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Molecular Epidemiology of Rhinovirus in Southern Palestine

By

** Hana Abdel-Motaleb Qaisi**

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By

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology.

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Molecular Epidemiology of Rhinovirus in Southern Palestine

By Hana Abdel-Motaleb Qaisi

ABSTRACT

Background

Rhinoviruses (RVs) are classified in the *Enteroviruses* genus of *Picornaviridae* family. RVs are a major cause of the common cold. Several studies have shown that the RV is associated with other respiratory diseases such as asthma and sinusitis in young children. RVs are highly communicable viruses, particularly during the spring and fall months. RVs are divided into three genetic diversity groups (A, B, and C) with more than 160 serotypes. In Palestine and other parts of the Middle East, the epidemiology of RVs is not well defined.

Objective

This study examines the molecular epidemiology of RV in patients admitted to Caritas Baby Hospital (CBH) during 2013 and evaluates the co-infection rates between RVs and other common respiratory viruses.

Materials and Methods

Nasopharyngeal aspirates (N=1,745) collected from patients admitted to CBH in 2013 with upper respiratory tract infection were tested by Direct Fluorescent Antibody (DFA) staining for eight common human respiratory viruses (influenza viruses A and B, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), parainfluenza 1, 2, and 3, and adenovirus). Nasopharyngeal aspirates collected from patients less than 14 years of age between January and December 2013 where included in the study. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) was used to evaluate the presence of RVs in the DFA tested samples. In addition, RV subgrouping (A, B, or C) was performed on 184 (26.3%) RV positive patients samples by RT-PCR. Finally, sequence analysis of partially amplified RV genome sequences were performed on different RV subgroups on positive patient's samples and used to determine the RV genotypes.

Results and Discussion

Of the 1,745 samples, 699 (40%) were positive for RV by qRT-PCR. Variable activity (23.4%-61.7%) was noted during the study of which the peak activity occurring in September. Moreover, the male to female ratio of positive samples was 1.1:1. RV activity was noted all children aged groups ranging between 31.6% and 47.1%. Subgrouping of RV (N=184) revealed group A (66%) circulated the most in the Southern part of Palestine followed by group C (15%) and group B (8%), while 11% of the tested RV positive samples could not be typed. Sequence analysis of several isolates revealed that there were multiple genotypes of RV circulating in Palestine. Finally, RSV appears to be the most common virus-causing co-infection with RV as 55 (7.9%) of RV patients were also positive for RSV particularly during the RSV respiratory season.

Conclusion

This study is the first to show the prevalence of RV in the southern part of Palestine, which is an important indicator for our physicians to evaluate the role that RVs play in mediating asthma and sinusitis in Palestinian children. In addition, the high prevalence of RV in the Palestinian population can serve as a criterion for physicians to control antibiotic usage.

Keywords: (RV) Rhinovirus, (CBH) Caritas Baby Hospital, (DFA) Direct Fluorescent Antibody, (RSV) Respiratory Syncytial Virus, human metapneumovirus (hMPV), (qRT-PCR) Quantitative Reverse Transcription PCR.

وباء فيروس الأنف في جنوب فلسطين

هناء عبد المطلب قيسي

ملخص

خلفية

الفيروس الأنفي يصنف في جنس الفيروسات المعوية من عائلة الفيروسات البيكورناوية. الفيروس الأنفي هو المسبب الرئيس للزكام. وقد أظهرت العديد من الدراسات أن الفيروس الأنفي مرتبط مع أمراض تنفسية أخرى لدى الأطفال مثل الربو والتهاب الجيوب الأنفية. الفيروس الأنفي هو فيروس شديد العدوى خاصة خلال أشهر فصلي الربيع والخريف. تقسم الفيروسات الأنفية إلى ثلاث مجموعات جينية متنوعة (أ، ب، ج) مع أكثر من مئة وستين نمط مصلي. دراسات علم الأوبئة للفيروسات الأنفية قليلة في فلسطين وبعض دول الشرق الأوسط.

الهَدَف

تهدف هذه الدراسة لفحص انتشار الفيروس الأنفي لدى المرضى المُدخلين إلى مستشفى الكاريتاس للأطفال خلال سنة ٢٠١٣، وتقييم معدل العدوى المرافقة بين الفيروسات الأنفية والفيروسات التنفسية الشائعة الأخرى.

المواد والطّرق

تم اخضاع ١٧٤٥ عينة من المستخرج البلعومي التي جُمعت من المرضى المصابين بالتهابات الجهاز التنفسي المُدخلين لمستشفى الكاريتاس للأطفال في عام ٢٠١٣. لفحص صبغ الأجسام المضادة الفلورية المباشرة لثماني فيروسات تنفسية (فيروس الانفلونزا أ، ب، الفيروس التنفسي المخلوي، الفيروس نظير الانفلونزا ١،٢،٣، الفيروس الغداني، الفيروس ميتا رئوي). لقد تم جمع عينات المستخرج البلعومي من الأطفال تحت سن الرابعة عشر في الفترة ما بين كانون الثاني وكانون الأول من العام ٢٠١٣. تم استخدام تفاعل البوليميراز المتسلسل الكمّي اللحظي العكسي لتقييم وجود الفيروس الأنفي في العينات المختبرة سابقًا لفحص صبغ الأجسام المضادة الفلورية المباشرة. بالإضافة إلى ذلك، لقد تم تنفيذ تفاعل البوليميراز المتسلسل اللحظي لتحديد المجموعات الفرعية على ١٨٤عينة. وأخيرًا ، تم استخدام تحليل تسلسل الحمض النووي للمنطقة الجينية ما بين البروتين الفيروسي٢ والبروتين الفيروسي ٤ في المجموع المورثي للفيروس الأنفي على عينات مختارة لتحديد النمط الجيني للفيروس.

النتائج والمناقشة

من ١٧٤٥ عينة كان هناك ٦٩٩عينة مصابة بالفيروس الأنفي عن طريق تفاعل البوليميراز المتسلسل الكمّي اللحظي العكسي. وقد لوحظ نشاط متغير للفيروس خلال الدراسة حيث كانت تتراوح بين (٤,٢٣٪) و (٧,٦١٪)، حيث كانت الذروة في شهر أيلول. علاوة على ذلك، كانت نسبة الذكور إلى الإناث ١,١:١. ولقد لوحظ النشاط الرئيسي للفيروس الأنفي في جميع أعمار الأطفال بنسبة تراوح ما بين ٦,٣١٪

و١,٤٧٪. وقد كشف تحليل النمط الجيني لـ١٨٤ عينة أن المجموعة أ من الفيروس الأنفي هي الأكثر تداولًا في الجزء الجنوبي من فلسطين بنسبة ٦٦٪، بينما ١٥٪ تنتمي للمجموعة ج، و٨٪ تنتمي للمجوعة ب، ولم تنجح محاولة التحليل لـ١١٪ منها. لقد كشف تحليل تسلسل الحمض النووي لعدد من العينات أن هناك عدة أنماط وراثية متداولة جنوبي فلسطين. وأخيرًا؛ يبدو أن الفيروس التنفسي المخلوي هو الأكثر شيوعًا بتسبُب العدوى المرافقة مع الفيروس الأنفي؛ حيث كان هناك ٥٥ مريضًا مصابون بالفيروسين في ذات الوقت.

الاستنتاج

بياناتنا هي الأولى التي تُظهر انتشار الفيروس الأنفي في الجزء الجنوبي من فلسطين، وهو مؤشر مهم لأطبائنا لتقييم الدور الذي تلعبه الفيروسات الأنفية في المساعدة على الإصابة بالربو والتهاب الجيوب عند الأطفال الفلسطينيين. بالإضافة إلى ذلك، فإن نسبة عالية من الفيروسات الأنفية في السكان الفلسطينيين هو مؤشر للسيطرة على استخدام المضادات الحيوية من قبل أطبائنا.

DECLARATION

I declare that the Master Thesis entitled "Molecular Epidemiology of Rhinovirus 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.

Name and signature: "Hana Abdel-Motaleb Qaisi"

Date: December, 2018

/

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for the joint master degree in biotechnology at Palestine Polytechnic University and Bethlehem University, I agree that the library shall make it available to borrowers under rules of the library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of the source is made.

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Date: December, 2018

Dedication

I would like to dedicate my thesis to those who most deserve my loyalty,

who directed me on this path,

and who taught me the unfathomable value in learning;

My Parents, You have been significant influences in my life

To the Spirit of my Father, who shared with my family and me the best of life's moments until 19.08.2013. My dear father, whom I miss deeply, serves as an icon of patience and compassion and

My incredible mother symbolizes sacrifice and perseverance.

I thank you both for your unconditional love and endless support.

To my brothers and sisters for constantly encouraging one another throughout our lives. My achievements would not be possible without you all.

To my colleague, companion, and husband, Muradi.

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I would also like to show gratitude to the Faculty of Science at Bethlehem University and the Faculty of Scientific Research and Higher Studies at Palestine Polytechnic University for their permission to carry out this research.

I would like to acknowledge the Augusta Victoria Hospital Molecular laboratory for the quality work done with the sequencing component of my research.

Abbreviations

A	**Adenine**
A	**Alanine**
Å	**Angstrom equal to 10^{-10} meter**
C	**Cytosine**
°C	**Celsius**
CBH	**Caritas Baby Hospital**
CDC	**Centers for Disease Control and Prevention**
CDHR	**Cadherin-related**
COPD	**Chronic Obstructive Pulmonary Disease**
CL	**Cloverleaf**
Cre	**Cis-acting replication element**
Ct	**Cycle threshold**
CV	**Coefficient of Variation**
CXCL	**Chemokine (C-X-C motif) ligand**
D	**Aspartic acid**
DFA	**Direct Fluorescent assay**
dH2O	**Distilled Water**
dNTPs	**Deoxy Nucleoside Tri Phosphate**
DNA	**Deoxyribonucleic Acid**

ds	**Double strand**
E	**Glutamic Acid**
ENA	**Epithelial cell-derived neutrophil-activating peptide**
FDA	**Food and Drug Administration**
FGF	**Fibroblast growth factor**
G	**Glycine**
G	**Guanine**
HEV	**Human enterovirus**
hMPV	**Human metapneumovirus**
HRV	**Human Rhinovirus**
Ig	**Immunoglobulin**
IL	**Interleukin**
ICAM	**Intercellular Adhesion Molecule**
ICTV	**International Committee on Taxonomy of Viruses**
I	**Isoleucine**
IFN	**Interferon**
ILI	**Influenza like illness**
IRES	**Internal ribosome entry site**
K	**Lysine**

kDa	**Kilo Dalton**
LDLR	**Low density lipoprotein receptor**
LFA	**Lymphocyte function-associated antigen**
LNA	**Locked Nucleic Acid**
MDA	**Melanoma Differentiation-Associated protein**
MgCl2	**Magnesium Chloride**
M2	**Matrix protein 2**
MRC	**Medical research committee**
mRNA	**Messenger Ribonucleic Acid**
M	**Methionine**
µL	**Microliter**
Ml	**Milliliter**
N	**Asparginine**
NPA	**Nasopharyngeal aspirates**
NA	**Neuraminidase**
NK	**Natural killer cells**
NO	**Nitric oxide**
NTPase	**Nucleoside-triphosphatase**
ORF	**Open reading frame**

PAMP	**Pathogen-associated molecular pattern**
PBS	**Phosphate Buffer solution**
pH	**Potential of Hydrogen**
PCR	**Polymerase Chain Reaction**
P	**Probe**
P	**Proline**
PRR	**Pathogen recognition receptor**
Q	**Glutamine**
qRT-PCR	**Quantitative Reverse Transcription Polymerase Chain Reaction**
R	**Arginine**
RANTES	**Regulated on activation normal T cell expressed and secreted**
RIG-I	**Retinoic acid-inducible gene I**
RNA	**Ribonucleic acid**
RNase P	**Ribonuclease P**
RT-PCR	**Reverse Transcription Polymerase Chain Reaction**
RSV	**Respiratory syncytial virus**
RTIs	**Respiratory tract infections**
RV	**Rhinovirus**
S	**Serine**

SA	**Sialic Acid**
SD	**Standard Deviation**
TBE	**Tris Buffer Solution**
T	**Threonine**
T	**Thymidine**
TGF	**Transforming growth factor**
TLR	**Toll-like receptor**
UTR	**Untranslated Region**
UV	**Ultra violet light**
USA	**United States of America**
V	**Valine**
V	**Voltage**
VEGF	**Vascular endothelial growth factor**
VP	**Viral protein**
VPg	**Viral protein genome-linked**
WHO	**World Health Organization**
Y	**Tyrosine**

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CHAPTER 1

Introduction and literature review

1.1 Introduction

Acute respiratory tract infections (RTIs) are a leading cause of morbidity and mortality worldwide in people of all ages (Wardlaw et al., 2006). Each year, nearly 1.4 million children die of acute lower RTIs, such as pneumonia and bronchiolitis (WHO, 2012). Upper RTIs, popularly known as the "common cold", are the most common acute illnesses within the industrialized world (Simasek and Blandino, 2007).

The common cold is caused by more than 250 virus serotypes belonging to at least five different families, including adenovirus, respiratory syncytial virus, and coronavirus, whereas rhinovirus (RV) is the causative agent of 35% of the cases (Nguyen et al., 2016). RV infections occur early and can reoccur during life, causing significant diseases in infants and young children (Jartti et al., 2008).

Globally, RVs are the primary cause of acute upper RTIs. Although most infections are mild and self-limiting, RVs can also replicate in the lower respiratory tract and cause other complications in young children, including acute otitis media and sinusitis (Winther, 2011). Lower RTIs such as pneumonia (Louie et al., 2009), bronchiolitis (Papadopoulos et al., 2002), and wheezing (Korppi et al., 2004) are commonly caused by RV. Also, several studies showed that RVs infection in infants could lead to the development of recurrent asthma later in childhood (Smuts, Workman and Zar, 2011) and exacerbation of chronic obstructive pulmonary disease (COPD) (Mallia et al., 2011).

RVs were first discovered in the 1950s and were originally classified into two distinct species; RV-A and RV-B, based on traditional serological and cell culture methods (Conant and Hamparian, 1968). However, in 2009, the development of highly sensitive molecular techniques led to the identification and designation of RV-C by the International Committee on Taxonomy of Viruses (ICTV) (Jacobs et al., 2013).

1.2 Virology

Rhinoviruses (RVs) are classified as members of the *Enterovirus* genus of the diverse family of *Picornaviridae*, which consists of 12 genera and 28 species (Knowles et al., 2010). Until 2009, RV was a genus, but was reclassified into the *Enterovirus* genus, which has 10 species to correct for phylogenetic inconsistencies (Knowles et al., 2010).

The virus was originally named as human rhinovirus (HRV), but in 2013, the ICTV renamed this species Rhinovirus A, B, and C (Picornaviridae website). After the discovery of species C rhinoviruses, the name "serotype" was changed to "genotype" or simply "type" (Royston and Tapparel, 2016).

RV is the smallest virus with a non-enveloped icosahedral capsid protected a single-stranded, positive sense, non-segmented RNA of approximately 7,200 base pairs. RVs have a 30 nanometer diameter and an external radius of approximately 150Å. The weight of the RNA is $2.6x10^6$ Daltons, with a total molecular weight of about $8.4x10^6$ Daltons (Medappa, McLean and Rueckert, 1971).

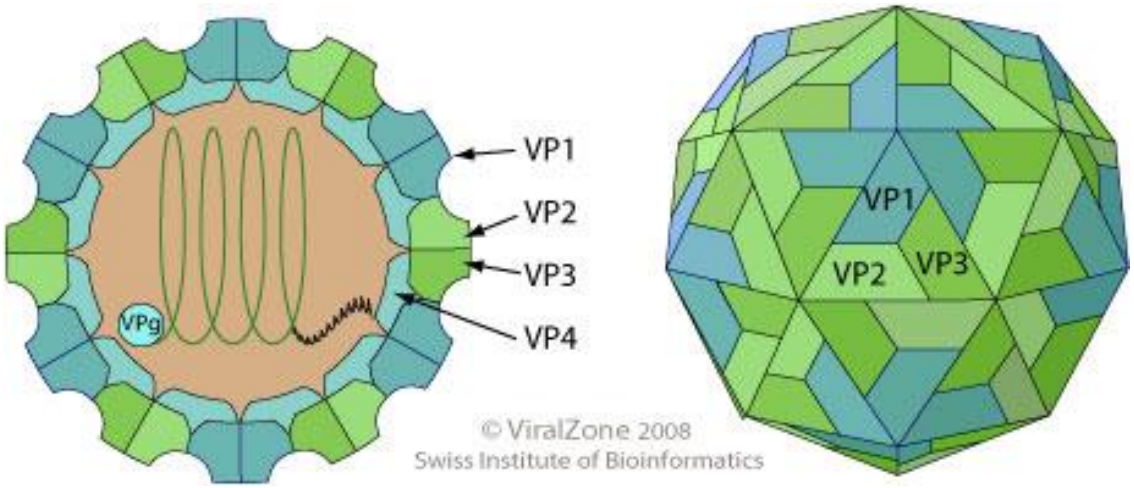

Figure1.1: The structure of Rhinovirus viroin. A non-enveloped, spherical and about 30 nanometer in diameter, with a capsid surrounding the RNA genome. The capsid consists of an icosahedral arrangement of 60 protomers, each consisting of 4 polypeptides VP1, VP2, VP3, and VP4. VP4 is located on the internal side of the capsid (Viralzone website).

RV species A, B, and C isolates were subdivided into numeric genotypes that were primarily based on the sequence comparisons of the VP1 protein or VP4/VP2 coding region (Palmenberg and Gern, 2015).

The genetic diversity of RVs is continuously changing; consequently the classification of RVs is regularly updated (Drysdale, Mejias and Ramilo, 2017). Within the RV-A species, for example, a discrete "clade D" has recently emerged, which may in time be classified separately from RV-A (Palmenberg and Gern, 2015).

The 2017 ICTV released over 160 genotypes of diverse RVs known to be circulating throughout the world; RV-A includes 80 species, RV-B has 32 species, and RV-C has 56 virus species (Zell et al., 2017). The main explanation for such an important genetic variability includes the high error rate of the viral RNA-dependent RNA polymerase and recombination events (Phuektes et al., 2011).

1.3 Structure

The RV genome contains a single open reading frame (ORF) which is flanked by 5′ and 3′ untranslated regions (UTR). The open reading frame encoding a polyprotein, which is normally cleaved during translation into eleven proteins; seven are a nonstructural proteins involved in viral genome replication and assembly, and the others form the capsid (Yang et al., 2011). The mutation rate estimated to range from 10^{-3} to 10^{-5} mutations per nucleotide per genome replication event (Domingo, 1994).

1.3.1 5' UTR

The 5' UTR is the most complex region in the genome due to its many secondary and tertiary motifs. At the 3' end of this region sits the VPg, the protein primer for RNA synthesis involved in the initiation of viral replication (Xiang, Paul and Wimmer, 1997). Adjacent to the VPg is a 5'-terminal cloverleaf-like motif (CL), that binds viral and cellular proteins for the initiation of RNA synthesis, and helps convert infecting genomes from translation to replication templates (Palmenberg, Rathe and Liggett, 2010). Associated with the CL is the internal ribosomal entry site (IRES), essential functioning as a 'landing pad' for ribosomes (Palmenberg, Rathe and Liggett, 2010).

1.3.2 ORF

The open reading frame of the genome codes a single polyprotein that cleaved to form P1 (containing structural proteins VP1-4), P2 (nonstructural proteins 2A-C), and P3 (nonstructural proteins 3A-C) (Hershenson, 2010).

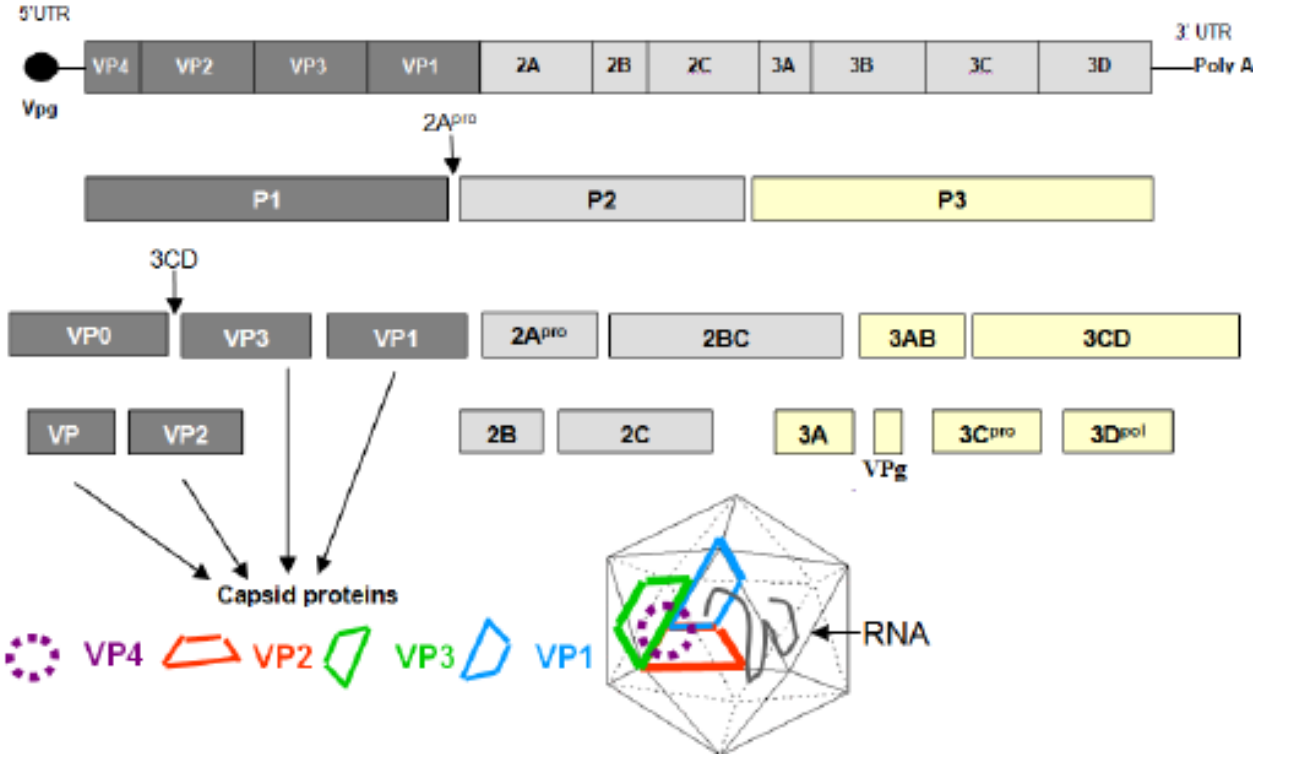

Figure 1.2: Organization of the enterovirus genome, polyprotein processing cascade and architecture of enterovirus capsid. The genome of enteroviruses contains one single open reading frame flanked by a 5' UTR and 3' UTR. A small viral protein, VPg, is covalently linked to the 5' UTR. The 3' UTR encoded poly (A) tail. The translation of the genome results in a polyprotein which is cleaved into four structural proteins (dark gray) and seven non- structural proteins (light gray and yellow). The sites of cleavage by viral proteinases are indicated by arrows. The four structural proteins adopted an icosahedral symmetry with VP1, VP2 and VP3 located at the outer surface of the capsid and VP4 at the inner surface. The single strand genomic RNA is located inside the capsid (Hober et al., 2013).

Region P1 comprises the four structural proteins VP 1-4 which make up 60 protomers composing the icosahedral structure of the capsid. Each contains a single molecule of four polypeptides; VP 1-3 exposed on the surface with approximately 30kDa, while VP4 is approximately 7kDa located internally in deep canyons with a hydrophobic pocket and is responsible for the assembly of the virus and infection of new cells (Bella and Rossman, 1999; Fuchs and Blaase, 2010; Palmenberg, Rathe and Liggett, 2010).

Region P2 comprises the three proteins 2A, 2B and 2C proteins. The 2A protein is a protease that involved in the polyprotein cleaving; it cleaves factors involved in translation initiation and in shutting down host cell translation (Racaniello, Knipe and Howley, 2007). The 2B protein inhibits the two cellular secretory pathways; the endoplasmic reticulum and Golgi apparatus. The 2C is involved in vesicle formation and has nucleoside triphosphatase (NTPase) activity (Racaniello, Knipe and Howley, 2007).

Region P3 comprises the four proteins 3A, 3B, 3C and 3D proteins. The 3A protein is involving in the inhibition of intracellular transport, while 3B codes for the subsequent VPgs. The 3C protease is responsible for generating replication proteins from the viral genome and inhibiting host transcription. The 3D protein encodes RNA-dependent RNA polymerases (Racaniello, Knipe and Howley, 2007), and $3D^{pol}$ replicates the poly-(A) tail of viral RNA, synthesizing VPg-linked poly (U) at the 5' ends of negative-strands (Steil, Kempf and Barton, 2010). The VPg-linked poly (U) then intervene function as templates for the polyadenylation of nascent positive-strand RNA (Steil, Kempf and Barton, 2010).

A cis-acting replication element (cre) is an RNA which harbors stem-loop structure and has been observed within the genome of several picornavirus genera. It has been implicated in negative strand RNA synthesis (Goodfellow et al., 2000). In RVs, this cre stem-loop structure is part of the ORF, and its location varies in the three RV species. It lies within the 2A coding region for RV-A, VP2 for RV-C and VP1 for RV-B (Cordey et al., 2008).

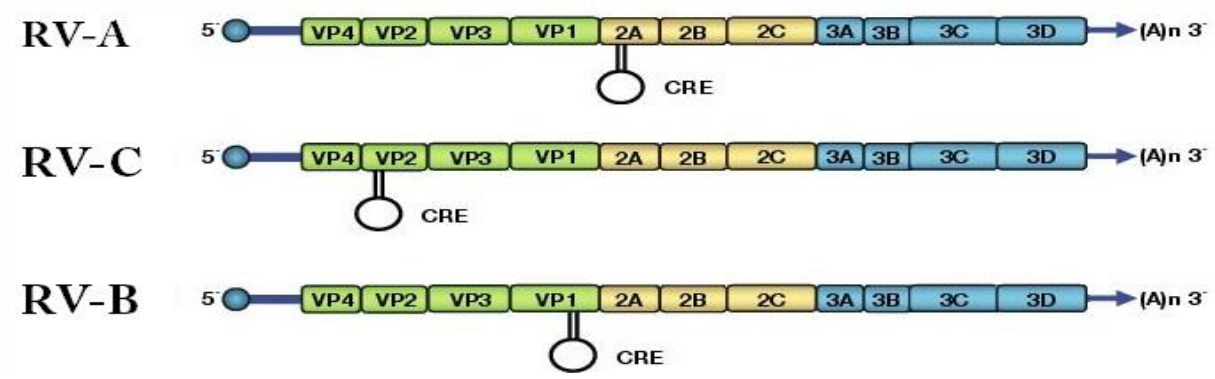

Figure 1.3: Schematic diagrams of Rhinovirus (RV) genomic organization showing the locations of known cre elements in RV-A, RV-B and RV-C (Cordey et al., 2008).

1.4 Pathogenesis

Most RV genotypes enter the host cell by receptor mediated endocytosis. RVs are acid labile, so the RNA genome is released when a pH of ~6.5 is reached in the endosomal compartment (Racaniello, Knipe and Howley, 2007) And as RV does not cause cytopathology like other respiratory viruses (i.e. influenza, respiratory syncytial virus), it's cycle is exclusively cytoplasmic (Jacobs et al., 2013).

1.4.1 Receptor Usage

Rhinoviruses utilize three major types of cellular membrane glycoproteins to gain entry into the host cell. The majority of RV-A and all RV-B enter by binding to intercellular adhesion molecule 1 (ICAM-1), and 12 RV-A types enter by binding to low density lipoprotein receptor (LDLR), while cadherin-related family member 3 (CDHR3) has been recently identified for RV-C. (Bochkov and Gern 2016; Palmenberg, 2017).

1.4.2 Cell Entry

Once the positive sense single stranded RNA (+ssRNA) is released into the cytoplasm, the VPg is removed, and the IRES promotes binding of the 40S ribosomal subunit initiating translation. The polyprotein is then subsequently cleaved by the viral 2A and 3C proteases, producing individual viral proteins (Quiner and Jackson, 2010). RV +ssRNA is then replicated by the viral RNA-dependent RNA polymerase to a –ssRNA form, using membrane vesicles as a scaffold. The -ssRNAs are then copied to more +ssRNAs which can then be used for further translation into more viral proteins or used for packaging into new virion particles, a process called morphogenesis. Due to the generation of –ssRNA, double stranded (ds) RNA is formed during the viral replication cycle and functions as pathogen-associated molecular patterns (PAMP) (Quiner and Jackson, 2010).

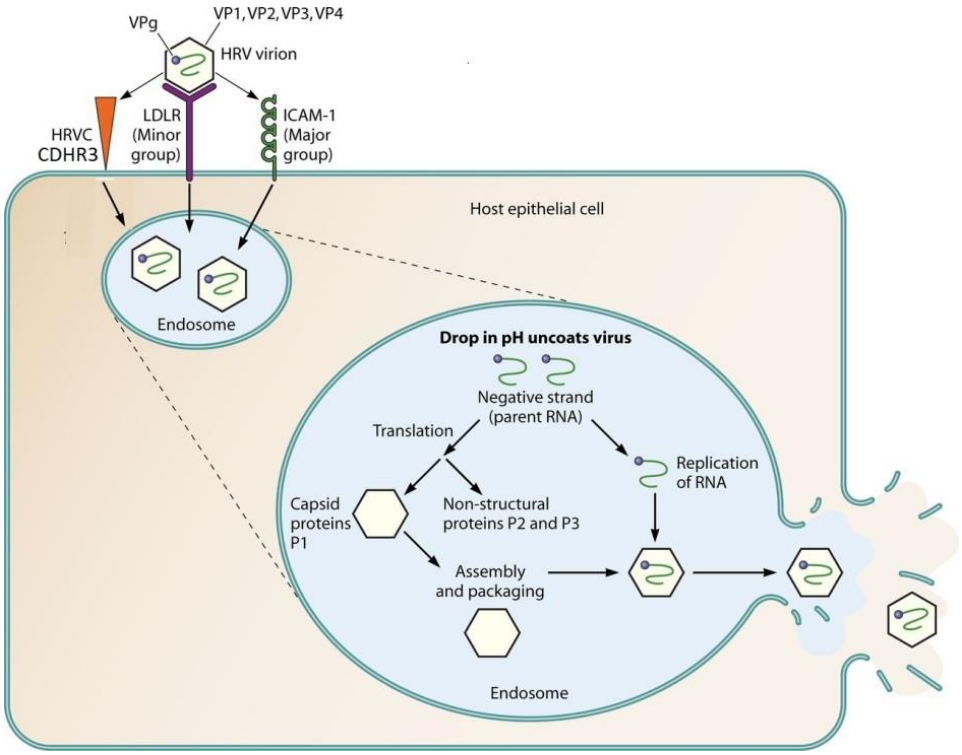

Figure 1.4: RV receptor usage and cell entry. After virus uptake, a drop in the pH leads to viral uncoating. Negative-strand (parental) RNA is replicated and translated into structural and nonstructural proteins. The virion is then assembled and packaged prior to cellular export via cell lysis (Jacobs et al., 2013).

The optimal condition for growth and replication for RVs is 33°C - 34°C at a pH of 7 - 7.2, making the upper airway an ideal candidate for RV propagation. RVs are sensitive to acidic pH and don't survive in the acidic gastrointestinal environment (Gern, 2010). However, some RV types were shown to grow equally or more prominently at 37°C (Papadopoulos et al., 1999), An increasing number of reports have found RV nucleic acids in stools (Harvala et al., 2012; Lau et al., 2012), while the virus could even isolated in stool samples of young children (Honkanen et al., 2013). These findings suggest that some RVs are able to survive in the higher temperature and acidic environment as in the gastrointestinal tract and cause the RV effect in lower respiratory diseases.

1.4.3 Immune Response

Host responses to RVs are thought to be initiated when viral proteins and nucleic acids trigger pathogen recognition receptors (PRRs) of the innate immune system responding to PAMPs (Wang et al., 2009; Slater et al., 2010).

Once the RV infects the epithelium, the innate immune system is initiated. Type I interferon production and a low airway pH occurs within 24 hours of experimental exposure to RV (Ngamtruakulpanit et al., 2003). The capsid is recognized by toll-like receptor-2 (TLR-2) on the epithelium and RV nucleic acids are detected by TLR3, TLR7, and TLR8. The activation of TLRs cause activation of the receptors genes melanoma differentiation associated gene-5 (MDA-5) and retinoic acid inducible gene-I (RIG-I) (Triantafilou et al., 2011; Slater et al., 2010). Subsequently, activation of these receptors results in the release of type I and type III interferons to inhibit virus replication. Cytokines like interleukin (IL)-12 and (IL)-15 recruit natural killer cells. Chemokines secreted (e.g. CXCL8 and CXCL10) early in the infection recruit neutrophils (Kennedy et al., 2012). Neutrophils in nasal secretions are one of the characteristic features of a symptomatic cold and also induce the production of bradykinin and kallidin and increase vasodilation (Kennedy et al., 2012).

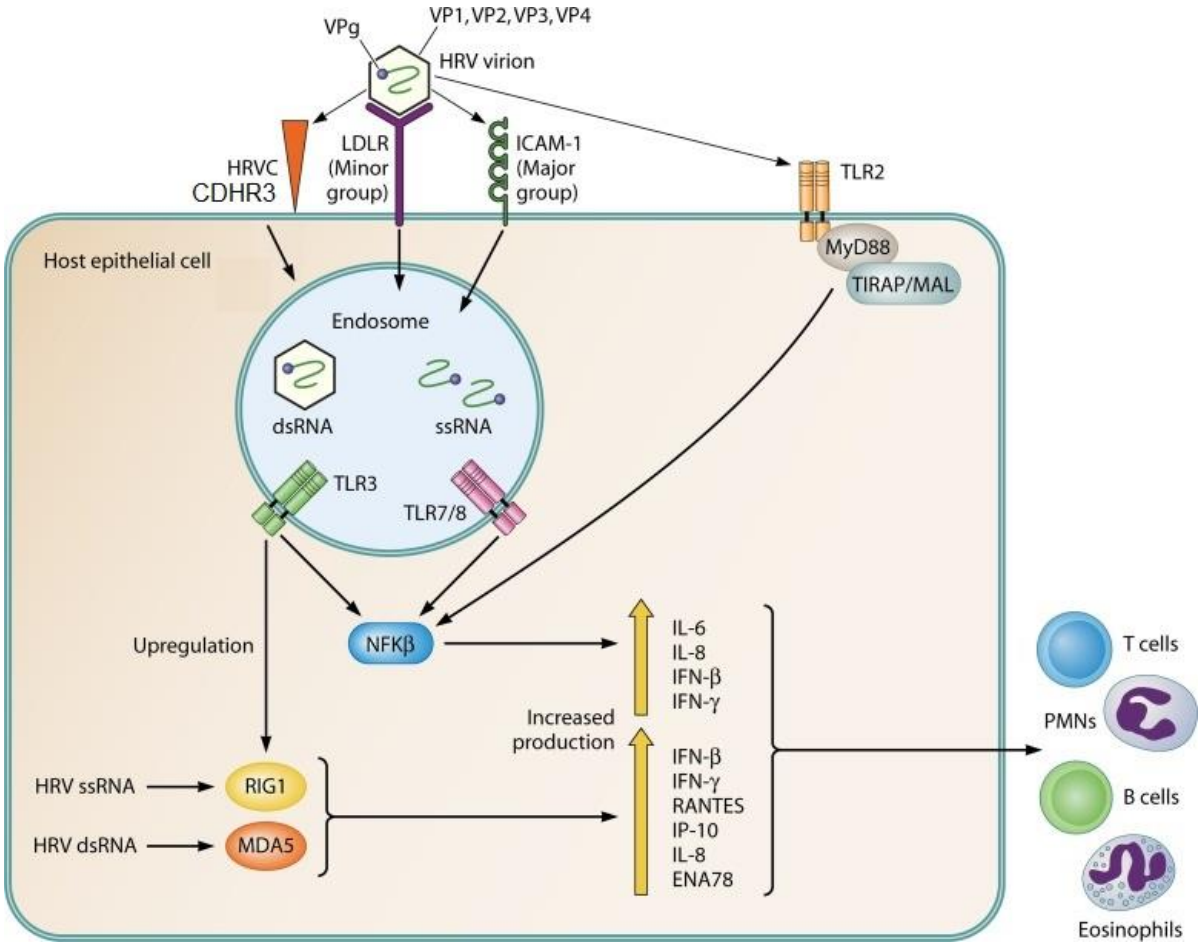

Figure 1.5: Signal transduction pathways and activation of the innate immune response. In the endosome, viral dsRNA and ssRNA are recognized by TLR3 and TLR7/8, respectively. An interaction with TLR3 triggers the upregulation of the pattern recognition receptors RIG-1 and MDA-5 (RNA helicases) in the intracellular compartment. RIG-1 and MDA-5 also recognize newly synthesized viral dsRNA and ssRNA in the cytoplasm. RIG-1 and MDA-5 stimulate RV-induced interferon (IFN) gene expression as well as the increased production of T cell and neutrophil cytokines, including regulated on activation normal T cell expressed and secreted (RANTES); IFN-γ-IP-10; IL-8; and epithelial cell-derived neutrophil-activating peptide 78 (ENA78). An interaction with TLR7/8 triggers IFN-β and IFN-γ production and activates the NF-κβ pathway. RV also interacts with TLR2 on the cell surface to initiate a proinflammatory cytokines response via a MyD88-dependent pathway (Jacobs et al., 2013).

The humoral immune response to RV infection is insufficiently understood. Patients with humoral immune failures experience severe episodes of RV (Kainulainen et al., 2010). In experimental RV infections of individuals with previous exposure, seven serotype-specific IgAs are detected by day 3 and IgG at day 7-8 (Johnston, 2001). Antibodies contribute to viral clearance by preventing the virus from entering the host cell, opsonizing the virus, and by initiating natural killer cell-mediated antibody dependent cellular cytotoxicity (Kennedy et al., 2012). Viral titers begin to decline approximately 72 hours post-infection and cell mediated immune responses reflect the activation of pre-existing memory T cells. Memory T cells respond to shared epitopes on the capsid of the infecting RV (Kennedy et al., 2012; Edlmayr et al., 2011). The role of CD8+ T cells in RV infections is not well documented. However, these cells are thought to drive the eradication of the virus through the production of IFN-γ (Kennedy et al., 2012; Sun et al., 2011).

Thus, it is assumed that innate immune responses play the dominant role in regulating symptomatic responses. It is thought that adaptive immunity can offer protection against subsequent infections with that particular strain of RV. Unfortunately, since there is such a large number of circulating RV genotypes with a high mutation rate, repeated infection can still occur in individuals (Drake and Holland, 1999).

1.5 Clinical Presentation and Complications

The incubation period of the infection is 8 - 12 hours (Harris and Gwaltney Jr, 1996). Within 2-3 days of infection, the local area becomes inflamed as immune cells invade the host, and the initial symptom is a sore throat beginning 10-16 hours post infection (Gwaltney Jr, 2002). There is often a local edema, or swelling caused by an increased permeability of capillaries and blood vessels in the area to plasma. At this point, mucous membranes in the nasal passages begin to secrete large volumes of fluid. The end results are symptoms that include: cough, rhinitis, sore throat, rhinorrhea, sneezing, and fever. In most cases, colds do not cause serious illness or complications. Most colds last for three to seven days, although many people have residual symptoms such as coughing, sneezing, nasal or chest congestion for up to two weeks. (Drysdale, Mejias and Ramilo, 2017).

RV disrupts tight junctions in the epithelial barrier causing increased vascular leakage and mucus secretions resulting in rhinorrhea (Kennedy et al., 2012). Coughing occurs in 30% of episodes and is

possibly the result of direct infection of the large airways (Kennedy et al., 2012). Increased vascular permeability can lead to secondary bacterial infections in the sinus openings and the Eustachian tube, resulting in acute secondary bacterial sinusitis and otitis media, respectively (Kennedy et al., 2012). RV can infect the lower respiratory tract and has been cultured from secretions in the lower respiratory tract (Halperin et al., 1983).

RV is commonly shed through nasal secretions and can spread to uninfected individuals. A review indicates that RV can be shed in nasal secretions for 11-days post-infection (Cate, Couch and Johnson, 1964; Van Elden, et al., 2008; Hendley and Gwaltney, 2004).

RV may cause otitis media complications due to blocked middle ear ventilation, mucociliary damage, increased mucus in the Eustachian tube, and secondary bacterial infections (Greenberg, 2011). In a study of 121 children susceptible to otitis media, RV was identified in 30% of specimens and was co-infected with *Moraxella catarrhalis* and/or *Streptococcus pneumoniae* (Alper et al., 2009). Less than 20% of infections have complications from a secondary bacterial infection (Greenberg, 2011; Gwaltney Jr, 2000). In addition, abnormalities in the maxillary and ethmoid sinuses can result in rhinosinusitis (Greenberg, 2011).

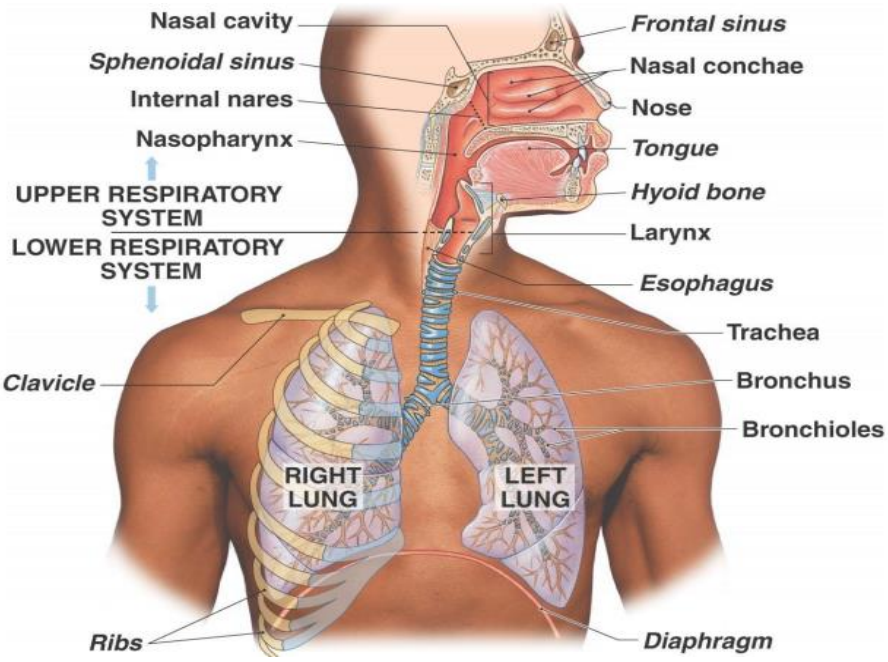

Figure 1.6: Diagram of respiratory tract. The respiratory system is divided into; upper respiratory system above the larynx, and lower respiratory system below the larynx (Martini and Nath, 2009). Rhinovirus has been typically associated with upper respiratory tract infections; however, with the development of molecular methods, RV has also been found to be commonly associated with lower respiratory tract infections. These findings are changing the long held view of RV as a minor pathogen, as it is now being involved in a wide variety of respiratory illnesses, ranging from mild common colds and asthma exacerbation to serious lower respiratory tract disease (Aponte et al., 2015).

RV infections may lead to asthma and exacerbations of chronic respiratory diseases. RV infections are common in patients with underlying asthma or chronic lung conditions such as chronic obstructive pulmonary disorder (COPD) (Kennedy et al., 2012). Members of RV-C are more likely to cause wheezing illnesses and asthma exacerbations compared with other RVs (Steinke and Borish, 2016). In asthma, innate immune responses such as decreases in IFN-α, IL-12, and IFN-γ are thought to be defective in bronchial epithelial cells (Greenberg, 2011). RV infections also induce vascular endothelial growth factor (VEGF), nitric oxide (NO), transforming growth factor-beta (TGF-β), and fibroblast growth factor (FGF) to induce airway remodeling (Greenberg, 2011). The mechanism induce COPD exacerbations is unknown. It is speculated that the result of ICAM-1 up-regulation in the bronchial mucosa or the adherence of bacteria to RV-infected epithelial cells causes ICAM-1 and TLR-3 up-regulation, leading to increased RV binding, therefore an increased RV-induced cytokine response (Sajjan et al., 2006).

In immunocompetent individuals, the virus is usually strictly restricted to the upper respiratory airways and typically induces nasal congestion and rhinorrhea, cough, sneezing, sore throat, and malaise with a spontaneous resolution within one to two weeks. Rare cases of extra-pulmonary illnesses related to RV have been recently described, including cases of gastroenteritis and pericarditis (Royston and Tapparel, 2016).

1.6 Transmission

RV is transmitted to humans horizontally from person to person via droplets that are expelled into the air from infected person by coughs, sneezes, respiratory secretions, and fomites (Bella and Rossman,

1999; Gwaltney, 2002). Some droplets also fall onto surfaces, such as telephones or door handles with which many others come into contact (Blomqvist et al., 2002).

Once touched, the cold virus is transferred to the person's hand, which can then come into contact with the mouth, thereby infecting the "host". Once within the host, the virus fuses to the plasma membrane of a permeable cell within the respiratory tract, particularly through the nose, mouth, and throat (Greenberg, 2011).

The virus then replicates its genome within the host cells. Once complete, virus particles leave the cell, enter into the surrounding tissues, and infect other cells within the host. This is a cyclical process (Jansen et al., 2011).

Once the virus has proliferated, it is shed from the mucous membranes into the saliva and the nasal discharge of the host. The infectious stage begins about one day before the presentation of symptoms and may continue for up to one week (Van Elden et al., 2008).

1.7 Diagnosis

Traditional procedures used for identification of picornaviruses in respiratory samples include cell cultures and acid liability testing through inactivation of virus at low pH (Santti, Vainionpää and Hyypiä, 1999).

Cell culture has several limitations including the need of expertise, the need to use different cell lines, an intrinsic delay of several days, a lack of sensitivity due to many strains, and the requirement of separate equipment (incubator set at 33˚C) (Lu et al., 2008). Cell culture was successful to isolate RV-A and RV-B, but unsuccessful for RV-C. The diversity of RVs makes serology unfeasible. As a result, molecular testing is the preferred method for RV detection (Santti, Vainionpää and Hyypiä, 1999).

Numerous molecular assays have been described for RV since 1988, primarily targeting the 5' UTR, which contains conserved motifs required for translation and replication (Lu et al., 2008; Savolainen, Blomqvist and Hovi, 2003; Santti, Vainionpää and Hyypiä, 2009). RT-PCR improved a sensitive detection and differentiation of RV serotypes through nasopharyngeal swabs or aspirates and tracheal aspirates (Lu et al., 2008).

1.8 Treatment

Despite common cold being regarded as harmless diseases, they can cause significant morbidity and are the most common acute reason for children to visit their primary care physician's office (Nadeem Ahmed et al., 2010; Rechsteiner and Schappert, 2011). There is no therapeutics have been developed for rhinovirus, patients need to take rest, adequate amount of fluids, antipyretic drugs and symptomatic treatment for cough and cold to recover from viral URTI (Bhanwra, 2013). Warm saline gargles and steam inhalation are inexpensive and relatively safe measures that provide temporary relief of throat symptoms. Non-steroidal anti-inflammatory drugs are useful for relieving fever, headache and malaise (Bhanwra, 2013).

The use of over-the-counter (OTC) symptomatic treatments can reduce symptoms in some patients. Antihistamines and nonsteroidal anti-inflammatory drugs may relieve some symptoms, but they do not shorten the duration of illness (Greenberg, 2003). Other medications have been reported to be beneficial in hastening recovery from the common cold, but review of controlled trials has failed to provide incontrovertible evidence of benefit. These other medications include zinc lozenges, Echinacea, and high-dose vitamin C (Greenberg, 2003).

The URTIs are the most common reason for inappropriate antibiotic use in children and adults, contributing to the emergence of resistant bacterial strains (Gonzales et al., 2001). It is well known that antibiotics are ineffective against viral infections and that inappropriate use can put patients at harm for allergic reactions and antibiotic-resistant infections (Grijalva, Nuorti and Griffin, 2009), so antibiotics have no role in the management of common cold or any mild URTI except when it is due to bacterial infections (Bhanwra, 2013). During the clinical course of a common cold, secondary bacterial infections may occur, however, antimicrobials are frequently used inappropriately for events that are normal during the clinical course of a viral infection (Dowell, Schwartz and Phillips, 1998). Recent studies have shown high rates of antibiotic prescription for viral respiratory infections, ranging from 20-60% (El Sayed et al., 2009; Nadeem Ahmed et al., 2010).

1.9 Prevention and vaccination

The search for an RV inhibitor began in the late 1980s, when the crystal structure of RV 14 was identified, targeting the protein capsid (Rossmann et al., 1985). The first known capsid binders are

"WIN" compounds. These compounds bind onto a hydrophobic depression on the floor of the viral capsid canyon where cell receptor binding occurs. This binding increases the rigidity of the virion, decreasing its linkage to the receptor (Thibaut, De Palma and Neyts, 2012). The "WIN" compound pleconaril was put into clinical trials in 1996 but was rejected by the Food and Drugs Administration (FDA) in 2002 due to safety concerns (Hershenson, 2010).

Other trials for RV prevention included targeting the proteolytic enzymes inhibitor, as in rupintrivir was one of the most potent compounds to inhibit the 3C protease *in vitro*. It was active against a broad panel of RVs and EVs, well tolerated and reduced viral loads and respiratory symptoms. But it did not decrease the clinical severity or viral load in natural infection (Patick et al., 2005).

This issue is still being researched, therefore, there is no approval for antiviral agents that can prevent RV infection. There is also a manifold of genotypes, making a vaccine against RV unrealistic. Also, the lack of epidemiological data to identify the most commonly circulating RV genotypes. There is an incomplete understanding of antigenic differences between RV genotypes, hindering the production of appropriate vaccines (Basta et al., 2014).

1.10 Epidemiology

RV infections are the prime causes of respiratory illness in children and adults. Infections can occur at any time during the year, but two peaks were reported to be in the spring and autumn (Winther, Hayden and Hendley, 2006).

Despite the usual self-limiting nature of the common cold, it places a considerable economic and social burden upon suffering populations, leading to an increased number of doctor visits as well as more absences from school and work than any other illness every year. In terms of lost productivity, the virus causes more purchases of drugs and unnecessary prescriptions of antibiotics (Fendrick et al., 2003).

Children are considered the major reservoirs for RV and experience up to 8 to 12 infections per year, whereas adults are infected two to three times per year (Madigan, Martinko and Parker, 2003). A study showed that RV-Cs demonstrate a different trend, with a peak of infection during winter months (Linder et al., 2013).

RVs were also be detected in nasal secretions and blood. Reports identified RVs in plasma, pericardial fluid, stool, and urine in neonates (Tapparel et al., 2009; Broberg et al., 2011). Viremia is common in enteroviruses infection, but rare in RV (Tapparel et al., 2009). Understanding the molecular epidemiology of RV has significantly improved over recent years with difficulties due to the high variability between RV genotypes. Until now, there are no former studies about RV genotypes circulating in Palestine. This study searches for the molecular epidemiology of circulating RV groups as well as their relationship with other respiratory virus complications in young children in southern Palestine.

CHAPTER 2

Objectives

The objectives of the research include:

- Studying the molecular epidemiology of RV in patients admitted to Caritas Baby Hospital (CBH) during the year 2013.
- Determining RV subtypes by RT-PCR.
- Determining the genotypes of selected RV subtypes.
- Evaluating the co-infection rate between RVs and other common respiratory viruses.

CHAPTER 3

Materials and Methods

3.1 Study location

This study was conducted at Caritas Baby Hospital (CBH) in Bethlehem, Palestine. CBH is a non-profit charitable hospital in the West Bank that caters exclusively to children. It serves children in the southern part of Palestine, mainly in Bethlehem and Hebron districts, and all surrounding camps. CBH aims to provide medical care for any child in need and involves mothers in order to prevent future illness. The hospital treats around 40,000 children annually with around 4,000 children admitted each year.

3.2 Study Group

The patient population studied included children admitted to CBH with respiratory infection between January 2013 and December 2013. Their age distribution per number of individuals in each age group, included newborn and children up to 14 years.

3.3 Clinical specimens

Nasopharyngeal aspirates (NPA) (N=1,745) were collected by well trained nurses using mucus extractors from children with respiratory symptoms to check for viral infections (Figure 3.1). The patient's age, sex, admission date, and geographical area were compiled in a database which also included Direct fluorescent antibody (DFA) test results that were routinely performed by CBH laboratory technologist for the presence of any common respiratory viruses in the patient's samples such as RSV, hMPV, influenza A, influenza B, adenovirus, and parainfluenza 1, 2, and 3. The analysis revealed that 27.7% of the 1,745 samples contained at least one of these viruses tested.

Figure 3.1: Mucous extractor used at CBH

The NPAs were well-labeled and saved at -70^{o}C. In this study, all NPAs of the study group were retrieved from the storage boxes to proceed with the molecular testing in order to achieve the goals of this research.

3.4 RNA extraction

RNA from all the NPAs was extracted using High Pure Viral Nucleic Acid Extraction kit (Roche Applied Science, Germany), according to manufacturer's instruction. The kit was reliable and guaranteed nucleic acid recovery of at least 2 x 10^5 RNA molecules / 200 µL in 10 minutes. RNA extraction was performed as described in Appendix 1. Extracted RNA was stored in well labeled sterile Eppendorf tubes at -70^{o}C pending analysis.

3.5 Detecting RV by qRT-PCR

The 5' UTR of RV was selected for amplifying a 207 base pairs region of RV genome using one step qRT-PCR on the 7500 Fast Real-Time PCR System, (Life Technologies, USA). The 12.5 µl reaction volume contained 0.8 µl (300nM) each of RV forward and reverse primers, 0.5 µl (200nM) of RV probe, 0.125 µl each of RNase P forward and reverse primers (300nM) and probe (200nM), in addition of 6.25 µl of master mix buffer containing dNTPs and the other constituents needed for reaction.

qRT-PCR cycling conditions were optimized to the following conditions: 48^{o}C reverse transcription for 30 minutes, an initial activation step of 3 minutes at 95°C for polymerase activation, and then 45 cycles of 15 seconds at 95°C and 1 minutes at 60°C. A panel of primers and hydrolysis probes were synthesized according to (Lu et al., 2008) listed in table 3.1 for 5' UTR and in table 3.2 for RNase P.

Table 3.1: RV detection primers and probe set

Primer / Probe[1]	**Sequence[2] (5' – 3')**	**Position**
Forward primer	CPX GCC ZGC GTG GC	356–369
Reverse primer	GAA ACA CGG ACA CCC AAA GTA	563–543
Probe	TCC TCC GGC CCC TGA ATG YGG C	444–465

[1]probe were 5' end labeled with 6-carboxyfluorescein and 3'-end labeled with black hole quencher

[2](Y=C, T; X=LNA-A; Z=LNA-T). The nucleotide numbering is based on that of the RV1B sequence (accession no. D00239). LNA: locked nucleic acid, synthesized at Metabion, Germany.

Each RNA sample had the endogenous RNase P reference gene primer/probe set run in parallel with the RV 5' UTR target. The human RNase P gene primer and probe set was used as an internal positive control for human nucleic acid to monitor sample quality and to indicate adequate isolation of nucleic acid resulted from extraction of the clinical specimen (Emery et al., 2004).

Table 3.2: RNase P primers and probe set.

Primer / Probe[1]	**Sequence (5' – 3')**
Forward primer	AGA TTT GGA CCT GCG AGC G
Reverse primer	GAG CGG CTG TCT CCA CAA GT
Probe	TTC TGA CCT GAA GGC TCT GCG CG

[1]probe were 5' end labeled with 6-Carboxyfluorescein and 3'-end labeled with black hole quencher

All clinical samples exhibit positive RNase P reaction curves that cross the threshold line before 40 Ct indicating that nucleic acid extraction and PCR amplification did occur. All samples have less than 40 Ct value considered positive for RV infection. Figure 3.2 shows the amplification plot of qPCR.

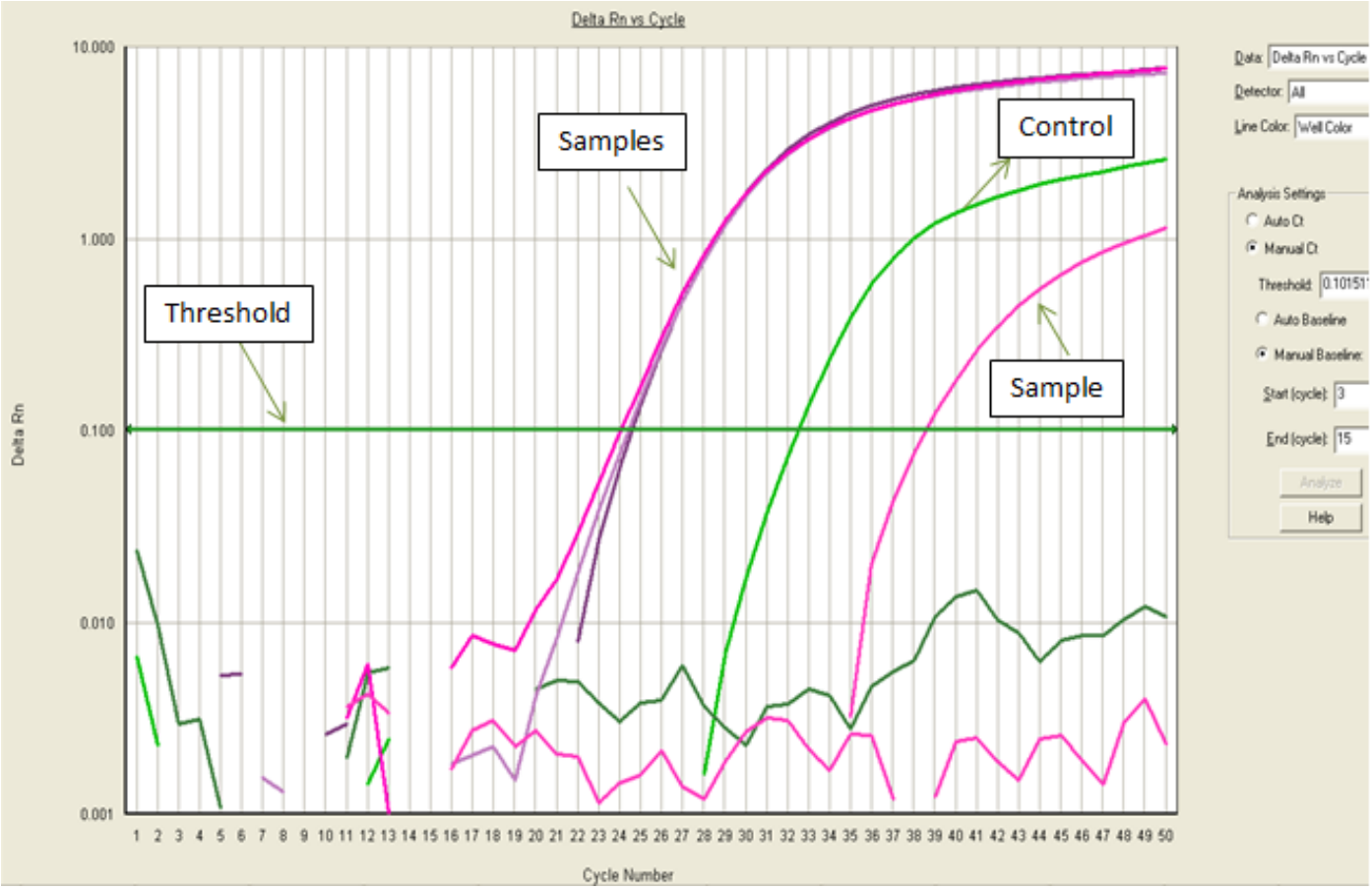

Figure 3.2: The amplification plot of qPCR.

3.5.1 RV detection Control

A positive RV RNA control from this study group was used as a control for the detection of RV genome. The control Ct value was set at 30 ±1 Ct. It was prepared by diluting and aliquoting the sample into 50 tubes. One new tube has been used for each PCR run. The means Ct values of the controls were 29.3 with Standard Deviation (SD) 1.1 and Coefficient of Variation (CV) 3.2%.

3.6 RV typing

RV, N=184 (26.3%) positive samples with less than RV 35 Ct value of qRT-PCR were typed by nested RT-PCR for VP1 gene. All group A negative samples were then tested for group C. Negative A and C samples for RV were also tested for group B.

The nested RT-PCR was performed to type samples for RV group A and C by the following conditions; 48ºC for reverse transcription for 30 minutes, 95°C for 15 minutes, followed by 40 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, with a final extension of 72°C for 5 minutes using an external set of primers, then the product subjected to two separate amplifications using A internal forward and C internal forward primer respectively and identical A/C internal reverse primer. Whereas, B-specific PCR products using external primers set was added to a second PCR using species B-specific internal primers. The second PCRs were performed with the same first PCR condition except using 55ºC as annealing temperature and running 35 cycles (Iwane et al., 2011). the primers were synthesized listed in table 3.3, and their positions represented in Figure 3.3.

Table 3.3: The sets of primers used for RV grouping.

Primer	**Sequence[2] (5' – 3')** [a]	**Position** [b]
A & C, External forward	GTA TTA AAT GAA GTT HTD SHD GTN CC	2360-2385
A & C External reverse	TTC ATA TCC ATC ATA RAA CAT RTA RTA	2938-2915
A, Internal forward	TTA GAT GCT GCT GAA ACN GGN CAY AC	2433-2457
C, Internal forward	AAT GCT CTG GGT GCT DTR GAR ATW GGD G	2398-2426
A & C, Internal reverse	TGG TGC CCC TGG TGG NAC RWA CAT	2791-2768
B, External forward	GAA TTG GAA GAA GTC ATT RTY GAN AAA	2342-2368
B, External Reverse	ATC ATC TGT GAG TAA CCR TCR TAR AA	2953-2927
B, Internal forward	AGC TTC TTG GGT AGA KCN GCN TGT GT	2534-2559
B, Internal Reverse	TGC ACC TGG AGG TAC ATA CAT DGC YTG	2802-2774

[a] IUB ambiguity codes: H=A,T,C ; D=A,T,G ; N=any base ; R=A,G ; Y=C,T ; W= A,T; K=T,G.

[b] Nucleotide numbering based on RV1B sequence (accession no. D00239) for species A, RV14 sequence (accession no. K02121) for B species and RVC strain 024 (NC_009996).

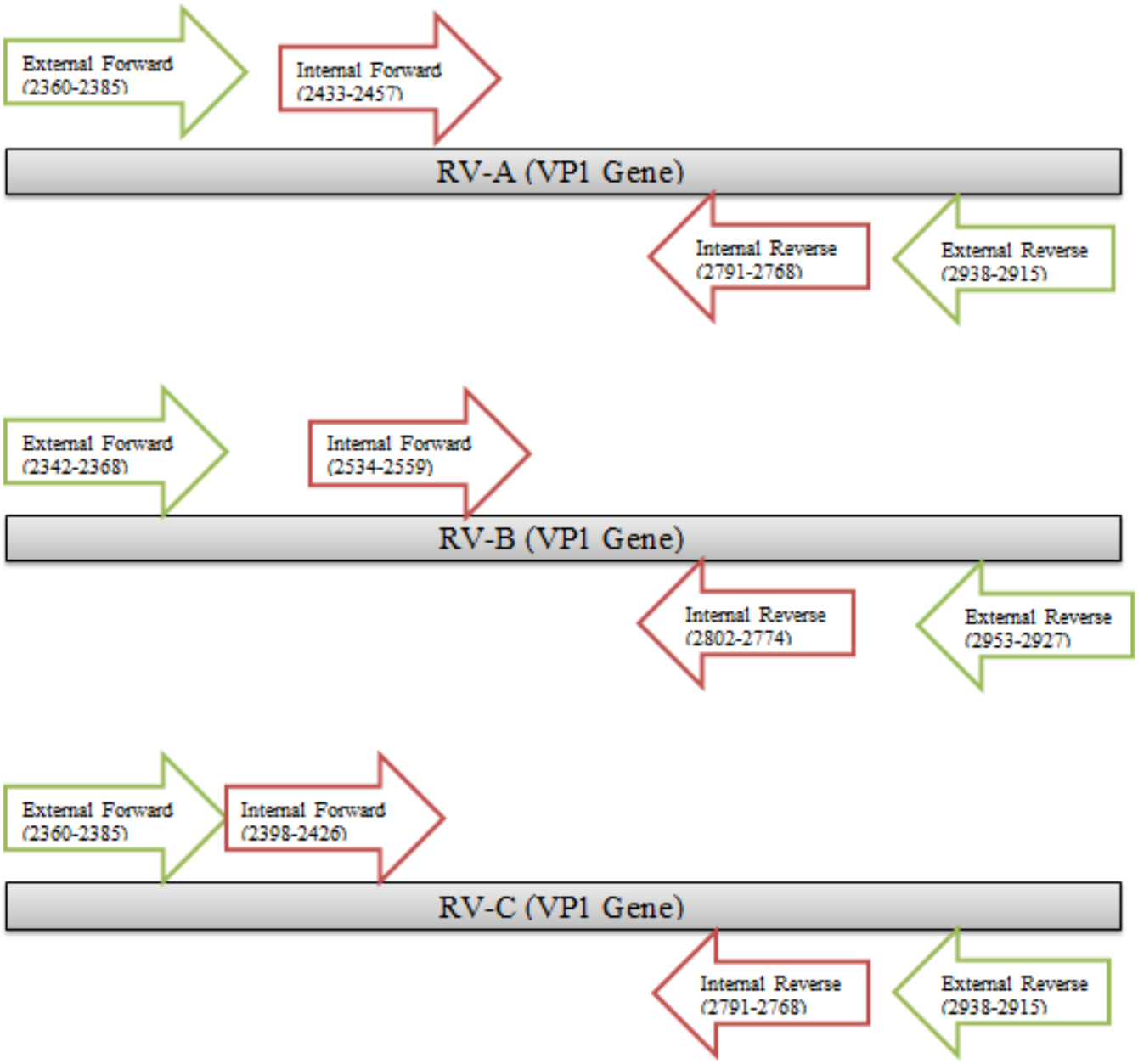

Figure 3.3: Positions of primers used for typing.

The PCR products were visualized by electrophoresis on a 1.5% agarose gel. Figure 3.4 shows band of RV-A typing products (358 base pairs).

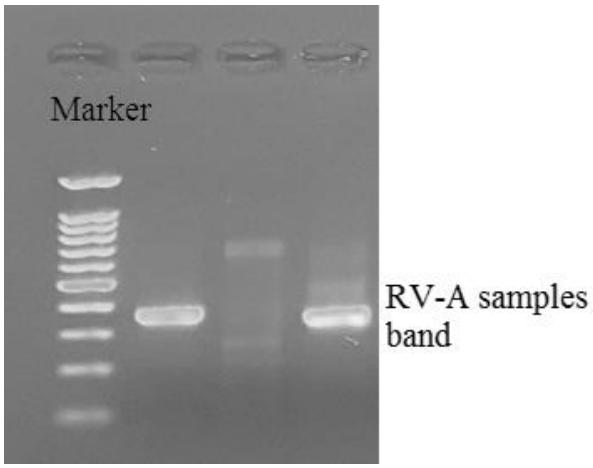

Figure 3.4: RV-A typing Gel

3.7 RV Genotyping by Either UTR or VP4/VP2 Partial Sequencing

RV (N=11) positive samples with a clear band from RV typing results (RV-A, B, and C groups) were randomly selected for genotyping by sequencing the appropriate gene. The extracted RNA of theses samples were used for PCR analysis. A conventional one-step PCR was performed for RV-A and RV-B, targeting 5' UTR, a 606 base pairs in length for RV-A, and 613 base pairs for RV-B (Lu et al., 2008) and semi-nested PCR was used for targeting the VP4-VP2 region of RV-C, a 542 base pairs in length (Iwane et al., 2011). Table 3.4 and figure 3.5 display the primers used for this step. The following conditions for conventional RT-PCR of RV-A and B were; 48^{o}C for reverse transcription, 95°C for 15 minutes, followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minutes, and 72°C for 1 minute with a final extension of 72°C for 5 minutes.

PCR cycling conditions for RV-C were; 48^{o}C for reverse transcription 95^{o}C for 2 minutes followed by 35 cycles of 95^{o}C for 30 seconds, 45^{o}C for 30 seconds and 72^{o}C for 30 seconds, with a final extension of 72^{o}C for 5 minutes. The primary product was then added to a semi-nested reaction containing semi nested forward and reverse primers. PCR cycling conditions were as above except using 50^{o}C as annealing temperature and running 30 cycles.

Table 3.4: The sets of primers used for sequencing

Primer	**Sequence[1] (5' – 3')**	**Position**
A, Forward	GTA CTC TGT TAT TCC GGT AAC TTT GYA YGC CA	49-80
A, Reverse	CCA ACA TTC TGT CTA GAT ACY TGD GCV CCC AT	655-623
B, Forward	ACT CTG GTA CTA TGT ACC TTT GTA CGC CTG TT	48-80
B, Reverse	CCA CTC TTC TGT GTA GAC ACY TGD GCD CCC AT	661-629
C, External forward	GGC CCC TGA ATG YGG CTA A	450-468
C, Semi nested forward	ACT ACT TTG GGT GTC CGT GTT TC	541-563
C, Reverse	GCA TCI GGY ARY TTC CAC CAC CAN CC	1083-1058

[1] IUB ambiguity codes: D=A,T,G ; R=A,G ; Y=C,T ; V= A,T.; I=inosine.

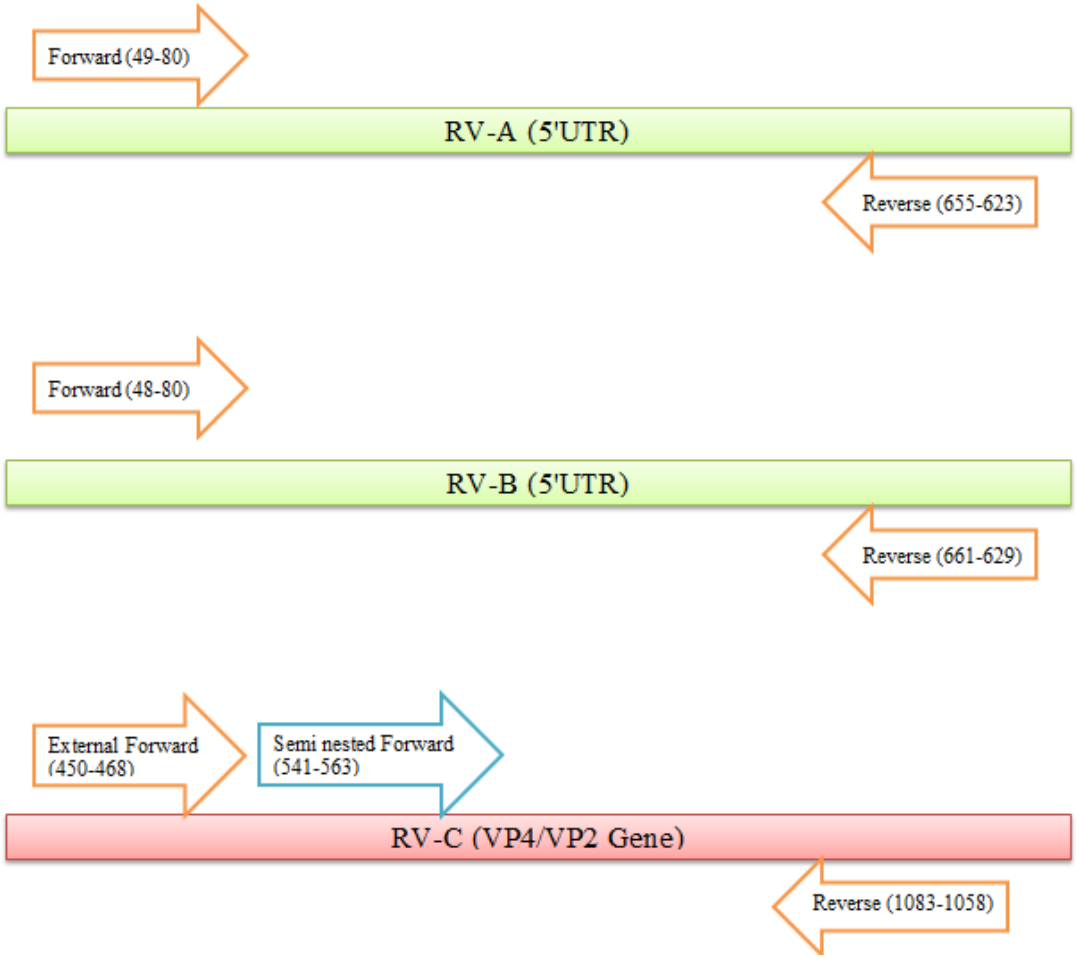

Figure 3.5: Positions of primers used for genotyping.

3.8 Band purification

After gel electrophoresis, the verified PCR products were sent for sequencing to Augusta Victoria Hospital Molecular Genetics Laboratory. Cleanup of the PCR products prior to sequencing was performed to eliminate primers, unincorporated dNTPs, DNA polymerases, and salts that would inhibit the downstream manipulations of products during sequencing. High Pure PCR Cleanup Micro Kit from Roche – Germany (Product number 04983955001) was used where nucleic acids were bound to the surfaces of glass fibers on the filter in the presence of salts. Therefore DNA amplicons were purified after several steps of washing and elution in a 50 µl of elution buffer at the end by the elution step. (Appendix 3).

Confirmation of the presence of the purified product was performed by running the purified amplicon on another agarose gel electrophoresis.

3.9 Sequencing

RV (N=11) samples were randomly selected from RV-A, RV-B, and RV-C. The samples were then purified and sent for sequencing to Augusta Victoria Hospital, Molecular Genetics laboratory. Sequencing was performed using forward and reverse primers (Table 3.4) using Applied Biosystems, 3500 gene analyzer, according to the manufacturer's instructions.

3.10 Sequence assembly and analysis

Sequences were aligned with the different RV references and cleaned using Sequencher program (Gencodes Corporation, Ann Arbor, MI). After sequence alignments, a FASTA text file was generated, and the program MEGA7 (manufacturer**)** was used to determine the genotypes that circulated in the Palestinian population. Phylogenetic trees were generated using the nearest neighbor analysis with 1,000 bootstraps. Phylogenetic trees were then visualized using the NJ plot.

CHAPTER 4

Results

This study was performed at Caritas Baby Hospital (CBH) in Bethlehem. The experimentation and findings were approved by the Caritas Baby Hospital Medical Research Committee (MRC-019). The study included 1,953 NPA specimen collected during the period January 2013 to December 2013. All of the samples came from hospitalized patients. All characteristics of the patient population size were well recorded. This included the patient's demographics, age, sex and infection. The study exclude 208 of 1953 patients samples; 31 had failed the internal control step as RNase P amplification was noted, 24 were from patients older than 14 years, and 3 samples from patients from Gaza and Israel. The other 150 samples were duplicate samples for children in less than a 30-day interval. In all, 1,745 samples were studied.

4.1 Rhinovirus frequency in Southern Palestine.

High percentage of RV infection was noted in our study population; 699 patient samples of the 1745 tested NPAs were diagnosed with RV infections, representing 40% of the samples. 21% of samples infected with other common respiratory viruses, while the rest of the patient samples (39%) could be infected with pathogenic bacteria or viruses types such as Bocavirus or Coronavirus. Depending on the number of hospitalized children at CBH, the frequency for overall RV hospitalization during the year was 49.5 episodes per 1,000 hospitalization days. Figure 4.1 shows the RV episodes to 1000 hospitalization days in each month during study period.

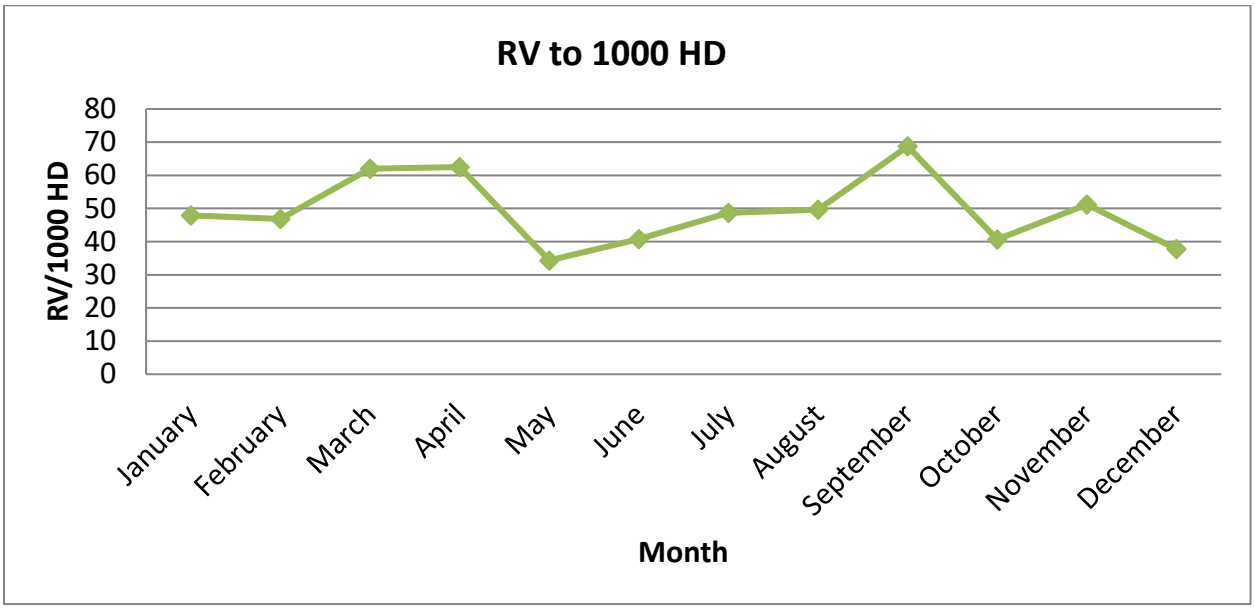

Figure 4.1: RV to 1000 hospitalization days

4.2 Geographical distribution

Table 4.1 shows that, the majority of samples tested and patients admitted to CBH came from southern Palestine, particularly residents of the Bethlehem district (50.7%) or the Hebron district (44.0%) and other minor districts (5.3%) including Jerusalem, Jericho, Ramallah, Nablus, and Jenin. A major cause of this distribution is the location of Caritas Baby Hospital in Bethlehem, a far distance for citizens of other parts of West Bank to visit. It should be noted that the census of Hebron in the study year was 662,454 and 204,929 in Bethlehem (Palestinian Central Bureau of Statistics).

Table 4.1: Geographical distribution of the study population and positive RV cases

	Bethlehem	Hebron	Jerusalem	Jericho	Ramallah	Nablus & Jenin	**Grand Total**
Positive RV	349	320	20	5	5	0	**699**
Negative	535	449	50	5	4	3	**1046**
Grand Total	**884**	**769**	**70**	**10**	**9**	**3**	**1745**

As illustrated in Figure 4.2, 349 (49.9%) samples of Bethlehem were positive for RV, while 320 (45.7%) out of 699 samples from Hebron were also positive for RV.

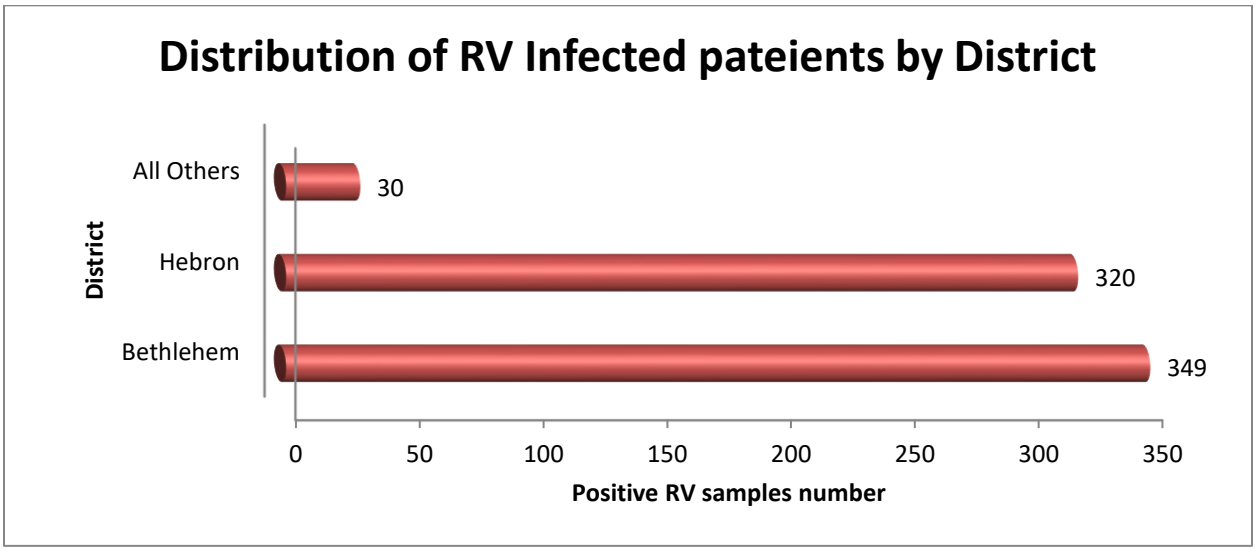

Figure 4.2: Distribution of RV infections by District.

Within each district, the distribution of RV infection is determined in villages and camps. The main areas where RV circulated during the study period in Hebron District were from Hebron, Sourif, Doura, and Beit Ummar cities as well as Bethlehem, Beit Jala, Dheisheh and E'beidiyeh cities in Bethlehem district as shown in Figure 4.3.

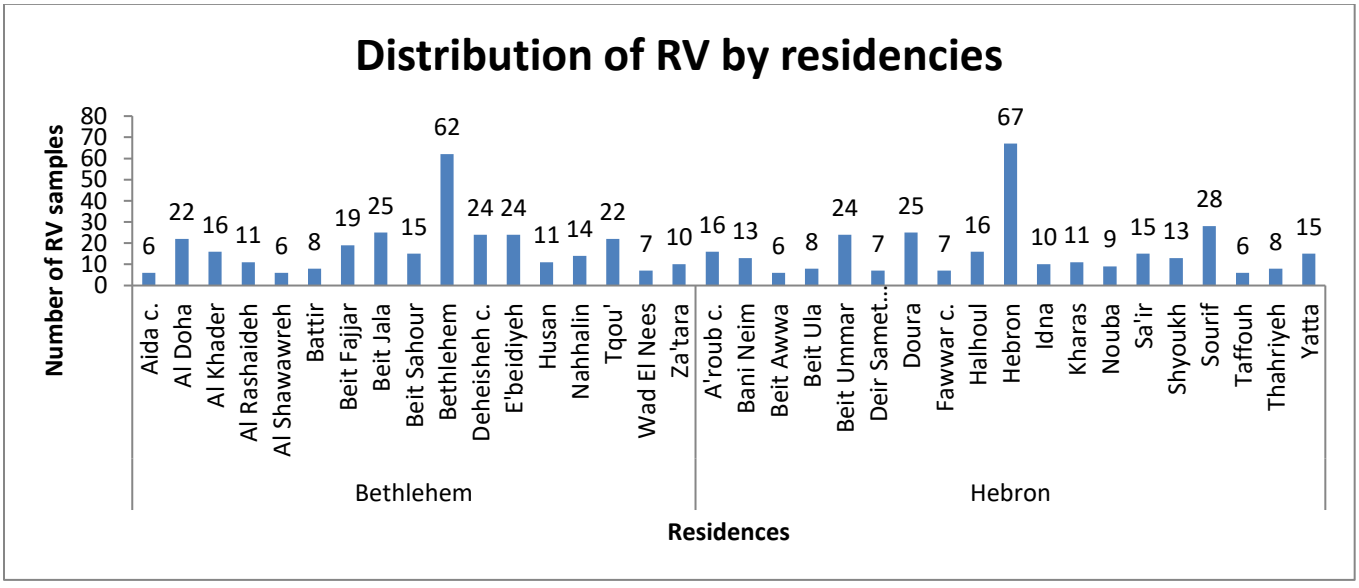

Figure 4.3: Distribution of RV infection by main residencies.

4.3 Distribution by age

One of the important determinants in this study was the age group of the patients. Age was considered an important variable that influences the results of RV infection. In this study, patients were grouped into 6-month interval age groups as shown in Table 4.2. Age group 1 includes patients in their first 6 month of life, Age group 2 includes patients in their second 6 months of life.

The patients' ages were from one day to less than 14 years. Stratifying the patients by age group revealed that most of the patients were less than three years old (90.2%).

Table 4.2: Distribution of study population and RV infection by age.

Age group (6 months intervals)		No. of samples	Positive	RV Percent per each age	RV Percent per total positive
Group 1	Up to 06 months	825	333	40.36%	47.28%
Group 2	06 to 12 months	360	152	42.22%	20.63%
Group 3	12 to 18 months	177	70	39.55%	10.14%
Group 4	18 to 24 months	102	48	47.06%	5.85%
Group 5	24 to 30 months	63	26	41.27%	3.61%
Group 6	30 to 36 months	47	16	34.04%	2.69%
Rest	Over 36 months	171	54	31.58%	9.80%
	Total	1,745	699		100.00%

RV infection was found mainly in younger infants. Almost 67.9% of tested samples are infected in their first year. When comparining the RV positivity rate of each age group, the RV positivity rate ranged between 31.58-47.06% (Table 4.2), mainly seen in the 18-24 months of age (Figure 4.4).

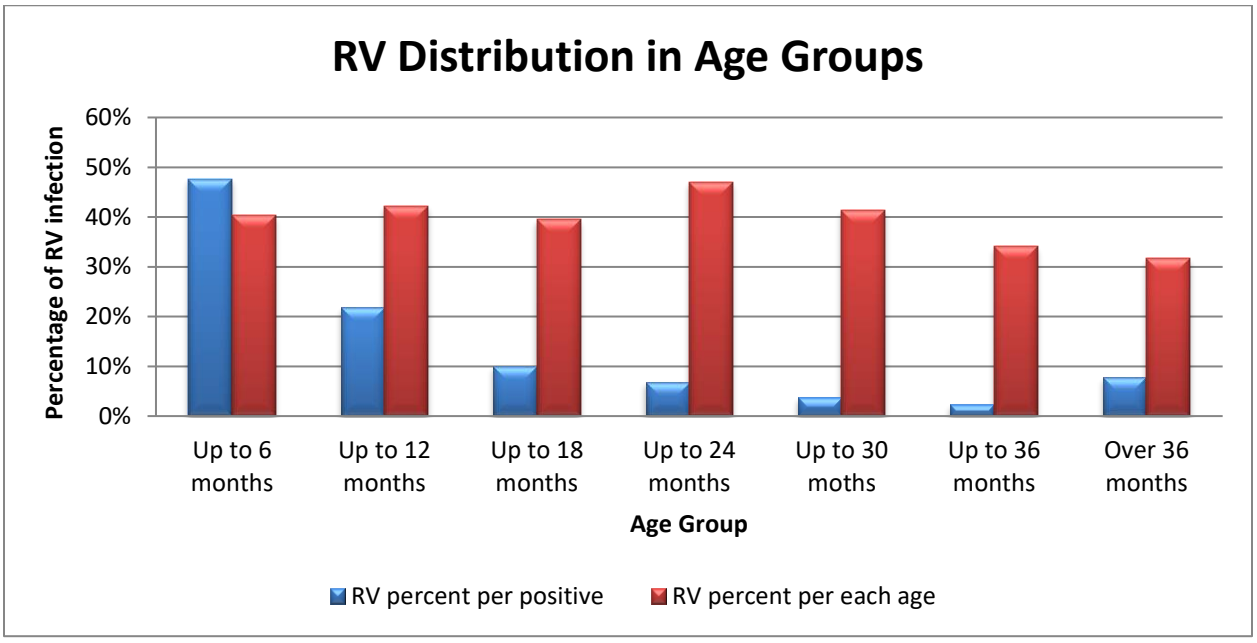

Figure 4.4: RV infection by age.

4.4 Distribution by Gender:

The male patients comprise 63% of study period population, while 37% were female. After analyzing the RV positive cases by gender, the RV male cases were 65% in comparison to female with 35%. Evaluating the percent RV infections in the male and female groups revealed that the male to female ration 1.1:1 (Table 4.3).

Table 4.3: Rhinovirus infection numbers in gender.

RV infection	All samples	Positive	Negative	Percent positive from each gender
Female	653	247	406	37.83%
Male	1092	452	640	41.40%
Total	1,745	699	1,046	40.00%

4.5 RV seasonality

Overall, NPA tested from patients admitted to the hospital due to a RV infection were distributed by months as in table 4.4, which shows that the RV infection increased during the autumn and spring seasons, and decreased prevalence during the summer in accordance to the year.

Table 4.4: Rhinovirus distribution in seasons.

Season	Positivity
Winter	%23
Spring	%30
Summer	%20
Autumn	%26

According to Figure 4.5, the highest activity of RV infection was in September with 61.7% of the tested samples in this month were positive for RV. This was followed by 52.7% in July, and 49.5% in March.

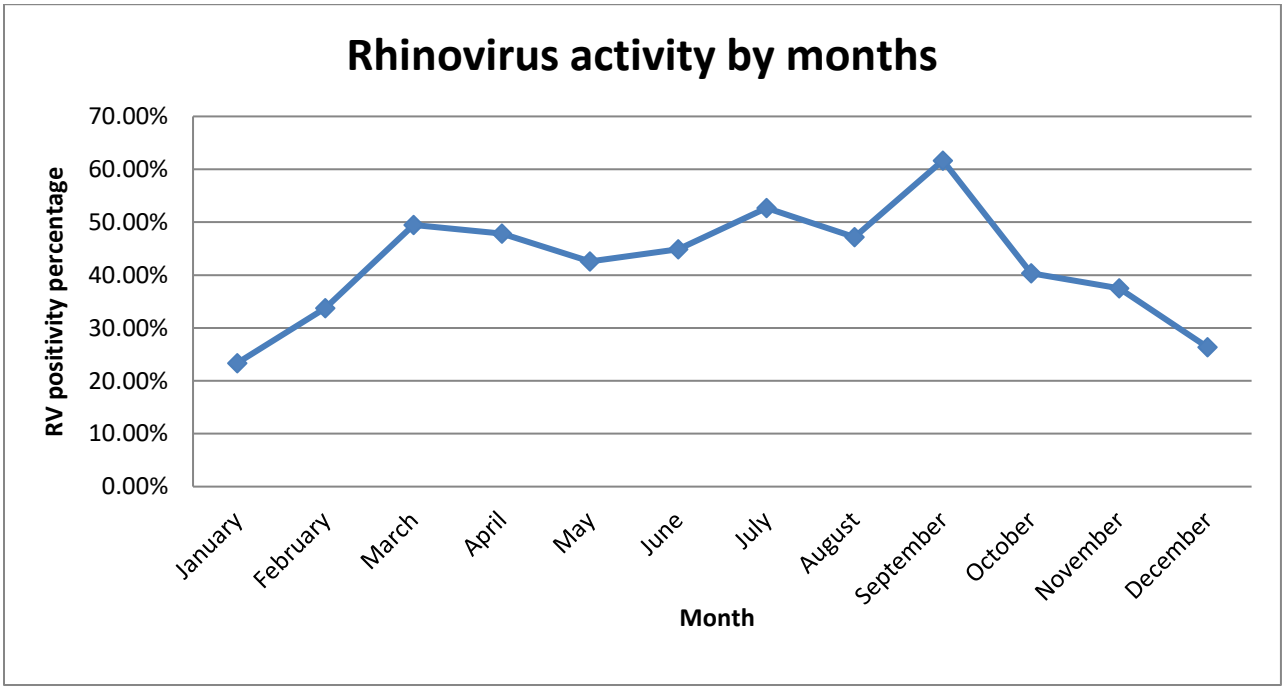

Figure 4.5: RV activity by month.

4.6 Recurrence

Of the 699 RV infected patient samples which were collected from 658 patients, 41 samples (5.9%) were from recurrent RV infections. Of this 41 samples, 36 had 2 episodes while 5 patients had three episodes. Of the 36 patients, 28 (77.8%) were male and 8 (22.2%) were female. While, 27 (75%) of those patients were less than one year old.

4.7 RV infection and Co-infection

All NPA collected in the hospital were routinely investigated for a panel of 8 respiratory viruses including influenza A and B viruses, RSV, parainfluenza 1,2 and 3,adenovirus and hMPV. There were 479 (27%) samples infected by one of these viruses within the study population. Results are shown in Table 4.5.

Table 4.5: Results of routine DFA.

DFA result	**Number of patients**
Adeno	53
Adeno + hMPV	3
hMPV	59
Influenza A	37
Influenza B	9
Parainfluenza 1	28
Parainfluenza 2	3
Parainfluenza 3	45
RSV	237
RSV + Influenza A	4
RSV + Parainfluenza 1	1
Grand Total	**479**

Depending on the results of DFA analysis, 109 (15.6%) of 699 RV-infected samples were co-infected with other respiratory viruses. As illustrated in Figure 4.6; RSV was the most frequent respiratory virus detected; 53 of 109 co-infected patients. The least detected pathogen was parainfluenza virus 2 and influenza B, two cases each. Whereas one patient had triple infection with RV, RSV, and influenza A.

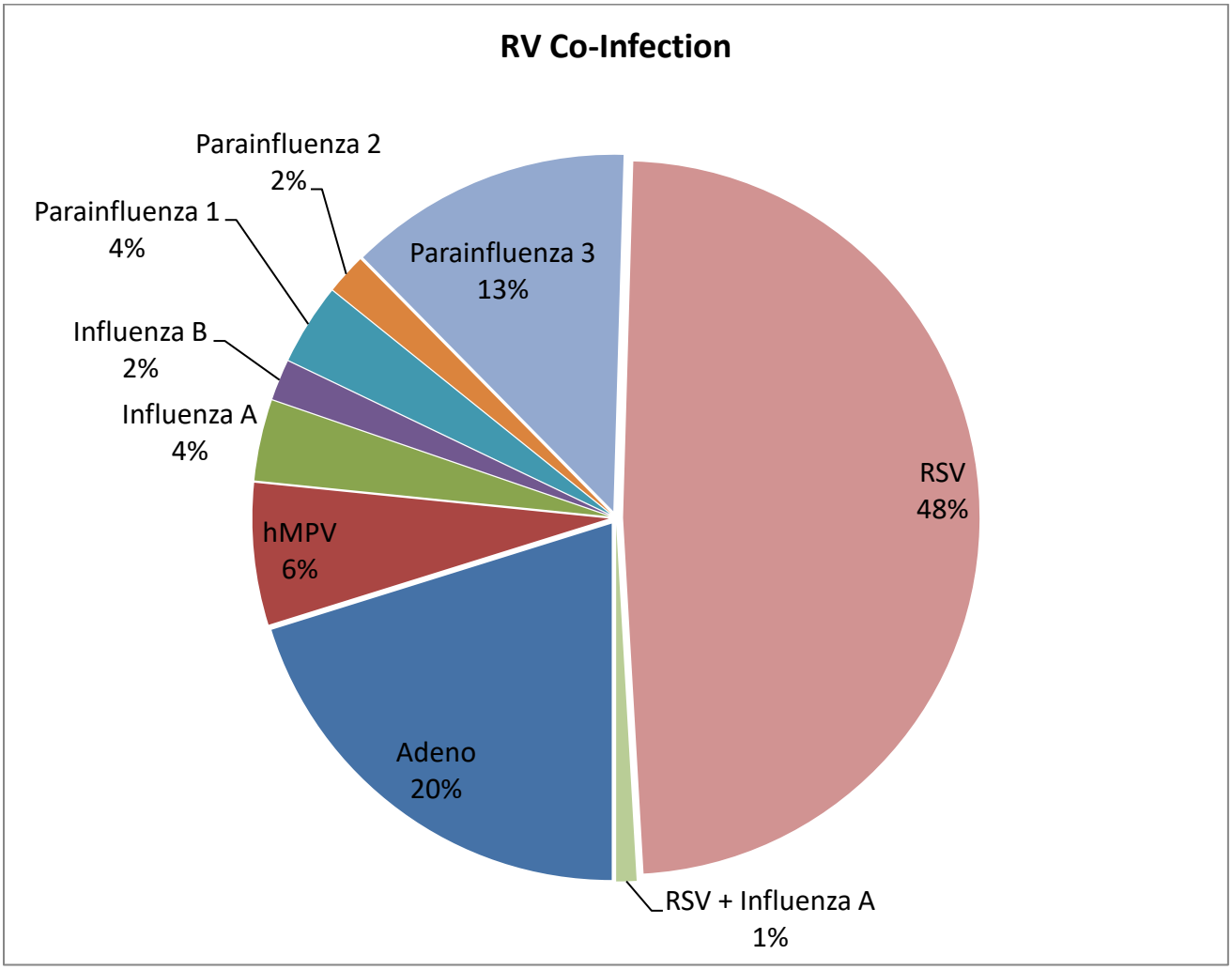

Figure 4.6: Co-infection viruses with Rhinovirus.

The predominance of RV RSV co-infection was limited in the winter and less in the spring, while showing a disappearing during the rest of the year, as shown in Figure 4.7.

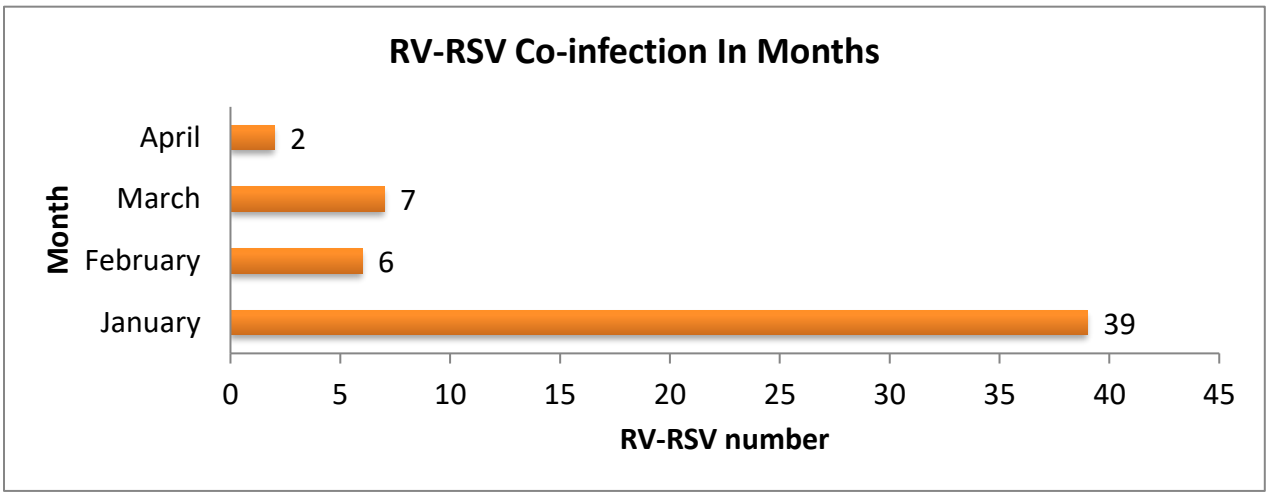

Figure 4.7: RV and RSV co-infection in the first four months of the year.

4.8 RV Typing

In an attempt to determine the presence of RV circulating groups, a representative sample (26%) of positives RV samples were analyzed using the conventional RT-PCR method. This analysis was conducted on 184 samples out of the 699 positive RV samples. Group A was found in 122 out of 184 samples, group C was found in 28 samples, and Group B was found in 19 patients. The other 22 samples failed to be grouped. Results shown in the Figure 4.8.

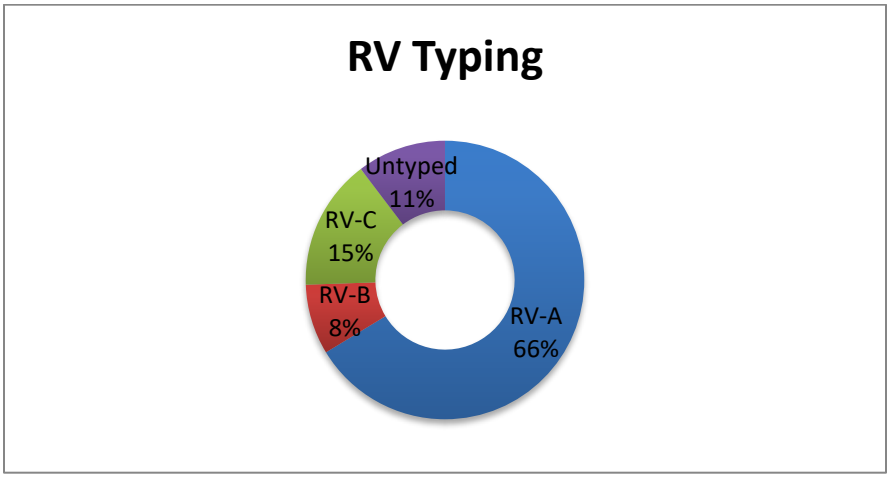

Figure 4.8: The percentage of RV groups.

4.9 Phylogenetic Analysis of RV A, B and C

Of the 699 RV strong positive samples, 11 (1.6%) were chosen randomly and subjected for sequencing. Figures 4.9, 4.10, 4.11 and 4.12 show the reference genomes in which samples belong.

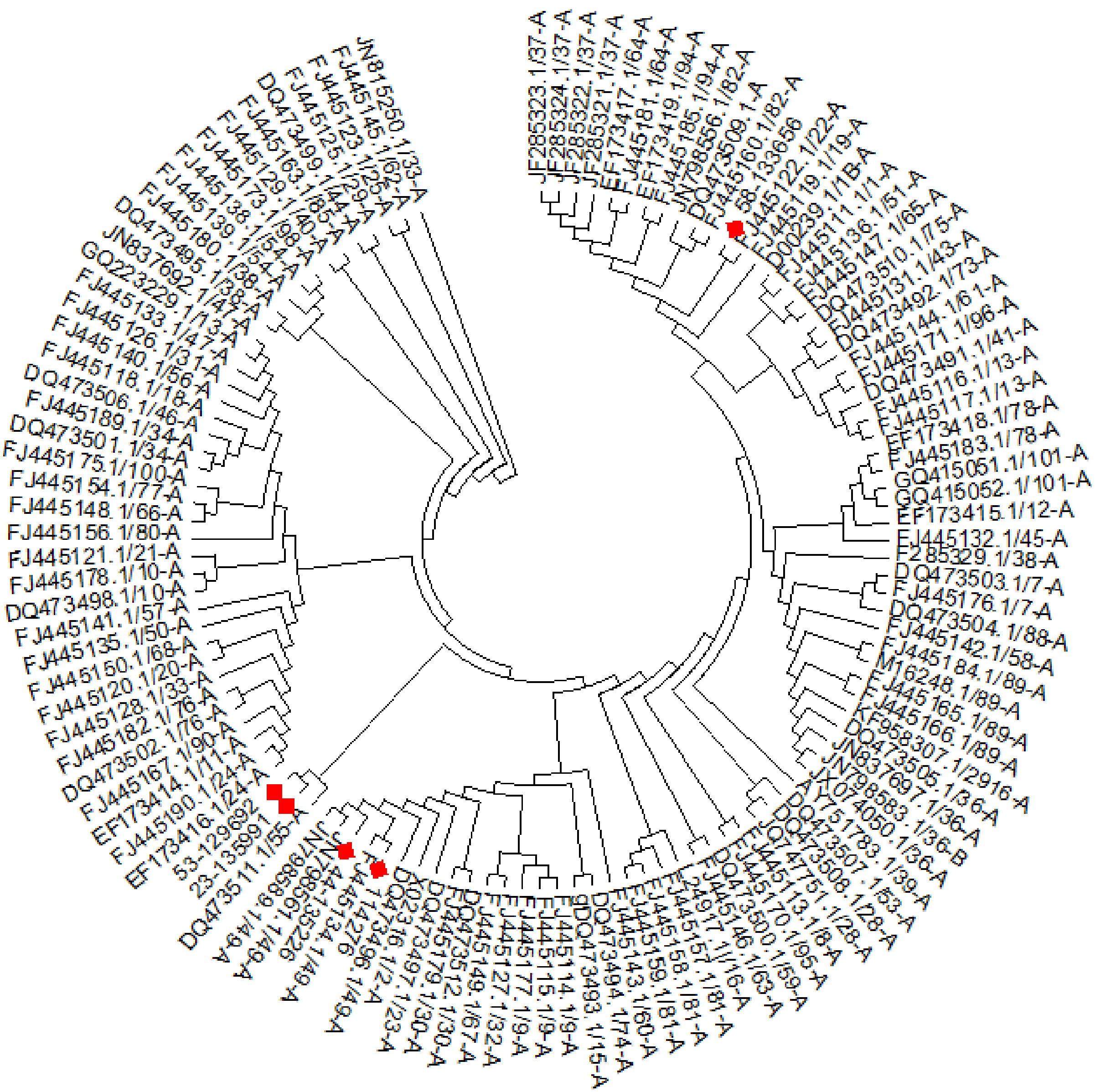

Figure 4.9: Circle phylogenetic tree of 613 base pairs of 5' UTR sequences of RV-A. The phylogenetic tree was constructed by means of the neighbor joining method. Reference strains of each species were obtained from GenBank, and the isolates are indicated by red rhombus.

Figure 4.10: A zoom of RV-A phylogenetic tree showing the isolates indicated by red rhombus and reference genes their belong.

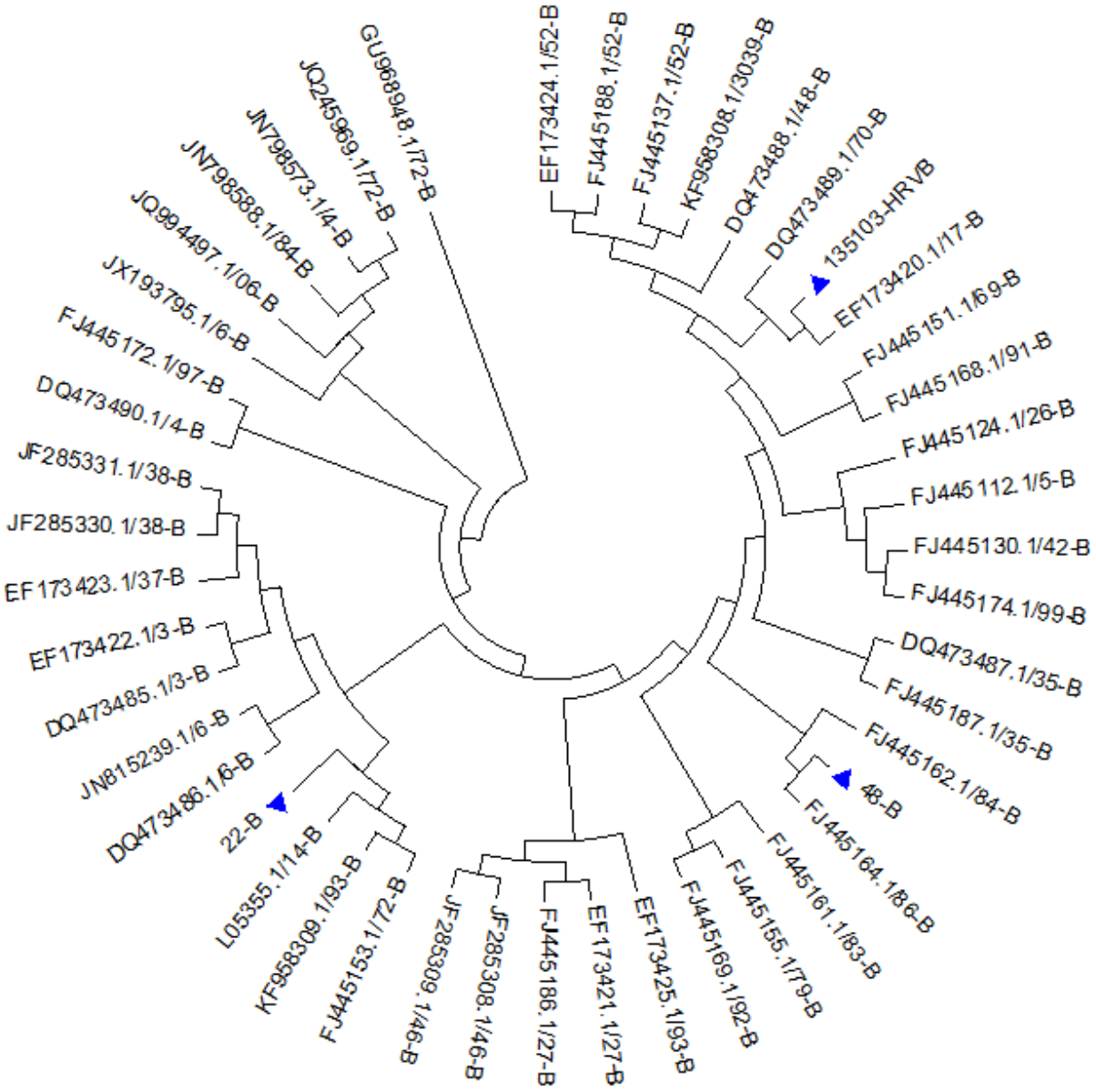

Figure 4.11: Circle phylogenetic tree of 613 base pairs of 5′ UTR sequences of RV-B. The phylogenetic tree was constructed by means of the neighbor joining method. Reference strains of each species were obtained from GenBank, and the isolates are indicated by blue triangle.

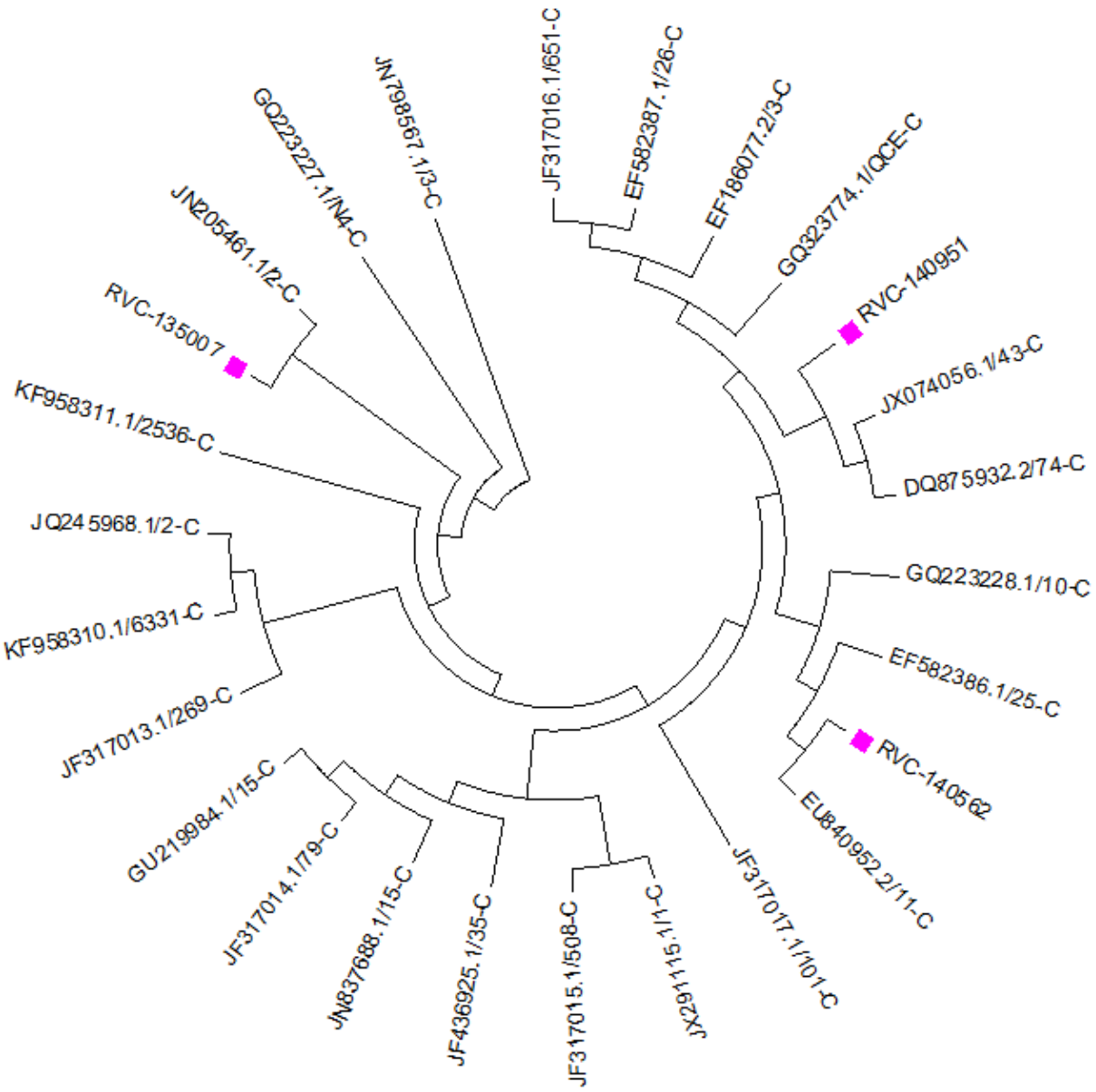

Figure 4.12: Circle phylogenetic tree of 542 base pairs of VP4/VP2 sequences of RV-C. The phylogenetic tree was constructed by means of the neighbor joining method. Reference strains of each species were obtained from GenBank, and the isolates are indicated by pink squares.

CHAPTER 5

Discussion

Over the recent years, RV has gained wider recognition as clinically relevant pathogens causing not only mild respiratory infections, but also severe respiratory disease have come to a rise. RV causes two to three self-limited episodes of the common cold per person each year (Hershenson, 2010). The infection is associated with recurrent episodes of wheezing, asthma, and severe lower respiratory disease. RV is a leading cause of respiratory virus infections causing hospitalizations in children: 25-50% of children with respiratory symptoms test positive for RV (Van der Zalm et al., 2011; Legg et al., 2005). RV exacerbates chronic respiratory diseases and increases morbidity rates in the elderly (Gerna et al., 2009; Jartti and Korppi, 2011; Longtin et al., 2010a). Viral infections cause 85% of asthma exacerbations in children, and RV infections are responsible for 50-80% of these exacerbations (Kelly and Busse, 2008; Wark et al., 2005). RV infection is associated with asthma exacerbation; a single report in southern Palestine indicated this association to be stronger than that of asthma and hMPV infection (Rishmawi, 2007). Surveillance of long-term care facilities in Ontario from July 1 to December 31 2009, found that of 297 respiratory outbreaks, RV was identified in 59% (Longtin et al., 2010b).

Rhinovirus is the most frequent cause of common cold infections worldwide. This study presents an overview of the molecular epidemiology of RV in respiratory samples from children admitted to CBH, Palestine during 2013. To our knowledge, this is the first study of RV epidemiology among Palestinian patients. Due to the laboratory diagnosis of RV infection is generally not available in clinical virology laboratories; its impact on health is often ignored.

RVs are not usually diagnosed by clinical laboratories. Traditional detection methods (i.e. antigen detection) are not feasible due to the large number of genotypes. Recently, molecular assays targeting the 5' UTR and commercial multiplex assays detecting human enterovirus (HEV) and RV have been described (Gambarino et al., 2009; Jokela et al., 2005; Lu et al., 2008). Molecular tests targeting the conserved 5' UTR have improved sensitivity and increased the coverage of picornaviruses. However, they do not distinguish between HEV and RV (Mahony, 2008). Not all resources, such as locked

nucleotide analog (LNA) primers which stabilize melting temperatures and allow for shorter primer sequences are available to everyone as there are limited manufacturers limiting the possibilities of developing RV-specific assays (Lu et al., 2008). qPCR assays have recently been developed for some respiratory viruses including RV (Hohaus et al., 2011; Ward et al., 2004). They are used to assess responses to antiviral agents and to predict patient outcomes (Franz et al., 2010; Utokaparch et al., 2011).

From an economic standpoint, this disease is seen as an illness episode and often does not require a physician visit, which may be burdensome for some families. Previous studies report that rhinovirus infection is associated with a high annual number of days of patients with respiratory symptoms, frequent OTC medication use, and half of all parental work days lost due to children's acute respiratory infections (Toivonen et al., 2016).

In this study, during the 12-months period, 40% of samples were diagnosed with RV infections N=699 of the 1745 tested NPAs, representing 49.5 rhinovirus per 1000 hospitalization days. Few studies in the Middle East have reported RV infections. In 2008, Rashid et al. detected RV in 13% (19/150) of UK pilgrims and 3% (3/110) of domestic pilgrims with URI in Mecca, Saudi Arabia. Also, In Jordan, Kaplan et al. identified RV in 11% (36/325) of hospitalized young children in 2008. We enrolled a larger cohort than these studies and discovered a greater burden of RV-associated illness in young children, likely because we used highly sensitive real-time RT-PCR (Lu et al., 2008).

The rhinovirus infection is most frequently found in children (Vesa et al., 2001), and its infection is typically transmitted from school-aged children to other family members via direct contact (Hendley, Gwaltney Jr and Jordan, 1969). We found that the prevalence of rhinovirus was the highest at 18-24 months of age with 47.06%, whereas it decreases down (31.5%) in patients over 36 months of age. Meanwhile, other studies have reported slightly different results. In Italy, for example, 31.1% of the children admitted to the emergency room with RV infection were aged less than 1 year old, 71 (47.0%) were aged 1-3 years, and 33 (21.9%) were aged 4 years or more (Daleno et al., 2013).

The majority of RV infected samples were proved in the current research to be male patients, and by evaluating the percent infections in the male and female groups revealed that the male to female ratio 1.1:1. The ratio may be a risk factor for other infections as Fish (2008) explain that male children are

more susceptible to severe disease infection than females, due to that females have stronger humoral and cellular immune responses to infection or antigenic stimulation than do males

Lau et al. (2010) in a review article indicated that RV infection occurs throughout the year, but pronounced seasonal patterns are varied geographically in different countries or regions depending on the type of the climate, and usually peaked in fall or winter in most temperate or subtropical countries. The incidence of rhinovirus infection peaks during spring 30% followed by autumn 26% of total infected patients. The overall rates of respiratory illness are low in summer, but rhinovirus is also isolated during the summer months and responsible for most of the illness with 48% of admitted patients in this period. This is accordance with previous reports (Wildenbeest et al., 2016).

Rates of RV infection were approximately threshold higher during peak prevalence months in September, April, and March (68.7, 62.5, 62 infections per 1000 hospitalization-days, respectively), compared with December and may (34.4, 37.8 infections per 1000 hospitalization-days). But the activity of the RV showing the highest in September with 61.7% positivity of collected samples in this month. This is similar to Markovich et al. (2015) when a study in Israel found that rhinovirus was the predominant in September causing back-to-school illness consisting of URI. Also in Israel, Scheuerman et al. (2009) found that there is a characteristic increase in asthma exacerbations and admissions in September in the pediatric age group; this might be explained by the increased exposure to respiratory viruses as rhinovirus. Other international studies showed that there are no RV seasonal variations (Fujitsuka et al., 2011; Kiyota et al., 2013).

Khatib (2006) had investigated some of the housing conditions at Jalazone Refugee Camp in Palestine and their impact on refugee's health inside the camp, especially those with respiratory symptoms and diseases by selection of randomized sample of 200 housing units, representing one fifth of the camp’s population, approximately half of the people in the sample were under 15 years. Depending on statistical data Khatib found that there is a significant relationship between common cold which caused by mainly by RV, spread by cough, close personal contact, and nasal mucous on the hand, and the transmission facilitated by overcrowding, poor ventilation, and conditions of houses. This might explain the high RV infections in Bethlehem and Hebron. (Khatib, 2006).

Common human respiratory viruses were detected by DFA in 479 (27%) of 1745 samples obtained. Overall collected samples, RSV were detected in 13.6%, hMPV in 3.4%, and adenovirus in 3%. Only 109 (15.6%) of the rhinovirus positive children were co-infected with another virus; the most frequent was 53 RSV co-infection, then 22 patients was co-infected with adenovirus. 90.1% of children with coinfections were less than 24 months. RSV coinfection children was limited to around 1 year age (43% of all co-infections) and mainly in winter months. This is consistent with other studies (Johnston, 1999; Rawlinson et al., 2003). There are few studies focused on coinfections between RSV and RV and the number of patients analyzed is still small. A previous study shown that coinfections had significantly more proportion of fever, and also more proportion of hypoxia than single infections of RSV or RV (Calvo et al., 2015). Other one shown that RV single infections were less severe than RV plus RSV coinfections. RV requiring less proportion of hospital admission, also patients with single RV were older than coinfections, and the most frequent diagnosis was recurrent wheezing (Costa et al., 2014).

After the analysis of 26% of the positive RVs; 66% belonged to RV-A being the most prevalent species, followed by 15% of RV-C, however we only 15 cases of 184 typed samples belonged to be RV-B, the failure to type the 22 samples could be due to the primers mismatches that mandate further future investigations. These results suggest that RV-A may be more prevalent than RV-C and RV-B in our population, and there are various types of RV-A and C strains which may be associated with various respiratory infections and higher hospitalization rate in southern Palestine, whereas RV-B seemed to be less virulent. These findings are similar to a study from the Middle East in Cyprus, from a total of 116 rhinovirus-positive samples, 68 samples were typed finding that 52.9% of samples were identified as RV-A and 39.7% as RV-C, with only 7.4% samples belonging to the RV-B species(Richter et al., 2015). These findings are similar to those reported in various countries (Mak et al., 2011; Lee et al., 2012; Franco et al., 2012; Pierangeli et al., 2013; Rahamat-Langendoen et al., 2013).

Based on the nucleotide sequences and phylogenetic tree of few samples, RV isolates belong to the A55, which was sequenced in 2007 in the USA (Kistler et al., 2007), A49 sequenced in 2009 in USA (Liggett et al., 2014), A22 and B86 sequenced in 2009 in USA (Palmenberg et al., 2009). B14 sequenced in 1993 in USA (Lee, Monroe and Rueckert, 1993), B17 sequenced in 2007 in Switzerland (Tapparel et al., 2007), C39 sequenced in 2011 in Australia, C11 sequenced in 2009 in Switzerland (Tapparel et al.,

2009), and C43 sequenced in 2014 in USA (Liggett et al., 2014). Data suggests that we have multiple types of rhinovirus and we don't have an outbreak of one single genotype

As illustrated in the literature review, there is no approved treatment for rhinovirus, so the inappropriate use of antibiotics for viral common colds is an important problem. Holding the spread of resistant bacteria by reasonable prescription of antibiotics has become an important target of medical organizations worldwide (Brandileone et al., 2006, Higashi and Fukuhara, 2009).

According to the highly rhinovirus infection in our population and the antibiotic over usage by people which is mentioned in the literature review, patients should be advised and educated about the existence of bacterial and viral infections and also that the antibiotics would be of no use in a viral illness and these drugs misused could become ineffective, even for a bacterial infection. Patient education can help in reducing the drug resistance problem. Also, the drug regulatory authorities must take adequate steps to curb the OTC availability of antibiotics and empirical use of broad spectrum antibiotics in URTIs.

CHAPTER 6

Conclusion

›

› This first report of the clinical and molecular epidemiology of RV infection in the Palestinian territories is considered a breakthrough that was not researched before of RV infection. Our data is the first to show the widespread infection of RV in the southern part of Palestine (40%). RV was mainly seen in the spring season in children less than one year of age. RV-Group A was the most common type of the virus seen in Palestine. The data suggests that we have multiple genotypes of rhinovirus and we don't have an outbreak of one single genotype. Such results serve as an important indicator in evaluating the role that RVs play in mediating asthma and sinusitis in Palestinian children. The high prevalence of rhinovirus in the community mandates that antimicrobial stewardship programs must be implemented in order to prevent the emergence of antibiotic resistant bacteria.

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Appendices

Appendix 1:

RNA extraction

Protocol:

Kit used: High Pure Viral RNA Kit (Cat # 11858882001; Roche Applied Science)

Usage: The High Pure Viral RNA Kit is used to purify viral RNA from serum, plasma, NPA and other body fluid samples.

Kit contents:

A- Ready to use contents is as the following:

- Binding Buffer (Green cap)
- Elution Buffer (White cap)
- High Pure Spin Filter Tubes
- Collection Tubes

B- 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 1

3. Inhibitor Removal Buffer (Vial 3; 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 4; blue cap)

- Add 40 ml ethanol 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

Procedure:

- Thaw one vial of 50 µl poly A carrier RNA for 12 preparations.
- Mix thoroughly with 5 ml Binding Buffer (vial 1).
- Aliquot 400 µL Binding buffer supplemented with Poly-A in separate Eppendorf tubes.
- Add 200 µL from each NPA sample to the mixture, mixed gently to avoid the aerosols.
- Add to the upper reservoir of High Pure Filter Tube.
- Centrifuge at 8000g (approximately 10,000 rpm) for one minute.
- Transfer the filter to another collection tube.
- Add 500 µL of inhibitor removal buffer to the filter.
- Centrifuge at 8000g for one minute.
- Transfer the filter to another clean collection tube.
- Add 450 µL of wash buffer.
- Centrifuge at 8000g for one minute.

- Repeat the wash buffer step one more time, at the end of this step the filter was centrifuged at the maximum speed at 13,000g for 15 seconds to dry the filter.
- In the last step, transfer the filter to an Eppendorf tube.
- Add 50 µL of the elution buffer.
- Soaked for 30 seconds and centrifuged at 8000g for one minute.
- The collect is in the Eppendorf tube.

Appendix 2:

Agarose gel electrophoresis to verify PCR products

Buffer preparation: A stock solution of 10% Tris Borate buffer is prepared by adding the following ingredients to 1000 ml distilled water to reach pH of 8.4; Trizma 121.1 g/L, Boric Acid 55.65 g/L, EDTA 3.725 g/L.

Buffer dilution: The 10% TBE is diluted to 1X with distilled water as 100 ml of 10% TBE is added to 900 ml DW in a well labeled bottle.

Gel preparation: 1% Agarose gel is used and prepared by adding 1 gram of Ultra-pure Agarose powder from Invitrogen (catalogue no. 15510-027) into 100ml (1X TBE) in an Erlenmeyer flask. The Agarose – buffer mixture is melted by heating in a microwave; the flask is swirled every 30 seconds until the Agarose has completely dissolved. The flask is allowed to cool on the bench top, then one drop of Ethidium Bromide solution from Thermo scientific (product no. 17896) is added.

The Electrophoresis unit (Scie Plas model): The gel tray is placed into the casting apparatus. The comb is placed into the gel mold to create the wells. The Agarose is poured into the tray and set to cool at room temperature. After 20 minutes, the comb is removed, and the tray holding the gel is transposed in the apparatus and covered by 1X TBE.

Gel apparatus set up

2.5 µL of the loading buffer (Invitrogen catalogue no. 1317514) is added to 5 µL of each PCR product. Since the loading buffer is heavy, it allows the DNA to sink in the wells and facilitates the DNA track upon travelling. The PCR products combined with the loading buffer is loaded into the wells; a ladder, DNA marker is loaded in the first well to mark the DNA size. The leads of the gel box are attached to the power supply by connecting the cathode (black) closer to the wells than the anode (red). The power is turned on at a voltage of 85 for 45 minutes. During that time, the migration is checked frequently and at the end, the power is turned off, the gel box is removed, and the gel tray is removed. Excess buffer is drained off and the tray is put on tissue to absorb the excess buffer. The gel is removed, exposed to UV light and scanned by the transillumniator from DINCO & RHENIUM Ltd industries before the gel is documented.

Appendix 3:

PCR product clean up

High Pure PCR Cleanup Micro Kit is used.

Kit contents: Binding buffer, Elution buffer, High pure micro filter tube, collection tubes and wash buffer. All the components are ready to use except for the wash buffer to which 20 ml of pure ethanol must be added before use.

Procedure

The PCR product is transferred to an eppendrof tube and the volume is completed to 100 µL with the elution buffer, 400 µL of the binding buffer is added with good mixing. The mixture is added onto High pure filter tube and centrifuged at 8000g for 30 seconds then the filter is transferred to another collection tube. The wash buffer is added in two steps 400 µL and 300 µL respectively and each step is centrifuged for 8000 g for 30 seconds followed by discarding the flow through and changing the collection tubes. The second wash step is followed by drying the filter by centrifugation one more time for a minute. The filter at a final step is soaked with 50 µL of the Elution buffer for 30 seconds and centrifuged at 8000g for one minute then the purified PCR product is collected in the eppendrof tube saved at -30C with an appropriate labeling.