CHAPTER ONE

INTRODUCTION

Lower Respiratory Infections (LRIs) ranked topmost among the cause of mortality due to infectious diseases globally. Only malaria associated deaths exceeds that caused by LRIs among children under 5 years in Nigeria (Global Burden of Diseases and Injuries in Children and Adolescents, 2017). Human respiratory syncytial virus (HRSV) has been identified as the most paramount viral pathogen of LRIs, with annual outbreaks in infants, young children and vulnerable adults worldwide (Falsey *et al.,* 2005; Nair *et al.,* 2010). Nearly two of every three infants are infected with HRSV within their first twelve months in live, and about 90% would have been infected at least once within their first twenty-four months in life (Collins and Graham, 2008). Infection with HRSV does not confer a lasting immune response, hence re-infection, even with the same strain of the virus is possible within few months after the first infection (Agoti *et al.,* 2015).

HRSV is one of the few childhood diseases without a licensed vaccine, however, there are ongoing efforts towards the development of an effective vaccine. Till date, various experimental vaccine candidates have been developed based on the prototype strains identified a long time ago (van Niekerk and Venter 2011). Previous research brought to fore the high burden of HRSV in Nigeria, with prevalence ranging from 8.8% to 54% (Nwankwo *et al.,* 1988; Olaleye *et al.,* 1992; Odaibo *et al.,* 2013; Faneye *et al.,* 2014) however, the subtype(s) and genotype(s) of the virus circulating in Nigeria is not known.

There are two subtypes of HRSV, each with multiple genotypes. Genotypic characterization of HRSV is based on the genetic diversity in G gene (Anderson *et al.,* 1985; Parveen *et al.,* 2006). The G and F genes are the only genes that elicit neutralizing antibodies, hence they are the target for vaccine development (Collins and Graham 2008). Most efforts for vaccine development are concentrated on the F gene because it is conserved among HRSV strains, hence antibodies produced against the fusion protein confer broad spectrum protection against the different subtypes and strains of the virus (Singh *et al.,* 2007).

Palivizumab (PZ), a humanized, murine monoclonal antibody, is the only licensed immunoprophylaxis used to reduce the severity of HRSV-induced respiratory disease among high-risk infants (Huang *et al.,* 2010). In places where PZ is in use, this passive immunization targeted against the F protein has recorded a considerable success (Zhao *et al.,* 2004; Cane, 2011). However, it has been shown in recent studies that strains of the virus that are monoclonal-antibody-resistant are emerging (Adams *et al.,* 2010, Zhu *et al.,* 2011). Although Palivizumab is not yet in use in Nigeria and most developing countries (mainly due to cost), there is the likelihood of its introduction for use in the country, especially as data on the burden of HRSV becomes available. There is therefore the need to determine if the mutation(s) that are suggestive of Palivizumab resistance already exist among the HRSV isolates circulating in Ibadan, Nigeria. This information is vital to inform policy on PZ use in Nigeria.

Information on the diversity of the virus, particularly the G and F genes in regions with high burden of HRSV infection is important in the molecular epidemiology, antiviral therapy and vaccine research hence the need to characterize the circulating strains of the virus in Nigeria.

1.1 **JUSTIFICATION**

Although high burden of HRSV infection has been reported in Nigeria, there is paucity of information on the genetic diversity of the virus circulating in the country. Since studies have shown that the circulating strains of HRSV vary from one community to another (Peret *et al.,* 1998), it is necessary to identify and characterize the circulating strains of the virus in Nigeria.

Currently, there is no approved vaccine or effective treatment option available against HRSV. Palivizumab (PZ), a humanized murine monoclonal antibody (mAb) currently in use for prophylaxis against HRSV (among infants and children with high risk of infection) binds to the F protein at site within the antigenic site II of the protein (Wang *et al*., 2011). However, some studies have shown that some HRSV strains have developed resistant mutations against PZ in the F gene binding site (Zhao *et al.,* 2004; Adams *et al.,* 2010; Xia *et al.,* 2013). It is therefore very important to determine if strains of the virus with the resistance mutation are circulating in Nigeria. This

information is very vital before the introduction and use of the monoclonal antibody in the country.

1.2 THE AIM OF THE STUDY

The aim of this study was to detect and characterize HRSV strains circulating among children aged 0 – 5 years presenting with symptoms of respiratory tract infection in Ibadan, Nigeria.

1.3 SPECIFIC OBJECTIVES

- To detect HRSV in children in Ibadan, Nigeria using the Matrix gene.
- To determine the subtype(s) and genotype(s) circulating among children in Ibadan.
- To detect the presence of Palivizumab-resistant mutants in the F gene of HRSV isolates from Ibadan, Nigeria.

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical background

The oldest known record of human RSV infection is traceable to the year 1931; when a one-month old child suffered from an atypical pneumonia with symptoms of cough and wheezing without bacterial co-infections (Johnson *et al.*, 2007). However, the agent responsible for the 1931 infection remained unraveled until 1955 when it was first isolated as the agent responsible for upper respiratory tract infection in an adolescent laboratory chimpanzee housed at the Walter Reed Army Institute of Research, in Washington D.C (Blount *et al*., 1956). The virus was also associated with symptoms of common cold in their human caretakers during the outbreak.

The virus, initially named 'Chimpanzee Coryza Agent' CCA, due to the manifestation of coryza in infected chimpanzee, was isolated a year later from two children, one with bronchopneumonia and the other with bronchiolitis (Chanock *et al*., 1957). In the year 1957, the name of the virus was changed to 'respiratory syncytial virus' (RSV) due to its predilection for cells of the respiratory tract and its characteristic cytopathic effect (CPE) in cell culture. HRSV formed multinucleated giant cells (syncytia) in cell culture, including liver epithelial and KB (derived from human carcinoma of the nasopharynx) cell cultures. Although there exist versions of RSV in animals, including the murine pneumonia virus (MPV) and bovine respiratory syncytial virus (BRSV), no animal reservoir of HRSV is known till date (Collins and Graham, 2008).

Following the demonstration with hyperimmune serum that HRSV isolates exhibit variations (Coates *et al*., 1966), the virus was subsequently subdivided into two antigenic groups (A and B) using panels of monoclonal antibodies (Anderson *et al.,* 1985; Mufson *et al.,* 1985). These subgroups have been shown to correlate with viruses that are genetically different (Cristina *et al.,* 1990).

2.2 Classification

The human respiratory syncytial virus is grouped into the Mononegavirales, a viral order that was established in the year 1991 (Pringle*,* 1991). The order consists of

viruses with similar features, including: linear, non-segmented, single-stranded, negative-sense RNA genomes (Pringle, 1991). Initially, members of this order were assigned into three families (*Filoviridae*, *Paramyxoviridae*, and *Rhabdoviridae*) (Pringle, 1991) with subsequent addition of two other families (*Bornaviridae* and *Nyamiviridae*) (Pringle 1996; Kuhn *et al.,* 2013; Adams *et al.,* 2014). However, as at 2017, the total number of families in the order had risen to eight, following the addition of the families *Myonaviridae*, *Sunviridae* and *Pneumoviridae* (Amarasinghe *et al*., 2017). Viruses belonging to this order share certain structural and functional features, including the underlisted: (Melero, 2007)

i. A bilayer of lipids in which the viral glycoproteins are inserted envelopes the viral particles.
ii. Their genomes are tightly-associated with the nucleoprotein (N), which results into the formation of a nucleocapsids that is RNase-resistant, and serve as templates for all RNA synthesis.
iii. Transcription proceeds from the 3'-end of the viral RNA in a sequential and polar order which entails termination and re-initiation at each of the junctions of the genes.
iv. Replication of the viral genome is done through the synthesis of an antigenome that is complementary to the mother strand.
v. The entry of viral nucleocapsids into the cells of the host occur by membrane fusion.

HRSV was previously classified as a member of the subfamily Pneumovirinae in the Paramyxoviridae family. There were two subfamilies (Paramyxovirinae and Pneumovirinae) in the family Paramyxoviridae. Members of the Paramyxovirinae included the human and animal parainluenza viruses (PIVs), mumps virus, and measles virus among others. The Pneumovirinae subfamily consisted of two genera: genus Pneumovirus consisting of HRSV, bovine RSV (BRSV), and pneumonia virus of mice (PVM), while the genus Metapneumovirus consists of the human metapneumovirus (HMPV) and the avian metapneumovirus (AMPV) (Collins and Karron, 2013).

In 2016, the International Committee on Taxonomy of Viruses (ICTV) elevated pneumovirinae subfamily into a full family, and also created the Mymonaviridae and

Sunviridae as two new families in the order Mononegavirales (Amarasinghe *et al.,* 2017). The elevation of the Pneumovirinae to a full family status was due to the close relatedness of members of the taxon to both the filoviruses and the paramyxoviral subfamily (Amarasinghe *et al.,* 2017).

2.3 The virion

HRSV virions are pleomorphic. Two types of viral particles: (i) spherical particles – with size ranging between 150 and 250 nm in diameter and (ii) filamentous particles up to 10μm in length, have been identified (Collins and Karron, 2013). Both particles are infectious, and are often found in cell culture, with the filamentous particles usually being the predominant (Collins and Karron, 2013). Both forms of the virion mostly remain cell associated. HRSV is very fragile and highly unstable, hence, its infectivity could easily and readily be lost during handling and freeze-thawing (Ausar *et al.,* 2007). When irradiated with ultraviolet (UV) light, infectivity is lost with single-hit kinetics (Dickens *et al.,* 1990). There are indirect evidences suggesting that the long filamentous shape of the particle, as well as the surface glycoproteins, especially F, are some likely factors that confer fragility (Rawling *et al.,* 2011). The fragility can be overcome partly by agents such as sugars (like sucrose) that reduce aggregation and improve thermal stability (Ausar *et al.,* 2007).

2.4 Structure of HRSV

The single negative strand of non-segmented RNA of RSV consists of 10 genes, encoding 11 proteins. RSV proteins can be clustered according to their function and location in the virus particle, except two nonstructural proteins (NS1 and NS2), that are not present in the virus particle. These two NS proteins are expressed early after infection and limit the anti-viral responses in infected cells. The HRSV virion comprise of a nucleocapsid packaged in a bilayer of lipid envelope (Figure 2.1) that originates from the host cell plasma membrane during the process of budding (Figure 2.2). There are three virally encoded surface glycoproteins spikes on the envelope. These include: the fusion protein (F), the attachment protein (G) and the small hydrophobic (SH) protein (Collins and Karron 2013).

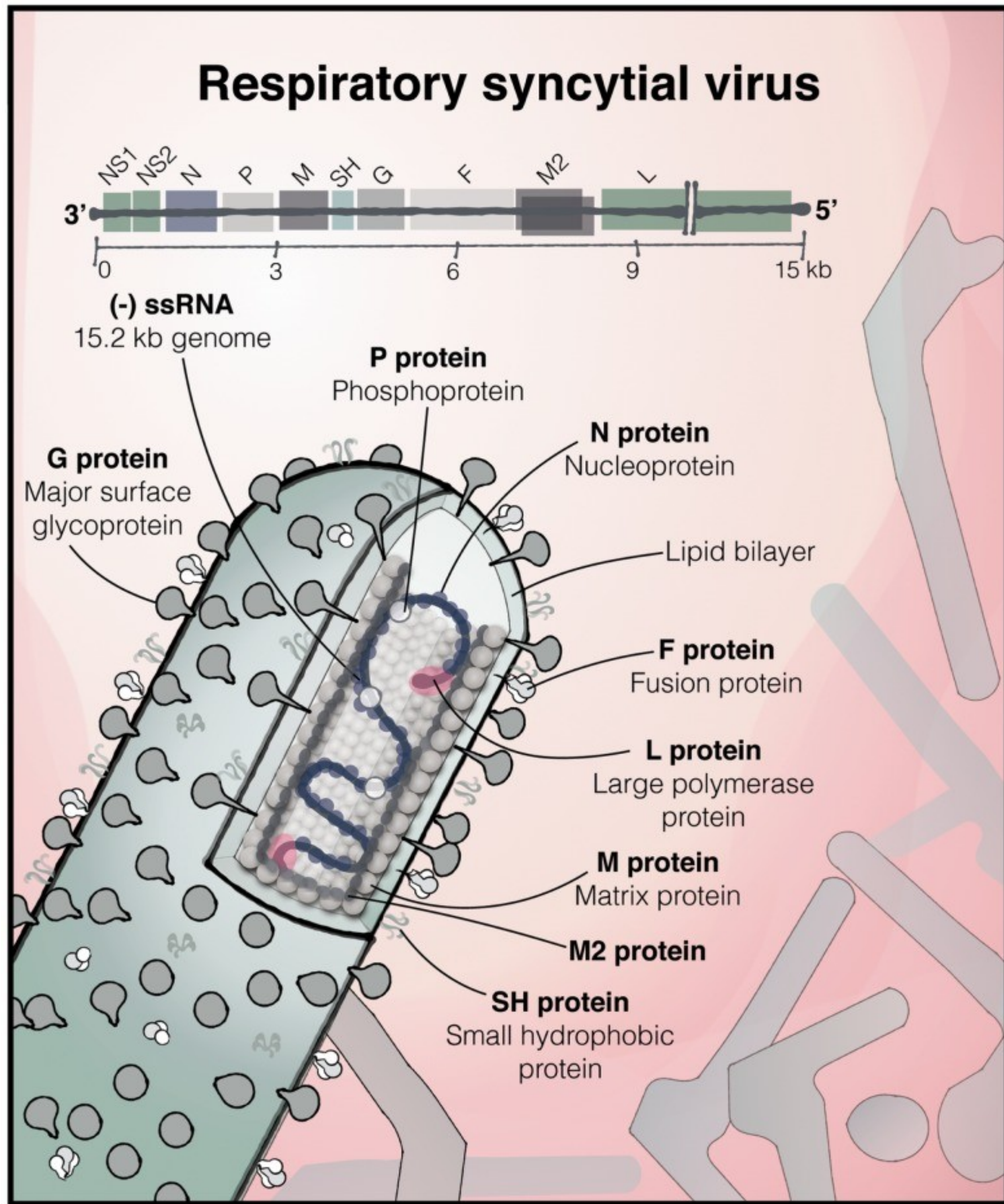


Plate 2.1. A schematic diagram of Human Respiratory Syncytial Virus.

(Source: Lambert *et al.,* 2014)

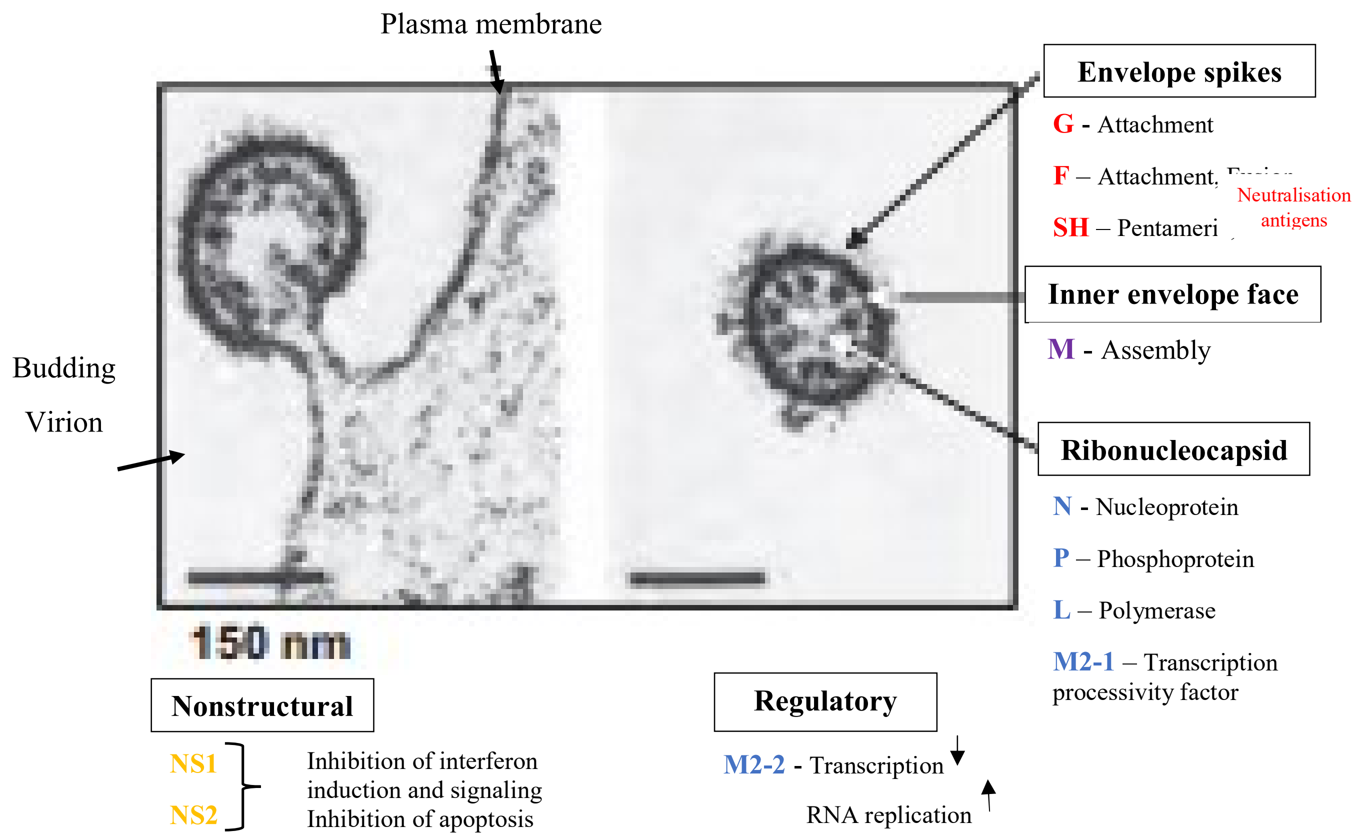


Plate 2.2 - Electron micrographs showing an RSV virion budding through the plasma membrane of an infected cell (left) and a free virion (right). The protein functions are indicated.

Source: Collins and Graham, 2008

Both the neuraminidase and hemagglutinin spikes found in other members of the paramyxoviridae family are lacking in RSV, and the F glycoprotein in RSV is known to be heavily sialylated possibly to compensate for the neuraminidase that is lacking (Collins *et al.,* 2013).

The nucleocapsid is found within the envelope and posterior to the M-protein. The nucleocapsid consist of the N-protein (a major nucleocapsid which binds to genomic RNA), a phosphoprotein (P), a transcription anti-terminator factor or transcriptase processivity factor (M2-1), and an RNA-dependent RNA polymerase called the large polymerase subunit (L) (Gottschalk, *et al.,* 1996).

The structure and properties of HRSV (McNamara and Smyth, 2002) is given below in summary:

1. The N protein encapsidates the viral genome tightly. The minimum unit for RNA replication comprises of the N protein, the phosphoprotein (P) as well as the large polymerase (L) subunit.
2. Replication of RNA involves the synthesis of a replicative intermediate called the antigenome. The antigenome is a positive-sense, exact-copy of the mother strand which is encapsidated and serves as the template for progeny genome.
3. The M2 open reading frame 1 (ORF-1) protein and the nucleocapsid-associated proteins are required for transcription. These proteins also ensure that messenger RNA of full length is synthesized.
4. The ORF-2 proteins are also encoded by the M2 gene. The negative regulatory effect of ORF-2 may render nucleocapsids quiescent before being incorporated into virions.
5. The virus has two non-structural proteins namely: NS1 and NS2. The functions of these proteins are not known.
6. A matrix protein (M), is encoded by the virus. During morphogenesis, the M protein mediates interaction of the envelope and nucleocapsid of the virion.

2.4.1 The attachment (G) glycoprotein

The G glycoprotein is responsible (although not solely) for the attachment of HRSV to cells (McNamara and Smyth, 2002). It is a type II glycoprotein ranging in length between 288 and 299 amino acids and consists of two highly variable regions in the extracellular domain, often referred to as the hypervariable regions (Peret, *et al.,* 1998; Reiche and Schweiger, 2009) (Figure 2.3). The mature 90-kDa form of the G protein is obtained via extensive addition of N-linked sugars to Aspartic acid residues and O-linked sugars to Serine and Threonine residues of the 32-kDa polypeptide precursor (Zlateva, *et al.,* 2005; Parveen, *et al.,*2006). However, the positions of the potential N- and O-linked glycosylation sites are poorly conserved.

The attachment G protein has three domains: the intracellular domain, transmembrane domain, and a large ectodomain. The ectodomain contains two hypervariable regions (HVR1 and HVR2) separated by a conserved 13 amino acid motif (Peret, *et al.,* 1998; McNamara and Smyth, 2002). The HVR1 is located in the amino (N)-terminal of the protein preceding the conserved region, while the HVR2 is located in the carboxy (C)-terminal end. The highest variability of the virus occurs in the ectodomain (Peret *et al.,* 1998; Peret *et al.,* 2000; Venter *et al.,* 2001; Zlateva *et al.,* 2004; Shobugawa *et al.,* 2009; Dapat *et al.,* 2010; Cane, 2011) which has only about 44% amino acid similarity between the two groups, unlike the percentage similarity of about 83% found in the other two domains (Cane and Pringle, 1991; Cane and Pringle, 1995). In general, variability in the G protein is greater than in other proteins, both between and within the major antigenic groups of RSV (Cane *et al*., 1991; Cane and Pringle, 1995; Cane, 1997; Sullender 2000; Zlateva *et al.,* 2005; Dapat *et al.,* 2010; Cane, 2011).

Amino acid variations of the protein exist in both RSV A and B groups, with variations subgroup A more pronounced. The inter-subtype variation in the G protein could be up to 47%, while the intra-subtype variation may not exceed 20% (McNamara and Smyth, 2002).

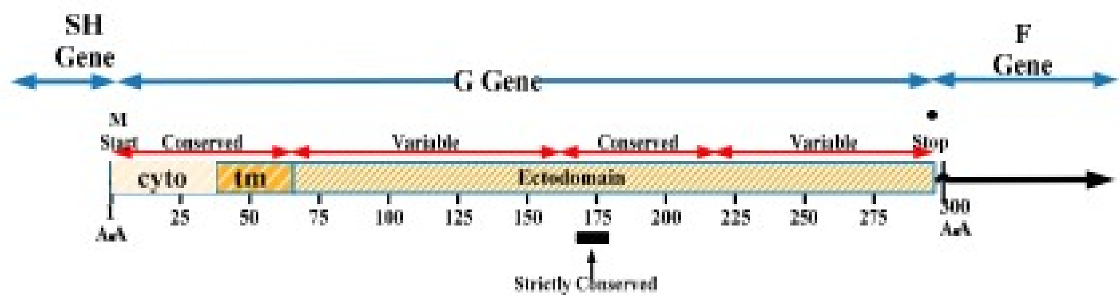


Figure 2.1: The map of the G Gene

The HVR2 has been mostly used in molecular and epidemiological studies to understand the genetic diversity of the virus (Peret, *et al.,* 1998; Peret, *et al.,* 2000, Dapat, *et al.,* 2010; Agoti *et al*., 2015).

The G protein elicits neutralizing antibodies, however, it does not (unlike the F protein) stimulate an appreciably significant cytotoxic T-lymphocyte responses (Sullender, 2000). It has been shown by Sullender *et al*. (1991) that the G protein differs in size and conformation from proteins (hemagglutinin or neuraminidase) used by other paramyxoviruses for similar functions as the G protein.

2.4.2 The fusion (F) protein

In its native form (F0), the RSV fusion protein comprises of five hundred and seventy-four amino acids and like other viral fusion proteins, it has a trimeric coiled structure (McNamara and Smyth, 2002). The F protein is a type I transmembrane glycoprotein having a transmembrane anchor near the C terminus and a cleaved N-terminal signal sequence. Activation of the F protein occurs when the F0 is cleaved into two subunits (F1and F2) with a disulphide-bond linkage (Sullender, 2000). The F protein facilitates the fusion of viral envelope and the host plasma membranes thereby allowing the movement of genetic material from the virus into the infected cell (Domachowske and Rosenberg, 1999). The process of fusion of the RSV and the host cell is depicted in figure 2.4. There are numerous F proteins (as well as other glycoproteins) rooted into the viral envelope by the transmembrane anchor. Following cleavage and conformational changes of the F0, the fusion peptide is inserted into the host cell membrane thereby bringing the viral and the host cell close (Griffiths *et al*., 2017).

The F protein is also involved in the fusion of infected cell membrane to the adjacent/neighbouring cell membranes causing the formation multinucleated cells called syncytia (Domachowske and Rosenberg, 1999). The syncytia formation is the characteristic cytopathic effect of RSV in tissue culture, and it is very vital for transmission of virus from infected cell to other cells.

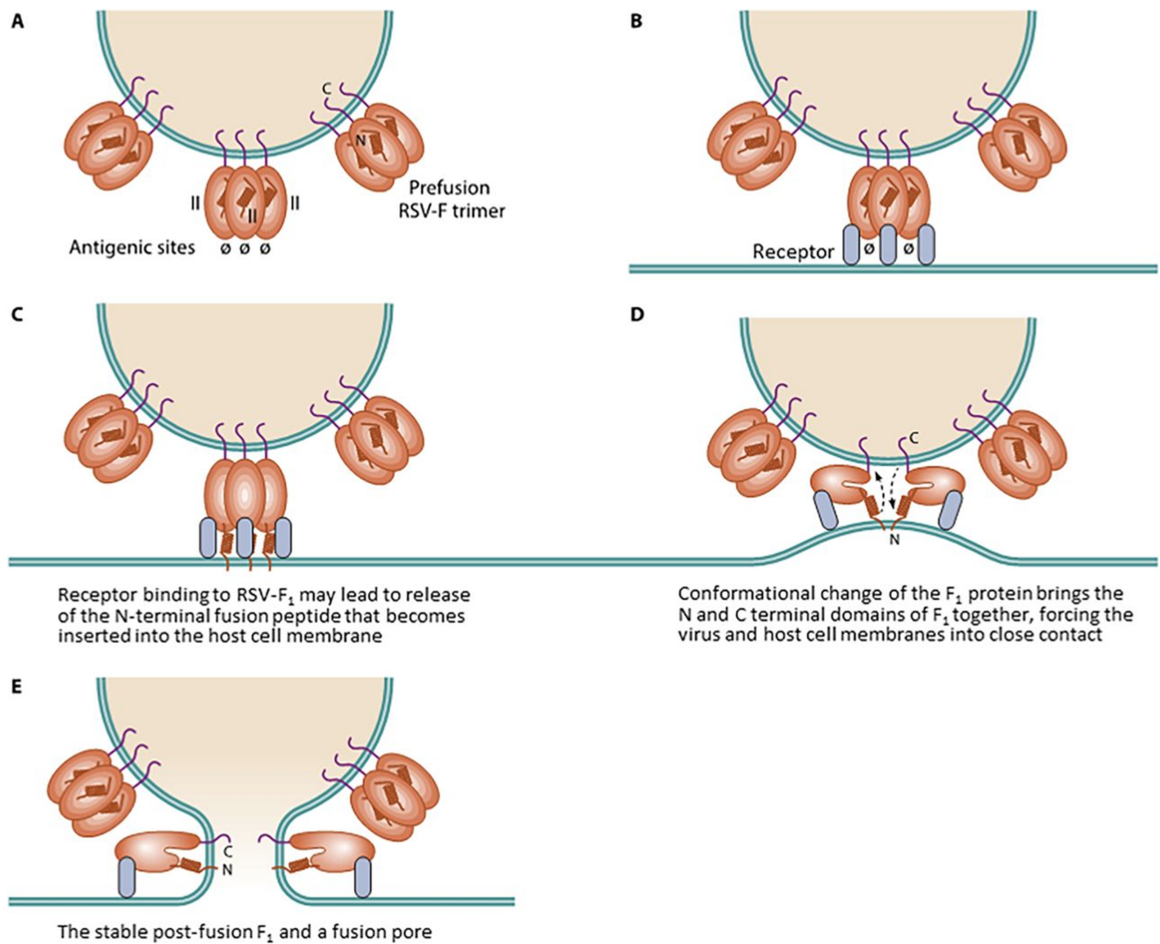


Plate 2.3. Process of fusion of RSV to host cell membrane.

Soure: Griffiths *et al*., 2017

2.4.3 The small hydrophobic (SH) Protein

The SH protein is the third of the surface proteins found on the envelope of RSV. This transmembrane protein is comprised of 64 amino acids. It is neither needed for viral replication nor required in the formation of syncytium (Domachowske and Rosenberg, 1999). In actual fact its precise role is not known (McNamara and Smyth, 2002).

2.4.4 The nucleocapsid proteins (N, P, L)

The nucleocapsid protein of HRSV is a major structural protein in the virus. This protein contains 391 amino acid residues and is about 93% identical to the N protein of bovine RSV (Stokes *et al*., 2003). The N gene is highly conserved, having about 96% amino acid sequence similarities between the two subtypes of HRSV (Collins and Karron, 2013) and only six nucleotides that vary substantially between the two groups (Hu *et al*., 2003). The N protein encapsidates the viral RNA genome, and together with both the P and L proteins are essential for genome replication (Stokes *et al.,* 2003). Together with the M2-1, the N protein is required for efficient genome transcription (Grosfeld *et al.,* 1995). Inside an RSV-infected cell, the N, P and M2-1 linked together in forming cytoplasmic inclusions (Lambert *et al.,* 2014). In the interaction of the N and P proteins of RSV, the C terminal of the P protein has been shown to be the most vital site for binding. The regions of the N protein involved in the binding are the amino acid positions 46–65, 241–260 and 305–335 or 334–352 (Stokes *et al*., 2003).

2.4.5 The matrix proteins M and M2

Like most other RNA viruses with non-segmented, negative-stranded genome, the pneumoviruses have matrix protein. However, they have two of such proteins M and M2 (Huang *et al.,* 1985). The former is thought to correspond to the M protein of the other viruses. However, with a length of about 256 amino acids, it is smaller in size than other members that were in the previously existing family of paramyxoviruses and lacks the unambiguous sequence relatedness with them (Huang *et al.,* 1985). In contrast, the M2 protein (194aa) does not have an apparent counterpart in the paramyxoviruses and other non-segmented viruses, with the exception of the filoviruses (Huang *et al.,* 1985).

Like the typical M proteins, the RSV M and M2 proteins are non-glycosylated and lack sufficient transmembrane hydrophobic sequences. They remain associated with the virion even when the envelope is stripped off by treatment with non-ionic detergent in low salt but are solubilsed if additional salt is added.

2.5 HRSV subgroups and genotypes

Human respiratory syncytial virus is classified into two subgroups namely A and B on the basis of antigenic and genetic variability (Peret *et al.,* 1998; Reiche and Schweiger, 2009; Shobugawa *et al.,* 2009; Dapat, *et al.,* 2010; Zhang *et al.,* 2010) when they react with monoclonal antibody targeting the attachment glycoprotein G (Cane and Pringle, 1995; Hall, 2001; Dapat *et al.,* 2010). Within each group, additional variability is also detected (Zhang *et al.,* 2010) leading to further subdivision into genotypes. The subtype A appeared to show more genetic variability (up to 14%) as suggested by Venter *et al.* (2001) and Parveen *et al*. (2006). There are currently 14 genotypes of subgroup A which include: NA1 to NA4, GA1 to GA7, CB-A, SAA1 and the ON1 (Ren *et al.,* 2014). The subgroup B is categorized into: SAB1 to SAB4, GB1 to GB4, URU1 to URU2, CB-B, CB-1, and the BA genotypes. The BA genotype has been further subdivided into: BA1 to BA12, BA-CCA, BA-CCB and BA-C (Zheng *et al.,* 2017).

The BA (Buenos Aires) genotype of HRSV subgroup B has a region of 60-nucleotide insertion in the 2nd hypervariable region of the G protein and was firstly reported in the Buenos Aires, Argentina in 1999. Since the time of the first report of the BA genotype, it has been the predominant HRSV B in other areas in the world (Trento, *et al.,* 2003; Zlateva, *et al.,* 2005; Dapat *et al.,* 2010). Like the BA genotype of the subtype B, the ON1 genotype of the subgroup A has a characteristic 72nt insertion at the HVR2 of the G glycoprotein. This genotype was name ON1, to depict Ontario, in Canada where it was first reported in the year 2012 (Eshaghi *et al.,* 2012).

Both subgroups A and B strains circulate at the same time but subgroup A is often reported to predominate (Hall, 2001; Scott *et al*., 2004; Zhang *et al*., 2007; Tran *et al*., 2013). According to a report by Oliveira *et al.* (2008), strains of HRSV belonging to the B subgroup tend to grow slower in tissue culture with the production of fewer virus particles and a loss of syncytial formation when compared with the strains of subgroup A.

2.6 RSV Infection

2.6.1 Infection in Children

About fifty to seventy percent of infants will experience primary HRSV infection within the first twelve months of their lives and almost all children would have been infected not less than once by time they are two years of age (Renato *et* al., 1999; Shobugawa *et al.,* 2009). Most researches on detection of HRSV focus on children under the age of five due to their not fully matured immunity, the bulk of the burden of infection is however found in younger age groups especially in children aged 6 months and below (Etemadi *et al*., 2013; Nolan *et al*., 2015). The clinical presentation in HRSV infection may vary from mild upper respiratory tract (URT) illness to severe and debilitating lower respiratory tract involvement including bronchiolitis (Figure 2.5) and pneumonia. In children, HRSV infection usually begins as URT infection which may then progress to the lower respiratory tract with clinical presentations including stuffy nose (rhinitis), cough and coryza (runny nose). Children with birth-related risk factors like prematurity and chronic lung or heart diseases together with children with weakened immune systems have the greatest risk of HRSV infection (Aujard and Fauroux, 2002; Resch, 2014). According to the report of a study conducted by Levin *et al.* (2010), there is higher than expected incidence of bacterial pneumonia that complicates HRSV infections in infants with respiratory failures.

2.6.2 Infection in Adults and Elderly

The HRSV is now seen as an important pathogen in adults as well (Peret *et al.,* 2000; Sullender, 2000; Hall, 2001). It causes LRTI in immunocompromised individuals and the elderly (Falsey, 2007; Sullender, 2000; Zhang *et al.,* 2010). In fact, it is now known to be an important cause of morbidity and mortality in the elderly, especially in adults greater than 65 years of age and those with impaired immunity and underlying illnesses including congenital heart disease (Falsey *et al*., 2005).

2.6.3 Reinfection

HRSV reinfection is a very common occurrence throughout the life of young children with ARTI (Zhang, *et al.,* 2010). In addition, HRSV reinfection is common throughout life in all age groups (Peret, *et al.,* 2000; Ohuma *et al*., 2012). Since HRSV does not have the antigenic diversity similar to what is found in RNA viruses

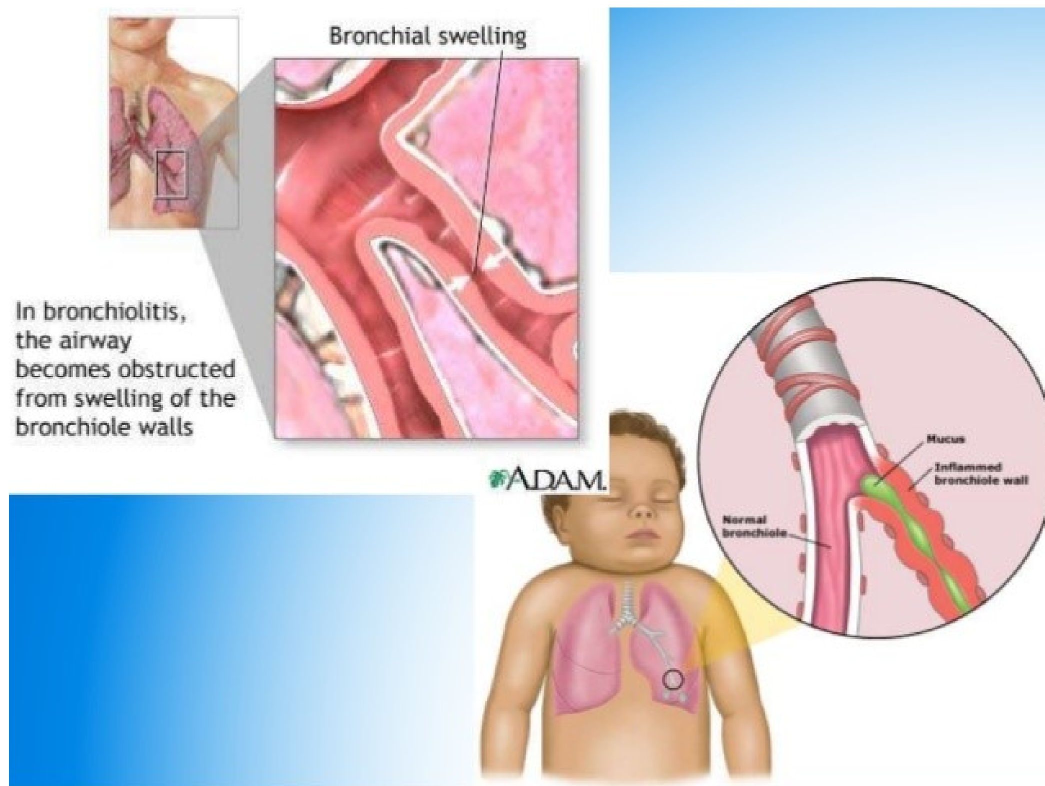


Plate 2.4. A schematic image of bronchiolitis.

(Ahmad, 2017)

with segmented genome like influenza viruses, the repeated reinfection therefore shows that immunity to reinfection is either not present at all or it is quick waning (Zlateva, *et al.,* 2005; Ohuma *et al*., 2012).

In a study conducted in North Carolina, Henderson *et al.* (1979) reported extremely important results on the rate of HRSV infection and reinfection in children. His study was also important in giving a better understanding of the acquired immunity to RSV infections. It was shown that 98% of children in day care facilities became infected with HRSV during their first year of life, 74% and 65% of whom were re-infected in their second and their third year respectively. It has been shown that reinfection with HRSV often result in less severe outcome compared to the primary infection (Collins and Karron, 2013).

2.7 Pathogenesis of HRSV

Humans are the only natural host of HRSV, although similar viruses in the *Pneumoviridae* family namely murine pneumonia virus and bovine pneumonia virus are known to infect mice and cattle respectively (Collins and Karron, 2013). The route of entry of HRSV into the body is usually the eye or nose and seldomly the mouth. The virus thereafter spreads through the epithelial cells from the upper respiratory tract to the lower respiratory tract, majorly by the characteristics cell to cell transfer (McNamara and Smyth, 2002). The incubation period of RSV is two to eight days (Hall, 2001). RSV infection is initiated as a mild upper respiratory tract infection. It takes about one to three days for the viral particles to spread to the lower respiratory tract where it may produce bronchiolitis and /or pneumonia. Signs of lower respiratory infection occurs about three days after the onset of rhinorrhea (Collins and Karron, 2013). The characteristics of RSV bronchiolitis include: necrosis and sloughing of the epithelium of the small airways; increased secretion of mucus which obstructs flow in the airways and edema.

Clinical findings include hyper-inflammation, atelectasis and wheezing. Recovery starts within few days after the symptoms appear. Hall (2001) explained that, although it is true that recovery starts after few days, the ciliated epithelial cells may not appear until after two weeks. Hall (2001) also confirmed that full recovery requires four to eight weeks.

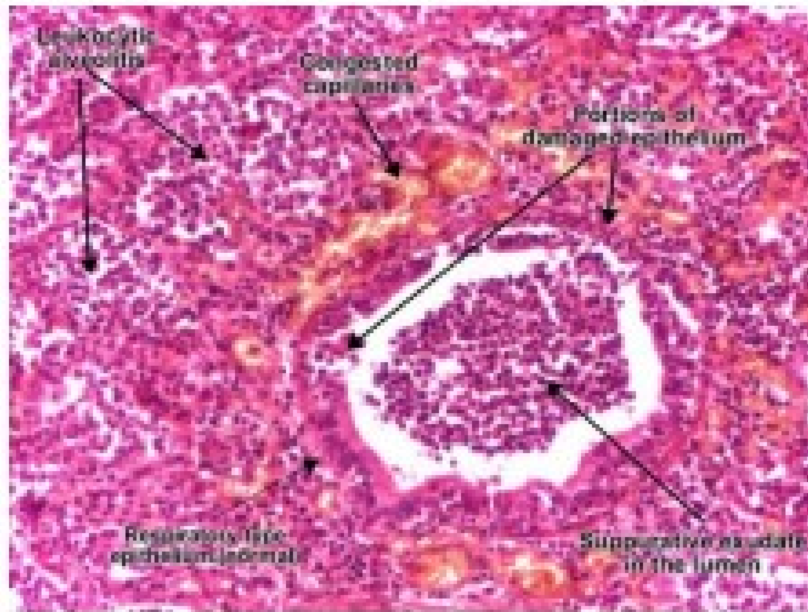


Plate 2.5: Histology of bronchiolitis in an infant with HRSV infection

Source: http://www.mdblogger.com/2011/04/histology-and-explanation-of_10.html

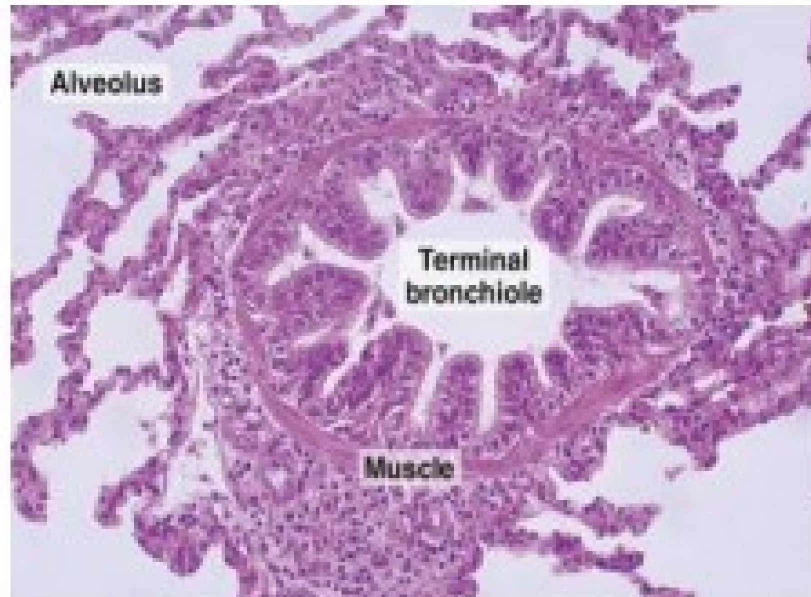


Plate 2.6. Histology of an uninfected bronchiole

Source:

http://www.mc.vanderdilt.edu/histology/labmanual2002/labsection2/Respiratory03.htm

2.8 RSV Epidemiology

HRSV infection is a menace both in developing as well as developed countries (Reiche and Schweiger, 2009).

2.8.1 Age Group

All age groups could be infected by HRSV, however infants below the age of five years, especially those below the age two years as well as the elderly (>65 years of age) are more prone to the infection (Collins and Karron, 2013). RSV infection is the leading cause of LRTI in infants, especially those less than age 6 months and is also a major cause of respiratory infection among the elderly (Sidwell and Barnard, 2006). The burden of HRSV infection appears to be inversely correlated with age, hence the highest rate of infection is commonly reported among children in the age group less than or equal to six months (Hall *et al*., 2009; Papenburg *et al*., 2012)

2.8.2 Seasonality

The HRSV season typically vary by region (Frogel *et al.,* 2010). Although infection may occur all year round, the virus has been found to cause epidemics during the winter months in the temperate regions and during the rainy season in the tropical regions (Trento *et al.,* 2006; Eshaghi *et al.,* 2012; Aamir *et al.,* 2013)

2.8.3 Patterns of HRSV subtype circulation

Both subtypes of HRSV are mostly found circulating together in the community (Papenburg *et al*., 2012; Aamir *et al.,* 2013; Bigogo *et al*., 2013; Gimferrer *et al*., 2015). However, it appears that the predominating subtype change from one season to another. Shobugawa (2009) showed that about 75% of their sample were HRSV-A while only 25% were HRSV-B. In a similar study conducted in Japan (Dapat, *et al.,* 2010) the subtype-A was predominant circulating in six seasons out of the nine season of the study, while HRSV-B was predominant only in three seasons. On the other hand, report from a study conducted in Germany by Reiche and Schweiger, (2009) showed that although both groups A and B can co-circulate in the same community, group A tends to dominate. According to Reiche and Schweiger (2009) the seasonal distribution of subgroup A viruses throughout the study period (1998 to 2007) except in the 1998-99 season indicated the RSV group A dominance.

Various studies have shown that the circulating strains of RSV vary from one community to another (Gottschalk *et al.,* 1996; Peret *et al*., 2000; Zlateva *et al.,* 2007). For instance, a research conducted in North America (Peret *et al.,* 2000) showed that the patterns of HRSV circulation is distinct from one community to another thereby suggesting that RSV outbreaks are more often a community phenomenon rather than a national one. This position was further substantiated by reports by other researchers (Gottschalk *et al.,* 1996; Zlateva *et al.,* 2007).

2.8.4 RSV Transmission

Transmission of infectious HRSV particles is possible via different means including by large droplet particles (Figure 2.8), by aerosolized small particles and by self-inoculation after contamination of the hands by fomites on surfaces including door-knobs (Hall *et al*., 1981). For direct contact transmission to be effective, close contact with an infected person is required, from whom the virus infect the mucous surfaces of the eye and the nose (Hall *et al*., 1981). On the other hand, transmission by aerosols does not require close contact due to the ability of the infectious aerosols to travel as far as a distance of six or more feet. It has been shown that the virus can remain infectious in fomites within the environment for up to a period of 6 hours (Hall *et al*., 1980). Due to the susceptibility of infants and young children, children holding facilities like day care centers play a role in the transmission. Hall *et al*. (1981) also showed the importance of nosocomial transmission in the spread of HRSV. It has been reported, that whereas infants are often carrying the highest clinical burden of infection, HRSV infection are often transmitted to the infants within the family either by the parents or the older siblings which may be asymptomatic (Heikkinen, *et al*., 2014).

2.9 Immunity to HRSV infection

The level of disease pathogenesis resulting from HRSV infection can be balanced through the nature of the host immune response elicited against the virus, thereby leading to viral elimination (Tripp, 2004). The immunological response to infections with HRSV can be categorised into either innate or adaptive (McNamara and Smyth, 2002; Tripp, 2004).

2.9.1 Innate Immunity

This is the first line and also the earliest phase of defense mounted by the body against foreign organisms. The innate immune system involves the use of cytokines, which, when released, directs the recruitment of effector molecules and phagocytic cells to the site of the infection (McNamara and Smyth, 2002). The toll-like receptor 4 (TLR-4) and CD14 are considered two important receptors in innate immunity. When protein A binds to the F protein of HRSV through these receptors, viral neutralization occurs. Neutralization does not occur if the protein binds to G protein of HRSV (Tripp, 2004).

How RSV is detected by cells of innate immunity.

In natural and experimental infections with RSV, the virus mainly infects ciliated airway epithelial cells from alveoli and small airways (Chanock *et al*., 1957). These epithelial cells, together with specialized dendritic cells (DC) and macrophages, are the first cells to detect and respond to RSV invasion (Lukens, 2009). The relative presence of different cell types at the site of infection determines the course of the immune response. For instance, increased numbers of plasmacytoid DC (pDC) compared to conventional DC have an anti-inflammatory effect on RSV disease and allergic responses, related to increased IFN-α production (Smit *et al*., 2008). Detection of viral pathogens, like RSV occurs through a family of pattern recognition receptors (PRR), consisting of Toll Like receptors (TLR) and cytoplasmic sensors of viral RNA, like RIG-1 and MDA-5 (Arruvito *et al*., 2015). Activation of PRR and subsequent signaling via downstream kinases leads to translocation of NFκB to the nucleus, where it acts as a transcription factor for pro-inflammatory cytokines like IL-6, IL-8 and TNF-α (Collins and Karron, 2013). Activation of RIG-1/MDA-5, TLR3 and in some cases TLR4 not only activates NFκB but also causes the translocation of IRF-3 to the nucleus, where it induces transcription of type I interferon (type I IFN) (Arruvito *et al*., 2015). The type I IFNs, like IFN-α and IFN-β are key cytokines in antiviral responses, they induce apoptosis and limit virus replication in infected and non-infected cells. Initial sensing of respiratory single stranded RNA viruses like RSV, is thought to be mediated by RIG-1 in airway epithelial cells detecting single stranded viral RNA and is followed by activation of TLR3 by double stranded RNA formed during replication of the virus (Lukens, 2009). Other pathogen recognition

receptors involved in detection of viral RNA are TLR7 and TLR8, however their role in the detection of RSV has not been tested thoroughly (Arruvito *et al*., 2015). Toll-like receptor 4 has also been implicated in the recognition of RSV. Different cell types express a different array of TLR, for instance TLR7 is mainly expressed by pDC, while monocytes use TRL8 for the detection of single stranded viral RNA (Smit *et al*., 2008).

The innate immune response triggered by RSV greatly depends on where and which cell types come in contact with (infectious) virus particles, and what is their current expression level of PRR. Furthermore, also the accessibility of viral components, for instance via antibodies, to different PRR influences the subsequent chemokine and cytokine production attracting different innate and adaptive immune cells (Lukens, 2009). In addition to the classical pattern recognition receptors involved in the detection of RNA viruses, it well established that the fusion protein of RSV could induce TLR4-dependent IL-6 production in human monocytes and murine macrophages (Tripp, 2004). The interaction between RSV-F and TLR4 is host species specific, requiring either intact human RSV or purified RSV-F, whereas bovine RSV derived F protein does not induce a TLR4 mediated response (Tripp, 2004).

Chemokine and cytokine production during RSV infection.

Upon infection of mice with RSV, expression of up to 1200 genes involved in anti-viral responses and antigen presentation are altered in the respiratory tract (Tripp *et al*., 1999). Compared to other respiratory infections (like influenza), RSV shows the greatest upregulation of chemokine/cytokine genes, leading to higher levels of chemokines/cytokines in the respiratory tract (Tripp *et al*., 1999). Enhanced production of CC (CCL2, CCL3 and CCL5), CXC (CXCL1, CXCL8 and CXCL10) and the CX3C (fractalkine, CX3CL) chemokines, leads to recruitment of neutrophils, monocytes, DC and T cells into the respiratory tract (Lukens, 2009).

The cellular composition of the lung after primary infection has a great impact on subsequent infections with unrelated pathogens. Alterations in ratios of different DC populations in the lungs affect primary and secondary responses, due to difference in chemokine/cytokine secretion and the attraction of different subsets of inflammatory cells (Smit *et al*., 2008). In patients with severe RSV infections high levels of TNF-α,

CCL5, IL-6 and IL-8 (CXCL8) were found in the airways, causing an influx of mainly neutrophils and activated T cells, possibly enhancing the disease (Smit *et al.*, 2008).

The important role of chemokine and cytokines in steering and controlling the immune responses led to a focus of genetic studies on associations of severe RSV disease and polymorphisms in these genes. Several associations have been found between the severity of RSV disease and single nucleotide polymorphisms (SNP) in chemokine genes. A SNP in the IL-8 promoter region leading to increased IL-8 production, which potentially increases the neutrophil influx in the lungs during RSV infection, is highly represented in children showing severe outcome of infection. Also, a SNP in the CCR5 promoter region is associated with increased chance of developing severe RSV disease (Lukens, 2009). The second group of SNPs linked with severity of RSV disease is formed by cytokine genes that can affect the effector functions of T cells. Associations between SNP in the IL-9, IL-10 and TNF-α genes and severe RSV disease are found (Lukens, 2009).

The important components of the innate immune response to HRSV include the following:

1. **Phagocytic cells**

This includes both the neutrophils and the macrophages.

a. Neutrophils

Strong neutrophil response is associated with RSV infection. The neutrophils are considered the predominant airway leukocyte during infection of HRSV due to the vital role they play in the pathological changes that occur in bronchiolitis. In fact, when bronchoalveolar lavage fluid collected from the lungs of infants presenting with bronchiolitis are tested, neutrophils were the major immune cells detected (Russell *et al.*, 2017). The height of neutrophil response during HRSV infection has been found to occur at the time of highest viral load as well as the period of most severe clinical symptoms (Russell *et al.*, 2017). The macrophages and respiratory epithelial cells produce interleukin 8 (IL-8) and chemokine. These cytokines dictate the chemotaxis of neutrophils during HRSV (McNamara and Smyth, 2002; Tripp, 2004). Neutrophils are recruited from the bloodstream into the site of infection through a four-step process which include: rolling, adhesion, extravasation and migration.

b. Macrophages

Upon migration of circulating monocytes into tissues, macrophages are formed. Macrophages, together with respiratory epithelial cells happen to be the first cells to encounter HRSV in the airways (McNamara and Smyth, 2002; Tripp, 2004).

The macrophages have been suggested to serve as antigen presenting cells and also perform immune-regulatory roles at local sites of HRSV infection (Russell *et al*., 2017). However, they can also destroy invading pathogens. They are able to direct the immune response to viral infection, through the production of cytokines and by direct interaction with both helper and cytotoxic T-cells.

2. **Eosinophils**

Eosinophils, as well as their induced RNases are known to have antiviral activities (Tripp, 2004). Activation of eosinophils occur during the acute phase of lower respiratory tract infection caused by HRSV. Eosinophils may play a significant role in recovery from HRSV infection, as shown by the high count of eosinophil detected in the blood in children recovering from bronchiolitis (Russell *et al*., 2017). RANTES and MIP-1a are chemo-attractants, that helps the migration of eosinophils to respiratory epithelial cells due to the up-regulation of these chemo-attractants by the HRSV infected cells. Degranulation of eosinophils have been reportedly detected in the lung parenchyma and the nasopharynx of people infected with human respiratory syncytial virus (Domachowske and Rosenberg, 1999; McNamara and Smyth, 2002; Tripp, 2004).

3. **Natural Killer cells**

Reduced natural killer (NK) cell counts have been detected in the blood during infection with HRSV, however the activated subset of NK cells lacking the expression of CD94 increases during the infection (Russell *et al*., 2017). Most of the early interferon (IFN) production during HRSV infection could be attributable to the NK cells that accumulate in the lungs early during the infection (Domachowske and Rosenberg, 1999; McNamara and Smyth, 2002; Tripp, 2004). It has been suggested that NK cells are redirected to enter other tissues including the lungs from the peripheral circulation (Domachowske and Rosenberg, 1999; McNamara and Smyth, 2002; Tripp, 2004).

2.9.2 Adaptive Immunity

Immune protection.

Two vital questions that are still begging for answers with regard to protective immunity to RSV are: (1) the contribution of adaptive immune responses to immune pathology and (2) why protective immunity against RSV is incomplete. Protective adaptive immunity against RSV is considered to be suboptimal, based on the frequent re-infections in humans (Hall *et al*., 1991). Adults and children have high titers of neutralizing antibodies after primary or secondary infections, which increase almost four-fold during re-infection and drop to pre-infection levels within one year (Collins and Karron, 2013). Antibodies produced by B cells are responsible for some protection against subsequent RSV challenges and maternally derived antibodies diminish disease severity during primary infection (Muelenaer *et al*., 1991). Upon infection respiratory DC transfer viral antigen to lymph nodes draining the site of infection and stimulate naïve and memory antigen specific T cells (Lukens, 2009). Naïve T cells rapidly expand upon activation, acquire effector functions and migrate towards a chemokine gradient to the inflamed site (Lukens, 2009). Protective T cell responses against live viruses usually consist of T helper cells producing Th1 type cytokines like IFN-γ or TNF-α. During acute viral infections in healthy adults, virus specific CD8+ T cells develop and peak within 14 days after infection (Collins and Karron, 2013). After elimination of the pathogen, inflammation wanes and the number of virus specific T cells contract to form a memory population (McNamara and Smyth, 2002). Memory T cells ensure an early and rapid response against secondary infections. Due to the fact that memory T cells are a heterogeneous population that differs in location, responsiveness and longevity, different kinetics and responses are found for memory subsets during secondary infections (McNamara and Smyth, 2002). Long lived memory T cells against respiratory viruses and RSV can be found in the lungs of healthy adults and mice (Lukens, 2009). It has been reported that RSV specific CD8+ T cells present in the lungs, are unable to exert their effector function (Lukens, 2009). This partial inactivation of RSV specific CD8+ T cells in the lungs of mice has therefore been implicated as a possible explanation for the lack of protective memory responses (Hall *et al*., 1991). However, both in humans and mice T cells are found to be vital in clearing RSV from the lungs (Collins and Karron, 2013). Moreover, this phenomenon of partial CD8+ T cell inactivation was not RSV specific

and was observed with many other respiratory viruses as well, suggesting that partial inactivation of T cells might be due to the local environment in the lung and possibly reflects a mechanism in the lung to prevent extensive immune damage (Lukens, 2009).

Immune pathology.

Adaptive T cell responses are not only associated with viral clearance but also implicated in immune pathology. The theory of T cell induced immune pathology during RSV infection is mainly based on mouse experiments, where T cells dominate the immune response (Lukens, 2009). Upon depletion of CD4+ and CD8+ T cells, mice shed RSV for a prolonged time without severe illness. When only one of the T cell subsets is depleted or transferred to persistently infected mice, disease becomes more evident (Lukens, 2009). Both CD4+ and CD8+ T cells are involved in rapid viral clearance but could under certain circumstances contribute to immune pathology. Further evidence of an important role of T cells in RSV disease is based on animal experiments reproducing enhanced disease after formalin-inactivated (FI-RSV) vaccination in humans. Mice subcutaneously primed with the FI-RSV vaccine used in 1960s showed similar enhanced disease symptoms upon challenge with live virus as humans. Especially Th2 type T cells played an important role in the vaccine induced immune pathology. To gain further insight in the contribution of individual RSV proteins to memory responses, mice were primed with recombinant vaccina viruses expressing individual RSV proteins. The enhanced disease, with eosinophilia and Th2 CD4+ T cell responses could be reproduced when mice were primed with recombinant vaccina virus expressing the RSV-G protein (Collins and Karron, 2013). This enhanced disease mimicking the response after FI-RSV priming and challenge was only observed when mice were primed with the G protein, while priming with the F or M2 protein induces a more protective Th1 CD8+ T cell response (Collins and Karron, 2013). Enhanced disease after priming with the G protein is largely caused by a single epitope in the conserved region, recognized by a subset of CD4+ T cells producing both IFN-γ and IL-5. Enhanced disease after G protein priming depends on the genetic background of the mice and MHC genes, mice resistant to enhanced disease respond with higher CD8+/CD4+ T cell ratios. Indeed, the balance between protective CD8+ T cells and Th2 CD4+ T cells determines whether enhanced disease develops or protective responses are initiated upon challenge infection

(Domachowske and Rosenberg, 1999). In contrast to mouse experiments, in immune compromised or suppressed human patients with reduced T cell responses, RSV disease is observed more often and disease is more severe compared to healthy subjects (Domachowske and Rosenberg, 1999).

Human autopsy material from severe fatal RSV infections also showed widespread viral replication with few T cells and NK cells. In contrast, pulmonary material from a 15-month-old less severe RSV patient killed in a traffic accident, showed extensive pulmonary monocyte and T cell infiltrations (Lukens, 2009). These observations indicated a possible difference in the involvement of T cells in disease severity between human and mice, where increased T cell responses might protect against severe RSV disease in humans. Also, in children hospitalized due to severe RSV infection and requiring mechanical ventilation at the intensive care unit (ICU), the majority of cells in the airways consisted of mainly neutrophils and low numbers of highly activated CD8+ T cells (Collins *et al*., 2013). However, kinetics of primary RSV specific T cell responses in children with severe RSV infections, hint at a role of viral or neutrophil mediated pathology as both peak together with disease severity, while T cell responses appear in the blood of these children at the end of stay at the ICU (McNamara and Smyth, 2002)

Modulation and evasion of immunity by RSV.

Despite high levels of chemokines and cytokines produced during RSV infection in mice, there is hardly any production of type I IFNs, key regulators of anti-viral responses (Lukens, 2009). This is probably due to two proteins (NS1 and NS2), that are not found in live virus particles, do not seem to be involved in the virus structure/particle and are therefore called nonstructural proteins (NS). The NS1/2 genes are situated at the 3'-end of the viral genome, sandwiched by the untranslated leader region and the nucleoprotein gene respectively and are the first and most abundant transcribed proteins in infected cells (Dickens *et al*., 1990). RSV deletion mutants lacking NS1 or NS2 are viable, suggesting that these proteins are not required for viral replication (Collins and Karron, 2013).

One of the essential transcription factors for type I IFN is IRF-3, which is C-terminally phosphorylated for translocation to the nucleus. NS proteins from RSV interfere with RIG-1 signaling, inhibit IRF-3 phosphorylation and translocation to the

nucleus, while NFκB activation remains untouched (Bossert and Conzelmann, 2002). The important role of functional NS proteins in increasing virulence makes these proteins an interesting target for anti-RSV therapeutics (Lukens, 2009). Mice treated prophylactic with siRNA nanoparticles directed against the NS1 protein, show decreased viral lung titres, reduced inflammation and airway reactivity and increased adaptive CD8+ T cell responses during infection with RSV (Bossert and Conzelmann, 2002). Increased CD8+ T cell responses are related to the function of DC, as these cells form the bridge between innate and adaptive immune response. Maturation and chemokine/cytokine production of DC is inhibited by RSV NS proteins, affecting the formation and quality of adaptive immune responses (Smit *et al*., 2008).

Besides the recognition of RSV-F by TLR4 and the type I IFN production inhibition by the NS proteins, two other proteins of RSV are also implicated in regulation of innate and adaptive immune responses. Using G/SH protein deficient recombinant RSV, it was observed that trafficking of neutrophils and NK cells was reduced with altered chemokine production (Tripp *et al*., 1999). Interestingly, the G protein is not only expressed as a membrane protein (mG), but also secreted from infected cells as a soluble protein (sG) (Collins and Karron, 2013). This is suggestive for an important role of the G protein in modulation of both the adaptive and innate immune responses. The G protein contains a conserved part from amino acids 169-191, which mimics the CX3C chemokine motif. Although fractalkine is the only known chemokine with a CX3C motif, it has little amino acid homology with the G protein, but in structure the two proteins are alike. It was found that the G protein could bind the fractalkine receptor (CX3CR1) and blocking of this receptor reduced RSV infection. CX3CR1 expressing leukocytes migrated towards both fractalkine as well as soluble RSV-G during RSV infection in mice, leading to increased cell influx in the lungs (Lambert *et al*., 2014).

It has been observed that antigen presentation and stimulation of T cells by RSV infected DC is inefficient. The exact mechanisms by which this occurs are unknown, but both contact dependent mechanisms as well as soluble factors have been implicated. RSV infected DC produce a soluble factor, that inhibits T cell proliferation and effector function (Lukens, 2009). The exact factor is unknown, although IFN-λ has been associated with RSV induced inhibition of T cell proliferation. Also contact dependent mechanisms contribute to reduced T cell

effector mechanisms. The F-protein on RSV infected cells can limit the proliferative response of T cells in a contact depended manner (Lukens, 2009).

2.10 Risk factors for HRSV infection

Studies reported that the following could be considered risk factors:

- History of pre-mature birth;
- Congenital heart disease;
- Crowded living conditions;
- Bronchopulmonary dysplasia;
- Cystic fibrosis;
- Immunodeficiency;
- Being male; Oliveira *et al*. (2008) reported that male children are more susceptible to severe disease infection than females, that RSV A-infected patients tends to be younger, and children who were less than one month of age were more prone to infection by subgroup A.
- Socioeconomic factors such as belonging to lower income families.

2.11 HRSV associated mortality

In some parts of the world, where medical services are not well developed (Parveen, *et al.,* 2006) approximately half a million children die each year because of ALRI which accounts for 25% of all children around the world. Most of the deaths occur among rural community dwellers and occur without reaching a hospital.

2.12 Diagnosis of HRSV infection

It is almost impossible to diagnose RSV infection based on clinical manifestations only, hence the need for specific laboratory diagnosis of the infection (Gottschalk, *et al.,* 1996). There are several methods used for diagnosis of HRSV including rapid methods of antigen detection by enzyme immune assay (EIA) or direct immunofluorescence assay (DFA), viral culture and the highly sensitive methods of RNA detection with RT-PCR.

2.12.1 Specimen Collection

The type and source of the specimen highly influences the success or otherwise of HRSV in patient samples (Abu-Diab, *et al.,* 2008). An array of techniques is often

used for collection of respiratory specimens from different sources. The samples used include nasal swabs, oropharyngeal (OP) swab, nasopharyngeal (NP) swabs, nasal washes, nasopharyngeal aspirates (NPAs), bronchoalveolar lavages and sputa specimens. Although NPAs may be the most sensitive specimens due to the high yield of ciliated epithelial cells (Loens *et al.,* 2009), collection of NPA requires more expertise and is often greeted with high level of rejection from patients than that of any swab. Also, due to the invasiveness of the procedure, it may not be realistic to collect NPAs in an outpatient or field setting (Kim *et al*., 2011).

Procedure for collection of Nasopharyngeal Aspirate

1- A suction tube is attached to disposable aspiration trap.

2- The length of the tube is measured to be as the distance between the nostril and the ear of the patient.

3- The tube is inserted gently into the nostril (Figure 2.9).

4- Irregular suction is applied.

5- The tube is removed slowly from the nasopharynx.

6- The specimen is washed with sterile saline and the contents are aspirated into the NPA container.

7- The container is to be tightly closed and immediately transported to the lab for viral testing and, if processing is delayed, the specimen must be kept in a refrigerator at 4°C.

Procedure for collection of Nasopharyngeal Swab

A flexible, fine-shafted polyester swab is inserted into the nostril parallel to the palate (Figure 2.10) (not upwards) until resistance is encountered or the distance is equivalent to that from the ear to the nostril of the patient indicating contact with the nasopharynx. The swab is left in place for a few seconds to absorb secretions before removing. It is then slowly withdrawn with a rotating motion. The tip of the swab is put into a vial containing 2–3 ml of virus transport medium and the shaft cut.

Plate 2.7. Collecting Vacuum-Assisted Nasopharyngeal Aspirates (NPAs)

(Berkshire and Surrey Pathology Service, 2016)

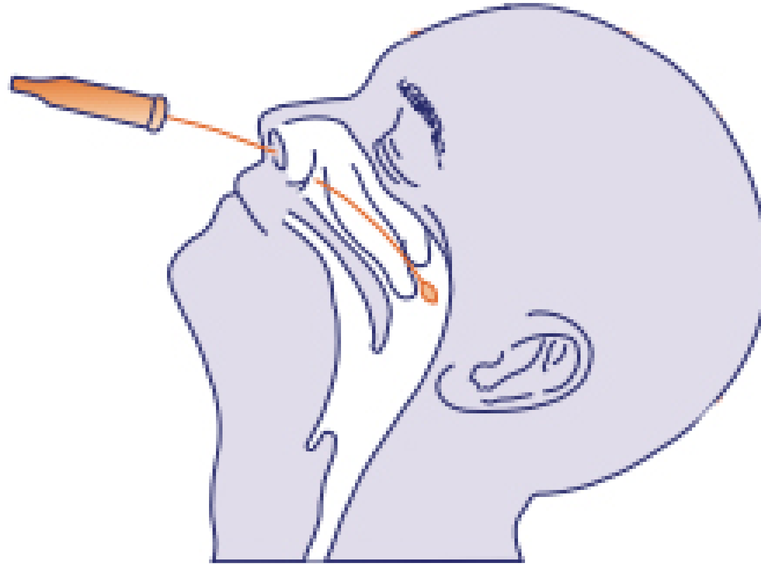


Plate 2.8. Collection of Nasopharyngeal swab.
(Diagnostic Laboratory of Oklahoma, 2017)

Procedure for collection of Oropharyngeal swabs

A pharyngeal swab is firmly inserted into the pharynx through the oral cavity (Figure 2.11), and mucosal epithelium collected by swabbing the posterior wall of the pharynx and the palatine tonsil several times, centering around the rubefacient portion. Touching of saliva should be avoided. The tip of the swab is put into a vial containing 2–3 ml of virus transport medium and the shaft cut.

2.12.2 Rapid Diagnosis

a. Immunofluorescence (IF) - both direct and indirect IF utilizing either polyclonal or monoclonal antibodies are available which possess a high degree of sensitivity and specificity. The general sensitivity of DFA for the detection of HRSV is > 95% and for monoclonal antibody 95 - 100%. IF techniques are fast and easy to perform but the interpretation of results is subjective and the specimen must contain adequate nasopharyngeal cells. This method is widely used to detect RSV in children (Gottschalk, *et al.,*, 1996) and can be performed in less than 30 minutes turn-around time.

Several kits are available for the detection of HRSV antigens on a solid phase. Kits techniques offer the advantages of objective interpretation, speed, and the possibility of screening a large number of specimens. Disadvantages include a generally poorer sensitivity and a "grey zone" of equivocal results, which requires confirmation by a time-consuming procedure.

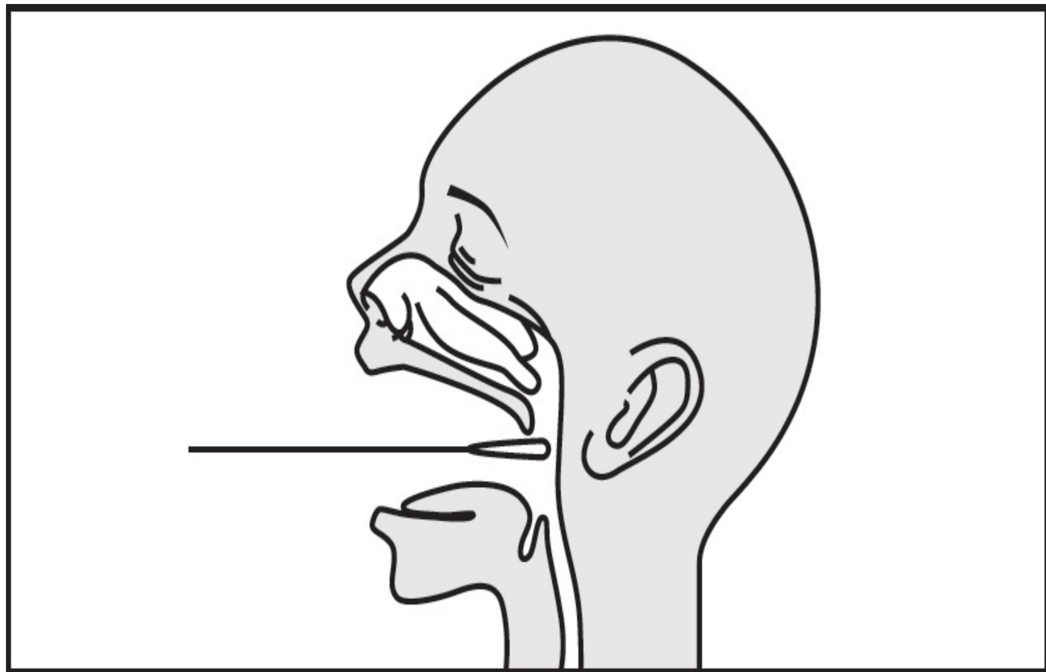


Plate2.9. Collection of Oropharyngeal swab

Source: http://capilia.co.jp/english/capilia_adeno_neo.html

2.12.3 Cell Culture

HRSV is a highly labile virus and any patient specimens should be transported to the laboratory promptly and inoculated into cell cultures. Nasopharyngeal aspirates, nasal washes or tracheal secretions are generally the best specimens for RSV isolation. Specimens should not be subjected to major temperature changes such as freezing and thawing. Human heteroploid cells, such as HEP-2 and HeLa generally provide the best tissue culture for the isolation of HRSV. HRSV produces a characteristic CPE consisting of syncytia formation and appears in 4 to 5 days.

2.12.4 Serology

Serological diagnosis can be made by detecting increase in antibody in acute and convalescent sera. It is unlikely to be of help in the management of the patient because of the length of time required. Furthermore, the serological response in young infants may be poor and not detectable by some antibody assays. Seroconversion does not occur for at least 2 weeks and may require 4 -6 weeks. Complement Fixation Tests (CFTs) are less sensitive than neutralization and kits assays.

2.12.5 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

This test is sensitive and helps substantiate HRSV diagnosis. Zhang et. al. (2010) performed RT-PCR for a fragment of HRSV G gene with NPAs collected from children with ARI who were hospitalized in a Children's Hospital in China. Falsey (2007) used this lab test on samples from nasal swabs and sputum. Sputum provided a high yield of ciliated epithelial cells in the research of Gottschalk*et al.,* (1996) in which they performed RT-PCR testing. This method is currently used successfully not only in pediatric patients but also in adults. Most assays use primers targeting the conserved portion of the M, F and N genes and can differentiate group A and B viruses. In a study of 1,000 nasal samples, RT-PCR was more sensitive compared to viral cultures (Gottschalk *et al.,* 1996). Although this is a sensitive and specific method, the RT-PCR is limited by expense, labor-intensiveness and it is not commercially available. Therefore, some researchers (Hu *et al.,* 2003) used Real-Time (RT-PCR), that helped them reduce the complexity of analysis by not performing post-PCR processing. This is done because the Real-Time PCR method permits the fluorescence signals to be analyzed and recorded during PCR cycling.

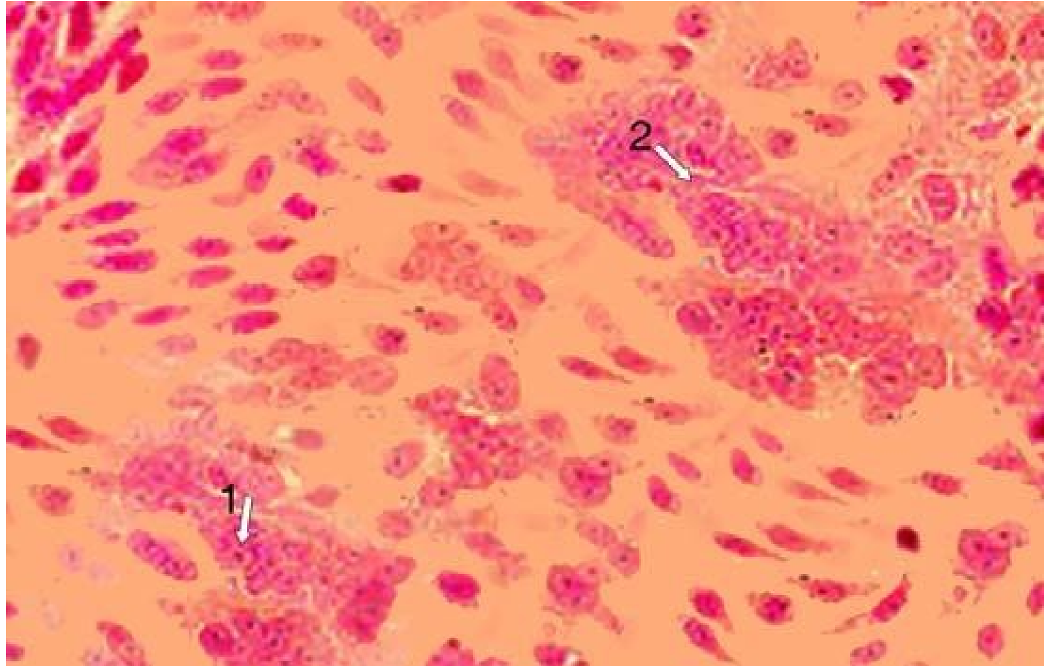


Plate 2.10. The characteristic cytopathic effect (Syncytium formation) of RSV in tissue culture. The virus was cultivated in Hep-2 cell culture. Arrows 1 and 2 show the syncytia formed under X400 magnification and stained by safranin T.

(Zahran, 2017)

2.13 Prevention of HRSV infection

Vaccines and protection

Vaccine failure.

In the early years following the discovery and isolation of RSV, there appear to have been a major breakthrough to checkmate the menace of the HRSV infection due to the development of a vaccine via a well-known protocol of virus inactivation with formalin in combination with aluminum hydroxide as adjuvant. This FI-RSV vaccine was used in the 1960s in a series of clinical trials with children with unforeseen consequences. Despite high levels of RSV specific antibodies, the children were not protected against natural RSV infections. Upon infection of the vaccinated children from natural exposure to HRSV, about 80 % of them had severe outcome necessitating hospitalization, out of which two deaths was recorded (Collins and Karron, 2013). The vaccinees suffered from bronchiolitis and pneumonia with high viral load and eosinophilic influx in the airways. Moreover, high lymphocyte proliferative responses against RSV antigens were observed (Collins and Karron, 2013).

In subsequent analysis applying animal experiments and reevaluating original patient material, it was discovered that high titer of non-neutralising antibodies with low avidity was induced by the vaccine and the same was deposited as immune complex with RSV in the lungs of patients (Lukens, 2009). Inactivation of RSV with formalin induces reactive carbonyl groups on RSV, which might have promoted non-protective Th2 immune responses (Collins and Karron, 2013). In mice Th2 associated cytokine producing CD4+ T cells are responsible for the enhanced disease, with limited induction of protective CD8+ T cell responses (Russell *et al.*, 2017). These data and subsequent experiments in animals indicated that excessive Th2 skewed immune responses were responsible for enhanced disease upon natural infection.

Protection and treatment

Despite much effort in the past 40 years, there still is no safe and effective licensed vaccine that can protect infants against severe RSV infection. There have been attempts to use live viruses attenuated by cold passage or chemical mutagenesis (Collins and Karron, 2013). Other strategies included reverse genetics to create

attenuated mutant viruses or use viral and bacterial vectors to induce protective T and B cell responses (Russell *et al*., 2017). Protective vaccination in the neonatal period is difficult, because the developing immune system reacts weaker to stimuli compared to adults and is Th2 skewed (Lukens, 2009). Neonatal B cell responses are delayed, leading to lower IgG levels and different isotype distribution compared to older children, thus causing the humoral arm of the immune system to be less protective (Lukens, 2009). Furthermore, maternally derived antibodies transferred to the child via breast milk and in utero, provide some early protection, but could also interfere with effective induction of protective immune responses by vaccines, in part due to B and T cell epitope masking or lowering antigenic load (Russell *et al*., 2017).

Despite the fact that maternally derived antibodies can hinder the induction of protective response during vaccination, there is also a clear inverse correlation between levels of RSV neutralizing IgG in cord blood and severe RSV infections (Lukens, 2009). This indicates that prophylactic treatment with neutralizing antibodies can confer protection against RSV in the neonatal period (Haynes *et al*., 2009). This is important because there is a clear association between the age of primary infection and subsequent responses during secondary infections at later age (McNamara and Smyth, 2002). Based on these observations a polyclonal human antibody preparation isolated from healthy donors, consisting of fractionated IgG with highly neutralizing capabilities (RespiGam) was given to high risk children. This treatment conferred some protection. The drawback was that infants required monthly intra-venous injections and that not all children with congenital heart disease or premature gestation were effectively protected (Collins and Karron, 2013).

The idea of protective IgGs led to the development of palivizumab, a monoclonal antibody directed against the F protein of RSV. Palivizumab, which is directed against epitope 4 of the antigenic site A of the F protein was selected from a panel of murine antibodies and was humanized to allow repetitive use in humans (Sidwell and Barnard, 2006). Palivizumab effectively reduces hospitalization rates of high-risk infants with 50 % compared to a placebo group (Zhu *et al*., 2011). Unfortunately, not all high-risk infants are protected by prophylactic treatment with palivizumab, these children still show viral replication in the upper respiratory tract. Currently, several approaches are in progress to enhance the protective effects of antibodies (Collins and Karron, 2013). RNA viruses are known to have high mutation rates because their

RNA polymerases lack RNA proofreading capabilities. Since palivizumab and a newly developed more potent derivative Motavizumab are based on the same parental mouse antibody, there is an increased chance that palivizumab escape mutants might develop (Zhu *et al*., 2012). Therefore, there still is a need for new developments in the protection of those at risk for severe RSV infection, were prophylactic antibodies seems to be the best option during the early months of life and in immune compromised individuals.

2.14 Treatment of HRSV

Despite over 60 years of continuous efforts, there are no effective means to control HRSV infections. The development of the vaccine has always been facing obstacles due to the lack of durable immunity in addition to the diversity of population at risk for infection (Hall, 2001). This means that infections may occur in the presence of preexisting immunity such as the presence of maternally derived antibodies as well as the reinfections throughout life. Up to this moment, studies are still conducted to reach for an effective treatment for HRSV. Haynes et. al. (2009) examined whether therapeutic treatment with an anti-RSV G mAb, 131-2G, that blocks the CX3C-associated activity of RSV G protein might decrease the pulmonary inflammation associated with infection in BALB/c mice. The results show that treatment with mAb 131-2G on day 3 after RSV infection reduces both inflammation and RSV titer in the lungs. Later administration of anti-RSV G mAb (day 5 after RSV infection) effectively reduced the viral titer but had a minimal effect on pulmonary inflammation. This study suggests that an anti-RSV G mAb might be an effective antiviral, either alone or in combination with anti-RSV F protein neutralizing antibodies, for decreasing the virus-induced host response to infection and improve treatment outcome.

In analyzing the risk factors for severe RSV infection in elderly persons, Walsh et.al. (2004) found that the level of neutralizing antibody was inversely correlated with the risk of hospitalization during RSV infection. They collected data from persons over 65 years of age. The data revealed that the induction of neutralizing serum antibody with an RSV vaccine may potentially reduce disease severity in adult population. This means that RSV vaccines that stimulate humoral immunity may play a role in reducing disease burden in adults.

2.14.1 Ribavirin

Ribavirin, a guanosine analogue with broad spectrum of antiviral activity is the only FDA approved treatment option available for HRSV (Falsey, 2007). The use of Ribavirin, which was approved in 1986 by Food and Drug Administration (FDA) (Sidwell and Barnard, 2006) was however limited to aerosolized use among infants (Gottschalk, *et al.,* 1996). Although the specific mechanism by which HRSV is inhibited by ribavirin is not well documented, the mechanism of viral inhibition by ribavirin is known to be multi-faceted and includes: inhibition of the 5'cap formation of messenger RNA, inhibition of the enzyme Inosine Monophosphate Dehydrogenase (IMPDH), and inhibition of viral polymerase by the phosphorylated forms of the compound (Sidwell and Barnard, 2006).

The enzyme IMPDH catalyzes the conversion of inosine monophosphate to xanthosine monophosphate. This step is very crucial in the de novo biosynthesis of guanine nucleotides leading to DNA and RNA synthesis. Inhibition of IMPDH therefore reduces the amount of intracellular guanine nucleotides available for RNA and DNA synthesis and consequently result in significant antiviral effects, although such effects may also be associated with inhibition of cell replication (Sidwell and Barnard, 2006).

2.14.2 Palivizumab

There are two antibodies that are approved for treatment of RSV disease: RSV-IGIV, which is RSV immune globulin, and palivizumab which is a chimeric humanized IgG monoclonal antibody (Sidwell and Barnard, 2006). Both of these two treatments are produced by MedImmune Inc. located in the USA. Sidwell and Barnard (2006) described the two treatments as follows:

1- **RSV-IGIV**:

a. An infusion of 750 mg/kg administered monthly to prematurely born infants. This has been reported to significantly decrease hospitalization and to reduce the number of hospital days with supportive oxygen.

b. However, the product is derived from blood and has the potential to transmit blood-borne pathogens; furthermore, its viscosity, coupled with required high volumes for administration, may lead to fluid overload. RSV-IGIV must be administered under

medical guidance. With the introduction of palivizumab, use of the product has dramatically declined.

2- **Palivizumab**:

Palivizumab is a humanized monoclonal antibody directed to an epitope in the A antigenic site of the F-protein of HRSV. It was approved in 1998 for the prophylaxis of infants at high risk for HRSV infection. It was reported that this product is 50–100 times more potent than RSV-IGIV and is now being used worldwide with considerable success. Palivizumab is licensed for intramuscular injection of 15mg/kg administered at monthly intervals throughout the HRSV season and all strains of HRSV appear to be neutralized by it. Infants with bronchopulmonary dysplasia or congenital heart disease have had a significantly lower rate of protection. Infants are required to take an intramuscular injection (15 milligrams of Palivizumab per kilogram) once every month for five months. In their study, Frogel *et al.* (2010) showed that the rate of compliance with this treatment could be as low as 25% due to factors such as access to care and parental perception as to its benefits. In addition to that, Motavizumab, a derivate of Palivizumab have also been developed with improved affinity (Huang *et al*., 2010).

2.14.3 Interferons

One other way of treatment that was actively pursued is the application of interferons (IFNs) (Bossert and Conzelmann, 2002). IFN /ß are induced by virus infection or double-stranded RNA in many cell types to work on inhibiting cell growth and promote apoptosis. This means that they can restrict virus spread. Therefore, treatment of RSV-infected children by IFNs is expected to help improve the health conditions of those children. Bossert and Conzelmann (2002) proved that application of IFNs /ß to infected children helped improve their clinical course but it did not prove to be effective as a therapeutic agent against RSV infection in adults.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area and population

Ibadan is the capital city of Oyo State, Nigeria and the third largest metropolitan area, by population in Nigeria. The city is located at latitude 7.3775601 and longitude 3.90591, in the northern hemisphere (with degree, minutes and seconds coordinates 7°22'39.2"N and 3°54'21.3"E). There are 11 Local Government Areas (LGAs) in Ibadan, consisting of 5 urban and six semi-urban LGAs. It has a tropical wet and dry climate, with a lengthy wet season that runs from March through October. Samples for the study were collected from March to October 2015 from apparently healthy children attending routine immunization clinics in two primary health centers (one each from Ibadan Southeast and Ido LGA) in Ibadan metropolis and showing signs of respiratory infection as well as from those seeking medical care due to respiratory infection at the Our Lady of Apostle Catholic Hospital, Oluyoro, a secondary health facility in Ibadan. Some of the patients attending Our Lady of Apostle Catholic Hospital were hospitalised while others were seen at the outpatient clinic.

3.2 Case definition

Cases of Respiratory Tract Infections (RTIs) were identified using WHO protocol for the identification of Influenza-like illnesses (ILIs) and/or Severe Acute Respiratory Infections (SARI) (WHO, 2014). Briefly, ILI was defined as a respiratory infection with acute onset, with any of the following clinical symptoms: fever (measured body temperature of ≥ 38°C) and cough and/or nasal discharge or congestion (Nyawanda *et al*., 2016) with day of onset not exceeding ten days. SARI was defined as an acute respiratory infection with clinical symptoms similar to that in ILI and severe enough to require hospitalization.

Children whose day of onset of symptoms was beyond seven days as well as those whose parental assent were declined were excluded from the study. The study

protocol was approved by the University of Ibadan/University College Hospital (UI/UCH) ethics committee with approval number UI/EC/14/0284

3.3 Collection of demographic and medical information

A structured questionnaire was used to capture the needed demographic and medical information from each participant after obtaining informed assent from the parent/care giver. Information captured include: sex, age, symptoms, day of onset of symptoms/illness etc. (Appendix 1 and 2).

3.4 Sample collection

The child was allowed to sit on the laps of the parent / guardian for calmness. Nasopharyngeal (NP) and Oropharyngeal (OP) samples were carefully collected (Kim *et al*., 2011) with different sterile Dracon/nylon tipped swab sticks with plastic shaft (Graceland Plus, Lot No.: 140903). The samples were collected, following the WHO guidelines for specimen collection (WHO, 2005).

Four hundred and sixty-two samples comprising of 231 each of Oropharyngeal (OP) and Nasopharyngeal (NP) swabs were collected from 231 participants. The NP and OP swab from same participant were put into same vial of transport medium, thereby resulting into 231 specimens tested.

3.4.1 Nasopharyngeal swab collection.

A flexible, fine-shafted polyester swab was inserted into the nostril parallel to the palate (not upwards) until resistance is encountered or the distance is equivalent to that from the ear to the nostril of the participant indicating contact with the nasopharynx. The swab was left in place for a few seconds to absorb secretions before removing. It was then slowly withdrawn with a rotating motion. The tip of the swab was put into a vial containing 2 ml of virus transport medium (VTM) and the shaft cut off.

3.4.2 Oropharyngeal swab collection.

Another sterile swab stick was used to vigorously swab both the tonsils and the posterior pharynx. The tip of the swab was put into the same vial containing 2 ml of virus transport medium and the shaft cut off.

Both the NP and OP swabs from same participants were placed into the same VTM. The samples were transported on ice to the Department of Virology, College of Medicine, University of Ibadan for processing. Where RNA extraction is not possible on same day of collection, samples were kept in -80°C until processed.

3.4.3 Viral Transport Medium (VTM) – The VTM was prepared at the Department of Virology, College of Medicine, University of Ibadan using Dulbecco's minimum essential medium (D-MEM), 2% fetal bovine serum (FBS) and 0.2% Penicilin-streptomycin.

3.5 Nucleic acid extraction

Viral RNA was extracted from the samples using the QIAamp Viral RNA Mini Kit according to the manufacturer's procedures. Nucleic acid extraction was done directly from the VTM containing both NP and OP swabs without putting in tissue culture for replication, although viral cultivation in culture could have resulted in boosting of the viral load in samples from which HRSV could not be detected due to insufficient nucleic acid as a result of very low viral load. It is however necessary to use samples without tissue culture replication to avoid any mutation in the nucleotide sequences of the isolates that could be selected for during the process of replication in tissue culture, especially on the F gene sequences that will be sequenced for analysis of monoclonal antibody resistance mutation.

3.5.1 Principle of Spin Column based nucleic acid extraction.

Spin columns contain a silica resin that binds nucleic acid from a pool of lysed specimen. The selective binding potentials of a silica-gel-based membrane and the speed of microspin are combined in the kit. The cells containing the nucleic acid of interest is first lysed with lysis buffer (AVL) which contains a chaotropic salt (guanidine thiocyanate). Two vital roles are performed by chaotropes during the

process of nucleic acid extraction. Firstly, they destabilize hydrogen bonds, van der Waals forces and hydrophobic interactions, leading to destabilization of proteins, including nucleases. Secondly, they disrupt the association of nucleic acids with water, thereby providing optimal conditions for their transfer to silica. The inactivation of RNases ensures isolation of intact viral RNA. In addition, the buffer AVL contains detergents that aid protein solubilization and cell lysis. The affinity of the viral nucleic acid to the QIAamp Mini (spin column) is further enhanced by the carrier RNA added to the lysis buffer (AVL). The addition of the carrier RNA also reduces the chances of losing the extracted RNA to degradation in the rare event that RNase molecules escape denaturation by the chaotropic salts and detergent in buffer AVL.

After adding the lysate to the column and centrifugation, the desired nucleic acids (RNA) binds to the column while protein and other cellular impurities remain in the flow-through. However, some residual proteins as well as salt residues may still remain bound to the membrane which are removed by the wash steps. The first wash involves buffer AW1 containing a low concentration of chaotropic salts to remove residual proteins. This is followed with a second wash with AW2 to remove the salts from AW1 which is crucial to getting high yields and purity as residual salt will impede the elution of nucleic acid, resulting in poor yield.

Materials

- QIAamp® Viral RNA Mini Kit (Qiagen, Hilden Germany)
- 1.5ml micro centrifuge tubes
- Sterile RNase-free pipette tips with aerosol barrier
- Vortex mixer.
- Table-top cold centrifuge
- Absolute alcohol (99 – 100% ethanol)
- Disposable, powder-free hand gloves.

Preparation of reagents.

All buffers, were equilibrated to room temperature. Reagents and buffers were prepared according to manufacturer’s instruction.

Addition of carrier RNA to buffer AVL

Buffer AVL (containing guanidine thiocyanate) was checked for any precipitate. When there were precipitates, buffer was incubated at 80°C until it dissolved. Appropriate volume of buffer AVL was added to tube containing lyophilized carrier RNA and vortexed. The dissolved carrier RNA was then transferred into the buffer AVL bottle and thoroughly mixed before use.

Preparation of buffer AW1 and AW2

Buffer AW1 contains guanidine hydrochloride. AW1 and AW2 were supplied as a concentrate. The appropriate amount of ethanol (96-100%) as indicated on the bottle was added to the buffers before using for the first time. To a 95ml concentrate of buffer AW1, 125ml of ethanol was added to make a final volume of 220ml. To a 6ml concentrate of buffer AW2, 160ml of ethanol was added to give a final volume of 226ml. The bottles were vortexed upon the addition of ethanol for thorough homogeneity.

3.5.2 Procedure of viral RNA extraction.

Viral RNA was extracted directly from the clinical specimen using QIAamp® Viral RNA Mini Kit (Qiagen, Hilden Germany) according to manufacturer's instruction. Briefly, reagents and buffers were allowed to attain room temperature. The wash buffers, carrier RNA and lysis buffer were reconstituted accordingly as recommended by the manufacturer. One hundred and forty microliters of each of the swab samples was added into labelled microcentrifuge tubes containing 560µl of reconstituted lysis buffer. The tubes were mixed by pulse vortexing for 15 seconds and incubated for 10 minutes at room temperature followed by brief centrifugation to pull down samples trapped inside the lids. Thereafter, 560µl of absolute ethanol was added into the sample and mixed by pulse vortexing, followed by brief centrifugation. Six hundred and thirty microliters of the sample solution were added into appropriately labelled QIAamp Mini spin column placed in a 2ml collection tube and centrifuged at 8000rpm (5510g) for 1 minute. The Mini spin column was transferred into a clean collection tube, the remaining sample solution was added and then centrifuged at the same condition. The Mini spin column was transferred into a clean collection tube for

the other two washing steps. Five hundred microliter of wash buffer 1 was added into the Mini spin column followed by centrifugation at 8000rpm (5510g) for 1 minute. Similarly, 500µl of wash buffer 2 was added followed by centrifugation at 14000rpm (16873g) for 3 minutes. The Mini spin column was thereafter placed in a clean, RNAse-free 1.7ml microcentrifuge tube for nucleic acid elution. Sixty microliters of elution buffer was transferred into the spin column and then centrifuged at 8000rpm (5510g) for 1 minute. The eluted RNA was either used immediately for RT-PCR or stored at -20°C until used.

3.6 cDNA synthesis

The RNA extracted from the swab samples were used as template for the synthesis of single strand complementary DNA (cDNA).

3.6.1 Principle of the cDNA synthesis assay.

The assay uses SCRIPT Reverse Transcriptase, a genetically engineered Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase with eliminated RNase H activity and increased thermal stability to synthesize first strand cDNA from an mRNA population. The enzyme is an RNA-dependent DNA polymerase that can synthesize a complementary DNA from a single-stranded RNA or DNA template. The reaction is initiated by Oligo-dT primers, Random Hexamers or sequence specific primers. While oligo-dT primers will initiate the synthesis of cDNA only from RNA with poly-A sequences, random hexamers will initiate cDNA synthesis using the entire RNA sequence present in the pool of RNA extract and specific primers will initiate cDNA synthesis of targeted RNA template.

All the necessary salts (e.g. $MgCl_2$) required to catalyze the RT activity are contained in the SCRIPT RT buffer complete. The DNA nucleotides (dATP, dTTP, dGTP and dCTP) that serve as the building blocks for the synthesis of the complementary DNA strand are contained in the dNTP mix. The DDT is a reducing agent which helps in facilitating the stability of RNA strand by breaking hydrogen bond, thereby making it impossible for RNA templates to form any secondary structure. The RNAse inhibitor helps to prevent RNAse activities known to degrade RNA template during cDNA

synthesis. The use of RNAse-free materials like powder-free gloves further enhance the prevention of RNA degradation.

3.6.2 Procedure of the cDNA synthesis assay.

First strand cDNA was generated by reverse transcriptase reaction using random hexamer primers and SCRIPT reverse transcriptase (Jena Bioscience, Germany) according to manufacturer's instruction. Briefly, 5µl of the RNA was used as the template in a 20µl reaction cocktail containing: reverse transcriptase, random hexamers, RNAse inhibitor, dNTP mix, DTT stock solution, SCRIPT RT buffer and RNAse-free water. The proportion of the constituents of the cocktail per reaction is shown in Table 3.1. The reaction mix was prepared on ice and then transferred into thermal cycler (Applied Biosystems GeneAmp® PCR system 900) and incubated at 42°C for 10 minutes, followed by 50°C for 60 minutes and then 70°C for 10 minutes to inactivate the reverse transcriptase.

3.7 Polymerase Chain Reaction (PCR).

Polymerase Chain Reaction was used for the detection of HRSV in the test samples using oligonucleotide primers that target the conserved region of the Matrix gene. In samples where HRSV were detected, the virus was subtyped by the amplification of the second hypervariable region of the G gene, using subtype-specific primers. Some HRSV-positive samples were also selected and their fusion (F) gene amplified by PCR.

3.7.1 Controls for PCR

Following approval for importation of PCR control by National Agency for Food and Drugs Administration and Control (NAFDAC), HRSV A2 (ATCC-1540) was obtained from National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG, WHO International Laboratory for Biological Standard, UK and used as positive control. PCR grade water was used as no template control in each step to validate the assay.

Table 3.1: Constituents of the 20µl reaction mix for cDNA synthesis.

Reagents	**Volume per 20µl**
RNase-free water	7µl
SCRIPT RT Buffer complete (1X)	4µl
DTT stock solution (5mM each)	1µl
dNTP Mix (500µM each)	1µl
RNase inhibitor (40 units)	1µl
Random hexamers (50pmol)	0.5µl
SCRIP Reverse Transcriptase	0.5µl
RNA template	5µl

3.7.2 Principle of Polymerase Chain Reaction

In PCR, there is geometric amplification of the starting DNA templates due to the doubling of all available DNA template at every step in a chain reaction as the name implies. The continuous geometric amplification of the DNA is made possible by specific proteins referred to as polymerases. Polymerases are enzymes that are able to couple together individual DNA building blocks to form a stretch of another DNA strand that is complementary to the mother strand. To carry out the job of DNA amplification, polymerases need a supply of the DNA building blocks called deoxynucleotide-triphosphates (dNTPs) i.e. the nucleotides consisting of the four bases adenine (A), guanine (G), cytosine (C) and thymine (T). A pair of short sequences of nucleotides that are complementary to the flanks of the region to be amplified are also required. It is unto these short sequences of nucleotides called oligonucleotides that dNTPs are attached. A DNA template to be amplified is also needed for the synthesis of new, complementary strands. If these three ingredients are supplied, the enzymes will construct exact copies of the templates. The polymerase chain reaction leverage on the ability of the polymerase enzymes (Taq polymerase - derived from *Thermus aquaticus*) to remain stable at high temperature. When a synthetic oligonucleotide is annealed to a single-stranded template at the region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3`-end to generate double stranded DNA that span the expected region.

Three major steps are involved in the PCR reaction: denaturation, annealing, and extension. In the first step, the double-stranded DNA template is heated to a temperature between 90 - 97°C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, thereby allowing the strands to unzip into single stranded amplification template. In the second step, the mixture is cooled to a temperature ranging between 50-70°C. This allows the primers to bind (anneal) to their complementary sequence in the template DNA. The specific annealing temperature is determined by the primer factors including the length and its GC percentage. The final step of the cycle involves heating to a temperature of 72°C, being the optimal temperature for DNA polymerase activities. DNA polymerase extends the primers,

adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

3.8 Test procedure.

3.8.1 Test procedure for the detection of HRSV

Five microliters of the cDNA were used as template in a 25µl PCR reaction, using Red Load Taq Master (Jena Bioscience, Germany) consisting of DNA Taq polymerase, dNTPs, KCl, and $MgCl_2$ in a premixed 5X concentrated ready-to-use solution. Detection of the presence of HRSV in the samples was done using 10pmol of a pair of primer (Table 3.2) that targets the conserved region of the viral matrix gene (Aamir *et al.,* 2013). Amplification was carried out under the following conditions: 94°C for 2 min, followed by 40 cycles of 94°C for 30s, 53°C for 30s and 72°C for 45s and then a final extension at 72°C for 5min in a conventional PCR procedure. The expected amplified products of 84bp were detected on a 2% agarose gel by electrophoresis.

3.8.2 Test procedure for HRSV subtyping.

Samples positive for HRSV were further analysed to determine the virus subtype using HRSV – A and HRSV – B specific primers targeting the second hypervariable region (HVR2) of the G gene as previously described (Aamir *et al.,* 2013) with slight modification. Samples positive for HRSV, but whose subtype could not be detected with the subtype-specific PCR protocols described above were further analysed by PCR using other sets of primers (ABG490, F164, AG655 and BG517) and conditions as previously described by Parveen et al., 2006.

3.8.2.1 Detection of HRSV A using nested PCR

The first-round PCR was performed using 5µl cDNA in a 25µl reaction cocktail containing 10pmol each of the external primer pairs (RSVA-G513-F and RSVA-F131-R) using Red Load Taq Master (Jena Bioscience, Germany) as shown in Table 3.2. Amplification was carried out in Applied Biosystems GeneAmp® PCR System 9700 at 94°C for 5 min, followed by 40 cycles, each consisting of 94°C for 30s, 58°C for 30s, and 72°C for 1min, then a final elongation step of 72°C for 10min. Five

Table 3.2: Oligonucleotide primers used for HRSV detection, HVR2 and F region amplification and sequencing.

SN	PRIMER NAME	PRIMER SEQUENCE (5'→3')	AMPLICON SIZE (bp)
1	***RT-PCR for HRSV detection***		84
	RSV – Forward*	GGCAAATATGGAAACATACGTGAA	
	RSV – Reverse*	TCTTTTTCTAGGACATTGTAYTGAACAG	
2	***RT-PCR and sequencing primers for HRSV-A G gene***		
	External Primers		583
	RSVA-G513-F*	AGTGTTCAACTTTGTACCCTGC	
	RSVA-F131-R*	CTGCACTGCATGTTGATTGAT	
	Inner Primers		391
	RSVA-G606-F*	AACCACCACCAAGCCCACAA	
	RSV-F22-R*	CAACTCCATTGTTATTTGCC	
3	***RT-PCR and sequencing primers for HRSV-B G gene***		801
	BGF*	GCAGCCATAATATTCATCATCTCT	
	BGR*	TGCCCCAGRTTTAATTTCGTTC	
4	***RT-PCR and sequencing primers for HRSV(A&B) G gene***		
	Eternal Primers (A/B)		610
	ABG490-F[ø]	ATGATTWYCAYTTTGAAGTGTTC	
	F164-R[ø]	GTTATAACACTGGTATACCAACC	
	Inner Primers		
	AG655-F[ø]	GATCYCAAACCTCAAACCAC	450
	BG517-F[ø]	TTYGTTCCCTGTAGTATATGTG	585
	F164-R[ø]	GTTATAACACTGGTATACCAACC	
5	***RT-PCR and sequencing primers for HRSV A&B F gene***		A: 1047 B: 1065
	F1-AB-FWD[#]	GGC AAA TAA CAA TGG AGT TG	
	F1-AB-REV[#]	AAG AAA GAT ACT GAT CCT G	

*(Aamir *et al.,* 2013) [ø](Parveen *et al*., 2006) #(Tapia *et al.,* 2014)

microliter of the 1st round PCR product was used as template for second round PCR. The 2nd round was performed in a 25µl reaction cocktail with 10pmol of inner primers (RSVA-G606-F and RSVA-F22-R). The cycling condition was the same as for the 1st round PCR, but for the annealing temperature, which was 53°C. The PCR product with expected band size of 391bp were detected by electrophoresis on 1.5% agarose gel and visualized using Bio-Rad Gel Doc™ XR+ System.

3.8.2.2 PCR for detection of HRSV B

Five microliters of cDNA in a 25µl reaction mixture containing 10pmol of each of HRSV-B specific primers (BGF and BGR) was prepared and heated in Applied Biosystems GeneAmp® PCR System 9700 thermal cycler under the following cycling condition: 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 63°C for 1 min, and 72°C for 1 min, then a final extension step of 72°C for 10 min. The amplified products with expected band size of 801bp were detected with 1.5% agarose gel electrophoresis and visualized using Bio-Rad Gel Doc™ XR+ System.

3.8.2.3 Other PCR for detection of HRSV A and B

Semi-nested PCR for detection of HRSV A and B

The external PCR was carried out with primers ABG490 and F164 (Parveen *et al*., 2006). Three microliters of cDNA were used in a 25µl reaction mixture containing 50pM each of the primers, 5µl of the 5X Red Load Taq Master (Jena Bioscience, Germany) and the volume was made up with PCR grade water. Amplification was carried out in Applied Biosystems GeneAmp® PCR System 9700 at 94°C for 1 min, followed by 35 cycles of 94°C for 40 s, 50°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 10 min. The amplified products with the expected band size (607/610bp and 670bp for group A/B and BA viruses, respectively) were analyzed by electrophoresis on a 2% agarose gel and visualized using Bio-Rad Gel Doc™ XR+ System.

One microliter of the ten-fold dilution of the external PCR products was used for the semi-nested PCR. Primers AG655 (for group A) and BG517 (for group B) were used as forward primers and F164 was used as the reverse primer. Amplification was carried out at 94°C for 1 min, followed by 25 cycles of 94°C for 40 s, 58°C for 45 s,

and 72°C for 45 s, with a final extension at 72°C for 10 min. The product of the nested reaction with the expected size of 450/585bp and 645bp for group A/B and BA viruses, respectively, were analyzed by electrophoresis on a 2% agarose gel and visualized using Bio-Rad Gel Doc™ XR+ System.

3.8.3 Amplification of the Palivizumab-binding site on the F gene.

Some of the HRSV – positive samples: (i) with relatively high viral copies (based on intensity on gel electrophoresis) and (ii) that were representative of the subtypes and genotypes detected in this study were selected. The region of the antigenic site II on their Fgene containing the binding site of Palivizumab was amplified by PCR for resistance mutation detection.

3.8.3.1 Test procedure for amplification of the Fusion Gene

Five microliters of cDNA in a 25µl reaction cocktail containing 10pmol of each of primers F1-AB-FWD and F1-AB-REV (Table 3.2) targeting the fusion antibody binding site was prepared and heated in Applied Biosystems GeneAmp® PCR System 9700 thermal cycler. A cycling condition which included 5 cycles of 94°C for 30 seconds, 48°C for 30 seconds and 72°C for I minute followed by 35 cycles of 94°C for 30 s, 55°C for 30 seconds, and 72°C for 1 min was used for the amplification (Tapia *et al*., 2014). The amplified products with expected band size of 1047bp for HRSV-A or 1065bp for HRSV-B were detected with 1% agarose gel electrophoresis and visualized using Bio-Rad Gel Doc™ XR+ System**.**

3.9 Gel Electrophoresis for detection of amplified PCR products

3.9.1 Principle of gel electrophoresis

Nucleic acid molecules are separated according to their molecular weight (which is related to their size in length) by the aid of an electric field where negatively charged molecules migrate towards the anode (positive) pole. The flow of the migration is determined solely by the molecular weight where small weight molecules expectedly migrate faster than larger ones. In order to visualize nucleic acid molecules in agarose gels, ethidium bromide (to be used with caution, being mutagenic) or SYBR Green

are commonly used dyes. Illumination of the agarose gels with 300nm UV light is subsequently used for visualizing the stained nucleic acids.

3.9.2 Preparation of Tris Borate EDTA (TBE) buffer

The TBE buffer used for the preparation of agarose gel and as the electrolyte in the electrophoresis setup was prepared from the constituent salts in appropriate proportion.

Materials needed for TBE preparation.

- Tris base
- Boric acid
- Stock solution of 0.5M EDTA
- Distilled water

Procedure for TBE preparation.

A 1x solution was prepared by dissolving 10.8g of tris base and 5.5g of boric acid in 900ml of distilled water. Four milliliters of 0.5M EDTA was added and the volume was adjusted to 1 liter.

3.9.3 Gel electrophoresis

Materials

- Agarose powder
- TBE buffer
- Conical flask
- Microwave oven
- Gel Staining Dye
- Gel casting tray and combs

3.9.3.1 Test procedure for gel electrophoresis

A 0.5x TBE buffer was prepared. The appropriate gram of agarose powder (2g for 2% gel etc.) was weighed into a conical flask and 100ml of the prepared 0.5x TBE was

added. The mixture was heated in an oven until the powder was completely dissolved. The slurry was allowed to cool down to about 55°C and 5µl of EvaGreen® Fluorescent Gel Stain (Jena Bioscience, Germany) was added while still in molten state. The molten agarose was poured into gel tray with gel comb fixed in place. The gel was left for about 30minutes to set and the comb carefully removed. The gel, together with the tray was fix into position in the gel tank and 05x TBE buffer was poured into the tank up to the marked level. Five microliters of the amplified PCR products were dispensed into the wells in the gel immersed in TBE using micropipette. Five microliter of an appropriate DNA ladder (low range or mid-range) was also dispensed into the central well in the gel. The tank was connected to electrophoretic power source and ran at 400AM, 120Volts for 30 minutes. The DNA bands were visualized using Bio-Rad Gel Doc™ XR+ System**.**

3.10 Purification of amplified PCR products

Spin-column based PCR purification kit produced by Jena Bioscience (Germany) was used for the clean-up of the amplified PCR products in preparation for the sequencing reactions.

3.10.1 Principles of spin column purification.

The spin column purification kit utilizes silica gel membrane which binds up to 10ug of DNA fragment selectively with the aid of specialized binding buffers. Nucleotides, oligos (<40-mer), enzymes, mineral oil and other impurities do not bind to the membrane and are washed away. The DNA fragments can thereafter be eluted off the column in small volume and used in downstream applications.

Materials.

- PCR purification kit (Jena Bioscience, Germany)
- 1.5ml microtubes
- Sterile RNase-free pipette tips with aerosol barrier
- Vortex mixer.
- Table-top cold centrifuge
- Absolute alcohol (99 – 100% ethanol)

- Isopropanol
- Disposable, powder-free hand gloves.

Preparation of reagents.

- All buffers, were equilibrated to room temperature. Before start, absolute alcohol was added to the washing buffer as indicated on the bottle.

Sample preparation.

- Three volumes of binding buffer and 2 volumes of Isopropanol was added to the PCR sample.

Column Activation.

- The Spin Column was placed into a 2-ml collection tube, 100µl of activation buffer was added and the column centrifuged at 10,000g for 30sec.

Column Loading.

- The prepared sample mixture was added into the activated spin column and centrifuged at 10,000g for 30sec. The flow-through was then discarded.

Column washing.

- The loaded spin column was placed into the used 2ml tube and 700µl of washing buffer was added.
- The column was centrifuged at 10,000g for 30sec and the flow-through discarded.
- Another 700µl of washing buffer was added to the spin column and the centrifugation step was repeated.
- After discarding the flow-through, the column was further centrifuged for 2min to remove residual washing buffer.

Elution.

- The spin column was placed into a clean 1.5 ml microtube and 50µl of elution buffer or double distilled water was added to the center of the column membrane.
- After a 1min incubation at room temperature, the column was centrifuged at 10,000g to elute the DNA.

3.11 Quantification of purified PCR product

The purified products of PCR amplification were quantified using the PicoGreen dsDNA quantitation assay to determine if the products contain sufficient quantity of DNA for downstream sequencing reaction.

Materials.

- 1X TE buffer
- 200ng/ml DNA standard
- Eppendorf tubes
- PicoGreen dye

3.11.1 Principle of PicoGreen dsDNA quantitation assay.

PicoGreen (PG) is an ultra-sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA) as little as 1 ng/mL. The free dye does not fluoresce, but upon binding DNA, PG fluorescence increases >1000-fold and this is proportional to the quantity of DNA present. PG has the ability to strongly bind not only to highly polymeric DNA but also to short duplexes <20 bp, likewise exhibiting a sensitivity in the picogram range.

3.11.2 Procedure of the PicoGreen dsDNA quantitation.

Preparation of calibration reagents.

100µl of the 200ng/ml DNA standard was aliquoted into a clean Eppendorf tube labelled “STANDARD”.

100µl of 1X TE was aliquoted into a clean Eppendorf tube and labelled “BLANK”.

Aliquot of PicoGreen was thawed in the dark and mixed gently by flicking. A working solution of the PG (wPG) was prepared by combining 5µl of PG with 995µl 1X TE. The preparation was vortexed and stored in the dark.

100µl of the wPG was added each to the STANDARD and the BLANK, vortexed and incubated for 2 – 5minutes in the dark.

50µl each of the STANDARD and BLANK was carefully aliquoted into separate clean mini-cuvettes and bubbles avoided. If any, bubbles were tapped out.

The Turner BioSystems (TBS - 380) fluorometer was turned on, while ensuring that the side of the cuvette mini-adaptor labelled BLUE was facing out.

Fluorometer calibration.

The <STD VAL> button was pressed. Where the LED display did not read 100, the up/down arrow was used to set it to 100. Then <ENTER> button was pressed followed by the <CAL> button. To start the calibration, the <ENTER> button was pressed, and the direction to insert the BLANK was followed then the <ENTER> button pressed. The direction to insert STANDARD was also followed, then <ENTER> button pressed. Upon completion of the calibration, the <ENTER> button was pressed to accept the calibration.

Quantification

An Eppendorf was labelled for each of the samples to be measured and 99µl of 1X TE was aliquoted into the tubes. Into each tube, 1µl of the experimental DNA was added and vortexed. 100µl of wPG was added into each of the tubes, vortexed and incubated in the dark for 2 – 5 minutes. From each of the sample preparation, 50µl was pipetted into clean mini cuvette while ensuring bubbles was avoided. To read each sample, the mini cuvette was placed into the fluorometer then <SET> was pressed. If the LED light did not read “discrete”, the up and down arrows were used to set it then <ENTER> was pressed after which <READ> was pressed. After waiting for few seconds, the reading displayed on the LED was taken.

3.12 DNA sequencing

Ten to fifteen microliters of the purified PCR products with DNA quantity ≥10ng/µl were aliquoted and used for the Sanger sequencing procedure. The same primers used for the PCR amplifications were used to sequence the DNA in the forward and the reverse directions on an ABI Prism 3130 genetic analyzer (Applied Bio Systems). The sequencing reactions were carried out at Inqaba Biotec Laboratory in South Africa.

3.12.1 Principle of DNA sequencing.

The chain termination method of DNA sequencing, otherwise known as Sanger sequencing was used in this study. This method is based on the principle that single-stranded DNA molecules with just a single nucleotide difference in length can be separated from one another using polyacrylamide gel electrophoresis.

The DNA to be sequenced, is first prepared as a single-stranded DNA. Next, a short oligonucleotide is annealed, or joined, to the same position on each template strand. The oligonucleotide acts as a primer for the synthesis of a new DNA strand that will be complimentary to the template DNA.

This technique requires that four nucleotide-specific reactions; one for each of the nucleotides (G, A, C, and T) be performed on four identical DNA samples. The four sequencing reactions require the addition of all the components necessary to synthesize and label new DNA, including:

- A DNA template;
- A primer tagged with a mildly radioactive molecule or a light-emitting chemical;
- DNA polymerase--an enzyme that drives the synthesis of DNA;
- Four deoxynucleotides (G, A, C, T); and
- One dideoxynucleotide, either ddG, ddA, ddC, or ddT.

After the first deoxynucleotide is added to the growing complementary sequence, DNA polymerase moves along the template and continues to add base after base. The strand synthesis reaction continues until a dideoxynucleotide is added, blocking further elongation. This is because dideoxynucleotides are lacking the 3'-hydroxyl group, needed to form a connection with the next nucleotide. Only a small amount of a dideoxynucleotide is added to each reaction, allowing different reactions to proceed

for various lengths of time, until, by chance, DNA polymerase inserts a dideoxynucleotide, terminating the reaction. Therefore, the result is a set of new chains, all of different lengths.

To read the newly generated sequence, the four reactions are run side-by-side on a polyacrylamide sequencing gel. The family of molecules generated in the presence of ddATP are loaded into one lane of the gel and the other three families, generated with ddCTP, ddGTP, and ddTTP, are loaded into three adjacent lanes. After electrophoresis, the DNA sequence can be read directly from the positions of the bands in the gel.

3.13 Phylogenetic Analysis

Electropherograms of the sequenced samples were assembled, and consensus sequences were generated using CLC Main Workbench 7.6.2 software (CLC bio, Cambridge, MA, USA). Multiple sequence alignments were conducted and then phylogenetic analysis with sequences of the HVR2 of the G gene using Clustal-W 1.6 method in MEGA 5.05 software (Tamura *et al.,* 2011). The search for sequences that were similar to those generated from this study was carried out using the Basic Local Alignment Search Tool (Blastn) from the NCBI BLAST web portal. Reference strains of existing HRSV-A and HRSV-B genotypes previously published and deposited in the GenBank (http://www.ncbi.lm.nih.gov) (as at January 16, 2017) were retrieved and used for phylogenetic tree construction together with the sequences generated in this study. The trees were generated using the neighbor-joining model, and the statistical significance of the tree topology tested by bootstrapping with 1,000 replicates on MEGA 5.05. Identical sequences were detected using ElimDupes (http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html). Using MEGA 5.05 and BioEdit sequence alignment editor, pairwise nucleotide distances were estimated for comparison of the differences within genotypes of subgroup A and B.

Deduced amino acid sequences were translated with the standard genetic code using MEGA 5.05. For comparison and identification of amino acid substitution, the second hypervariable region of the G protein of the Nigeria (NGR) sequences of HRSV-A and HRSV-B, generated in this study were aligned with prototype ON1 strain ON6-1210A (GenBank accession number JN257693) for HRSV-A and prototype BA strain BA4128/99B (GenBank accession number AY333364) for HRSV-B.

3.14 Amino acid glycosylation site analysis.

Potential N-glycosylation sites were predicted if the encoded amino acids were N-X-T/S, where X was not a proline and accepted if the glycosylation potential was ≥0.5 using NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc). The O-glycosylation residues were predicted using the NetOGlyc 3.1 server (http://www.cbs.dtu.dk/services/NetOGlyc-3.1/) and accepted if the G-score was ≥0.5 (Jelenius *et al.,* 2005).

3.15 Selective pressure analysis

Positively and negatively selected sites among the ON1 and BA9 strains were calculated in order to evaluate the selective pressure on the HVR2 of the G glycoprotein.
Out of the 906 ON1 sequences available in GenBank as at 10th January, 2017, 529 with identical sequences were detected and eliminated using ElimDupes (http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html). The 377 unique sequences (including the Nigeria sequences) were then used for the selective pressure analysis. Three sequences, being identical, were further removed by Datamonkey, hence, 374 unique global sequences were analysed. The synonymous (dS) and nonsynonymous (dN) substitution rates at each codon was calculated using Datamonkey (http://www.datamonkey.org). Three methods: Fixed Effects Likelihood (FEL), Single Likelihood Ancestor Counting (SLAC) and Internal Fixed Effects Likelihood (IFEL) (Pond *et al.,* 2006; Pond and Frost 2005), with an HKY 85-nucleotide substitution bias model were used to identify the diversifying selection. A P-value <0.1 was used to define the sites that were positively (dN>dS) or negatively (dN<dS) selected.

The procedures used for the ON1 was repeated to detect the selective pressure in the BA9 genotypes. Two hundred and four unique BA9 sequences were used for the analysis, having filtered out eighteen and five sequences that were exact duplicates of other sequences contained in the 204 sequences. The duplicate sequences (including NGR/OR1/15-RSVB and NGR/OR75/15-RSVB from this study) were detected and one each of the duplicates were removed using ElimDupes

(http://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html) and datamonkey (http://www.datamonkey.org) respectively.

3.16 Disease severity scoring of HRSV infection

The severity of infection due to HRSV in the study participants was scored according to the methods of Gern *et al.,*, 2002; Lemanske *et al.,*, 2005 and as used by Houben *et al.,*, 2010. The scoring of the disease severity is shown in Table 3.3.

3.17 Isolate identification name

The isolates were given unique name that depicts features of the isolates including the country, study site and sample number in the site, year of sample collection and the HRSV subtype. Each of the features used in naming the isolates were abbreviated as shown below:

Country: Nigeria – NGR

Study sites: Our Lady of Apostle Catholic Hospital, Oluyoro – OL

Primary Health Center, Oranyan – OR

Primary Health Center, Omi-Adio – OA

3.18 Data analysis

Statistical analyses were done using SPSS20 software. Chi square was used to test the level of significance of differences in proportions of various demographic and clinical features and p-value of < 0.05 was considered statistically significant.

Table 3.3: Severity score attributed to symptoms of respiratory infection.

Item	**Severity score**
Fever (≥38°C)	1
Cough	1-2-3
Rhinorrhea	1-2
Hoarseness	1
Duration of illness >4days	1
Apnea	3
Wheezing	5
Cyanosis	5
Retractions	5
Tachypnea	5
Severity score (sum)	**0-31**

NB: Severity of infection is proportional to the summation of the severity scores of symptoms manifested. 1 – 4 indicates least severity, 5 – 8 is moderate severity while ≥9 implies high severity.

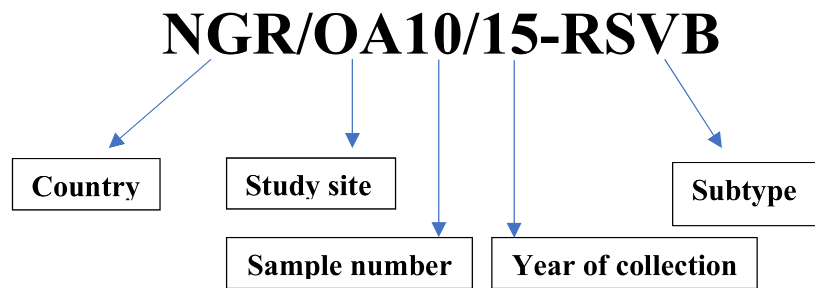


Figure 3.1: Isolate identification name.

CHAPTER FOUR

RESULTS

4.1 Characteristics of the study participants

Of the 231 participants, 150 (64.9%) were those attending Primary Healthcare Centers (PHCs), while 81 (35.1%) were participants seeking care for respiratory tract infection in a Secondary Healthcare Facility (SHF). The demographic characteristics of the study participants are shown in Table 4.1. The ages of the participants ranged between 1 and 72 months with the mean age of 9.76 months. The maximum age of the participants attending PHCs was 12 months. In all, children, aged 0 - 6 months constituted the majority [128 (55.4%)] of the study participants. The participants were almost equally spread relative to their gender with 120 (≈52%) and 111 (≈48%) being male and female respectively. The male to female ratio was 1.08.

4.2 HRSV detection

Figure 4.1 shows the agarose gel electrophoresis image of HRSV-positive PCR products. Overall, HRSV was detected in 41 (17.7%) of the study participants (Table 4.2). The HRSV positivity detected among the apparently healthy participants and the participants seeking care for respiratory infection; 8.7% and 34.6% respectively were significantly different ($p<0.01$). However, the difference in HRSV positivity between male and female participants was not significant ($p=0.558$).

Four and two of the participants seeking care for respiratory infection were hospitalised on clinical diagnosis of bronchopneumonia and bronchiolitis respectively. Human respiratory syncytial virus was detected in all the participants hospitalised on account of bronchopneumonia and in one of the two participants with bronchiolitis.

Table 4.1: Demographic characteristics of the study participants.

	PHC (Apparently healthy participants)	**SHF** (Seeking care for respiratory infection)	P value
Gender, n (%)			>0.05
Male	75 (50)	45 (55.6)	
Female	75 (50)	36 (44.4)	
TOTAL	150	81	
Age in months, mean ± SD	4.97±6.49	18.63±15.32	<0.001
Age group, n (%)			
0 – 6 months	114 (76.0)	14 (17.3)	
7 – 12 months	36 (24.0)	38 (46.9)	
13 – 24 months	0 (0.0)	14 (17.3)	
>24 months	0 (0.0)	15 (18.5)	
TOTAL	150	81	

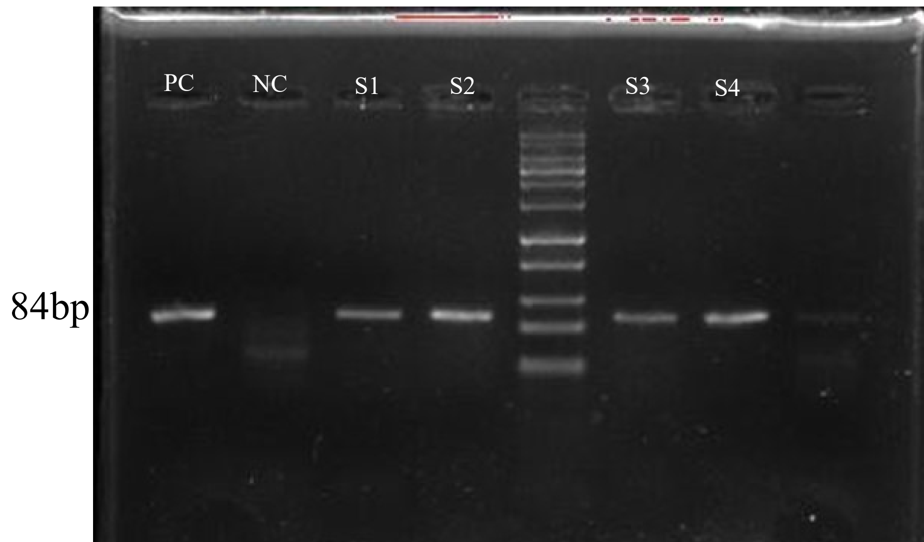


Plate 4.1: Agarose electrophoresis image showing HRSV-positive PCR products with band size of 84bp. Low range DNA Ladder (Jena Bioscience, Germany) was used to detect the expected DNA size. PC= Positive Control, NC= Negative Control, S1 – S4 were test samples.

Table 4.2: Detection of HRSV among the studied population.

	Number tested	Number (%) Positive	P - value
Health Status			<0.01
Apparently healthy	150	13 (8.7)	
Seeking health care	81	28 (34.6)	
Total	231	41 (17.7)	
Gender			>0.05
Male	120	23 (19.2)	
Female	111	18 (16.2)	
Age Group			0.002
0 – 6 months	128	15 (11.7)	
7 – 12months	74	13 (17.6)	
13 – 24months	14	6 (42.9)	
>24months	15	7 (46.7)	

4.3 Symptoms observed in study participants.

Among the study participants, cough was the most common symptoms observed. All participants with HRSV-positive samples were seen to present with cough as well as 84.7% of HRSV-negative participants (Table 4.3). Apnea and wheezing were not common symptoms, howbeit, they were significantly observed in participants with HRSV positivity.

4.4 HRSV subtypes detected

Both HRSV subtypes A and B were detected among the study population. Overall, subtype A was the predominant subtype, being 53.7% of the total HRSV detected. However, 8 of the 41 HRSV-positive samples could not be subtyped. No case of coinfection of an individual with HRSV A and B was found. HRSV-A was also the predominant subtype detected among participants who were seeking care for respiratory infection, while subtype B was predominant among apparently healthy participants. (Table 4.4). All the HRSV detected from the hospitalised participants, with clinical diagnosis of bronchopneumonia and bronchiolitis were subtype A.

The highest disease severity score recorded was associated with HRSV – A infection (Table4.5).

4.5 Sequence alignments and phylogenetic analysis

The representative image of the electropherogram from the Sanger Sequencing is shown in Figure 4.2. Fourteen out of the 22 (65.6%) subtype A and all of the subtype B samples with appreciably high DNA yield (39.9 ng/5µl – 260.0 ng/5µl) were successfully sequenced and analyzed. Other samples with lower DNA yield (3.007 ng/5µl – 21.7 ng/5µl) could not be successfully sequenced.

Nucleotide alignment of the sequences in MEGA software showed that 11 of the 14 HRSV A had 72 nucleotide insertions (Figure 4.3) between positions 849 and 850 relative to the prototype A2 strain (accession number M11486) while all the HRSV B had 60 nucleotide insertions (Figure 4.4).

Table 4.3: Distribution of HRSV by observed symptoms.

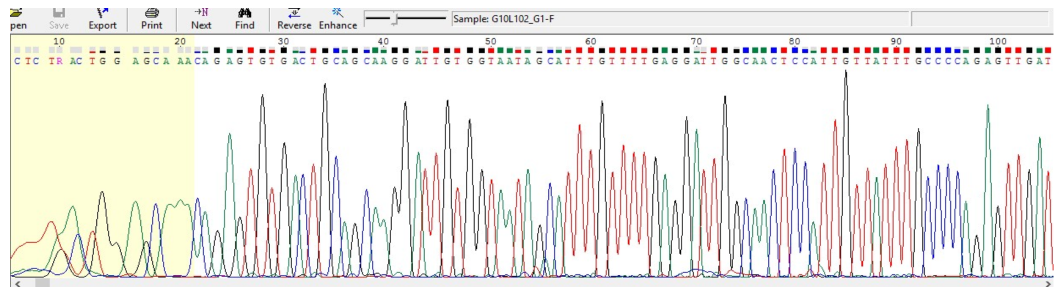
Symptoms	**RSV - positive N (%)**	**RSV - negative N (%)**	**P - value**
Cough	41 (100)	161 (84.7)	0.007
Rhinorrhea	34 (82.9)	164 (86.3)	0.574
Fever	24 (58.5)	90 (47.4)	0.195
Apnea	7 (17.1)	13 (6.8)	0.035
Wheezing	5 (12.2)	3 (1.6)	0.001
Nasal congestion	2 (4.9)	56 (29.5)	0.001

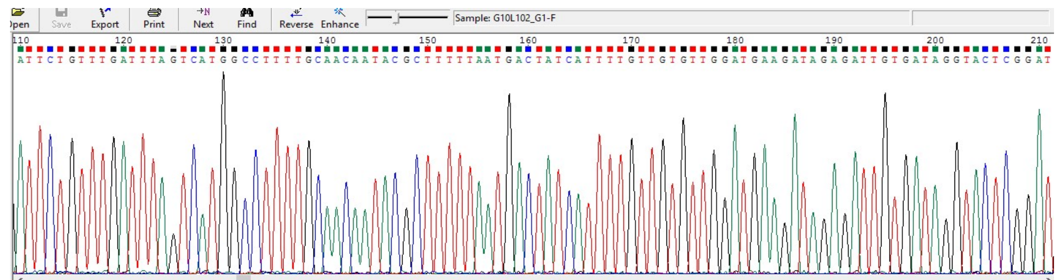
Table 4.4: HRSV subtypes status and clinical diagnosis.

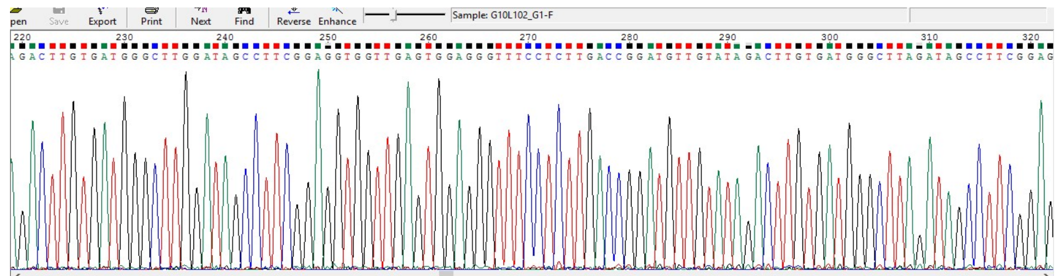
	HRSV SUBTYPES			P value
	HRSV positive	Subtype A	Subtype B	
Health facility				<0.001
Apparently healthy	13	2 (15.4)	10 (77.0)	
Seeking health care	28	20 (71.4)	1 (3.6)	
Total	41	22 (53.7)	11 (26.8)	
Clinical Diagnosis				
Bronchopneumonia	4	4 (100)	0 (0)	
Bronchiolitis	1	1 (100)	0 (0)	

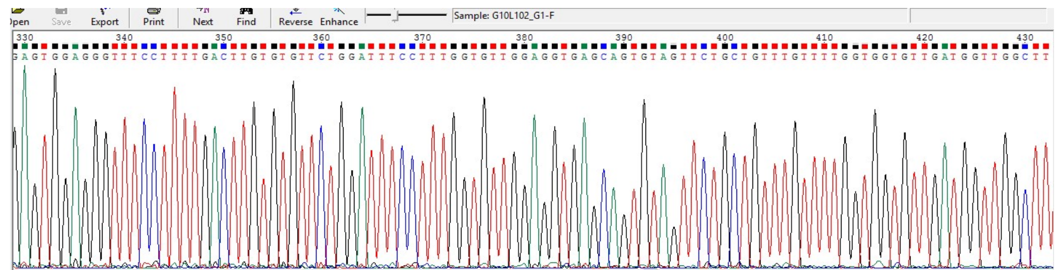
Table 4.5: HRSV types by disease severity scoring.

SEVERITY SCORE	**HRSV TYPE $P>0.05$ n (%)**		
	No Tested	**HRSV-A**	**HRSV - B**
3	19	12 (63.2)	4 (21.1)
4	12	5 (41.7)	4 (33.3)
6	2	2 (100.0)	0 (0.0)
7	1	0 (0.0)	1 (100.0)
8	1	1 (100.0)	0 (0.0)
9	4	1 (25.0)	2 (50.0)
11	2	1 (50)	0 (0.0)
Total	41	22 (53.7)	11(26.8)









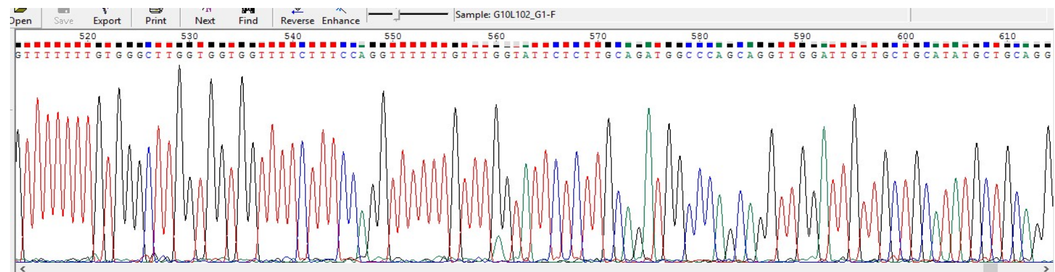


Plate 4.2: Electropherogram of the sequenced G gene of isolate NGR/OL102/15-RSVA.

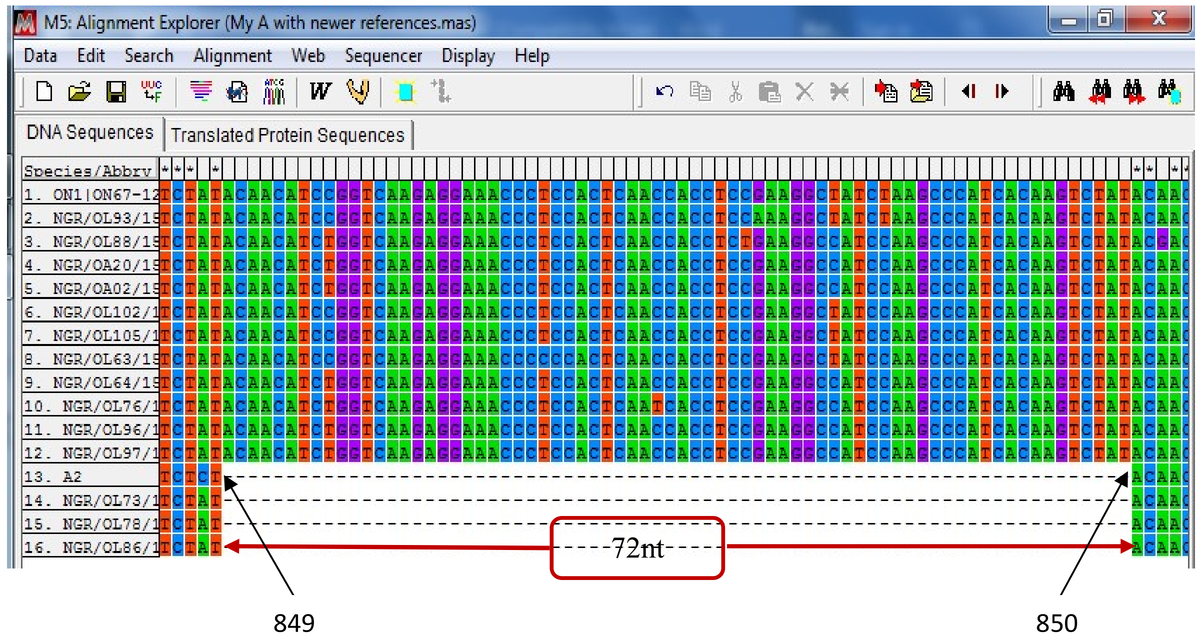


Plate 4.3: Image of nucleotide sequence alignment of HRSV A on MEGA tools. The prototype ON1 was shown with serial number 1 on the left side while the prototype A2 is numbered 13. The alignment showed 72 nucleotides inserted within position 849 and 850 relative to the prototype A2.

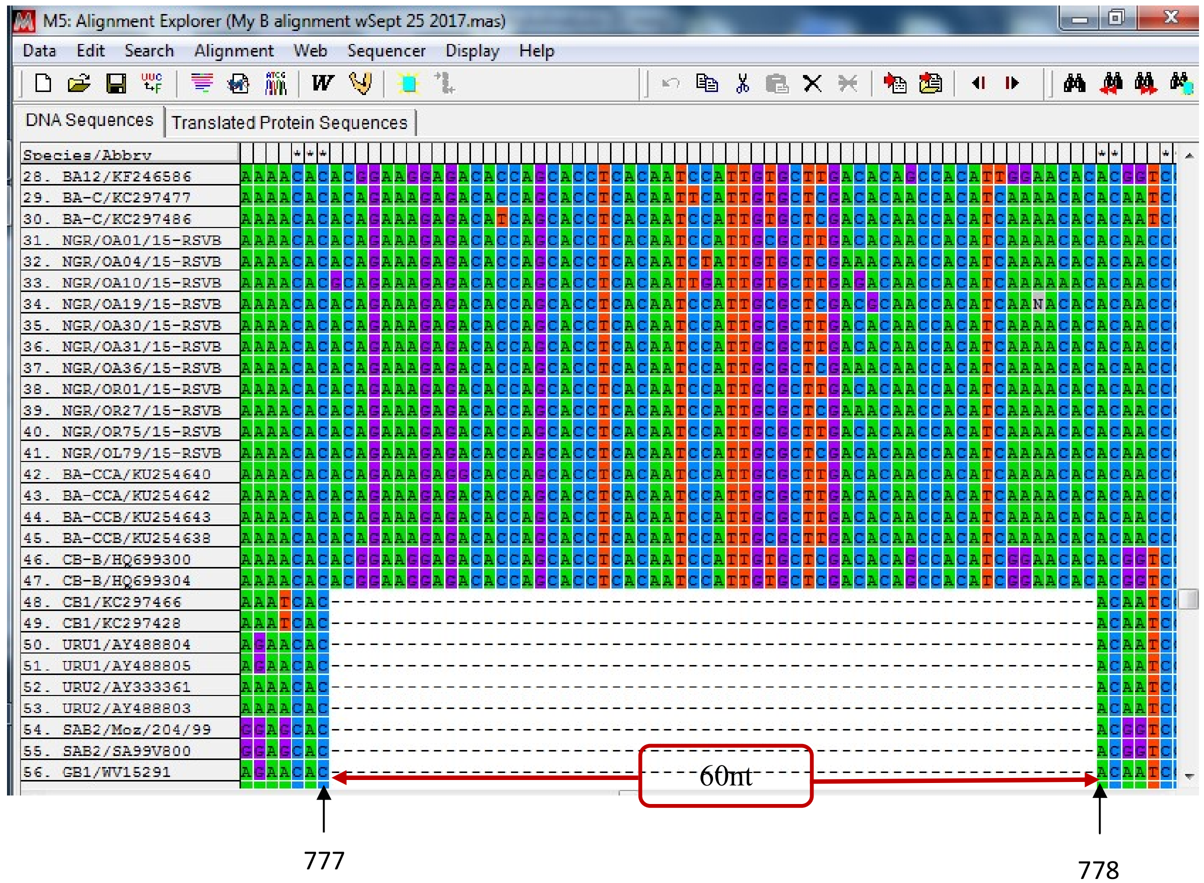


Plate 4.4: Image of nucleotide sequence alignment of HRSV B on MEGA tools. The alignment showed 60 nucleotides inserted within position 777 and 778 relative to the prototype GB1.

The phylogenetic analysis showed that the HRSV A with 72nt insertion clustered with the ON1 genotype (Figure 4.5), while the other 3 HRSV-A sequences clustered with strains previously assigned to genotype NA2. All HRSV-B sequences obtained clustered with strains previously assigned to genotype BA (Figure 4.6).

The similarity in the sequences of the HRSV-A ON1 isolates from Nigeria and the prototype ON1 reference strain (accession number JN257693) ranges between 96.5% and 98.8% at the nucleotide level and 92.7% to 97.2% at the amino acid level respectively (Table 4.6). These showed that the HRSV-A were truly ON1 genotypes. The HRSV-A NA2 genotype from Nigeria had 92.3% to 92.5% and 77% to 78.2% similarities with the prototype A2 strain (accession number M11486) at the nucleotide and amino acid level respectively. This relatedness showed that the isolates belonged to the NA2 genotype. The level of sequence similarities in the isolates belonging to the NA2 genotype appeared to be more than those in the ON1 genotype as shown in their p-distance. The overall nucleotide p-distance between the ON1 sequences from Nigeria was 1.8% while that for the NA2 genotype was 0.8%.

The HRSV-B from Nigeria, belonging to the BA genotype had 86.3% to 95.2% and 84.9% to 92.5% similarities with the prototype BA (accession number AY333364) at the nucleotide and amino acid levels respectively. The isolates belonging to the BA genotypes had the least sequence similarity as revealed in their p-distance. The overall nucleotide p-distance between the BA sequences from Nigeria was 5.6%.

4.6 Deduced amino acid sequence analysis

In Figure 4.7-A, the deduced amino acid sequences of HRSV-A ON1 from Ibadan, Nigeria as well as ON1 sequences from other parts of the world aligned with the prototype ON1 strain was shown. The deduced amino acid sequences of the NA2 from Ibadan, Nigeria aligned with sequence of the prototype NA2 was shown in Figure 4.7-B. In the ON1 sequences, the 72 nucleotides (24 amino acids) insertions culminated into an extension in the amino acid from a length of 297 (as found in the prototype A2 strain) to 321. The amino acid insertions in the ON1 isolates from this study, unlike the prototype ON1 strain (JN257693), are not exact duplicates of the preceding 23aa region. Different amino acid substitutions were identified in both

GA1|NY108
GA1|MO48
100
SAA1|SA97D669
SAA1|SA98V603
GA5|BE-1556-01
GA5|AL19556-3
GA4|CH09
GA4|BE-8078-92
GA7|SA99V360
GA7|CN1973
GA6|NY20
GA6|AL19452-2
GA3|MO16
GA3|CN2395
NA4|KJ130577
NA4|KC297374
GA2|AL19376-1
GA2|TX69564
NA3|KC297260
NA3|KJ130599
NA1|PG/09/12
NA1|ON160-0111A
NGR/OL78/15-RSVA
NGR/OL73/15-RSVA
NGR/OL86/15-RSVA
CB-A|HQ699281/CB929/10
CB-A|HQ699278 -CB824/09
NA2|JX513386
NA2|JX513384
NA2|JX513383
NA2|JX513371
ON1|AB754590-Japan
ON1|KF246641-India
ON1|KF057865-Croatia
ON1|KC858211-Italy
ON1|AB700370-Japan
ON1|KF587959-Kenya
ON1|AB808774-Japan2
ON1|AB808757-Japan
ON1|KF246640-India
ON1|AB761610-Japan
ON1|KC858245-Italy
ON1|JX912364-Germany
ON1|KJ672468-USA
NGR/OL97/15-RSVA
NGR/OL88/15-RSVA
NGR/OL64/15-RSVA
NGR/OA02/15-RSVA
NGR/OA20/15-RSVA
NGR/OL96/15-RSVA
NGR/OL76/15-RSVA
NGR/OL102/15-RSVA
NGR/OL63/15-RSVA
NGR/OL105/15-RSVA
NGR/OL93/15-RSVA
ON1|JX627336-Korea
ON1|KM873424-PHILP
ON1|JX256871-Malaysia
ON1|ON67-1210A
ON1|KF030154-Latvia
ON1|JX988448-Italy
ON1|JX912357-Germany
ON1|KC559440-China
ON1|JX988452-Italy
ON1|KJ627264-PERU
ON1|JX885730-SA
ON1|KC461212-China
ON1|KC559442-China
ON1|ON1|BJ/40507
ON1|ON1|BJ/39979
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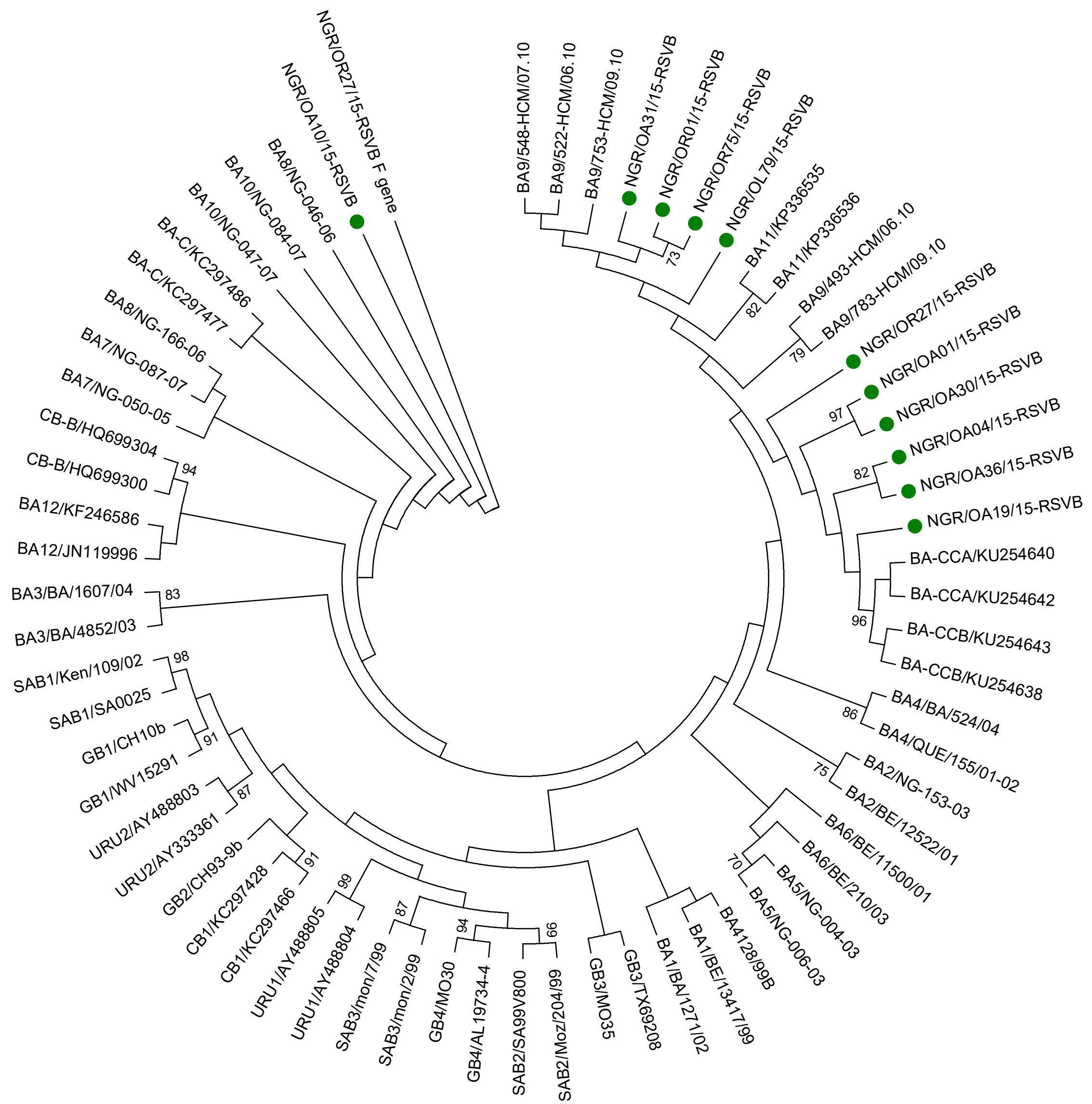

Figure 4.2. Phylogenetic tree of the second hypervariable region of the G gene of HRSV B. Genotypes represented by the reference strains are indicated before the strain ID. The genotypes circulating in Ibadan, Nigeria are indicated by solid circles. Multiple sequences alignment and phylogenetic tree were constructed using Clustal W and neighbor-joining algorithm in MEGA 5.05 software. Statistical significance of the tree topology was tested by 1000 bootstrap replication. Only bootstrap values above 70% are displayed at the nodes.

Table 4.6: Relatedness between Nigeria isolates and the prototype sequences.

Similarity index	Percentage score
Overall p distance btw NGR	
ON1	1.8
NA2	0.8
BA	5.6
Similarity with prototype (nt)	
ON1	96.5 – 98.8
NA2	92.3 – 92.5
BA	86.3 – 95.2
Similarity with prototype (aa)	
ON1	92.7 – 97.2
NA2	77.0 – 78.2
BA	84.9 – 92.5

copies of the 23aa duplicate when compared to the prototype ON1 strain (Figure 4.7 and Table 4.7). These differences in amino acid substitutions include: E262K common to all and L298P common to all but one ON1 strains found in this study. G272S was found in three strains, L274P in seven of the 11 ON1 strains, while L289P and T292I were found in single but different strains.

Upon comparison of the NA2 strains with the reference A2 strain, specific amino acid substitutions were identified in the Nigeria strain (Table 4.7). The amino acid substitution P310L is within the antigenic site at amino acid position 283 – 291 relative to HRSV A2, which is equivalent to the aa positions 283 and 308 – 315 in Figure 4.7

The deduced amino acid of the HRSV-B strains from this study, like that of the prototype BA strain has 20aa insertion (Figure 4.8), resulting in increased length of 312 in some of the strains and 319 in others instead of the 296aa length found in other genotypes of subtype B. The regions of the 20aa duplicates found in this study are not exact repeats as obtained in the prototype BA strain. When compared to the prototype BA strain, some mutations were found (Table 4.7) including T270I and I281T common to all the Nigerian BA strains.

A

B

Figure 4.3. Alignment of deduced amino acid sequences of 2nd hypervariable region of HRSV-A strains. A) Alignment of HRSV-A genotype ON1 sequences from different parts of the world. Alignments are shown and residues numbered relative to sequences of prototype ON1 strain ON67-1210A (GenBank accession no. JN257693). The strains from this study are highlighted in green. The country of isolation of other strains used in the alignment are highlighted in yellow. Identical residues are indicated by dots and stop codons by asterisks. Potential N-glycosylation sites (N-X-T/S, where X is not a proline) are indicated by gray-shaded rectangles. Potential O-glycosylation sites of the prototype ON1 strain are indicated by unfilled circles, while black circles indicated the predicted O-glycosylation sites common in all Nigeria strains. Other predicted O- glycosylation sites, not found in all the strains in Nigeria are underlined. The two copies of the 23 amino acids duplicated sequences are framed by rectangles. B) Alignment of HRSV-A subtype NA2 from this study. Alignments are shown relative to the sequence of prototype strain A2 (GenBank accession number M11486). The identifier of strains from this study are highlighted in green. Residues are numbered relative to the amino acid sequences of prototype ON1 strain ON67-1210A (GenBank accession no. JN257693). Identical residues are indicated by dots, alignment gaps by dashes and stop codons by asterisks. Potential N-glycosylation sites (N-X-T/S, where X is not a proline) are indicated by shaded rectangles. Potential O-glycosylation sites of the prototype A2 strain were indicated by unfilled circles, while black circles indicated the predicted O-glycosylation sites in the Nigeria NA2 strains.

Table 4.7: Amino acid substitutions found on G - HVR2 of isolates when compared to the prototype strains.

Genotype	Substitution
ON1	L226H, G232R[z], T241I, I243S*, T245I[#], T249A[b],T259K, E262K*, G272S[bz], L274P, L289P[a], T292I[a], E295K [a], Y297H[a], L298P*[a]
NA2	S222P, P226L, E233N[z], P234L, I244R, L258H[z], M262E, F265L[z], S269T[z], N273Y[zg-], P274L[g-], S280Y, P286L[z], P289S[z], S290P[z], P292S, P293S, P296T [g+]
BA	D215V, P216L, K218T, L219P, T222P, L223P*, E226K[s], P231A/S, R242K, D243E, T244I, S247P, S249F, T254I/R, T260A [a],S269L [a], T270I*[a], V271A [a], D273E [a], T274A [a],H279N [a], I281T*, L287Y, T290A, N293Y, E305K, P306S, T312N [g-], K314R

Mutations that occurred on antigenic sites were denoted with superscript 'z', 'a' denoted mutation within region of amino acid insertion, '*' showed mutations common to the isolates. Mutations found in samples from apparently healthy participants were denoted with '#', 'b' showed mutations found in samples from participants with clinical diagnosis of bronchopneumonia, 's' denotes mutations found in samples of sick participants. Mutations denoted with 'g- 'resulted in loss of N-glycosylation with that denoted with 'g+' led to its gain.

FIGURE 4.4. Amino acid alignments of the 2nd hypervariable region of the G protein from HRSV-B. Residues are numbered relative to the sequences of prototype BA4128/99B (GenBank accession number AY333364). Identical residues are indicated by dots; gaps are indicated by dash and stop codons by asterisks. The two copies of the duplicated 20-amino acids are framed by rectangles. Potential N-glycosylation sites (NXT/S, where X is not a proline) are indicated by shaded rectangles. Potential O-glycosylation sites of the prototype BA strain are indicated by unfilled circles, while black circles indicated the predicted O-glycosylation sites common to all Nigeria strains. Other predicted O – glycosylation sites that are not found in all the strains from Nigeria are underlined. The genotypes are shown on the right by brackets

4.7 Glycosylation sites

Similar to the reference ON1 strain, all Nigeria isolates had two amino acid positions (237 and 318) predicted to carry N-linked sugars (Figure 4.7). However, the position and frequency of predicted O- glycosylation with G-score >5.0 slightly vary from those in the ON1 reference strain. The frequency of O – glycosylated sites in the isolates range between 38 and 41. All the amino acid positions with O- glycosylation potentials in the prototype ON1 strain were also predicted O- glycosylation sites in the Nigeria strains. Predicted O- glycosylation sites, not found in the prototype ON1 strains were seen in some of the Nigeria ON1 strains.

The three NA2 strains had three predicted N- glycosylation sites at amino acid positions 237, 251 and 318 (numbered relative to the prototype ON1) (Figure 4.7). The amino acid substitution N273Y and P274L led to the loss of N glycosylation site while substitution P320T resulted in acquisitions of an additional N glycosylation site in the Nigerian strains relative to the prototype A2. Thirty-three predicted O-glycosylation sites were found in the NA2 strains. All these sites were also found on the reference A2 strain except four positions i.e. aa positions 313, 316, 317 and 320. Three of the positions with predicted O- glycosylation found in the reference A2 strain were not found on the Nigerian NA2 strains. Two of these were due to mutations (S222P and S280Y) while the other position had G- score <5.0.

Seven of the eleven BA isolates found in this study, (including 6 isolates with lengths of 319aa) had two predicted N- glycosylation sites at positions 296 and 310 (Fig. 4.8) like the prototype BA strain. The other four BA isolates with 312aa length had single predicted N- glycosylation sites at position 296 as the amino acid substitution T312N led to the loss of the second N- glycosylation site. The number and positions of predicted O- glycosylation with G-score >5.0 vary in the BA strains, ranging from 42 to 44 sites. Two of the predicted O- glycosylation sites found in the Nigeria isolates which were not found in the reference strain resulted from mutation K218T and I281T.

4.8 Selective Pressure Analysis of the ON1 genotype.

The positive and negative selection sites for the ON1 genotypes are shown in Table 4.8 and Table 4.9. A total of ten positive selection sites were detected in the hypervariable region two (amino acid positions 212 to 321) by all the three methods used. All the methods detected positive selection on amino acid positions 274 and 298 (L274P/Q and L298P). Three positively selected substitutions (L274P, Y297H and L298P) were detected in the Nigeria isolates. A total of twenty-three negative selection sites were detected by all the three methods used, ten of which were commonly identified by all the methods. 212 - 321

Tables 4.10 and 4.11 show the positive and negative selection sites within amino acid positions 213 to 322 on the BA9 genotypes. Altogether, six positively selected positions were detected in BA9 by all the methods used. Isolates from Nigeria were found to possess positively selected substitutions at three positions (L219P, L223P and N293Y). Twenty-two negatively selected sites of substitutions were detected on the BA9, fourteen of which were commonly detected by all the methods used.

4.9 Analysis of the Fusion gene for Palivizumab-resistance mutation.

Eleven HRSV-positive samples were selected and the region of their F gene carrying the antigenic site II was amplified by PCR. The samples were selected using criteria which include: sex (six females and five males), patient status (four apparently healthy and seven from among those seeking care for respiratory infection) and HRSV subtype (six subtype A and five subtype B). The alignment of the deduced amino acid sequence of the antigenic site II of the F gene is shown in Figure 4.9. All the isolates had substitutions on amino acid position 276.

4.10 GenBank accession numbers

The nucleotide sequences of the second hypervariable region of the G gene and the antigenic site II of the F protein obtained in this study were deposited in GenBank. Accession numbers assigned to the sequences of the isolates are shown in table 4.12.

Table 4.8: Positive selection sites within the HVR2 in the G protein of HRSV- A, ON1 genotype.

Model	**Positive selection site**									
SLAC	V225A/G/L/E		S267P/L		L274P/Q		L298P*	L310P	L314P	T318A/K
FEL		K233Q		Y273H	L274P/Q	Y297H*	L298P*			T318A/K

P-value <0.1

* Represents sites within the region of 24-amino acid duplication

Green colour signify sites that were positively selected in Nigeria isolates.

Table 4.9: Negative selection sites within the HVR2 in the G protein of HRSV- A, ON1 genotype.

Model	**Negative selection sites**															
SLAC	Q218	P222	T227	P230	T239	T245	Y280	Q285*	S291*	T292*	T305*	S307*	S317			
FEL	Q218	P222	T227	P230	T239	G259	T264	E271	S277	Q285*	S291*	T292*	Q302	T305*	S307*	S317
IFEL	Q218	P222	T227	P230	T231	G232	T239	S250	G254	S277	Q285*	S291*	T292*	S294*	T305*	S307*

P-value <0.1

* Represents sites within the region of 24-amino acid duplication

Table 4.10: Positive selection sites within the HVR2 in the G protein of HRSV- B, BA9 genotype.

Model	Positive selection sites					
SLAC	L219P/V	L223P	-	S267P/L*	-	T302A
FEL	L219P/V	-	S265G/N/I/R*	S267P/L*	N293Y	T302A

P-value ≤0.1

* Represents sites within the region of 24-amino acid duplication.

Green colour signify sites that were positively selected in Nigeria isolates.

Table 4.11: Negative selection sites within the HVR2 in the G protein of HRSV- B, BA9 genotype.

Model	SLAC	FEL
Negatively selected sites	P216	P216
	-	K221
	I229	I229
	N230	-
	K238	K238
	T239	T239
	T250	-
	L252	L252
	D263*	D263*
	T266*	T266*
	L272*	L272*
	-	Q283
	-	Q284
	S285	S285
	P295	P295
	-	Q299
	P301	P301
	-	A303
	S304	S304
	S307	S307
	-	N310
	S311	S311

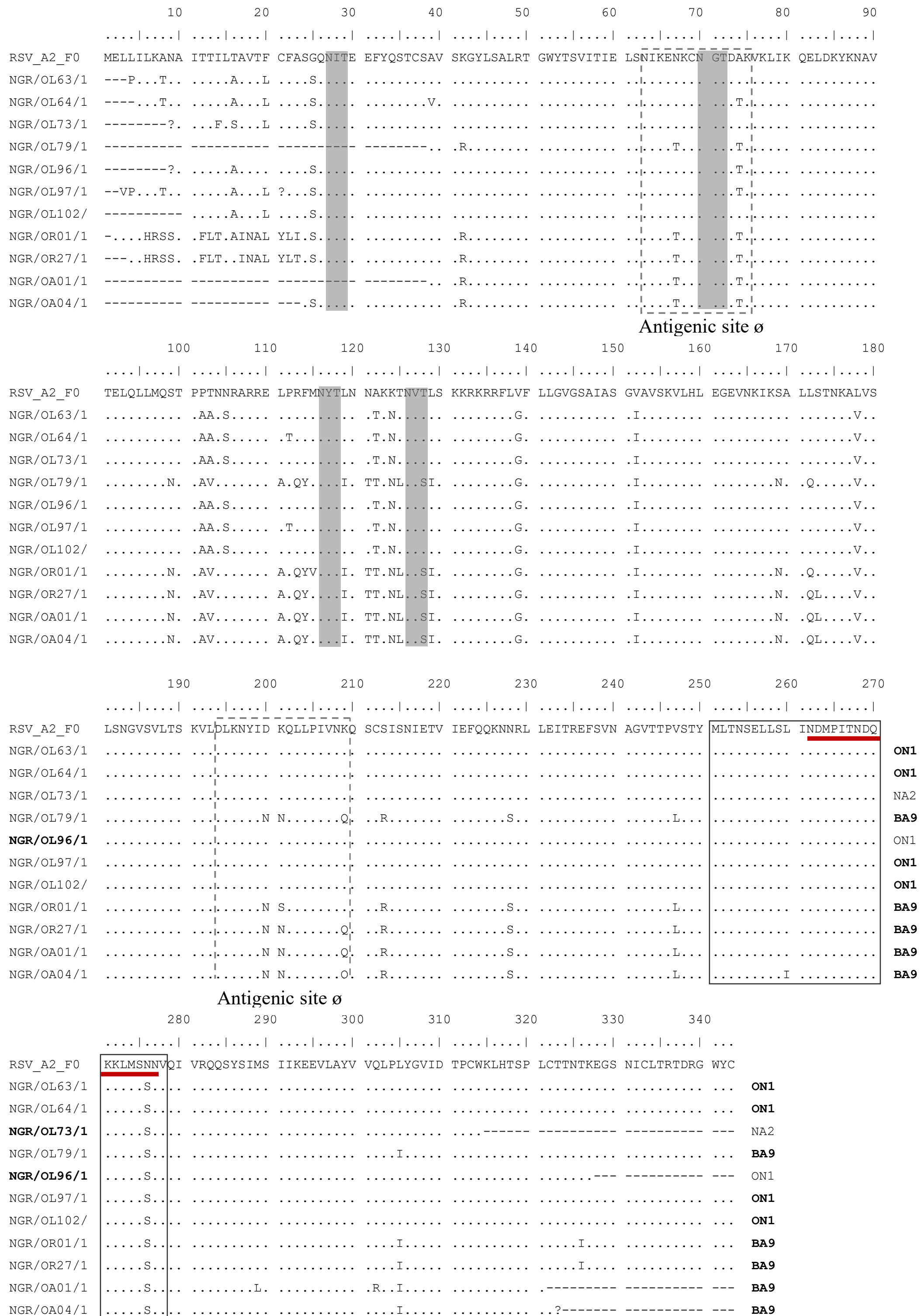

FIGURE 4.5. Amino acid alignments of partial fusion protein of selected isolates. Residues are numbered relative to the sequences of prototype strain A2 (GenBank accession number M11486). Identical residues are indicated by dots and gaps indicated by dash. The region of the antigenic site II is framed by rectangles and the Palivizumab binding site underlined in red. Potential N-glycosylation sites (NXT/S, where X is not a proline) are indicated by shaded rectangles. The genotypes are written on the right side.

Table 4.12-a: Accession numbers of partial sequences of the Glycoprotein gene of HRSV type A deposited in GenBank.

Isolate ID	Genotype	GenBank accession number
NGR/OL63/15-RSVA	ON1	KU736767
NGR/OL64/15-RSVA	ON1	KU736768
NGR/OL73/15-RSVA	NA2	KU736769
NGR/OL76/15-RSVA	ON1	KU736770
NGR/OL78/15-RSVA	NA2	KU736771
NGR/OL96/15-RSVA	ON1	KU736772
NGR/OL97/15-RSVA	ON1	KU736773
NGR/OL102/15-RSVA	ON1	KU736774
NGR/OL105/15-RSVA	ON1	KU736775
NGR/OA02/15-RSVA	ON1	KU736776
NGR/OL86/15-RSVA	NA2	MG014703
NGR/OL88/15-RSVA	ON1	MG014704
NGR/OL93/15-RSVA	ON1	MG014705
NGR/OA20/15-RSVA	ON1	MG014706

Table 4.12-b: Accession numbers of partial sequences of the Glycoprotein gene of HRSV type B deposited in GenBank.

Isolate ID	**Genotype**	**GenBank accession number**
NGR/OL79/15-RSVB	BA9	KU736777
NGR/OR01/15-RSVB	BA9	KU736778
NGR/OR75/15-RSVB	BA9	KU736779
NGR/OA01/15-RSVB	BA9	KU736780
NGR/OA04/15-RSVB	BA9	KU736781
NGR/OA19/15-RSVB	BA9	KU736782
NGR/OA31/15-RSVB	BA9	KU736783
NGR/OA36/15-RSVB	BA9	KU736784
NGR/OA10/15-RSVB	BA9	MG014707
NGR/OA30/15-RSVB	BA9	MG014708
NGR/OR27/15-RSVB	BA9	MG014709

Table 4.12-c: Accession numbers of partial sequences of the Fusion gene of HRSV types A and B deposited in GenBank.

Subtype	Isolate ID	Genotype	GenBank accession number
A	NGR/OL63/15-RSVA	ON1	KU893253
A	NGR/OL64/15-RSVA	ON1	KU893254
B	NGR/OL79/15-RSVB	BA9	KU893255
A	NGR/OL97/15-RSVA	ON1	KU893256
A	NGR/OL102/15-RSVA	ON1	KU893257
B	NGR/OR01/15-RSVB	BA9	KU893258
B	NGR/OR27/15-RSVB	BA9	KU893259
B	NGR/OA01/15-RSVB	BA9	MG323925
B	NGR/OA04/15-RSVB	BA9	MG323926
A	NGR/OL73/15-RSVA	NA2	MG323927
A	NGR/OL96/15-RSVA	ON1	MG323928
A	NGR/OL78/15-RSVA	NA2	MG323929
A	NGR/OL88/15-RSVA	ON1	MG323930

CHAPTER FIVE

DISCUSSION

The percentage of HRSV positivity (17.7%) observed among the study participants is an indication that the virus is still a significant cause of respiratory infection in Ibadan, as previously reported (Olaleye *et al.,* 1992; Odaibo *et al.,* 2013; Faneye *et al.,* 2014). Although some of the previous studies reported a higher percentage of HRSV detection, most of these studies employed serological techniques that captured both old and recent infections. Since only recent infections are required in this study, a more sensitive technique of PCR (Loens *et al*., 2009) was employed. Prevalence similar to that reported in this study was also reported among children less than 5 years in Kenya (Bigogo *et al.,* 2013) as well as Sudan (Khalil *et al.,* 2015). These studies similarly used PCR techniques for their viral detection. The observed HRSV detection rate in this study is distantly higher than the 0.41% positivity detected by Akinloye *et al.,* (2011) among relatively similar population in Ibadan. This may be due to the fact that here, PCR for HRSV detection was done using two different sets of primers that target the conserved Matrix and Nucleoprotein of the virus.

Varying HRSV positivity/prevalence has been recently reported from different parts of Africa, including 60.4% from Ghana (Obodai *et al.,* 2014), 21.2% from Madagascar (Razanajatovo *et al.,* 2011), 11.9% from Gabon (Lekana-Douki *et al.,* 2014), 11.4% from Senegal (Fall *et al.,* 2016) and 5.7% from Cameroon (Njouom *et al.,* 2012). The discrepancies in the reported prevalence could be linked to geographical differences in virus burden, variation in technical approaches, the number of patients tested, the periods during which samples were collected as well as the type of sample collected and even the period and length of the study (Yoshida *et al*., 2012).

The prevalence of HRSV vary sharply in the two groups of participants in this study. The participants attending primary health centers (PHCs) were adjudged apparently healthy by their parents / guardian and were only brought to the hospital to receive vaccine, unlike their counterparts that presented at the secondary health facility (SHF) purposely for care on respiratory tract illness. It is therefore not surprising that the

prevalence of HRSV (34.6%) among children seeking care for respiratory infection was significantly higher than the 8.7% obtained among apparently healthy children. The prevalence of HRSV among those seeking care for respiratory tract infection showed that HRSV infection is a major reason for hospital visits among children aged 5 years and below in Nigeria. Reports from other parts of the world has also shown that HRSV infection is a major reason for hospital visit in developing (Okiro *et al*., 2012) as well as developed (Shay *et al*., 1999) countries. Laboratory diagnosis as well as treatment regimen for respiratory infection in Nigeria and perhaps in other developing countries more often focus on bacterial etiological agents than viral agents. The prevalence of HRSV among those seeking care for respiratory tract illness therefore brings to fore, among other things, the need to test for virus in addition to the bacterial screening, for better management of the patients**.**

HRSV subtype A was the predominant subtype detected in Ibadan, Nigeria. To the best of our knowledge, there is no previous report published on HRSV subtype detection in any part of Nigeria, to which comparison of this study could be made. However, the subtype A predominance has similarly been reported in Vietnam (Tran *et al.,* 2013), Kenya (Scott *et al.,* 2004) and Philippines (Malasao *et al.,* 2015) among other places. Available evidence in the literature (Peret *et al.,* 1998; Mlinaric-Galinovic *et al.,* 2009; Bose *et al.,* 2015) shows that the predominant subtype varies and alternate from time-to-time as well as place to place. One possible explanation for the alternation in predominance is the development of specific immunity against a specific RSV type that is prevalent in a given area at the preceding year.

Interestingly, subtype B was the predominant subtype detected among the apparently healthy children attending PHCs. This is in concordance with the findings of Tran *et al.,* (2013) that subgroup B infected children were admitted to the hospital less often than subgroup A infected children. Although 8 of 41 (19.5%) samples detected to be HRSV-positive by the matrix gene amplification could not be successfully subtyped, most of which were those collected from children seeking medical care due to respiratory infection, we can confidently draw inference from the available data that subtype A was predominant among children seeking medical care due to respiratory infection. This shows that HRSV A was more associated to hospital visits and admission than HRSV B.

All the participants with disease severity score ≥8 were those infected with HRSV subtype A, thus supporting previous reports (Tran *et al*., 2013) that subtype A resulted in more severe disease than that of subtype B. The result of the severity scoring further corroborates the result of the HRSV subtype detected among the few children diagnosed by clinicians and hospitalized on account of bronchopneumonia and bronchiolitis. All HRSV detected from participants with these clinical diagnoses belong to the subtype A. The number of participants diagnosed of bronchopneumonia and bronchiolitis are very small hence we cannot conclude that HRSV subtype B may not also be implicated in such clinical outcome. However, our report agrees with most studies (Mufson *et al.,* 1988; McConnochie *et al.,* 1990; Walsh *et al.,* 1997; Tran *et al.,* 2013) that HRSV subtype A is associated with more severe clinical disease than subtype B.

Phylogenetic analyses showed that two genotypes of HRSV-A: ON1 and NA2 were circulating among children in Ibadan, with ON1 genotype being the predominant of the subtype A detected. Genetic analyses have shown that the ON1 which evolved from genotype NA1 (Hirano *et al.,* 2014); has signature 72nt insertion at the C – terminal end of the attachment glycoprotein and was first reported in Ontario in Canada in the year 2012 (Eshaghi *et al.,* 2012). The prototype of the ON1 genotype is the strain ON67-1210A with the characteristic 72nt insertion in G, translating into 24 additional amino acids, 23 (QEETLHSTTSEGYLSPSQVYTTS) of which are exact duplicates of the preceding amino acids at positions 261–283. Unlike the prototype strain, ON1 strains detected in Ibadan Nigeria did not have their nucleotide insertions translated into duplicate of the amino acid at positions 261-283.

The ON1 genotype has been increasingly detected in many countries (Cui *et al.,* 2013; Agoti *et al.,* 2014; Ren *et al.,* 2014; Tabatabai *et al.,* 2014; Fall *et al.,* 2016) since it was first reported in Canada, and is now replacing other HRSV A genotypes in different parts of the world. Like its ancestor that emerged in 2004 and became predominant in many areas within a period of 10 years (Tan *et al.,* 2012; Cui *et al.,* 2013; Etemadi *et al.,* 2013), in no distant time, the ON1 genotype may become the predominating HRSV-A globally, going by the reports from different parts of the world (Agoti *et al.,* 2014; Kim *et al.,* 2014; Panayiotou *et al.,* 2014 Duvvuri *et al.,* 2015). As at the time of collection of the last sample from which an ON1 isolate was

detected in this study, sequence information available in GenBank showed that this genotype had been previously reported at one time or the other in about twenty-one countries that spread across almost all the continents of the world (Duvvuri *et al.,* 2015) and possibly still circulating undetected or unreported in many other countries.

All the HRSV-B viruses found in this study belong to the BA genotype which was first reported in Buenos Aires, Argentina in 1999 (Trento *et al.,* 2003) with characteristic 60 nucleotides (20 amino acids) duplication in the C-terminal end of the G glycoprotein. Similar to the ON1 genotype, the BA genotype has been detected in many countries nearly predominating and replacing previously circulating genotypes of HRSV subtype B including the SAB1, SAB2 and SAB3 previously detected in some parts of Africa (Venter *et al.,* 2001; Scott *et* al., 2004; Shobugawa *et al.,* 2009; Dapat *et al.,* 2010; van Niekerk and Venter 2011; Panayiotou *et al.,* 2014). Although there are no prior data on the genotypes of HRSV circulating in Nigeria, based on reports from other countries, we could also say that the BA genotype is most likely replacing previously circulating genotypes of HRSV B in Ibadan and possibly other parts of Nigeria.

The BA genotype has shown high diversity resulting in emergence of different lineages, including the BA1 – BA12, and the recently classified BA-CCA and BA-CCB (Zheng *et al.,* 2017). The isolates of the BA genotype appeared to be the most divergent of the genotypes detected in this study as shown by the 'between isolates' p – distance. Phylogenetic analysis showed that the BA genotypes from this study clustered in three different groups. Group 1 (NGR/OA31/15-RSVB, NGR/OR01/15-RSVB, NGR/OR75/15-RSVB and NGR/OR79/15-RSVB), Group 2 (NGR/OA01/15-RSVB, NGR/OA04/15-RSVB, NGR/OA19/15-RSVB, NGR/OA30/15-RSVB, NGR/OA36/15-RSVB and NGR/OR27/15-RSVB), Group 3 (NGR/OA10/15-RSVB). The group one appeared in same cluster with the BA genotype isolated from Vietnam and previously assigned to the sub-genotype BA9 (Tran *et al.,* 2013). Although the group 1 also appeared in the same clade with isolates previously assigned to lineage BA11, nucleotide blast results further showed their 99% similarity with the BA9 lineage including those isolated from China (GenBank accession number KT781359). The Nigeria isolates in the group 2 appeared in the same cluster with the BA genotype from North-Eastern China recently designated as the BA-CCA and BA-CCB (Zheng

et al., 2017). However, nucleotide blast of the sequences of the group 2 in GenBank showed their high relatedness to viruses isolated from China, and assigned to the BA9 lineage, with accession number KT781370 and KT781379 (Unpublished).

Genotype overlap and disagreement in assigning to a particular sub-genotype or lineage is a common occurrence in the BA genotype. This often results due to the different reference sequences used by different studies. For example, Bose *et al* (2015) reported that sequences previously classified as belonging to the BA4 lineage were intermixed with those reported as belonging to the BA7, BA8, BA9, and BA10 lineages. The BA9 lineage was first identified during the 2006-2007 season in Niigata, Japan (Dapat *et al.,* 2010) and was subsequently reported to be the predominating HRSV-B globally in the 2009-2010 season (Ohno *et al.,* 2013). Since the time of the first report of the BA9, it has been detected in more than twenty-three countries across different continents of the world (Haider *et al*., 2018). Whereas Haider *et al*. (2018) suggested that the global transmission of the BA9 lineage of the BA genotype could be associated with travelers, the accumulation of positively selected mutations especially at the region of the twenty amino acid insertions could also confer a fitness advantage on the BA9. This HRSV-B genotype has also been reported in West Africa, and so far, it is the only HRSV-B genotype reported in this region of Africa (Fall *et al.,* 2016).

The third group of the BA genotype found in this study consists of a single isolate that did not form cluster with any of the reference BA genotype on the phylogenetic tree, perhaps pointing to the likelihood of the emergence of a distinct/new BA sub-genotype. It is however not surprising that the isolate did not appear in similar clades with other isolates from this study as the said isolate had seven unique amino acid substitutions (at positions 231, 243, 254, 260, 269, 279 and 294) which were not found in any of the other isolates. Raghuram *et al*. (2015) made a similar report where two of the BA sequences from their study could not be assigned to any of the BA sub-genotypes.

The length of the attachment glycoprotein of the BA genotype from Nigeria differ from one isolate to the other. Variability in the attachment glycoprotein of HRSV subtype B is known to often result from mechanism which include: amino acid substitution, insertion, deletion, duplication, and change in stop codon usage (Zlateva

et al., 2005; Zhan *et al*., 2010). Whereas all the isolates had the 60 nucleotides (translating into 20 amino acids) insertions characteristic of the BA genotype, and there were no deletions, the variations in the glycoprotein length resulted from the variability in their stop codon usage. The isolates using 'TAA' as stop codon had 312 amino acid lengths while those using 'TAG' had 319 amino acids. This is not an unexpected occurrence as BA genotypes are known to be highly variable in their stop codon usage, with previous reports of the use of TAA and TAG (Tran *et al.,* 2013; Bose *et al*., 2015; Raghuram *et al*., 2015) as well as CAG (Trento *et al.,* 2003) stop codons usage. Premature stop codons in RSV has been suggested to have drastic antigenic changes on the attachment glycoprotein, perhaps by affecting the protein folding pattern thereby leading to absence of reactivity with most anti-G antibodies (Rueda *et al.,* 1991). The isolates with 312 amino acids could therefore be more evasive to antibody neutralization. The stop codon variability observed among the BA isolates were not found in the ON1 isolates. This among other reasons may account for the relative conservation known with the ON1 genotype unlike the variability in the BA leading to the multiple BA lineages like BA1 – BA12 (Zlateva *et al*., 2005, Zheng *et al*., 2017)

Upon comparison of the nucleotides as well as the deduced amino acid sequences of the 2nd hypervariable region of HRSVA, it is clear that the ON1 subtype from this study vary considerably from the prototype ON1 (GenBank accession no. JN257693) due to accumulation of mutations. The substitution of amino acid proline for leucine at position 274 (L274P) was found in most of the Nigerian ON1 isolates while similar amino acid substitution at position 298 (L298P), within the duplicated region of the attachment glycoprotein of the virus was detected in all but one of the isolates. Substitutions at these two sites (i.e. L274P and L298P) have been reported to often occur concurrently (Agoti *et al.,* 2014). Although the entire functional effect(s) of most of the substitutions we found are not well known, substitution L274P is often found in vaccine escape mutants (Gardinassi *et al*., 2012) and has been well reported to be associated with resistance to neutralizing antibody (Rueda *et al.,* 1991; Botosso *et al.,* 2009).

The substitution of amino acid arginine for glycine at position 232 (G232R) was found in one of our isolates (NGR/OL63/15-RSVA). The effect of this substitution

was not established in our study, however substitution at this position has been suggested to improve the probability of RSV survival (Zheng *et al.,* 2017). Howbeit, Zheng *et al*. (2017) detected the substitution with glutamic acid at the position 232 (G232E) rather than the arginine substitution that we detected. Similarly, we detected the amino acid substitution of lysine for glutamic acid at position 262 (E262K) which is reported to help RSV evade human immunity, thereby enhancing viral survival (Zheng *et al.,* 2017). One ON1 isolate (NGR/OL76/15-RSVA) had the substitution of isoleucine for threonine at amino acid position 292 (T292I) which is within the 24 amino acid insertion regions. As far as can be ascertained, this substitution has not been previously reported, therefore showing the genetic diversity of the isolate. Amino acid substitutions also occurred on some of our isolates at positions 249, 272, 274. These positions were previously predicted to bear positively selected substitutions (Agoti *et al.,* 2014), hence these isolates may be well adapted to evade host immunity (Botosso *et al.,* 2009; Tapia *et al.,* 2014)

It is expected that the G glycoprotein will be under immune selection pressure due to its importance in viral attachment and as a major target of neutralising antibodies (Botosso *et al*., 2009). The bulk of the variation and the positive selection for amino acid changes are concentrated in two hypervariable regions of the G protein which is also known to contain multiple epitopes (Kim *et al.,* 2014). Our analysis of the positive selections acting on the HVR2 of the G glycoprotein showed the different amino acid substitutions that are involved in the variability of the ON1 genotype of RSV-A and the BA9 genotype of RSV-B globally. Among all the ON1 genotypes globally, ten positive selection substitutions were detected, three of which were found in the Nigeria isolates. It is interesting to note that the three positive selection sites found in the Nigeria isolates were located within two of the common epitopes of ON1. There are about four B-cell epitopes within the 2nd hypervariable region of the ON1: aa221-242, 251-265, 272-284 and 287-299 (Kim *et al.,* 2014). Seven of the eleven ON1 isolates (NGR/OL64/15-RSVA, NGR/OL76/15-RSVA, NGR/OL88/15-RSVA, NGR/OL96/15-RSVA, NGR/OL97/15-RSVA, NGR/OA02/15-RSVA and NGR/OA20/15-RSVA) had positively selected substitutions of proline for leucine at amino acid position 274 and histidine for tyrosine at amino acid position 297. These substitutions were within the third and fourth B cell epitopes.

All the isolates (except NGR/OL93/15-RSVA) also had the substitution of proline for leucine at amino acid position 298. These sites of positive selections are under adaptive evolution in the G protein and the substitutions may enhance the sustained transmission of this genotype in Nigeria as it makes the virus better fit to evade hosts neutralising antibody. The presence of amino acid substitutions on two or more of the epitopes from this study coupled with the characteristics 72 nucleotide insertion may mean that the ON1 genotype will remain the predominant RSV-A genotype in Nigeria, just like it has been in other parts of the world (Cui *et al.,* 2013; Agoti *et al.,* 2014; Ren *et al.,* 2014; Tabatabai *et al.,* 2014; Fall *et al.,* 2016). Six putative positive selection substitutions were found in all the available BA9 sequences used for the in-silico analysis, three of which were detected in the BA9 isolates from Nigeria. These substitutions (Leu219Pro, Leu223Pro and Asn293Tyr) as well as other substitutions (Ser247Pro, Thr270Ile, His287Tyr and Glu305Lys) found in the isolates are known to enhance the immune evasion potential of the virus and eventually its sustained transmission (Haider *et al*., 2018).

There were numerous sites of negative selection on both the ON1 and the BA9 isolates. This is not surprising since most amino acid mutations in RNA viruses are deleterious, hence purifying selection is very vital in their evolution (Holmes, 2013). Although the role(s) of negative selection in the G glycoprotein of HRSV is not clearly elucidated, it is known that it plays major roles in the prevention of functional deterioration in some viruses (Domingo *et al*., 2006). It is likely that the numerous negative selections observed in the isolates were similarly involved in the prevention of deterioration of antigenic function (Garcia *et* al., 1994; Domingo, 2006; *et al*., 2013; Hirano *et al*., 2014).

Four distinct antigenic sites have been previously identified in the second hypervariable region of HRSV-A (aa229-240; 250-258; 265-273 and 283-291) and numbered relative to the prototype A2 strain (Cane 1997). The NA2 isolates from this study had some point mutations on some of these antigenic sites. These mutations may have vital antigenic and immunogenic implication for the survival of the virus**.** Similarly, three ON1 isolates (NGR/OL64/15-RSVA, NGR/OL88/15-RSVA and NGR/OL97/15-RSVA) have amino acid substitution of serine for glycine at position 272 which is within the antigenic site 265-273, thereby showing the antigenic

diversity among the ON1 strains. Interestingly, isolates belonging to the subtypes ONA as well as the NA2 that had substitutions on their antigenic sites were predominantly implicated in relatively more severe clinical outcomes, unlike the BA isolates that do not have mutations on their antigenic sites. This may suggest the importance of these mutations to disease severity.

While the ON1 isolates supposedly predisposed to more severe outcome as they were predominantly detected among those presenting for care of respiratory infection at the secondary health facility, two of the ON1 isolates were detected among the apparently healthy participants that came for vaccination at the PHCs. It is worthy of note, that the isolates NGR/OA02/15-RSVA and NGR/OA20/15-RSVA had an amino acid substitution of isoleucine for threonine at position 245 that was not found in the other isolates. It remains to be known, whether this substitution mitigate virulence and predispose to less severe outcome of infection. Although a follow up on the children from whom the specimen were collected could have better informed us as to the eventual outcome of the infection, it was not feasible to get this information due to the lapse of time between the sample collection and the availability of the sequence information.

The HRSV-B isolates could be thought to cause less severe disease outcomes as they were detected predominantly among apparently healthy participants. However, a single isolate of the subtype B (NGR/OL79/15-RSVB) was detected among those presenting for care of respiratory tract infection at the SHF. This isolate interestingly had a unique amino acid substitution of lysine for glutamic acid at position 226 (E226K) that was not found in the isolates from the apparently healthy participants. One could therefore cautiously hypothesize that the amino acid substitution could predispose to more severe outcome of infection.

N - and O – linked glycosylation of the G protein may influence the antigenicity of HRSV by masking the epitope expression thereby affecting antibody recognition and by extension aiding immune evasion (Garcia-Beato *et al*., 1996; Khan *et al.,* 2014; Raghuram *et al*., 2015). Whereas the isolates belonging to the ON1 genotype have similarity with the prototype strain in the number and sites of N – linked glycosylation, the occurrence of more sites of O – linked glycosylation on the isolates showed the diversity in the antigenicity of the ON1 genotype. In the NA2 isolates,

substitutions of tyrosine for asparagine, as well as leucine for proline at positions 273 and 274 respectively culminated into the loss of an N – linked glycosylation site. Although the number of N – linked glycosylation in the isolates relative to the prototype is same, as the loss of glycosylation was compensated for by glycosylation at another site due to the substitution of threonine for proline at position 320. It remains to be known whether the alteration in the site of the N – linked glycosylation has a debilitating effect on the pathogenicity of the NA2 genotype or not. It has been shown that alteration of site(s) of glycosylation could have drastic impacts on the survival and transmissibility of HIV and hepatitis C virus both positively and negatively (Land and Braakman, 2001; Slater-Handshy *et al*., 2004; Vigerust and Shepherd, 2007). The fact that alteration of the site(s) of glycosylation affects viral interactions with receptor (Vigerust and Shepherd, 2007) may be one of the reasons for the low circulation of the NA2 genotype in Ibadan, Nigeria. Some isolates of the BA genotypes in Nigeria have two N-linked glycosylation sites, while others have a single glycosylation site because the substitution T312N resulted into the loss of potential N – glycosylation sites in some isolates.

To date, the only licensed prophylaxis for the prevention of infection with HRSV is the passive administration of RSV-specific immunoglobulin Palivizumab. Palivizumab (PZ; brand name Synagis®, MedImmune Inc., Gaithersburg) is an HRSV neutralizing humanized murine monoclonal antibody (MAb) reactive with a defined epitope in the antigenic site II of HRSV fusion (F) protein (Zhao *et al.,* 2006). Although the immunoprophylaxis has been approved for use since 1998 by the US Food and Drug Administration for prevention of severe lower respiratory tract disease caused by HRSV in pediatric patients who are at increased risk of severe disease (The IMpact-RSV Study Group 1998; American Academy of Pediatrics, 2009), and it is being used in more than sixty countries (Hashimoto and Hosoya, 2017), it is not yet in use in Nigeria. Reports from various studies have described mutations in the putative binding site of palivizumab in cell culture, cotton rats and in human population (Zhao and Sullender 2005; Boivin *et al.,* 2008; Adams *et al.,* 2010). There have been divergent reports on the amino acid positions that represent the putative palivizumab binding site. Whereas some reported amino acid positions 262 – 276 (Beeler *et al*., 1989; Johnson *et al*., 1997; Crowe *et al*., 1998; Huang *et al*., 2010) and 262 – 270 (Zhao *et al*., 2006), other recent researchers reported positions 256 – 276 (Xia *et al*.,

2013) as well as 256 – 277 (Hashimoto and Hosoya, 2017). We found in this study that the region of antigenic site II, within which is the Palivizumab-binding site, is relatively conserved among both subtypes of HRSV. However, at amino acid position 276, there was the substitution of serine for asparagine (N276S) in all of the Ibadan subtype A isolates when compared to the prototype A2 strain. The N276S mutation was not known anywhere in the world prior to the introduction of palivizumab (Hashimoto and Hosoya, 2017). However, the mutation has been reported in countries including Israel and Turkey in the 2007 – 2008 season (Zhu *et al*., 2012) as well as in Canada between 2008 and 2010 (Papenburg *et al*., 2012). The detection of substitution N276S in Nigeria is similar to the reports by Xia *et al*. (2013) who detected the same substitution in 95.2% and 96.9% of HRSV A during the 2010 – 2011 and 2011 – 2012 seasons respectively when Palivizumab was not yet in use in China. All the HRSV B isolates from Ibadan also had serine at amino acid position 276. Whereas the occurrence of serine at position 276 on the fusion glycoprotein results due to mutation in the subtype A of HRSV (Adams *et al.,* 2010), asparagine and serine are the consensus amino acids in the wildtype HRSV subtypes A and B respectively at that position (Adams *et* al., 2010; Zhu *et al*., 2012). It has been hypothesized, that the N276S and the S276N mutations in RSV-A and RSV-B respectively appeared due to hybridization between the subgroups or due to natural mutation.

There are controversies over the functional implication of the observed substitution N276S on palivizumab effectiveness. While some suggest that the said substitution alters the ability of palivizumab to bind to F protein thereby resulting in the emergence of clinical isolates that were totally resistant to the antibody (Adams *et al.,* 2010), other reports showed that the substitution cannot, as a stand-alone, confer complete resistance to Palivizumab (Zhu *et al.,* 2012; Papenburg *et al*., 2012, Xia *et al*., 2013). They submitted that N276S substitution in the subtype A of HRSV predisposes to another substitution of lysine for glutamic acid or glutamine at amino acid position 272 (K272E) which then led to complete resistance against palivizumab. Other palivizumab resistant mutations including substitution of isoleucine for asparagine at position 268 (N268I), glutamine or methionine, or asparagine or threonine or glutamic acid/glutamate for lysine at position 272 (K272Q,M,N,T,E) and phenylalanine or leucine for serine at position 275 (S275F,L) (Zhao *et al*., 2004; Zhu

et al., 2011) were not detected in the Ibadan isolates. Unlike the N276S which has been reported in areas with and without palivizumab usage, these substitutions appeared to be restricted only to regions where palivizumab are in use. The non-detection of these other substitutions in Ibadan, Nigeria may further suggest that the palivizumab-resistant isolates in Ibadan evolved due to natural mutation that is positively selected, and not due to international human movements. The fact that Crowe *et al*. (1998) reported that substitution at the same amino acid position 276 (tyrosine for asparagine) was found with the murine RSV-neutralising monoclonal antibody 1237 showed that the position is sensitive to selection pressure. However, the reason why the N276S selection is only seen after the introduction of palivizumab in several regions of the world is not clear. It is also not clear why the mutation on the amino acid position 276 that could predispose to palivizumab resistance was only detected in the subtype A isolates but not in the subtype B isolates from Ibadan (Adams *et* al., 2010; Zhu *et al*., 2012).

Most of the participants in this study were within the age group 12 months and below, with the bulk of them within the age group 6 months and below. This agrees with the known fact that children age group 12 months and below are more susceptible to respiratory infection compared to children aged above 12 months. Unlike most studies where the highest prevalence was reported in younger age groups, we found here that the prevalence of HRSV increased with age. This is likely due to the fact that the samples collected tapered significantly as the age group increased, together with the fact that the participants in age groups 12 months and below were mostly apparently healthy.

Gender was not significantly associated with susceptibility to HRSV infection in our study, like in other studies (Aamir *et al.,* 2013; Tran *et al.,* 2013) although the prevalence was slightly higher in male children. Various studies suggest that male children are more susceptible to severe HRSV infection than females (Goto-Sugai *et al.,* 2010; Eshaghi *et al.,* 2012). Although all the two children admitted into the secondary health facility with clinical diagnosis of bronchiolitis during the course of this study were male, on the other hand, 75% of participants clinically diagnosed with bronchopneumonia were females. These small numbers of participants with severe outcomes are therefore not sufficient to make inferences on the association of gender to disease severity.

HRSV was detected in all the participants clinically diagnosed of bronchopneumonia as well as 50% of those diagnosed of bronchiolitis. This is in tandem with other reports that HRSV is the leading etiology of pneumonia and bronchiolitis globally (Shay *et al.,* 1999; Venter *et al.,* 2011; Lamarao *et al.,* 2012; Khalil *et al.,* 2015; Shi *et al.,* 2017). All the children with clinical diagnosis of bronchiolitis and bronchopneumonia from whom HRSV were detected were 12 months and below, with 3 of the 5 (60%) being below 6 months of age. This is similar to the finding by Lamarao *et al.,* (2012), and further lends credence to available evidence in the literature (D´Elia *et al.,* 2005; Lee *et al.,* 2007; Midulla *et al.,* 2010; Sricharoenchai *et al.,* 2016) suggesting that HRSV is the main pathogen responsible for bronchiolitis and pneumonia during the first 12 months of life. Illness resulting from HRSV infection in this age group is also known to be severe (Lamarao *et al.,* 2012).

CHAPTER SIX

SUMMARY AND CONCLUSIONS

The results of this study have shown a higher prevalence of subtype A of HRSV among pediatric population in Ibadan, Nigeria. Subtype A predisposed to relatively more severe disease outcome and is the major HRSV infection necessitating hospital visits as it the predominant subtype detected among children seeking medical care due to respiratory infection. On the other hand, subtype B predominated among apparently healthy children.

Three genotypes: ON1, NA2 and BA9 were found to be circulating among children in Ibadan, Nigeria with ON1 and BA being the most detected genotypes. All the genotypes detected vary considerably from the prototype strains due to accumulation of mutations, including amino acid substitutions on some of the antigenic sites. The isolates belonging to the BA9 genotype appeared to be more variable than the ON1 and NA2 isolates. The variability of the BA9 genotype was shown in their multiple stop codon usage, variable G glycoprotein length as well as difference in N glycosylation patterns.

Relative to the prototype A2 strain, mutations were found on the antigenic site II of the F gene. The substitution of amino acid asparagine for serine that has been shown in the literature to confer partial or complete resistance to Palivizumab (the only approved immunoprophylaxis used to reduce severity of HRSV infection among high risk infants) was found in most of the isolates. It is most likely that the isolates from Ibadan will be resistant to Palivizumab due to the substitution on the fusion protein at amino acid position 276.

It is therefore recommended that additional study with samples collected from other parts of the country be done to determine the extent of genotype diversity of HRSV circulating in Nigeria. It may also be necessary to do microneutralization assay with the approved monoclonal antibody using the isolates from different parts of the country before it is used in Nigeria.

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Appendix1

RESEARCH QUESTIONNAIRE

GENOTIPIC CHARACTERISATION OF HUMAN RESPIRATORY SYNCYTIAL VIRUS IN CHILDREN PRESENTING WITH RESPIRATORY TRACT INFECTIONS IN IBADAN, NIGERIA

Institutional Review Committee (IRC) approval number: UI/EC/14/0284

Dear Sir / Madam

I seek your consent to participate in this study titled "Genotypic characterization of Human Respiratory Syncytial Virus in children presenting with respiratory tract infection in Ibadan, Nigeria". The specific aim of this study is to detect and characterize the RSV strains circulating among children aged 0 – 5 years presenting with acute respiratory tract infection in Ibadan, Nigeria.

Case definition: Cases of Respiratory Tract Infections (RTIs) were identified using WHO protocol for the identification of Influenza-like illnesses (ILIs) and/or Severe Acute Respiratory Infections (SARI) (WHO, 2014). Briefly, ILI was defined as a respiratory infection with acute onset, with any of the following clinical symptoms: fever (measured body temperature of ≥ 38°C) and cough; with day of onset not exceeding ten days. SARI was defined as an acute respiratory infection with clinical symptoms similar to that in ILI and severe enough to require hospitalization.

Volunteer ID………………………………

Hospital/Site………………………………….

Date of sample collection…………………

DEMOGRAPHIC DATA

Age (in months) …………………………...

Gender Male [ ] Female [ ]

Nationality Nigerian [ ] Others [ ]

OBSERVED SYMPTOMS

Fever: Yes [ ] No [ ]

Cough: Yes [ ] No [ ]

Difficulty breathing Yes [ ] No [ ]

Fast breathing Yes [ ] No [ ]

Wheezing (whistling sound at end inspiration or early expiration): Yes [ ] No [ ]

Stridor (rough crowing sound): Yes [ ] No [ ]

Apnoea (temporary cessation of breath): Yes [ ] No [ ]

Chest in-drawing: Yes [ ] No [ ]

Congested/runny nose: Yes [ ] No [ ]

Others (specify)…………………………………………….

Days of onset of symptoms…………………………………..

Gestational age at birth………………………………………

Birth weight………………………………………………….

Duration of breast feeding……………………………………

Exclusive breastfeeding? Yes [ ] No [ ]

Does this child have any underlying disease? Yes [ ] No [ ]

If yes, specify……………………………………………….

Appendix 2

INFORMED CONSENT FORMS

(ENGLISH VERSION)

IRC Research Approval Number: UI/EC/14/0284

I seek your consent to participate in this study entitled: Genotypic characterisation of human respiratory syncytial virus in children presenting with respiratory tract infections in Ibadan, Nigeria

The purpose of this research is to detect and characterize the RSV strains circulating among children aged 0 – 5 years presenting with respiratory tract infection in Ibadan, Nigeria. Submission of locally circulating virus strains would facilitate adequate representation in the globally acceptable RSV vaccine development.

Nasal swab shall be collected from each child whose parent/guardian give assent to participate with a procedure not exceeding 10 minutes. The study poses no risk and there is no monetary implication for your participation.

All information generated in the course of this study will be coded. Hence, demographic information of participants cannot be linked to individuals. Participation in the study attracts no monetary compensation and refusal to participate in the study will not affect services due to you in the hospital.

Participants can decide to withdraw from the study at any point. However, it should be noted that information collected prior withdrawal may have been used in reports thus cannot be retracted. Furthermore, in the course of this study, no participant will be subjected to act capable of inflicting injury.

I have fully explained this research to ____,

And have given sufficient information to enable him/her make an informed decision.

DATE:________________________

SIGNATURE: __________________________

NAME:

____

I have read or listened to translation of description of the study and understand that my participation is voluntary. In addition, I know enough about the purpose, risks and benefits of the study, thus I have decided to participate voluntarily with the conviction that my participation can be withdrawn at any point. Furthermore, I have received a copy of this consent and additional information sheet to keep for myself.

DATE:___________________________

SIGNATURE: ______________________

NAME:

____

(YORUBA VERSION)

IRC Research Approval Number: UI/EC/14/0284

A beere boya e fi ara mo lati kopa ninu ise iwadi ti a gbe kale labe akori: **Mimo oniruuru kokoro aifojuri ti o n s'okunfa otutu aya laarin awon omode jojolo ni ilu Ibadan, ti orile ede Naijiria.**

Idi ti a fi gbe ise iwadi yi kale ni lati se iwadi ijinle lori kokoro aifi oju ri tio nfa otutu ayalaarin ọmọ owo ati omo irinse ti ojo ori won ko ju odun marun lo ni ilu Ibadan. Ise iwadi yi ni yio ran wa lowo lati fi oniruuru kokoro aifojuri ti a ri ni esekuku sowo si awon ti n se abere ajesara ki won ba le se abere ti yio koju awon kokoro yi kaakiri agbaaye.

A o fi nkan ro imu enikeni ti obi tabi alagbato re ba fi ara mo ti o si kun oju osuwon lati kopa ninu ise iwadi yi. Gbogbo eto ayewo naa ki yio gba wa ju iseju mewa lo.O seese ki omo ti a nfi nkan ro ni imu ki o sin jade lakoko ti a n roo ni imu, sugbon mo fi da yin loju wipe ko si ewu tabi ipalara kankan ti o ro mo kikopa ninu ise iwadi yii, beni e ko ni lati san owo kankan lati le kopa ninu ayewo naa.

Gbogbo akosile lati inu ise iwadi yii ni a o se ni ipamo ni ona ti enikeni ti ko leto ki yio lee mo itumo re tabi eniti akosile naa tokasi. A ko nii fun enikeni ni owo lati kopa ninu ise iwadi yii, beeni ko si ijiya kankan fun eniti o ba kọ lati kopa.

Enikeni ti o kopa lee jawo ninu ise iwadi yii ni igbakugba ti o ba wu. Sugbon, ki o di mimo wipe o seese ki a ti lo lara awon akosile ti a ti gba sile, eleyi ti o lee ma see da pada. Sugbon, mo fi n da yin loju wipe, ninu ise iwadi yii, a ki yio se ohunkohun ti o le mu ipalara fun enikeni ti o kopa ninu ise iwadi naa.

Mo ti se alaye nipa ise iwadi yii fun____, mo si se alaye lekunrere nipa awon ewu ati ere ti o wa ninu kikopa ninu ise iwadi naa fun lati lee yan ninu okan re lati kopa.

DATE:________________________

SIGNATURE: ________________________

NAME:

____

Mo ti ka tabi teti si akosile nipa ise iwadi yii, o si ye mi yekeyeke wipe ikopa mi ninu ise iwadi naa gbodo je atinuwa. A ti wipe, mo ni imo ti o to nipa ewu ati anfaani ti o ro mo kikopa ninu ise iwadi naa. Nitori idi eyi, mo ti gba lati kopa ninu ise iwadi naa pelu idaniloju wipe mo ni etọ lati yi ipinu mi pada ni igbayiowu ti mo ba fe. Atipelu wipe a ti fun mi ni eda iwe yii, ati awon akosile miiran lati fi pamo fun ara mi.

DATE:________________________

SIGNATURE: ________________________

NAME:

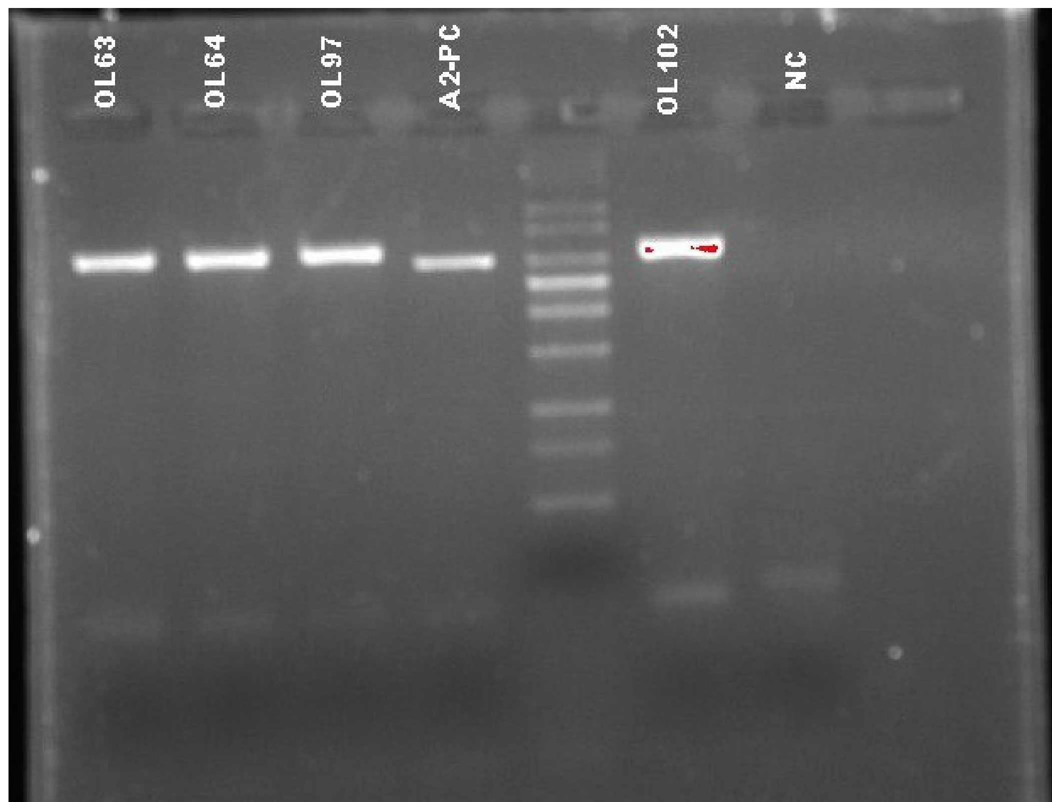
____

Appendix 3

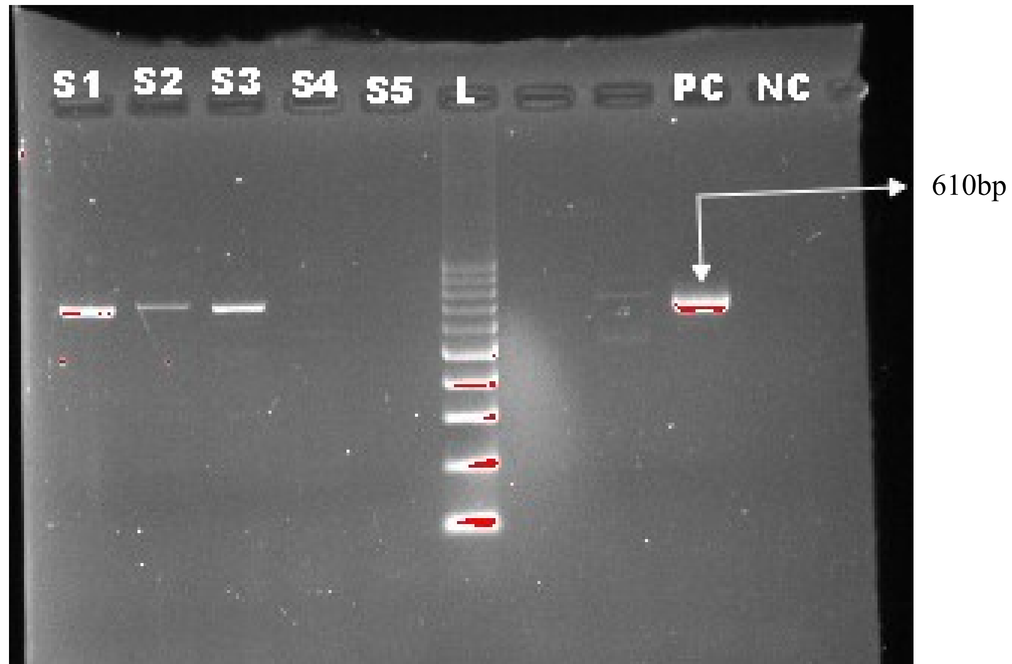
Agarose electrophoresis images of:

(A1 – A3) HRSV G gene amplification with subtype-specific primers.

(B) F gene amplification.

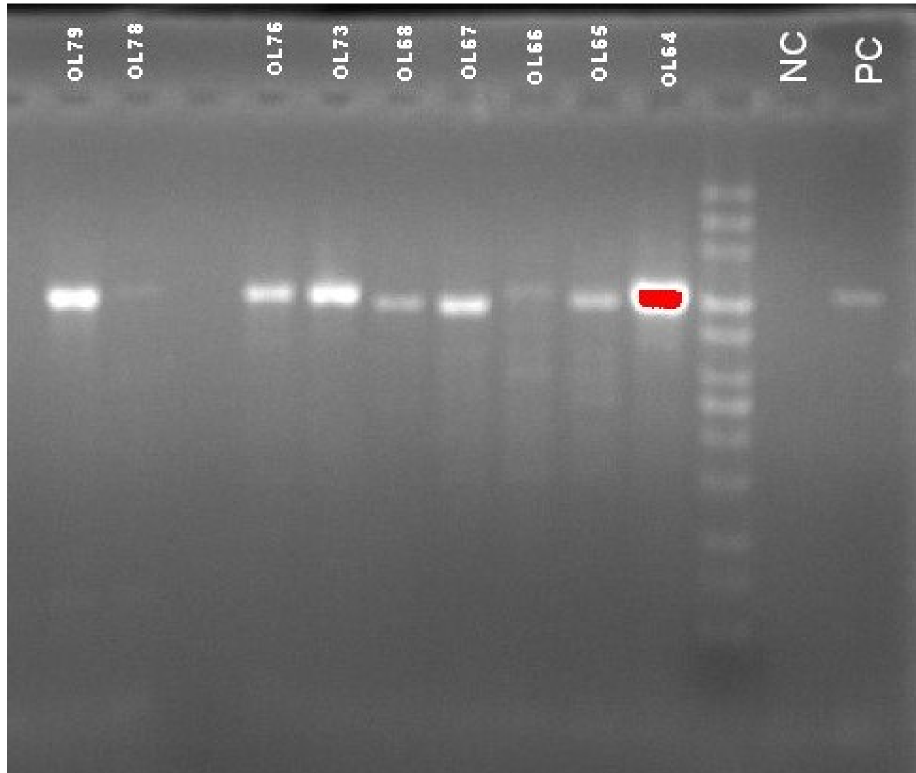


A1: Agarose electrophoresis image showing amplification of subtype A of HRSV using primers: RSVA-G513-F and RSVA-F131-RPositive. PCR products have band size of 583bp. Mid Range DNA Ladder (Jena Bioscience, Germany) was used to detect the expected DNA size. PC= Positive Control, NC= Negative Control, OL63, OL64, OL97 and OL102 were test samples.



A2: Agarose electrophoresis image showing amplification of subtype A and B of HRSV using primers: ABG490-F and F164-R. Positive. PCR products have band size of 610bp. 100 bp DNA Ladder (Jena Bioscience, Germany) was used to detect the expected DNA size. PC= Positive Control, NC= Negative Control, S1 – S5 were test samples.

A3: Agarose electrophoresis image showing amplification of subtype B of HRSV using primers: BGF and BGR. Positive. PCR products have band size of 801bp. GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific) was used to detect the expected DNA size. PC= Positive Control, NC= Negative Control, OA01 – OA02 were test samples.



B: Agarose electrophoresis image showing amplification of F gene of HRSV. PCR products have band size of 1047bp / 1065bp. Mid Range DNA Ladder (Jena Bioscience, Germany) was used to detect the expected DNA size. PC= Positive Control, NC= Negative Control, OL64 – OL79 were test samples.

Appendix 4

DNA copies of G gene amplified and sequenced. The subtype of the isolate is depicted as the last part of the isolate ID.

Isolate ID	**DNA copies ng/ul**	**GenBank accession number**	**Isolate ID**	**DNA copies ng/ul**	**GenBank accession number**
NGR/OL63/15-RSVA	70.05	KU736767	NGR/OL79/15-RSVB	75.10	KU736777
NGR/OL64/15-RSVA	89.10	KU736768	NGR/OR01/15-RSVB	234.00	KU736778
NGR/OL73/15-RSVA	161.30	KU736769	NGR/OR75/15-RSVB	139.10	KU736779
NGR/OL76/15-RSVA	165.00	KU736770	NGR/OA01/15-RSVB	131.30	KU736780
NGR/OL78/15-RSVA	99.00	KU736771	NGR/OA04/15-RSVB	196.50	KU736781
NGR/OL96/15-RSVA	125.00	KU736772	NGR/OA19/15-RSVB	96.50	KU736782
NGR/OL97/15-RSVA	205.00	KU736773	NGR/OA31/15-RSVB	141.00	KU736783
NGR/OL102/15-RSVA	174.42	KU736774	NGR/OA36/15-RSVB	39.90	KU736784
NGR/OL105/15-RSVA	75.04	KU736775	NGR/OA10/15-RSVB	33.10	MG014707
NGR/OA02/15-RSVA	359.80	KU736776	NGR/OA30/15-RSVB	36.20	MG014708
NGR/OL86/15-RSVA	78.40	MG014703	NGR/OR27/15-RSVB	230.10	MG014709
NGR/OL88/15-RSVA	50.50	MG014704			
NGR/OL93/15-RSVA	56.28	MG014705			
NGR/OA20/15-RSVA	70.00	MG014706			

DNA copies of F gene amplified and sequenced. The subtype of the isolate is depicted as the last part of the isolate ID.

Isolate ID	DNA copies ng/ul	GenBank accession number
NGR/OL63/15-RSVA	135.20	KU893253
NGR/OL64/15-RSVA	292.20	KU893254
NGR/OL79/15-RSVB	43.90	KU893255
NGR/OL97/15-RSVA	136.20	KU893256
NGR/OL102/15-RSVA	124.80	KU893257
NGR/OR01/15-RSVB	76.61	KU893258
NGR/OR27/15-RSVB	176.40	KU893259
NGR/OA01/15-RSVB	20.82	MG323925
NGR/OA04/15-RSVB	22.17	MG323926
NGR/OL73/15-RSVA	20.42	MG323927
NGR/OL96/15-RSVA	32.04	MG323928
NGR/OL78/15-RSVA	20.15	MG323929
NGR/OL88/15-RSVA	15.53	MG323930

DNA copies of G gene amplified but not yielding good reads in sequencing reactions.

Isolate ID	**DNA copies/5ul**
G gene	
NGR/OL89/15-RSVA	8.238
NGR/OL49/15-RSVB	5.966
NGR/OL54/15-RSVA	6.685
NGR/OL55/15-RSVA	9.148
NGR/OL65/15-RSVA	6.432
NGR/OL48/15-RSVA	7.488
NGR/OL58/15-RSVA	11.56
NGR/OL61/15-RSVA	5.697
NGR/OL65/15-RSVA	5.507

Appendix 5

Nucleotide sequences of isolates submitted to genbank.

The accession numbers are in red colour, while the names of isolates are underlined.

The nucleotides are arranged in sixes for convenience of management only.

KU736767 Human respiratory syncytial virus strain NGR/OL63/15-RSVA attachment glycoprotein (G) mRNA, partial cds
ACCACC ACCAAG CCCACA AAAAAA CCAACC CTCAAG ACAACC AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACT ACCAAG CCTACA AGAAAG CCAACC ATCAAC ACCACT AAAACA AACAGC AGAACT ACACTG CTCACC TCCAAC ACCAAA GGAAAT CCAGAA CACACA AGTCAA AAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCTAT CTAAGC CCATCA CAAGTC TATACA ACATCC GGTCAA GAGGAA ACCCCC CACTCA ACCACC TCCGAA GGCTAT CCAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CTATCT TCATCC AACACA ACAAAA TGATAG

KU736768 Human respiratory syncytial virus strain NGR/OL64/15-RSVA attachment glycoprotein (G) mRNA, partial cds
ACCACC ACCAAG CCCACA AAAAAA CCAACC CTCAAG ACAACC AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACT ACCAAG CCTACA GGAAAG CCAACC ATCAAC ACCACT AAAATA AACAGC AGAACT ACACTG CTCGCC TCCAAC ACCAAA GGAAAT CCAGAA CACACA AGTCAA AAGGAA ACCCTC CACTCA ACCACC TCCGAA AGCTAT CCAAGC CCATCA CAAGTC TATACA ACATCT GGTCAA GAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCCAT CCAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CTATCT TCATCC AACACA ACA

KU736769 Human respiratory syncytial virus strain NGR/OL73/15-RSVA attachment glycoprotein (G) mRNA, partial cds
ACCACC ACCAAG CCCACA AAAAAA CCTACC CTCAAG ACAACC AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACC ACCAAG CCTACA GAAAAT CCAACC ATCAAC ACCACC AAAACA AACATC AGAACT ACACTG CTCACC TCCAAC ACCACA GGAAAT CCAGAA CACACA AGTCAA GAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCTAT CTAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CCATCA TCATCC AACACA ACAAAA TTATAG

KU736770 Human respiratory syncytial virus strain NGR/OL76/15-RSVA attachment glycoprotein (G) mRNA, partial cds
ACCACC ACCAAG CCCACA AAAAAA CCAACC CTCAAG ACAACC AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACT ACCAAG CCTACA GGAAAG CCAACC ATCAAC ACCACT AAAATA AACAGC AGAACT ACACTG CTCACC TCCAAC ACCAAA GGAAAT CCAGAA CACACA AGTCAA AAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCTAT CCAAGC CCATCA CAAGTC TATACA ACATCT GGTCAA GAGGAA ACCCTC CACTCA ATCACC TCCGAA GGCCAT CCAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CTATCT TCATCC AACACA ACAAAA TGATAG

KU736771 Human respiratory syncytial virus strain NGR/OL78/15-RSVA attachment glycoprotein (G) mRNA, partial cds
ACCACC ACCAAG CCCACA AAAAAA CCAACC CTCAAG ACAACC AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACC ACCAAG CCTACA GAAAAT CCAACC ATCAAC ACCACC AAAACA AACATC AGAACT ACACTG CTCACC TCCAAC ACCACA GGAAAT CCAGAA CACACA AGTCAA GAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCTAT CTAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CCATCA TCATCC AACACA ACAAAA TTATAG

KU736772 Human respiratory syncytial virus strain NGR/OL96/15-RSVA attachment glycoprotein (G) mRNA, partial cds
ACCACC ACCAAG CCCACA AAAAAA CCAACC CTCAAG ACAACT AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACT ACCAAG CCTACA GGAAAG CCAACC ATCAAC ACCACT AAAATA AACAGC AGAACT ACACTG CTCACC TCCAAC ACCAAA GGAAAT CCAGAA CACACA AGTCAA AAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCTAT CCAAGC CCATCA CAAGTC TATACA ACATCT GGTCAA GAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCCAT CCAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CTATCT TCATCC AACACA ACAAAA TGATAG

KU736773 Human respiratory syncytial virus strain NGR/OL97/15-RSVA attachment glycoprotein (G) mRNA, partial cds
ACCACC ACCAAG CCCACA AAAAAA CCAACC CTCAAG ACAACC AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACT ACCAAG CCTACA GGAAAG CCAACC ATCAAC ACCACT AAAATA AACAGC AGAACT ACACTG CTCGCC TCCAAC ACCAAA GGAAAT CCAGAA CACACA AGTCAA AAGGAA ACCCTC CACTCA ACCACC TCCGAA AGCTAT CCAAGC CCATCA CAAGTC TATACA ACATCT GGTCAA GAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCCAT CCAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CTATCT TCATCC AACACA ACAAAA TGATAG

KU736774 Human respiratory syncytial virus strain NGR/OL102/15-RSVA attachment glycoprotein (G) mRNA, partial cds
ACCACC ACCAAG CCCACA AAAAAA CCAACC CTCAAG ACAACC AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACT ACCAAG CCTACA GGAAAG CCAACC ATCAAC ACCACC AAAACA AACAGC AGAACT ACACTG CTCACC TCCAAC ACCAAA GGAAAT CCAGAA CACACA AGTCAA AAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCTAT CTAAGC CCATCA CAAGTC TATACA ACATCC GGTCAA GAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCTAT CCAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CTATCT TCATCC AACACA ACAAAA TGATAG

KU736775 Human respiratory syncytial virus strain NGR/OL105/15-RSVA attachment glycoprotein (G) mRNA, partial cds
ACCACC ACCAAG CCCACA AAAAAA CCAACC CTCAAG ACAACC AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACT ACCAAG CCTACA GGAAAG CCAACC ATCAAC ACCACT AAAACA AACAGC AGAACT ACACTG CTCACC TCCAAC ACCAAA GGAAAT CCAGAA CACACA AGTCAA AAGGAA ACTCTC CACTCA ACCACC TCCGAA GGCTAT CTAAGC CCATCA CAAGTC TATACA ACATCC GGTCAA GAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCTAT CCAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CTATCT TCATCC AACACA ACAAAA TGATAG

KU736776 Human respiratory syncytial virus strain NGR/OA02/15-RSVA attachment glycoprotein (G) mRNA, partial cds
CCCACA AAAAAA CCAACC CTCAAG ACAACC AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACT ACCAAG CCTACA GGAAAG CCAACC ATCAAC ACCACT AAAATA AACAGC AGAATT ACACTG CTCACC TCCAAC ACCAAA GGAAAT CCAGAA CACACA AGTCAA AAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCTAT CCAAGC CCATCA CAAGTC TATACA ACATCT GGTCAA GAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCCAT CCAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CTATCT TCATCC AACACA ACAAAA TGATAG

MG014703 Human orthopneumovirus strain NGR/OL86/15-RSVA attachment glycoprotein (G) gene, partial cds
AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACC ACCAAG CCTACA GAAAAG CTAACC ATCAAT ACCACC AAAACA AACATC AGAACT ACACTG CTCACC TCCAAC ACCACA GGAAAT CCAGAA CACACA AGTCAA GAGGAA ACCCTC CACTCA ACCACC TCCGAA

GGCTAT CTAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CCATCA TCATCC AACACA ACAAAA TTATAG

MG014704 Human orthopneumovirus strain NGR/OL88/15-RSVA attachment glycoprotein (G) gene, partial cds
AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACT ACCAAG CCTACA GGAAAG CCAACC ATCAAC ACCACT AAAATA AACAGC AGAACT ACACTG CTCGCC TCCAAC ACCAAA GGAAAT CCAGAA CACACA AGTCAA AAGGAA ACCCTC CACTCA ACCACC TCCGAA AGCTAT CCAAGC CCATCA CAAGTC TATACA ACATCT GGTCAA GAGGAA ACCCTC CACTCA ACCACC TCTGAA GGCCAT CCAAGC CCATCA CAAGTC TATACG ACATCC GAGTAC CTATCA CAATCT CTATCT TCATCC AACACA ACAAAA TGA

MG014705 Human orthopneumovirus strain NGR/OL93/15-RSVA attachment glycoprotein (G) gene, partial cds
AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CACACT ACCAAG CCTACA GGAAAA CCAACC ATCAAC ACCACT AAAACA AACATC AGAACT ACACTG CTCACC TCCAAC ACCAAA GGAAAT CCAGAA CACAAA AGTCAA AAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCTAT CTAAGC CCATCA CAAGTC TATACA ACATCC GGTCAA GAGGAA ACCCTC CACTCA ACCACC TCCAAA GGCTAT CTAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CTATCT TCATCC AACACA ACAAAA TGA

MG014706 Human orthopneumovirus strain NGR/OA20/15-RSVA attachment glycoprotein (G) gene, partial cds
AAAAAA GATCCC AAACCT CAAACC ACAAAA CCAAAG GAAGTA CTCACT ACCAAG CCTACA GGAAAG CCAACC ATCAAC ACCACT AAAATA AACAGC AGAATT ACACTG CTCACC TCCAAC ACCAAA GGAAAT CCAGAA CACACA AGTCAA AAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCTAT CCAAGC CCATCA CAAGTC TATACA ACATCT GGTCAA GAGGAA ACCCTC CACTCA ACCACC TCCGAA GGCCAT CCAAGC CCATCA CAAGTC TATACA ACATCC GAGTAC CTATCA CAATCT CTATCT TCATCC AACACA ACAAAA TGA

KU736777 Human respiratory syncytial virus strain NGR/OL79/15-RSVB attachment glycoprotein (G) mRNA, partial cds
GATGAT TACCAT TTTGAA GTGTTC AACTTT GTTCCC TGTAGT ATATGT GGCAAC AATCAA CTCTGC AAATCC ATTTGC AAAACA ATACCA AGCCAT AAACCA AAGAAA AAACCA ACTACA AAACCC ACAAAC AAACCA CCTACC AAAACC ACAAAC AAAAGA GACCCC AAAACA CTAGCC AAAACA CCAAAA AAGAAA ACCACC ATTAAC CCAACA AAAAAA CCAACC CCCAAG ACCACA GAAAGA GACACC AGCACC CCACAA TCCACT GTGCTC GACACA ACCACA TCAAAA CACACA GAAAGA GACACC AGCACC TCACAA TCCATT GCGCTC GACACA ACCACA TCAAAA CACACA ACCCAA CAGCAA TCTCTC TACTCA ACCACC CCCGAA AACACA CCCAAC TCCACA CAAACA CCCACA GCATCC GAGCCC TCCACA TCAAAT TCCACC CAAAAA CTCCAG TCATAT GCTTAG TTATTT

KU736778 Human respiratory syncytial virus strain NGR/OR01/15-RSVB attachment glycoprotein (G) mRNA, partial cds
GATGAT TACCAT TTTGAA GTGTTC AACTTC GTTCCC TGTAGT ATATGT GGCAAC AATCAA CTCTGC AAATCC ATTTGC AAAACA ATACCA AGCAAT AAACCA AAGAAA AAACCA ACCACA AAACCC ACAAAC AAACCA CCTACC AAAACC ACAAAC AAAAGA GACCCC AAAACA CTAGCC AAAACA CCGAAA AAAGAA ACCACC ATTAAC CCAACA AAAAAA CCAACC CCCAAG ACCACA GAAAGA GACACC AGCACC CCACAA TCTACT GTGCTC GACACA ACCACA TCAAAA CACACA GAAAGA GACACC AGCACC TCACAA TCCATT GCGCTT GACACA ACCACA TCAAAA CACACA ACCCAA CAGCAA TCTCTC TACTCA ACCGCT CCCGAA TACACA CCCAAC TCCACA CAAACA CCCACA GCATCC GAGTCC TCCACA TCAAAT TCCAAC TAAAAA CTCCAG TCATAT GCTTAG TTATTT

KU736779 Human respiratory syncytial virus strain NGR/OR75/15-RSVB attachment glycoprotein (G) mRNA, partial cds
GATGAT TACCAT TTTGAA GTGTTC AACTTC GTTCCC TGTAGT ATATGT GGCAAC AATCAA
CTCTGC AAATCC ATTTGC AAAACA ATACCA AGCAAT AAACCA AAGAAA AAACCA ACCACA
AAACCC ACAAAC AAACCA CCTACC AAAACC ACAAAC AAAAGA GACCCC AAAACA CTAGCC
AAAACA CCGAAA AAAGAA ACCACC ATTAAC CCAACA AAAAAA CCAACC CCCAAG ACCACA
GAAAGA GACACC AGCACC CCACAA TCTACT GTGCTC GACACA ACCACA TCAAAA CACACA
GAAAGA GACACC AGCACC TCACAA TCCATT GCGCTT GACACA ACCACA TCAAAA CACACA
ACCCAA CAGCAA TCTCTC TACTCA ACCGCT CCCGAA TACACA CCCAAC TCCACA CAAACA
CCCACA GCATCC GAGTCC TCCACA TCAAAT TCCAAC TAAAAA CTCCAG TCATAT GCTTAG
TTATTT

KU736780 Human respiratory syncytial virus strain NGR/OA01/15-RSVB attachment glycoprotein (G) mRNA, partial cds
GATGAT TACCAT TTTGAA GTGTTC AACTTT GTTCCC TGTAGT ATATGT GGCAAC AATCAA
CTCTGC AAATCC ATTTGC AAAACA ATACCA AGCAAC AAACCA AAGAAA AAACCA ACTACA
AAACCC ACAAAC AAACCA CCTACC AAAACC ACAAAC AAAAGA GACCCC AAAACA CCAGCC
AAAACA CCGAAA AAAGAA ACCACC ATTAAC CCAACA AAAAAA CCAACC CCCAAG ACTACA
GAAAGA GACACC AGCACC TCACAA TCCACT GTGCTC GACATA ACCACA TCAAAA CACACA
GAAAGA GACACC AGCACC TCACAA TCCATT GCGCTT GACACA ACCACA TCAAAA CACACA
ACCCAA CAGCAA TCTCTC CACTCA ACCACC CCTGAA AACACA CCCAAC TCCACA CAAACA
CCCACA GCATCC GAGCCC TCCACA TCAAAT TCTACC CAAAGA CTCCAG TCATAT GCTTAG
TTATTT

KU736781 Human respiratory syncytial virus strain NGR/OA04/15-RSVB attachment glycoprotein (G) mRNA, partial cds
GATGAT TATCAT TTTGAA GTGTTC AACTTT GTTCCC TGTAGT ATATGT GGCAAC AATCAA
CTCTGC AAATCC ATTTGC AAAACA ATACCG AGCAAT AAACCA AAGAAA AAACCA ACTACA
AAACCC ACAAAC AAACCA CCTACC AAAACC ACAAAC AAAAGA GACCTC AAAACA CTAGCC
AAAACA CCGAAA AAAGAA ACCACC ATTAAC CCAACA AAAAAA CCAACC CCCAAG ACTACA
GAAAGA GACACC AGCACC CCACAA TCCACT GTGCTC GACATA ACCACA TCAAAA CACACA
GAAAGA GACACC AGCACC TCACAA TCTATT GTGCTC GAAACA ACCACA TCAAAA CACACA
ACCCAA CAGCAA TCTCTC TACTCA ACCACC CCCGAA AACACA CCCAAC TCCACA CAAACA
CCCACA GCATCC GAGCCT TCCACA TCAAAC TCTACC CAAAGA CTCCAG TCATAT GCTTAG
TTATTT

KU736782 Human respiratory syncytial virus strain NGR/OA19/15-RSVB attachment glycoprotein (G) mRNA, partial cds
GATGAT TATCAT TTTGAA GTGTTC AACTTT GTTCCC TGTAGT ATATGT GGCAAC AATCTA
CTCTGC AAATCC ATTTGC AAAACA ATACCA AGCAAT AAACCA AAGAAA AAACCA ACTACA
AAACCC ACAAAC AAACCA CCTACC AAAACC ACAAAC AAAAGA GACCTC AAAACA CTAGCC
AAAACA CCGAAA AAAGAA ACCACC ATTAAC TCAACA AAAAAA CCAACC CCCAAG ACTACA
GAAAGA GACACC AGCACC CCACAA TCCACT GTGCTC GACACA ACCACA TCAAAA CACACA
GAAAGA GACACC AGCACC TCACAA TCCATT GCGCTC GACGCA ACCACA TCAANA CACACA
ACCCAA CAGCAA TCTCTC TACTCA ACCACC CCCGAA AACACA CCCAAC TCCACA CAAACA
CCCACA GCATCT GAGCCC TCCACA TCAAAT TCTACC TAAAGA CTCCAG TCATAT GCTTAG
TTATTA

KU736783 Human respiratory syncytial virus strain NGR/OA31/15-RSVB attachment glycoprotein (G) mRNA, partial cds
GATGAT TACCAT TTTGAA GTGTTC AACTTC GTTCCC TGTAGT ATATGT GGCAAC AATCAA
CTCTGC AAATCC ATTTGC AAAACA ATACCA AGCAAT AAACCA AAGAAA AAACCA ACCACA
AAACCC ACAAAC AAACCA CCTACC AAAACC ACAAAC AAAAGA GACCCC AAAACA CTAGCC
AAAACA CCGAAA AAAGAA ACCACC ATTAAC CCAACA AAAAAA CCAACC CCCAAG ACCACA
GAAAGA GACATC AGCACC CCACAA TCTACT GTGCTC GACACA ACCACA TCAAAA CACACA
GAAAGA GACACC AGCACC TCACAA TCCATT GCGCTT GACACA ACCACA TCAAAA CACACA
ACCCAA CAGCAA TCTCTC TACTCA ACCACC CCCGAA AACACA CCCAAC TCCACA CAAACA

CCCACA GCATCC AAGTCC TCCACA TCAAAT TCCAAC TAAAAA CTCCAG TCATAT GCTTAG TTATTT

KU736784 Human respiratory syncytial virus strain NGR/OA36/15-RSVB attachment glycoprotein (G) mRNA, partial cds
GATGAT TATCAT TTTGAA GTGTTC AACTTT GTTCCC TGTAGT ATATGT GGCAAC AATCAA CTCTGC AAATCC ATTTGC AAAACA ATACCA AGCAAT AAACCA AAGAAA AAACCA ACTACA AAACCC ACAAAC AAACCA CCTACC AAAACC ACAAAC AAAAGA GACCTC AAAACA CTAGCC AAACCA CCGAAA AAAGAA ACCACC ATTAAC CCAACA AAAAAA CCAACC CCCAAG ACTACA GAAAGA GACACC AGCACC CCACAA TCCACT GTGCTC GACATA ACCACA TCAAAA CACACA GAAAGA GACACC AGCACC TCACAA TCCATT GCGCTC GAAACA ACCACA TCAAAA CACACA ACCCAA CAGCAA TCTCTC CACTCA ACCACC CCCGAA AACACA CCCAAC TCCACA CAAACA CCCACA GCATCC GAGCCT TCCACA TCAAAC TCTACC CAAAGA CTCCAG TCATAT GCTTAG TTATTT

MG014707 Human orthopneumovirus strain NGR/OA10/15-RSVB attachment glycoprotein (G) gene, partial cds
AAAAGA GACCCC AAAACA CTAGCC AAAACA CCGAAA AAAGAA ACCACC ATTAAC GCAACA AAAAAA CCAACC CCAAAG ACTACA GAAAGA GAGATC AGCACA CCACAA TCTACT GTGCTA GACAGA ACCACA TCAAAA CACGCA GAAAGA GACACC AGCACC TCACAA TTGATT GTGCTT GAGACA ACCACA TCAAAA AACACA ACCCAA CAACAA TCTTTA CATTCA ACAACC CCCGAA AACAAA CCCAAC TCCACA CAAACA CCCACA GCATCT GAATCC TCCACA TCAAAT TCAAAC TAAAAA CTGTAG TCATAT GCTTAG TTATTT

MG014708 Human orthopneumovirus strain NGR/OA30/15-RSVB attachment glycoprotein (G) gene, partial cds
AAAAGA GTCCCC AAAACA CCAGCC AAAACA CCGAAA AAAGAA ACCACC ATTAAC CCAACA AAAAAA CCAACC CCCAAG ACTACA GAAAGA GACACC AGCACC TCACAA TTCACT GTGCTC GACATA ACCACA TCAAAA CACACA GAAAGA GACACC AGCACC TCACAA TCCATT GCGCTT GACACA ACCACA TCAAAA CACACA ACCCAA CAGCAA TCTCTC CACTCA ACCACC CCCGAA AACACA CCCAAC TCCACA CAAACA CCCACA GCATCC GAGCCC TCCACA TCAAAT TCTACC CAAAGA CTCCAG TCATAT GCTTAG TTATTT

MG014709 Human orthopneumovirus strain NGR/OR27/15-RSVB attachment glycoprotein (G) gene, partial cds
AAAAGA GACCCT AAAAAA CTAGCC AAAACA CTGAAA AAAGAA ACCACC ATCAAC CCAACA AAAAAA CCGACC CCCAAG ACCACA GAAAAG GACACA AGCACC CCACAA TCCACT GTGCTC GACATA ACCACA TCAAAA CACACA GAAAGA GACACC AGCACC TCACAA TCCATT GCGCTC GAAACA ACCACA TCAAAA CACACA ACCCAA CAGCAA TCTCTC TACTCA ACCACC CCCGAA AACACA CCCAAC TCCACA CAAACA CCCACA GCATCC GAGCCT TCCACA TCAAAC TCTACC CAAAGA CTCCAG TCATAT GCTTAG TTATTT

KU893253 Human respiratory syncytial virus A isolate NGR/OL63/15-RSVA-F fusion glycoprotein (F) gene, partial cds
CCAATC CTCAAA ACAAAT GCTATT ACCACA ATCCTT GCTGCA GTCACA CTCTGT TTTGCT TCCAGT CAAAAC ATCACT GAAGAA TTTTAT CAATCA ACATGC AGTGCA GTTAGC AAAGGC TATCTT AGTGCT CTAAGA ACTGGT TGGTAT ACTAGT GTTATA ACTATA GAATTA AGTAAT ATCAAG GAAAAT AAGTGT AATGGT ACAGAC GCTAAG GTAAAA TTAATA AAACAA GAATTA GATAAA TATAAA AATGCT GTAACA GAATTG CAGTTG CTCATG CAAAGC ACACCA GCAGCC AACAGT CGAGCC AGAAGA GAACTA CCAAGA TTTATG AATTAT ACACTC AACAAT ACCAAA AACACC AATGTA ACATTA AGTAAG AAAAGG AAAAGA AGATTT CTTGGA TTTTTG TTAGGT GTTGGA TCTGCA ATCGCC AGTGGC ATTGCC GTATCC AAGGTC CTGCAC CTAGAA GGGGAA GTGAAC AAAATC AAAAGT GCTCTA CTATCC ACAAAC AAGGCT GTAGTC AGCTTA TCTAAT GGAGTC AGTGTC TTAACC AGCAAG GTGTTA GACCTC AAAAAC TATATA GATAAA CAGTTG TTACCT ATTGTT AACAAG CAAAGC TGCAGC ATATCA AACATT GAAACT GTGATA GAGTTC CAACAA AAGAAC AACAGA CTACTA GAGATT ACCAGA GAATTT AGTGTT AATGCA GGTGTA

ACTACA CCTGTA AGCACT TATATG TTAACT AATAGT GAGTTA TTATCA TTAATC AATGAT ATGCCT ATAACA AATGAT CAGAAA AAGTTA ATGTCC AGCAAT GTTCAA ATAGTT AGACAG CAAAGT TACTCT ATCATG TCAATA ATAAAA GAGGAA GTCTTA GCATAT GTAGTA CAATTA CCACTA TATGGT GTAATA GATACT CCTTGT TGGAAA CTACAC ACATCC CCTCTA TGTACA ACCAAC ACAAAG GAAGGA TCCAAC ATCTGC TTAACA AGAACC GACAGA GGATGG TACTGT GA

KU893254 Human respiratory syncytial virus A isolate NGR/OL64/15-RSVA-F fusion glycoprotein (F) gene, partial cds
ATCCTC AAAACA AATGCT ATTACC ACAATC CTTGCT GCAGTC ACACTC TGTTTT GCTTCC AGTCAA AACATC ACTGAA GAATTT TATCAA TCAACA TGCAGT GTAGTT AGCAAA GGCTAT CTTAGT GCTCTA AGAACT GGTTGG TATACT AGTGTT ATAACT ATAGAA TTAAGT AATATC AAGGAA AATAAG TGTAAT GGTACA GACACT AAGGTA AAATTA ATAAAA CAAGAA TTAGAT AAATAT AAAAAT GCTGTA ACAGAA TTGCAG TTGCTC ATGCAA AGCACA CCAGCA GCCAAC AGTCGA GCCAGA AGAGAA CTAACA AGATTT ATGAAT TATACA CTCAAC AATACC AAAAAC ACCAAT GTAACA TTAAGT AAGAAA AGGAAA AGAAGA TTTCTT GGATTT TTGTTA GGTGTT GGATCT GCAATC GCCAGT GGCATT GCCGTA TCCAAG GTCCTG CACCTA GAAGGG GAAGTG AACAAA ATCAAA AGTGCT CTACTA TCCACA AACAAG GCTGTA GTCAGC TTATCT AATGGA GTCAGT GTCTTA ACCAGC AAGGTG TTAGAC CTCAAA AACTAT ATAGAT AAACAG TTGTTA CCTATT GTTAAC AAGCAA AGCTGC AGCATA TCAAAC ATTGAA ACTGTG ATAGAG TTCCAA CAAAAG AACAAC AGACTA CTAGAG ATTACC AGAGAA TTTAGT GTTAAT GCAGGT GTAACT ACACCT GTAAGC ACTTAT ATGTTA ACTAAT AGTGAG TTATTA TCATTA ATCAAT GATATG CCTATA ACAAAT GATCAG AAAAAG TTAATG TCCAGC AATGTT CAAATA GTTAGA CAGCAA AGTTAC TCTATC ATGTCA ATAATA AAAGAG GAAGTC TTAGCA TATGTA GTACAA TTACCA CTATAT GGTGTA ATAGAT ACTCCT TGTTGG AAACTA CACACA TCCCCT CTATGT ACAACC AACACA AAGGAA GGATCC AACATC TGCTTA ACAAGA ACCGAC AGAGGA TGGTAC TGTGAC AT

KU893255 Human respiratory syncytial virus B isolate NGR/OL79/15-RSVB-F fusion glycoprotein (F) gene, partial cds
GCAGTT AGCAGA GGTTAC TTGAGT GCTTTA AGAACA GGTTGG TATACC AGTGTC ATAACA ATAGAA TTAAGT AATATA AAAGAA ACCAAA TGCAAT GGAACT GACACT AAAGTA AAACTT ATAAAA CAAGAA TTAGAT AAGTAT AAGAAT GCAGTA ACAGAA TTACAG TTACTT ATGCAA AACACA CCAGCT GTCAAC AACCGG GCCAGA AGAGAA GCACCA CAGTAT ATGAAC TACACA ATCAAT ACCACT AAAAAC CTAAAT GTATCA ATAAGC AAGAAG AGGAAA CGAAGA TTTCTG GGCTTC TTGTTA GGTGTA GGATCT GCAATA GCAAGT GGTATA GCTGTA TCCAAA GTTCTA CACCTT GAAGGA GAAGTG AACAAG ATCAAA AATGCT TTGCAG TCTACA AACAAA GCTGTA GTCAGT CTATCA AATGGG GTCAGT GTTTTA ACCAGC AAAGTG TTAGAT CTCAAG AATTAT ATAAAC AACCAA TTATTA CCTATA GTAAAT CAACAG AGTTGT CGCATA TCCAAC ATTGAA ACAGTT ATAGAA TTCCAG CAGAAG AACAGC AGATTG TTGGAA ATCACC AGAGAA TTTAGT GTCAAT GCAGGT GTAACG ACACCT TTAAGC ACTTAC ATGTTA ACAAAC AGTGAG TTACTA TCATTA ATCAAT GATATG CCTATA ACAAAT GATCAG AAAAAA TTAATG TCAAGC AATGTT CAGATA GTAAGG CAACAA AGTTAT TCTATC ATGTCT ATAATA AAGGAA GAAGTC CTTGCA TATGTT GTACAG CTACCT ATCTAT GGTGTA ATTGAT ACACCT TGCTGG AAATTA CACACA TCACCT CTGTGC ACCACC AACACC AAAGAA GGATCA AATATT TGTTTA ACAAGG ACTGAT AGAGGA TGGTAC TGTGAT AA

KU893256 Human respiratory syncytial virus A isolate NGR/OL97/15-RSVA-F fusion glycoprotein (F) gene, partial cds
GTGCCA ATCCTC AAAACA AATGCT ATTACC ACAATC CTTGCT GCAGTC ACACTC TNTTTT GCTTCC AGTCAA AACATC ACTGAA GAATTT TATCAA TCAACA TGCAGT GCAGTT AGCAAA GGCTAT CTTAGT GCTCTA AGAACT GGTTGG TATACT AGTGTT ATAACT ATAGAA TTAAGT AATATC AAGGAA AATAAG TGTAAT GGTACA GACACT AAGGTA AAATTA ATAAAA CAAGAA TTAGAT AAATAT AAAAAT GCTGTA ACAGAA TTGCAG TTGCTC ATGCAA AGCACA CCAGCA GCCAAC AGTCGA GCCAGA AGAGAA CTAACA AGATTT ATGAAT TATACA CTCAAC AATACC AAAAAC ACCAAT GTAACA TTAAGT AAGAAA AGGAAA AGAAGA TTTCTT GGATTT TTGTTA GGTGTT GGATCT GCAATC GCCAGT GGCATT GCCGTA TCCAAG GTCCTG CACCTA GAAGGG GAAGTG AACAAA ATCAAA AGTGCT CTACTA TCCACA AACAAG GCTGTA GTCAGC TTATCT

AATGGA GTCAGT GTCTTA ACCAGC AAGGTG TTAGAC CTCAAA AACTAT ATAGAT AAACAG
TTGTTA CCTATT GTTAAC AAGCAA AGCTGC AGCATA TCAAAC ATTGAA ACTGTG ATAGAG
TTCCAA CAAAAG AACAAC AGACTA CTAGAG ATTACC AGAGAA TTTAGT GTTAAT GCAGGT
GTAACT ACACCT GTAAGC ACTTAT ATGTTA ACTAAT AGTGAG TTATTA TCATTA ATCAAT
GATATG CCTATA ACAAAT GATCAG AAAAAG TTAATG TCCAGC AATGTT CAAATA GTTAGA
CAGCAA AGTTAC TCTATC ATGTCA ATAATA AAAGAG GAAGTC TTAGCA TATGTA GTACAA
TTACCA CTATAT GGTGTA ATAGAT ACTCCT TGTTGG AAACTA CACACA TCCCCT CTATGT
ACAACC AACACA AAGGAA GGATCC AACATC TGCTTA ACAAGA ACCGAC AGAGGA TGGTAC
TGTGAC AATGCA

KU893257 Human respiratory syncytial virus A isolate NGR/OL102/15-RSVA-F fusion glycoprotein (F) gene, partial cds
ATTACC ACAATC CTTGCT GCAGTC ACACTC TGTTTT GCTTCC AGTCAA AACATC ACTGAA
GAATTT TATCAA TCAACA TGCAGT GCAGTT AGCAAA GGCTAT CTTAGT GCTCTA AGAACT
GGTTGG TATACT AGTGTT ATAACT ATAGAA TTAAGT AATATC AAGGAA AATAAG TGTAAT
GGTACA GACGCT AAGGTA AAATTA ATAAAA CAAGAA TTAGAT AAATAT AAAAAT GCTGTA
ACAGAA TTGCAG TTGCTC ATGCAA AGCACA CCAGCA GCCAAC AGTCGA GCCAGA AGAGAA
CTACCA AGATTT ATGAAT TATACA CTCAAC AATACC AAAAAC ACCAAT GTAACA TTAAGT
AAGAAA AGGAAA AGAAGA TTTCTT GGATTT TTGTTA GGTGTT GGATCT GCAATC GCCAGT
GGCATT GCCGTA TCCAAG GTCCTG CACCTA GAAGGG GAAGTG AACAAA ATCAAA AGTGCT
CTACTA TCCACA AACAAG GCTGTA GTCAGC TTATCT AATGGA GTCAGT GTCTTA ACCAGC
AAGGTG TTAGAC CTCAAA AACTAT ATAGAT AAACAG TTGTTA CCTATT GTTAAC AAGCAA
AGCTGC AGCATA TCAAAC ATTGAA ACTGTG ATAGAG TTCCAA CAAAAG AACAAC AGACTA
CTAGAG ATTACC AGAGAA TTTAGT GTTAAT GCAGGT GTAACT ACACCT GTAAGC ACTTAT
ATGTTA ACTAAT AGTGAG TTATTA TCATTA ATCAAT GATATG CCTATA ACAAAT GATCAG
AAAAAG TTAATG TCCAGC AATGTT CAAATA GTTAGA CAGCAA AGTTAC TCTATC ATGTCA
ATAATA AAAGAG GAAGTC TTAGCA TATGTA GTACAA TTACCA CTATAT GGTGTA ATAGAT
ACTCCT TGTTGG AAACTA CACACA TCCCCT CTATGT ACAACC AACACA AAGGAA GGATCC
AACATC TGCTTA ACAAGA ACCGAC AGAGGA TGGTAC TGTGA

KU893258 Human respiratory syncytial virus B isolate NGR/OR01/15-RSVB-F fusion glycoprotein (F) gene, partial cds
GAGTTG CTGATC CATAGA TCAAGT GCAATC TTCCTA ACTCTT GCTATT AATGCA TTGTAC
CTCATC TCAAGT CAGAAC ATAACC GAGGAG TTTTAC CAATCA ACATGT AGTGCA GTTAGC
AGAGGT TACTTG AGTGCT TTAAGA ACAGGT TGGTAT ACCAGT GTCATA ACAATA GAGTTA
AGTAAT ATAAAA GAAACC AAATGC AATGGA ACTGAC ACTAAA GTAAAA CTTATA AAACAA
GAATTA GATAAG TATAAG AATGCA GTAACA GAATTA CAGTTA CTTATG CAAAAC ACACCA
GCTGTC AACAAC CGGGCC AGAAGA GAAGCA CCACAG TATGTG AACTAC ACAATC AATACC
ACTAAA AACCTA AATGTA TCAATA AGCAAG AAGAGG AAACGA AGATTT CTGGGT TTCTTG
TTAGGT GTAGGA TCTGCA ATAGCA AGTGGT ATAGCT GTATCC AAAGTT CTACAC CTTGAA
GGGGAA GTGAAC AAGATC AAAAAT GCTTTG CAGTCT ACAAAC AAAGCT GTAGTC AGTCTA
TCAAAT GGGGTC AGTGTT TTAACC AGCAAA GTGTTA GATCTC AAGAAT TATATA AACAGC
CAATTA TTACCT ATAGTA AATAAA CAGAGT TGTCGC ATATCA AACATT GAAACA GTTATA
GAATTC CAGCAG AAGAAC AGCAGA TTGTTG GAAATC ACCAGA GAATTT AGTGTC AATGCA
GGTGTA ACGACA CCTTTA AGCACT TACATG TTAACA AACAGT GAGTTA CTATCA TTAATC
AATGAT ATGCCT ATAACA AATGAT CAGAAA AAATTA ATGTCA AGCAAT GTTCAG ATAGTA
AGGCAA CAAAGT TATTCT ATCATG TCTATA ATAAAG GAAGAA GTCCTT GCATAT GTTGTA
CAGCTA CCTATC TATGGT GTAATT GATACA CCTTGC TGGAAA TTACAC ACATCA CCTCTG
TGCACC ACCAAC ATCAAA GAAGGA TCAAAT ATTTGT TTAACA AGGACT GATAGA GGATGG
TACTGT GATAAT GCA

KU893259 Human respiratory syncytial virus B isolate NGR/OR27/15-RSVB-F fusion glycoprotein (F) gene, partial cds
CTGATC CATAGA TCAAGT GCAATC TTCCTA ACTCTT ACTATT AATGCA TTGTAC CTTACC
TCAAGT CAGAAC ATAACT GAGGAG TTTTAC CAATCA ACATGT AGTGCA GTTAGC AGAGGT
TACTTG AGTGCT TTAAGA ACAGGT TGGTAT ACCAGT GTCATA ACAATA GAATTA AGTAAT
ATAAAA GAAACC AAATGC AATGGA ACTGAC ACTAAA GTAAAA CTTATA AAACAA GAATTA
GATAAG TATAAG AATGCA GTAACA GAATTA CAGTTA CTTATG CAAAAC ACACCA GCTGTC

AACAAC CGGGCC AGAAGA GAAGCA CCACAG TATATG AACTAC ACAATC AATACC ACTAAA
AACCTA AATGTA TCAATA AGCAAG AAGAGG AAACGA AGATTT CTGGGC TTCTTG TTAGGT
GTAGGA TCTGCA ATAGCA AGTGGT ATAGCT GTATCC AAAGTT CTACAC CTTGAA GGAGAA
GTGAAC AAGATC AAAAAT GCTTTG CAGCTT ACAAAC AAAGCT GTAGTC AGTCTA TCAAAT
GGGGTC AGTGTT TTAACC AGCAAA GTGTTA GATCTC AAGAAT TATATA AACAAC CAGTTA
TTACCT ATAGTA AATCAA CAGAGT TGTCGC ATATCC AACATT GAAACA GTTATA GAATTC
CAGCAG AAGAAC AGCAGA TTGTTG GAAATC ACCAGA GAATTT AGTGTC AATGCA GGTGTA
ACGACA CCTTTA AGCACT TACATG TTAACA AACAGT GAGTTA CTATCA TTAATC AATGAT
ATGCCT ATAACA AATGAT CAGAAA AAATTA ATGTCA AGCAAT GTTCAG ATAGTA AGGCAA
CAAAGT TATTCT ATCATG TCTATA ATAAAG GAAGAA GTCCTT GCATAT GTTGTA CAGCTA
CCTATC TATGGT GTAATT GATACA CCTTGC TGGAAA TTACAC ACATCA CCTCTG TGCACC
ACCAAC ATCAAA GAAGGA TCAAAT ATTTGT TTAACA AGGACT GATAGA GGATGG TACTGT
GATAT

MG323925 Human respiratory syncytial virus B isolate NGR/OA01/15-RSVB-F fusion glycoprotein (F) gene, partial cds

GCAGTT AGCAGA GGTTAC TTGAGT GCTTTA AGAACA GGTTGG TATACC AGTGTC ATAACA
ATAGAA TTAAGT AATATA AAAGAA ACCAAA TGCAAT GGAACT GACACT AAAGTA AAACTT
ATAAAA CAAGAA TTAGAT AAGTAT AAGAAT GCAGTA ACAGAA TTACAG TTACTT ATGCAA
AACACA CCAGCT GTCAAC AACCGG GCCAGA AGAGAA GCACCA CAGTAT ATGAAC TACACA
ATCAAT ACCACT AAAAAC CTAAAT GTATCA ATAAGC AAGAAG AGGAAA CGAAGA TTTCTG
GGCTTC TTGTTA GGTGTA GGATCT GCAATA GCAAGT GGTATA GCTGTA TCCAAA GTTCTA
CACCTT GAAGGA GAAGTG AACAAG ATCAAA AATGCT TTGCAG CTTACA AACAAA GCTGTA
GTCAGT CTATCA AATGGG GTCAGT GTTTTA ACCAGC AAAGTG TTAGAT CTCAAG AATTAT
ATAAAC AACCAA TTATTA CCTATA GTAAAT CAACAG AGTTGT CGCATA TCCAAC ATTGAA
ACAGTT ATAGAA TTCCAG CAGAAG AACAGC AGATTG TTGGAA ATCACC AGAGAA TTTAGT
GTCAAT GCAGGT GTAACG ACACCTT TAAGCA CTTACA TGTTAA CAAACA GTGAGT
TACTAT CATTAA TCAATG ATATGC CTATCA CAAATG ATCAGA AAAAAT TAATGT CTAGCA
ATGTTC AGATAG TAAGGC AGCAAA GTTATT CTATCC TGTCTA TAATTA AGGAGG AAGTCC
TTGCTT ATGTTG TACGGC TACCTA TCTATG GTGTAA TTGATA CACCTT GCTGGA AATTAC
ACACAT CCCCTC TG

MG323926 Human respiratory syncytial virus B isolate NGR/OA04/15-RSVB-F fusion glycoprotein (F) gene, partial cds

TCAAGT CAGAAC ATAACT GAGGAG TTTTAC CAATCA ACATGT AGTGCA GTTAGC AGAGGT
TACTTG AGTGCT TTAAGA ACAGGT TGGTAT ACCAGT GTCATA ACAATA GAATTA AGTAAT
ATAAAA GAAACC AAATGC AATGGA ACTGAC ACTAAA GTAAAA CTTATA AAACAA GAATTA
GATAAG TATAAG AATGCA GTAACA GAATTA CAGTTA CTTATG CAAAAC ACACCA GCTGTC
AACAAC CGGGCC AGAAGA GAAGCA CCACAG TATATG AACTAC ACAATC AATACC ACTAAA
AACCTA AATGTA TCAATA AGCAAG AAGAGG AAACGA AGATTT CTGGGC TTCTTG TTAGGT
GTAGGA TCTGCA ATAGCA AGTGGT ATAGCT GTATCC AAAGTT CTACAC CTTGAA GGAGAA
GTGAAC AAGATC AAAAAT GCTTTG CAGCTT ACAAAC AAAGCT GTAGTC AGTCTA TCAAAT
GGGGTC AGTGTT TTAACC AGCAAA GTGTTA GATCTC AAGAAT TATATA AACAAC CAATTA
TTACCT ATAGTA AATCAA CAGAGT TGTCGC ATATCC AACATT GAAACA GTTATA GAATTC
CAGCAG AAGAAC AGCAGA TTGTTG GAAATC ACCAGA GAATTT AGTGTC AATGCA GGTGTA
ACGACA CCTTTA AGCACT TACATG TTAACA AACAGT GAGTTA CTATCA ATAATC AATGAT
ATGCCT ATAACA AATGAT CAGAAA AAATTA ATGTCA AGCAAT GTTCAG ATAGTA AGGCAA
CAAAGT TATTCT ATCATG TCTATA ATAAAG GAAGAA GTCCTT GCATAT GTTGTA CAGCTA
CCTATC TATGGT GTAATT GATACA CCTTGC TGGAAA TTACAC ACATCA CCTCTG TGCA

MG323927 Human respiratory syncytial virus A isolate NGR/OL73/15-RSVA-F fusion glycoprotein (F) gene, partial cds

TGCTAT TACCAC ATTCCT TTCTGC AGTCAC ACTCTG TTTCGC TTCCAG TCAAAA TATCAC
TGAGGA ATTTTA TCAATC AACATG CAGTGC AGTTAG CAAAGG CTATCT TAGTGC TCTAAG
AACTGG TTGGTA TACAAG TGTTAT AACTAT AGAATT AAGTAA TATCAA GGAAAA TAAGTG
TAATGG TACAGA CGCTAA GGTAAA ATTAAT AAAACA AGAATT AGATAA ATATAA AAATGC
TGTAAC AGAATT GCAGTT GCTCAT GCAAAG CACACC AGCAGC CAACAG TCGAGC CAGAAG
AGAACT ACCAAG ATTTAT GAATTA TACACT CAACAA TACCAA AAACAC CAATGT AACATT
AAGTAA GAAAAG GAAAAG AAGATT TCTTGG ATTTTT GTTAGG TGTTGG ATCTGC AATCGC
CAGTGG CATTGC CGTATC CAAGGT CCTGCA CCTAGA AGGGGA AGTGAA CAAAAT CAAAAG

TGCTCT ACTATC CACAAA CAAGGC TGTAGT CAGCTT ATCTAA TGGAGT CAGTGT CTTAAC
CAGCAA AGTGTT AGACCT CAAAAA CTATAT AGATAA ACAGTT GTTACC TATTGT TAACAA
GCAAAG CTGCAG CATATC AAACAT TGAAAC TGTGAT AGAGTT CCAACA AAAGAA CAACAG
ACTACT AGAGAT TACCAG AGAATT TAGTGT TAATGC AGGTGT AACTAC ACCTGT AAGCAC
TTATAT GTTAAC TAATAG TGAGTT ATTATC ATTAAT CAATGA TATGCC TATAAC AAATGA
TCAGAA AAAGTT AATGTC CAGCAA TGTTCA AATAGT TAGACA GCAAAG TTACTC TATCAT
GTCAAT AATAAA AGAGGA AGTCTT AGCATA TGTAGT ACAATT ACCACT ATATGG TGTAAT
AGATAC TCCTTG TTGG

MG323928 Human respiratory syncytial virus A isolate NGR/OL96/15-RSVA-F fusion glycoprotein (F) gene, partial cds
TGCTAT AACCAC AATCCT TGCTGC AGTCAC ATTCTG TTTTGC TTCCAG TCAAAA CATCAC
TGAAGA ATTTTA TCAATC AACATG CAGTGC AGTTAG CAAAGG CTATCT TAGTGC TCTAAG
AACTGG TTGGTA TACTAG TGTTAT AACTAT AGAATT AAGTAA TATCAA GGAAAA TAAGTG
TAATGG TACAGA CACTAA GGTAAA ATTAAT AAAACA AGAATT AGATAA ATATAA AAATGC
TGTAAC AGAATT GCAGTT GCTCAT GCAAAG CACACC AGCAGC CAACAG TCGAGC CAGAAG
AGAACT ACCAAG ATTTAT GAATTA TACACT CAACAA TACCAA AAACAC CAATGT AACATT
AAGTAA GAAAAG GAAAAG AAGATT TCTTGG ATTTTT GTTAGG TGTTGG ATCTGC AATCGC
CAGTGG CATTGC CGTATC CAAGGT CCTGCA CTTAGA AGGGGA AGTGAA CAAAAT CAAAAG
TGCTCT ACTATC CACAAA CAAGGC TGTAGT CAGCTT ATCTAA TGGAGT CAGTGT CTTAAC
CAGCAA GGTGTT AGACCT CAAAAA CTATAT AGATAA ACAGTT GTTACC TATTGT TAACAA
GCAAAG CTGCAG CATATC AAACAT TGAAAC TGTGAT AGAGTT CCAACA AAAGAA CAACAG
ACTACT AGAGAT TACCAG AGAATT TAGTGT TAATGC AGGTGT AACTAC ACCTGT AAGCAC
TTATAT GTTAAC TAATAG TGAGTT ATTATC ATTAAT CAATGA TATGCC TATAAC AAATGA
TCAGAA AAAGTT AATGTC CAGCAA TGTTCA AATAGT TAGACA GCAAAG TTACTC TATCAT
GTCAAT AATAAA AGAGGA AGTCTT AGCATA TGTAGT ACAATT ACCACT ATATGG TGTAAT
AGATAC TCCTTG TTGGAA ACTACA CACATC CCCTCT ATGTAC AACCAA CACAAA G

MG323929 Human respiratory syncytial virus A isolate NGR/OL78/15-RSVA-F fusion glycoprotein (F) gene, partial cds
GAACTG GTTGGT ATACTA GTGTTA TAACTA TAGAAT TAAGTA ATATCA AGGAAA ATAAGT
GTAATG GTACAG ACACTA AGGTAA AATTAA TAAAAC AAGAAT TAGATA AATATA AAAATG
CTGTAA CAGAAT TGCAGT TGCTCA TGCAAA GCACAC CAGCAG CCAACA GTCGAG CCAGAA
GAGAAC TACCAA GATTTA TGAATT ATACAC TCAACA ATACCA AAAACA CCAATG TAACAT
TAAGTA AGAAAA GGAAAA GAAGAT TTCTTG GATTTT TGTTAG GTGTTG GATCTG CAATCG
CCAGTG GCATTG CCGTAT CCAAGG TCCTGC ACCTAG AAGGGG AAGTGA ACAAAA TCAAAA
GTGCTC TACTAT CCACAA ACAAGG CTGTAG TCAGCT TATCTA ATGGAG TCAGTG TCTTAA
CCAGCA AAGTGT TAGACC TCAAAA ACTATA TAGATA AACCGT TGTTAC CTATTG TTAACA
AGCAAA GCTGCA GCATAT CAAACA TTGAAA CTGTGA TAGAGT TCCAAA CAAAAA ACAACA
GACTAC TAGAGA TTACCA GAGAAA TTAGTG TTAATG CAGGTG TAACTA CGCCTG CAAGCA
CTTATA TGGTAA CTAATA GTGAGT TAATAT CATTAA TCAATG ATGTGC CTATAA CAAATG
ATCAG

MG323930 Human respiratory syncytial virus A isolate NGR/OL88/15-RSVA-F fusion glycoprotein (F) gene, partial cds
GTAATA TCAAGG AAAATA AGTGTA ATGGTA CAGACA CTAAGG TAAAAT TAATAA AACAAG
AATTAG ATAAAT ATAAAA ATGCTG TAACAG AATTGC AGTTGC TCATGC AAAGCA CACCAG
CAGCCA ACAGTC GAGCCA GAAGAG AACTAA CAAGAT TTATGA ATTATA CACTCA ACAATA
CCAAAA ACACCA ATGTAA CATTAA GTAAGA AAAGGA AAAGAA GATTTC TTGGAT TTTTGT
TAGGTG TTGGAT CTGCAA TCGCCA GTGGCA TTGCCG TATCCA AGGTCC TGCACC TAGAAG
GGGAAG TGAACA AAATCA AAAGTG CTCTAC TATCCA CAAACA AGGCTG TAGTCA GCTTAT
CTAATG GAGTC

KU893253 Human respiratory syncytial virus A isolate NGR/OL63/15-RSVA-F fusion glycoprotein (F) gene, partial cds
CCAATC CTCAAA ACAAAT GCTATT ACCACA ATCCTT GCTGCA GTCACA CTCTGT TTTGCT
TCCAGT CAAAAC ATCACT GAAGAA TTTTAT CAATCA ACATGC AGTGCA GTTAGC AAAGGC
TATCTT AGTGCT CTAAGA ACTGGT TGGTAT ACTAGT GTTATA ACTATA GAATTA AGTAAT
ATCAAG GAAAAT AAGTGT AATGGT ACAGAC GCTAAG GTAAAA TTAATA AAACAA GAATTA
GATAAA TATAAA AATGCT GTAACA GAATTG CAGTTG CTCATG CAAAGC ACACCA GCAGCC
AACAGT CGAGCC AGAAGA GAACTA CCAAGA TTTATG AATTAT ACACTC AACAAT ACCAAA

AACACC AATGTA ACATTA AGTAAG AAAAGG AAAAGA AGATTT CTTGGA TTTTTG TTAGGT GTTGGA TCTGCA ATCGCC AGTGGC ATTGCC GTATCC AAGGTC CTGCAC CTAGAA GGGGAA GTGAAC AAAATC AAAAGT GCTCTA CTATCC ACAAAC AAGGCT GTAGTC AGCTTA TCTAAT GGAGTC AGTGTC TTAACC AGCAAG GTGTTA GACCTC AAAAAC TATATA GATAAA CAGTTG TTACCT ATTGTT AACAAG CAAAGC TGCAGC ATATCA AACATT GAAACT GTGATA GAGTTC CAACAA AAGAAC AACAGA CTACTA GAGATT ACCAGA GAATTT AGTGTT AATGCA GGTGTA ACTACA CCTGTA AGCACT TATATG TTAACT AATAGT GAGTTA TTATCA TTAATC AATGAT ATGCCT ATAACA AATGAT CAGAAA AAGTTA ATGTCC AGCAAT GTTCAA ATAGTT AGACAG CAAAGT TACTCT ATCATG TCAATA ATAAAA GAGGAA GTCTTA GCATAT GTAGTA CAATTA CCACTA TATGGT GTAATA GATACT CCTTGT TGGAAA CTACAC ACATCC CCTCTA TGTACA ACCAAC ACAAAG GAAGGA TCCAAC ATCTGC TTAACA AGAACC GACAGA GGATGG TACTGT GA

KU893254 Human respiratory syncytial virus A isolate NGR/OL64/15-RSVA-F fusion glycoprotein (F) gene, partial cds
ATCCTC AAAACA AATGCT ATTACC ACAATC CTTGCT GCAGTC ACACTC TGTTTT GCTTCC AGTCAA AACATC ACTGAA GAATTT TATCAA TCAACA TGCAGT GTAGTT AGCAAA GGCTAT CTTAGT GCTCTA AGAACT GGTTGG TATACT AGTGTT ATAACT ATAGAA TTAAGT AATATC AAGGAA AATAAG TGTAAT GGTACA GACACT AAGGTA AAATTA ATAAAA CAAGAA TTAGAT AAATAT AAAAAT GCTGTA ACAGAA TTGCAG TTGCTC ATGCAA AGCACA CCAGCA GCCAAC AGTCGA GCCAGA AGAGAA CTAACA AGATTT ATGAAT TATACA CTCAAC AATACC AAAAAC ACCAAT GTAACA TTAAGT AAGAAA AGGAAA AGAAGA TTTCTT GGATTT TTGTTA GGTGTT GGATCT GCAATC GCCAGT GGCATT GCCGTA TCCAAG GTCCTG CACCTA GAAGGG GAAGTG AACAAA ATCAAA AGTGCT CTACTA TCCACA AACAAG GCTGTA GTCAGC TTATCT AATGGA GTCAGT GTCTTA ACCAGC AAGGTG TTAGAC CTCAAA AACTAT ATAGAT AAACAG TTGTTA CCTATT GTTAAC AAGCAA AGCTGC AGCATA TCAAAC ATTGAA ACTGTG ATAGAG TTCCAA CAAAAG AACAAC AGACTA CTAGAG ATTACC AGAGAA TTTAGT GTTAAT GCAGGT GTAACT ACACCT GTAAGC ACTTAT ATGTTA ACTAAT AGTGAG TTATTA TCATTA ATCAAT GATATG CCTATA ACAAAT GATCAG AAAAAG TTAATG TCCAGC AATGTT CAAATA GTTAGA CAGCAA AGTTAC TCTATC ATGTCA ATAATA AAAGAG GAAGTC TTAGCA TATGTA GTACAA TTACCA CTATAT GGTGTA ATAGAT ACTCCT TGTTGG AAACTA CACACA TCCCCT CTATGT ACAACC AACACA AAGGAA GGATCC AACATC TGCTTA ACAAGA ACCGAC AGAGGA TGGTAC TGTGAC AT

KU893255 Human respiratory syncytial virus B isolate NGR/OL79/15-RSVB-F fusion glycoprotein (F) gene, partial cds
GCAGTT AGCAGA GGTTAC TTGAGT GCTTTA AGAACA GGTTGG TATACC AGTGTC ATAACA ATAGAA TTAAGT AATATA AAAGAA ACCAAA TGCAAT GGAACT GACACT AAAGTA AAACTT ATAAAA CAAGAA TTAGAT AAGTAT AAGAAT GCAGTA ACAGAA TTACAG TTACTT ATGCAA AACACA CCAGCT GTCAAC AACCGG GCCAGA AGAGAA GCACCA CAGTAT ATGAAC TACACA ATCAAT ACCACT AAAAAC CTAAAT GTATCA ATAAGC AAGAAG AGGAAA CGAAGA TTTCTG GGCTTC TTGTTA GGTGTA GGATCT GCAATA GCAAGT GGTATA GCTGTA TCCAAA GTTCTA CACCTT GAAGGA GAAGTG AACAAG ATCAAA AATGCT TTGCAG TCTACA AACAAA GCTGTA GTCAGT CTATCA AATGGG GTCAGT GTTTTA ACCAGC AAAGTG TTAGAT CTCAAG AATTAT ATAAAC AACCAA TTATTA CCTATA GTAAAT CAACAG AGTTGT CGCATA TCCAAC ATTGAA ACAGTT ATAGAA TTCCAG CAGAAG AACAGC AGATTG TTGGAA ATCACC AGAGAA TTTAGT GTCAAT GCAGGT GTAACG ACACCT TTAAGC ACTTAC ATGTTA ACAAAC AGTGAG TTACTA TCATTA ATCAAT GATATG CCTATA ACAAAT GATCAG AAAAAA TTAATG TCAAGC AATGTT CAGATA GTAAGG CAACAA AGTTAT TCTATC ATGTCT ATAATA AAGGAA GAAGTC CTTGCA TATGTT GTACAG CTACCT ATCTAT GGTGTA ATTGAT ACACCT TGCTGG AAATTA CACACA TCACCT CTGTGC ACCACC AACACC AAAGAA GGATCA AATATT TGTTTA ACAAGG ACTGAT AGAGGA TGGTAC TGTGAT AA

KU893256 Human respiratory syncytial virus A isolate NGR/OL97/15-RSVA-F fusion glycoprotein (F) gene, partial cds
GTGCCA ATCCTC AAAACA AATGCT ATTACC ACAATC CTTGCT GCAGTC ACACTC TNTTTT GCTTCC AGTCAA AACATC ACTGAA GAATTT TATCAA TCAACA TGCAGT GCAGTT AGCAAA GGCTAT CTTAGT GCTCTA AGAACT GGTTGG TATACT AGTGTT ATAACT ATAGAA TTAAGT

AATATC AAGGAA AATAAG TGTAAT GGTACA GACACT AAGGTA AAATTA ATAAAA CAAGAA
TTAGAT AAATAT AAAAAT GCTGTA ACAGAA TTGCAG TTGCTC ATGCAA AGCACA CCAGCA
GCCAAC AGTCGA GCCAGA AGAGAA CTAACA AGATTT ATGAAT TATACA CTCAAC AATACC
AAAAAC ACCAAT GTAACA TTAAGT AAGAAA AGGAAA AGAAGA TTTCTT GGATTT TTGTTA
GGTGTT GGATCT GCAATC GCCAGT GGCATT GCCGTA TCCAAG GTCCTG CACCTA GAAGGG
GAAGTG AACAAA ATCAAA AGTGCT CTACTA TCCACA AACAAG GCTGTA GTCAGC TTATCT
AATGGA GTCAGT GTCTTA ACCAGC AAGGTG TTAGAC CTCAAA AACTAT ATAGAT AAACAG
TTGTTA CCTATT GTTAAC AAGCAA AGCTGC AGCATA TCAAAC ATTGAA ACTGTG ATAGAG
TTCCAA CAAAAG AACAAC AGACTA CTAGAG ATTACC AGAGAA TTTAGT GTTAAT GCAGGT
GTAACT ACACCT GTAAGC ACTTAT ATGTTA ACTAAT AGTGAG TTATTA TCATTA ATCAAT
GATATG CCTATA ACAAAT GATCAG AAAAAG TTAATG TCCAGC AATGTT CAAATA GTTAGA
CAGCAA AGTTAC TCTATC ATGTCA ATAATA AAAGAG GAAGTC TTAGCA TATGTA GTACAA
TTACCA CTATAT GGTGTA ATAGAT ACTCCT TGTTGG AAACTA CACACA TCCCCT CTATGT
ACAACC AACACA AAGGAA GGATCC AACATC TGCTTA ACAAGA ACCGAC AGAGGA TGGTAC
TGTGAC AATGCA

KU893257 Human respiratory syncytial virus A isolate NGR/OL102/15-RSVA-F fusion glycoprotein (F) gene, partial cds
ATTACC ACAATC CTTGCT GCAGTC ACACTC TGTTTT GCTTCC AGTCAA AACATC ACTGAA
GAATTT TATCAA TCAACA TGCAGT GCAGTT AGCAAA GGCTAT CTTAGT GCTCTA AGAACT
GGTTGG TATACT AGTGTT ATAACT ATAGAA TTAAGT AATATC AAGGAA AATAAG TGTAAT
GGTACA GACGCT AAGGTA AAATTA ATAAAA CAAGAA TTAGAT AAATAT AAAAAT GCTGTA
ACAGAA TTGCAG TTGCTC ATGCAA AGCACA CCAGCA GCCAAC AGTCGA GCCAGA AGAGAA
CTACCA AGATTT ATGAAT TATACA CTCAAC AATACC AAAAAC ACCAAT GTAACA TTAAGT
AAGAAA AGGAAA AGAAGA TTTCTT GGATTT TTGTTA GGTGTT GGATCT GCAATC GCCAGT
GGCATT GCCGTA TCCAAG GTCCTG CACCTA GAAGGG GAAGTG AACAAA ATCAAA AGTGCT
CTACTA TCCACA AACAAG GCTGTA GTCAGC TTATCT AATGGA GTCAGT GTCTTA ACCAGC
AAGGTG TTAGAC CTCAAA AACTAT ATAGAT AAACAG TTGTTA CCTATT GTTAAC AAGCAA
AGCTGC AGCATA TCAAAC ATTGAA ACTGTG ATAGAG TTCCAA CAAAAG AACAAC AGACTA
CTAGAG ATTACC AGAGAA TTTAGT GTTAAT GCAGGT GTAACT ACACCT GTAAGC ACTTAT
ATGTTA ACTAAT AGTGAG TTATTA TCATTA ATCAAT GATATG CCTATA ACAAAT GATCAG
AAAAAG TTAATG TCCAGC AATGTT CAAATA GTTAGA CAGCAA AGTTAC TCTATC ATGTCA
ATAATA AAAGAG GAAGTC TTAGCA TATGTA GTACAA TTACCA CTATAT GGTGTA ATAGAT
ACTCCT TGTTGG AAACTA CACACA TCCCCT CTATGT ACAACC AACACA AAGGAA GGATCC
AACATC TGCTTA ACAAGA ACCGAC AGAGGA TGGTAC TGTGA

KU893258 Human respiratory syncytial virus B isolate NGR/OR01/15-RSVB-F fusion glycoprotein (F) gene, partial cds
GAGTTG CTGATC CATAGA TCAAGT GCAATC TTCCTA ACTCTT GCTATT AATGCA TTGTAC
CTCATC TCAAGT CAGAAC ATAACC GAGGAG TTTTAC CAATCA ACATGT AGTGCA GTTAGC
AGAGGT TACTTG AGTGCT TTAAGA ACAGGT TGGTAT ACCAGT GTCATA ACAATA GAGTTA
AGTAAT ATAAAA GAAACC AAATGC AATGGA ACTGAC ACTAAA GTAAAA CTTATA AAACAA
GAATTA GATAAG TATAAG AATGCA GTAACA GAATTA CAGTTA CTTATG CAAAAC ACACCA
GCTGTC AACAAC CGGGCC AGAAGA GAAGCA CCACAG TATGTG AACTAC ACAATC AATACC
ACTAAA AACCTA AATGTA TCAATA AGCAAG AAGAGG AAACGA AGATTT CTGGGT TTCTTG
TTAGGT GTAGGA TCTGCA ATAGCA AGTGGT ATAGCT GTATCC AAAGTT CTACAC CTTGAA
GGGGAA GTGAAC AAGATC AAAAAT GCTTTG CAGTCT ACAAAC AAAGCT GTAGTC AGTCTA
TCAAAT GGGGTC AGTGTT TTAACC AGCAAA GTGTTA GATCTC AAGAAT TATATA AACAGC
CAATTA TTACCT ATAGTA AATAAA CAGAGT TGTCGC ATATCA AACATT GAAACA GTTATA
GAATTC CAGCAG AAGAAC AGCAGA TTGTTG GAAATC ACCAGA GAATTT AGTGTC AATGCA
GGTGTA ACGACA CCTTTA AGCACT TACATG TTAACA AACAGT GAGTTA CTATCA TTAATC
AATGAT ATGCCT ATAACA AATGAT CAGAAA AAATTA ATGTCA AGCAAT GTTCAG ATAGTA
AGGCAA CAAAGT TATTCT ATCATG TCTATA ATAAAG GAAGAA GTCCTT GCATAT GTTGTA
CAGCTA CCTATC TATGGT GTAATT GATACA CCTTGC TGGAAA TTACAC ACATCA CCTCTG
TGCACC ACCAAC ATCAAA GAAGGA TCAAAT ATTTGT TTAACA AGGACT GATAGA GGATGG
TACTGT GATAAT GCA

KU893259 Human respiratory syncytial virus B isolate NGR/OR27/15-RSVB-F fusion glycoprotein (F) gene, partial cds
CTGATC CATAGA TCAAGT GCAATC TTCCTA ACTCTT ACTATT AATGCA TTGTAC CTTACC
TCAAGT CAGAAC ATAACT GAGGAG TTTTAC CAATCA ACATGT AGTGCA GTTAGC AGAGGT
TACTTG AGTGCT TTAAGA ACAGGT TGGTAT ACCAGT GTCATA ACAATA GAATTA AGTAAT
ATAAAA GAAACC AAATGC AATGGA ACTGAC ACTAAA GTAAAA CTTATA AAACAA GAATTA
GATAAG TATAAG AATGCA GTAACA GAATTA CAGTTA CTTATG CAAAAC ACACCA GCTGTC
AACAAC CGGGCC AGAAGA GAAGCA CCACAG TATATG AACTAC ACAATC AATACC ACTAAA
AACCTA AATGTA TCAATA AGCAAG AAGAGG AAACGA AGATTT CTGGGC TTCTTG TTAGGT
GTAGGA TCTGCA ATAGCA AGTGGT ATAGCT GTATCC AAAGTT CTACAC CTTGAA GGAGAA
GTGAAC AAGATC AAAAAT GCTTTG CAGCTT ACAAAC AAAGCT GTAGTC AGTCTA TCAAAT
GGGGTC AGTGTT TTAACC AGCAAA GTGTTA GATCTC AAGAAT TATATA AACAAC CAGTTA
TTACCT ATAGTA AATCAA CAGAGT TGTCGC ATATCC AACATT GAAACA GTTATA GAATTC
CAGCAG AAGAAC AGCAGA TTGTTG GAAATC ACCAGA GAATTT AGTGTC AATGCA GGTGTA
ACGACA CCTTTA AGCACT TACATG TTAACA AACAGT GAGTTA CTATCA TTAATC AATGAT
ATGCCT ATAACA AATGAT CAGAAA AAATTA ATGTCA AGCAAT GTTCAG ATAGTA AGGCAA
CAAAGT TATTCT ATCATG TCTATA ATAAAG GAAGAA GTCCTT GCATAT GTTGTA CAGCTA
CCTATC TATGGT GTAATT GATACA CCTTGC TGGAAA TTACAC ACATCA CCTCTG TGCACC
ACCAAC ATCAAA GAAGGA TCAAAT ATTTGT TTAACA AGGACT GATAGA GGATGG TACTGT
GATAT

MG323925 Human respiratory syncytial virus B isolate NGR/OA01/15-RSVB-F fusion glycoprotein (F) gene, partial cds
GCAGTT AGCAGA GGTTAC TTGAGT GCTTTA AGAACA GGTTGG TATACC AGTGTC ATAACA
ATAGAA TTAAGT AATATA AAAGAA ACCAAA TGCAAT GGAACT GACACT AAAGTA AAACTT
ATAAAA CAAGAA TTAGAT AAGTAT AAGAAT GCAGTA ACAGAA TTACAG TTACTT ATGCAA
AACACA CCAGCT GTCAAC AACCGG GCCAGA AGAGAA GCACCA CAGTAT ATGAAC TACACA
ATCAAT ACCACT AAAAAC CTAAAT GTATCA ATAAGC AAGAAG AGGAAA CGAAGA TTTCTG
GGCTTC TTGTTA GGTGTA GGATCT GCAATA GCAAGT GGTATA GCTGTA TCCAAA GTTCTA
CACCTT GAAGGA GAAGTG AACAAG ATCAAA AATGCT TTGCAG CTTACA AACAAA GCTGTA
GTCAGT CTATCA AATGGG GTCAGT GTTTTA ACCAGC AAAGTG TTAGAT CTCAAG AATTAT
ATAAAC AACCAA TTATTA CCTATA GTAAAT CAACAG AGTTGT CGCATA TCCAAC ATTGAA
ACAGTT ATAGAA TTCCAG CAGAAG AACAGC AGATTG TTGGAA ATCACC AGAGAA TTTAGT
GTCAAT GCAGGT GTAACG ACACCTT TAAGCA CTTACA TGTTAA CAAACA GTGAGT
TACTAT CATTAA TCAATG ATATGC CTATCA CAAATG ATCAGA AAAAAT TAATGT CTAGCA
ATGTTC AGATAG TAAGGC AGCAAA GTTATT CTATCC TGTCTA TAATTA AGGAGG AAGTCC
TTGCTT ATGTTG TACGGC TACCTA TCTATG GTGTAA TTGATA CACCTT GCTGGA AATTAC
ACACAT CCCCTC TG

MG323926 Human respiratory syncytial virus B isolate NGR/OA04/15-RSVB-F fusion glycoprotein (F) gene, partial cds
TCAAGT CAGAAC ATAACT GAGGAG TTTTAC CAATCA ACATGT AGTGCA GTTAGC AGAGGT
TACTTG AGTGCT TTAAGA ACAGGT TGGTAT ACCAGT GTCATA ACAATA GAATTA AGTAAT
ATAAAA GAAACC AAATGC AATGGA ACTGAC ACTAAA GTAAAA CTTATA AAACAA GAATTA
GATAAG TATAAG AATGCA GTAACA GAATTA CAGTTA CTTATG CAAAAC ACACCA GCTGTC
AACAAC CGGGCC AGAAGA GAAGCA CCACAG TATATG AACTAC ACAATC AATACC ACTAAA
AACCTA AATGTA TCAATA AGCAAG AAGAGG AAACGA AGATTT CTGGGC TTCTTG TTAGGT
GTAGGA TCTGCA ATAGCA AGTGGT ATAGCT GTATCC AAAGTT CTACAC CTTGAA GGAGAA
GTGAAC AAGATC AAAAAT GCTTTG CAGCTT ACAAAC AAAGCT GTAGTC AGTCTA TCAAAT
GGGGTC AGTGTT TTAACC AGCAAA GTGTTA GATCTC AAGAAT TATATA AACAAC CAATTA
TTACCT ATAGTA AATCAA CAGAGT TGTCGC ATATCC AACATT GAAACA GTTATA GAATTC
CAGCAG AAGAAC AGCAGA TTGTTG GAAATC ACCAGA GAATTT AGTGTC AATGCA GGTGTA
ACGACA CCTTTA AGCACT TACATG TTAACA AACAGT GAGTTA CTATCA ATAATC AATGAT
ATGCCT ATAACA AATGAT CAGAAA AAATTA ATGTCA AGCAAT GTTCAG ATAGTA AGGCAA
CAAAGT TATTCT ATCATG TCTATA ATAAAG GAAGAA GTCCTT GCATAT GTTGTA CAGCTA
CCTATC TATGGT GTAATT GATACA CCTTGC TGGAAA TTACAC ACATCA CCTCTG TGCA

MG323927 Human respiratory syncytial virus A isolate NGR/OL73/15-RSVA-F fusion glycoprotein (F) gene, partial cds

TGCTAT TACCAC ATTCCT TTCTGC AGTCAC ACTCTG TTTCGC TTCCAG TCAAAA TATCAC TGAGGA ATTTTA TCAATC AACATG CAGTGC AGTTAG CAAAGG CTATCT TAGTGC TCTAAG AACTGG TTGGTA TACAAG TGTTAT AACTAT AGAATT AAGTAA TATCAA GGAAAA TAAGTG TAATGG TACAGA CGCTAA GGTAAA ATTAAT AAAACA AGAATT AGATAA ATATAA AAATGC TGTAAC AGAATT GCAGTT GCTCAT GCAAAG CACACC AGCAGC CAACAG TCGAGC CAGAAG AGAACT ACCAAG ATTTAT GAATTA TACACT CAACAA TACCAA AAACAC CAATGT AACATT AAGTAA GAAAAG GAAAAG AAGATT TCTTGG ATTTTT GTTAGG TGTTGG ATCTGC AATCGC CAGTGG CATTGC CGTATC CAAGGT CCTGCA CCTAGA AGGGGA AGTGAA CAAAAT CAAAAG TGCTCT ACTATC CACAAA CAAGGC TGTAGT CAGCTT ATCTAA TGGAGT CAGTGT CTTAAC CAGCAA AGTGTT AGACCT CAAAAA CTATAT AGATAA ACAGTT GTTACC TATTGT TAACAA GCAAAG CTGCAG CATATC AAACAT TGAAAC TGTGAT AGAGTT CCAACA AAAGAA CAACAG ACTACT AGAGAT TACCAG AGAATT TAGTGT TAATGC AGGTGT AACTAC ACCTGT AAGCAC TTATAT GTTAAC TAATAG TGAGTT ATTATC ATTAAT CAATGA TATGCC TATAAC AAATGA TCAGAA AAAGTT AATGTC CAGCAA TGTTCA AATAGT TAGACA GCAAAG TTACTC TATCAT GTCAAT AATAAA AGAGGA AGTCTT AGCATA TGTAGT ACAATT ACCACT ATATGG TGTAAT AGATAC TCCTTG TTGG

MG323928 Human respiratory syncytial virus A isolate NGR/OL96/15-RSVA-F fusion glycoprotein (F) gene, partial cds
TGCTAT AACCAC AATCCT TGCTGC AGTCAC ATTCTG TTTTGC TTCCAG TCAAAA CATCAC TGAAGA ATTTTA TCAATC AACATG CAGTGC AGTTAG CAAAGG CTATCT TAGTGC TCTAAG AACTGG TTGGTA TACTAG TGTTAT AACTAT AGAATT AAGTAA TATCAA GGAAAA TAAGTG TAATGG TACAGA CACTAA GGTAAA ATTAAT AAAACA AGAATT AGATAA ATATAA AAATGC TGTAAC AGAATT GCAGTT GCTCAT GCAAAG CACACC AGCAGC CAACAG TCGAGC CAGAAG AGAACT ACCAAG ATTTAT GAATTA TACACT CAACAA TACCAA AAACAC CAATGT AACATT AAGTAA GAAAAG GAAAAG AAGATT TCTTGG ATTTTT GTTAGG TGTTGG ATCTGC AATCGC CAGTGG CATTGC CGTATC CAAGGT CCTGCA CTTAGA AGGGGA AGTGAA CAAAAT CAAAAG TGCTCT ACTATC CACAAA CAAGGC TGTAGT CAGCTT ATCTAA TGGAGT CAGTGT CTTAAC CAGCAA GGTGTT AGACCT CAAAAA CTATAT AGATAA ACAGTT GTTACC TATTGT TAACAA GCAAAG CTGCAG CATATC AAACAT TGAAAC TGTGAT AGAGTT CCAACA AAAGAA CAACAG ACTACT AGAGAT TACCAG AGAATT TAGTGT TAATGC AGGTGT AACTAC ACCTGT AAGCAC TTATAT GTTAAC TAATAG TGAGTT ATTATC ATTAAT CAATGA TATGCC TATAAC AAATGA TCAGAA AAAGTT AATGTC CAGCAA TGTTCA AATAGT TAGACA GCAAAG TTACTC TATCAT GTCAAT AATAAA AGAGGA AGTCTT AGCATA TGTAGT ACAATT ACCACT ATATGG TGTAAT AGATAC TCCTTG TTGGAA ACTACA CACATC CCCTCT ATGTAC AACCAA CACAAA G

MG323929 Human respiratory syncytial virus A isolate NGR/OL78/15-RSVA-F fusion glycoprotein (F) gene, partial cds
GAACTG GTTGGT ATACTA GTGTTA TAACTA TAGAAT TAAGTA ATATCA AGGAAA ATAAGT GTAATG GTACAG ACACTA AGGTAA AATTAA TAAAAC AAGAAT TAGATA AATATA AAAATG CTGTAA CAGAAT TGCAGT TGCTCA TGCAAA GCACAC CAGCAG CCAACA GTCGAG CCAGAA GAGAAC TACCAA GATTTA TGAATT ATACAC TCAACA ATACCA AAAACA CCAATG TAACAT TAAGTA AGAAAA GGAAAA GAAGAT TTCTTG GATTTT TGTTAG GTGTTG GATCTG CAATCG CCAGTG GCATTG CCGTAT CCAAGG TCCTGC ACCTAG AAGGGG AAGTGA ACAAAA TCAAAA GTGCTC TACTAT CCACAA ACAAGG CTGTAG TCAGCT TATCTA ATGGAG TCAGTG TCTTAA CCAGCA AAGTGT TAGACC TCAAAA ACTATA TAGATA AACCGT TGTTAC CTATTG TTAACA AGCAAA GCTGCA GCATAT CAAACA TTGAAA CTGTGA TAGAGT TCCAAA CAAAAA ACAACA GACTAC TAGAGA TTACCA GAGAAA TTAGTG TTAATG CAGGTG TAACTA CGCCTG CAAGCA CTTATA TGGTAA CTAATA GTGAGT TAATAT CATTAA TCAATG ATGTGC CTATAA CAAATG ATCAG

MG323930 Human respiratory syncytial virus A isolate NGR/OL88/15-RSVA-F fusion glycoprotein (F) gene, partial cds
GTAATA TCAAGG AAAATA AGTGTA ATGGTA CAGACA CTAAGG TAAAAT TAATAA AACAAG AATTAG ATAAAT ATAAAA ATGCTG TAACAG AATTGC AGTTGC TCATGC AAAGCA CACCAG CAGCCA ACAGTC GAGCCA GAAGAG AACTAA CAAGAT TTATGA ATTATA CACTCA ACAATA CCAAAA ACACCA ATGTAA CATTAA GTAAGA AAAGGA AAAGAA GATTTC TTGGAT TTTTGT TAGGTG TTGGAT CTGCAA TCGCCA GTGGCA TTGCCG TATCCA AGGTCC TGCACC TAGAAG GGGAAG TGAACA AAATCA AAAGTG CTCTAC TATCCA CAAACA AGGCTG TAGTCA GCTTAT CTAATG GAGTC