nt) lengths; those of the extragenic leader (le), trailer (tr), and intergenic regions are underlined, and that of the HRSV gene overlap is in parentheses. Italicized numbers above each map indicate amino acid (aa) lengths (Straus, 2013).

2.1.3 **Proteins:**

HRSV mRNA encodes for 11 proteins (NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2 and L).

The G, F and SH proteins are envelope proteins, the M resides on the inside of the envelope, the N, P, L and M2-1 proteins are nucleocapsid proteins, M2-2 protein is a regulatory protein and NS1 and NS2 are non-structural proteins.

- The H**RSV G protein**, a type II membrane protein formed by polymerization of 289 to 299 amino-acids (aa) (depending on the virus strain), is the major attachment protein of HRSV (Levine *et al.*, 1987; Ghildyal *et al.*, 2005; Melero *et al.*, 2016). This protein is also known for its role in infectivity and antigenicity of HRSV. Among all the HRSV proteins, the G protein is the most variable gene product between HRSV isolates. Antigenic differences of this protein have been used to classify HRSV isolates into two groups (A and B) (Melero *et al.*, 2016).

- The H**RSV F protein** is a class 1 fusion protein, consisting of 574 aa, and it mediates binding of HRSV to cellular receptors and induces pH-independent fusion between the viral envelope and the cellular plasma membrane (Eckardt-Michel *et al.*, 2008).
- The **SH protein** is a short type II transmembrane glycoprotein containing 64 to 65 aa residues (Araujo *et al.*, 2016). It has been shown that SH plays a role in fusion mediation (Heminway *et al.*, 1994) and apoptosis inhibition in cell lines (Fuentes *et al.*, 2007).
- The **M protein** contains 256 aa residues. It is present between the outer envelope and inner ribonucleoproteins. It plays a role in virus assembly and budding (Bajorek *et al.*, 2014).
- **NS1 and NS2** are non-structural proteins containing 139 and 122 aa residues respectively. These two proteins interfere strongly with host innate immunity by inhibiting IRF 1 and 3 signalling pathways (Ren *et al.*, 2011).
- The **N protein** contains 391 aa. This protein is tightly bound to the single-stranded RNA of the HRSV, forming a nuclease-resistant helical ribonucleoprotein complex, called nucleocapsid (Tawar *et al.*, 2009). The N protein gene is the highly conserved gene in the HRSV genome (Zhang *et al.*, 2009). Also, there is a strong interaction between the P protein and the N protein in the formation of the nucleocapsid (Slack & Easton, 1998).
- The **P protein** contains 241 aa and is a phosphoprotein. This protein is known to confer stability to the L protein (Simabuco *et al.*, 2011).
- The **L protein**, containing 2165 aa, is an RNA-dependent RNA-polymerase that is involved in mRNA transcription and genome replication (Tremaglio *et al.*, 2013).
- The **M2-1 protein**, containing 194 aa, is an essential transcription processivity factor that prevents premature termination during transcription (Collins *et al.*, 1996). It also acts as a transcription anti-terminator by enhancing the ability of the polymerase to read through transcription termination signals (Fearns & Collins, 1999).
- The **M2-2 protein** is a small protein of 90 aa residues. It is encoded by a second ORF in the M2 mRNA that overlaps the 5'-proximal M2-1 ORF (Collins *et al.*,

1990). This protein is particularly known for its importance in the regulation of the viral RNA transcription and replication.

2.1.4 **The Replication Cycle:**

To initiate the replication cycle, HRSV has to bind first to a receptor on the host cell. In 1987, using mono-specific anti-G serum induced in rabbits, the G protein was demonstrated to be the HRSV attachment protein (Levine *et al.*, 1987). Additionally, cellular glycosaminoglycans (GAG) appeared to be the main cellular attachment site of HRSV G protein. The most important GAG types for HRSV infection in HEp-2 cells are heparin sulphate and chondroitin sulphate B (Hallak, Collins, *et al.*, 2000). The negative electrostatic charges on GAG chains provided by the sulphate groups are known to facilitate this binding process (Hallak, Spillmann, *et al.*, 2000). Apart from GAG, the following surface molecules had been identified as potential HRSV receptors: CX3CR1 (Johnson *et al.*, 2015), ICAM-1 (Arnold *et al.*, 1995) and RhoA, which is also known to play a role in the syncytium formation (Gower *et al.*, 2005).

After attachment to the host cell, HRSV undergoes the process of entry, which is modulated by the fusion protein F. This protein mediates the fusion of the HRSV envelope with the host cell membrane in a pH-independent fashion (Zhao *et al.*, 2000). Several studies have suggested that the HRSV F protein undergoes conformational changes after binding to the host cell membrane. Its fusion peptide is then inserted into the cell membrane where its 2 main domains interact to form a stable core structure resulting in membrane apposition. Finally, the fusion pore opens and viral genetic material enters the host cell. The entry can also be mediated by endosomal membranes following clathrin-mediated endocytosis (Baker *et al.*, 1999; Zhao *et al.*, 2000; Sun *et al.*, 2013).

Genome transcription and replication occur concomitantly in the cytoplasm. Figure 2.3 shows an overview of HRSV genome transcription and replication. It is known that the HRSV genome is bordered by two short extragenic regions: a 3’ leader region (Le) formed by 44 nucleotides and a 5’ trailer region (Tr) formed by 155 nucleotides (Mink *et al.*, 1991). The polymerase initiates transcription at position 3 of the Le promoter (Tremaglio *et al.*, 2013) and transcription progresses along the length of the genome by a sequential termination-reinitiation (stop-start) mechanism.

This stop-start transcription is directed by gene start (GS) and gene end (GE) signals found at the upstream and downstream boundaries of each gene (Kuo *et al.*, 1997). The GE also initiates the 3'-polyadenylation as the GS initiates the 5'-capping. Subgenomics mRNAs (which are polyadenylated at the 3' end and capping at the 5' end) are then released and translated into their corresponding viral proteins using the host cell machinery.

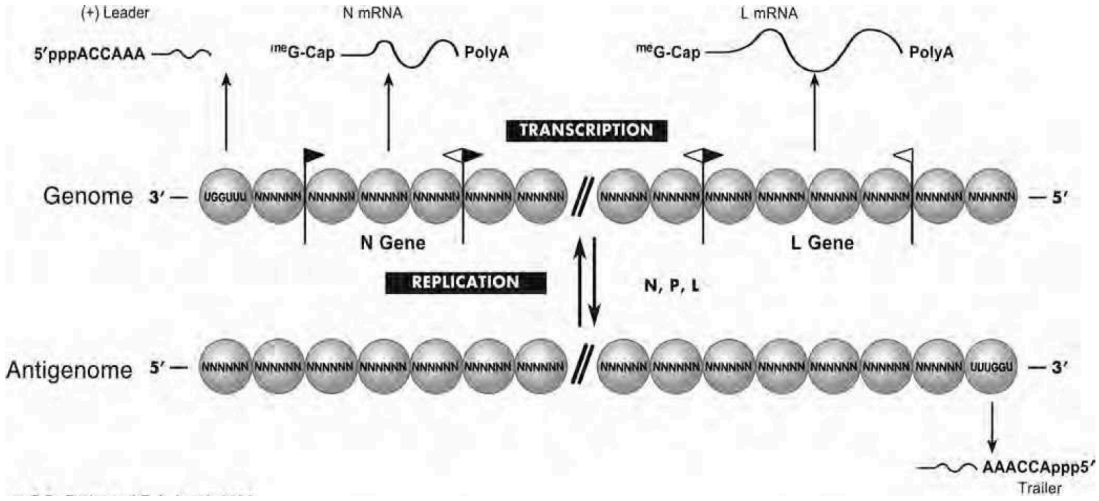

Figure 2.3: Paramyxovirus RNA synthesis. Viral nucleocapsids, the templates for RNA synthesis, are shown as a linear array of N subunits (ovals), with arrowheads indicating the gene junctions. Note that N protein binds six nucleotides, resulting in complete encapsidation of the RNA if it has a chain length that is an even multiple of six. The viral polymerase (P-L) transcribes the genome template, starting at its 3' end, to generate the positive leader RNA and the successive capped (meG-Cap) and polyadenylated (PolyA) mRNAs, by stopping and restarting at each junction. Once these primary transcripts have generated sufficient viral proteins, unassembled N (as a P-N complex) begins to assemble the nascent leader chain, and the coordinate assembly and synthesis of the RNA cause the polymerase to ignore the junctions, yielding the antigenome nucleocapsid (bottom). The P-L polymerase can also initiate RNA synthesis at the 3' end of the antigenome in the absence of sufficient P-N; however, only a 5' trailer RNA is made in this case. Note that positive leader RNA is not capped or polyadenylated and that genomic and antigenomic RNA never appear as naked RNA (Lamb & Parks., 2006).

Replication, as with transcription, begins by the binding of the polymerase to the Le promoter. In this case however, it does not respond to GE and GS signals but

instead generates a full-length complementary positive-sense RNA (the antigenome) which will serve as a template for the synthesis of the viral negative-sense RNA genome. In its 3' region, the antigenome has a complement of the trailer (TrC). The polymerase binds to a promoter in the TrC and initiates the synthesis of the viral genome (Cowton *et al.*, 2006).

Assembly and budding occur at the plasma membrane. It has been shown that the cytoskeleton plays a role in the transport of the ribonucleoprotein complex to the budding site (Santangelo & Bao, 2007) as well as in the optimization of HRSV budding and release (Shahriari *et al.*, 2016).

2.2 **HRSV Immunity and Pathology:**

2.2.1 **Innate Immunity**

Respiratory epithelial cells are recognized as initial targets of HRSV infection and as the first site for innate immune response activation (Bueno *et al.*, 2008). As soon as HRSV contacts airway respiratory cells, it is recognized by toll-like receptors (TLR) present on the plasma membrane. Various TLR are involved in HRSV immunity. TLR4 plays the major role. Stimulation of TLR4 by HRSV F protein leads to activation of intracellular signalling pathway NF-kB and thus inflammatory cytokine production such as interleukin (IL)-6, IL-8 and IL-10 (Haeberle *et al.*, 2002).

It was shown that, compared to wild-type mice, TLR4-deficient mice were unable to mount an innate immune response to HRSV F protein and to clear infection (Kurt-Jones *et al.*, 2000). In addition, some studies have revealed that genetic polymorphisms of TLR4 genes can predict HRSV infection risk and disease severity (Zhu *et al.*, 2015), and the TLR4 mutations $Asp^{299}Gly$ and $Thr^{399}Ile$ have been correlated with the gravity of HRSV infection in children (Tulic *et al.*, 2007).

In addition to TLR4 (Kurt-Jones *et al.*, 2000), TLR3 (Rudd *et al.*, 2006), TLR7 (Schlender *et al.*, 2005) and TLR2 (Murawski *et al.*, 2009) can also activate immune responses upon HRSV recognition.

Respiratory epithelial cells exposed to HRSV produce a broad range of pro-inflammatory cytokines. Although CXC chemokines (particularly CXCL10 / interferon [IFN]-γ inducible protein [IP]-10 and CXCL8 / IL-8) are the most abundant

chemokines during HRSV infection. CC chemokines (CCL2 / monocyte chemotactic protein [MCP]-1 and CCL3 / macrophage inflammatory protein [MIP]-1α) are also present (McNamara *et al.*, 2005). These chemokines act as chemo-attractants that guide the migration of cells of the immune system (neutrophils, monocytes, memory T cells, and eosinophils) from blood to infected tissues. These recruited cells contribute to the cytokine production (Jafri *et al.*, 2004) which concurrently helps to contain HRSV infection, but also enhance tissue damage (Janssen *et al.*, 2007). Many studies have shown that by reducing pro-inflammatory cytokine production, lung injury was considerably decreased (Foronjy *et al.*, 2014; Ren *et al.*, 2016; Schuijs *et al.*, 2016).

Another important first line defence is airway mucus production. Mucus is secreted by epithelial goblet cells. Its main role is to protect respiratory airways from injury due to its viscosity and through mucocilliary clearance. Even though airway mucus production is considered a part of innate defence, it can actually worsen the disease by obstructing respiratory airways when it is secreted abundantly (Johnson *et al.*, 2007).

2.2.2 The Role of Dendritic Cells in the Link of Innate and Adaptive Immunity

Dendritic cells (DC) are antigen-presenting cells (APC) of the mammalian immune system. They play a key role in the regulation of the immune response by connecting innate and adaptive immunity (Kim & Lee, 2014). There are 2 main lineages of DC: conventional $CD11c^+$ DC (cDC; previously called myeloid DC) and $CD11c^{low}$/mPDCA-1^+ plasmacytoid DC (pDC) (Wikstrom & Stumbles, 2007; Satpathy *et al.*, 2012).

In the mouse lung, there are two major cDC populations: $CD11c^+$ / $CD103^-$ / $CD11b^{high}$ cDC ($CD11b^{hi}$ cDC) are located in the lung parenchyma and $CD11c^+$/$CD103^+$/$CD11b^{low}$ cDC ($CD103^+$ cDC) are located in the basal lamina of the airway epithelium and spread their dendrites all over the epithelium (Lukens *et al.*, 2009).

After their maturation, cDC activate adaptive immune system cells (Manicassamy & Pulendran, 2011). $CD103^+$ cDC are important for the induction of $CD8^+$ T-cell responses, while $CD11b^{hi}$ cDC mainly activates naïve $CD4^+$ T cells (Lukens *et al.*,

2009). cDC in lymph nodes present HRSV-antigen to $CD8^+$ and $CD4^+$ T cells via Major histocompatibility complex (MHC)-I and MHC-II respectively (Lukens *et al.*, 2009). It is known that MHC-I molecules present endogenous antigen and MHC-II exogenous ones (Ryan & Cobb, 2012).

Lung pDC activate the antiviral function of other cells by producing type I IFN, when the receptor TLR7 recognizes the antigen (Johnson *et al.*, 2011; Garg *et al.*, 2012). It has been shown that pDC play a role in the limitation of lung pathology during acute HRSV infection by limiting T helper (Th) 2 cytokine production (Smit *et al.*, 2006, 2008).

2.2.3 **Adaptive Immunity**

2.2.3.1 T Cell Response

Once activated by infected DC, CD8 T cells migrate from the lymph nodes to the site of infection. This migration is governed by the chemokine CXCL10/CXCR3 (Lindell *et al.*, 2008) and the inhibition of the production of this chemokine can lead to an exacerbated HRSV disease. CD8+ T cells control the infection by cytokine production and by lysing the infected cells.

Cytotoxic T lymphocytes (CTL) can lyse infected cells by two ways. The first is the perforin-granzyme pathway. When the CTL receptor binds to the target cell, CTL releases perforin and granzyme proteins. The perforin protein binds to the target cell and forms a pore by polymerization. The granzyme protein then penetrates into the target cell and induces nucleic acid fragmentation (Kägi *et al.*, 1996; Fan & Zhang, 2005). The second pathway is the Fas Ligand pathway. Fas ligand is a type II transmembrane protein that can cause cell apoptosis when binding to its receptor (Fas). It has been shown that ligation of Fas causes assembly of an intracellular death inducing signalling complex through the activation of the cysteine protease caspase-8 and activation of the caspase cascade (Strasser *et al.*, 2009; van den Berg *et al.*, 2011).

Th cells, also known as $CD4^+$ T cells, are implicated in HRSV immunity as well as in the immune-mediated pathology during HRSV infection (Johnson *et al.*, 2007). A process of activation occurs when a naïve Th cell interacts with a DC. This process is

performed in three different signals. The first signal is the recognition of the antigen (carried by the MHC II protein of the DC) by the Th cell receptor (Janeway CA *et al.*, 2001). The second signal involves the interaction between the DC molecules CD80 and CD86 which bind to CD28 on the Th cell (Linsley *et al.*, 1990; Thauland *et al.*, 2014). The third signal is mediated by cytokines binding to the Th cell receptor. Depending on the cytokine environment, Th cells proliferate either into Th1 cells or Th2 cells. IFN-γ drives Th1 proliferation as Th2 proliferation is driven by IL-4 (Tanaka *et al.*, 2000; Wurster *et al.*, 2002; Zhu, 2015). It has been shown that a Th2-mediated immune response to HRSV infection is associated with mucus production and airway hyperactivity. Comparing the ratio of IL-4 to IFN-γ from infants with HRSV acute bronchiolitis to those with HRSV upper respiratory tract infection (URTI), Legg *et al.* (2003) found that this ratio was markedly higher in the first group than in the second one. Thus, Th2 cytokines can be considered as makers of HRSV severe disease.

2.2.3.2 B Cell Response and Antibodies

Naïve B cells are activated by two different mechanisms: the T cell-dependent activation and the T cell-independent activation (Parker, 1993; Hess *et al.*, 2013). In HRSV immunity, naïve B cells are activated through the T cell-dependent pathway (Varga & Braciale, 2013). These activated B cells will produce antibodies. Even though HRSV infection can induce antibody responses against several viral antigens, it has been shown that only the two major surface glycoproteins, F and G, induce antibodies that have a major role in protection (Groothuis, 1994). Although antibody responses are vital for protection against HRSV infection, cellular immune responses may have a greater role in virus clearance (Oshansky *et al.*, 2009).

2.2.4 HRSV Mechanisms of Immune Escape (Modulation)

HRSV NS1 and NS2 proteins can inhibit type I IFN production and signalling (Spann *et al.*, 2004; Ramaswamy *et al.*, 2006).

HRSV G protein is known to bind neutralizing antibodies, thereby preventing neutralization or opsonization of viral particles. G protein can also serve as a soluble chemokine receptor agonist (Varga & Braciale, 2013).

2.2.5 **Pathology**

By assessing the HRSV-specific immune response in humanized mice (mice with a human immune system) with functional human $CD4^+$ T and B cells, Sharma *et al.* (2016) proved that HRSV infection induces the following pathological features: peribronchiolar inflammation with neutrophil predominance in the bronchiolar lavage fluid and enhanced airway mucus production. Comparing epithelial cell pathology from 250 autopsies of young children with ARI including HRSV infection, adenoviruses (ADV), *influenza virus* and PIV, Zinserling (1972) noticed that HRSV was causing the greatest degree of bronchiolar involvement. Many researchers agree that HRSV can lead to the formation of multi-nucleated polypoid epithelial cells casting off into the airway lumen (Shedden & Emery, 1965; Delage *et al.*, 1984; Neilson & Yunis, 1990).

2.3 **Epidemiology**

2.3.1 **HRSV Burden:**

2.3.1.1 Worldwide and Western Countries

HRSV is the most common cause of respiratory tract infection in children worldwide (Bont *et al.*, 2016; Fall *et al.*, 2016; Gamino-Arroyo *et al.*, 2016). By estimating incidence for industrialized and developing countries for 2005, and summing these estimates, Nair *et al.* (2010) has indicated that during that year, 33.8 million new cases of non-severe HRSV-associated infection occurred globally in children younger than 5 years. Of these, 96% arose from developing countries (more than 90% of the world's population aged younger than 5 years live in developing countries). Moreover, the author indicated that the 2005 estimated global incidence of severe HRSV-associated disease in children younger than 5 years was approximately 3.4 million cases, among which 91% occurred in developing countries. Furthermore, by combining data from 25 published and 6 unpublished studies, the author estimated the number of deaths due to HRSV-associated severe LRTI in children admitted to hospital. He found that 66 000 to 155 000 children younger than 5 years died in 2005 as a result of HRSV-associated severe LRTI.

HRSV prevalence was determined by Nolan *et al.* (2015) in a cohort of children aged from 6 months to 10 years with influenza-like illness who were enrolled in a randomized clinical trial of 2 pandemic *influenza virus* H1N1 vaccines conducted in 17 centres all over the world. The authors reported an overall HRSV prevalence of 9.7%.

Stein *et al.* (2016) has also revealed, through a meta-analysis that included 55 studies from 32 countries all over the world, the burden of severe HRSV infection. The author mentioned that in children younger than 5 years the global HRSV ARI hospitalization estimate was reported to be 4.37 per 1 000 children per year. This estimate was 19.19 per 1 000 among children younger than 1 year and 20.01 per 1 000 among children younger than 6 months, while among premature children under 1 year it was 63.85 per 1 000 children. Among children younger than 5 years, it was 6.21 per 1 000, 6.60 per 1 000 among those younger than 1 year and 1.04 per 1 000 among preterm children younger than 1 year.

In a systematic review done in 2016 by Bont *et al.* (2016), the author analysed the burden of severe HRSV infection among infants and children in Western countries by collecting data from 98 studies published between January the 1st 1995 and December the 31st 2015. The data showed that HRSV was associated with 12-63% of all ARI and 19-81% of viral ARI leading to hospitalization in children. It was concluded that in comparison to *influenza virus*, HRSV lead up to 16 times more hospitalizations and emergency department visits in children younger than 5 years.

In the United States of America (USA), HRSV has led to an average of 2.1 million outpatient visits among children younger than 5 years old and 57 527 hospitalizations among those children in 2009 (Hall *et al.*, 2009).

2.3.1.2 Africa

The epidemiology of HRSV ARI across Africa varies from country to country, as shown in Table 1.

HRSV prevalence in children under 5 years in Kenya is around 17% (Bigogo *et al.*, 2013; Nyawanda *et al.*, 2016). Bigogo *et al.* (2013) has reported incidence rates of severe HRSV ARI in Kenya among infants under 1 year of age and among children

younger than 5 years. In the first group, the HRSV ARI incidence rate was 62.8 to 86.9 episodes per 1 000 person-years while, in the second group, this incidence rate was 23.7 to 26.9 episodes per 1 000 person-years. Emukule *et al.* (2014), on the other hand, analysed the HRSV ARI hospitalization incidence rate at two health facilities in western Kenya. The author found an incidence rate of 5.2 per 1 000 children younger than 5 years.

In South Africa, many scientists have worked on HRSV. Among children younger than 5 years and suffering from severe ARI, Pretorius *et al.* (2016) reported a very high significant attributable factor for HRSV (>90%), with an adjusted prevalence of 22.4%. Kyeyagalire *et al.* (2014) estimated the HRSV-associated all respiratory hospitalization rate among children younger than 1 year to be 7 601 per 100 000 person-years. Tempia *et al.* (2014) found in children younger than 5 years of age, the mean annual number of HRSV-associated all respiratory deaths was 10 per 100 000 person-years.

Table 2.1 HRSV prevalence in Africa

Reference	Country	Prevalence	Cohort
Pretorius *et al.* (2016)	South Africa	10.7% (N= 5743)	Outpatients and inpatients
Bigogo *et al.* (2013)	Kenya	17% (N=2050)	Outpatients and inpatients
Fodha *et al.* (2004)	Tunisia	21.6% (N= 815)	Inpatients
Ouédraogo *et al.* (2014)	Burkina Faso	11.9% (N= 209)	Inpatients and outpatients
Loscertales *et al.* (2002)	Mozambique	9.8% (N= 1172)	Inpatients and outpatients
Kwofie *et al.* (2012)	Ghana	14.1% (N= 128)	Inpatients
Weber *et al.* (2002)	Gambia	8.7% (N= 4799)	Inpatients

2.3.2 HRSV and Risk for ARI to be Diagnosed as LRTI

Most cases of HRSV ARI are presented as URTI. In some cases however, URTI can extend and lead to LRTI. Certain factors are known to increase the risk of severe HRSV ARI. The major risk factors include: premature birth (Helfrich *et al.*, 2015), low birthweight (Parejo *et al.*, 2016), increased exposure (day-care attendance, siblings

younger than 5 years of age, admission to the hospital during HRSV season) (Anderson *et al.*, 1988), passive smoking (Gurkan *et al.*, 2000; DiFranza *et al.*, 2012), family history of atopy (Trefny *et al.*, 2000), low parental education and socio-economic income (Glezen *et al.*, 1981), indoor air pollution (Al-Sonboli *et al.*, 2006), no breastfeeding (Nishimura *et al.*, 2009), chronic lung disease (Paes *et al.*, 2016), congenital heart disease (Friedman *et al.*, 2017), neuromuscular diseases(Resch *et al.*, 2009) and primary immunodeficiency disorders (Lanari *et al.*, 2014).

In 2015, Shi *et al.* (2015) carried out a systematic review with meta-analysis aimed to identify risk factors for HRSV-associated LRTI in children younger than 5 years of age. The author included 20 studies with good quality that investigated 18 risk factors of HRSV-associated LRTI, among which 8 were significantly associated. The meta-estimates of their odds ratios (OR) were as following: prematurity - 1.96 (95% confidence interval [CI] 1.44-2.67), low birthweight - 1.91 (95% CI 1.45-2.53), male gender - 1.23 (95% CI 1.13-1.33), having siblings - 1.60 (95% CI 1.32-1.95), maternal smoking - 1.36 (95% CI 1.24-1.50), history of atopy - 1.47 (95% CI 1.16-1.87), no breastfeeding - 2.24 (95% CI 1.56-3.20) and crowding - 1.94 (95% CI 1.29-2.93).

2.3.3 **HRSV and Viral Co-Infection**

The progress of molecular diagnostic tools for the detection of respiratory viruses has provided insight into the viral respiratory dynamics during respiratory infection. Hence, some researchers have focused their attention on the weight of viral co-infection during ARI.

Even though epidemiologic data had shown that when the rate of HRSV infection is high, the *influenza virus* infection rate tends to be low (and vice versa) (Wang *et al.*, 2013), Mazur *et al.* (2017) has reported that HRSV-*influenza virus* co-infection increases the odds for prolonged hospitalization when compared to HRSV mono-infection. The author also reported that HRSV-ADV co-infection had a 3.4 increased odds of life-threatening disease compared with RSV mono-infection. However, the role of HMPV in severe HRSV infection is still controversial. While Van Woensel *et al.* (2006) reported that HMPV is not frequent in severe HRSV LRTI, McNamara *et al.*

(2007) suggested that HRSV-HMPV co-infection is clinically symptomatic for longer than RSV mono-infection.

Other viruses, such as HRV, CoV, BoC, PIV and EV are also implicated in HRSV co-infection, but the direction of this relationship is mostly unclear.

2.4 **Clinical Features and Diagnosis:**

2.4.1 **Clinical Features:**

HRSV transmission is through direct or close contact with contaminated secretions. The incubation period is 2 to 8 days and the period of viral shedding is usually 3 to 8 days, but it may continue for as long as 3 to 4 weeks (American Academy of Pediatrics [AAP], 2009).

In infants and children, HRSV infection can manifest either as URTI (rhinitis, pharyngitis, acute bronchitis) or LRTI (bronchiolitis, pneumonia) (Glezen *et al.*, 1986).

HRSV URTI usually occurs in older children (mostly as reinfection) and can manifest as cough, coryza, rhinorrhoea and conjunctivitis, although ears (Kristjansson *et al.*, 2010) and sinuses(Ramadan *et al.*, 1997) can also be affected.

LRTI is characterized by signs of increased respiratory effort (tachypnea, nasal flaring, chest retraction), wheezing, shortness of breath and rales. Severe HRSV LRTI can lead to respiratory failure and even death (Geoghegan *et al.*, 2016).

Many studies have reported a correlation between HRSV LRTI and subsequent respiratory function impairment characterized by recurrent wheezing and asthma later in life (Fauroux *et al.*, 2017; Kabego & Beer, 2017). Moreover, it was shown that prevention of HRSV LRTI using palivizumab has the effect of decreasing the odds of recurrent wheezing late in childhood (Simoes *et al.*, 2007).

2.4.2 **Laboratory Diagnosis:**

The laboratory diagnosis of HRSV is made by analysing respiratory secretions. Different types of sample types, such as nasopharyngeal aspirates and swabs, as well as nasal and throat swabs, can be used (Gray *et al.*, 1968; Stensballe *et al.*, 2002).

HRSV culture is traditionally known as the gold standard for HRSV laboratory diagnosis (Popow-Kraupp & Aberle, 2011). However, culture can be disadvantageous due to virus lability, the time taken to produce results and the storage of specimens at cold temperatures (Tregoning & Schwarze, 2010). HEp-2, Vero, HeLa, and A549 cells are the most sensitive cell lines for primary isolation (Mahony, 2008; Corry *et al.*, 2016).

Diagnosis can be made more rapidly by direct immunofluorescence assay (DFA) for viral antigen detection or antigen-capture enzyme immunoassay (EIA). In the DFA, a primary antibody is linked to a fluorophore that will recognize the HRSV antigen within the sample. A wavelength of light is produced during the reaction and is visualized by fluorescent microscopy (White *et al.*, 1988). In the EIA, the sample is added to a microplate coated with immobilized antibodies that bind to the HRSV antigen. A secondary antibody linked to an enzyme is then added, followed by the addition of a substrate, which is then converted to a detectable form through colour change. The reaction plate is read spectrophotometrically at a specific wavelength (Wren *et al.*, 1990) and the optical density is proportional to the concentration of the antigen in the sample.

Nucleic acid assays are the most sensitive and specific methods for the detection of HRSV (Falsey *et al.*, 2003). Of the different nucleic acid amplification techniques, reverse transcription polymerase chain reaction (RT-PCR) is the most frequently used (Popow-Kraupp & Aberle, 2011).

2.5 **Prevention and Treatment of HRSV:**

2.5.1 **Prevention**

HRSV has a very high basic reproduction number (the average number of uninfected people who can become infected from one infectious person), which is estimated to be between 5 and 25 (Weber *et al.*, 2001). This virus is one of the major nosocomial hazards in paediatrics wards. IPC measures and passive immunoprophylaxis of high-risk individuals can be helpful to prevent HRSV infection.

2.5.1.1 Infection Control:

In the community, HRSV infection can be reduced by hand hygiene and avoiding settings with increased risk of exposure, such as childcare centres.

In health settings, the following practices may reduce HRSV infection: hand hygiene (Weedon *et al.*, 2013), the use of personal protective equipment (gowns, gloves and goggles) by staff (French *et al.*, 2016), isolating or cohorting infants with HRSV infection (Groothuis *et al.*, 2008), excluding visitors with current respiratory tract infections, excluding staff with respiratory tract illness or HRSV infection from caring for susceptible infants and limiting young sibling visitation during the HRSV season (AAP, 2009).

2.5.1.2 Passive Immunoprophylaxis:

Presently, palivizumab is the only available option for HRSV prophylaxis. However, due to its high cost and restricted clinical benefits, its use has been limited to a group of high-risk infants (Rezaee *et al.*, 2017). Wegzyn *et al.* (2014) conducted a systematic review and summarized the evidence of the effectiveness of palivizumab. The author reported that there were low HRSV-related hospitalization rates in palivizumab recipients when compared to placebo with an OR of 0.41. In addition, among palivizumab-prophylaxed subjects, rates of intensive care unit admission and mechanical ventilation from HRSV hospitalization were also low.

2.5.1.3 Vaccines:

There is no licensed vaccine available yet for HRSV infection. However, many vaccine candidates are currently being evaluated for their effectiveness and safety (Glenn *et al.*, 2013, 2016; Malkin *et al.*, 2013; Taylor *et al.*, 2015). The main strategies of HRSV vaccine development are: attenuating the virus or using recombinant technology to present viral antigens.

2.5.2 Treatment:

Here, the focus will mostly be on the treatment of RSV bronchiolitis, which has a considerable burden in child health.

2.5.2.1 Supportive Care:

Hydration (fluid management) and oxygenation remain the cornerstones of bronchiolitis clinical management (Mazur *et al.*, 2015). According to recommendations of the American Academy of Paediatrics (AAP), supplemental oxygen is recommended if oxyhaemoglobin saturation falls persistently below 90% in previously healthy infants. Moreover, the AAP recommends the administration of fluid to children suffering from bronchiolitis, depending on their ability, orally or through a gastric tube or intravenous catheter (American Academy of Pediatrics, 2006; Ralston *et al.*, 2014).

The AAP recommends not using the following therapeutics options: corticosteroids (strong recommendation), epinephrine (strong recommendation), albuterol or salbutamol (strong recommendation), chest physiotherapy (moderate recommendation) and antibacterial drugs (unless a concomitant bacterial infection is present or strongly suspected) (strong recommendation). However, nebulized hypertonic saline may be administered by the clinician to infants and children with bronchiolitis.

Other therapeutic options are currently being evaluated for their effectiveness in the management of HRSV bronchiolitis. These include exogenous surfactants (Jat & Chawla, 2015) and a helium/oxygen mixture (heliox) (Seliem & Sultan, 2017).

2.5.2.2 Antiviral Intervention:

Ribavirin, a purine analogue, is an antiviral medication approved by the USA Food and Drug Administration (FDA) for the treatment of HRSV bronchiolitis (Marcelin *et al.*, 2014). However, the high cost, difficulty to administer make the use thereof controversial (Wright *et al.*, 2008). Therefore, the AAP does not recommend its routine use in children with bronchiolitis (Turner *et al.*, 2014).

2.6 Summary

HRSV, a single-stranded RNA virus, is a major cause of ARI in children worldwide and is responsible of a substantial number of children deaths each year. There is neither a licensed vaccine to prevent HRSV infection nor an effective drug to treat it. However, in community as well as in hospital settings, HRSV can be contained

during the HRSV period and even prevented when its epidemiology is well known. This study will provide much needed information on HRSV and ARI epidemiology in DRC which can be used to improve the healthcare system.

CHAPTER 3: MATERIALS AND METHODS

3.1 Ethical Aspects:

Ethics approval was obtained from the Health Research Ethics Committee of Stellenbosch University (Appendix 1) and from the Ethics Committee of the Catholic University of Bukavu (Appendices 2 and 3).

3.2 Study Design

An analytical cross-sectional study design was used and its main purposes were:

- To determine the prevalence of HRSV ARI and non-HRSV ARI in children under the age of 5 years.
- To investigate the relationship between independent variables (socio-economic factors, clinical data and RT-PCR results) and the dependent variable (ARI).

3.3 Study Settings

Bukavu is a city in the eastern region of DRC. It is the capital of the South Kivu province with an estimated population of 870 954 in 2003 (Yogolelo, 2013).

PGHB is a main healthcare facility of Bukavu. It also serves as the health reference centre for the province of South Kivu and as the teaching hospital of the Catholic University of Bukavu. It has 385 beds with 6 400 admissions and 4 900 outpatients per year.

3.4 Participants

In a consecutive manner, 146 patients visiting the PGHB for ARI were prospectively recruited between August and December 2016.

Inclusion criteria for the study were all children under 5 years, who were experiencing an episode of ARI within the week prior to visiting the paediatric department of PGHB either as an outpatient or an inpatient; regardless of gender, provenance, religion and

social status. Before enrolling a participant, the parent or guardian had to sign the consent form after reading and understanding it properly.

Exclusion criteria were children older than 5 years and those who visited the PGHB for other symptoms not relating to ARI.

3.5 **Variables**

A clinical examination, performed by a paediatrician and a study questionnaire (Appendix 5) allowed the collection of clinical data and socio-demographic characteristics of the participants.

3.5.1 **Outcome**

The outcome of this study was ARI. It was classified in two groups: URTI and LRTI. Participants with bronchiolitis or pneumonia belonged to the LRTI group. Bronchiolitis was defined as a set of clinical symptoms occurring in children less than 2 years of age, characterized by upper respiratory symptoms (rhinorrhoea, cough) followed by wheezing. Pneumonia was defined as the presence of signs of LRTI (breathing fast, lower chest indrawing, crackles, fever) and characteristic signs of pneumonia on chest radiography.

Children who presented with cough, nasal secretion or difficulties in breathing, but no other signs of LRTI, were considered as suffering from URTI. These children had one of the following diagnoses: rhinitis, laryngitis, otitis or bronchitis. LRTI and URTI were mutually exclusive.

3.5.2 **Explanatory Variables or Predictors**

Baseline explanatory or independent variables included:

- **Socio-demographic characteristics**: age, gender, weight, settings (urban or rural), income level, maternal education, number of siblings in the household, alcohol exposure during pregnancy, cigarette and cooking smoke exposure.

- **Medical features**:
  - Medical history: birthweight, specific gestational age, history of neonatology stay, as well as oxygen therapy, family history of atopy
  - Clinical findings: nutritional status (malnutrition), antibiotic use before visit, symptoms duration, immunization status, exclusive breastfeeding, and admission.
  - Management: antibiotic use, oxygen therapy, nasogastric intubation.
- **RT-PCR result.** The following viruses were screened for: HRSV A and B, *influenza virus* A and B, HRV A/B/C, CoV OC43 and CoV 229E/NL63, PIV 1, 2, 3 and 4, HMPV, HBoV, ADV and EV. Mono-infection was defined as the detection of only one virus, while co-infection was defined as the detection of at least two viruses.

According to their household income, participants were classified in 3 groups: low income (< 100 USD per month), middle income (100 to 500 USD per month) and high income (>500 USD per month). Moreover, they were classified according to the level of education of their mother in 4 groups: illiterate (can't read and write), primary (1 to 6 years at primary school), secondary (1 to 6 years at secondary school) and university (≥ 1 year at university). Cigarette smoke exposure was defined as the presence of smokers in the household. Cooking smoke exposure was considered when biomass fuel was used for cooking in the house. Participants were regarded as being exposed to alcohol during pregnancy if their mothers were drinking any kind of alcoholic beverage while pregnant. Malnutrition was defined as a weight-for-age z-score ≤ 2. The z-score was calculated using the *WHO anthro software* version 3.2.2. All children born before 37 weeks gestational age were considered to be premature; children who had weighed less than 2.5 kg at birth were considered having a low birthweight. Exclusive breastfeeding was defined by the number of months that children were receiving only breastfeeding. There was a family history of atopy when the mother, father or sibling(s) reported ever having had asthma or atopy (allergic rhinitis or eczema). All the children who received any kind of treatment for at least 24 hours in a neonatology care unit had a neonatology stay, among where some

received oxygen as part of their treatment. The investigator checked for immunization status of the participants according to the DRC health ministry (Miso *et al.*, 2014).

3.6 **Procedures**

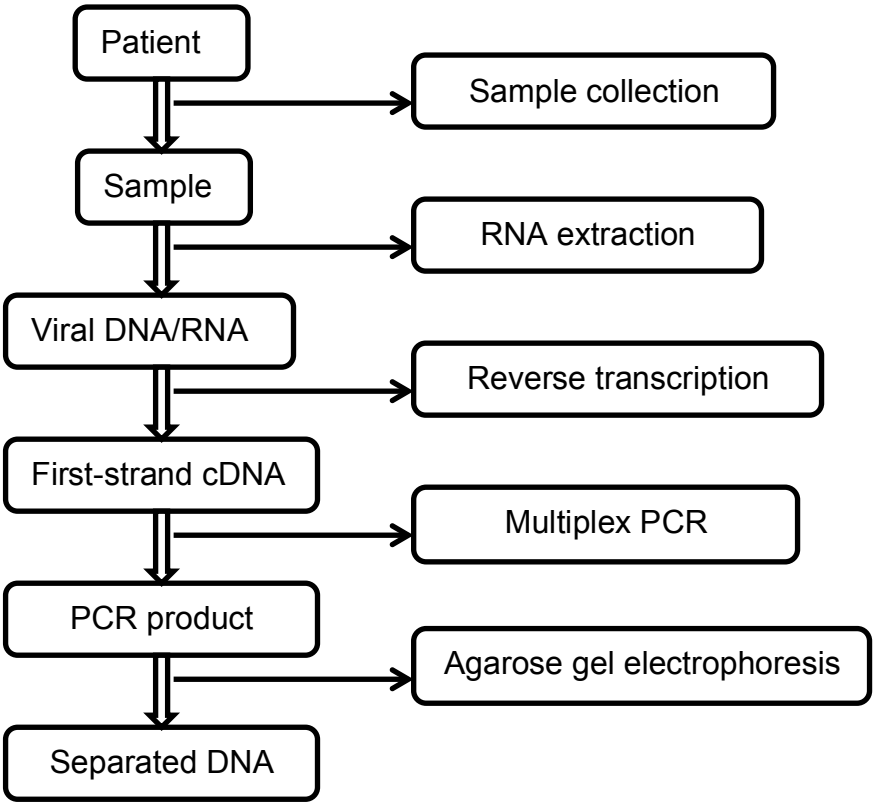

Figure 3.1 Workflow diagram of the viral identification process

3.6.1 **Specimen Collection, Storage and Transfer**

a. Materials:

- Swabs flocked paediatric 100MM B/Point Dry Tube ST (Lasec, Cape Town).
- Tube Universal Transport Media (UTM) 16X100MM 3 ml (Lasec, Cape Town).
- 2.0 ml graduated tube with Flat Top Cap (Whitehead Scientific, Cape Town)
- 100-1 000 µl standard Tip Blue (Whitehead Scientific, Cape Town)
- Sterile gloves, a paper surgical mask, a marker, a biohazard bag, facial tissues, eye protection, a vortex machine, a pipette, a cartoon ruler.

b. Methods:

The parent or guardian was fully informed about the procedure that was to take place. One assistant restrained the child's arms and another his head.

The distance between the base of the nose and ear loop of the child was measured with a disposable cartoon ruler. With a marker, the investigator marked the depth of the insertion of the swab, which was the half distance of the ear loop-base nose distance. The child's head was tilted to a 70° angle and the flocked swab was inserted horizontally in one of the two nostrils until it reached the nasopharynx. Once there, it was left for a few seconds (2 to 3 seconds), pulled out with a rotating motion and inserted into a tube containing 3 ml UTM. The tube was transported to the laboratory on wet ice; it was mixed thoroughly by a vortex, the swab was removed, the specimen was placed into two 1.5 ml tubes and stored at -70°C.

After the collection period, all the samples were sent on dry ice by DHL Express to the Tygerberg Virology Laboratory in Cape Town, South Africa. For that purpose, the investigator applied for a South African biological material import permit (Appendix 6), as well as a biological export permit from DRC (Appendix 7).

3.6.2 Nucleic Acid Extraction:

a. Materials:

Viral nucleic acid was extracted from respiratory swab samples using the QIAamp *cador* Pathogen Mini Kit (Whitehead Scientific, Cape Town) according to the manufacturer's instructions with a few modifications. This kit contains:

- QIAamp Mini Columns
- Collection Tubes (2 ml)
- Buffer VXL
- Buffer ACB (concentrate)
- QIAGEN® Proteinase K
- Carrier RNA (poly A)
- Buffer AW1 (concentrate)
- Buffer AW2 (concentrate)
- Buffer AVE
- Quick-Start Protocol

Buffer ACB, Buffer AW1 and Buffer AW2 were supplied as concentrates. Before using, 8 ml of isopropanol was added to 12 ml of Buffer ACB, 25 ml of 96% ethanol to 19 ml of AW1 and 40 ml of 96% ethanol to 17 ml of AW2.

b. Methods:

In brief, the process was as follows: 20 µl of proteinase K (digest proteins, which usually contaminate nucleic acid preparations) was pipetted into a 2 ml microcentrifuge tube. 200 µl of the sample was added to the tube. To enhance the lysis of the sample, 100 µl of Buffer of VXL containing 1 µl of carrier RNA was added and the solution was mixed by pulse-vortexing. The 2 ml tube was incubated at 20-25°C for 15 minutes and briefly centrifuged. 350 µl of Buffer ACB (adjusts the binding conditions for the co-purification of DNA and RNA) was added to the sample and mixed thoroughly by pulse-vortexing. The 2 ml tube was briefly centrifuged. The lysate was transferred to the QIAamp Mini column placed in a 2 ml collection tube and then centrifuged at 8 000 rpm for 1 minute. The mini column was placed into a clean 2 ml collection tube. 600 µl Buffer AW1 was added into the QIAamp Mini column and centrifuged at 8 000 rpm for 1 minute. The QIAamp Mini column was placed in a clean 2 ml collection tube. Buffer AW2 was added into the QIAamp Mini column and centrifuged at 8 000 rpm for 1 minute. The QIAamp Mini column was placed in a clean 2 ml collection tube. The 2 ml collection tube was centrifuged at 13 200 rpm for 3 minutes. The QIAamp Mini column was placed in a clean 1.5 ml microcentrifuge tube. 40 µl Buffer AVE (an elution buffer) was added to the centre of the membrane, the tube was incubated at room temperature (15-25°C) and centrifuged at 13 200 for 1 minute.

After extraction, the nucleic acid concentration of each sample was measured on a NanoDrop spectrophotometer ND1000 (NanoDrop Technologies, USA)

3.6.3 Complementary DNA

RNA viruses and subtypes were analysed using complementary DNA (cDNA).

a. Materials:

RNA samples were reverse-transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermofisher Scientific, Cape Town). This kit contains random

hexamers, nuclease-free water, Revert Aid Premium Reverse Transcriptase enzyme, ribolock, dNTP mix and 5x RT Buffer.

b. Methods:

In brief, cDNA was performed as follows: after putting 8 µl of the sample into a 0.2 ml Quality Scientific Plastics (QSP) PCR tube, 2 master mix solutions were successively added. The first contains: 1 µl of random hexamers and 3 µl nuclease-free water. The second contains: 4 µl of 5XRT buffer, 1 µl of Ribolock, 2 µl of dNTP mix and 1 µl of Revert Aid Premium reverse transcriptase. cDNA synthesis was then done on a Gene Amp PCR system 9700. The reverse-transcription was initiated with an incubation of 5 minutes at 25°C and continued with a 60 minutes incubation at 42°C and ending with a 5 minute incubation at 70°C.

3.6.4 PCR:

a. Materials:

cDNA was screened for 15 viruses using the Seeplex RV15 Ace Detection kit (supplied by Inqaba Biotechnical Industries). This kit contains:

- Positive control
- Negative control
- 5XRV15 Ace
- Multiplex Master mix
- 8-Mop solution

b. Methods:

In brief, a master mix containing 4 µl of 5XRV15 Ace, 10 µl of 2X Multiplex Master mix, 3 µl 8-Mop solution and 3 µl of the sample was prepared in a 0.2 ml PCR tube, mixed and briefly centrifuge. Amplification reaction was performed on a Gene Amp PCR system 9700 using the PCR protocol listed in Table 3.1. A positive and negative control was included in each run.

Table 3.1 Seeplex RV15 ACE PCR reaction program

Step	No of cycles	Temperature	Duration
Initiation	1	94°C	15 minutes
Denaturation		94°C	0.5 minutes
Annealing	40	60°C	1.5 minutes
Elongation		72°C	1.5 minutes
Final elongation	1	72°C	10 minutes

3.6.5 Visualization of PCR Products

Agarose gel electrophoresis was used to separate PCR products according to Sambrook & Russell (2001) with slight modifications.

a. Materials:

Buffers and solutions:

- 2% Agarose solution
- TAE (Tris base, acetic acid and EDTA) was used as the electrophoresis buffer. A 1X TAE working solution was prepared by mixing 20 ml of 50X TAE (Bio Basic Canada Inc) and 980 ml of water.
- Gel loading buffer: GeneDirex® Novel juice (Biocom Biotech, Cape Town)

Nucleic acids and Oligonucleotides:

- PCR product
- Positive and negative controls (provided with Seeplex RV15 Kit)
- DNA molecular weight markers (provided with Seeplex RV15 Kit)

Special equipment:

- Horizontal gel electrophoresis apparatus
- Gel combs and rubber gaskets (end gates)

- Basin with cold water
- Microwave oven
- Power supply device: BG Power6000 electrophoresis system (BayGene®, China)
- Lab rotator (Digisystem laboratory instruments Inc. Taiwan)
- UVITEC computer

b. Methods:

Preparing the gel:

2 g of Lonza SeaKem® LE agarose (Whitehead Scientific, Cape Town) were dissolved in 100 ml 1X TAE and melted in a microwave oven for 3 minutes. Melted agarose was then cooled in a basin containing cold water before pouring it onto a horizontal gel electrophoresis apparatus (care was taken to make sure that the comb and rubber gaskets were placed). All bubbles on the surface of the agarose were removed and the gel was allowed to cool. After approximately 20 to 30 minutes, the gel was solidified and became slightly opaque. End gates were removed and we submerged the gel by adding 1X TAE buffer to cover the gel by about 0.5 cm. The comb was carefully removed by lifting it gently at one end.

Loading and running the gel:

5 µl of the PCR product, 5 µl of molecular markers, 2 µl of the positive control and 2 µl of the negative control were each mixed with 1 µl of novel juice and loaded into individual wells with a gel loading tip and a P-20 Pipette. Once the loading was done, the cover was placed on the gel apparatus and leads were connected (the negative lead is at the end of the gel containing the wells) to the power supplier. The gel was run at 60 volts for 40 minutes and gently shaken on a lab rotator.

Visualization of PCR products:

The gel was examined using an UVITEC computer by regulating the scale under white light and visualizing bands under the transilluminator.

Table 3.2 Amplification information

Set A	**Size (bp)**	**Target gene**
Internal control	850	CesA3
ADV	534	Major core protein
CoV 229E/NL63	375	Spike
PIV 2	264	HN
PIV 3	189	F
PIV 1	153	HN
Set B	**Size (bp)**	**Target gene**
Internal control	850	CesA3
CoV OC43	578	ORF1b
HRV A/B/C	394	5'UTR
HRSV A	269	F
Influenza virus A	206	M1
HRSV B	155	F
Set C	**Size (bp)**	**Target gene**
Internal control	850	CesA3
HBoV 1/2/3/4	579	NS1
Influenza virus B	455	NA
HMPV	351	F
PIV 4	249	HN
EV	194	5'UTR

bp: base pairs

3.7 Statistical Analyses

3.7.1 Sample Size Calculation:

Published literature has reported HRSV ARI prevalence of 11.9% in Burkina Faso (Ouédraogo *et al.*, 2014) and 10.7% in South Africa (Pretorius *et al.*, 2016). The expected prevalence of HRSV in DRC might be as high as 10%. With a level of significance at 95% and level of precision at 5%, the sample size must be 138 patients.

The following formula was used for sample size calculation (Leslie, 1965):

$$n = \frac{(1.96^2 x\ SD^2)}{d^2}$$

$$SD = \sqrt{p(1-p)}$$

n: simple size

d: level of significance

p: expected prevalence

3.7.2 **Summary Statistics**

When analysed with the Shapiro test for normality (α level: 0.05), numerical data of this study displayed a non-parametric distribution and therefore, the median was used for the central tendency measure and the interquartile range (IQR) as the measure of dispersion. Categorical data, on the other hand, were summarized as proportions.

3.7.3 **Analytical Analysis**

Proportions and medians of the baseline characteristics variables were compared between the two groups of ARI (URTI versus LRTI) using the Chi-square test and the Mann-Whitney U test (two samples Wilcoxon test) respectively. The Fisher test was used to compare proportions when the criteria for the Chi-square test were not met. Simple logistic regression was used to compute the OR ratio with 95% CI. The difference between two groups was statistically significant when the p-value was less than 0.05.

To analyse independent association between baseline characteristics and LRTI, a multiple logistic regression model was built. This model included variables with a cut-off of p-value < 0.1 in bivariate analyses. The null deviance and residual deviance were used to evaluate the overall performance of the model. The results of the model were reported as odds ratios and corresponding 95% CI.

R software was used for statistical analyses.

CHAPTER 4: RESULTS

4.1 Baseline Characteristics of the Study Population:

A total of 146 children under the age of 5 years that were visiting the Paediatrics Department of the PGHB for ARI were recruited between August and December 2016. The male to female ratio was 1 : 0.97. The median age of the study group was 14.5 months (IQR 7-28), while the median gestational age was 38 weeks (range: 29-41) and the median birthweight was 3 300g (range: 1 400-5 200g). The median number of siblings was 3 (IQR: 2-5) and 41 (28.1%) reported a family history of atopy. Other demographic factors included exposure to cigarette and cooking smoke in 19 (13.0%) and 61 (41.8%) respectively and exposure to alcohol during pregnancy in 32 (21.9%) of children.

Table 4.1 summarizes the socio-demographic and relevant clinical information collected from the study group.

Antibiotic treatment was initiated by the paediatrician in 60 children among which a viral infection was confirmed in 36 (60%) cases. The median symptom duration before the visit was 96 hours (range: 16-192).

4.2 HRSV Prevalence, non-HRSV ARI and Co-Infection

Of 146 samples analysed, 84 (57.5%) displayed a positive result for at least one of the 15 viruses. The prevalence of HRSV ARI was 21.2% (95% CI: 14.9-28.8). HRSV was detected in LRTI and URTI in 40.5% (95% CI: 24.8-57.9) and 14.7% (95% CI: 8.6-22.7) of cases respectively.

A total of 95 viruses were detected in 84 participants (Figure 4.1). HRSVA (30, 20.5%) was the virus the most often detected, followed by HRV (24, 16.4%), PIV3 (20, 16.7%) and ADV (7, 4.8%). Co-infection with two viruses occurred in 11 (7.5%) cases, with HRV being detected most often in the co-infected cases (Table 4.2).

Table 4.1 Sociodemographic and clinical information collected from the study group

	Frequency	Percentage
Urban settings	129	88.4
Household income level		
Low	47	32.2
Middle	54	37.0
High	45	30.8
Maternal level of education		
Illiterate	22	15.1
Primary school	30	13.7
Secondary school	34	23.3
University	70	48.0
Malnutrition	40	27.4
Admission to neonatology healthcare at birth	29	19.9
Received oxygen	10/29	34.5
Immunization up to date	120	82.2
Exclusive breast feeding	71	48.6
Antibiotic use prior to medical examination	47	32.2
Antibiotic treatment initiated at medical examination	60	41.1
Hospitalization	30	20.6
Received oxygen	17/30	56.7
Nasogastric intubation	12/30	40.0
Diagnosis: LRTI	37	25.3
Pneumonia	21/37	56.8
Bronchiolitis	16/37	43.2
Diagnosis: URTI	109	74.66
Pharyngitis	38/109	34.9
Otitis media	2/109	1.8
Laryngitis	2/109	1.8
Rhinitis	67/109	61.5

URTI: Upper respiratory tract infection. LRTI: Lower respiratory tract infection.

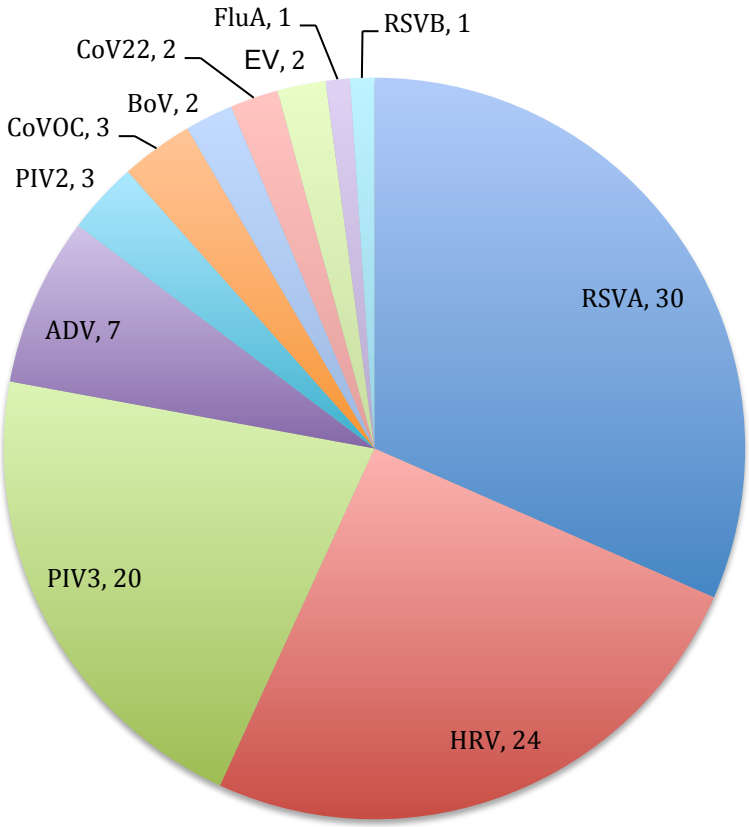

Figure 4.1 Viruses detected by RT-PCR. Abbreviation of viruses detected and frequency.

Table 4.2 Frequency of viruses detected in co-infected cases

	Frequency
ADV and HRV	1
CoV OC43 and HRV	1
PIV2 and ADV	1
PIV2 and HRV	1
PIV3 and HRV	3
HRSVA and CoV OC43	1
HRSVA and PIV3	2
HRSVB and PIV3	1

4.3 **Risk Factors for ARI to be Diagnosed as LRTI**

Table 4.3 summarizes bivariate analyses of the effect of different factors (demographics, clinical factors and HRSV status) on the risk of ARI being diagnosed as LRTI. Children with malnutrition had 2.67 times higher odds of ARI to be diagnosed as LRTI when compared to children without malnutrition (OR = 2.67, 95% CI: 1.1-6.3, p-value = 0.01). HRSV infection was associated with a 3.91-fold increase in the odds of LRTI (95% CI: 1.55-9.97, p-value < 0.001). Table 4.4 summarizes the effects of all the viruses that were detected in this study in relation to the odds of LRTI.

The OR for LRTI in children from low versus high-income households was 3.13 (95% CI: 1.17-8.36, p-value = 0.010). This OR in children with illiterate mothers compared to children with mothers who attended university was 3.34 (95% CI: 1.14-9.79, p-value = 0.020). Older children (by one month) had 5% lower odds of LRTI than younger children (OR = 0.95, 95% CI: 0.92-0.98, p-value = 0.006).

A multiple logistic regression was performed to ascertain the effect of age, HRSV infection, immunization, malnutrition, history of atopy, maternal education, household income and settings on the likelihood of ARI to be diagnosed as LRTI (Table 4.5). Of the eight factors, only two were statistically significant: age and HRSV infection. Children with HRSV infection had 6.45 times higher odds to exhibit LRTI when compared to children without HRSV infection (adjusted OR (aOR) = 6.64, 95% CI: 2.28-19.47, p-value < 0.001). Older children were less likely to have LRTI when compared to younger children (aOR = 0.94, 95% CI: 0.90-0.97, p-value = 0.004).

Table 4.3 Demographics and clinical findings of the population study grouped by the type of acute respiratory infection

	URTI (N=109)	LRTI (N=37)	P-value	OR	95% CI
Gender (male)	56(51.3)	16(43.2)	0.39	0.72	0.31-1.69
Settings (rural)	8(7.3)	9(24.3)	0.01	4	1.24-13.18
Income					
Low	28(25.6)	19(51.3)	0.01	3.13	1.17-8.36
Middle	44(40.3)	10(27)	0.92	1.05	0.36-2.99
High (ref.)	37(33.9)	8(21.6)			
Maternal education					
Illiterate	13(11.9)	9(24.3)	0.02	3.34	1.14-9.79
Primary	14(12.8)	6(16.2)	0.21	2.07	0.64-6.67
Secondary	24(22)	10(27)	0.15	2.01	0.75-5.38
University (ref.)	58(53.2)	12(32.4)			
Cigarette smoke exposure	14(12.8)	5(13.5)	0.91	1.05	0.27-3.43
Cooking smoke exposure	46(42.2)	15(40.5)	0.85	0.93	0.40-2.12
Alcohol exposure	25(22.9)	7(18.9)	0.61	0.78	0.25-2.12
History of atopy	35(32.1)	6(16.2)	0.06	0.41	0.12-1.12
Neonatology stay	21(19.2)	8(21.6)	0.75	1.15	0.39-3.08
Oxygen therapy at birth	7(6.4)	3(8.1)	0.71	1.28	0.20-6.01
Malnutrition	24(22)	16(43.2)	0.01	2.67	1.12-6.30
Immunization status	93(85.3)	27(72.9)	0.08	0.46	0.17-1.29
Exclusive breast feeding	58(53.2)	18(48.6)	0.99	1	0.44-2.25
HRSV	16(14.6)	15(40.5)	<0.001	3.91	1.55-9.97
Age *	16	10	0.006	0.95	0.92-0.98
Number of siblings *	3	3	0.74	0.99	0.82-1.18
Birthweight (gram)*	3 300	3 300	0.85	1.00	0.99-1.00
Gestational age (weeks)*	34	34	0.64	1.09	0.00-1.72
Symptoms duration (hours)*	96	96	0.48	0.99	0.99-1.00

Data are presented as n (%) or median*

Ref. : parameter of reference

Table 4.4 Effects of respiratory viral infection confirmed by RT-PCR on the risk of LRTI among children under the age of 5 years at the Provincial General Hospital of Bukavu

	RT-PCR	URTI (n=109)	LRTI (n=37)	odds ratio (95% CI)	P-value
HRSVA	Positive	15	15	4.22 (1.65-10.89)	<0.001
HRSVB	Positive	1	0		
HRV	Positive	17	7	1.92 (0.40-3.59)	0.63
PIV2	Positive	3	0		
PIV3	Positive	15	5	0.97 (0.25-3.13)	0.96
ADV	Positive	5	2	1.18 (0.10-7.65)	1.00
BoV	Positive	1	1	2.97 (0.03-237.15)	0.44
CoV229E	Positive	2	0		
CoV OC43	Positive	2	1	1.48 (0.02-29.25)	1.00
EV	Positive	2	0		
Influenza virus A	Positive	0	1		

URTI: Upper respiratory tract infection, LRTI: Lower respiratory tract infection, RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

Table 4.5 Logistic regression predicting likelihood of LRTI

	B	SE	aOR	CI	p-value
Age	-0.06	0.20	0.94	0.90-0.97	0.004
HRSV infection	1.86	0.54	6.45	2.28-19.47	<0.001
History of atopy	-0.65	0.57	0.51	0.15-1.53	0.25
Immunization	-0.37	0.66	0.62	0.18-2.60	0.57
Income					
Low	1.53	1.07	4.64	0.58-41.97	0.15
Middle	0.03	1.63	1.03	0.29-3.67	0.95
Malnutrition	0.41	0.74	1.51	0.35-6.73	0.57
Maternal education					
Illiterate	-0.42	1.17	0.65	0.05-6.10	0.71
Primary	-1.42	1.14	0.24	0.02-2.07	0.21
Secondary	-0.05	0.66	0.94	0.24-3.45	0.93
Settings	1.39	0.79	4.02	0.89-20.75	0.07

B: Slope, SE: Standard Error, aOR: Adjusted Odds Ratio

CHAPTER 5: DISCUSSION

This cross-sectional study undertaken over 5 months at the PGHB is the first study to determine the overall burden of viral ARI in DRC. It aimed to analyse the epidemiological characteristics of ARI in children under the age of 5 years at the PGHB by setting two hypotheses. The first one stipulated that compared to other respiratory viruses the prevalence of HRSV is higher in ARI. The second hypothesized that HRSV, as well as some socio-demographic factors and medical features, is associated with the risk of ARI to be diagnosed as LRTI in children under the age of 5 years. These two hypotheses were confirmed: HRSV was the most prevalent virus in ARI and in bivariate analyses, HRSV infection, malnutrition, younger age, rural settings, low income and mother illiteracy were associated with the risk of ARI to be diagnosed as LRTI. However, in multivariate analyses, only HRSV infection and younger age predicted LRTI.

5.1 HRSV ARI Prevalence and Co-Infection

This study reports an overall HRSV prevalence (21.23%) similar to that reported in Kenya (17%) by Bigogo *et al.* (2013). However, this prevalence is slightly higher than what was reported in other countries of Africa: 14.1% in Ghana (Kwofie *et al.*, 2012), 11.9% in Burkina Faso (Ouédraogo *et al.*, 2014), 10.7% in South Africa (Pretorius *et al.*, 2016), 9.8% Mozambique (Loscertales *et al.*, 2002) and 8.7% in Gambia (Weber *et al.*, 2002). When considering only children with LRTI, the prevalence of HRSV is 40.5%, a value much higher in respect to the prevalence reported in Tunisia (21.6%) by Fodha *et al.* (2004). Only one case of HRSV B infection was found, which is much lower than the 30 cases of HRSV A observed. This predominance of HRSV A over HRSV B has been previously observed in Burkina Faso as well as in Cyprus and in China even though there was a monthly distribution pattern involved (Ouédraogo *et al.*, 2014; Panayiotou *et al.*, 2014; Zheng *et al.*, 2017).

Some studies have suggested that the peak of HRSV prevalence appears to occur during the rainy season (Chan *et al.*, 2002; Kwofie *et al.*, 2012), other studies have reported that this peak occurs during the dry hot season (Forgie *et al.*, 1991; Yoshida *et al.*, 2013) and finally, others had failed to demonstrate any seasonality feature (Okiro *et al.*, 2012; Haynes *et al.*, 2013). Given that our findings are based on data

collected only during the rainy season of 2016, these results should be interpreted against the background of the season. It is therefore important to gain more insight into the HRSV seasonality in order to address prevention strategies of HRSV ARI in DRC.

In this study, four cases of HRSV co-infection were detected, three of them with PIV3 and one with CoV. Previously, PIV3 and CoV have been reported in cases of co-infection with HRSV (Yoshida *et al.*, 2013; Calvo *et al.*, 2015). Yoshida *et al.* (2013) worked on children under 2 years of age hospitalized in Vietnam for LRTI and found that HRSV-PIV3 co-infection led to a high risk of pneumonia than single infection of any of these two viruses.

No case of HRSV-*influenza virus* co-infection was observed during the study period and *influenza virus* was detected in just one case. In a study on the prevalence of co-infection between HRSV and *influenza virus* in children conducted by Meskill *et al.* (2016) in Texas, the author observed 6 times less cases of HRSV-*influenza virus* co-infection than expected during the seasons where these two viruses were co-circulating and in the 2011-2012 season, the HRSV and *influenza virus* seasons did not overlap at all. Though, the theory of niche competition between these two viruses at the host level observed in several studies can be suggested. Using a mathematical model, Xicoténcatl Velasco-Hernández *et al.* (2015) has explained the alternating pattern of HRSV and *influenza virus* through the ecological hypothesis of competitive interaction between theses two viruses and niche availability. The author reported that HRSV is competitively superior to *influenza virus* and though, HRSV superinfects *influenza virus* allowing it to survive. In the case that *influenza virus* would superinfect HRSV, this would result in the extinction of the inferior competitor (*influenza virus*) leading a system of epidemics of HRSV only. These findings can lead us to hypothesize that there was probably an undocumented epidemic of *influenza virus* early during the year of our study period.

HRV was the virus the most detected after HRSV in this study (16.4%); however, no case of HRSV-HRV co-infection has been observed. This virus is known to be involved in ARI in children (Onyango *et al.*, 2012; Pretorius *et al.*, 2012). Our findings contrast with previous results reported by Luchsinger *et al.* (2014) who found a sustainable degree of co-infection (22%) between these two viruses. However, the

work of Achten *et al.* (2017) on the interference of HRSV and HRV infection in infancy can explain our findings. The author has found evidence that support HRSV and HRV interference by demonstrating a negative association between these two viruses in ARI in infancy. He reported that children with HRSV infection had 83% lower odds of HRV infection than children without HRSV infection. Furthermore, he said that children who were on HRSV immunoprophylaxis had higher odds of HRV infection than those who were not on HRSV prophylaxis. Many other studies have demonstrated this negative association pattern between HRSV and HRV (Greer *et al.*, 2009; Wisdom *et al.*, 2009; Martin *et al.*, 2013; Karppinen *et al.*, 2016).

PIV3 and ADV were the viruses that were detected most often in this study after HRSV and HRV. PIV3 was the virus most often involved in co-infection with HRSV. PIV3 is known to be an important agent of ARI in children in both URTI and LRTI (Mizuta *et al.*, 2012; Frost *et al.*, 2014). Although PIV3 infections are naturally mild and self-limiting, they can frequently cause severe diseases in immunocompromised people resulting in high rates of morbidity and mortality (Harvala *et al.*, 2012). ADV is involved not only in respiratory infection, but also in gastroenteritis and conjunctivitis outbreaks (Dashti *et al.*, 2016; Zhang *et al.*, 2016). However, these conditions were not investigated during this study and no comment can be made on the possible association with ADV found.

Two cases of BoV and EV were found in this study. BoV, a single-stranded DNA virus, was first discovered in 2005 and shown to be a cause of respiratory infection and gastroenteritis (Jartti *et al.*, 2012), even though its role in human disease has not been well established yet. EV on the other hand, is a well-known virus involved in a wide spectrum of illness in children. Depending on the species, it can cause ARI, viraemia, herpangina, gastroenteritis, encephalitis and Hand-Foot-Mouth disease (Tapparel *et al.*, 2013).

PIV 1 and 4, *influenza virus* B, and HMPV were not detected this study at all, but are recognized in the literature as usual pathogens of the respiratory tract in children.

5.2 Risk Factors for LRTI

5.2.1 Age

This study highlights the independent association between younger age and the risk of LRTI. This has been documented in previous studies and supports findings in the literature (Ujunwa & Ezeonu, 2014). The relatively immature immune system and poor compliance of immature bronchioles can explain why young children are more at risk for LRTI when compared to older children (Nathan *et al.*, 2014). In a rat model, Martin *et al.* (1995) has revealed the evidence of postnatal impairment of TLR2 and TLR4 expression when lungs epithelium was exposed to exogenous antigens. In infants, the level of adaptive immunity is also reduced and it was reported that both CD4 and CD8 functions are deficient which can lead to reduced viral clearance (Tregoning & Schwarze, 2010).

5.2.2 HRSV Infection

A very important finding of this study was that HRSV was independently associated with the risk of ARI to be diagnosed as LRTI. This substantiates previous studies in the literature all around the world (Singleton *et al.*, 2010; Al-Ayed *et al.*, 2014; Ouédraogo *et al.*, 2014). HRSV displays some specific characteristics that make it very prevalent and highly infectious. This includes high infectivity, tropism to the superficial respiratory epithelium (that reduces the effectiveness of the host immunity response), expression of type I IFN antagonists, proteins that inhibit apoptosis, capacity of reinfection throughout the life, expression of fractalkine and TLR antagonists, interference with normal macrophage and DC function (Collins & Graham, 2008).

5.2.3 Other Factors

Even though malnutrition, rural settings, low income and mother illiteracy were associated with the risk of LRTI in bivariate analyses, this study failed to find an independent association with any of these factors. This is in contrast to a study done in Kenya, Okiro *et al.* (2008) that has demonstrated an independent association between malnutrition, the presence of 2 or more smokers in the household, illiteracy

of primary caretakers, a number of family children of eleven or more and the risk of LRTI. Moreover, our study also failed to demonstrate any kind of association between LRTI and gender, cigarette and smoke exposure, alcohol exposure during pregnancy, family history of atopy, exclusive breastfeeding, immunization status, high numbers of siblings, prematurity or low birthweight. Jackson *et al.* (2013) published a meta-analysis on the risk factors for severe LRTI in children. The author identified 36 studies that investigated 19 risk factors among which 7 were significantly associated with severe LRTI: low birth weight, lack of exclusive breast feeding, crowding (more than 7 persons per household), exposure to indoor air pollution, incomplete immunization, undernutrition (weight for age < 2 standard deviation) and HIV infection.

Weight for age was used in this study as the nutritional index because in the database the height measurement was missing in some participants. It was therefore not possible to assess stunting (low height for age) and wasting (low weight for height).

5.3 **Strengths and Limitations:**

5.3.1 **Strengths:**

This study was performed in a provincial referral hospital and therefore included patients from different areas. The findings of this study can therefore be generalizable to all the Province of South Kivu. Moreover, the sample size used was representative of the study population.

In contrast to many studies in the literature which usually investigate only a few viral agents involved in ARI in children, this study investigated 15 different viruses at the same time.

This is the first study on viral aetiology of ARI in children in DRC. Standardized approaches were used and therefore, this study can be replicated in other places of the country.

5.3.2 **Limitations:**

This study did not assess the role of bacteria in the aetiologies of ARI. There are some gaps that remain on the epidemiology of ARI.

It was not possible to assess seasonality of viruses involved in ARI, due to limited time available for this study. This should be followed up with bigger studies over longer periods of time to elucidate the effect of seasons on different individual viruses and co-infections.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS:

6.1 Conclusion

As expected, the prevalence of HRSV is high among children visiting the PGHB for ARI. HRSV infection and lower age are independently associated with the risk of ARI to be diagnosed as LRTI.

6.2 Recommendations

After thorough analysis of the findings, the following recommendations are offered:

- Medical practitioners should use antibiotics cautiously in children with ARI.
- IPC measures should be reinforced:
  - Caregivers should wash their hands before and after touching a patient.
  - Caregivers should use gloves and gowns.
  - Rapid methods for early detection of HRSV infection should be implemented.
  - Infected patients and caregivers should be cohorted.
  - Visitors should be advised about a possible risk of transmission.
- A national active surveillance program for HRSV infection should be set up and performed during high risk seasons to identify disease patterns.
- Further studies on ARI in children with a lengthy period of study to should be carried out so as to determine the seasonality of HRSV and other viruses involved in ARI.
- Studies on the role of bacteria in ARI should be conducted.

CHAPTER 7: REFERENCES

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CHAPTER 8: APPENDICES

APPENDIX 1: Stellenbosch University RSV study ethical approval

UNIVERSITEIT·STELLENBOSCH·UNIVERSITY
jou kennisvennoot • your knowledge partner

Approved with Stipulations
Response to Modifications- (New Application)

11-Jul-2016
Cihambanya, Kabego KL

Ethics Reference #: S16/03/051

Title: **Prevalence and risk factors of respiratory infection by Respiratory Syncytial Virus in children at Provincial General Hospital of Bukavu**

Dear Dr Kabego Cihambanya,

The **Response to Modifications** - ***(New Application)*** received on **17-May-2016**, was reviewed by members of **Health Research Ethics Committee 2** via Expedited review procedures on **11-Jul-2016**.

Please note the following information about your approved research protocol:

Protocol Approval Period: **11-Jul-2016 -10-Jul-2017**

The Stipulations of your ethics approval are as follows:
1. Please let the institutional Ethics Committee of the Catholic University of Bukavu determine whether it is necessary to provide medical insurance for research related injury to study participants in the local setting.
2. Please provide some form of compensation to each child after a nasal swab such as a lollipop or cool drink.

Please remember to use your **protocol number** (**S16/03/051**) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard HREC forms and documents please visit: www.sun.ac.za/rds

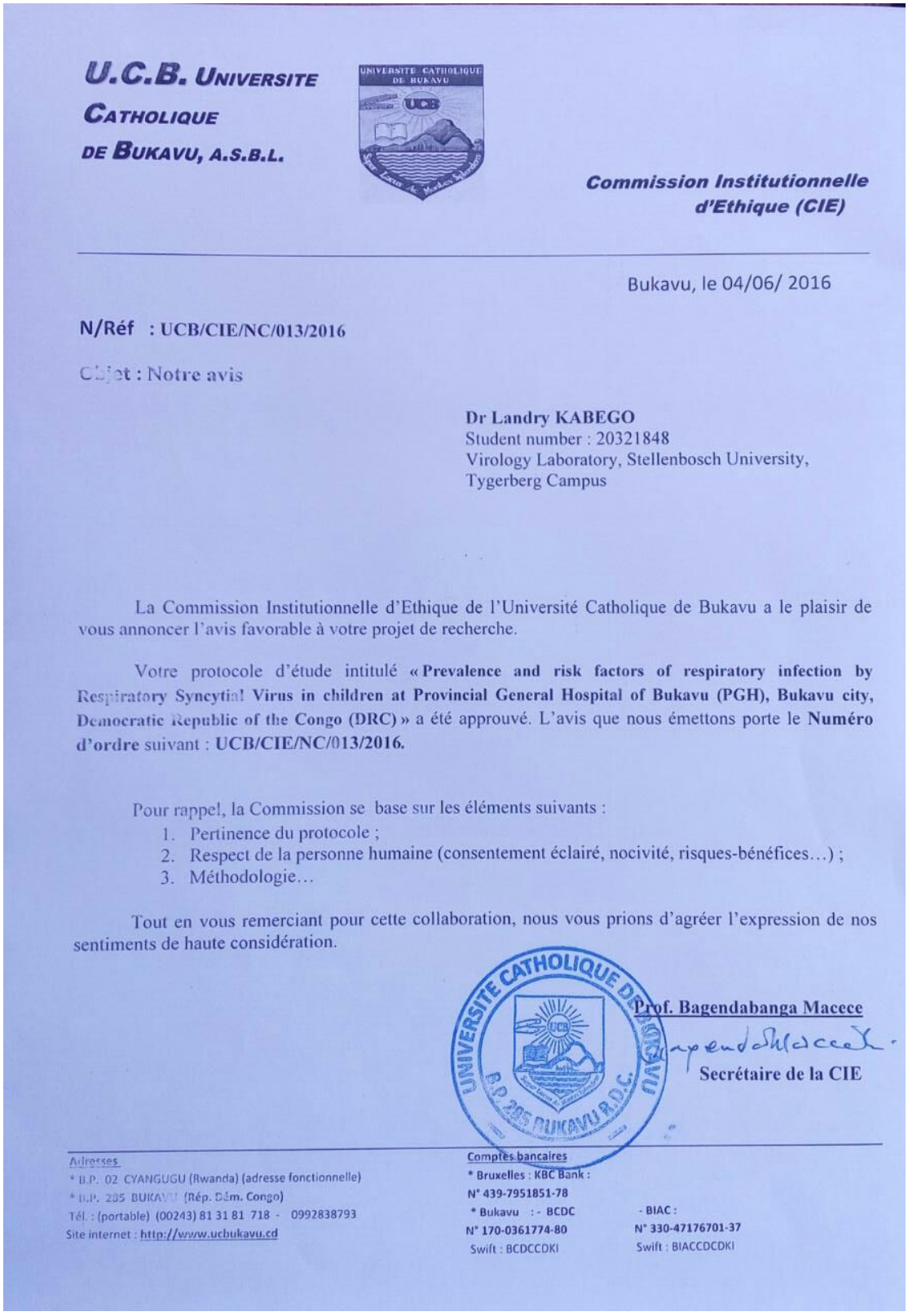
If you have any questions or need further assistance, please contact the HREC office at .

Included Documents:
20160524 MOD Application form
Application form signature page.pdf
20160524 MOD Protocol
Declaration LK Cihambanya.pdf
Informed Consent General Eng.pdf
20160608 REC approval Catholic University (French)
Declaration E Vardas.pdf
Checklist.pdf
Protocol Synopsis.pdf
CV C de Beer.pdf
CV E Vardas.pdf
HREC Application Form V9 11 November 2015 (Eng) (3).doc
20160524 MOD Cover letter
Application Form.pdf
Letter re Prof Vardas declaration.pdf
20160608 REC approval Catholic University (Eng)
Declaration C de Beer.pdf
20160524 MOD Consent
CV LK Cihambanya.pdf

Sincerely,

Francis Masiye
HREC Coordinator
Health Research Ethics Committee 2

APPENDIX 2: Catholic University of Bukavu RSV study ethical approval (French version)



U.C.B. UNIVERSITE CATHOLIQUE DE BUKAVU, A.S.B.L.

UNIVERSITE CATHOLIQUE DE BUKAVU

Commission Institutionnelle d'Ethique (CIE)

Bukavu, le 04/06/ 2016

N/Réf : UCB/CIE/NC/013/2016

Objet : Notre avis

Dr Landry KABEGO
Student number : 20321848
Virology Laboratory, Stellenbosch University,
Tygerberg Campus

La Commission Institutionnelle d'Ethique de l'Université Catholique de Bukavu a le plaisir de vous annoncer l'avis favorable à votre projet de recherche.

Votre protocole d'étude intitulé « **Prevalence and risk factors of respiratory infection by Respiratory Syncytial Virus in children at Provincial General Hospital of Bukavu (PGH), Bukavu city, Democratic Republic of the Congo (DRC)** » a été approuvé. L'avis que nous émettons porte le **Numéro d'ordre** suivant : **UCB/CIE/NC/013/2016.**

Pour rappel, la Commission se base sur les éléments suivants :

1. Pertinence du protocole ;
2. Respect de la personne humaine (consentement éclairé, nocivité, risques-bénéfices…) ;
3. Méthodologie…

Tout en vous remerciant pour cette collaboration, nous vous prions d'agréer l'expression de nos sentiments de haute considération.

UNIVERSITE CATHOLIQUE DE BUKAVU B.P. 285 BUKAVU R.D.C.

Prof. Bagendabanga Macece
Secrétaire de la CIE

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N° 330-47176701-37
Swift : BIACCDCDKI

APPENDIX 3: Catholic University of Bukavu RSV study ethical approval (English version)

Catholic University of Bukavu
Institutional Ethics Committee
BP 285 BUKAVU (Democratic Republic of Congo)
Phone number: (00243) 813181718 and 0992838793
Site: www.ucbukavu.cd

Ref number: UCB/CIE/NC/013/2016
Object: Our point

Dr Landry Kabego
Student number: 20321848
Virology Laboratory, Stellenbosch University
Tygerberg Campus

The Institutional Ethics Committee of the Catholic University of Bukavu has the pleasure to let you know that your research project was approved.

Your study protocol named: **Prevalence and risk factors of respiratory infections by respiratory syncytial virus in children at Provincial General Hospital of Bukavu (PGHB), Bukavu City, Democratic Republic of Congo** (DRC) was approved and the reference number for the protocol is UCB/CIE/NC/013/2016.

We remind you that the committee makes its decisions on the following features:

1. The pertinence of the protocol
2. Respect of human beings (informed consent, harmfulness, risk-benefit ratio…)
3. The methodology

Many thanks for your collaboration.

Kind regards

Date and place of Issue: The 4th of June 2016, Bukavu
Stamp: Catholic University of Bukavu.
Signature: Professor Bagendabanga Macece, secretary of the Institutional Ethics committee of the Catholic University of Bukavu.

APPENDIX 4: RSV study consent form

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:

Prevalence and risk factors of respiratory infection by Respiratory Syncytial Virus in children at Provincial General Hospital of Bukavu

REFERENCE NUMBER: S16/03/051

PRINCIPAL INVESTIGATOR: Dr Landry Kabego C.

ADDRESS: Division of Medical Virology, Faculty of Medicine & Health Sciences, Tygerberg Campus

CONTACT NUMBER: +243992048827, +27626386130, 021 938 9354

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Ethics Committee of the Catholic University of Bukavu and** the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

- *This study will be conducted at the Provincial General Hospital of Bukavu, Democratic Republic of Congo. Between 1st June 2016 and 31st May, all children under 5 years of age with signs of respiratory infections will be approached to take place to this study. We expect to enrol approximately 152 children in this study.*
- *Respiratory infections are very frequent in childhood, and the number of deaths is very high. Viruses are a major cause of respiratory infections in children. Here in Bukavu, little baseline information is available on acute respiratory infection. The aim of this study is to identify the role of viruses in acute respiratory infection in children and therefor to manage them correctly. Moreover, this study will allow us to set a system of infection control and any outbreak that may arrived will be managed to avoid infection propagation.*
- *When your children will be approached, a medical doctor will do a complete examination. Routine samples will be taken and, if you accept that your child participates, a Perinasal Flocked swab will be done on him/her. The process is very simple. The physician will introduce gently a swab in your child's nose and will rotate it five times and pull it out. It will take no longer than 10 seconds. It can be uncomfortable procedure and can cause slight pain however.*
- *No medication will be used.*

Why have you been invited to participate?

- Your child has been invited to participate because he or she has a respiratory infection which may be caused by viruses.

What will your responsibilities be?

- *You will be expected to answer questions about your child's history*

Will you benefit from taking part in this research?

- *You will not benefit directly from this study. However, the results obtained may considerably improve children's health in this region.*

Are there in risks involved in your taking part in this research?

If perinasal flocked swab is performed improperly it can cause minor trauma. However, our medical staffs are well trained and therefore we don't expect any injury to occur. But if any form of injury occurs, the medical staff will look after your child.

If you do not agree to take part, what alternatives do you have?

Your participation is completely voluntary. If you do not want to participate, it will not affect your child's care in any way. You are also free to withdraw from the project at any point, even if you did agree to take part.

Who will have access to your medical records?

The information collected will be confidential. Your name will not appear in the data base and will be replaced by a code, so you will remain anonymous. Only the research staff will have access to the information.

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

If any injury occurs, your child will be looked after by the medical staff.

Will you be paid to take part in this study and are there any costs involved?

You will not be paid to take part to this study. You won't have to pay for the tests being done for this study.

Is there any thing else that you should know or do?

- *You can contact Dr Landry Kabego at tel +24399204882 / +27626386130 if you have any further queries or encounter any problems.*

- *You can contact the Ethics Committee of the Catholic University of Bukavu at +243 813181718 and the Health Research Ethics Committee at +27 21-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.*

- *You will receive a copy of this information and consent form for your own records.*

Declaration by participant

By signing below, I ……..…… agree to take part in a research study entitled *(insert title of study)*.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) .....…..... on (*date*) ……...…….. 201...

....

Signature of participant **Signature of witness**

Declaration by investigator

I *(name)* ……..……. declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .....…..... on (*date*) ……...…….. 201...

....

Signature of investigator **Signature of witness**

Declaration by interpreter

I *(name)* ……..……. declare that:

- I assisted the investigator (*name*) ……..……. to explain the information in this document to (*name of participant*) ……..…….. using the language medium of French/Swahili.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .....…..…… on (*date*) ……..

....

Signature of interpreter **Signature of witness**

APPENDIX 5: RSV study questionnaire

QUESTIONNAIRE

Prevalence and risk factors of respiratory infection by Respiratory Syncytial Virus in children at Provincial General Hospital of Bukavu

ID :

Age: months

Gender: Male Female

Weight: gr **Height**: cm

Malnutrition: Acute ☐ ☐ Chronic

Birthweight: gr **Gestational age**: weeks

Neonatology stay: Yes ☐ ☐ No

Oxygen treatment in neonat.: Yes ☐ ☐ No

Socio-economic status: ☐ Poor ☐ Middle ☐ Rich

Mother education: Illiterate ☐ Primary ☐ Secondary ☐ University ☐

Antibiotics before visit: Yes ☐ No ☐

Symptoms duration (hours):

Immunization up to date : Yes No

Breastfeeding (months):

Siblings:

Smoke exposure: Cigarette Cooking smoke

Alcohol exposure during pregnancy: Yes ☐ No ☐

Congenital heart diseases:

Chronic lung diseases:

Familial history of atopy: ☐ Asthma ☐ Urticaria ☐ Others

URTI: Rhinitis ☐ Otitis ☐ Pharyngitis ☐ Laryngitis ☐

LRTI: Pneumonia ☐ Bronchiolitis ☐

Antibiotic treatment: Yes ☐ No ☐

Oxygen treatment: Yes ☐ No ☐

Nasograstric intubation: Yes ☐ No ☐

APPENDIX 6: South Africa import permit

health

Department:
Health
REPUBLIC OF SOUTH AFRICA

Private Bag X828, PRETORIA, 0001. 27th Floor, Room 2710, Civitas, Cnr Thabo Sehume & Struben Street, PRETORIA, 0001
Tel: +27 (0) 12 395 8000, Fax: +27 (0) 12 395 8422

(012) 395 8366/8965 Ms Lineo Motopi

importexportpermit@health.gov.za J1/2/4/1 No 1/16

IMPORT PERMIT

In terms of Section 68 of the National Health Act 2003 (Act No. 61 of 2003) –

Landry Kabego Cihambanya
MSc Student
Medical Virology
Stellenbosch University
CAPE TOWN
8000
Tel. No.: 021 938 9453 and 021 93 89 354 Fax. No.: 021 9389361

is hereby authorised to import into the Republic of South Africa –

300 Nasopharyngeal swab

from –

Joe Bwija Kasengi
Catholic University of Bukavu
P O Box 285
Bukavu
DR OF CONGO
Tel: (00243) 817520258 Fax:

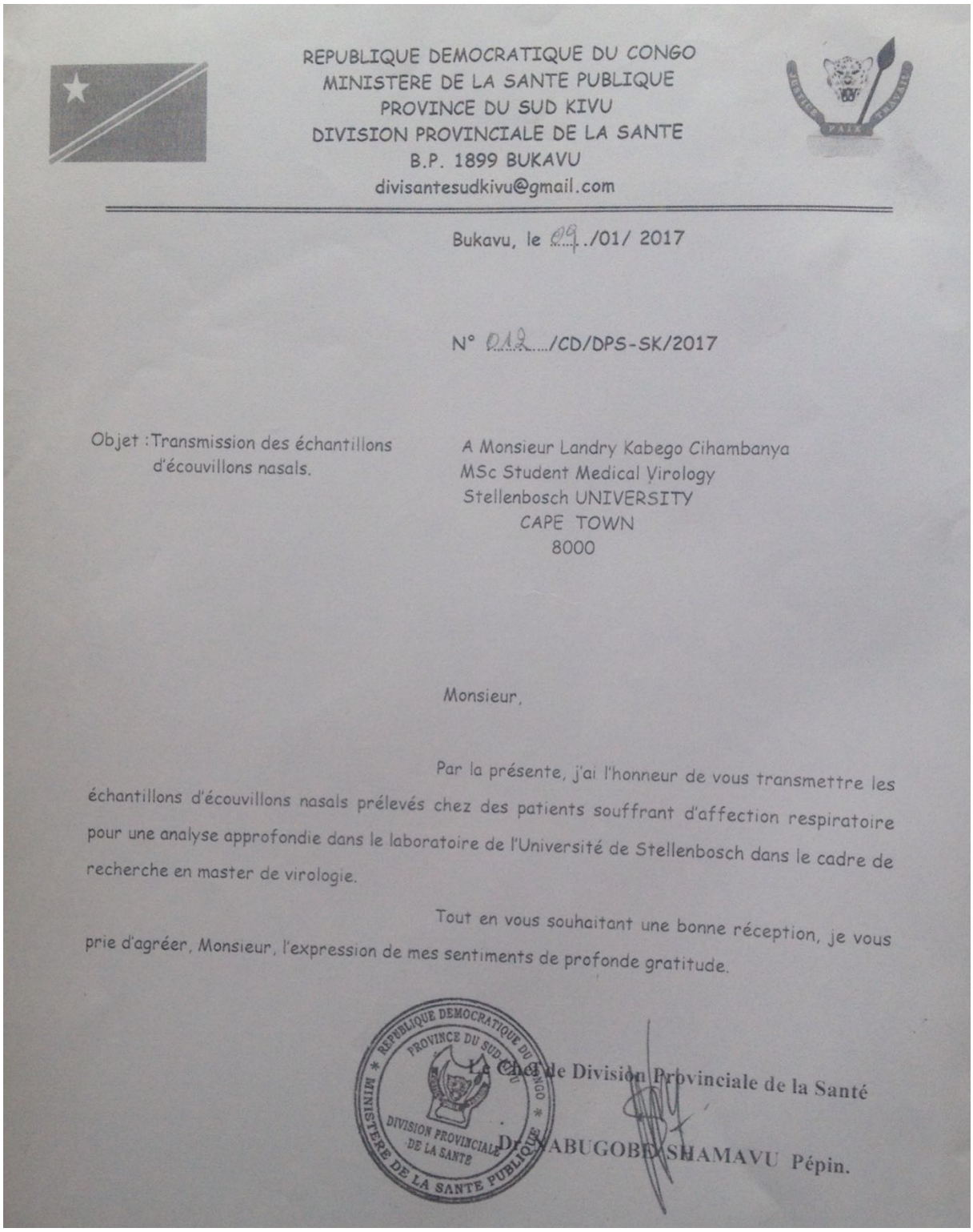
For – Research

This import permit is subject to the following conditions:

1. The material shall be exported from the country specified above, within the legal requirements of that country.
2. The material shall be imported into South Africa and handled in accordance with the provisions of the National Health Act 2003 (Act No. 61 of 2003), and the regulations made in terms of the Act.
3. The import permit shall not be used for any trade or advertising purposes.
4. **This import permit shall expire on 31 July 2017.**

DIRECTOR-GENERAL: HEALTH
Date: 28/07/2016
Ms P Netshidzivhani

APPENDIX 7: DRC export permit



REPUBLIQUE DEMOCRATIQUE DU CONGO
MINISTERE DE LA SANTE PUBLIQUE
PROVINCE DU SUD KIVU
DIVISION PROVINCIALE DE LA SANTE
B.P. 1899 BUKAVU
divisantesudkivu@gmail.com

Bukavu, le 09./01/ 2017

N° 012 /CD/DPS-SK/2017

Objet : Transmission des échantillons d'écouvillons nasals.

A Monsieur Landry Kabego Cihambanya
MSc Student Medical Virology
Stellenbosch UNIVERSITY
CAPE TOWN
8000

Monsieur,

Par la présente, j'ai l'honneur de vous transmettre les échantillons d'écouvillons nasals prélevés chez des patients souffrant d'affection respiratoire pour une analyse approfondie dans le laboratoire de l'Université de Stellenbosch dans le cadre de recherche en master de virologie.

Tout en vous souhaitant une bonne réception, je vous prie d'agréer, Monsieur, l'expression de mes sentiments de profonde gratitude.

Le Chef de Division Provinciale de la Santé

Dr ABUGOBO SHAMAVU Pépin.