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Molecular Epidemiology of RSV

In Southern Palestine

By

Randa Said Jadallah Kattan

In Partial Fulfillment of the Requirements for the Degree
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In Southern Palestine

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Molecular Epidemiology of Respiratory Syncytial Virus in Southern Palestine

By Randa Said Jadallah Kattan

ABSTRACT

Respiratory Syncytial Virus (RSV) is one of the major respiratory tract viral pathogens throughout the world, causing acute lower respiratory infections among infants and children less than 5 years of age. Two major antigenic groups of RSV (A and B) have been identified based on monoclonal antibodies to the major structural glycoproteins G and F. Children initially infected with a group A RSV are relatively protected against group A infection, and re-infections are more likely to be due to heterologous group B RSV.

The aims of this study were to investigate the epidemiology of RSV in the southern part of Palestine between January 2006 and December 2009, identify which RSV serotype has circulated and determine the genotypes of selected RSV isolates.

Laboratory diagnosis of RSV by direct fluorescent antibody (DFA) staining was performed on 5,820 nasopharyngeal aspirates (NPA) collected from patients admitted to Caritas Baby Hospital with respiratory tract infection. Typing of RSV was performed on extracted viral RNA by Reverse-Transcriptase Polymerase Chain reaction (RT-PCR) using different sets of primers, while, RSV genotyping was performed by sequencing part of the RSV A and B glycoprotein G gene.

Of the 5,820 NPA samples analyzed, 1,388 (23.8%) were positive for RSV, of which, 70% were from children in the first six months of their life and the male to female ratio was 1.6:1. In Southern Palestine, RSV major activity varies between the winter and spring seasons. Molecular typing of RSV revealed that 68% belonged to group A, while 28% belonged to group B and 2.9% could not be typed. RSV-A appears to have circulated in the southern part of Palestine between January 2006 and July 2007, while RSV-B started to circulate after January 2008. RSV genotyping revealed the presence of several RSV A and B genotypes.

Phylogenetic analysis revealed that the genotypes in Palestine for RSV A belong to GA5 and GA2 which are known worldwide. Interestingly, newly proposed lineages Pal/GA8 and Pal/GA9 were detected within the samples tested. RSV B genotyping revealed that the circulating groups belong to BA9/GB13 that were also found in other areas in the world. In addition new proposed lineages were identified as Pal/GB14.

RSV is an important viral pathogen in southern Palestine causing more than 25% of respiratory infections. RSV-A appears to be the most common RSV type during the study period. Because of the high prevalence of RSV infections in young children, hospital laboratories must bring laboratory detection methodologies to their laboratories.

Key words: Respiratory Syncytial Virus (RSV), nasopharyngeal aspirates (NPA), RSV Group A and RSV Group B.

وباء ال (RSV) في المناطق الجنوبية في فلسطين

ملخص

يعد فيروس ال (RSV) أحد أهم مسببات الأمراض الفيروسية التنفسية في جميع أنحاء العالم حيث يتسبب في الالتهابات الحادة في الجهاز التنفسي السفلي بين الرضّع والأطفال الذين تقل أعمارهم عن خمس سنوات. وقد تم تحديد مجموعتين رئيسيتين من هذا الفيروس (RSV-A) و (RSV-B) وذلك عن طريق التحليل باستخدام الأجسام المضادة للجليوكوبروتينات G و F. فالأطفال الذين يصابون بالمجموعة (RSV-A) تقل احتمالات إعادة إصابتهم بنفس المجموعة غير أن الإصابة الثانية قد تنتج عن المجموعة الثانية (RSV-B).

تهدف هذه الدراسة إلى البحث في مدى انتشار وباء ال (RSV) في المناطق الجنوبية في فلسطين ما بين كانون ثاني 2006 وكانون أول 2009، تحديد النوع الفرعي (RSV-A or B) المنتشر في تلك الفترة إضافة إلى تحديد الصفات الوراثية لعينات معزولة من هذا الفيروس.

أجري التحليل المخبري باستخدام طريقة (DFA) على 5820 عينة من مستخرج البلعوم (NPA) والتي تم جمعها من اطفال مصابين بالتهابات الجهاز التنفسي أدخلوا إلى مستشفى كاريتاس للأطفال في بيت لحم. وقد تم تصنيف جينات ال RSV بطريقة (RT-PCR) وذلك باستخدام مجموعات مختلفة من ال Primers. بينما تم تصنيف جينات الفيروس عن طريق تحديد تسلسل القواعد النووية للجليكوبروتين G وذلك لكل من (RSV-A) و(RSV-B).

بينت الدراسة أن 1388 (23,8%) من العينات المدروسة والتي كان عددها 5820 عينة، هي عينات مصابة بفيروس ال RSV يعود 70% منها إلى أطفال لم يتجاوزوا من العمر 6 أشهر كما وكانت نسبة الإصابة في الذكور إلى الإناث تعادل 1:1.6.

كما وتبين أن ذروة النشاط لفيروس RSV كانت في موسمي الشتاء والربيع. كما وتبين من التصنيف الجيني للفيروس أن 68% منه تنتمي إلى المجموعة A و28% تنتمي إلى المجموعة B ولم تتمكن الدراسة من تصنيف 2,9% من العينة المدروسة. كما وتبين أن الفيروس RSV-A قد انتشر في جنوب فلسطين بين كانون ثاني 2006 وتموز 2007 بينما بدأ

RSV-B بالانتشار بعد كانون ثاني 2008. كما وبين التحليل الجيني انتشار عدد من الأنماط الجنينة للفيروس المنتشر سواء كان ينتمي إلى المجموعة الأولى أو الثانية.

بيَّن تحليل السلالات الجينية أن التصنيف الجيني لفيروس RSV-A ينتمي إلى سلالات GA5 وGA2 المعرّفة عالمياً. ومن المثير للاهتمام أن تقترح هذه الدراسة وجود سلالتين جديدتين للمجموعة A انتشرتا في فلسطين في السنوات الخاضعة للدراسة. تقترح الباحثة تسميتها بـِ PAL/GA8 و PAL/GA9. وبالمقابل تبين أن التصنيف الجيني لفيروس RSV-B ينتمي إلى سلالات BA9/GB13 المعرّفة عالمياً إضافة إلى بروز سلالة جديدة منتشرة في فلسطين في سنوات الدراسة تقترح الباحثة تسميتها PAL/GB14.

يعتبر RSV التهاب فيروسي ينتشر في جنوب فلسطين ويسبب ما يزيد عن 25% من الالتهابات في الجهاز التنفسي. وتبين أن RSV-A كان الأكثر انتشاراً في السنوات الخاضعة للدراسة. وبسبب هذا الانتشار بين الأطفال الصغار، على المستشفيات أن تجهز مختبراتها بأساليب ومنهجيات خاصة للكشف عن هذا الفيروس.

Declaration

I declare that the Master Thesis entitled “Molecular Epidemiology of Respiratory Syncytial Virus in Southern Palestine” is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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Dedication

To my mother for her understanding….

To my brother for his support….

To my sisters, nephews and nieces for their patience….

Acknowledgment

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List of Abbreviations

ABI	Applied Biosystems
ALRI	Acute Lower Respiratory Infection
AR	Acute Respiratory
BA	Buenos Aires
BPD	Bronchopulmonary Dysplasia
bRSV	Bovine RSV
CBH	Caritas Baby Hospital
cDNA	Complimentary Deoxyribonucleic Acid
CF	Cystic Fibrosis
CFT	Compliment Fixation Test
CPE	Cytopathic Effect
CX3C	Chemokine Receptors
DC (USA)	Washington, D.C
EIA	Enzyme Immuno Assay
ELISA	Enzyme-Linked Immunosorbent Assay
F	Fusion Protein
FDA	Food and Drug Administration
GTPase	An Enzyme That Hydrolyze Guanosine Triphosphate
HeLa	Cell Type in an Immortal Cell Line
hRSV	Human RSV
IFA	Immunofluorescence Antibody
IFN	Interferons
IL	Interleukins
IMPDH	Inosine Monophosphate Dehydrogenase
KDa	Kilo Dalton
LRTI	Lower Respiratory Tract Infection
M	Matrix Protein
mAb	Monoclonal Antibody

Mr	Marker Protein
mRNA	Massenger Ribonucleic Acid
NK	Natural Killer
NPA	Nasopharangeal Aspirate
NS	Nephrotic Syndrome
OFR	Open Reading Frame
P	Phosphoprotein
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
RhoA	Ras Homolog Gene Family, Member A
RNA	Ribonucleic Acid
RPM	Round Per Minute
RSV	Respiratory Syncycial Virus
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TLR4	Toll-Like Receptor 4
URT	Upper Respiratory Tract
USA	United State Of America
WB	West Bank

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CHAPTER 1 – Introduction and Literature Review

1 Introduction

Respiratory Syncytial Virus (RSV) is the most common cause of serious Acute Lower Respiratory Infection (ALRI) among infants and young children in the world (Zlateva, et al., 2005). This virus remains to be a significant public health problem in the world although there are some developments in the field of anti-RSV neutralizing antibodies.

This thesis intends to highlight the both, the epidemiology and molecular epidemiology of RSV in the southern parts of Palestine, Bethlehem and Hebron districts. It is based on analyzing patient respiratory samples for the presence of different RSV strains among children in several cities of the West Bank (WB).

This thesis is divided into several chapters. The first chapter discusses the history of RSV, which includes elaborations on RSV structure, pathogenesis, transmission, immunology, treatment and RSV vaccination. A literature review is presented in this chapter to highlight major findings of previous research in this field.

The second chapter of the thesis describes the objectives of the research and its importance in this part of the world, as well as the description of the methodology used in collecting and analyzing data, the population's characteristics and samples' description. The results of the research are presented in chapter three of this thesis, followed by the discussion of the results as well as conclusions in chapter four.

1.1 Historical Overview

RSV was discovered in 1956 when a group of chimpanzees in Washington, DC (USA) were noted to have developed cold-like illness. Ogra (2004) reported that Morris and colleagues recovered a cytopathic agent by cell culture from one of these chimpanzees, which presented with an upper respiratory tract (URT) illness, runny nose and malaise. What was also noted that any human who was in contact with the sick chimpanzees also developed similar symptoms.

1.2 Virology

RSV belongs to the family *Paramyxoviridae*, genus *Pneumovirinae*. This family has a non segmented, single stranded, negative-sense 15KDa RNA genome. RSV has a pleomorphic morphology (spherical or filamentous) (see Figure 1.1 and Figure 1.2) ranging in diameter between 150-300 nm (Barends, et al., 2004; Cane, 2001; Gottschalk, et al., 1996; Hall, 2001; Zlateva, et al., 2005). The *Paramyxoviridae* family includes two other genera: *Morbillivirus* (measles virus) and the *paramyxoviruses* (mumps virus and *parainfluenza* viruses). RSV genome is made of 10 genes that encode for 11 proteins (Bossart and Broder, 2000; Gottschalk, et al., 1996). RSV has a quite mutable genome by virtue of its dependence on an RNA dependent RNA polymerase that lacks the capacity for proofreading and editing. Populations of RNA viruses exist in equilibrium around a theoretical consensus sequence as quasispecies, with a distribution of related but non-identical genomes. This genetic heterogeneity is then shaped by the selective pressures of the environment, providing for great adaptability among these viruses (Sullender, 2000).

Figure 1.1: RSV Pleomorphic Shape[1]

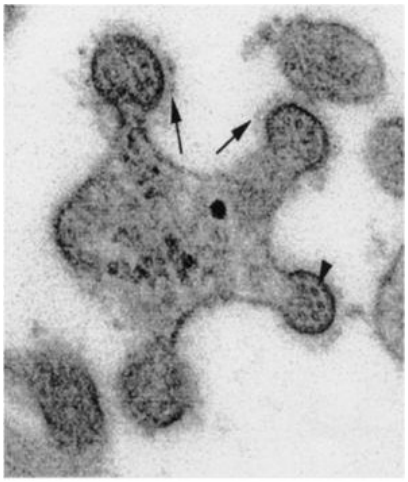


Figure 1.2: RSV Budding Virus[2]

1.3 RSV Structure and Proteins

The 11 RSV encoded proteins (Gottschalk, et al., 1996; Hall, 2001; McNamara and Smyth, 2002; Mufson, et al., 1985) are visualized in the cartoon illustrated in Figure 1.3. Although the figure shows 10 proteins only, the M2 protein is divided into two proteins M2-1 and M2-2 which will increase the number of proteins to 11 as discussed in the following page. A description of the proteins is shown in Table 1.1 below:

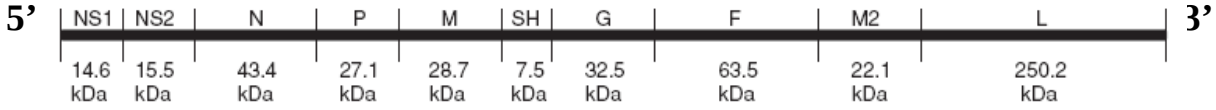


Figure 1.3: The RSV Genome Showing the Location and Size of the Protein (adapted from McNamara and Smyth (2002))

[1] Source: http://www.ncbi.nlm.nih.gov/ICTVdb/WIntkey/Images/em_param.htm
[2] Source: http://www.ncbi.nlm.nih.gov/ICTVdb/WIntkey/Images/em_param.htm

Table 1.1: CHARACTERISTICS OF RSV PROTEINS
(ADAPTED FROM (HALL, 2001))

PROTEIN	MOLECULAR MASS IN RSV (KILODALTONS)	FUNCTIONS
STRUCTURAL PROTEIN		
SURFACE		
FUSION (F)	68	PENETRATION; MAJOR PROTECTION ANTIGEN
ATTACHMENT (G)	90	VIRAL ATTACHMENT; MAJOR PROTECTIVE ANTIGEN
SMALL HYDROPHOBIC (SH[1A])	4.8-30*	UNKNOWN
MATRIX		
MATRIX (M)	28	MEDIATES ATTACHMENT OF NUCLEOCAPSID TO ENVELOPE
SMALL ENVELOPE (M2)	22*	TRANSCRIPTIONAL REGULATION; UNIQUE TO PNEUMOVIRUSES
NUCLEOCAPSID-ASSOCIATED		
NUCLEOPROTEIN (N, NP)	44	MAJOR RNA-BINDING NUCLEOCAPSID PROTEIN
PHOSPHOPROTEIN (P)	37	MAJOR PHOSPHORYLATED PROTEIN; RNA-DEPENDENT RNA POLYMERASE ACTIVITY
LARGE POLYMERASE COMPLEX (L)	200	LARGE NUCLEOCAPSID-ASSOCIATED PROTEIN; MAJOR POLYMERASE SUBUNIT; RNA-DEPENDENT RNA POLYMERASE ACTIVITY
NONSTRUCTURAL PROTEIN		
NONSTRUCTURAL (NS1 [1C])	15.6*	FUNCTION UNKNOWN; UNIQUE TO PNEUMOVIRUSES
NONSTRUCTURAL (NS2 [1B])	14.7*	FUNCTION UNKNOWN; UNIQUE TO PNEUMOVIRUSES

* THERE ARE FOUR GLYCOSYLATED AND NON-GLYCOSYLATED FORMS WITH MOLECULAR MASSES OF 4.8, 7.5, 13 TO 15, AND 21 TO 30 KDA.

The RSV genome as well as the location and the size of each of the proteins as encoded by the individual RNA genes are shown in Figure 1.3 above.

RSV genes encode for two nonstructural proteins that precede nine structural proteins in the following order: N, P, M, SH, G, F, M2-1, M2-2, and L (Gottschalk, et al., 1996). The viral structure consists of a nucleocapsid core surrounded by a lipid layer where three glycoproteins (G, F, and SH) are embedded (see Figure 1.4). The G protein is responsible for viral attachment to host cells, while the F protein promotes syncytia formation (McNamara and Smyth, 2002). The F and G glycoproteins are the major targets of neutralizing antibodies (Gottschalk, et al., 1996). RSV infection into the host cell begins with the G protein binding to the host cell, followed by F protein-mediated fusion of the viral envelope into the cell membranes and penetration of the nucleocapsid complex into the cytoplasm. The F- and G-proteins stick from the virus envelope and are responsible for attachment (G-protein) and fusion (F-protein) to host cells.

SH is a small integral membrane protein whose function is not clear. It seems to be phosphorylated by an MAPK p38-dependant tyrosine kinase to achieve its normal cellular distribution in infected cells (Sidwell and Barnard, 2006).

RSV inner portion of the envelope interacts with the mature (M) protein, which directs the assembly of virions within the inner side of the host cell membrane from which the viral envelope is derived. Within the envelope, posterior to the M-protein, is the nucleocapsid, which is made up of the major nucleocapsid N-protein that binds to

genomic RNA, a phosphoprotein (P), a transcription anti-terminator factor or transcriptase processivity factor (M2-1), and the large polymerase subunit (L), which is a RNA-dependant RNA transcriptase. In addition, there are two non-structural proteins referred to as NS1 and NS2 and a regulatory protein known as M2-2.

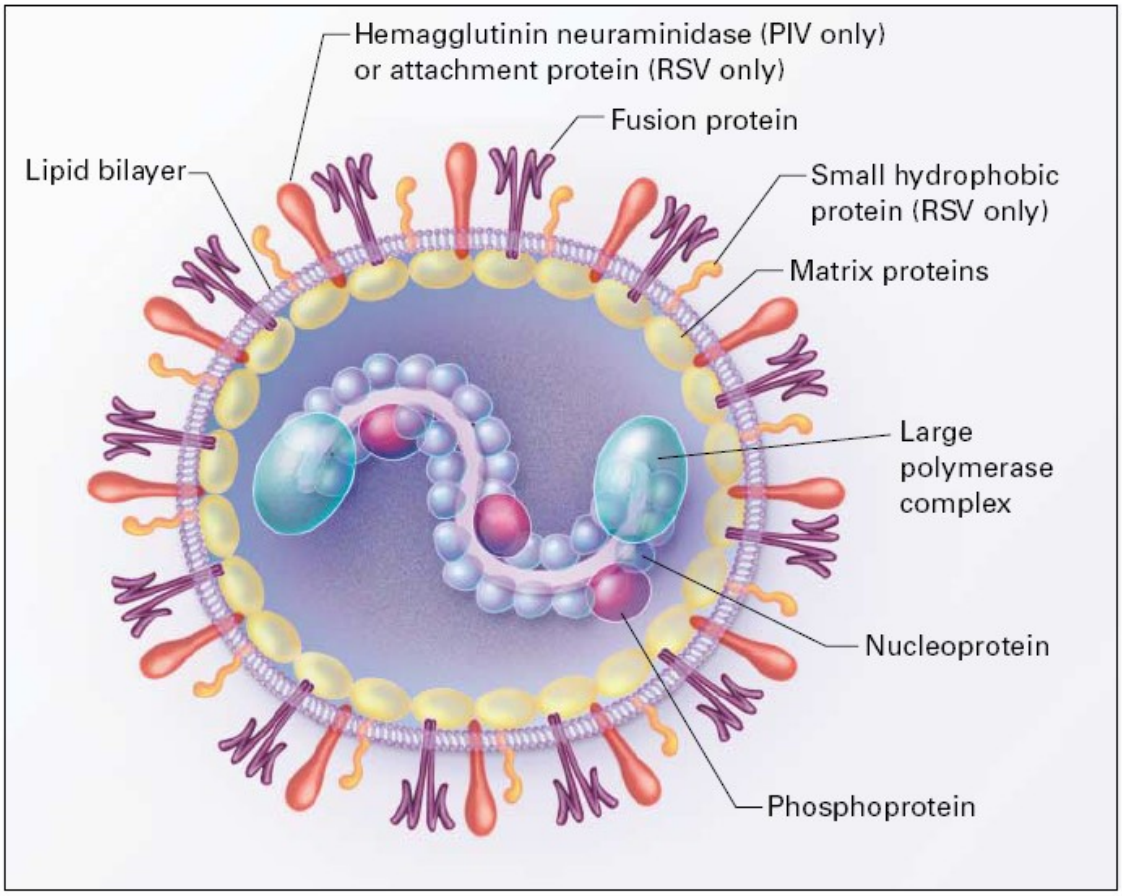


Figure 1.4: Structure of RSV and Parainfluenza Virus (PIV)
Adapted from Hall (2001)

Looking into the properties of the virus (McNamara and Smyth, 2002), the following could be summarized:

1. The viral genome is tightly encapsidated by the nucleocapsid N protein, which, together with the phosphoprotein P and the large polymerase subunit L, forms the minimum unit for RNA replication.

2. RNA replication involves the synthesis of a positive-sense, exact-copy, encapsidated, replicative intermediate called the antigenome, which serves as the template for progeny genome.
3. Transcription requires the M2 open reading frame 1 (ORF-1) protein as well as the nucleocapsid-associated proteins that ensure efficient production of full-length mRNA.
4. The M2 gene also encodes for the open reading frame 2 (ORF-2) protein that has a negative regulatory effect and may render nucleocapsids quiescent prior to incorporation into virions.
5. RSV encodes a matrix protein M, which is thought to mediate interaction between the nucleocapsid and envelope during virion morphogenesis.
6. There are two non-structural proteins, NS1 and NS2, whose functions are unknown, although NS1 appears to be a negative regulatory factor for RNA synthesis.
7. RSV encodes three surface envelope proteins that are components of the virion: the attachment protein G, the fusion protein F, and the small hydrophobic protein SH.

Further discussions of the relevant RSV proteins G and F protein are included due to their importance to this study.

1.3.1 The G Protein:

The G protein which is responsible for viral attachment to cells (McNamara and Smyth, 2002). It is a glycoprotein of 288-299 amino acids in length and it consists of two hypervariable regions in the extracellular domain (Peret, et al., 1998; Reiche and

Schweiger, 2009) (see Figure 1.5 below). The mature 90-kDa form of the G protein is obtained through extensive glycosylation of the 32-kDa polypeptide precursor by the addition of N-linked sugars to Aspartic acid residues and O-linked sugars to Serine and Threonine residues which are potential acceptor sites for O-linked sugars (Parveen, et al., 2006; Zlateva, et al., 2005). The attachment G protein can be divided into an intracellular domain, a transmembrance domain, and a large ectodomain. Diversity occurs mainly in the G ectodomain which has only 44% amino acid identity between the two groups, compared with 83% for the transmembrane and cytoplasmic domains. However, the amino acid sequence positions of potential N-linked and O-linked glycosylation sites are poorly conserved, although the general locations of the latter are similar.

Cane and Pringle (1995) highlighted that the attachment G protein shows the most genetic variability among all RSV proteins .

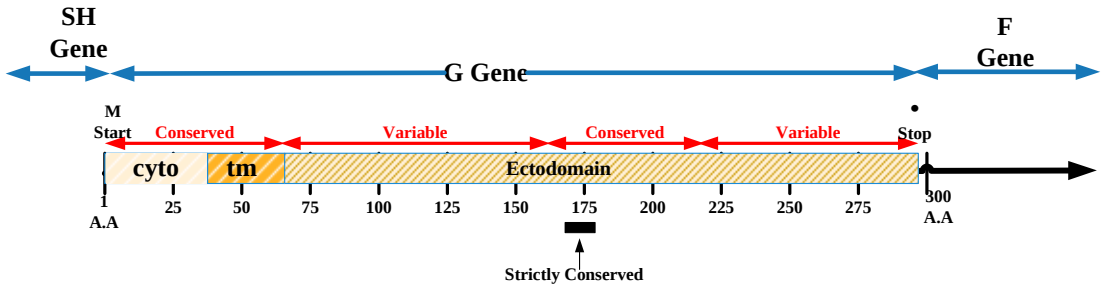


Figure 1.5: The G Gene (Created by the author)

The G protein is the most variable RSV protein because it contains two hypervariable regions shown in Figure 1.5 (Peret, et al., 1998; Peret, et al., 2000; Shobugawa, et al., 2009; Venter, et al., 2001). Variability in the G protein is greater than in other RSV proteins, both between and within the major antigenic groups of RSV (Cane and Pringle, 1995; Cane, 1997; Cane, 2001; Dapat, et al., 2010; Sullender, 2000; Zlateva, et al., 2005).

Amino acid variations of the protein exist in both RSV A and B groups, but variations are more pronounced in RSV group A. The G protein of the A and B strains can differ by up to 47%, while the G protein from the same strain can differ by a maximum of 20% (McNamara and Smyth, 2002). G protein variability is concentrated in the ectodomain, which contains two hypervariable regions separated by a conserved 13 amino acid motif (McNamara and Smyth, 2002; Peret, et al., 1998). The first is located in the amino (A)-terminal part of the protein preceiding the conserved region, and the second variable region is located in the carboxy (C)-terminal end. The second variable region, which corresponds to the C-terminal region of the G protein, reflects overall gene variability and has been analyzed in molecular epidemiological studies (Peret, et al., 1998; Peret, et al., 2000). The C-terminal region which accounts for strain-specific epitopes (Cane and Pringle, 1995; Cane, 1997; Cane, 2001; Dapat, et al., 2010) is widely used to study the genetic diversity of RSV.

The G protein is recognized by neutralizing antibodies but, opposite to the F protein, does not stimulate significant cytotoxic T-lymphocyte responses (Sullender, 2000). Sullender et. al (2000) indicated that the G protein lacks the hemagglutinin or neuraminidase activity of the other paramyxovirus attachment proteins and it also differs in size and biochemistry from these proteins. Akerlind and Norrby (1986) used monoclonal antibody (mAbs) which recognized the G, F, N, P, and M2 proteins to analyze viruses isolated in Stockholm.

1.3.2 The F protein

The RSV F protein in its inactivated form (F_0) comprises 574 amino acids and has a trimeric coiled-coil structure, similar to other viral fusion proteins (McNamara and Smyth, 2002). The F protein is a type I transmembrance glycoprotein with a cleaved N-terminal signal sequence and a transmembrance anchor near the C terminus. Activation occurs through cleavage of F_0 into two disulphide linked subunits, F_1 and F_2. After synthesis and modification by the addition of N-linked sugars, it is cleaved into two subunits, F_1 and F_2, that are linked by disulphide bonds (Sullender, 2000).

The F protein promotes both fusion of viral and cell membranes resulting in the transfer of viral genetic material, and fusion of infected and adjacent cell membranes causing the formation of syncytia (Domachowske and Rosenberg, 1999). These syncytia are the hallmark of the RSV cytopathic effect and are necessary for cell-to-cell viral transmission.

The interaction between the F protein and a small GTPase, RhoA, facilitates RSV-induced syncytium formation (Pastey, et al., 1999). Syncytia formation is also associated with the expression of cytokeratin-17 by RSV-infected respiratory epithelial cells. Cytokeratins are among the major components of the filament networks that make up the cytoskeleton. Cytokeratin-17 expression in RSV infection is neutralized by anti-RSV F protein antibody (Tripp, et al., 1999).

1.3.3 The SH Protein

The SH protein is the third transmembrance protein and is comprised of 64 amino acids. Its precise role is still unknown (McNamara and Smyth, 2002) and it is not required for viral replication or syncytium formation although it does facilitate fusion (Domachowske and Rosenberg, 1999).

1.3.4 The N protein

The N gene is one of the most conserved genes in the RSV genome. The nucleotide sequences from this gene could be used to detect a large variety of RSV strains. Six nucleotides in the N gene differ substantially between groups A and B (Hu, et al., 2003). The nucleocapsid (N) protein of the pneumovirus RSV is a major structural protein which encapsidates the RNA genome and is essential for replication and transcription of the RSV genome (Stokes, et al., 2003).

Within RSV infected cells, the N protein links with the P protein and M2-1 protein to form cytoplasmic inclusions (García, et al., 1993). N, P and L proteins are required for replication, while the M2-1 protein is required for efficient transcription (Grosfeld, et al., 1995). The interaction of the RSV N and P proteins has been well documented and the C terminal of the P protein was found to be the most important site for binding to the N protein. The N protein in human RSV (hRSV) and bovine RSV (bRSV) are 93% identical at the amino acid level and both are 391 residues in length. The regions (46–65, 241–260 and 305–335 or 334–352) of the N protein involved in binding to the P protein have been variously mapped for human RSV. The N proteins of pneumoviruses do not

possess a hypervariable C-terminal tail required for interaction with the polymerase (Stokes, et al., 2003).

The study of Murphy et al. (2003) revealed that a protein containing only the residues 1–91 of the N protein is able to bind to RNA and form nucleocapsid-like structures. Marriott et al. (1999) assayed the N protein for its function in RNA replication, transcription and encapsidation using a minigenome replication assay, in which plasmids encoding the minigenome and the N, P, L and M2-1 proteins are transfected into an appropriate cell line. Khattar et al. (2000) mapped the important regions on the bRSV N protein by means of nucleotide deletions and found that all internal deletions resulted in inactivity, and deletion of as little as two residues from the N terminus or one residue from the C terminus which was enough to reduce activity by 95–98% which means that the structural integrity of the N protein is compromised by deletions which in turn leads to a loss of the active conformation.

1.4 RSV Strains

The RSV is classified into subgroups A and B based on antigenic and genetic variability (Peret, et al., 1998; Reiche and Schweiger, 2009; Zhang, et al., 2010). They are named as RSV-A and RSV-B (Dapat, et al., 2010; Shobugawa, et al., 2009). The basis of this grouping is the virus's reactivity with monoclonal antibodies that are directed against the G protein which is known as the attachment glycoprotein (Cane and Pringle, 1995; Dapat, et al., 2010; Hall, 2001). Within each group, additional variability is also detected (Zhang, et al., 2010). The major difference between the two subtypes is identified as being on the G protein. Venter et. al (2001) studied RSV over four consecutive seasons

(1997-2000) in a single tertiary hospital in South Africa to reveal that subgroup A revealed more variability and displacement of genotypes while subgroup B remained more consistent. Almost the same results were reported in India by Parveen et. al. (2006) who proved that the level of genetic variability was higher among the group A viruses (up to 14%) than among the group B viruses (up to 2%).

Both of group A and group B strains circulate at the same time but group A usually dominates (Hall, 2001). Sequence studies of nucleoprotein (N), phosphoprotein (P), small hydropholic (SH) protein and attachment (G) protein genes have confirmed the division of RSV into two groups and also have identified numerous variants within each group (Peret, et al., 1998).

It is evident from various studies that RSV A prevails over RSV B in some epidemic seasons. Zhang et. al. (2007) studied the variability of RSV in China using nucleotide sequencing of the hypervariable C-terminal region of the G protein gene. Phylogenetic analysis of 80 isolates obtained from three different hospitals over a period of three epidemic seasons 1990/1991, 2000/2001, and 2003/2004 showed that group A prevailed over group B.

In a comparison between the two groups, Oliveira et. al. (2008) reported that group B strains tend to grow more slowly in tissue culture, to produce fewer virus particles, and to loose the characteristic of syncytial formation. All of this could reduce the ability to

isolate the virus from clinical specimens and as a result will reduce the ability to perform group determination.

RSV A and B subgroups have also been subdivided further into genotypes, by genetic analysis as follows:

1- RSV-A is divided into seven genotypes (GA1 to GA7). An additional RSV-A genotype, SAA1 (South Africa A1), has also been proposed (Dapat, et al., 2010; Shobugawa, et al., 2009).

2- RSV-B is divided into four genotypes (GB1 to GB4). An additional RSV-B genotypes SAB1 to SAB3, has also been proposed as well by (Shobugawa, et al., 2009; Venter, et al., 2001). In addition, RSV-B genotype Buenos Aires (BA) type strain, which has a 60-nucleotide insertion in the second hypervariable region of the G protein and has been reported in Buenos Aires in 1999 as well as other areas in the world (Dapat, et al., 2010; Trento, et al., 2003; Zlateva, et al., 2005). The BA strains are further subdivided into six clusters (BA-1 to BA-6) (Trento, et al., 2006). Dapat et. al. (2010) were able to identify and distinguish four new clusters BA7, BA8, BA9 and BA10 which contained strains from 2005-2006 season to 2009 to 2010 season. This clustering of viruses that circulated in the same year observed in BA7 to BA10 genotypes supports the temporal nature of RSV.

The following graph (Figure 1.6) was adapted from Sato, et. al. (2005) depicting the different subgroups described above:

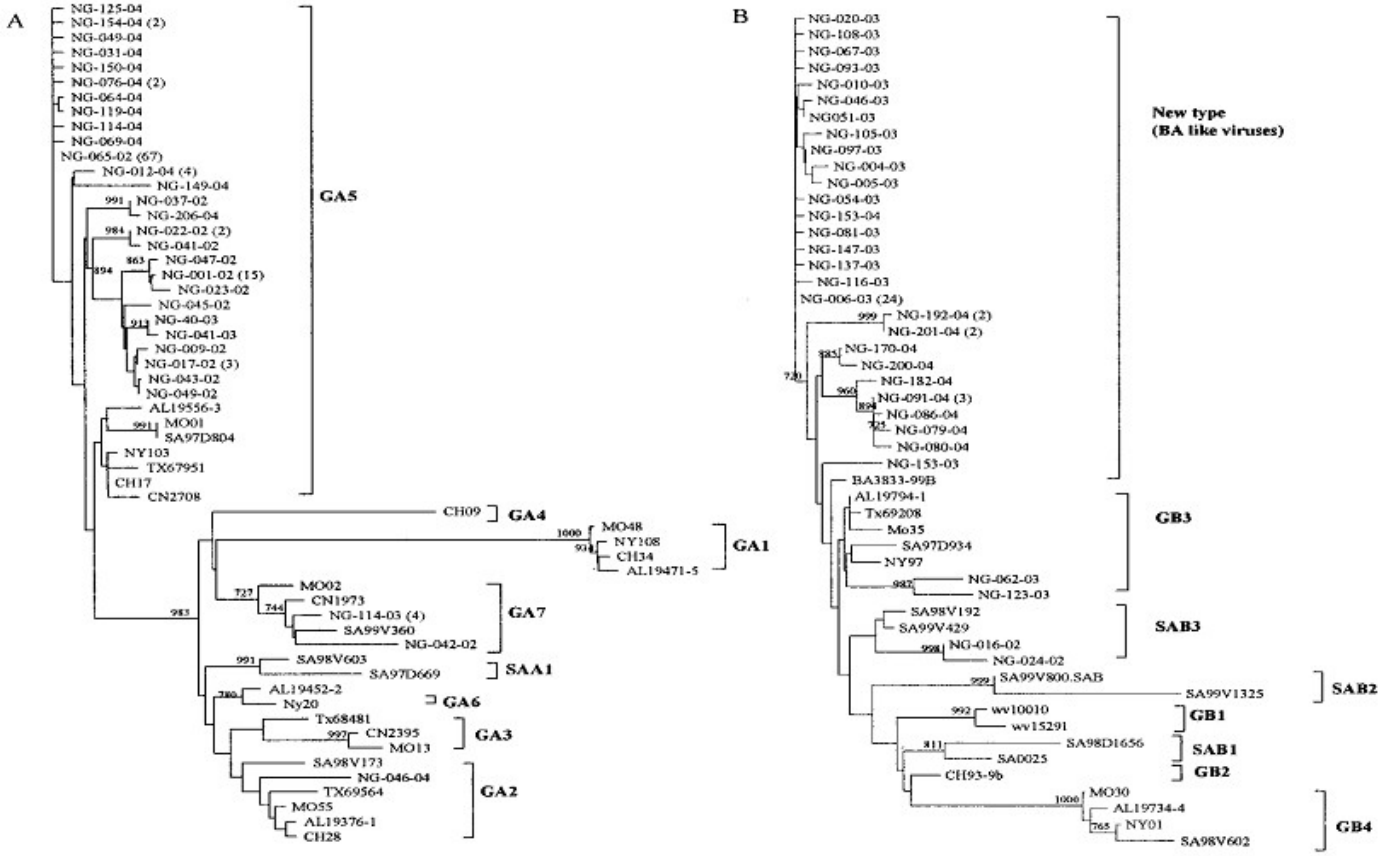


Figure 1.6: The different subgroups of strains A and B
Adapted from Sato et. al. (2005)

Distinct genotypes within each strain might dominate within a community and this will change annually (Hall, 2001). This annual shift suggests that reinfection is highly possible because immunity from previous infection can be evaded.

1.4.1 RSV Evolution

Some studies focused on the evolution of the RSV over time. Such studies considered the analysis of RSV isolates in certain countries over a long period of time. In New Zealand, Matheson et al. (2006) analyzed viral attachment glycoprotein gene (the G gene)

sequences from 106 RSV subgroup A isolates collected between 1967 and 2003, as well as 38 subgroup B viruses collected between 1984 and 2004. Their analysis revealed that the mutation rate calculated for RSV B ($3.5 \times 10^{-3} \pm 4 \times 10^{-4}$ changes per nucleotide per year) G gene was significantly higher than RSV A ($2.6 \times 10^{-3} \pm 1 \times 10^{-4}$ changes per nucleotide per year). A major finding of that was that there are different patterns of evolution between subgroups A and B, with subgroup B viruses evolving faster than A. The study reported that these rates of change are significantly different at a 95% confidence interval. This difference suggests that RSV B is evolving faster than RSV A.

Studies have shown that reinfection occurs during life (Glezen, et al., 1986; Henderson, et al., 1979; Lee, et al., 2005; Peret, et al., 2000; Zhang, et al., 2010). This indicates that infection not only induces partial immunity (Galiano, et al., 2005; Zlateva, et al., 2005), but also led researchers to think that there is some patterns of change and evolution that take place over time which permit the RSV to escape from host neutralizing antibodies (Hall, et al., 1991; Matheson, et al., 2006). Over 50% of the nucleotide mutations introduced during replication result in amino acid changes, indicating active selective pressure is present (Johnson, et al., 1987; Sullender, et al., 1991). Nucleotide changes in the variable domains suggest that there may be considerable immunological pressure for change in certain areas of the G protein and this may account for the ability of this virus to reinfect individuals repeatedly (Cane, et al., 1991). Matheson et al. (2006) reported that changes corresponding to predicted amino acid substitutions occurred very frequently for both subtypes A and B, with a greater degree of change seen in RSV B, implying selective pressure on both subtypes of RSV (Matheson, et al., 2006; Sullender,

et al., 1991). Amino acid substitutions corresponding to predicted O-glycosylation changes occurred more frequently for RSV B than for RSV A, indicating stronger selective pressure acting on RSV B.

1.4.1.1 Evolutionary Pattern of RSV A

Matheson et al. (2006) found in a research conducted in New Zealand that RSV subgroups exhibit different patterns of evolution, with subgroup B viruses evolving faster than A even though they come from a similar environment. His analysis revealed substantial conflict in the RSV A alignment for which close to 15 thousand equally parsimonious trees were found. Conducting a majority consensus did not help the researchers in New Zealand to see the genetic distances between isolates. That had led the researchers to construct a network of RSV A alignment which helped inspect the degree and nature of repeated mutations or conflicts in the data as well as the genetic distances separating isolates. These methods were also used by other researchers who emphasized the fact that network analyses are more appropriate to describing similarities in form and structure, recombination and sequence space (Matheson, et al., 2006; Salemi, et al., 2003; Wain-Hobson, et al., 2003).

In there study, Matheson et al. (2006) revealed that although there was a genetic similarity between isolates, yet they proved that evolution occurs over time. They proved that although isolates described prior to their research continued to exist and reappear over time, which implies persistence and recurrence of similar isolates, clustering of isolates by year of isolation demonstrated some drift which indicates progressive viral

evolution. The NZA1 genotype was a new genotype described by their study, while GA5 was predominating over time.

1.4.1.2 Evolutionary Pattern of RSV B

Research over time revealed the progressive linear evolution of RSV B (Matheson, et al., 2006). In their study, Matheson et al. (2006) described, through genotyping of RSV B, considerable clustering by year of isolation indicating substantial change from year to year. Despite the greater percentage of splits displayed in the RSV B network, Matheson et al. (2006) showed that there is only one closed cycle compare to six in RSV A network which indicates differing levels of conflict within the networks of the two RSV subgroups. In addition to that, isolates are strongly separated into contemporaneous clades within RSV B isolates compare to the RSV A networks.

Although the majority of RSV B G gene isolates were 295 amino acids in length, some exceptions were identified in the study of Matheson et al. (2006). For example, the New Zealand G protein isolates (NZB2 genotype) was only 292 amino acids long, isolate 04^{02} had the 60-bp insertion[3] (which was first observed in Argentina and reported by Trento et al. (2003) in 1999 and later in Japan as reported by Sato et al. (2005) in 2003). In addition, isolate 04^{02} had two amino acid deletions as observed in Belgian in 1982 and reported by Zlateva et al. (2005).

[3] RSV strain related to G protein group B13.

1.5 RSV Infection

Although this study focuses on RSV infection in children, it was reported by different researchers that RSV infection is also known to cause infection in adults and elderly as well. This section gives an overview of research results in the area of RSV infection in children followed by infection in adults and elderly. Since reinfection is an attribute of RSV, a separate subsection will focus on research done in the area of reinfection.

1.5.1 Infection in Children

Some 50% to 70% of infants experience RSV infection in the first year of life and virtually all are infected by 2 years of age (Shobugawa, et al., 2009). This fact was also reiterated in prior studies such as the one by Barends, et al. (2004) who, although emphasized that the clinical presentation can vary from mild upper respiratory–tract (URT) illness to severe bronchiolitis and pneumonia, were able to prove using their data that almost all children become infected with RSV during their first or second year of life and that the incidence of RSV bronchiolitis reaches a maximum at the age of 2 months. The infection with RSV is a disease in both developing as well as developed countries (Reiche and Schweiger, 2009). It was proven by many researchers (such as: Dapat, et al., 2010; Reiche and Schweiger, 2009; Shobugawa, et al., 2009) that virtually all children have been infected at least once with RSV. In a recent study conducted by Levin et. al. (2010), it was noted that there is higher than expected incidence of bacterial pneumonia that complicates RSV infections in infants with respiratory failures.

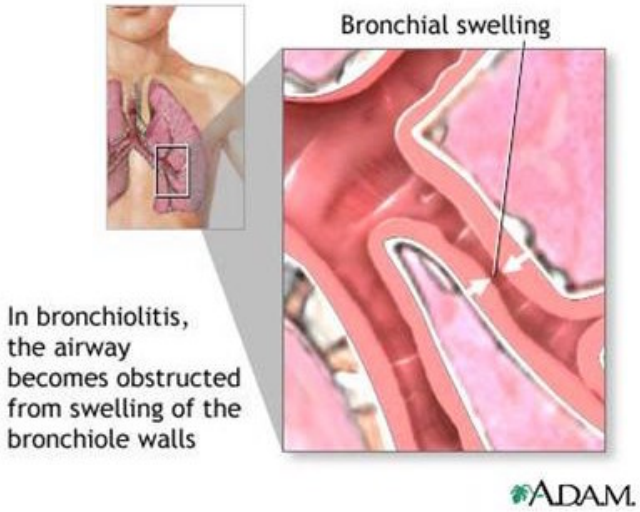


Figure 1.7: Bronchiolitis[4]

McNamara and Smyth (2002) reported that by the age of 18 months, 87% of children have antibodies to RSV and that by the age of 3 years, virtually all children are infected. These children, when infected, suffer from URT symptoms, which usually is followed by LRT symptoms, such as rhinitis (stuffy nose), cough and coryza (runny nose).

1.5.2 Infection in Adults and Elderly

The RSV is now seen as an important pathogens in adults as well (Hall, 2001; Peret, et al., 2000; Sullender, 2000). The RSV causes LRTI for immunocompromised individuals and the elderly (Falsey, 2007; Sullender, 2000; Zhang, et al., 2010). Researchers (Such as: Englund, et al., 1996) referred to patients who receive intense immunosuppression after undergoing transplantation of bone marrow and solid organs as immunocompromised individuals. Falsey (2007) reported that RSV is an important pathogen in elderly who suffer from chronic lung disease or those with impaired immunity. Children with underlying illness such as congenital heart disease and

[4] *Source: www.nlm.nih.gov/.../ency/fullsize/17098.jpg*

bronchopulmonary dysplasia are at increased risk for severe infections due to RSV. Those researchers have referred to RSV virus as potential opportunistic pathogen.

1.5.3 Reinfection

RSV reinfection is a very common event throughout the life of young children with ARTI (Zhang, et al., 2010). In addition, RSV reinfection is common throughout life, especially in immunocompromised and the elderly (Peret, et al., 2000), which indicates that RSV infections induce only partial immunity (Zlateva, et al., 2005).

In a study conducted in North Carolina, Henderson et. al. (1979) reported extremely important results on the rate of RSV infection and reinfection in children. His study was also important in giving a better understanding of the acquired immunity to RSV infections. Henderson et. al. (1979) analyzed data from a 10-year study of respiratory illness in normal children who were followed longitudinally from early infancy in order to better measure immunity in terms of failure to become infected or reduction in severity of clinical illness upon reinfection. In that study, Henderson et. al. (1979) reported that 98% of children attending day care during their first year of life became infected with RSV, 74% were reinfected in their second year, and 65% during their third year.

Age and history of infection both influenced illness. In a similar study, Glezen et. al. (1986) reported that 69% of children in Houston acquired RSV infection during their first year, 83% were reinfected during the second year, and 46% were reinfected in their third year. Immunity induced by a single infection had no demonstrable effect on illness associated with reinfection one year later; however, a considerable reduction in severity

occurred with the third infection. Even in infected people with the highest antibody levels, the risk of reinfection was 25% (Hall, et al., 1991).

1.5.4 Dual Infection

Semple et. al. (2005) conducted a study to examine the association between severe bronchiolitis and dual infection which can happen with different respiratory viruses of which human metapneumovirus (hMPV) is the most common. This was investigated in a group of less than 2-year-old infants with bronchiolitis who were admitted to the hospital during the 2001–2002 winter season. They concluded that there is high frequency of dual infection by hMPV and RSV in infants receiving mechanical ventilation for severe bronchiolitis. The virus is found to be extremely infectious too. Other studies (Such as: Zlateva, et al., 2007) that will be discussed in more depth later, have also shown the possibilities of double infection with both RSV groups A and B. Rishmawi et al. (Rishmawi, 2007) conducted a study in Palestine covering the period from November 2005 to October 2006 on the hMPV and reported that During the study period 50 patients (6.3%) had double infection between hMPV and RSV.

1.5.5 Hospitalization and Cost

The RSV is the main cause of hospitalization for respiratory tract illness in young children and it caused an estimated number of 100,000 children to be hospitalized in the USA in 1980, 250,000 emergency room visits in 1991 (Hall, 2001). Hospitalization for RSV is significantly more likely in the first months of life (Levin, et al., 2010).

Among children, RSV infection is the cause of 50 to 90% of hospitalizations for bronchiolitis, 5 to 40% of those for pneumonia, and 10 to 30% for tracheobronchitis (Hall, 2001). In the USA, it is estimated that RSV infections causes 50,000 to 80,000 hospitalizations each year and the death of 500 infants annually. The cost of hospitalizing RSV infected patients is approximately $365 - $585 million per year (McNamara and Smyth, 2002). In poor countries such as India (See Parveen, et al., 2006), a large number of children die every year because of ALRI while most of those deaths happen without reaching a hospital.

In Brazil, Oliveira et. al. (2008) investigated a possible association between RSV groups and severity of disease. RT-PCR was used to characterize 128 RSV nasopharyngeal specimens from children less than five years old experiencing acute respiratory disease. A total of 82 of 128 samples (64.1%) could be typed, and, of these, 78% were group A, and 22% were group B. Severity was measured by clinical evaluation associated with demographic factors: for RSV A-infected patients, 53.1% were hospitalized, whereas for RSV B patients, 27.8% were hospitalized. Around 35.0% of the patients presented risk factors for severity (e.g., prematurity). For those without risk factors, the hospitalization occurred in 47.6% of patients infected with RSV A and in 18.2% infected with RSV B. There was a trend for RSV B infections to be milder than those of RSV A. Even though RSV A-infected patients, including cases without underlying condition and prematurity, were more likely to require hospitalization than those infected by RSV B, the disease severity was not attributed exclusively to the RSV group type.

1.6 RSV Pathogenesis

The incubation period of RSV is two to eight days (Hall, 2001). After that period, the RSV virus replicate in the nasopharyngeal epithelium and will, one to three days later, spread to the lower respiratory tract. The characteristics of RSV bronchiolities include:

1- Necrosis and sloughing of the epithelium of the small airways;

2- Edema;

3- Increased secretion of mucus which obstructs flow in the airways.

4- This is illustrated in Figure 1.8 below:

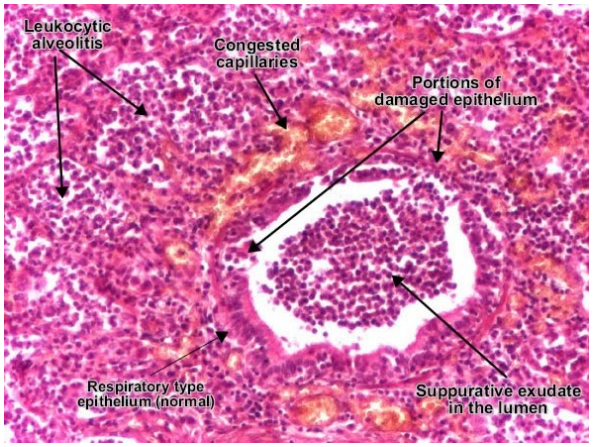


Figure 1.8: Bronchiolitis in an Infant with RSV Infection
Source:
http://www.mdblogger.com/2011/04/histology-and-explanation-of_10.html

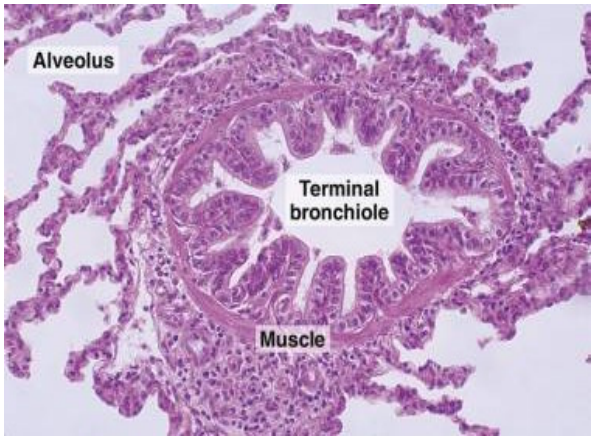


Figure 1.9: A normal bronchiole for an uninfected individual
Source:
http://www.mc.vanderbilt.edu/histology/labmanual2002/labsection2/Respiratory03.htm

For comparison purposes, Figure 1.9 is presented to show a normal bronchiole for an uninfected individual.

Clinical findings include hyper-inflammation, atelactasis 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 rarely appear before two weeks. Hall (2001) also confirmed that full recovery requires four to eight weeks.

Hall (2001) indicated that recognition of RSV infection in older children and adults is confounded by the fact that other respiratory agents cause similar clinical manifestations. In literature some other studies evaluated the role of the RSV infections. Those can be summarized as follows:

1- In children with nephrotic syndrome (NS), RSV infection is the most frequent cause of relapses associated with URTI (Noni, et al., 1986). In their study, Noni et. al. (1986) worked to determine whether respiratory virus infections are associated with exacerbation of NS in childhood. They conducted a two-winter study of 32 children with NS. In their experiment they obtained pre- and post-season viral serologic studies, biweekly nose and throat viral cultures, daily urinalysis, biweekly telephone follow-up for URI and renal complaints, and clinical assessments. When a URI occurred, the researchers did viral cultures weekly if the child was at home and twice weekly if the child was hospitalized. During the period of their study, 61 URIs occurred; the agent was identified in 33 (51.6%) cases (RSV 14, influenza virus 5, parainfluenza virus 5, varicella zoster virus 4, adenovirus 3, Mycoplasma pneumoniae 1, and Chlamydia trachomatis 1). Forty-one exacerbations occurred, 71% with URI; 29% had no URI during

the preceding 10 days. Total relapse occurred in 29 of 41 exacerbations, 69% with URI and 31% without URI. Patients with unstable NS had more exacerbations than those with stable NS (15 of 19 (79%) vs. 4 of 13 (31%)) and more URI (2.32 vs. 1.46 per child). Exacerbations in patients with minimal change, mesangioproliferative, and focal glomerulosclerosis occurred in 40%, 60%, and 64%, respectively. They conclude that exacerbations and relapses of childhood NS are temporally related to URI. Inasmuch as multiple viral agents were associated with exacerbations, nonspecific host response to infection, not viral antigen or antibody response, may be the link to NS.

2- In those with chronic pulmonary disorders, RSV virus infections lead to complications that are indistinguishable from those resulting from other infectious or noninfectious causes (Arnold, et al., 1999). Arnold et. al. (1999) compared outcomes of (RSV) infection in children with bronchopulmonary dysplasia (BPD) with those with other pulmonary disorders such as cystic fibrosis, recurrent aspiration pneumonitis, pulmonary malformation, neurogenic disorders, and tracheoesophageal fistula. They conducted their research by analyzing and following children with RSV infection who were hospitalized at 7 Canadian pediatric tertiary care hospitals in 1993 through 1994 and 9 hospitals in 1994 through 1995. They conducted analysis of secondary data. Out of the 1,516 admitted patients, there were no significant differences among the 7 groups (BPD, CF, recurrent aspiration pneumonitis, pulmonary malformation, neurogenic disorders, tracheoesophageal fistula, and other) for the morbidity

measures: duration of hospitalization, intensive care unit (ICU) admission, duration of ICU stay, mechanical ventilation and duration of mechanical ventilation. Patients using home oxygen were more likely to be admitted to the ICU than those who had never or previously used home oxygen (current 57.1%, past 23.8%, never 33.3%). This suggests that children with other underlying diseases have morbidity similar to those with BPD.

3- RSV infection appears particularly detrimental in patients with CF, resulting in reduced lung function and a greater rate of hospitalization (43%) than any other viral infection (Hall, 2001). Several research studies (such as: Armstrong, et al., 1998; Hiatt, et al., 1999) reached this conclusion as well. Hiatt et. al. (1999), for example, conducted a study to determine the effect of respiratory viral infections on pulmonary function in infants with CF after the respiratory virus season (October through March). They concluded that Infants with CF incurring respiratory virus infection are at significant risk for lower respiratory tract infection (LRTI), for hospitalization, and for deterioration in lung function that persists months after the acute illness. In another research study, Armstrong et. al. (1998) concluded in a similar study that covered the period 1991-1996 that respiratory viruses are important causes of hospitalization in CF infants. While viral infections were self-limited, they were accompanied by airway inflammatory changes, and admission to hospital was associated with early acquisition of *Pseudomonas aeruginosa* and persistent respiratory symptoms.

4- The current epidemic of asthma, marked by increasing severity of disease and hospitalization rates, has spotlighted the possibility of a pathogenic link between asthma and viral infections, primarily those due to RSV (Ball, et al., 2000). About 40 to 50 % of infants hospitalized with RSV bronchiolitis have subsequent episodes of wheezing. Furthermore, exacerbations of asthma in children and adults are primarily associated with viral infections (Ball, et al., 2000; Crain, et al., 1994; Gottschalk, et al., 1996; Martinez, et al., 1995; Teichtahl, et al., 1997). In their study conducted at Western Hospital in Melbourne, Australia, Teichtahl et. al. (1997) concluded that 37% of adult patients with acute asthma admitted to the Department of Respiratory Medicine over a 12-month period had evidence of recent respiratory tract infections. Openshaw and Walzl (1999) reported that there is good evidence that interactions with some respiratory viruses, particularly RSV, are followed by an increase in pulmonary symptoms. This association remains for at least 10 years and childhood acute respiratory illness may even be associated with respiratory morbidity and mortality in adult life. They have also reported that although respiratory infections have not conclusively been causally linked to later atopic disease, strong evidence from animal studies makes this highly plausible.

5- The role of respiratory viruses in wheezing is also proved by the similarity of the inflammatory response elicited by asthmatic attacks and that elicited by viral infections (Hall, 2001; Openshaw and Walzl, 1999). RSV infection has been associated with a T-cell response characterized primarily by the production of

cytokines by type 2 helper T cells, the same response observed during episodes of asthma. Both are characterized by the recruitment of T cells and eosinophils and the release of soluble mediators, such as histamine, kinins, and other leukotrienes. This suggests that viral infections may induce inflammatory responses that closely resemble those characteristic of asthma, including airway infiltration with lymphocytes and eosinophils and release of mediators of airway obstruction (Welliver, 1999). Welliver (1999), in the same research reported that among children with bronchiolitis, more frequent and severe wheezing has been correlated with elevated levels of IgE antibody to RSV and parainfluenza virus, suggesting that virus-induced antibodies augment the release of inflammatory mediators that are important in reactive airway disease. Larsen and Colasurdo (1999) conducted their study on rats and reported that RSV causes acute and chronic changes in neural control of airways. When infection occurs early in life, the alterations persist for long periods. This means that RSV influences and affects wheezing by causing alterations in the neural pathways which in turn affects airway responsiveness.

6- Some other studies revealed that there is a high correlation between the RSV genotype and the severity of illness. In their study, Martinello et. al. (2002), showed that there is no statistical difference in the severity of illness between group A and B, but the significant difference was found when comparing the severity of illness between clades GA2 and GA3, where clade GA3 was associated with significantly greater severity of illness compared to clade GA2.

Therefore, the study concluded that the specific genotype within subgroup A is associated with greater severity of illness. In another study conducted at Rochester General Hospital in New York (Walsh, et al., 1997) on 265 hospitalized infants between 1998 – 2001 confirmed that group A RSV infection results in greater disease severity than group B infection.

1.7 RSV Epidemiology

1.7.1 Age Group

RSV infection is the cause of LRTI in infants, particularly those less than 6 months of age and is also a significant cause of respiratory infection among the elderly (Sidwell and Barnard, 2006).

The study of (Shobugawa, et al., 2009) showed that the average age of people infected with genotypes GA5 and NA2[5] was 1.00 ± 0.8 years old versus 1.7 ± 1.1 years old. The average age of patients infected with genotype RSV-B and RSV-A strains were not statistically different. In a study conducted in Germany, Reiche and Schweiger (2009) showed that the majority of positive RSV infected individuals (51%) were younger than 3 months of age, while 47% were between 3 to 6 months.

1.7.2 Seasonality

The RSV season typically occurs between November and March every year, but it could vary by region (Michael P. Frogel, et al., 2010). In the USA, most RSV infections occur during a period of about 22 weeks from November to May, with the peak being in

[5] NA1 and NA2 emerged are genetically close to GA2 strains and were discovered in Niigata, Japan.

January and February (Hall, 2001). The study of Shobugawa (2009) showed that the number of RSV infected patients increased dramatically in Japan for unknown reasons in the 2005-2006 and 2006-2007 seasons.

RSV is the most common cause of serious ALRI disease among infants and young children, and is found mainly in late fall, winter, and spring in temperate zones in the world (Cane and Pringle, 1995; Cane, 2001; Dapat, et al., 2010; Gottschalk, et al., 1996; McNamara and Smyth, 2002; Michael P. Frogel, et al., 2010; Peret, et al., 2000; Shobugawa, et al., 2009; Zlateva, et al., 2005). The study of (Shobugawa, et al., 2009) showed that the disease was most prevalent and clearly active during autumn and early winter.

1.7.3 Circulation Patterns of RSV A and RSV B

The circulation patterns of RSV A and B including their different genotypes is not well studied worldwide, however few studies have been reported. The study of Shobugawa (2009) showed that about 75% of the sample were RSV-A while only 25% were RSV-B of which RSV-A strains were clustered into 4 genotypes (GA5, GA7, NA1 and NA2) and that the RSV-B strains were clustered into 3 genotypes (GB3, SAB3 and BA). In a similar study conducted in Japan (Dapat, et al., 2010) the RSV-A was dominant in six of the nine seasons, while RSV-B was dominant in three seasons only. One other study conducted in Germany (Reiche and Schweiger, 2009) showed that although both groups, A and B, can be present in the same community, group A tends to dominate. According to Reiche and Schweiger (2009) the seasonal distribution of RSV group A viruses throughout the study period (1998 to 2007) indicates that RSV group A viruses

predominated in all seasons except for 1998-1999 and 2002-2003, and their analysis revealed that all RSV group A viruses belonged to three genotypes: (33%) viruses to genotype GA2, (62%) to genotype GA5 and (5%) to GA7.

Research conducted in North America (Peret, et al., 2000) discussed patterns of circulating strains in communities are often distinct, suggesting that RSV outbreaks are a community phenomenon, not a national one. This was also reiterated by other researchers (such as: Gottschalk, et al., 1996; Zlateva, et al., 2007). This study found out that several genotypes were circulating during the period of the study, as well as a variety of distinct sequences within each group of RSV. Each community had had 1 or 2 dominant genotypes that accounted for more than 50% of the isolates during the season. However it was also found that RSV group A predominated in 80% of the communities under study.

The researchers (Peret, et al., 2000) identified instances in which strains spread between communities. It was found that in the majority of communities under study, distinct patterns of circulating genotypes were found. In most instances, the researchers found that isolates of the same genotype circulating within the community are less diversified than those circulating among communities. This suggests, once more, that the RSV infection has community characteristics not necessarily national characteristics. The researchers proved that regional spread of one predominant genotype does not occur. This is an indicator that there might be an influence of the immunity induced by prior years' strains infection. In their analysis of different strains taken from different

communities, the researchers found out that there are a number of identical RSV strains with identical sequences. This fact supports the concept that some virus strains can be transmitted over broad geographic regions during a single epidemic season and that these strains may be suited for widespread transmission during some epidemic seasons.

The same results were reached by an earlier study (Sato, et al., 2005). Although Peret et. al. (2000) conducted their study by sequencing of the attachment protein from a single community in the USA during 5 successive years while Sato et. al. (2005) conducted their study in Japan, conclusions were very similar showing that the relative ratio of group A and group B strains shifted every year with group A dominating in certain periods, while group B dominating in others. The study also revealed that multiple genotypes circulated each year with a subtype predominating in each year.

These studies suggest that the occurrence of the shift in RSV strains is a consistent and important feature of the epidemiology of RSV infection. These shifts logically will result in the lack of immunity in the population to the new virus which allows the virus to transmit more efficiently or more pathogenic.

Zhang et. al. (2010) investigated the genetic variability of the attachment G protein gene among RSV strains prevalent in China. In their study, they performed reverse transcription polymerase chain reaction (RT-PCR) for a fragment of RSV G gene from nasopharyngeal aspirates (NPAs) samples collected from children with acute respiratory tract infection (ARTI) who were hospitalized in a Children's Hospital in China. Out of a

sample of 1,387 cases, 31.7% tested positive for RSV infection. Out of those infected children, 79.5% were infected with RSV-A while only 19.8% were identified as RSV-B infected. Their study had also revealed that subgroup A viruses were dominant during two seasons, while subgroup B was predominant in one season only. Zhang et. al. (2010) added that through their phylogenetic analysis all of the group A strains were clustered into one genotype which is GA2, while the B strains were clustered into GB1, GB3 and BA genotypes. All of the findings suggest that group A is a predominant RSV in China.

In a very similar study conducted in South Africa, using the methods and techniques Venter et. al (2001) studied RSV over four consecutive seasons (1997-2000) in a single tertiary hospital in South Africa: 225 isolates were subgrouped by RT-PCR and the resulting products sequenced. The result was that subgroup A predominated in two seasons, while A and B co-circulated approximately equally in the other seasons. The nucleotide sequences of the C-terminal of the G-protein were compared to sequences representative of previously defined RSV genotypes. South African subgroup A and subgroup B isolates clustered into four and five genotypes respectively. Different genotypes co-circulated in every season. Different circulation patterns were identified for group A and B isolates. Subgroup A revealed more variability and displacement of genotypes while subgroup B remained more consistent.

Almost the same results were reported in India by Parveen et. al. (2006). The study reported on the genetic variability in the glycoprotein G gene among RSV isolates from India. RT-PCR of a region of the RSV G protein gene was done from nasopharyngeal

aspirates (NPAs) samples collected in a prospective longitudinal study in two rural villages near Delhi and from children with Acute Respiratory Infection (ARI) seen in an urban hospital. Nucleotide sequence comparisons among 48 samples demonstrated a higher prevalence of group A (77%) than group B (23%) RSV isolates. The level of genetic variability was higher among the group A viruses (up to 14%) than among the group B viruses (up to 2%). RSV genotypes were prevalent during the 2002-2003 season and that genotype GA5 was predominant in the 2003-2004 season, whereas during the 2004-2005 season both genotype GA5 and genotype BA cocirculated in almost equal proportions.

Another longitudinal study was conducted in Germany, Reiche and Schweiger (2009) analyzed 1,400 RSV isolates from human nasopharyngeal aspirates or nasal or throat swabs from patients with respiratory illness using RT-PCR. The results were identical to all other studies and showed that RSV group A was dominant in seven out of nine epidemic seasons. The analysis revealed that RSV group A genotypes GA2 and GA5 circulated from 1998 to 2007.

Arbiza et. al. (2005) conducted a similar study in Uruguay. The variability of the G glycoprotein from RSV (groups A and B) isolated during 17 consecutive epidemics in Montevideo, Uruguay were analyzed. The researchers studied several annual epidemics. The conclusion they reached was that strains from groups A and B circulated together throughout the epidemics with predominance of one of them. Group A predominated, but in some epidemics group B was more frequently detected.

In a study conducted in Belgium (Zlateva, et al., 2007), the researchers reported that both subgroups can cocirculate during epidemics; however, their frequencies might differ between seasons. The researchers investigated subgroup prevalence and genotype distribution patterns of RSV strains during 10 successive epidemic seasons (1996 to 2006). RSV A strain was predominant in two successive periods while B strain was predominant in the third season. RSV infections with both subgroups were more prevalent among children younger than 6 months and had a peak incidence in December. The most frequently detected genotypes were GA5 and GB13. Furthermore, GA5 remained the dominant RSV genotype in two consecutive epidemic seasons twice during the study period which extended over 10 years. Additional variability was detected among the GB13 isolates, due to the usage of a novel termination codon in the G gene. An interesting outcome of their research was the dual infections with both RSV subgroups that were detected in 9 patients (See Figure 1.10 below), and subsequent infections with the heterologous RSV subgroup that was documented for 15 patients. The results supported the hypothesis that the overall prevalence of RSV-A over RSV-B could be due to a more-transient subgroup A-specific immune protection.

Epidemic season	No. of samples[a]	No. (%) of typed samples	No. (%)[b] of HRSV infections with the following subgroup(s):		
			HRSV-A	HRSV-B	HRSV-A and HRSV-B
1996–1997	104	86 (83)	57 (66)	28 (33)	1 (1)
1997–1998	129	97 (75)	62 (64)	33 (34)	2 (2)
1998–1999	124	102 (82)	26 (25)	76 (75)	0
1999–2000	146	97 (66)	73 (75)	23 (24)	1 (1)
2000–2001	136	84 (62)	69 (82)	15 (18)	0
2001–2002	129	86 (67)	13 (15)	73 (85)	0
2002–2003	173	131 (75)	72 (55)	59 (45)	0
2003–2004	178	123 (69)	75 (61)	46 (37)	2 (2)
2004–2005	132	90 (68)	20 (22)	68 (76)	2 (2)
2005–2006	141	83 (59)	70 (84)	12 (15)	1 (1)

[a] Total number of HRSV antigen-positive samples.
[b] Percentages apply to the total number of typed HRSV antigen-positive samples for the season.

Figure 1.10: Results of Subgroup distributions of HRSV-A and -B in Belgium
Source: (Zlateva, et al., 2007)

1.8 RSV Transmission

Transmission of the RSV virus occurs by direct inoculation of contagious secretions from the hands or by large-particle aerosols into the eyes and nose, but rarely the mouth (Hall, et al., 1981). The findings of Hall et. al. (1981) suggest that RSV may infect humans after inoculation in the eyes or nose, and that both of these routes appear equally sensitive. In comparison, the mouth appears to be an insensitive route of inoculation. This study was conducted using thirty two volunteers who received serial dilutions of safety-tested live strain of RSV instilled into nose, eye, or mouth.

Figure 1.11: Transmission of RSV through fomites and large droplets[6]

As for the indirect contact, a study evaluating possible RSV transmission and spread, Caroline and Douglas (1981) examined the rates of infection in hospital staff who had cuddled infected infants, those who had touched only contaminated toys and subsequently touched their own eyes noses, and those who had sat close to but no more than about 1 m away from infected infants. The result of the comparison was that those who had cuddled infants or touched toys became infected, but those who had only sat near the infants did not. The same conclusions were confirmed by Zlateva et. al (2007).

1.9 RSV Immunity

The level of disease pathogenesis resulting from RSV infection can be balanced through virus elimination and the nature of the immune response to infection (Tripp, 2004). Sato et.al. (2005) reported that although there are a number of studies that have demonstrated

[6] Source: http://pluweb.wordpress.com/2009/05/07/tis-the-season-to-be-sneezing/

immunologically important differences between group A and group B viruses, but very few studies have looked into the immunological differences between the strains within each group. The immunological response to RSV infection can be divided into either innate or adaptive (McNamara and Smyth, 2002; Tripp, 2004).

1.9.1 Innate Immunity

This is the earliest phase of the body defense against foreign organisms. The innate immune response uses effector molecules and phagocytic cells and moves them to the site of the infection through the release of cytokines (McNamara and Smyth, 2002). This type of defense is memoryless. Protein A neutralizes RSV by binding to the F protein but not to the G protein through CD14 and Toll-like receptor 4 (TLR4) which are considered two important receptors in innate immunity (Tripp, 2004).

Following are three important cellular components of the innate response:

1. ***Phagocytic cells***

This includes both the neutrophils and the macrophages.

   a. ***Neutrophils***

The neutrophils play an important role in the pathological changes that take place in the RSV bronchiolitis and are considered its predominant airway leukocytes. In RSV infection, neutrophil chemotaxis is dependent on the production of the potent chemokine, IL-8, by respiratory epithelial cells and macrophages (McNamara and Smyth, 2002; Tripp, 2004). The process of neutrophil recruitment from the bloodstream into the infected tissue can be divided into four steps: rolling, adhesion, extravasation and migration.

b. Macrophages

Macrophages are formed when circulating monocytes migrate into tissue. They play an important part in controlling the immune response to viral infection, not only by direct interaction with helper and cytotoxic T-cells but also by the production of cytokines. They can destroy invading pathogens but more commonly act as antigen presenting cells. Macrophages, along with respiratory epithelial cells are the first cells to encounter RSV in the airways (McNamara and Smyth, 2002; Tripp, 2004).

2. Eosinophils

Both eosinophils and eosinophil RNases possess antiviral activity, and RSV infected respiratory epithelial cells up-regulate the expression of the eosinophil chemo-attractants, RANTES and MIP-1a. Several groups have shown evidence of eosinophil degranulation in both nasopharynx and lung parenchyma during RSV infection (Domachowske and Rosenberg, 1999; McNamara and Smyth, 2002; Tripp, 2004).

3. Natural Killer cells

Natural killer (NK) cells accumulate in the lung in the first few days of RSV infection and are responsible for much of the early production of IFN (interferons). As reported by several researchers, (see: Domachowske and Rosenberg, 1999; McNamara and Smyth, 2002; Tripp, 2004), in children with RSV bronchiolitis admitted to hospitals, blood NK cell counts are significantly lower than controls. This suggests that NK cells are redirected from the peripheral circulation into other tissues such as the lungs.

1.9.2 Adaptive Immunity

The adaptive immune response features immunological memory and is based on the clonal selection of lymphocytes bearing antigen specific receptors (Domachowske and

Rosenberg, 1999). Adaptive immunity is divided into humoral (mediated by B cell) and cell-mediated (mediated by T cell) responses. In RSV infection, the humoral response is primarily involved in protective immunity while, on the other hand, the cell-mediated response helps in viral clearance (McNamara and Smyth, 2002; Tripp, 2004).

1. Humoral

All newly born babies have specific RSV neutralising antibodies through the transfer of maternal immunoglobulin. Most severe RSV disease occurs between 2–6 months of age, when protection from maternal antibody should be present. This suggests that anti-RSV antibodies may have a role in the immunopathogenesis of RSV bronchiolitis (Renato, et al., 1999). In spite of the existence of this systematic and local antibody titre, there is however evidence of serious RSV disease in infants younger than 6 weeks of age. This means that humoral immunity is not playing a major role in protecting against this infection.

Evidence from animal models indicates that humoral antibody protects against RSV disease (Domachowske and Rosenberg, 1999), but once infection is established, it is the cell-mediated response that promotes viral clearance.

2. Cell-mediated

Cell-mediated immunity is an immune response that does not involve antibodies but rather involves the activation of macrophages, natural killer cells (NK), antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen. In cellular immunity, the protective function of immunization is associated with cells. CD4 cells or helper T cells provide protection against different pathogens. T cells cause

death by apoptosis without using cytokines, therefore in cell mediated immunity cytokines are not always present.

Cellular immunity protects the body by activating antigen-specific cytotoxic T-lymphocytes that are able to induce apoptosis in body cells displaying epitopes of foreign antigen on their surface, such as virus-infected cells; activating macrophages and natural killer cells, enabling them to destroy intracellular pathogens; and stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive immune responses and innate immune responses (McNamara and Smyth, 2002; Tripp, 2004). Cell-mediated immunity is directed primarily at microbes that survive in phagocytes and microbes that infect non-phagocytic cells. It is most effective in removing virus-infected cells such as RSV.

1.10 RSV Risk Factors

Studies reported that the following could be considered risk factors:

- History of pre-mature birth;
- Bronchopulmonary dysplasia;
- Congenital heart disease;
- Cystic fibrosis;
- Immunodeficiency;
- Crowded living conditions;
- Being male; Oliveira et. al. (2008) reported that male children are more susceptible to severe disease infection than females, that RSV A-infected patients

tended to be younger, and children who were less than one month of age were infected solely by group A.

- Socioeconomic factors such as belonging to lower income families.

1.11 RSV and Death

Death from bronchiolitis is rare in children without underlying cardiorespiratory or immunological conditions, and the vast majority of children infected with RSV make a full recovery. However, some infants with RSV bronchiolitis subsequently develop recurrent episodes of wheeze and cough, suggestive of asthma (Renato, et al., 1999). In some parts of the world, where medical services are not well developed such as India (see: 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 (1.9 million) who die annually because of ALRI. Since 70% of the India's population lives in rural areas, most of those deaths happen without reaching a hospital.

1.12 RSV Investigation Methods

In their study, Peret et. al. (2000) discussed the variability of the human RSV strains which is a main reason for repeated infection. Although researchers have identified two groups of the virus, there are still several strains within each group. Initially, researchers used monoclonal antibodies to characterize differences between RSV groups and strains within each group, but more recently, genetic characterization has been used to differentiate the human RSV strains (Peret, et al., 2000). Both of these methods have demonstrated that several distinct strains from group A and B often circulate during the

same season (Mufson, et al., 1985; Peret, et al., 1998; Peret, et al., 2000). These two methods are discussed in this section.

1.12.1 Monoclonal Antibodies Methods for RSV Detection

Investigators in the 1980s (Such as: Akerlind, et al., 1988; Mufson, et al., 1985) heavily used the Monoclonal antibodies to differentiate between the two groups of RSV.

Mufson et. al. (1985) analyzed the antigenic variation of human RSV strains using a collection of 9, 6, 6, 9 and 1 monoclonal antibodies respectively directed against the large glycoprotein (G), fusion protein (F), matrix protein (M), nucleoprotein (NP) and phosphoprotein (P) components of the Long strain of RSV. They compared the results with 7 other strains isolated during different years in radioimmune precipitation analyses and immune fluorescence tests. Two different subtypes of the virus were demonstrable. Subtype A included the prototype strains Long and A2 and virus isolates from 1973, 1983 and 1984; subtype B included 4 virus strains isolated in successive years from 1979 to 1982. Subtype A viruses reacted with all the antibodies, whereas subtype B viruses showed different epitope characteristics in 4 structural components. The number of altered epitopes were 5/6, 1/2, 2/6 and 1/6 in the G, F, M and NP components, respectively. The researchers concluded that the two subtypes have evolved separately.

In the second study conducted by Akerlind et. al. (1988), the researchers investigated possible structural differences among the two subgroups of RSV, by analyzing the antigenic characteristics and size of structural proteins of 20 subgroup A and 43 subgroup B strains. The researchers examined the reactions of the strains with monoclonal

antibodies (MAbs) directed against the proteins of RSV using immunofluorescence, ELISA and radioimmunoprecipitation assays which enabled the researchers to determine the size of different structural components. The 37 MAbs employed were generated by immunization with both subgroup A and B strains. They represented specificities for distinct epitopes on 5 different structural proteins. The subgroup A strains proved to be relatively uniform. The fusion protein (F), nucleoprotein (NP) and matrix proteins (M) of all strains tested had the same M_r (marker protein[7]) and all except one strain had a phosphoprotein (P protein) of the same M_r. The F and P proteins were lower in M_r in B strains compared to A strains. The M_r of the large surface glycoprotein (G protein) of subgroup A strains varied slightly, probably on the basis of differing glycosylation as explained by the researchers. By contrast, the subgroup B strains exhibited substantial variation in the M_r of the G and also the P proteins and in reactivity with MAbs directed against the G and F proteins. Three size classes of the P protein were identified in B strains: 33K to 34K, 32K to 33K, and 31K to 32K. Twenty-seven subgroup B strains failed to react with 4 anti-G MAbs representing a single epitope, G2; the remaining 16 strains reacted with these MAbs. The B1 strains also varied in the size of the G and P proteins. In contrast, all B2 strains had large G proteins and all except two strains had relatively large P proteins (33K to 34K). All subgroup B1 and B2 strains exhibited the same sizes of NP, F and M proteins. The study of Akerlind (1988) is an important one because it concluded that the subgroup B strains of RSV include two variants, B1 and B2, and that the major difference between them resides in the G and P proteins.

[7] Protein Marker is a mixture of purified proteins with known amino acid sequences. The protein marker is a marker for a specific protein and is used when running an experiment to determine the presence, absence or size of specific proteins.

The importance of the understanding of the extent of antigenic variability of RSV was recognized by earlier investigators of the virus starting in 1956 to 1962 (Sullender, 2000). The studies suggested that, based on antigenic differences, strains which vary antigentically probably circulate simultaneously in the world. Studies also revealed that antigenic variations do not explain the reinfections that occur with RSV.

1.12.2 Nucleic Acid Detection (NAT) Methods

In recent research, investigators started analyzing the genetic characteristics of RSV in differentiating the groups and the strains embedded within each group. Several techniques were used. The cloning of cDNAs and the determination of nucleotide sequences for the genes helped provide some insight in the antigenic as well as the genetic variability of the virus. Using this technique, the G proteins of the different group A viruses show a minimal variability (6% amino acid differences), whereas differences between group A and group B viruses had extensive differences (47% amino acid differences) (Akerlind and Norrby, 1986). Other techniques were later developed to investigate the above mentioned differences between the G proteins of the two groups. Examples include:

a- Nucleic acid hybridization assay;

b- MAbs Synthetic oligonucleotide probes[8]; and

c- Polymerase Chain Reaction (PCR) technology

d- Amplification of the N gene and its analysis by restriction endonuclease digestion.

[8] MAbs Synthetic oligonucleotide probes is an approach to RSV subgroup differentiation and is based on the hybridization of subgroup specific synthetic oligonucleotides with viral mRNA. It becomes a useful method by preparing pure cDNA to use as probes.

The above mentioned techniques revealed that groups A and B viruses have different patterns. This makes the techniques very useful to differentiate between the two groups of viruses (Akerlind and Norrby, 1986).

Cane and Pringle (1991) amplified the genes encoding the small hydrophobic (SH) proteins of a series of RSV strains using the polymerase chain reaction (PCR), which were later cloned and sequenced. Their analysis of the SH gene sequences from 12 RSV strains isolated between 1956 and 1989 confirmed the homogeneity with little variation in deduced amino acid sequences within the subgroups, A and B, previously defined serologically; although there was only 76% deduced amino acid sequence identity of SH proteins between the subgroups they analyzed. Cane and Pringle (1991) also showed that nucleotide homologies within the subgroups ranged between 93% and 99%. Forty-two isolates of RSV from a single epidemic season (autumn/winter 1989) were also examined to determine their relatedness. For these isolates regions of both the SH and nucleocapsid protein genes of each isolate were amplified and these regions were further analyzed by direct nucleotide sequencing or restriction mapping. From this analysis, Cane and Pringle (1991) were able to find at least six different sub-strains of RSV circulating at the same time and in the same locality.

Peret et. al. (1998) conducted a study on human RSV aiming at characterizing the molecular epidemiology of RSV strains over time. Their analysis was through the performance of sequencing of a variable region of the attachment protein gene (G gene) from a single community in the United States during 5 successive years. Phylogenetic

analysis revealed distinct genotypes that were further classified in subtypes. Five genotypes and 22 subtypes among 123 group A RSV isolates, and 4 distinct genotypes and 6 subtypes among 81 group B RSV isolates were identified. A shift in the predominant genotype or subtype occurred each year such that no genotype or subtype predominated for more than 1 of the 5 study years. The researchers found out, as hypothesized, that the 'newly emerged or evolved' strain is better able to evade previously induced immunity in the population and consequently either circulates more efficiently or is more pathogenic. This yearly shift in RSV strains contributes to the ability of RSV to consistently cause yearly outbreaks of the disease. This means that the consistent shifts in the predominating strains help the virus to evade preexisting immunity (Peret, et al., 2000). These results also suggest that isolates may need to be characterized as to both group and genotype to fully understand protective immunity after natural infection and efficacy studies of candidate vaccines.

In another study conducted in 1991 (Sullender, et al., 1991), the researchers reiterated the fact that RSV causes repeated infections throughout life, and that between the two main antigenic subgroups of RSV, there is antigenic variation in the attachment protein G. The antigenic differences between the subgroups appear to play a role in allowing repeated infections to occur. The researchers also reported that there are antigenic differences that also occur within subgroups; however, neither the extent of these differences nor their contributions to repeat infections are known. The researchers reported molecular analysis of the extent of diversity within the subgroup B RSV attachment protein genes of viruses isolated from children over a 30-year period. Amino acid sequence differences as high as

12% were observed in the ectodomains of the G proteins among the isolates, whereas the cytoplasmic and transmembrane domains were highly conserved. The changes in the G-protein ectodomain were localized to two areas on either side of a highly conserved region surrounding four cysteine residues. What was importantly reported was that single-amino-acid coding changes generated by substitution mutations were not the only means by which change occurred. The researchers reported that changes also occurred by

(i) substitutions that changed the available termination codons, resulting in proteins of various lengths, and

(ii) a mutation introduced by a single nucleotide deletion and subsequent nucleotide insertion, which caused a shift in the open reading frame of the protein in comparison to the other G genes analyzed.

Supporting this study, Zlatevia and a group of other researchers conducted a research and reported its results in two research papers (Zlateva, et al., 2005; Zlateva, et al., 2004). The first paper published in 2004 discussed the genetic changes in the G protein of subgroup A RSV, while the second paper reported on the changes in the G protein of subgroup B RSV. In their research, they analyzed the genetic differences by sequencing the G protein for the two groups. Those studies revealed that the RSV-B evolutionary rate (1.95×10^3 nucleotide substitutions/site/year) is similar to that previously estimated for RSV-A in 2004 (1.83×10^3 nucleotide substitutions/site/year). However, natural RSV-B isolates appeared to accommodate more drastic changes in their attachment G proteins. These changes might result from amino acid substitutions, insertions, deletions, duplications, changes in the stop codon usage, and one frame shift mutation. In Zlateva

et. al. (2007), the researchers reported that subgroup B viruses have a greater potential for divergence by accumulating substantial modifications in their attachment G protein genes, such as 60-nt duplications, 6-nt and 3-nt deletions and insertions, frameshift mutations, and premature stop codons. Furthermore, Zlateva et. al. (2005; 2004) had identified 12 positively selected sites in the G protein ectodomain, suggesting that immune-driven selective pressure operates in certain codon positions. They also concluded that both strains have similar phylodynamic patterns since both are characterized by global spatiotemporal strain dynamics, where novel strain of HSRV variants spread rapidly.

Fifty-one percent of the G-gene nucleotide changes observed among the isolates resulted in amino acid coding changes in the G protein, indicating a selective pressure for change. Maximum-parsimony analysis in that study demonstrated that distinct evolutionary lineages existed. These data show that sequence diversity exists among the G proteins within the subgroup B RSV, and this diversity may be important in the immunobiology of the RSV.

Garcia et. al. (1994), used genetic analysis in their study. They analyzed the genetic and antigenic variability of the G glycoproteins from 76 RSV (subgroup A) isolated during 6 consecutive epidemics in either Montevideo, Uruguay, or Madrid, Spain. Genetic diversity was evaluated for all viruses by the RNase A mismatch cleavage method and for selected strains by dideoxy sequencing for the G gene. The sequences reported were added to those published for 6 isolates from Birmingham, United Kingdom, and for two

reference strains (A2 and Long), to derive a phylogenetic tree of subgroup A viruses that contained two main branches and several subbranches. During the same epidemic, viruses from different subgroups were isolated. In addition, closely related viruses were isolated in distant places and from different years. These results illustrated the capacity of the virus to spread worldwide, influencing its mode of evolution. The antigenic analysis of all isolates was carried out with a panel of anti-G monoclonal antibodies that recognized strain-specific (or variable) epitopes. A close correlation between genetic relatedness and antigenic relatedness in the G protein was observed. These results, together with an accumulation of amino acid changes in a major antigenic area of the G glycoprotein, suggest that immune selection may be a factor influencing the generation of RSV diversity. The pattern of RSV evolution is thus similar to that described for influenza type B viruses, expect that the level of genetic divergence among the G glycoproteins of RSV isolates is the highest reported for an RNA virus gene product (Garcia, et al., 1994).

Development of monoclonal antibodies is not always possible in a routine laboratory with limited financial and laboratory resources (Gottschalk, et al., 1996). Commercial availability of monoclonal antibodies specific for RSV G protein is not certain either. This was the reason why many researchers used RT-PCR assay for subtyping RSV. The workload for subtyping of RSV with RT-PCR is higher than that with immunofluorescence. This drawback is limited in epidemiological investigations, but it would be unacceptable in routine diagnosis with large numbers of viral strains. RT-PCR

for determination of RSV subtypes A and B is therefore a very good method for epidemiological investigations and is the method of choice in some situations like ours.

1.13 RSV Diagnosis

Diagnosis of RSV on clinical grounds alone is rarely possible, and thus, laboratory testing is required for specific viral diagnosis (Gottschalk, et al., 1996). Several methods of diagnosis are generally available and include viral culture, antigen detection by direct immunofluorescence assay (DFA) or enzyme immune assay (EIA), RNA detection by RT-PCR, or serology.

RSV infection may be diagnosed by cell culture techniques or by the identification of viral antigen through rapid diagnosis techniques. Rapid diagnosis is important for the initiation of proper infection control procedures and for possible antiviral chemotherapy. The diagnosis may be backed up by serological tests, acute and convalescent samples, but these require a long time for analysis. Sections 1.14.2 to 1.14.5 describe possible diagnosis tools as described by T. Hadzhiolova (2005) and some were also described by Hall (2001).

1.13.1 Specimen Collection

A variety of samples can be tested using methods such as nasopharyngeal swabs, nasal washes, sputum, bronchoalveolar lavages or serum. Research has proven that the source of the specimen highly influences the identification of the respiratory viruses in patient samples (Abu-Diab, et al., 2008). More recently, collected pernasal flocked swabs have

been shown to give similar results to Nasopharyngeal Aspirates (NPAs) (Abu-Diab, et al., 2008).

The most common specimens' collection method is the NPAs due to the high yield of ciliated epithelial cells. Specimens, using this method can be collected using the following procedure[9]:

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 1.12).
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 $4^{o}C$.

[9] Source: http://www.nhspathology.fph.nhs.uk/core-service/Test-Directory/microbiology/CollectingVacuumAssisted.aspx

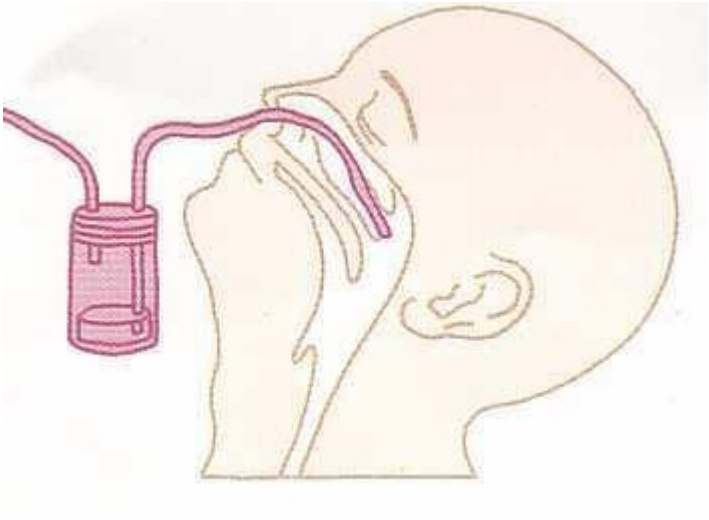
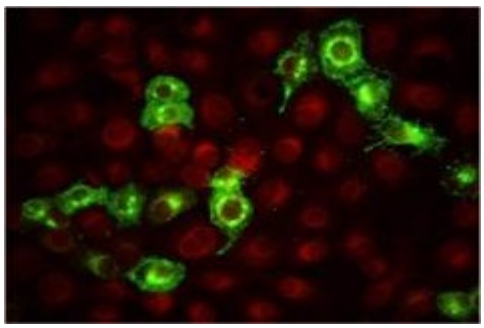


Figure 1.12: Collecting Vacuum-Assisted Nasopharyngeal Aspirates (NPAs)

1.13.2 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 RSV 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.

Figure 1.13: RSV positive cells – DFA analysis[10]



[10] Taken at CBH

b. Several kits are available for the detection of RSV 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.

1.13.3 Cell Culture

RSV 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 RSV. RSV produces a characteristic CPE consisting of syncytia formation and appears in 4 to 5 days.

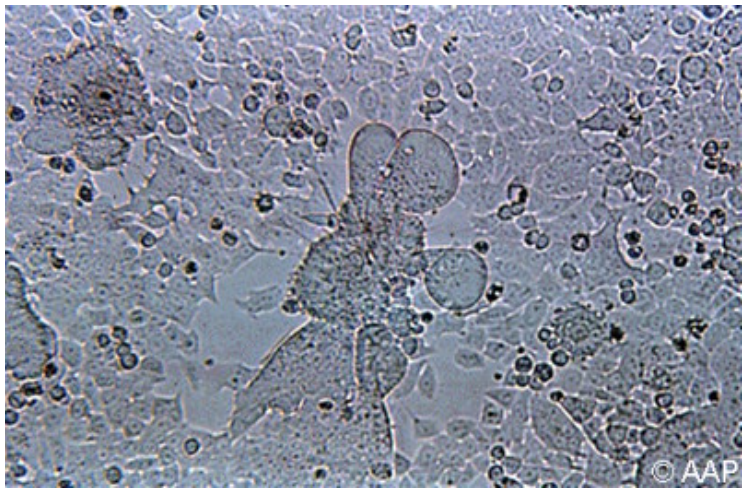


Figure 1.14: The characteristic cytopathic effect of RSV in tissue culture[11].

[11] http://aapredbook.aappublications.org/week/iotw022111.dtl

1.13.4 Serology

Serological diagnosis can be made by detecting antibody rises 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.

1.13.5 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

This is a test that is sensitive and helps substantiate RSV diagnosis. Zhang et. al. (2010) performed RT-PCR for a fragment of RSV 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 from the conserved portion of the F and N genes and can differentiate group A and B viruses. In a study of 1,000 nasal samples (Gottschalk, et al., 1996), RT-PCR was more sensitive compared to viral cultures.

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.

1.14 RSV Infection Prevention

The prolonged survival of RSV virus on skin, cloth, and other objects emphasizes the importance of hand washing in controlling infection. Michael et al. (1990) conducted a study in which RSV strains were placed on non-absorptive (stainless steel, laminated plastic, skin) and absorptive (hospital gown, facial tissue, laboratory coat) surfaces to assess persistence of virus recovery at 0, 0.5, 1, 2, 4, 6, 8, and 10 hours. Virus persisted longest on stainless steel. It was also clear that the higher the concentration of the virus, the higher its recovery will be. Drying of the inoculum on surfaces reduced but did not immediately eliminate the ability to recover virus. Cleaning the contaminated surface with a number of commonly available disinfectant or antiseptic agents reduced or eliminated virus with only short exposure times. It is likely that removal of contaminated material by vigorous cleaning was as important as the actual disinfecting substance.

According to Gottschalk (1996), RSV is believed to spread by large droplets and fomites and can survive on nonporous surfaces, skin, and gloves for many hours. Therefore, close person to person contact or indirect contact with contaminated environmental surfaces are required for transmission. Various infection control strategies, such as hand washing can limit the spread of nosocomial RSV. Some could implement the strategy of wearing gloves and gowns in pediatric wards, or even isolating infected patients could be recommended.

It is important to notice too that there are many clinical trials that evaluate the effect of some of the inhibitors of the RSV compounds. Most of those are still in the early clinical

evaluation phases such as VP-14637, BMS-433771, RFI-641, JNJ-2408068, MBX-300 and small peptide fusion inhibitors that target both the attachment and fusion proteins. Other clinical trials of inhibitors of other proteins are also undergoing evaluation at the clinical level (for detailed discussion, see: Sidwell and Barnard, 2006).

1.15 RSV Treatment

Despite over 40 years of continuous efforts, there are no effective means to control RSV virus 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 RSV. Haynes et. al. (2009) examined whether therapeutic treatment with an anti-RSV G mAb, 131-2G, that blocks the CX3C[12]-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.

[12] A Chemokine which is part of a family of small cytokine.

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 old. 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.

In the following subsections, three different treatments will be described.

1.15.1 Ribavirin

Falsey (2007) reported that Aerosolized Ribavirin (approved in 1986 by Food and Drug Administration (FDA)) is approved for RSV infections in infants. Ribavirin is a guanosine analogue with broad antiviral activity including treatment for RSV (Sidwell and Barnard, 2006) but its approval is limited to infants (Gottschalk, et al., 1996).

Many studies referred to Ribavirin as the only antiviral drug currently approved for treatment of RSV infections. The mechanism of viral inhibition by the drug is best described as multi-faceted and includes inhibition of the enzyme Inosine Monophosphate Dehydrogenase (IMPDH), inhibition of the 5' cap formation of mRNA, and inhibition of viral polymerase by the phosphorylated forms of the compound, although the specific mechanism by which RSV is inhibited is not well documented (Sidwell and Barnard, 2006).

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

1.15.2 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; further, its viscosity, coupled with required high volumes for administration, may lead to fluid overload. RSV-IGIV must be administered under medical surveillance. With the introduction of palivizumab, use of the product has dramatically declined.

2- Palivizumab: is a humanized monoclonal antibody directed to an epitope in the A antigenic site of the F-protein of RSV. It was approved in 1998 for the prophylaxis of infants at high risk for RSV 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 15 mg/kg administered at monthly intervals throughout the RSV season and all strains of RSV 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, parental perception as to the benefits and transportation problems.

In addition to that, there are efforts currently taking place to develop an improved version of palivizumab that is under clinical evaluation. This antibody appears to be more potent than the old version and can neutralize RSV in vitro (Sidwell and Barnard, 2006).

According to Frogel et. al. (2010), Palivizumab (that was invented and approved in 1998), a humanized monoclonal antibody directed against RSV, is the only immunoprophylaxis therapy approved by the FDA (Food and Drug Administration) for

prevention of serious lower respiratory tract disease caused by RSV in infants up to 2 years of age who meet 1 or more of the following criteria for high risk:

(a) Gestational age up to 35 weeks;

(b) Diagnosis of chronic lung disease or

(c) Diagnosis of cyanotic or complex congenital heart disease.

1.15.3 Interferons

One other way of treatment that was actively pursued is the application of interferons (IFNs) (Bossert and Conzelmann, 2002). IFN ά/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 ά/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.

1.16 RSV Vaccination

Antigenic variability contributes to the ability of the virus to reinfect individuals throughout their life time which might cause additional challenges and difficulties in developing vaccines (Zhang, et al., 2010). It is also noticed in several studies that the multiplicity of RSV strains and the constant shift in circulating strains in a community have made it difficult to define circulation patterns of strains and to understand the relationships between strains and RSV disease (Cane and Pringle, 1991; Peret, et al.,

1998; Peret, et al., 2000). Therefore, it is vital to better understand the genetic composition and the variability within a population targeted for vaccination.

It was noted in several studies during conducting this review that the antigenic differences among RSV isolates contribute to the ability of these viruses to establish infections in the presence of preexisting immunity. In addition to the fact that there is evidence that there is a sequential accumulation of change in the G protein of RSV in response to immune pressure (Cane and Pringle, 1995). All of this makes immunization a difficult task.

Sullender (2000) reported that the F protein provided nearly complete protection against viral replication in the lungs after group A or group B virus challenge in cotton rats. Immunization with RSV A virus G protein provided much better protection against group A than group B viral challenge. These results were confirmed by the observation that immunization with RSV B virus G protein protected against group B and not against group A viral challenge. Thus, a subunit vaccine based on the G protein might need G proteins of both antigenic groups to provide broad protection. An F protein vaccine might protect equally well against viruses of both antigenic groups. This means that antibody responses to the F protein were found to be cross-reactive between the two antigenic groups, whereas responses to the G protein were largely group-specific. In his study, Sullender (2000) used a chimeric FG protein to immunize cotton rats which reduced viral titers after challenge with either group A or group B viruses.

Muelenaer et al. (1991) found that antibody responses after infections with group A viruses were more cross-reactive than were the responses which followed primary infection by group B viruses. They suggested that this one-way pattern of cross-reactivity might contribute to the predominance of group A over group B RSV in epidemiologic studies.

The results of a study conducted by Connors et. al. (1991) demonstrate that the F and G proteins expressed by recombinant vaccinia viruses are the most effective RSV protective antigens. Therefore, their study suggested that RSV vaccines need only contain the F and G glycoproteins, because the immunity conferred by other proteins is less effective and appears to diminish rapidly with time.

CHAPTER 2 – Objectives, Materials and Methods

2 Introduction

Respiratory syncytial virus (RSV) is an important causative agent that causes a serious lower respiratory infection in almost all children by the age 2 years (Zlateva, et al., 2005; 2004; 2007). This fact was reiterated in some studies conducted in third world countries (Parveen, et al., 2006; Venter, et al., 2001) as well as in developed countries (Rafiefard, et al., 2004). Sometimes, the RSV infection can occur more than once. It, therefore, has an unusual characteristic by the possibility of repeatedly infecting the patient in spite of the development of the immune system. Most frequently, and during the first few months of life, it causes bronchiolitis and pneumonia. RSV is also considered an important respiratory pathogen for elderly that are either immunocompromised or having problems in their respiratory system (Zlateva, et al., 2005; 2004; 2007).

RSV is an enveloped virus with a nonsegmented, negative-stranded RNA genome of 15,200 nucleotides. It belongs to the genus *Pneumovirus* and is classified in the family *Paramyxoviridae*. RSV has two major groups according to the antigenic and sequencing studies; RSV-A and RSV-B that has 2 antigenic glycoproteins G and F. The differences and the variability within the glycoprotein G is the reason for inducing immune response as well as facilitating repeated RSV infection. It's C-terminal region, which is the second hypervariable, accounts for three strain-specific epitopes which are: (1) conserve found in subgroup A and B, (2) subgroup specific epitop, (3) strain-specific (variable) epitope (Zlateva, et al., 2004).

Reports and epidemiological studies have shown that both groups (RSV-A and RSV-B) can be found within the same outbreak but may vary in proportion (Sato, et al., 2005). Their genotype distribution pattern can be unique for each community affected by the immunity of a specific strain (Zlateva, et al., 2007).

In our hospital, Caritas Baby Hospital (CBH) in Bethlehem, the researcher dealt with many patients that have respiratory infections that are mainly due to virus rather than bacterial infections. Our nasopharyngeal aspirate testing revealed that these viruses are circulating over the year cycle in different percentages and with seasonal outbreaks. The main viruses studied and noted were RSV, influenza virus A, influenza virus B, parainfluenza virus 1, 2, and 3, adenovirus and human Metapneumovirus (hMPV).

This study aims at identifying the prevalence of RSV as well as the subgroups that are floating within the southern part of Palestine, and to identify the genotypes of the subgroups within each outbreak season. This study will be the first in Palestine that may shed light on the prevalence of the common viruses that we have in our communities, their subgroups and genotypes.

2.1 Research Objectives

The objectives of this research include:

1- To identify the seasonality of the infection.

2- To identify the pattern of infection based on certain demographic criteria such as age, sex, and place of residence.

3- To identify the different RSV groups.

4- To identify and characterize RSV lineages.

2.2 Hypothesis

The following are the hypothesis examined and validated through the selected research methods:

1- RSV endemics have a constant yearly patterns.

2- RSV groups A and B are evenly distributed.

3- Presence of different RSV lineages.

2.3 Research Inputs and Output

2.3.1 Inputs

Based on our review of the literature, and due to the focus on the children sector in the Bethlehem and Hebron districts, the inputs for this research include the following:

1- A database of all the nasopharyngeal aspirates tested at CBH from January 2003 till December 2010. Data is collected from 9,486 patients at CBH in Bethlehem over the period from January 2003 to December 2010.

2- Perform molecular analysis of all RSV positive samples. This included 1,388 NPAs (covering the period 2006-2009) on which RT-PCR testing was performed to differentiate between group A and B of RSV.

3- For genotyping, a representative sample of 10%-15% from both RSV A and B were analyzed.

4- Other demographic data needed for the research was collected from participating patients who were tested in CBH.

5- In conducting this research several types of materials were used in the examination of the samples and in the analysis stage of the research. These materials will be further described in the materials and methods section of this research.
6- In order to achieve the desired results, 6 months of labor hours were needed in the analysis of the sample and the results.
7- The sequencing machine (ABI machine used is the 3130 Genetic Analyser produced in the USA by Applied Biosystems) available at Bethlehem University was used for the sequencing of the samples.

2.3.2 Outputs

The outcomes of this research include the following:

1- A complete data base that contains data about the sample items. It should include all demographic data as well as data regarding viruses that infected the patients.
2- After the analysis of all collected samples and data from participating patients, the researcher prepared a complete database about the molecular epidemiology of RSV in the southern part of Palestine.
3- A clear understanding of the serotypes and genotypes of RSV within different endemic outbreaks.

2.4 Target Group

The population for this study was children of the southern Palestinian districts. The expected age of the participating patients includes new born children up to the age of 5 years.

2.4.1 Patients' Demographics

The patients were mostly from either Bethlehem district (58.8%) or Hebron district (38.2%). The ages of the RSV-infected patients range between 2 days and 5 years. The majority of patients were infants and children less than 2 years of age while the female to male ratio is nearly 1:2.

2.5 Materials and Methods

In conducting this research, several materials and methods were used at its different stages. In this section, a description of those materials and methods is briefly explained.

2.5.1 Clinical Specimens

Respiratory samples (N = 9,486) represented by nasopharyngeal aspirates (NPA) were collected by well trained CBH nurses using mucus extractors Figure 2.1, from patients with severe respiratory tract infection. The NPA specimens were stored at -20°C pending further use. The period covered started in January 2003 and ended in December 2010.

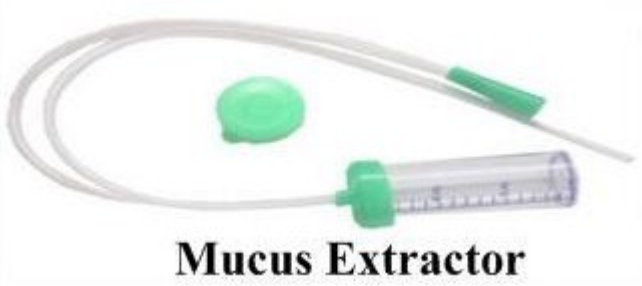


Figure 2.1: Mucus Extractor[13]

[13] Taken at CBH

2.5.2 Sample Preparation for Immunofluorescence Staining

The following procedures were followed to prepare the NPA samples for testing. This procedure was implemented on all of the 9,486 samples available.

1. Centrifuge nasopharyngeal aspirate at 300 x g for 10 minutes at 2-8°C.
2. Remove the supernatant into a new clean tube by carefully pouring the supernatant into a new vial.
3. Add 5ml PBS (Phosphate Buffer Solution) (4°C) to the cells and wash the cells by gentle shaking.
4. Centrifuge the samples at 300 x g for 10 minutes.
5. Remove the PBS by aspirating the fluid using the vacuum trap or pour off supernatant.
6. Add 5ml PBS (4°C) to the cells and wash the cells by gentle shaking.
7. Centrifuge the samples at 300 x g for 10 minutes.
8. Re-suspend the cells in 0.2ml cold PBS.
9. Add 1 drop of cells to 10 well slide cleaned with acetone to remove oil droplets.
10. Allow the sample to dry in the biological safety hood.
11. Fix the cells to the slide by putting the slide in ice-cold acetone for 10 minutes.
12. Allow the slide to dry.
13. Store the slide at -20°C or proceed to the staining procedure.

2.5.3 Direct Immunofluorescence Staining Procedure

The following procedure was followed to test patient's samples for RSV:

1. Allow the control slide and test slides to equilibrate to room temperature.
2. Add sufficient amount (1 drop) of DFA Respiratory Screen (Millipore respiratory DFA viral screening and identification kit (Cat # 3137) – produced by Research Development Production - USA) to the well.
3. Incubate at 37°C for 30 minutes in a humid chamber.
4. Rinse slide thoroughly with PBS / Tween for 10 to 15 seconds in the indicated bath.
5. Repeat step 4 three times.
6. Rinse slide thoroughly with dH_2O for 10 to 15 seconds in the indicated bath.
7. Remove the excess water on the slide by using the vacuum trap. Make sure not to aspirate the cells from their wells.
8. Allow the slide to dry.
9. Apply mounting fluid to the slide.
10. Place a 22x22 cover slip on the wells.
11. Read sides immediately after staining, if not possible, store slides at 2-8°C in a secure container in the dark.
12. Examine slides, using a fluorescence microscope (Nikon Eclipse 80i) at 160 to 200 X for the presence of cells exhibiting fluorescence.
13. Detailed examination may be carried out at 400 X magnification.

2.5.4 Preparation of 10X Phosphate Buffer Saline (PBS)

The following solution was prepared and was used for the preparation and the washing of the NPA samples for further DFA and molecular diagnosis. The components, as well as the concentration mix is shown in Table 2.1.

PBS components	Concentration = 10X
NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4	14.4 g
Deionized H_2O	Till 1 L
Adjust pH to 7.4 with HCl or NaOH	

Table 2.1: Phosphate Buffer Saline (10X)

2.5.5 Preparation of Tween 20 (Polyoxyethylene-Sorbitan Monolaurate)

The tween 20 was prepared using 1X PBS (997 ml) + 0.03% tween 20 (3ml). That is; 100ml 10X PBS + 900 ml H_2O.

2.5.6 Viral RNA Extraction

RNA was extraction from NPA samples were done by using 2 extraction protocols.

1. QIAamp DNA Blood Mini Kit (Qiagen Cat # 51104) according to the manufacturer's manual as follow:

    a. **Buffer AVL solution for RNA extraction preparation:**

    - Take 1 ml from the solution.
    - Add the 1 ml to the RNA carrier.
    - Shake and spin the RNA carrier.
    - Pour the mix into the big solution bottle and shake.
    - Distribute 560 µl into sterile eppendorf tubes and stored at 4°C.

b. RNA extraction:

- Incubate AVL eppendorf tube @ 56°C for 10 min. (Make sure all the crystals dissolve).
- Add 140 µl NPA sample into the AVL tube and mix up and down by pipetting 15 times.
- Incubate for 10 min at room temperature.
- Add 560 µl of absolute Ethanol (dehydrated 95%) and mix by pipetting 15 times.
- Pour the mixture into the Qiagen extraction column in 2 folds (630 µl each) and centrifuge each time for 1 min @ 8000 rpm. (Each time discard the 2 ml collection tube).
- Add 500 µl AW1 (Wash Buffer) solution and centrifuge for 1 min @ 13000 rpm. (Discard the 2 ml collection tube).
- Add 500 µl AW2 (Wash Buffer) solution and centrifuge for 3 mins @ 13000 rpm. (Discard the 2 ml collection tube).
- Centrifuge again (blank) for 1 min @ 13000 rpm. (Discard the 2 ml collection tube).
- Replace the 1.5ml eppendorf tube and place the Qiagen filter column into it.
- Add 40 µl elution buffer and incubate for 1 min.
- Centrifuge for 1 min @ 13000 rpm.
- Place the extracted RNA tube into -70°C freezer to be used later.

2. High Pure Viral RNA Kit (Cat # 11858882001; Roche Applied Science) as follow:

    a. **Before beginning, the preparation of working solutions is needed.**

    Beside the ready-to-use solutions supplied with this kit, you will need to prepare the following working solution:

        1. **Poly (A) carrier RNA (Vial 2)**

            - Dissolve poly A carrier RNA (vial 2) in 0.4 ml Elution Buffer (vial 4).
            - Prepare aliquots of 50 µl into 1.5 eppendorf tubes for running 8 x 12 purifications.
            - Store at -15 to -25°C.
            - **For the preparation of working solution**

        2. **Working solution:**

            - For 12 purifications, thaw one vial with 50 µl poly A carrier RNA and mix thoroughly with 5 ml Binding Buffer (vial 1)
            - Prepare always fresh before use! Do not store!
            - Protocol Step l

        3. **Inhibitor Removal Buffer (Vial 3a; black cap)**

            - Add 20 ml absolute ethanol to Inhibitor Removal Buffer and mix well.
            - Label and date bottle accordingly after adding ethanol.
            - Store at +15 to +25°C. (Stable through the expiration date printed on kit label).

- Protocol Step 5: To remove PCR inhibitors

4. **Wash Buffer (Vial 3; blue cap)**
    - Add 40 ml ethanol p.a. to each vial Wash Buffer before use and mix well.
    - Label and date bottle accordingly after adding ethanol.
    - Store at +15 to +25°C. (Stable through the expiration date printed on kit label).
    - Protocol Step 6 and 7: Removal of residual impurities

2.5.7 Nucleic Acid Testing (NAT)

2.5.7.1 Multiplex RSV A and B RT-PCR

RSVs were typed to group A or group B by using a multiplex reverse transcriptase PCR (RT-PCR) assay. While some samples, were confirmed by repeating the RT-PCR in separate singleplex reactions for subgroups A and B.

RT-PCR was performed by using QIAGEN one step RT-PCR (USA Cat # 210212) and PTC-100 Peltier Thermal Cycler (From MJ Research) was used to amplify the PCR product under the following conditions: 50 C° for 30 min, 95 C° for 15 min, then 94 C° for 45 sec; 56 C° for 45 sec; and 72 C° for 1 min; 40 cycles. Then 72 C° for 10 min for the final extension step.

Two sets of primers were used and were prepared as follows:

- Stock Primer = 80 nmol = 80,000 pmol = 80 pmol / µl.

- To dilute 80 pmol (4 times concentration) to 20 pmol.
- 1µl primer + 3µl H_2O
- 10µl primer + 30µl H_2O
- 100µl primer + 300µl H_2O = 400µl (20pmol/µl primer)
- 400µl aliquot into 10 (40µl) tubes.

1. **Primers set #1** (Zlateva, et al., 2007)

RSV-A (G267): atgcaacaagccagatcaag

RSV-A (F164): gttat**R**acactggtataccaacc

– The expected size is 914 bps

– The reverse primer for the RSV-A was ordered to contain nucleotide that would catch the mismatch of C.

```
Query  1    GTTATCACACTGGTATACCAACC  23
            ||||| ||||||||||||||||
Sbjct  173  GTTATGACACTGGTATACCAACC  151
```

The melting point: = 2(A+T) + 4(C+G)
((22+36=58) + (26+40=66)/2) – 5 = 57

RSV-B (BGF): gcagccataatattcatcatctct

RSV-B (BGR): tgccccag**R**tttaatttcgttc

– The expected size is 800 bps

The melting point: = 2(A+T) + 4(C+G)
((30+28=58) + (24+28=52)/2) - 5 = 52

- PCR product visualization was done by running the PCR product in 1% agarose.
- Electrophoresis was performed at 80 volt for 45 min.

2. **Primers set # 2** (Hu, et al., 2003)

A (N gene) (A21): GCTCTTAGCAAAGTCAAGTTGAATGA

A (N gene) (A102): TGCTCCGTTGGATGGTGTATT

– The expected size is 82 bps

B (N gene) (B17): GATGGCTCTTAGCAAAGTCAAGTTAA

B (N gene) (B120): TGTCAATATTATCTCCTGTACTACGTTGAA

– The expected size is 104 bps

- PCR product visualization was done by running the PCR product in 2.6% agarose.
- Electrophoresis was performed at 80 volt for 45 min.

Figure 2.2 illustrates one of the gels used to type RSV. It shows that RSV B had a size of 104 bps while RSV A had 82 bps.

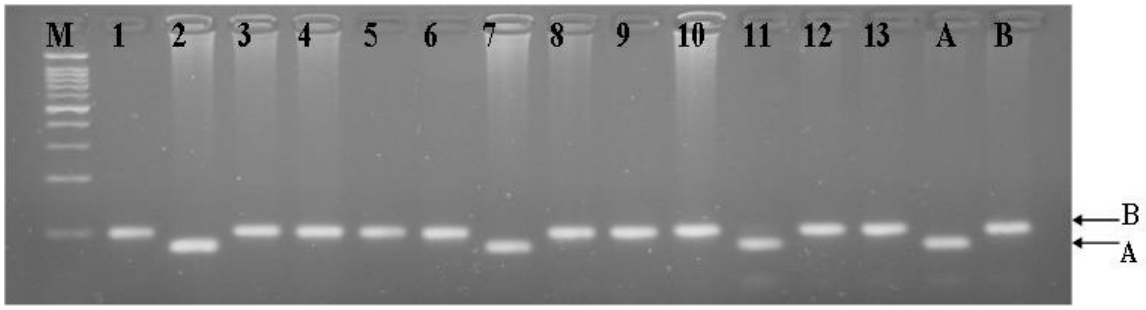


Figure 2.2: Multiplex RT-PCR

Following is a summary of the primers used in the RSV typing part of this research. Table 2.2 focuses on G-gene while Table 2.3 focuses on the N-gene.

RT-PCR primers	Gene	Positions	Sequence (5'-3')	Polarity
RSV-A				
G267	G	248–267	ATGCAACAAGCCAGATCAAG	+
F164	F	164–186	GTTATCACACTGGTATACC AACC	-
RSV-B				
BGF	G	169–192	GCAGCCATAATATTCATCATCTCT	+
BGR	G-F	5637–5658	TGCCCCAGRTTTAATTTCGTTC	-

Table 2.2: Oligonucleotide primers of RSV-A and -B used for G-gene

RT-PCR primers	Positions	Sequence (5'-3')
A (N-gene)		
A21	19-45	GCTCTTAGCAAAGTCAAGTTGAATGA
A102	81-101	TGCTCCGTTGGATGGTGTATT
B (N-gene)		
B17	15-41	GATGGCTCTTAGCAAAGTCAAGTTAA
B120	90-119	TGTCAATATTATCTCCTGTACTACGTTGAA

Table 2.3: Oligonucleotide primers of RSV-A and -B used for N-gene

2.5.8 PCR Product Sequencing

Twenty seven randomly selected RSV-A and RSV- B PCR products 914 bps and 800 bps, respectively, were purified with a QIAquick gel purification kit (QIAGEN, Germany) (Cat # 28704) according to the manufacturer's instructions as follow:

1. Excise the DNA fragment from the agarose gel (1%) with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless 1.5ml eppendorf tube.
3. Add 3 gel volumes of buffer QG to 1 volume of the excised agarose (1%) containing the PCR product.
4. Incubate at 50 ℃ for 10 minutes or until the gel slice has completely dissolved. To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation.

5. After the gel slice has dissolved completely, check that the color of the mixture is yellow
6. Add 1 gel volume of isopropanol to the sample and mix.
7. Place the QIAquick spin column in a provided 2 ml collection tube.
8. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.
9. Discard flow-through and place QIAquick column in a clean 2 ml collection tube.
10. Add 500 µl of QG buffer to QIAquick column and centrifuge for 1 min.
11. To wash, add 750 of buffer PE to QIAquick column, incubate for 1 min, and then centrifuge for 1 min.
12. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at the higher speed.
13. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
14. To elute add 50 µl EB to the QIAquick column membrane and let the column stand for 1 min, and then centrifuge for 1 min.
15. If the purified DNA is to be analyzed on a gel, add 1 volume of loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

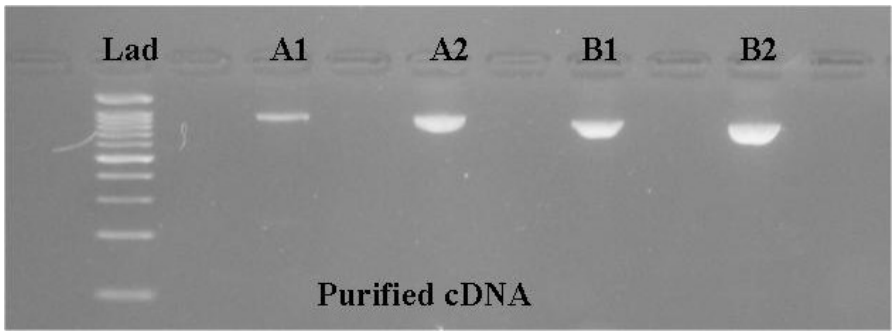


Figure 2.3: Purified RSV cDNA for PCR product

2.5.9 Nucleic Acid Detection

The purified PCR products were sequenced at Bethlehem University in the forward and the reverse directions using the ABI (Applied Biosystem) protocol:

1. Purify the sample for sequencing using PCR Clean-up System (Promega Kit Cat #: A9282).
2. Sequencing PCR using Big Dye Terminator V1.1 sequencing kit Cat # 433-7451-100 and PIN # 4336772.

   The following table shows PCR recipe for sequencing (ABI):

Materials	**1X**
5XBuffer	4 ul
Big Dye	2ul
Primer F or R	0.5 ul from (10 pml /ul) stock
Template	According to the template size and concentration.
H_2O	up to 20 ul

Table 2.4: PCR recipe for sequencing

   a) Do this for the control DNA provided with the kit to see if the PCR working or not.

   b) Do PCR for the sample.

3. Clean the sequencing PCR product using EDTA method precipitation using Qiagen DyeEx 2.0 Spin Kit (Cat # 63206) or Edge Biosystem Kit (Cat # 42453).

   a) Add 5 µl of 125 nM EDTA to the 20 µl PCR product total volume.

   b) Add 100 µl of absolute ethanol.

   c) Incubate at room temperature for 1 hr.

   d) Centrifuge for 30 min at 4 °C at 3800 rpm.

   e) Discard supernatant

f) Add 60 µl of 70 % ethanol then spin at 4 ℃ at 3800 rpm for 20 min and then discard the supernatant.

4. Dry the product on speed vacuum or at 70°C.
5. Denaturation: add 16 µl of Hidye-Formamid (Cat # 4311320) to the dried product, mix well, spin, put on 95 °C for 5 min directly, and then put on ice 5 min.
6. Run the sample on the ABI machine.
7. To run Standard: (we use standard to test the machine),
    a) Add 30 µl of Formamid to the Standard tube, mix, spin.
    b) Then take 3 µl from it and add to it 15 µl Formamid, mix, spin, put on 95°C 5min, then put on ice for 5 min.
    c) Run on the ABI machine (3130 Genetic analyzer).

The amplicons were sequenced in both directions by using the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) and the RT-PCR primers were used.

2.5.10 Sequence Analysis

The Sequencher program (Gencodes corporation, Ann Arbor, MI) was used to clean and align both RSV A and B nucleotide sequences. After sequence alignments, a FASTA text file was generated and the program Clustal X was used to generate the phylogenetic trees using the nearest neighbor analysis using with 1,000 bootstraps. Phylogenetic trees were then visualized using the NJ plot.

CHAPTER 3 – Results

3 Introduction

This research was conducted at Caritas Baby Hospital (CBH) in Bethlehem. Caritas is a pediatric hospital that provides services for the population of Palestine, serving especially the citizens of the southern Palestine. Most of the patients that were admitted to the hospital are inhabitants of the Bethlehem and the Hebron districts.

Patients admitted to the hospital with suspected viral infection were considered for this study. Samples were collected over a long period of time covering different epidemic seasons from the year 2003 to 2010. In this chapter, the researcher describes the characteristics of the population of the research as well as the analysis of the samples collected. Demographics, infection, dual infection as well as distribution of RSV disease and groups over the various study's seasons are also discussed.

3.1 Optimization of the PCR Reaction Volume

Before moving into depth, analyzing the data and the results of the research, the researcher performed an optimization of the PCR reaction volume in order to reduce the cost of running the typing due to the large number of positive RSV samples tested. The master mix total volumes were reduced gradually from 25µl, 12.5µl to 6.25µl with no difference in the result as shown in the following graph (Figure 3.1). Therefore, the researcher decided to use 6.25µl.

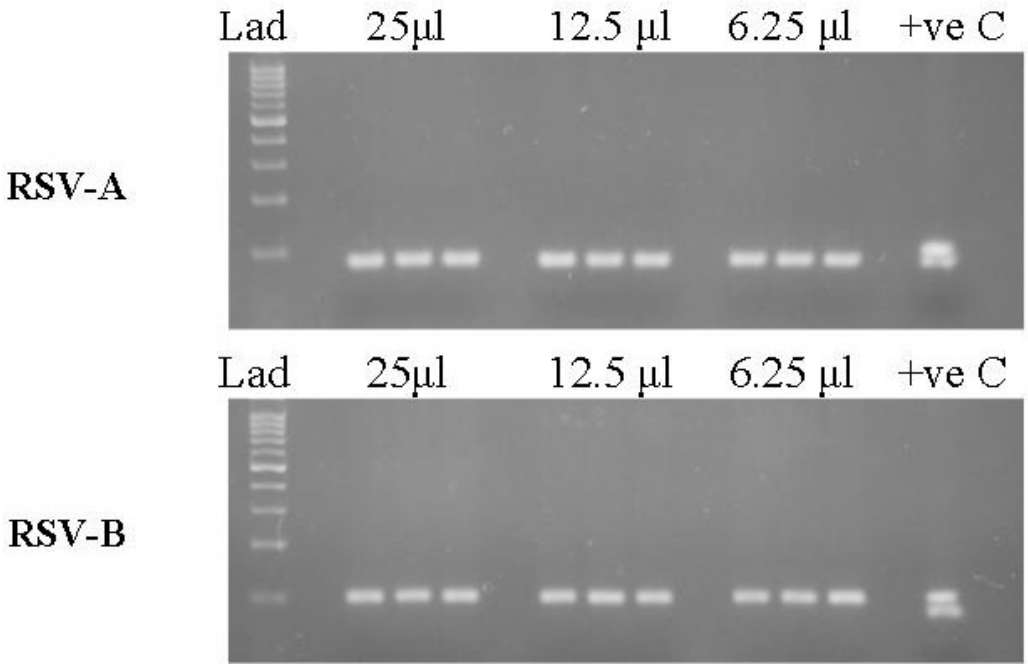


Figure 3.1: Differences in RT-PCR products between different Master Mix Reaction volumes

3.2 Population Characteristics

Data was collected from 9,486 patients at CBH in Bethlehem over the period 2003 to 2010. Out of those patients, 150 (2%) were not hospitalized while the rest (98%) were hospitalized. Analysis was based on the DFA method to determine whether there was an RSV viral infection or not.

3.2.1 Distribution by Age

Another important determinant for this study was the age group of the patients. In this study, age was considered as an important variable that influences the results of RSV infection.

In this research, patients were grouped into age groups as shown in the following table (Table 3.1). Grouping was made, for the purposes of this study by 6-month intervals.

That is, a patient in group 1 is in his first 6 months of life while a patient in group 2 is in his second 6 months of life (over six months up to one year of age).

Age group (In 6 months intervals)	Number of samples	Total
Up to 6 months	4,883	51.48%
Up to 12 months	2,267	23.90%
Up to 18 months	960	10.12%
Up to 24 months	455	4.80%
Up to 30 months	268	2.83%
Up to 36 months	173	1.82%
Over 36 months	480	5.05%
Total	**9,486**	**100.00%**

Table 3.1: Distribution of Population by age group

The following graph (Figure 3.2) shows the distribution by age group:

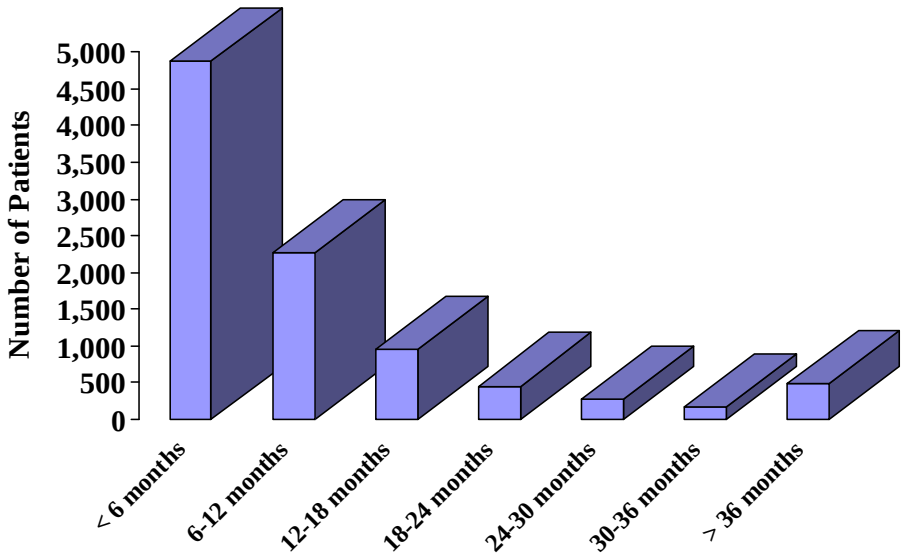


Figure 3.2: Distribution of population by age group

From Table 3.1 above, it is obvious that over 90% of the suspected patients with viral infection are in their first two years of life.

3.2.2 Distribution by Gender

Figure 3.3 depicts the distribution of the population of samples by gender. Female patients comprise 37% of the population while 63% were taken from male patients over the seven years in which samples were collected.

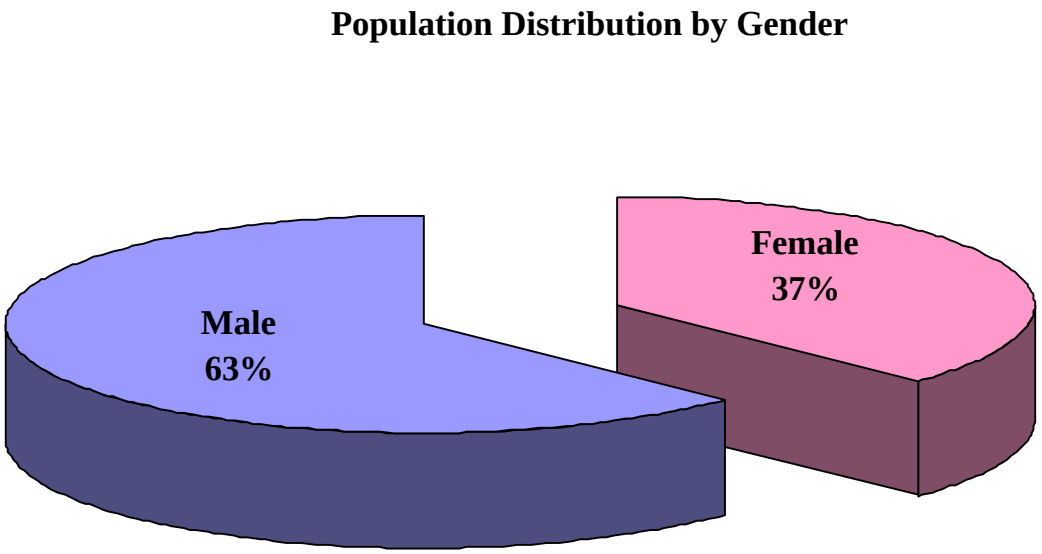


Figure 3.3: Distribution of Population by gender

3.2.3 Distribution by Year

Samples were collected from patients over the period from January 2003 to December 2010. Those samples were distributed as shown in Table 3.2 and as presented in Figure 3.4 below:

Year	Number of Samples	Percentage
2003	334	3.52%
2004	726	7.65%
2005	739	7.79%
2006	925	9.75%
2007	1,339	14.12%
2008	1,763	18.59%
2009	1,793	18.90%
2010	1,867	19.68%
Total	9,486	100.00%

Table 3.2: Distribution of Population by Year

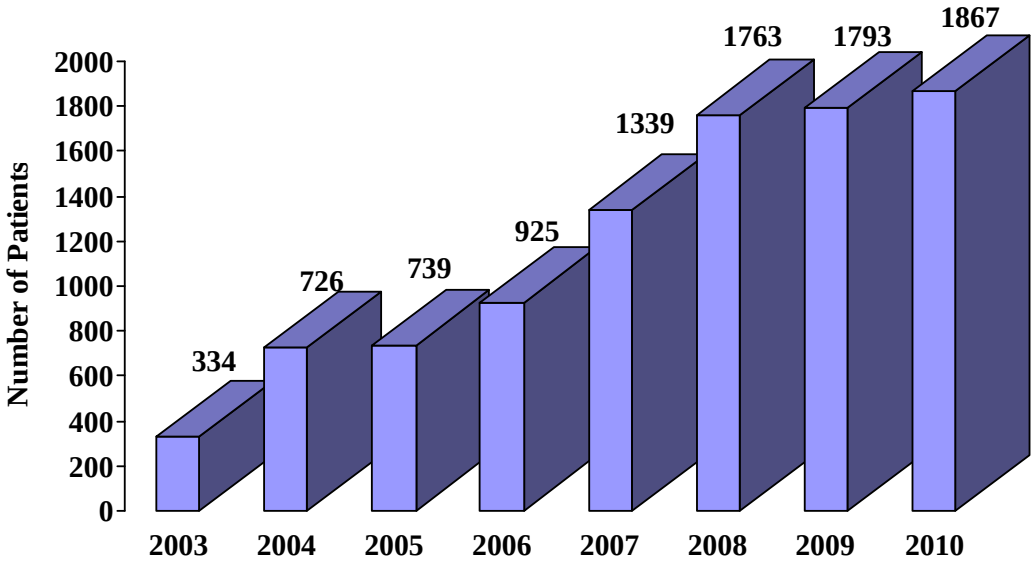


Figure 3.4: Distribution of the population by year from which samples were taken

3.2.4 Distribution by Season

Seasonality of RSV was reported in the literature which was the reason that led the researcher to look into the population distribution over the months of the year. Overall, samples tested from patients with suspected viral infection were distributed by month as shown in Table 3.3 and Table 3.4. The data distribution shows that the suspected viral infection increases around winter time and it is less severe in summer. Table 3.4 as well as Figure 3.6 show the same distribution per month and year of the population. The researcher hypothesize, based on the literature review that the epidemic seasons of the disease may be more severe in some years and less severe in other years.

Month	Number of samples	Percentage
January	1,505	15.87%
February	1,176	12.40%
March	1,015	10.70%
April	847	8.93%
May	726	7.65%
June	511	5.39%
July	403	4.25%
August	372	3.92%
September	414	4.36%
October	542	5.71%
November	870	9.17%
December	1,105	11.65%
Total	9,486	100.00%

Table 3.3: Distribution of Population by Month

To show the clear variation in number of cases admitted to hospital with suspected RSV, the following figure (Figure 3.5) shows the distribution of cases per month of the year:

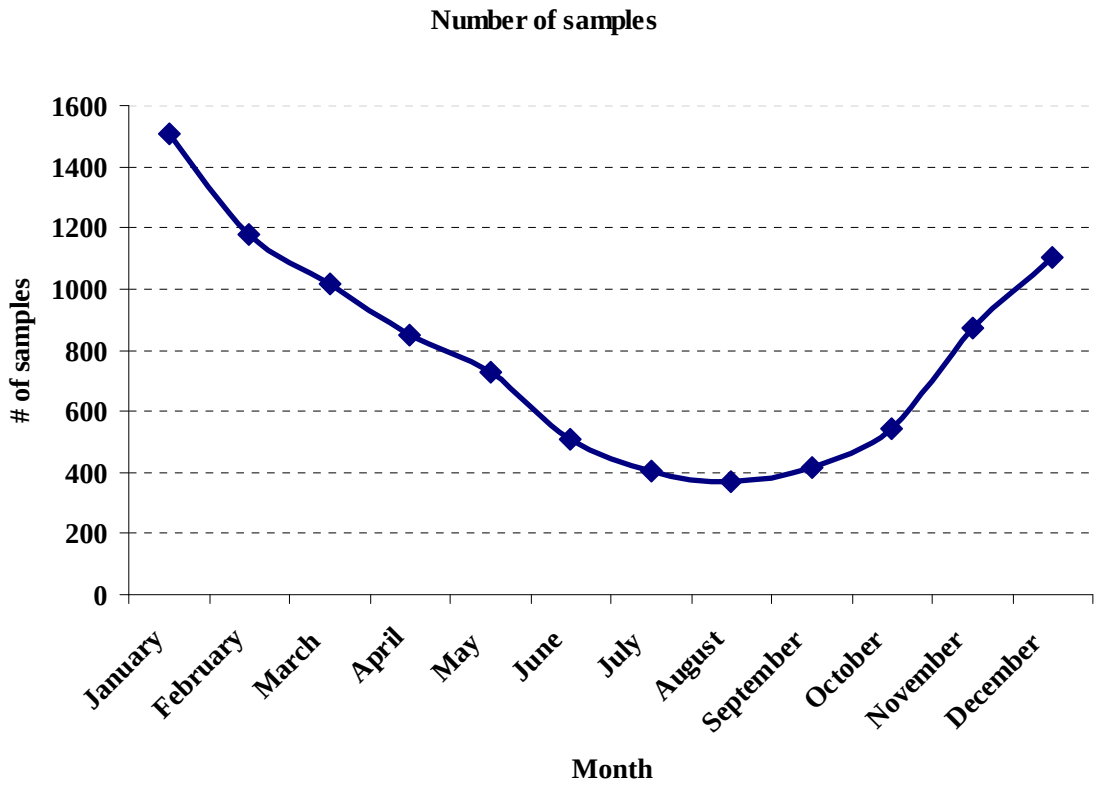


Figure 3.5: Distribution of samples per month from which the sample is selected

Month	2003	2004	2005	2006	2007	2008	2009	2010	Total
January	112	68	245	71	299	137	241	332	1,505
February	33	124	106	137	201	168	113	294	1,176
March	11	116	67	151	118	200	111	241	1,015
April	12	74	61	115	116	174	118	177	847
May	11	44	32	76	91	202	157	113	726
June	10	15	28	31	62	123	118	124	511
July	11	6	8	24	44	67	121	122	403
August	6	11	13	21	57	60	76	128	372
September	5	26	36	34	74	78	87	74	414
October	18	24	29	32	81	120	156	82	542
November	54	32	43	75	84	181	258	143	870
December	51	186	71	158	112	253	237	37	1,105
Total	**334**	**726**	**739**	**925**	**1,339**	**1,763**	**1,793**	**1,867**	**9,486**

Table 3.4: Distribution of Population by Month and Year

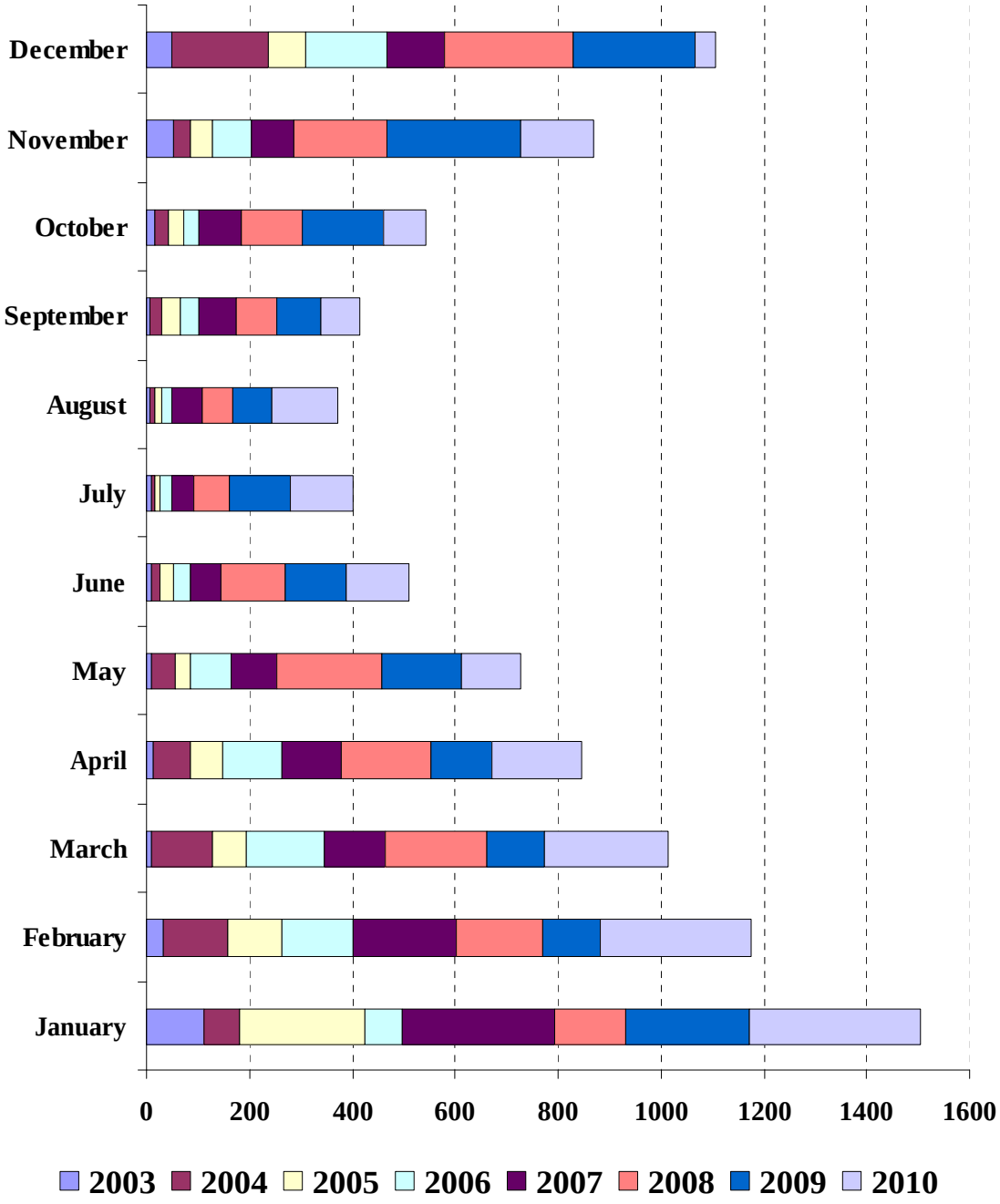


Figure 3.6: Distribution of samples by month and year from which each was taken

3.2.5 Distribution by District

Since it was shown in prior studies that the RSV infection varies in different communities, it was important to differentiate between samples collected based on the district in which the patient resides.

Analyses of the sample of suspected viral infected patients is distributed as follows (Table 2-5). The category "others" in Table 3.5 includes some samples taken from patients registered as Israeli patients (11), USA citizens (1) and the others (9) had no complete record indicating their place of residence. It is obvious from the distribution that the majority of the samples tested and patients admitted come from the southern part of Palestine, mainly from the Bethlehem and the Hebron districts. A major reason for this distribution is the location of the Caritas Baby hospital in Bethlehem that makes it quite difficult for citizens of other parts of the West Bank (WB) to visit.

District	Number of sample
Bethlehem	5,557
Hebron	3,632
Jenin	1
Jericho	47
Jerusalem	191
Nablus	4
Ramallah	31
Tulkarem	2
Others	21
Grand Total	**9,486**

Table 3.5: Distribution of Population by District

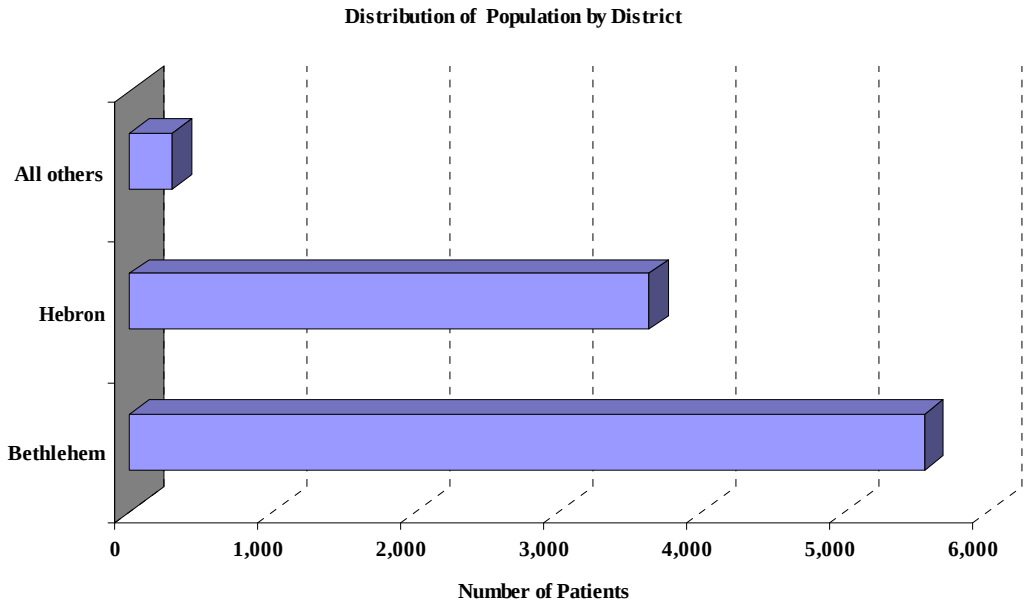


Figure 3.7: Distribution of population by district

3.3 Preliminary Viral identification Results from patient samples

In the initial analysis of all samples collected from patients over the seven years under study, the researcher examined the reactions of the strains with monoclonal antibodies (MAbs) directed against the proteins of RSV using immunofluorescence assays. This direct fluorescence assay technique was used to determine whether a patient has viral infection or not. This method was used by several researchers as reported in various prior studies cited in the literature review chapter of this thesis.

3.3.1 RSV Distribution by Age

One of the main determinants of the RSV infection is the age group of the patients. RSV infection was found mainly in younger infants. The age groups are clustered in 6 months intervals. Almost 99% of those tested are infected in the first three years of their life, while 88% are tested positive with RSV infection in the first year of their life. Table 3.6 as well as Figure 3.8 show a summary of those results.

Age group	Number of RSV infected patients	Percentage
Up to 6 months	1,636	67.72%
Up to 12 months	494	20.45%
Up to 18 months	153	6.33%
Up to 24 months	54	2.24%
Up to 30 months	32	1.32%
Up to 36 months	22	0.91%
Over 36 Months	25	1.03%
Total	**2,416**	**100.00%**

Table 3.6: Distribution of infection by Age

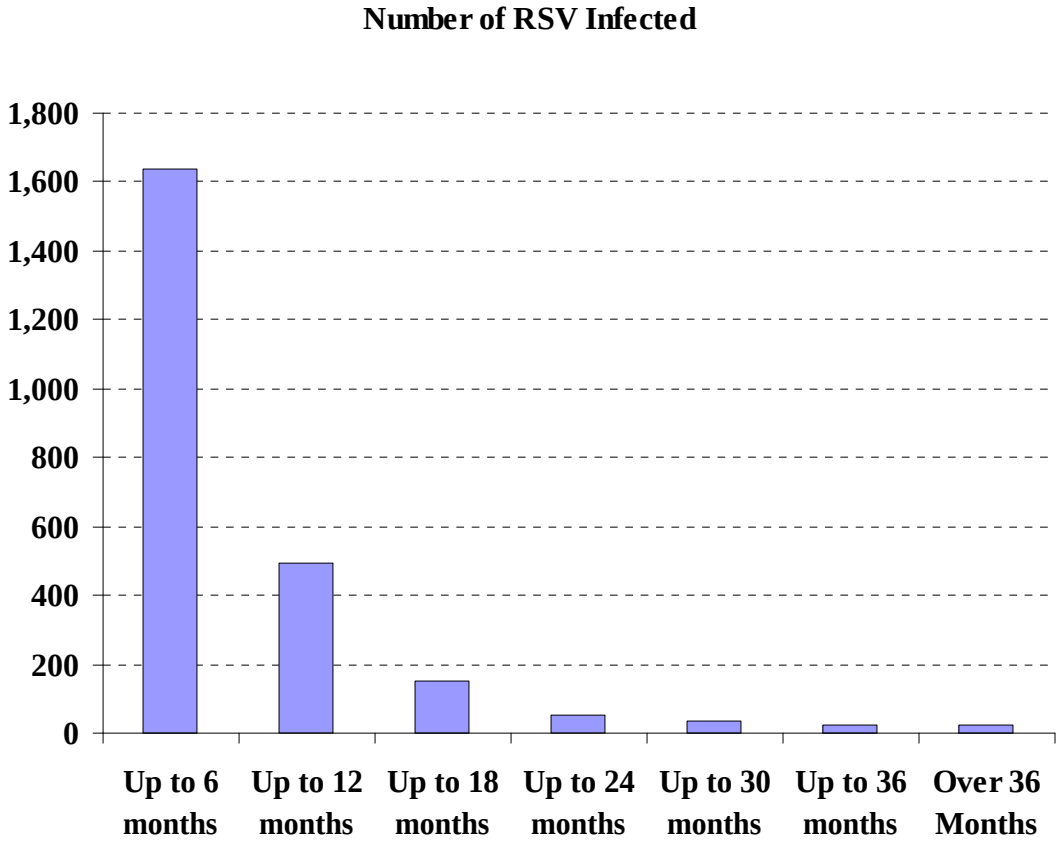


Figure 3.8: Distribution of RSV infected children by age group

3.3.2 RSV Infection and Dual Infection

The results of the DFA analysis revealed that 4,052 (42.9%) of the samples tested showed positive results for Respiratory virus infection while the other 5,383 (57.1%) test results were negative. Samples that tested positive did not only have an RSV infection, but

different viruses were detected of which some were of interest to the current research. For the current research, patients with RSV infection or dual infection that includes RSV as one of the viruses tested was further evaluated in this study. Fifty one (0.54%) patient samples not suitable for this analysis because of insufficient patient epithelial cells in the sample.

Samples that showed positive result for the presence of a respiratory virus are shown in Table 3.7:

Viral result		Number of samples	Total	
Adeno		348	8.59%	
Adeno + hMPV		13	0.32%	
Adeno + Influenza B		1	0.02%	
hMPV		453	11.18%	
hMPV + Para influenza 1		1	0.02%	
Influenza A		286	7.06%	
Influenza A + Adeno		1	0.02%	
Influenza A + Para influenza 3		2	0.05%	
Influenza B		27	0.67%	
Influenza B + hMPV		2	0.05%	
Para influenza 1		98	2.42%	
Para influenza 2		49	1.21%	
Para influenza 2 + hMPV		2	0.05%	
Para influenza 3		341	8.42%	
Para influenza 3 + hMPV		12	0.30%	
RSV	**2,416**	**2,347**	**57.92%**	**59.61%**
RSV + Adeno		**1**	**0.02%**	
RSV + hMPV		**67**	**1.65%**	
RSV + Para influenza 1		**1**	**0.02%**	
Total		**4,052**	**100.00%**	

Table 3.7: Distribution of infection by virus type

Of those patients diagnosed with a positive virus infection, 2,416 had a positive RSV result. Some of them had dual infection as shown in Table 3.7 above. The majority of the patients (N = 67) who developed dual infection had both RSV and hMPV infection.

3.3.3 Gender Distribution

On the other hand, further investigation of those infected with RSV was conducted shows that the majority are male patients as shown in Table 3.8. The male to female ration was 1.6:1.

Gender	Number of infected	Percentage
Female	926	38%
Male	1,490	62%
Total	2,416	

Table 3.8: Distribution of infection by gender

3.3.4 Epidemic Season

Looking into the distribution of those patients infected with RSV (including those with dual infection) based on the month of infection, winter time is the season for the RSV infection to spread. Over 96% of the infected patients were reported in the period between November and May. This result is shown in Table 3.9 and also depicted in Figure 3.9.

Month	Year								Total	Percentage
	2003	2004	2005	2006	2007	2008	2009	2010		
January	99	4	188	4	232	16	127	111	781	32.33%
February	15	32	55	32	134	53	37	113	471	19.50%
March		75	6	87	12	44	14	78	316	13.08%
April		44	1	68	2	47	3	22	187	7.74%
May		19		21		44	1	3	88	3.64%
June		2		2		22			26	1.08%
July				1		2			3	0.12%
August		2		1		2			5	0.21%
September		1				7			8	0.33%
October		5				36		2	43	1.78%
November		8		16		90		2	116	4.80%
December		134		65	1	145	20	7	372	15.40%
Grand Total	114	326	250	297	381	508	202	338	2,416	100.00%

Table 3.9: Distribution of infection by month/year

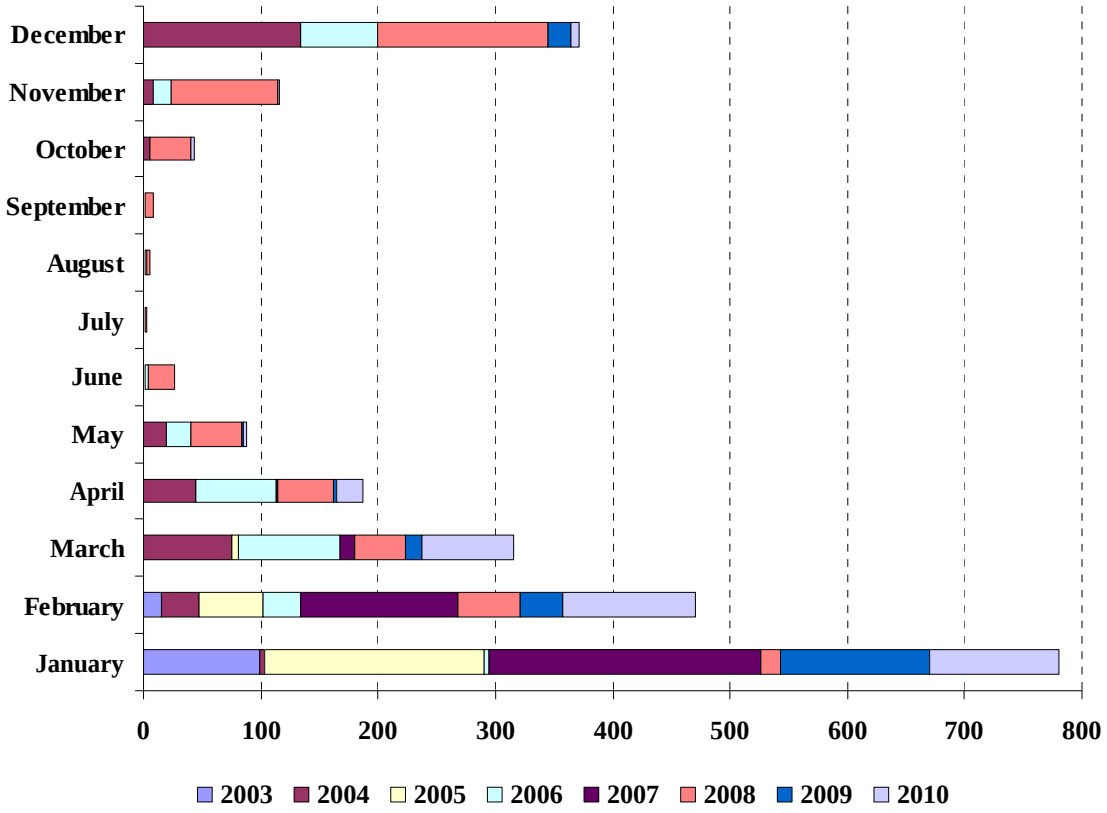


Figure 3.9: Infected children by month & year in which RSV was detected

The research revealed some pattern in the RSV infection monthly distribution over the period of study. RSV infection is highly clustered around January and March of every year. This is shown in Figure 3.10.

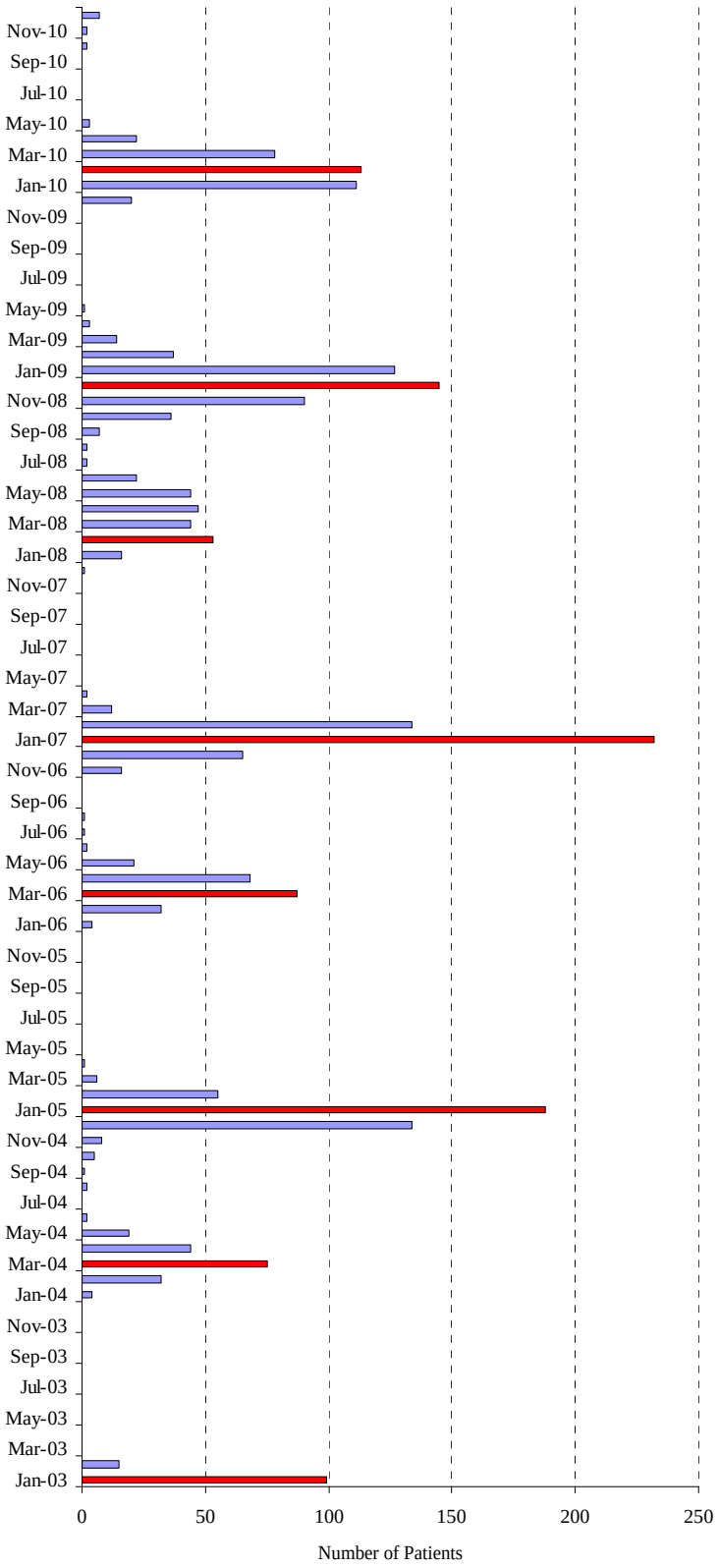


Figure 3.10: RSV infection – Monthly distribution

3.3.5 RSV Distribution by Community

Building on the fact that the RSV infection is a community phenomenon, not a national one (Gottschalk, et al., 1996; Peret, et al., 2000; Zlateva, et al., 2007), a distribution of those infected is prepared by city as shown in Table 3.10 and is depicted in Figure 3.11. Three of the infected patients were unknown as of their place of residence.

District	Number of patients	Percentage
Bethlehem	1,468	60.76%
Hebron	872	36.09%
Israel	3	0.12%
Jericho	10	0.41%
Jerusalem	55	2.28%
Nablus	1	0.04%
Ramallah	4	0.17%
Unknown	3	0.12%
Grand Total	2,416	100.00%

Table 3.10: Distribution of infection by District

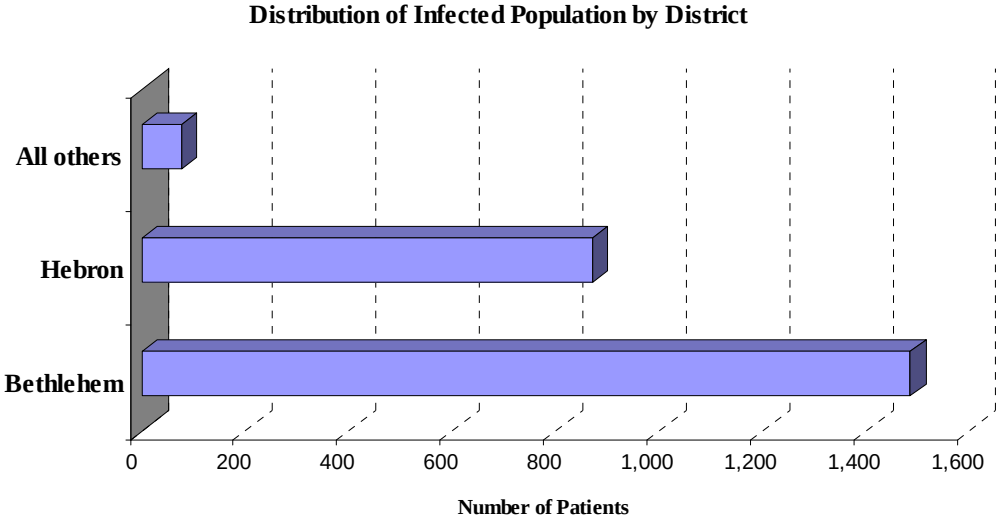


Figure 3.11: Distribution of infected by District

Within each district, the distribution of RSV infection is determined by village and/or refugee camp. The distribution within the two major districts is shown in Table 3.11

below followed by two graphs (Figure 3.12 and Figure 3.13). This makes up almost 97% of the cases analyzed.

Bethlehem							
Village	#	Village	#	Village	#	Village	#
Abu Njeim	8	A'sion	4	Deheisheh camp	93	Nahhalin	71
Aida camp	37	Azzeh camp	21	E'beidiyeh	96	Rakhama	9
Al Doha	70	Battir	15	Harmala	8	Ta'amreh	37
Al Khader	96	Beit Fajjar	84	Husan	46	Tqou'	75
Al Ma'sara	11	Beit Jala	90	Jab'a	6	Um Salamona	21
AL Rashaideh	21	Beit Sahour	42	Joret Al Shama'a	13	Urtas	40
Al Shawawreh	20	Bethlehem	246	Kisan	3	Wad El Nees	19
Al Walajeh	19	Creche (Unknown)	1	Marah Im'alla	10	Wad Foukin	13
A'sakreh	8	Dar Salah	30	Marah Rabah	24	Wad Rahhal	23
						Za'tara	38
						Total	**1,468**
Hebron							
Village	#	Village	#	Village	#	Village	#
Al Rihiyeh	4	Beit Ummar	125	Idna	8	Samou'	10
A'roub c.	107	Deir Samet (Doura)	7	Karma Doura	2	Shyoukh	18
Bani Neim	25	Doura	53	Kharas	26	Sourif	69
Beit Awwa	12	Fawwar c.	22	Nouba	20	Taffouh	9
Beit Kahel	9	Halhoul	60	Ramadin El Thahriyeh	2	Tarqoumia	13
Beit Ula	17	Hebron	181	Sa'ir	29	Thahriyeh	18
						Yatta	26
						Total	**872**

Table 3.11: Distribution of infection within major Districts

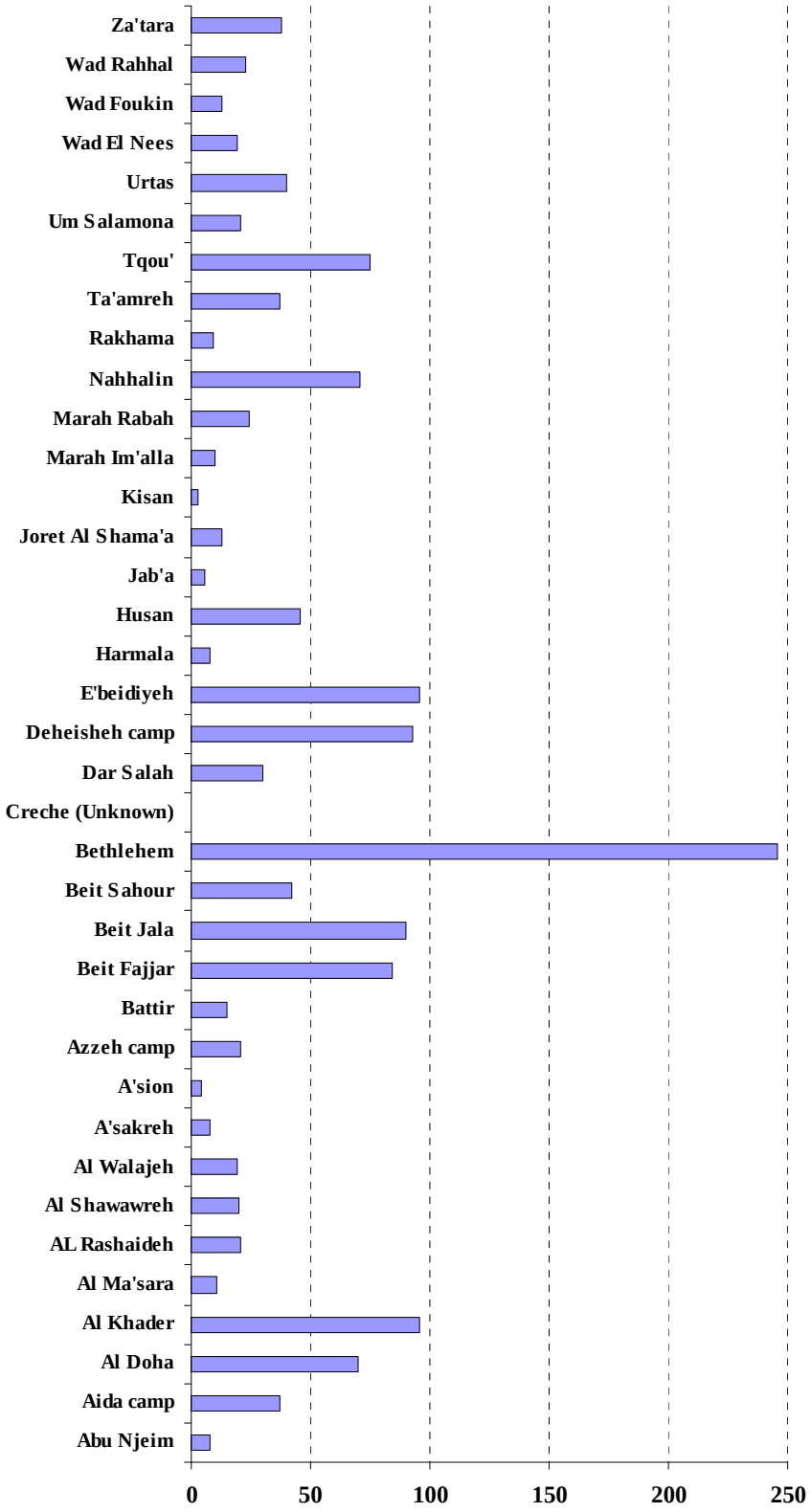


Figure 3.12: Distribution of positive RSV samples within the district of Bethlehem

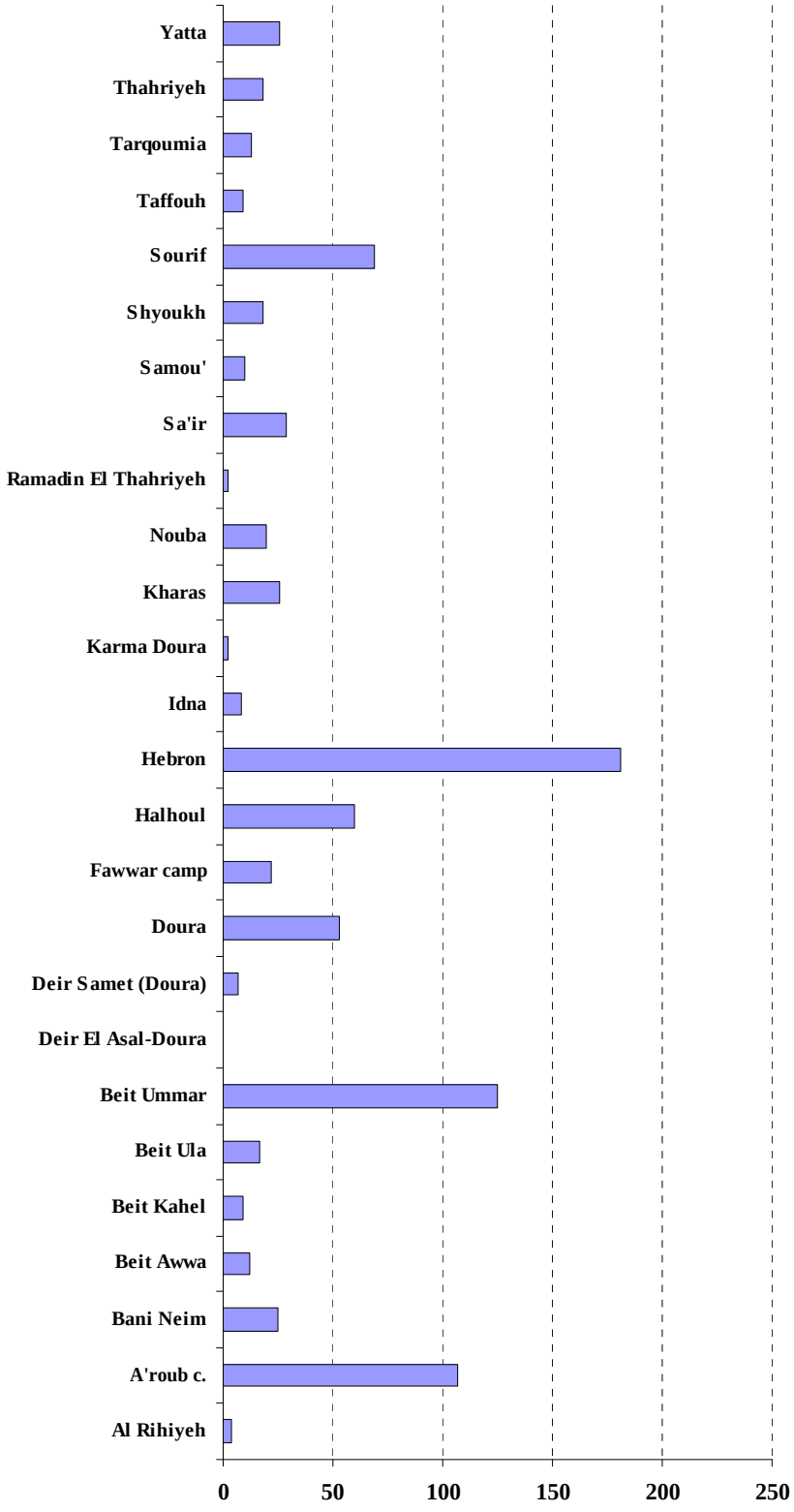


Figure 3.13: Distribution of positive RSV samples within the district of Hebron

3.4 RSV Groups

In an attempt to determine the group of RSV, available samples from the period of 2006 to 2009 were analyzed using typing by RT-PCR method. This analysis was conducted on 1,388 samples out of the 5,820 available samples. This sample was used because the other 4,432 available samples were either tested negative for RSV infection (4,416 samples) or not enough cells were available in a sample to conduct the proper testing (16 Samples).

3.4.1 RSV Groups by Age

The samples that were tested using the RT-PCR method for group type are distributed by age group as in Table 3.12 and are depicted in Figure 3.14 that follows as well.

Age group	RSV type A cases and (%)	RSV type A&B cases and (%)	RSV type B cases and (%)	Total cases and (%)
Up to 6 months	647 (71%)	9 (1%)	257 (28%)	913 (100%)
Up to 12 months	184 (67%)	4 (1%)	87 (32%)	275 (100%)
Up to 18 months	64 (71%)	1 (1%)	25 (28%)	90 (100%)
Up to 24 months	23 (79%)	1 (4%)	5 (17%)	29 (100%)
Up to 30 months	9 (53%)		8 (47%)	17 (100%)
Up to 36 months	10 (91%)		1 (9%)	11 (100%)
Over 36 months	5 (38%)	1 (8%)	7 (54%)	13 (100%)
Grand Total	**942 (70%)**	**16 (1%)**	**390 (29%)**	**1348** (100%)

Table 3.12: RSV group predominance by age group (2006 – 2009)

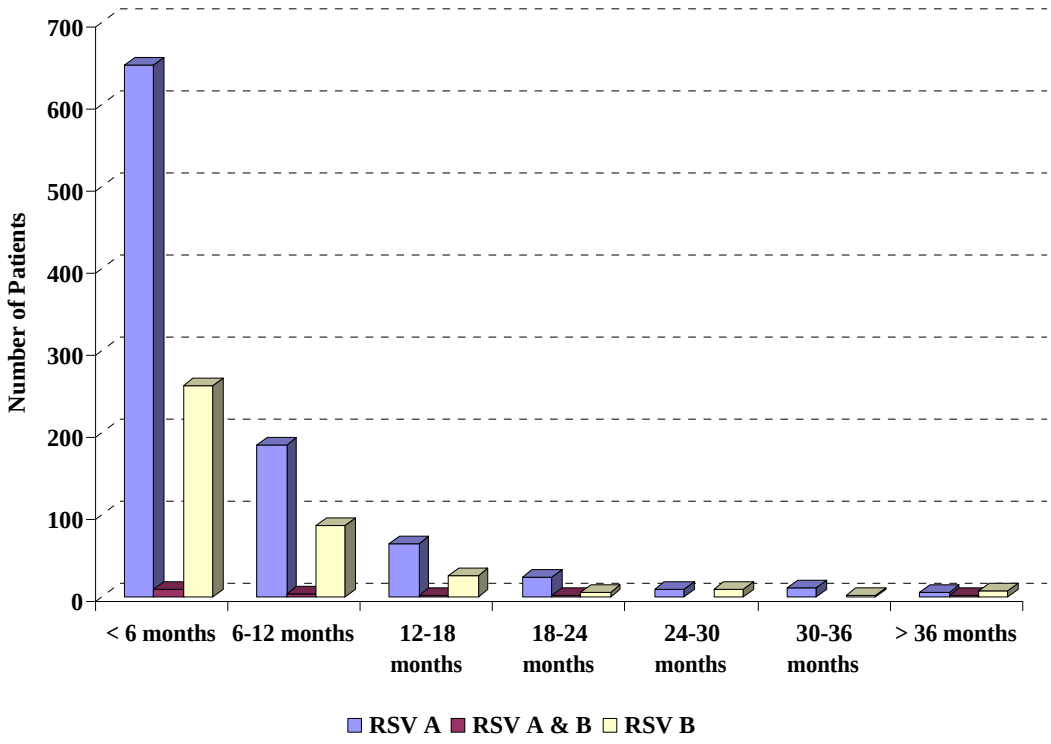


Figure 3.14: RSV group predominance by age group (2006 – 2009)

From Table 3.12 above, the following can be summarized:

1. Group A prevailed over group B.
2. Both strains, A and B, circulate at the same epidemic season.
3. The peak infection appears to be the first few months of the infants life.
4. Some patients develop both infections (A and B) at the same time (see Figure 3.15).

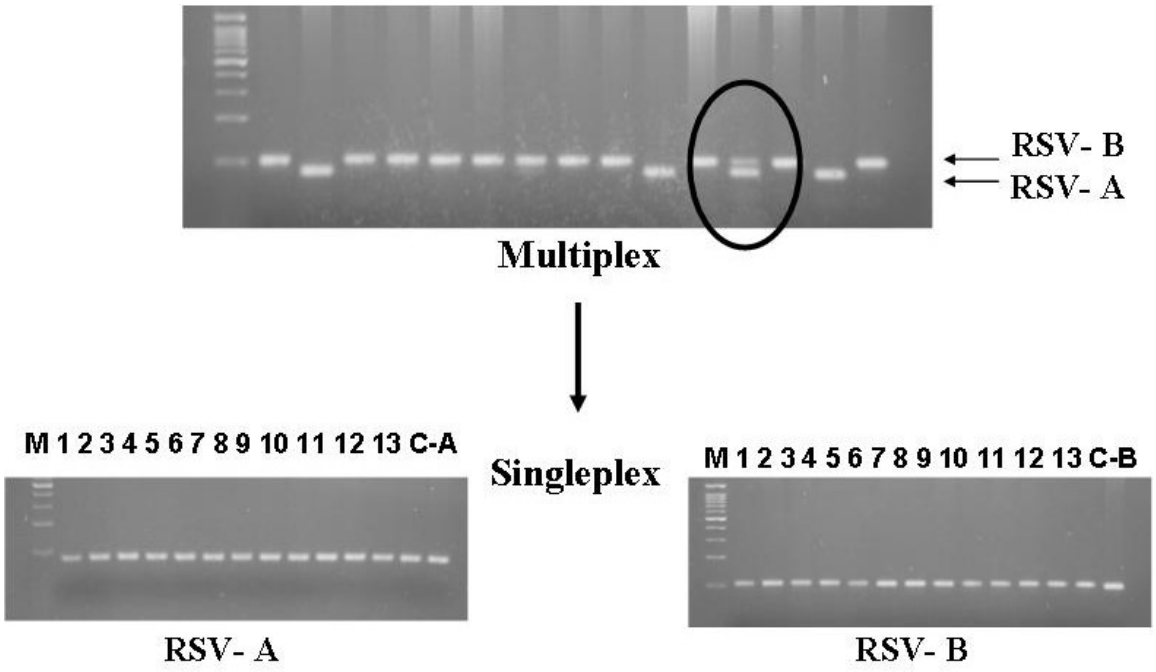


Figure 3.15: Example of Dual infection with RSV A and B in the samples tested

3.4.2 The Characteristics of the Sample Used for RSV Group Testing

The distribution of the available samples and the samples that were used for further testing for RSV group type is shown in Table 3.13 below.

Year	Number of Negative RSV Samples (%)	No cells for proper testing	Number of Positive RSV Samples (%)	Total number of samples (%)
2006	626 (14%)	2	297 (21%)	925 (16%)
2007	952 (22%)	6	381 (27%)	1,339 (23%)
2008	1,249 (28%)	6	508 (37%)	1,763 (30%)
2009	1,589 (36%)	2	202 (15%)	1,793 (31%)
Total	**4,416 (100%)**	**16**	**1,388 (100%)**	**5,820 (100%)**

Table 3.13: Distribution of RSV Results (2006 – 2009)

Out of these 1,388 samples, 856 (62%) were from male patients and 532 (38%) from female patients. Again, this is in line with the results of prior research as cited in the literature chapter.

As for the season of infection during each year, the sample is distributed as in Table 3.14 below. These results are depicted very clearly in Figure 3.16 below showing the distribution of infection around winter and autumn of the year. Over 94.7% of those infected with RSV had the infection in the months between November and May.

Month	Number of positive RSV samples	% per month
January	379	27.31%
February	256	18.44%
March	157	11.31%
April	120	8.65%
May	66	4.76%
June	24	1.73%
July	3	0.22%
August	3	0.22%
September	7	0.50%
October	36	2.59%
November	106	7.64%
December	231	16.64%
Total	**1,388**	**100.00%**

Table 3.14: Distribution of RSV Results per month (2006 – 2009)

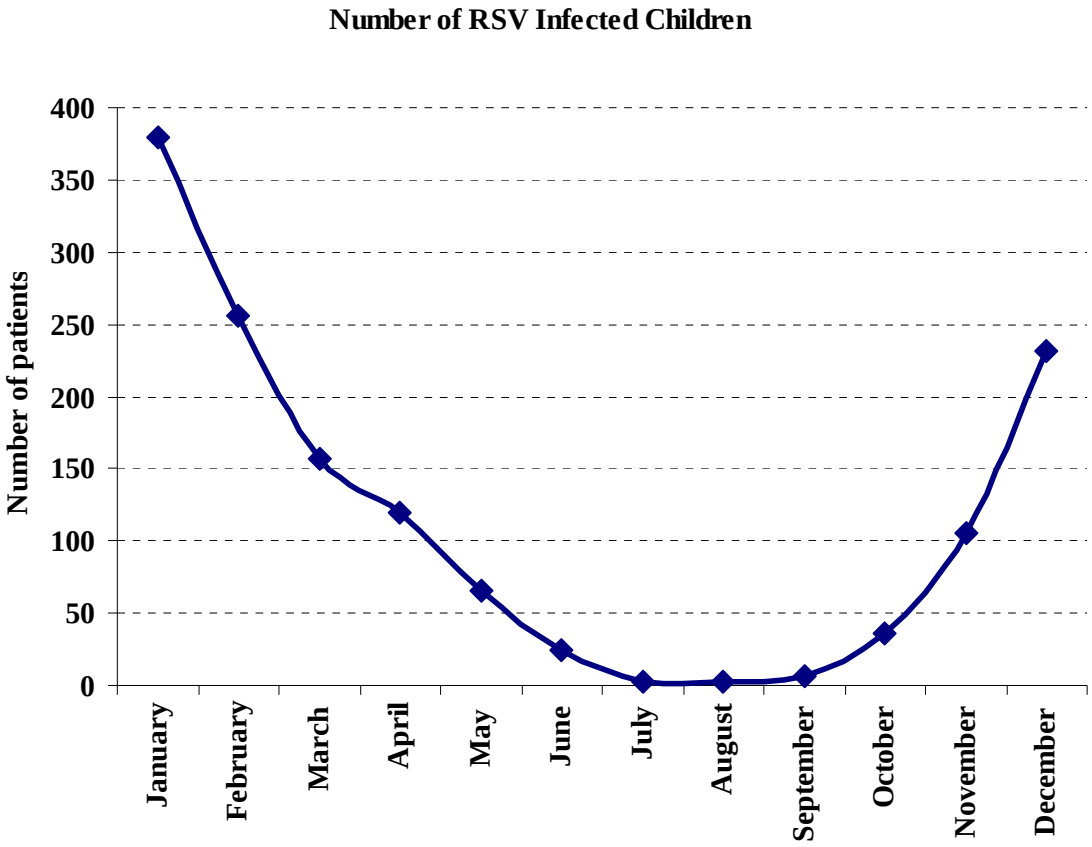


Figure 3.16: Number of infected children distributed by month of the year

Almost 95% of those infected with RSV between 2006 and 2009, that were further tested for group types, are younger than 1.5 years. This is shown in Table 3.15 below.

Age group	Number of Patients	% of total infected
Up to 6 months	935	67.36%
Up to 12 months	284	20.46%
Up to 18 months	92	6.63%
Up to 24 months	31	2.23%
Up to 30 months	21	1.51%
Up to 36 months	12	0.86%
Over 36 months	13	0.94%
Total	**1,388**	**100.00%**

Table 3.15: Distribution of RSV Results per patients' age (2006 – 2009)

Breaking down the first age group, table (Table 3.16) shows the distribution of infection among children whose age is up to six months:

Age group	Number of Patients	% of total infected
< 1 month	160	11.53%
Up to 2 months	256	18.44%
Up to 3 months	194	13.98%
Up to 4 months	141	10.16%
Up to 5 months	100	7.20%
Up to 6 months	84	6.05%
Over 6 months	453	32.64%
Total	**1,388**	**100.00%**

Table 3.16: RSV Results per age (2006 – 2009) focusing on the 1st six months of life

The sample is distributed geographically by district as shown in Table 3.17 below.

District	Number of Positive RSV	% of Positive RSV
Bethlehem	797	57.42%
Hebron	539	38.83%
Israel	2	0.14%
Jericho	8	0.58%
Jerusalem	35	2.52%
Nablus	1	0.07%
Ramallah	3	0.22%
Unknown	3	0.22%
Total	1,388	100.00%

Table 3.17: Distribution of RSV Results By district (2006 – 2009)

3.4.3 RSV Groups within the Sample Population

RSV is classified into antigenic subgroups A and B based on antigenic and genetic variability (Peret, et al., 1998; Reiche and Schweiger, 2009; Zhang, et al., 2010). The sample of 1,388 patients was used for further analysis to determine the type of RSV infection in the southern part of Palestine. Of the 1388 samples available, 1,348 (97.1%) samples were processed for the determination of RSV group type using RT-PCR method. The other 40 samples are classified as follows:

1- Three samples that were tested positive for RSV were not further analyzed due to shortage in RNA needed for that type of test.
2- The other 37 samples were tested positive for RSV but were not typable with RT-PCR method.

RT-PCR testing revealed the prevalence of group A among those tested with positive RSV. Group A infection was found in almost 70% (942 out of 1,348 samples) of the patients, while group B was found in almost 29% (390 out of 1,348 samples). The rest of the patients (1% or 16 out of 1,348 patients) were dually infected by both groups A and B RSV at the same time.

Although group A prevailed in the sample over group B, the analysis shows that there are certain variability. In some seasons, strain A is predominant while things could shift in other seasons as shown in Table 3.18 and Figure 3.17 below. It is also important to notice that both strains often circulate during the same season. This fact was also documented by several researchers in different parts of the world (Mufson, et al., 1985; Peret, et al., 1998; Peret, et al., 2000).

Year	A	A + B	B	Total	Predominance
2006	280		15	295	A (95%)
2007	347		6	353	A (98%)
2008	231	3	267	501	B (53%)
2009	84	13	102	199	B (51%)
Total	**942**	**16**	**390**	**1,348**	**A (70%)**

Table 3.18: RSV group predominance (2006 – 2009)

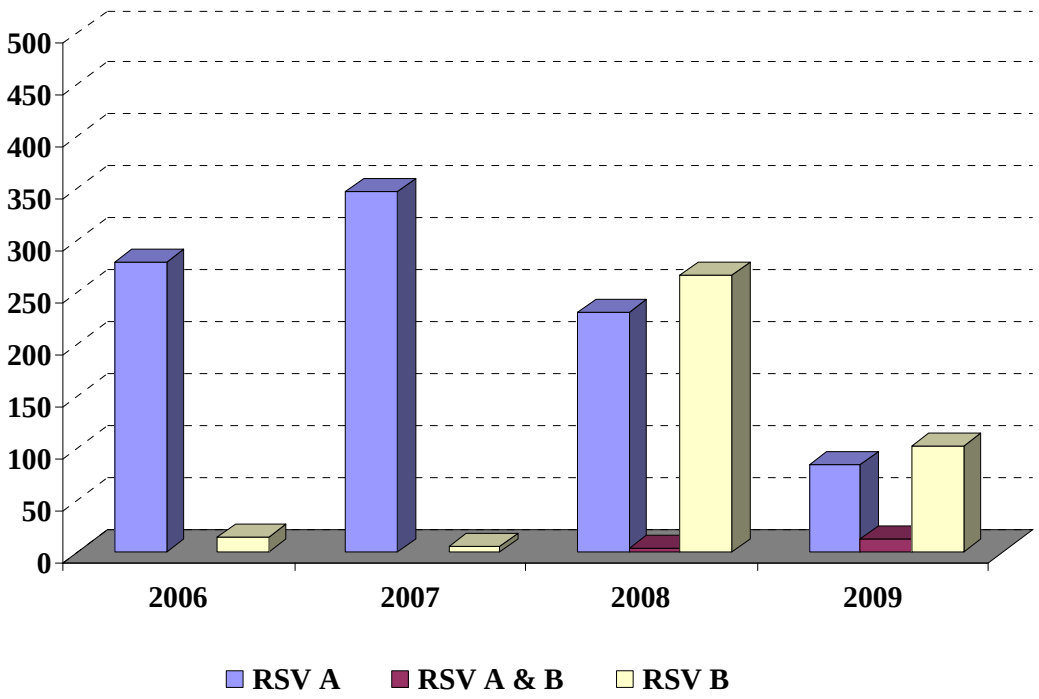


Figure 3.17: RSV group predominance in number of infected children (2006 – 2009)

3.4.4 RSV Groups Distribution by District

As previously mentioned, the samples were collected at the CBH in Bethlehem and most of the samples tested come from patients residing in the southern part of Palestine. This resulted in focusing the analysis on samples taken from the Bethlehem and the Hebron districts. Some other samples came from other districts, but the researcher considers those samples as not representative of the other districts. Distribution of the 1,348 samples by district is shown in Table 3.19. The same results are also depicted in Figure 3.18 below. The caption "others" in the table and the figure that follows refer to samples taken from Jerusalem (35 samples), Jericho (8 samples), Nablus (1), Ramallah (3) and (3) samples were received by the hospital for testing without determining the place of residence.

District	RSV group A	RSV A and B	RSV group B	Total
Bethlehem	563 (73%)	10	203 (26%)	776
Hebron	345 (66%)	5	172 (33%)	522
Others	34 (68%)	1	15 (30%)	50
Total	**942 (70%)**	**16**	**390 (29%)**	**1,348**

Table 3.19: RSV group predominance by district in number of cases (2006 – 2009)

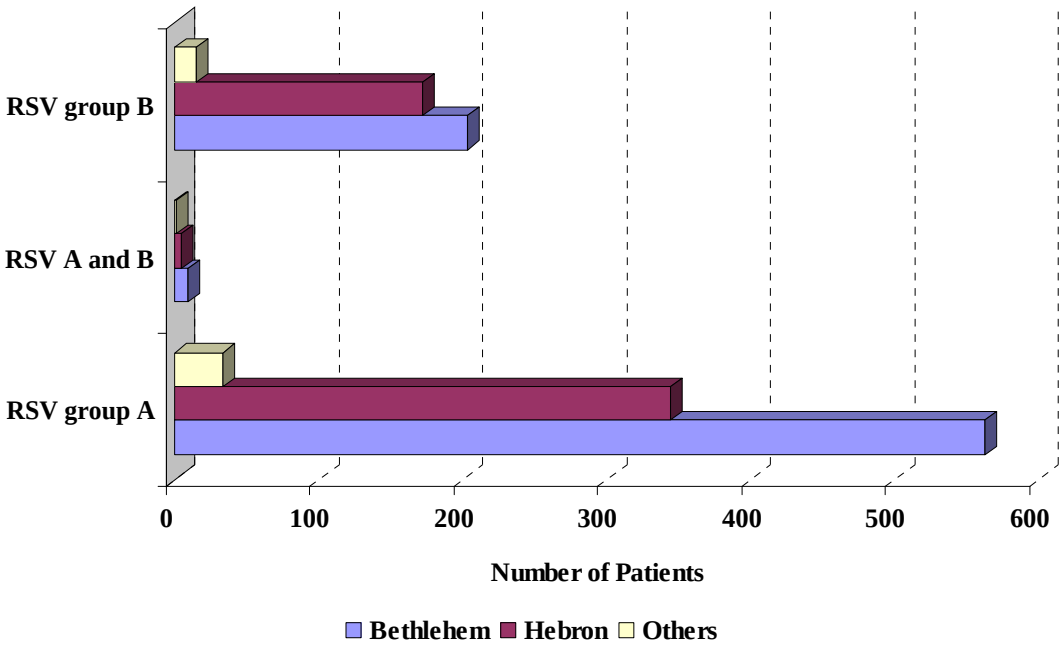


Figure 3.18: Predominance of RSV groups by district (2006 – 2009)

As for the distribution of the cases and RSV group infection, the data was distributed as in Table 3.20 and Table 3.21 within both the Bethlehem and the Hebron districts respectively.

Bethlehem District				
Village or Camp	**Group A cases (%)**	**Group B cases (%)**	**Dual infection A&B cases (%)**	**Total Cases**
Abu Njeim	3 (100%)	(0%)	(0%)	3
Aida camp	11 (69%)	5 (31%)	(0%)	16
Al Doha	31 (76%)	10 (24%)	(0%)	41
Al Khader	48 (89%)	6 (11%)	(0%)	54
Al Ma'sara	3 (50%)	3 (50%)	(0%)	6
AL Rashaideh	14 (93%)	1 (7%)	(0%)	15
Al Shawawreh	6 (50%)	6 (50%)	(0%)	12
Al Walajeh	8 (100%)	(0%)	(0%)	8
A'sakreh	2 (67%)	1 (33%)	(0%)	3
A'sion	(0%)	1 (100%)	(0%)	1
Azzeh camp	9 (69%)	3 (23%)	1 (8%)	13
Battir	3 (75%)	1 (25%)	(0%)	4
Beit Fajjar	38 (75%)	13 (25%)	(0%)	51
Beit Jala	39 (83%)	8 (17%)	(0%)	47
Beit Sahour	15 (71%)	6 (29%)	(0%)	21
Bethlehem	91 (67%)	41 (30%)	3 (2%)	135
Dar Salah	8 (53%)	7 (47%)	(0%)	15
Deheisheh camp	31 (61%)	18 (35%)	2 (4%)	51
E'beidiyeh	46 (85%)	6 (11%)	2 (4%)	54
Husan	21 (70%)	9 (30%)	(0%)	30
Jab'a	2 (67%)	1 (33%)	(0%)	3
Joret Al Shama'a	5 (71%)	2 (29%)	(0%)	7
Kisan	2 (67%)	1 (33%)	(0%)	3
Marah Im'alla	3 (60%)	2 (40%)	(0%)	5
Marah Rabah	12 (67%)	6 (33%)	(0%)	18
Nahhalin	18 (56%)	13 (41%)	1 (3%)	32
Rakhama	1 (17%)	5 (83%)	(0%)	6
Ta'amreh	11 (85%)	2 (15%)	(0%)	13
Tqou'	30 (70%)	13 (30%)	(0%)	43
Um Salamona	7 (100%)	(0%)	(0%)	7
Urtas	10 (59%)	7 (41%)	(0%)	17
Wad El Nees	9 (82%)	2 (18%)	(0%)	11
Wad Foukin	4 (67%)	2 (33%)	(0%)	6
Wad Rahhal	10 (100%)	(0%)	(0%)	10
Za'tara	12 (80%)	2 (13%)	1 (7%)	15
Total	563 (73%)	203 (26%)	10 (1%)	776

Table 3.20: RSV group predominance by Locality – Bethlehem District (06 –09)

Hebron District				
Village or Camp	**Group A cases (%)**	**Group B cases (%)**	**Dual infection A&B cases (%)**	**Total cases**
Al Rihiyeh	1 (33%)	2 (67%)	(0%)	3
A'roub camp	36 (69%)	16 (31%)	(0%)	52
Bani Neim	15 (88%)	2 (12%)	(0%)	17
Beit Awwa	6 (75%)	2 (25%)	(0%)	8
Beit Kahel	2 (50%)	2 (50%)	(0%)	4
Beit Ula	5 (71%)	2 (29%)	(0%)	7
Beit Ummar	45 (58%)	31 (40%)	1 (1%)	77
Deir Samet (Doura)	3 (100%)	(0%)	(0%)	3
Doura	21 (68%)	9 (29%)	1 (3%)	31
Fawwar c.	9 (75%)	3 (25%)	(0%)	12
Halhoul	20 (51%)	19 (49%)	(0%)	39
Hebron	74 (64%)	41 (35%)	1 (1%)	116
Idna	4 (80%)	1 (20%)	(0%)	5
Karma Doura	1 (50%)	1 (50%)	(0%)	2
Kharas	13 (76%)	4 (24%)	(0%)	17
Nouba	7 (70%)	3 (30%)	(0%)	10
Ramadin El Thahriyeh	1 (50%)	1 (50%)	(0%)	2
Sa'ir	13 (65%)	7 (35%)	(0%)	20
Samou'	8 (89%)	1 (11%)	(0%)	9
Shyoukh	10 (71%)	4 (29%)	(0%)	14
Sourif	21 (58%)	15 (42%)	(0%)	36
Taffouh	5 (100%)	(0%)	(0%)	5
Tarqoumia	6 (67%)	2 (22%)	1 (11%)	9
Thahriyeh	9 (100%)	(0%)	(0%)	9
Yatta	10 (67%)	4 (27%)	1 (7%)	15
Total	**345 (66%)**	**172 (33%)**	**5 (1%)**	**522**

Table 3.21: RSV group predominance by Locality – Hebron District (06 – 09)

3.4.5 Phylogenetic Analysis of RSV A and B

Of the 1,388 RSV positive samples, 54 (4%) RSV RNA samples were amplified by either of the RSV-A (G267 and F164) or RSV-B (BGF and BGR) genotyping primers (Table 3.22). The PCR products were purified and sent to Bethlehem University for sequence analysis of the G gene. Of the 54 samples analyzed, 25 (46%) belonged to the RSV-A group while 29 (54%) were from the RSV-B group.

			Subgroups			
Group	**Year**	**Total**	**GA2**	**Pal/GA8**	**GA5**	**Pal/GA9**
A	2006	11	3	2	6	
	2007	9		1	6	2
	2008	3			1	2
	2009	2				2
	Number	**25**	**3**	**3**	**13**	**6**
	%	**100%**	**12%**	**12%**	**52%**	**24%**
			Subgroups			
	Year	**Total**	**BA9/GB13**		**Pal/GB14**	
B	2006	10	5		5	
	2007	4	2		2	
	2008	9	8		1	
	2009	6	6			
	Number	**29**	**21**		**8**	
	%	100%	72%		28%	

Table 3.22: Sequenced samples (Groups A and B)

Sequence analysis of 521 base pair of RSV-A G gene (see Figure 3.19) which included parts of the two variable regions at the 3'-end of the G gene showed that 52% (N = 13) clustered in the GA5 group and 12% (N = 3) clustered in the GA2 group (Figure 3.23). Interestingly, 37% (N = 10) did not belong to any of the known types and the bootstrap values were greater than 900 indicating that these are true braches representing possibly new genotypes. Stratifying these 10 samples with unknown RSV-A genotype revealed that 12% (N = 3) belonged to the newly proposed genotype Pal-GA8, and 24% (N = 6) belonged to the newly

proposed group Pal-GA9. To confirm these results, sequence alignment of the 261 base pairs from the second variable region at the 3'-end of the G gene from amino acid 216-298 (see

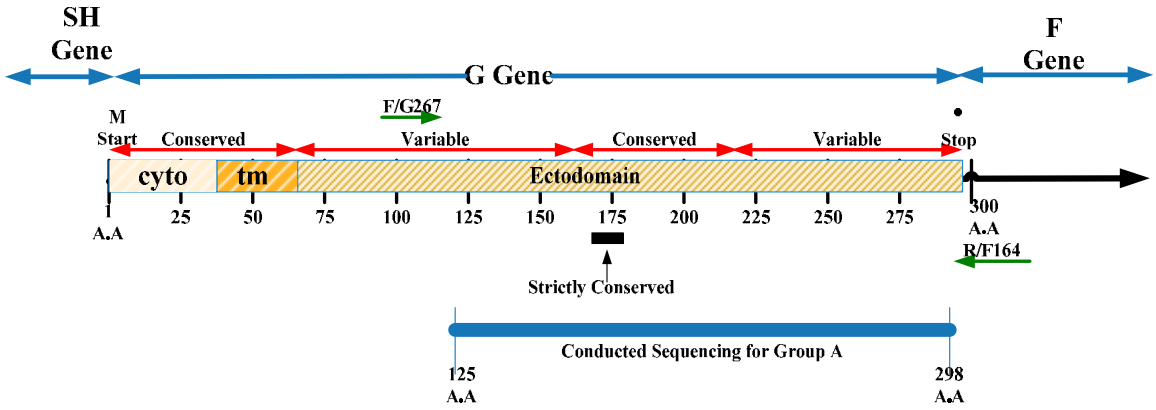


Figure 3.20) confirmed the description of the three new GA genotypes since the bootstrap values were greater than 900 indicating that they are true braches. (Figure 3.24).

Figure 3.19: RSV- Genotype A

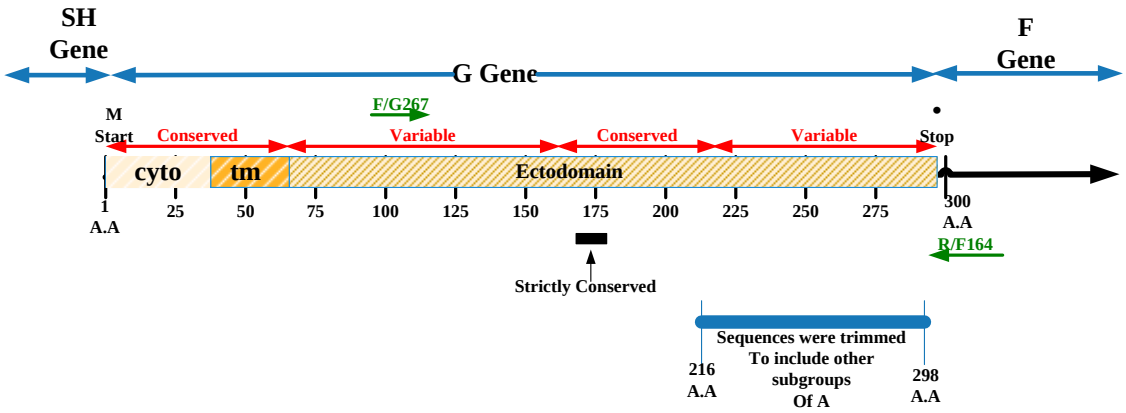


Figure 3.20: RSV- A G Gene

Similarly, sequence analysis of 515 base pairs of RSV-B G gene (see Figure 3.21 and Figure 3.22) which also included parts of the two variable regions at the 3'-end of the G gene showed that 72% (N = 21) belonged to the group GB13. Indeed, sequence alignment of three RSV-B sequences representing this group with all the Buenos Aires (BA) genotypes (BA1-BA13) , confirmed that they belong to the group GB13 and in

particular to the group Buenos Aires 9 (Figure 3.25), while 28% (N = 8) do not cluster with any reported groups. Indeed, we propose that this group be assigned to the newly proposed group Pal-GB14 (Figure 3.26).

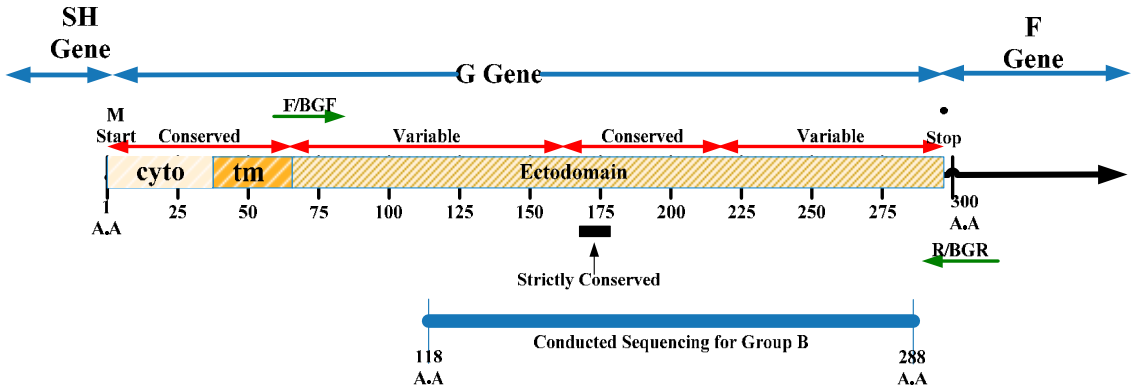


Figure 3.21: RSV- Genotype B

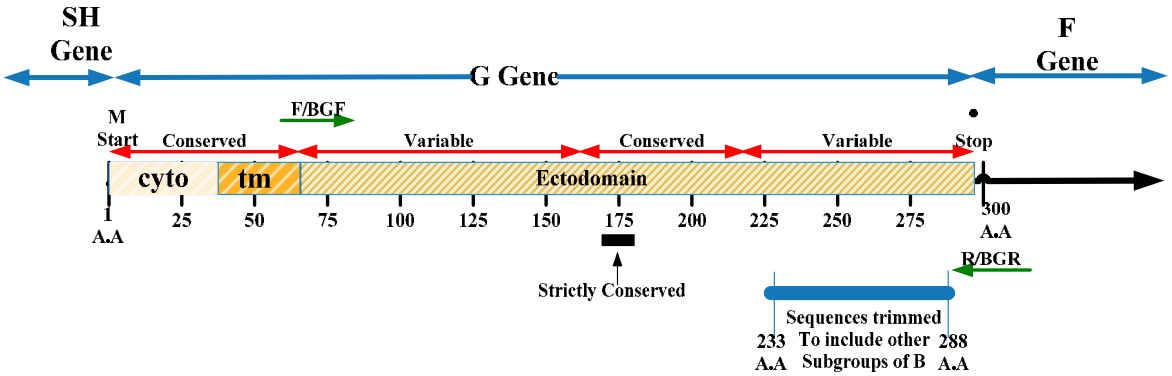


Figure 3.22: RSV- B G Gene

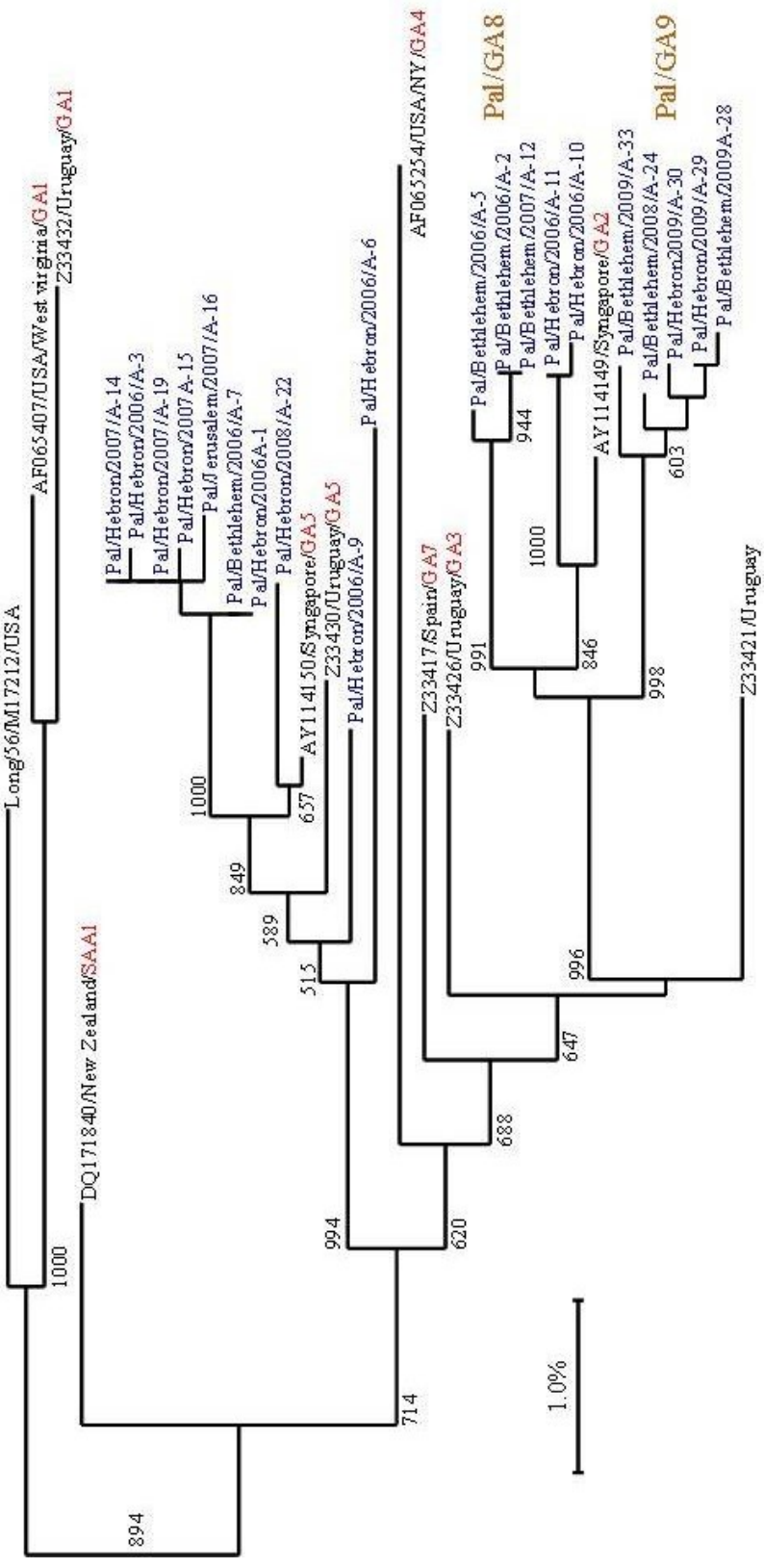


Figure 3.23: Phylogenetic Analysis of RSV-A (521bp)

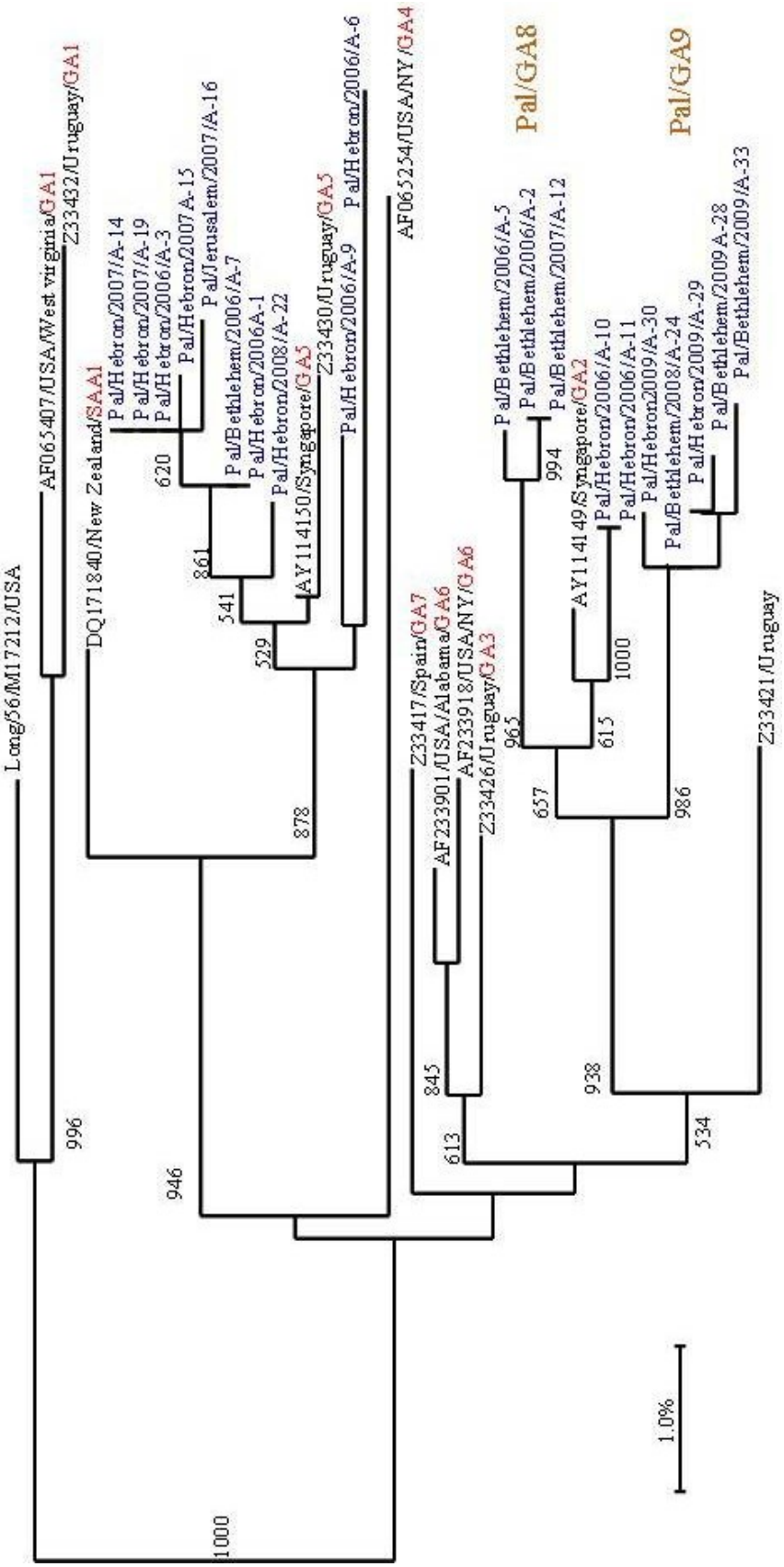


Figure 3.24: Phylogenetic Analysis of RSV-A (261bp)

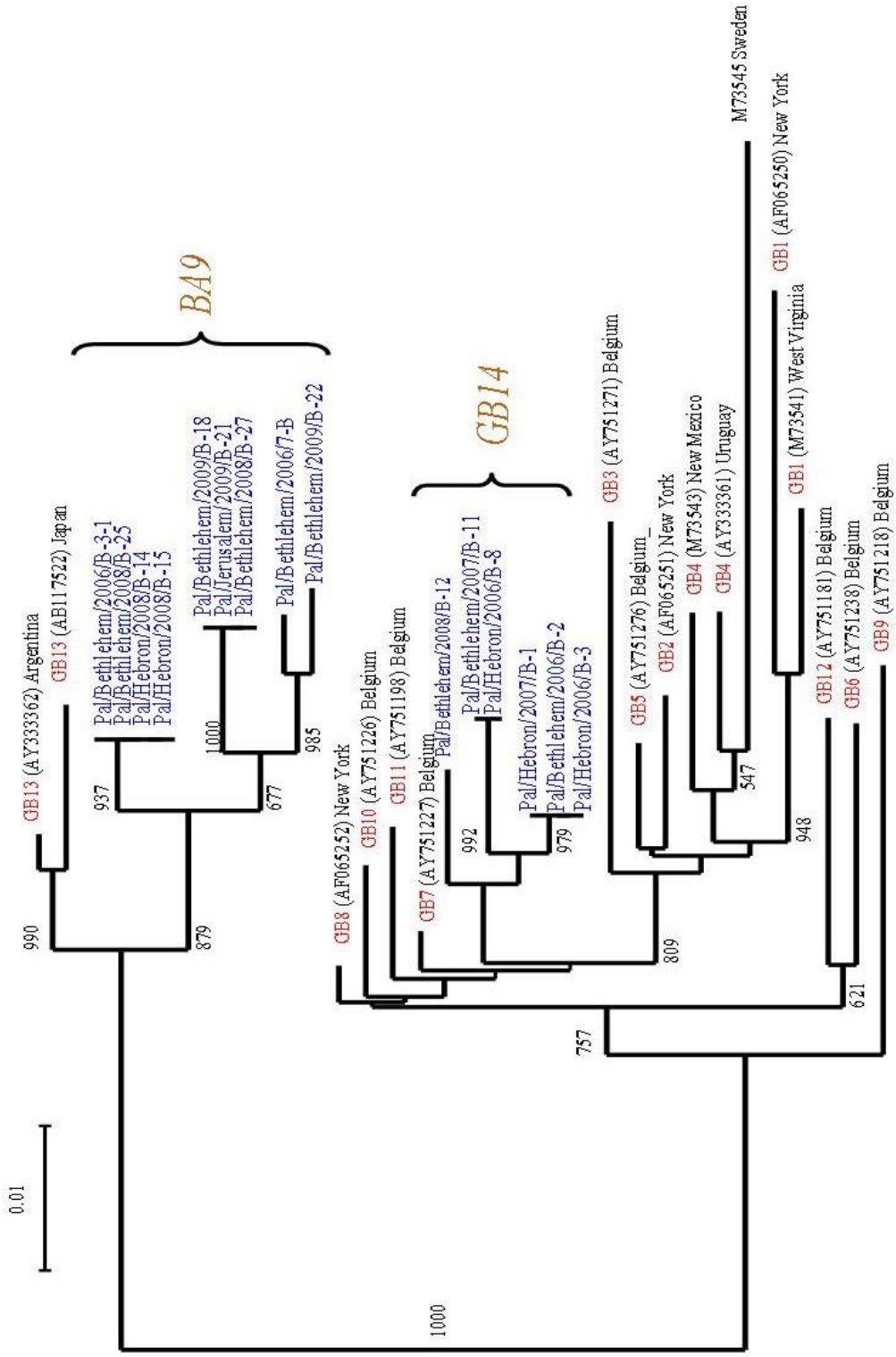


Figure 3.25: Phylogenetic Analysis of RSV-B (515bp)

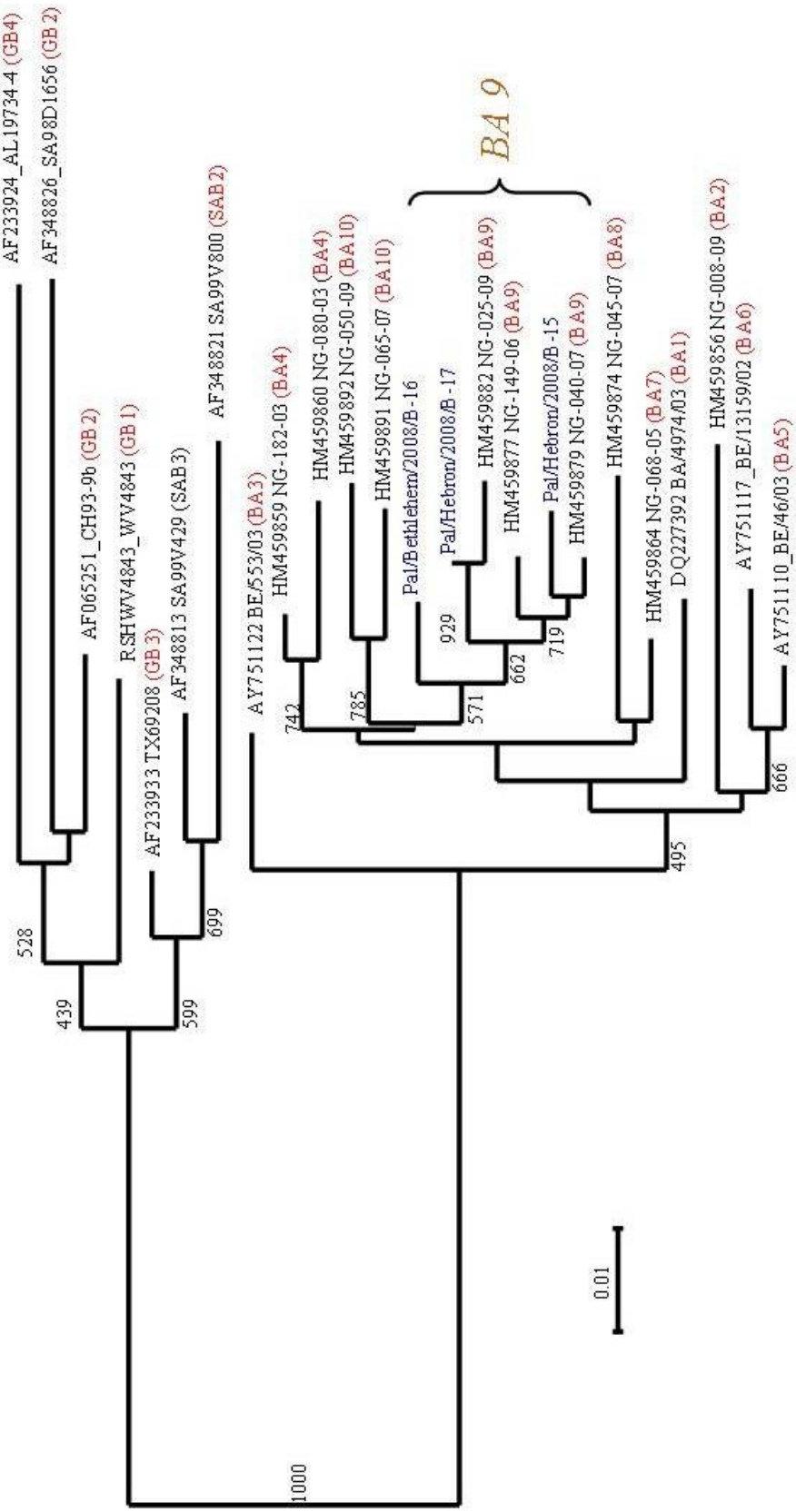


Figure 3.26: Phylogenetic Analysis of RSV-B (260bp)

CHAPTER 4 – Discussion and Analysis

4 Introduction

This chapter looks into the results of the research in depth and compares the findings with those reported by several researchers cited in the literature review chapter of this thesis. Although several results confirm findings of prior researchers, yet this research is considered a breakthrough in the Palestinian territories because of the geographic area that was not researched before for RSV infection. This research considered several factors for analysis as described in the following subsections. In the initial analysis, all samples within the population were examined using monoclonal antibodies (MAbs) directed against the proteins of RSV using immunofluorescence assays. This was used to determine whether a patient has a viral infection or not.

4.1 RSV Infection and Gender

The researcher evaluated a sample of specimens collected from children admitted to Caritas Baby Hospital in Bethlehem between 2003 and 2010. The ratio of male to female in this population was approximately 1.6:1. The majority of those infected with RSV were proved in the current research to be male patients (Table 3.8). This is in line with the literature that mentioned the gender of the patient, specifically being a male, is a risk factor. Several studies reviewed in the literature chapter listed “being male” as a risk factor for RSV infection. Oliveira et. al. (2008) noticed that male children are more susceptible to severe disease infection than females.

4.2 RSV Infection and Seasonality

Most studies proved that the epidemic season is mainly in late fall, winter, and spring in temperate zones in the world (Cane and Pringle, 1995; Cane, 2001; Dapat, et al., 2010; Gottschalk, et al., 1996; McNamara and Smyth, 2002; Michael P. Frogel, et al., 2010; Peret, et al., 2000; Shobugawa, et al., 2009; Zlateva, et al., 2005). The study of (Shobugawa, et al., 2009) showed that the disease was most prevalent and clearly active during autumn and early winter (November through March). As expected, the distribution of the population of children admitted to Caritas Baby Hospital with suspected RSV infection increased around winter time and was less prevalent in summer. Table 3.4 as well as Figure 3.6 show this fact. This fact remained constant over the seven years (2003 to 2010) covered by this study as shown in Figure 3.6. It was also noticed that children who were diagnosed with positive RSV infection might have also developed dual infection with hMPV infections (Table 3.7). This was evident in the literature as well. Semple et. al. (2005) concluded that there is high frequency of dual infection by hMPV and RSV in infants.

Over the period from which the data was collected (2003-2010), Table 3.9 as well as Figure 3.9 prove the same results as reported by various researchers in the world (See for example: Cane and Pringle, 1995; Cane, 2001; Dapat, et al., 2010; Gottschalk, et al., 1996; McNamara and Smyth, 2002; Michael P. Frogel, et al., 2010; Peret, et al., 2000; Shobugawa, et al., 2009; Zlateva, et al., 2005). Over 96% of the infected patients were reported in the period between November and May.

4.3 RSV Infection, not a National Phenomenon

As expected, differences in epidemics are in existence among communities. Several prior studies proved that this infection has community characteristics but not necessarily national characteristics. Examples of these studies include a research conducted in North America (Peret, et al., 2000) that discussed patterns of circulating strains in communities, suggesting that RSV outbreaks are a community phenomenon, not a national one. Other studies found out that several genotypes were circulating during a period, as well as a variety of distinct sequences within each group of RSV that are different from one community to the other (such as: Gottschalk, et al., 1996; Zlateva, et al., 2007). The population of suspected RSV infected patients is distributed based on communities within the southern part of Palestine as shown in Figure 3.7. In this research, analysis included the detection of the distribution within each of the major districts in the southern part of Palestine. This analysis showed a different pattern in each of the communities covered as shown in Table 3.11.

In further analyzing the RSV infected specimens to determine the group of RSV in each district, both of the major districts tested showed that RSV A is more prevalent compared to RSV B. In both areas, RSV A infection was close to 70% while RSV B made up the remainder of the cases (Table 3.19). But within each of the districts, these percentages differ which emphasizes the fact that RSV infection is a phenomenon of the locality not a national one (see Table 3.20 and Table 3.21 for data).

4.4 Age as a Risk Factor for RSV Infection

Most of the prior research conducted around the world, and reviewed for the purpose of this study, revealed that RSV infection is more serious in early months of the life of infants. The younger the infant is, the highest the probability of his/her infection. The majority of infants experience infection in the first year of life and virtually all are infected by 2 years of age (Shobugawa, et al., 2009). This fact was also reiterated in prior studies such as the one of Barends, et al. (2004) who were able to prove that almost all children become infected with RSV during their first or second year of life and that the incidence of RSV bronchiolitis reaches a maximum at the age of 2 months. McNamara and Smyth (2002) reported that by the age of 18 months, 87% of children have antibodies to RSV and that by the age of 3 years, virtually all children are infected. These children, when infected, suffer from upper respiratory tract symptoms, which usually is followed by lower respiratory tract symptoms, such as rhinitis (stuffy nose), cough and coryza (runny nose). Various studies referred to in the literature chapter reached the same results in various parts of the world. Sidwell and Barnard (2006), Shobugawa et. al. (2009), Reiche and Schweiger (2009), as well as many others reached the conclusion that RSV infection is most prevalent in the first year of an infant's life.

In this research close to 90% of children who were admitted to hospital for suspected RSV infection are of a maximum of 2 years of age.

Age is a risk factor in Palestine as well. Table 3.6 shows that the almost 99% of those tested are infected in the first three years of their life and almost 88% of those tested are infected in the first year of their life.

4.5 RSV Groups in Palestine

In testing to determine the group of RSV, a smaller sample was considered for the analysis. The sample included 1,388 specimens that were tested with positive RSV (Table 3.13) and the samples were taken from the years 2006 to 2009. 62% of the samples were from male patients. Of that sample, only 1,348 specimens were appropriate for further analysis using the RT-PCR method.

RT-PCR testing revealed the prevalence of group A among those tested with positive RSV. Group A infection was found in almost 70% (942 out of 1,348 samples) of the patients, while group B was found in almost 29% (390 out of 1,348 samples). The rest of the patients (1% or 16 out of 1,348 patients) were dually infected by both groups A and B RSV at the same time. This is of no surprise because similar results were found in other countries as well (Zlateva, et al., 2007).

It is obvious from the analysis above that both of group A and group B strains circulate at the same time but group A dominates. This was in vein with the results reached by Hall (2001). The results in Palestine are mirroring the results reached by several researchers in the world. Examples include:

1- Zhang et. al. (2007) studied RSV in China over a period of three epidemic seasons 1990/1991, 2000/2001, and 2003/2004. His results showed that group A prevailed over group B.

2- Oliveira et. al. (2008) conducted a study in Brazil and concluded that 78% of the RSV infected patients were infected by group A strains.

3- Arbiza et. al. (2005) conducted a similar study in Uruguay. He concluded that strains from groups A and B circulated together throughout the epidemics with predominance group A strains.

4- Zlateva et. al. (2007) conducted a study in Belgium to conclude that RSV A strain was predominant in two successive periods while B strain was predominant in the third season.

5- Sato et. al. (2005) conducted their study in Japan. The study revealed that the relative ratio of group A and group B strains shifted every year with group A dominating in certain periods, while group B dominating in others.

Although group A prevailed in the sample over group B, the analysis shows that there are certain variability. What is interesting to mention is that although the overall result shows the predominance of RSV A, RSV B was almost equally circulating as RSV A in the years 2007 and 2008. In some seasons, strain A is predominant while things could shift

in other seasons as shown in Table 3.18 and Figure 3.17. It is also important to notice that both strains often circulate during the same season. This fact was also documented by several researchers in different parts of the world (Mufson, et al., 1985; Peret, et al., 1998; Peret, et al., 2000).

4.6 RSV genotypes in Palestine

Nucleotide sequence analysis for 25 group A viruses isolated during a 4-year period measured the variability of the attachment protein G. Nucleotide sequences were determined for the majority of two variable regions of the G-protein gene. Phylogenetic analysis revealed that close clustering tended to be related to the date of isolation. Interestingly, RSV-A isolates belonging to the GA5, GA2 and the newly proposed lineage Pal-GA8 were isolated from the years 2006 and 2007. While the other newly proposed lineage Pal-GA9 was isolated from the three seasons 2007, 2008 and 2009. Cane et al. (Cane, et al., 1991) reported similar RSV-A distributions in the United Kingdom over 38-year period. RSV-A genotypes are widespread and have been shown to be changing with time. Thus, RSV-A vaccine development will be challenging as the viruses become antigenically less and less similar with time. With this study being the first from the researcher's region, no publications are available to compare the isolates that were detected in Palestine with others present in neighboring countries. However, similar RSV-A genetic variability in the GA5 and GA2 groups was previously reported from the US (Sullender, 2000), Belgium (Zlateva, et al., 2007), India (Parveen, et al., 2006), and South Africa (Venter, et al., 2001)..

Unlike RSV-A, less studies have been published worldwide about the genetic variability of RSV-B. From the 29 RSV-B 515 base pair PCR products analyzed in this study, 21 (72%) belonged to the GB13 group (Figure 3.25). RSV-B GB13 is a common lineage in Japan and in Argentina. This genotype of RSV-B has 60-nucleotide duplication in the G-protein gene. This genotype also referred to as genotype BA (Buenos Aeries). Sequence analysis of 3 GB13 RSV-B isolates revealed that they belong to the BA9 classification based on the study reported by Dapat et al. (2010). Interestingly, 8 RSV-B isolates did not belong to the GB13 or BA grouping and indeed they did not belong to any known groups. These 8 isolates were included in the newly designated group GB14 based on the sequences reported by Dapat et al. (2010) and Zlateva et al. (2007).

In summary, the research came as a first research in evaluating RSV infection in the region. The results were very much in vein with those of the prior research conducted in various other countries. RSV Group A prevailed over group B in most of the areas in the southern part of Palestine; both strains, A and B, circulate at the same epidemic season with the possibility of dual infection (along with hMPV) and double infection (RSV A and B at the same time), the peak infection appears to be the first few months of the infants life and is concentrated around winter months.

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