



**Clinical Laboratory Science Program**

**Molecular Detection and Characterization of Human  
Metapneumovirus (hMPV) in Hospitalized Children Residing in  
Southern Palestine**

By

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Supervisors

Dr. Musa Hindiyeh

Dr. Mohammad Farraj

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To Emili Rishmawi, my beloved mother

*“Mother, no matter what I do, I will never be able to express my  
gratitude to you ... Thank you!”*

# TABLE OF CONTENTS

<b>Acknowledgments</b>	<b>VII</b>
<b>List of Tables</b>	<b>VIII</b>
<b>List of Figures</b>	<b>IX</b>
<b>List of Abbreviations</b>	<b>X</b>
<b>Abstract</b>	<b>XI</b>
<b>Abstract</b>	<b>XII</b>
<b>1. Introduction</b>	<b>1</b>
1.1 Background	1
1.2 The classification of hMPV	3
1.3 hMPV Proteins	4
1.4 Current Model for Transcription	7
1.5 Current Model for Genome Replication	10
1.6 Packaging and Assembly	12
1.7 Taxonomy	12
1.8 hMPV Lineages	14
1.9 Epidemiology	17
1.10 Pathogenesis	18
1.11 Diagnosis	22
1.12 Treatment	25
1.13 Vaccination Against hMPV	26
<b>2. Materials and Methods</b>	<b>27</b>
2.1 Study Location	27
2.2 Patient Demographics and Study Period	27
2.3 Patients Samples Collection and Preparation	28
2.4 Direct Fluorescent Antibody Staining (DFA)	30
2.5 Identification of Positive Cells	30
2.6 Virus and Bacterial Stocks	31
2.7 RT-PCR Sensitivity	31
2.8 Molecular Diagnosis	32
2.9 Phylogenetic Analysis	35

<b>3. Results</b>	<b>36</b>
<b>3.1 Validation of an RT-PCR Assay for the Detection of hMPV RNA</b>	<b>36</b>
<b>3.2 Specificity of the hMPV NL-N RT-PCR Assay</b>	<b>38</b>
<b>3.3 Evaluation of the hMPV NL-N RT-PCR Assays Sensitivity</b>	<b>39</b>
<b>3.4 Prevalence of hMPV RNA in Patient Samples</b>	<b>39</b>
<b>3.5 Distribution of hMPV RNA in the Southern Palestinian Districts</b>	<b>40</b>
<b>3.6 Distribution of hMPV Positive Patients by Cities and Villages</b>	<b>42</b>
<b>3.7 Distribution of hMPV by Gender</b>	<b>42</b>
<b>3.8 Distribution of Positive hMPV Patients by Age</b>	<b>43</b>
<b>3.9 Distribution of Positive Patients by Month</b>	<b>44</b>
<b>3.10 The Phylogenetic Analysis of hMPV Positive Samples</b>	<b>46</b>
<b>4. Discussion</b>	<b>50</b>
<b>5. References</b>	<b>56</b>

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## LIST OF TABLES

<b>Table 1:</b> Clinical characteristics and outcomes among children with acute respiratory tract infections.	<b>20</b>
<b>Table 2:</b> Characteristics of 32 children hospitalized with hMPV infection.	<b>21</b>
<b>Table 3:</b> Age distribution of patients infected with hMPV	<b>28</b>
<b>Table 4:</b> List of primers sequences of the NL-N and ALT-N RT-PCR assays.	<b>32</b>
<b>Table 5:</b> Protocol used for hMPV detection and amplification	<b>34</b>
<b>Table 6:</b> Distribution of hMPV RNA in the Southern Palestine Districts	<b>41</b>



## LIST OF FIGURES

<b>Figure 1:</b> Electronic Microscope picture of hMPV	<b>3</b>
<b>Figure 2:</b> hMPV genomic organization	<b>4</b>
<b>Figure 3:</b> Phylogenetic analysis of hMPV	<b>16</b>
<b>Figure 4:</b> RT-PCR amplification of hMPV genome using the NL-N primers	<b>37</b>
<b>Figure 5:</b> RT-PCR amplification of hMPV genome using the ALT-N primers	<b>37</b>
<b>Figure 6:</b> RT-PCR amplification of hMPV genome using the NL-N and ALT-N primers	<b>37</b>
<b>Figure 7:</b> RT-PCR amplification of hMPV genome using the NL-N primers on divers bacterial strains	<b>38</b>
<b>Figure 8:</b> RT-PCR amplification of hMPV genome using the NL-N primers on external quality control 2006 batch	<b>39</b>
<b>Figure 9:</b> RT-PCR amplification of hMPV genome using the NL-N primers on patient samples	<b>40</b>
<b>Figure 10:</b> Distribution of hMPV RNA in the southern Palestine Districts	<b>41</b>
<b>Figure 11:</b> Distribution of patients infected with hMPV by cities and villages	<b>42</b>
<b>Figure 12:</b> Distribution of hMPV by Gender	<b>43</b>
<b>Figure 13:</b> Distribution of positive hMPV patients by age	<b>44</b>
<b>Figure 14:</b> Distribution of positive patients by month	<b>44</b>
<b>Figure 15:</b> Distribution of positive patients by weeks	<b>45</b>
<b>Figure 16:</b> Distribution of positive patients for hMPV and RSV	<b>46</b>
<b>Figure 17:</b> Electrophoresis of hMPV RT-PCR reaction using the NL-N (Forward)/ALT-N (Reveries) primers, before purification	<b>47</b>
<b>Figure 18:</b> Electrophoresis of hMPV RT-PCR reaction using the NL-N (Forward)/ALT-N (Reveries) primers after purification	<b>47</b>
<b>Figure 19:</b> Phylogenetic analysis of N-gene of hMPV virus	<b>49</b>

## List of Abbreviations

APV	Avian Metapneumovirus
CBH	Caritas Baby Hospital
DFA	Direct Fluorescent Antibody Staining
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
F protein	Fusion protein
FITC	Fluorescein isothiocyanate
G protein	Major attachment protein
hMPV	Human Metapneumovirus
IFA	Immunofluorescence assays
L protein	Large polymerase protein
LRTI	Lower respiratory tract infections
M protein	Matrix protein
mRNA	Messenger Ribonucleic acid
N protein	Nucleocapsid binding protein
NPA	Nasopharyngeal aspirates
ORFs	Open reading frames
P protein	Phosphoprotein
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PVM	Pneumonia virus of the mice
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RTI	Respiratory tract infections
RT-PCR	Reverse transcriptase – Polymerase chain reaction
SH	Small hydrophobic proteins
URTI	Upper Respiratory tract infections

# **Molecular Detection and Characterization of Human Metapneumovirus (hMPV) in Hospitalized Children Residing in Southern Palestine**

Nabil Rishmawi

## **ABSTRACT**

The recent discovery of Human Metapneumovirus (hMPV) by Van Den Hoogen from the Netherlands in 2001 has added a new member to the group of viruses which can cause respiratory tract infections. hMPV is a pleomorphic enveloped virus which contains single-stranded RNA with negative polarity. hMPV has been classified in the *Paramyxoviridae* family. Since its discovery, both hMPV lineages A and B and their sub-lineages have been reported in many parts of the world with a detection rate between 2-25%. Moreover, hMPV has been reported to be associated with respiratory tract illness in children less than 5 years of age.

In this study, we investigated the frequency of hMPV in 790 nasopharyngeal aspirates of Palestinian children, age one day to 13 years, hospitalized at Caritas Baby hospital between November 2005 and October 2006. Viral RNA was extracted using QIAamp RNA extraction kit (Qiagen GmbH, Hilden, Germany), and amplified for hMPV using an in-house RT-PCR assay. Moreover 1019 bp from the N gene was amplified and partially sequenced in order to perform phylogenetic analysis.

hMPV was detected in 251 (31.8%) of the patients samples analyzed. This positivity rate was one of the highest percents reported to date and might suggest that there was an outbreak of hMPV during the study period. Phylogenetic analysis of 34 (14%) positive hMPV amplified RNA of the N-gene showed that two serotypes of hMPV A lineage circulated in Southern Palestine. Indeed, one of the serotypes was not the same as that reported for the reference strains. Of the sequenced samples, 43% belonged to a novel clade of the lineage A. Upon determining the homology of the newly discovered clade, 95% homology was observed. Having this 5% difference from the 377 base pair sequences allowed to hypothesize that we are dealing with a new clade of the A lineage.

This study is the first to report and describe the epidemiology of hMPV in Southern Palestine. Moreover; it identified the genetic lineages and clades that circulated in Southern Palestine. The discovery of hMPV and its incorporation to routine diagnostic procedures has allowed us to identify the cause of a significant number of respiratory tract infections (31.8%) during the study period.

## الكشف الجيني ومعرفة خصائص آل Human Metapneumovirus (hMPV) في الأطفال الذين ادخلوا المستشفى من القاطنين بجنوب فلسطين

نبيل ر شماوي

### ملخص

الاكتشاف الذي حصل حديثاً من قبل فان دن هوجن للفيروس (human Metapneumovirus (hMPV) في هولندا عام ٢٠٠١ اضافة عضواً جديداً لهذه المجموعة من الفيروسات والتي تسبب التهابات في الجهاز التنفسي. hMPV عبارة عن فيروس متعدد الأشكال ويحتوي على غلاف خارجي وحامض نووي منفرد من نوع مركب RNA وذو قطبيه ساليه. صنف فيروس hMPV في عائلته Paramyxoviridae. منذ اكتشافه تم التبليغ عن وجودها كلا السلالتين (أ و ب) وما ينشق عنهما في أماكن كثيرة في العالم وكان انتشارها بمعدل ٢-٢٥%. أضافه فان التقارير تشير إلى أن hMPV مرتبط مع أمراض الجهاز التنفسي في الأطفال الذين أعمارهم أقل من خمسة سنوات .

في هذه الدراسة تم التحقيق من نسبه وجود hMPV في ٧٩٠ عينه مأخوذة من الأنف والبلعوم من أطفال فلسطينيين تتراوح أعمارهم من يوم واحد إلى ١٣ سنة في الفترة الزمنية من تشرين الثاني ٢٠٠٥ حتى تشرين الأول ٢٠٠٦. لقد تم الحصول على الحامض النووي للفيروس باستعمال QIAamp RNA extraction kit (Qiagen GmbH, Hilden, Germany), وضوعف hMPV باستعمال an in-house RT-PCR assay. أيضاً, ١٠١٩ قاعدة مزدوجة من الجين N وضوعفت وعرف ترتيب القواعد النووية لتحديد النسب الجيني لها .

كان وجود hMPV في ٢٥١ عينه بنسبه ٣١,٨% من العينات التي تم فحصها. هذه النسبة تعتبر من أعلى النسب التي تم نشرها حتى الآن, والذي يشير إلى وجود وباء لهذا الفيروس خلال فتره الدراسة. النسب الجيني ل ٣٤ (١٤%) من hMPV الذي وضوعفت أحماضه النووية للجين N أظهرت وجود نوعين ل hMPV من نوع (أ) في جنوب فلسطين . وحقا إن احد هذين النوعين يختلف عن النوع المرجعي. وتحليل العينات اظهر أن ٤٣% من hMPV ينتمي إلى نوع جديد من سلالة (أ). وكان لهذا النوع الجديد تشابه بنسبه ٩٥% إلى سلالة (أ) . الاختلاف بقيمه ٥% في الترتيب ل إل ٣٧٧ قاعدة نووية يسمح بافترض وجود نوع جديد ينسب إلى السلالة (أ) .

هذه الدراسة هي الأولى التي تقرر و تصف التوزيع البيئي ل hMPV في جنوب فلسطين. أضافه, تم تعريف السلالة الجينية ونسب هذا الفيروس في جنوب فلسطين. إن اكتشاف hMPV وجعل الفحوصات التشخيصية له من ضمن الفحوصات الروتينية سمح لنا بتشخيص لنسبه مهمة من التهابات الجهاز التنفسي (٣١,٨%) خلال فترة الدراسة.

# 1. INTRODUCTION

## 1.1 Background

Respiratory tract infections (RTI) are one of the main causes of morbidity and mortality worldwide (1). RTI ranked second as the leading cause of death in children less than 5 years of age, regardless of the country they live in (2). When age and gender are not taken into consideration, severe respiratory tract infections (pneumonia, influenza, and influenza-like illnesses) are considered to be the most common sicknesses; ranked as the sixth mortal illnesses in the United States, with greater than 45,000 deaths reported annually (3).

Reports from the US have showed that \$14.6 billion is the estimated cost for 4 million patients presenting with severe respiratory tract infections requiring on-sight medical care from which 500,000 cases require hospital intervention (4). In the Netherlands, as high as 5,445 RTI per 10,000 persons are reported annually (5). One major issue about severe respiratory tract infections is that, although clinical symptoms can easily be identified, the original cause of the disease remains often unidentified. Less than 50% of community-acquired pneumonia cases can be microbiologically diagnosed (6, 7). In children, bronchiolitis and lower respiratory tract infections (LRTI) are mainly due to respiratory syncytial virus (RSV). However, parainfluenza viruses, and influenza viruses have also been reported to cause RTI's (8, 9). In most cases, LRTI infections are said to be caused by a virus (10); nevertheless, and even with the presence of state of the art genomic amplification, the virus can be identified in 40% of the cases (11).

One of the pathogens implicated in causing RTI, has been recently identified as the Human Metapneumovirus (hMPV). Human Metapneumovirus (hMPV) a member of the Paramyxoviridae, subfamily pneumovirinae is a pleomorphic enveloped virus with an average diameter of approximately 200 nm (range, 150-1000 nm). hMPV genome is single-stranded RNA (13 kb) with negative polarity and based on sequence analysis the virus has two genetic lineages A and B. Respiratory Syncycial Virus (RSV), another member of the Paramyxoviridae family has similar genetic organization and clinically produces similar symptoms in infected patients.

It is closely related to the avian metapneumovirus (APVs) and has existed in the human population for at least 50 years (12). In the year 2001, Van Den Hoogen was the first to identify this virus (12). The virus was first isolated from children with acute respiratory tract diseases. It would be worth noting that, the hMPV was also identified in other age groups (13-16).

Van Den Hoogen evaluated nasopharyngeal aspirates (NPA), from patient samples stored for over 20 years where hMPV was isolated and characterized from 28 samples. Since this initial discovery, many reports have confirmed the prevalence of hMPV in the world Netherlands (12), Canada (17), France (18), Australia (19), Denmark (20), Argentina (21), Tunisia (22), Italy (23), USA (14), Japan (24), and Israel (25, 26), and identified hMPV as an important respiratory pathogen in children.

In 2-25% of the cases, children who were reported to present with acute bronchiolitis have shown the presence of hMPV in their nasopharyngeal samples. Human respiratory syncytial virus (hRSV) infection has a similar clinical syndrome, but with some cases characterized by respiratory tract infection and others by severe bronchiolitis and pneumonia. Out of a large number of reports few reports have shown that hMPV infection is associated with asthma exacerbation (18, 27, 28) and a single report indicated this association to be less strong than that of asthma and rhinovirus infection (29). There are several ways for the hMPV to be transmitted from one individual to another: by direct contact with respiratory secretions, by contact via large aerosolized droplet, and by contact via handled surfaces.

## **1.2 The classification of hMPV**

The human Metapneumovirus (hMPV) member of the Paramyxoviridae, subfamily pneumovirinae is a pleomorphic virus. It is an enveloped virus with an average diameter of approximately 200nm (range, 150-1000 nm) (Figure 1). hMPV genome is single-stranded RNA (13 kb) with negative polarity. hMPV have a helical nucleocapsid, and has glycoprotein surface with projections.

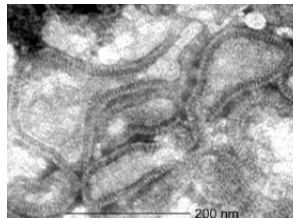


Figure 1: hMPV picture taken by Electronic Microscope

The Metapneumovirus genome encodes eight distinct transcription units in a linear array, with each unit separated by a short segment of untranscribed sequence. The nucleotide sequence analysis of the virus showed that the gene order from 3` end to 5` end as follows: 3`- N-P-M-F-M2-SH-G-L- 5` (Figure 2).



Figure 2: Genomic structures of human Metapneumovirus (hMPV)

### **1.3 hMPV proteins:**

**a. Nucleocapsid binding protein (N):** The N protein responsible for the helical structure of the RNA through its direct association with the virus RNA genome forms an integral part of the nucleocapsid complex of the virion and is an essential element of the polymerase complex.

The N gene encoding region is 1185 bases in length, the gene start sequences contains (GGGACAAG/AU) motif that is identical to the gene start signal characterized for the Avian pneumovirus (APV) subgroup A, B, and C. (30-33). The putative gene end signal for the hMPV N gene (AGUAAUUA6), is identical to the one characterized for the APV-C (30).

Deletion mutant analysis expressed in cells suggests that a large segment of N protein is required for interacting with the P protein (34). Barr and Easton (35) demonstrated that the amino-terminal half of the pneumonia virus of mice (PVM) N protein was unable to bind to immobilized P protein while the carboxy-



terminal half of the N protein bound with only 17.6% of the affinity seen with the full-length protein, results suggesting that the complete N protein is important for this interaction.

**b. Phosphoprotein (P):** One of the essential components in the replication and transcription complexes of pneumoviruses is the P protein (31, 36, 37). The carboxy terminus of the P protein contains most of the elements necessary to bind to the N protein (28, 34, 38, 39); analysis of the P protein has shown that when using different start codons, the P-protein genes of the Pneumovirinae encode several different proteins.

Moreover, and as demonstrated by Villanueva et al. (40), at specific serine residues (41), the P protein is phosphorylated and most of the phosphorylation, occurring at residues 116, 117, 119, and 232, is not essential for transcription or replication.

**c. Large polymerase protein (L):** Contain 2000 amino acids (37, 42). The L protein is thought to be responsible for mRNA methylation and capping, as well as one major component of the viral RNA-dependent RNA polymerase complex, responsible for the synthesis of all viral RNA, including mRNA, replicative intermediates, and the progeny RNA genomes.

**d. M2 proteins:** the mRNA, encoded by the M2 gene, contains two overlapping open reading frames (ORFs) M2-1 and M2-2 (37, 43, 44): M2-1 being the transcription elongation factor and M2-2, the RNA synthesis regulatory factor.

The first open reading frame, initiated by the 5'-proximal AUG initiation codon, encodes the M2-1 protein, which is involved in virus RNA synthesis while the expression of the M2-2 open reading frame resulted in the inhibition of virus gene expression from synthetic genomes. This protein is believed to switch the virus from replicative to assembly mode, prior to the release of the virion (43).

**e. Matrix protein (M):** The M protein, including a carboxy-terminal hydrophobic domain, is linked with the inner face of the lipid membrane of infected target cells (45). Analysis show that M proteins of nonsegmented minus-strand viruses function by promoting the association of the nucleocapsid complex with the nascent envelope (46), and rendering the nucleocapsid transcriptionally inactive before packaging.

**f. Major attachment glycoprotein (G):** Observations showed that G-specific polyclonal antibody blocked the absorption of the virus to the surface of target cells (47), therefore leading to its identification as a viral attachment protein. The role of pneumovirus G proteins, heavily glycosylated with both N- and O-glycosylated sites, have not been completely clarified. If the extra-cellular domain of the G protein witnesses a broad glycosylation, its antigenicity will then be reduced by shielding the virus protein with host-specified sugars. On the other hand, if glycosylation sites are to be partially utilized, then antigenic heterogeneity might occur, representing a source of diversity within a genetically homogenous virus population.

**g. Fusion protein (F):** The F protein is considered as being an inactive precursor (F<sub>0</sub>) accumulating in the rough endoplasmic reticulum (ER) into a homo-oligomer forming a structure seemingly representing a single virion spike (48). Then the F<sub>0</sub> is sub-divided by cellular protease into two subunits, F<sub>1</sub> and F<sub>2</sub> (49, 50). The pneumovirus F proteins have several identical structures as other members of the paramyxoviridae family, although they only show limited amino acid sequence.

**h. Small hydrophobic proteins (SH):** The SH proteins of pneumoviruses are small integral membrane proteins, of the size of 183 amino acids (51). Several analysis of hMPV mutants, lacking the SH and/or G genes, state that SH protein is not necessary for attachment, infectivity, or virion assembly.

#### **1.4 Current Model for Transcription**

Details of the present model for transcription and replication of the pneumovirus genomes are similar to those of the non-segmented negative-sense RNA viruses. The virus genome contains a single polymerase entry site, labeled the leader or promoter sequence, at the 3' end of the RNA. At the beginning of each transcribed gene is a conserved transcriptional start sequence which is initiated by the polymerase complex. The latter then travels along the genome until reaching a conserved signal that marks the point at which transcription ends. At some point during the transcription process, the embryonic mRNA is methylated to produce a cap structure that ensures translation on host cell ribosomes; there are no reports directly addressing this process. The end of transcription among the pneumoviruses is marked by a signal containing a short

run of U residues, pointing the position at which the polyadenylated tail is added to the mRNA. At the completion phase of the polyadenylation process, the mRNA is released for translation on either free or membrane-bound ribosomes, depending on the nature of the protein product. At this point, only a proportion of the active polymerase molecules progress along the genome, with estimates suggesting that approximately 50% of the polymerase molecules are able to continue. Dissociated polymerases can reinitiate transcription at the first gene by translocation to the 3'-terminal leader region. Without transcription, the remaining polymerases traverse the genome until they encounter the conserved transcriptional start sequence of the second gene. At the end of this gene, the remaining polymerases repeat the dissociation-versus-continuation process. The result is that genes proximal to the 3' leader region are represented in mRNA more abundantly than are those closer to the 5' end in a gradient of transcription (52). The inter-genic regions of the pneumoviruses vary in length and tolerate mutation and insertion of additional nucleotides (37, 53, 54).

Based on the RSV model, the M2-1 protein has proved its necessity in the production of full-length virus mRNA (48, 55, 56). The anti-termination activity during transcription allows the viral polymerase to remain associated with the template, thus increasing the production of full-length mRNA and bettering the production of products of genes that are distal to the 3' leader region. Although they vary in their sensitivity to the presence of the M2-1 protein and in their ability to terminate transcription, the eight RSV gene junctions all direct the production of more read through mRNA in the presence of M2-1 protein (57).

Nevertheless, the genome structure of hRSV contains an exception to the above explained process. At the 68 nucleotides downstream of the transcriptional start sequence for the L gene is the transcriptional stop signal for the M2 gene(58). Therefore, a large proportion of the mRNA initiated at the L-gene start sequence terminates at the M2 transcriptional stop sequence (58).

Elements within inter-genic regions play a major role in the regulation of transcription. In fact there are important sequence diversity in length in the inter-genic regions of the Pneumovirinae, vary from 2 to 56 nucleotides. The transcriptional start sequences are thought to be 10 nucleotides in length, and the consensus sequences, while conserved, are slightly different for each pneumovirus (59). The RSV or APV transcriptional start signals are less variable than that of the PVM since they are absolutely conserved with the exception of the L gene (60).

The transcriptional stop signals of hRSV consist of a conserved pentanucleotide sequence together with a 1- to 4-nucleotide A U-rich region and a 4- to 7-nucleotide poly (U) tract. Saturation mutagenesis of the hRSV gene transcriptional start sequences showed that residues 1, 3, 6, 7, and 9 were critical in directing transcription initiation, although there was some variability in efficiency, particularly with the NS1 and NS2 gene start sequences, which were approximately 40% less efficient than the others(59, 61).

As a result from the deletion of a transcriptional stop signal from an upstream gene a transcriptional read-through to the next gene without termination

was made. After analyzing the hRSV M-gene transcriptional stop sequence, Harmon et al. (62) determined that the integrity of the pentanucleotide and AU-rich regions are very important for efficient termination. The residue following the poly (U) tract might also be important for efficient transcription termination (63).

Nevertheless, there wasn't any significant difference in transcriptional activity or efficiency with different sized RSV intergenic sequence (61). An analysis of Bukreyev et al. (64) showed the recovery of a recombinant hRSV containing an intergenic region of 160 nucleotides, which had little effect on sequential transcription.

### **1.5 Current Model for Genome Replication**

The current model, based on that determined for other nonsegmented negative-sense RNA viruses, proposes that the nucleocapsid complex is the functional unit for both replication and transcription and that the L, N, and P proteins are involved in both processes. There is in fact a big similarity between the transcription and replication promoters of hRSV. In the replicative mode, the polymerase binds to the 3' end of the genome RNA and initiates RNA synthesis de novo. Once begun, the polymerase produces an antigenome at the end of the template. The polymerase complex then uses the antigenome as a template, binding to and initiating RNA synthesis at the 3' end of the antigenome RNA. In the genome sense RNA, the trailer region contains the anti-sense copy of the sequence necessary for initiating replication to produce more genome RNA from the antigenome.

So far, no study has clearly identified how the polymerase complex alters its activity and switches from the transcription to the replication mode. Fearn's et al. (65) showed that hRSV replication was stimulated by increased expression of the N protein. Nevertheless, conflicting data suggest that high levels of N protein may also stimulate transcription (66).

The 3' and 5' termini of the pneumovirus genome contain the sequences that direct replication. Reverse genetics studies showed that the 3'-terminal 44-nucleotide leader region and the 5'-terminal 40 nucleotides of the trailer region of hRSV were necessary for replication, encapsidation, and assembly (67). The immediate termini of the pneumovirus genomes have complementary sequences, as has been described for other negative-sense RNA viruses (37). The trailer sequences are more efficient than the leader sequences at directing replication, as would be expected from their role in replication and the requirement for the leader region to direct both replication and transcription (68).

Analysis of the leader and trailer sequences suggested that nucleotide positions 1, 2, 3, 5, 6, and 7 were particularly important for replication, while position 4 was found to be tolerant of alteration. Using synthetic chimeric minigenomes with terminal sequences from both APV and RSV in virus-infected cells, Marriott et al. (69) showed that paired termini from the same virus were required for replication to occur. It is not clear whether the critical step is replication or encapsidation, but the data suggest that an association between the leader and trailer is necessary for productive infection (69).

## **1.6 Packaging and Assembly**

Reverse genetics experiments suggest that the accumulation of the M protein may be a component of the trigger to initiate the assembly of virions. It is anticipated that the nucleocapsid complex/M-protein structure associates with the internal tails of the major glycoproteins that are inserted into the membrane of the infected cell after processing through the Golgi complex. In parallel with studies performed with other viruses, a progressive series of interactions between the glycoproteins and the M protein, which acts as a bridge with the nucleocapsids, results in the progeny virions budding from the surface of the cell in the form of filament structures. In polarized cells, such as those of the respiratory tract, hRSV buds from the apical surface and is shed into the airways, from which it can be transmitted to new hosts(70).

## **1.7 Taxonomy**

Genetically speaking, the closest relative to the hMPV, member of the Paramoxiviridae, subfamily pneumovirinae, is the avian pneumovirus type C (See below). hMPV is the only known human pathogen of the genus Metapneumovirus. However, clinically, it is quite similar to the Respiratory syncytial virus (RSV), a common respiratory pathogen classified in the family Paramoxiviridae, subfamily Pneumovirinae. Following is the diagram detailing the taxonomy of this family.



Order: Mononegavirales

Family: Paramyxoviridae

Subfamily: Paramyovirinae

Genus: Respirovirus

Species: Human parainfluenza type 1 and 3

Genus: Rubulavirus

Species: Human parainfluenza type 2 and 4

Genus: Morbillivirus

Species: Measles virus

Genus: Henipavirus

Species: Hendra virus. Nipah virus

Subfamily: Pneumovirinae

Genus: Pneumovirus

Species: Respiratory syncytial virus

Subgroup: A and B

Genus: Metapneumovirus

Species: Human Metapneumovirus

Subgroup: 1 and 2

### **1.8 hMPV Lineages:**

Several sequence analysis of hMPV isolates showed that there are two main genetic lineages (A and B) each representing two sub lineages, A1, A2, B1 and B2 (Figure 3). However lineage A2 appears to have two clusters, tentatively named A2a and A2b. This division of subgroup A2 is supported by bootstrap values (94% and 98%), considered high when compared to those found for widely accepted partition of A1 and A2.

In Germany, a new variant of hMPV isolated from a 6.5 years old child was reported by Schildgen et al, where alignment of 302 nucleotide human metapneumovirus (hMPV) sequences amplified from brain and lung tissues of the patient and from the supernatant of infected Vero cell culture and the sequence of the hMPV strain NLD00-1 was used as a prototype sequence.

The two viral genotypes have a similar genomic organization. The major differences between genotypes 1 and 2 are nucleotide polymorphisms and the G and SH protein contains the highest concentration of these polymorphisms. The G gene of hMPV shows an important strain-to-strain variability, resulting in significant amino acid variability, mostly observed in the putative extra-cellular domain of the protein.

The amino acid identity of the expected determined proteins between the two strains is 96 % (N protein), 85% (p protein), 97% (M protein), 95% (F

protein), 96% (M2-1 protein), 89% (M2-2 protein), 59% (SH protein), 37% (G protein), and 94% (L protein).

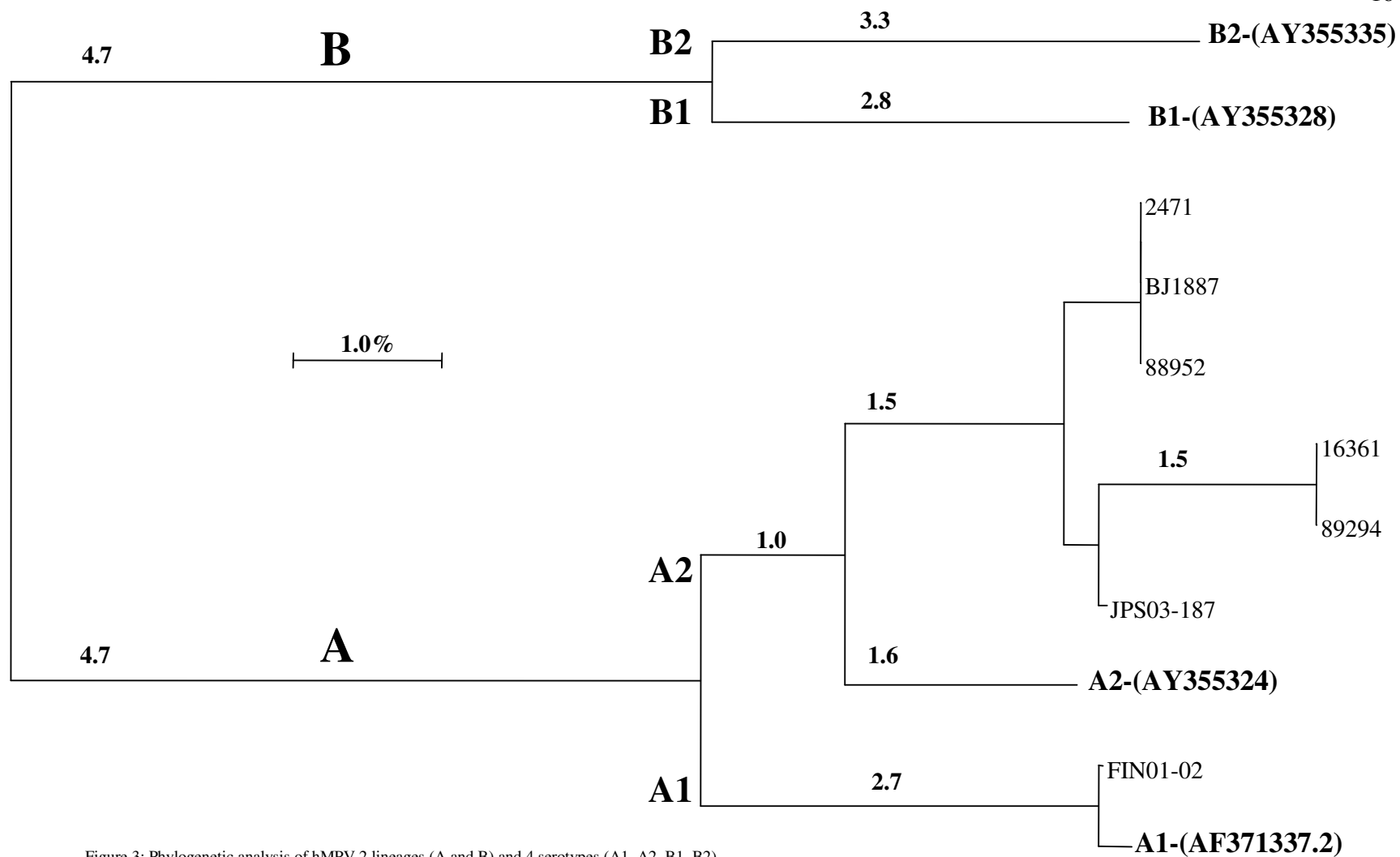


Figure 3: Phylogenetic analysis of hMPV 2 lineages (A and B) and 4 serotypes (A1, A2, B1, B2)

## **1.9 Epidemiology**

hMPV (be consistent, either hMPV or HMPV) has been detected in patient samples from the following countries: Netherlands (12), Canada (17), France (18), Australia (19), Denmark (20), Argentina (21), Tunisia (22), Italy (23), USA (14), Japan (24), and Israel (25, 26).

hMPV was mostly found to circulate in the late winter and spring, as well as in the peak of activity at any given location, often coinciding with or following the peak of the RSV activity.

It would be worth noting that hMPV is also detectable at lower levels during late spring, summer and fall. Its phylogenetic analysis of strains reveals that the epidemiology of this local phenomenon is complex and dynamic. Strains of hMPV differ from one community to another, and strains identified in one location may be quite similar or different from other identified in other locations in different years.

RSV and hMPV has similar epidemiological features, where subgroups of the virus co-circulate each year and the strains vary from area to another area and from different years as well. Moreover switching from one strain to another strain of same virus is observed.

### **1.10 Pathogenesis**

Ciliated respiratory epithelial cells changes and increased inflammatory cell infiltrates, predominantly mononuclear cells, in the lung interstitium was observed in experimental animals infected with hMPV. Moreover, hMPV virus uses specific strategies to overcome host defenses, similar to RSV, this virus can persist for several weeks in the lungs, despite an established immune response. Furthermore, hMPV infection causes increased myofibroblast thickening adjacent to the airway epithelium and staining of cellular debris.

Airway epithelium are the first infected cells with hMPV, that results in cell degeneration and/ or necrosis, with ciliacytophthoria and round, red cytoplasmic inclusions on a background of haemosiderinladen macrophages, abundant neutrophils and prominent mucus.

Lung biopsies performed at least one month after a positive hMPV nasal assay, have shown that later stages of the disease caused by hMPV include expansion of peribronchiolar lymphoid tissue, squamous metaplasia, haemosiderin and accumulation of intra-alveolar foamy macrophages. These features indicate chronic/ healing airway obstruction and impairment of the mucociliary escalator, and correlate well with the bronchiolitis and wheezing noted clinically in patients with hMPV infection.

**a. Clinical manifestation of the hMPV infection**

hMPV is responsible of a substantial proportion of LRTI in infants and young children and is second to RSV as a cause of bronchiolitis in early childhood. The incidence estimated range from 5 to 15% in most studies.

In most cases, an association between the LRTI and the hMPV in infants and young children requires clinical intervention. Severe cases of LRTI require intensive care, and one case required of extracorporeal membrane oxygenation.

Several signs and symptoms may help diagnose the hMPV infection; these range from rhinopharyngitis to bronchitis and pneumonia - some patients may even be admitted to intensive care units. A summary of the clinical data for a group of children seen in an emergency department for acute respiratory infection, grouped according to virus diagnosis is shown in (Table 1). Signs and symptoms caused by hMPV resemble those of RSV infection (bronchiolitis, asthma exacerbation and pneumonia) and sometimes those of influenza (fever and upper respiratory tract infection). The clinical presentation of hMPV infection have also been associated with febrile seizures, rash, diarrhea, enlarged liver, and altered liver function test results (Table 2).

<b>Characteristics</b>	<b>hMPV positive (n=41)</b>	<b>RSV positive (n=117)</b>	<b>Influenza positive (n=209)</b>
<b>Clinical presentation</b>			
Common cold, no.(%)	3 (7.3)	20 (17.1)	43 (20.6)
Pharyngitis, no (%)	11 (26.8)	20 (17.1)	73 (34.9)
Acute otitis media no (%)	5 (12.2)	10 (8.5)	34 (16.3)
Croup, no (%)	3 (7.3)	4 (3.4)	7 (3.3)
Acute bronchitis no (%)	4 (9.8)	15 (12.8)	20 (9.6)
Wheezing, no (%)	10 (24.4) a	30 (25.7)a	14 (6.7)
Pneumonia, no (%)	5 (12.2)	18 (15.4)	18 (8.6)
<b>Clinical outcome</b>			
Hospitalization, no (%)	2 (4.8)	16 (13.7)	11 (5.3)
School absence Median days (range)	10 (3-15)	10 (3-12)	12 (5-15)

Table 1. Clinical characteristics and outcomes among children seen for acute respiratory infection in an emergency department, grouped by virus RNA detection (71).



<b>Characteristics</b>	<b>hMPV positive/Total (%)</b>	<b>RSV positive /Total (%)</b>	<b>Influenza positive /Total (%)</b>
Influenza-like illness in family contact	10/19 (52.6)	7/29 (24.1)	19/24 (79.1)
Febrile seizure	5/32 (15.6)	1/32 (3.1)	3/32 (9.4)
Congested pharynx	12/32 (37.5)	11/32 (34.4)	11/32 (34.4)
Rash	4/32 (12.5)	1/32 (3.1)	4/32 (12.5)
Enlarged liver	2/32 (6.3)	0/32 (0.0)	4/32 (12.5)
Otitis media	4/32 (12.5)	0/32 (0.0)	0/32 (0.0)
Diarrhoea	2/32 (6.3)	1/32 (3.1)	3/32 (9.4)
Creptitations	18/32 (56.3)	14/32 (43.8)	3/32 (9.4)
Wheezing	9/32 (28.1)	12/32 (37.5)	2/32 (6.3)
Asthma exacerbation	6/32 (18.8)	2/32 (6.3)	2/32 (6.3)
Acute bronchiolitis	3/32 (9.4)	10/32 (31.3)	0/32 (0.0)
Pneumonia	12/32 (37.5)	5/32 (15.6)	1/32 (3.1)
Abnormal chest X-ray	17/25 (68.0)	11/18 (61.1)	1/17(5.9)
Lymphopenia (<1.5 * 10 /L)	9/31 (29)	2/27 (7.4)	12/29 (41.4)
Neutropenia (ANC <1* 10/L)	2/31 (6.5)	0/27 (0.0)	4/29 (13.8)
Elevated transaminase	2/15 (13.3)	0/5 (0.0)	3/11 (27.3)

Table 2 Characteristics of 32 children admitted with human Metapneumovirus infection compared to age-matched controls with respiratory syncytial virus or influenza A infection (71).

hMPV was found to cause severe symptoms in infants and patients with chronic illnesses; one death was reported in an immuno-suppressed patient (72). Laboratory analysis reported 32 hospitalized children under the age of 18 months, of which 29% had lymphopenia and 6% had elevated transaminases (28). In

another group of 10 hospitalized children, normal White Blood Counts and elevated C- reactive protein levels were reported (27).

In the Netherlands most of the people over 5 years of age have been exposed to the hMPV (12), and in southern part of Israel, over 50% of children that are less than 2 years of age have been infected with hMPV (25).

The infection of a patient by both the hMPV and other respiratory viruses may contribute to severe illnesses. Moreover, the co-infection with hMPV and RSV has been hypothesized to cause severe disease, often-requiring intensive care admission, and ventilatory support (73).

### **1.11 Diagnosis:**

Amongst the available diagnostic tests for hMPV are: the viral culture, immunofluorescence assays (IFA), and polymerase chain reaction (PCR). Other tests can be used to determine if the patient might have viral infections includes Complete Blood Count and C-Reactive Protein.

#### **a. Viral culture:**

The hMPV virus only grows slowly in tertiary monkey kidney cells supplemented by trypsin for propagation as described by van Den Hoogen. It is difficult to isolate it since it does not grow readily in tissue culture cells routinely used in the viral diagnostic laboratory.

Following is a small brief of how the process functions: The virus isolates replicated slowly in tertiary monkey kidney (tMK) cells, very poorly in Vero cells and human respiratory epithelial cell line (A549 cells), and cannot be propagated in Madin Darby canine kidney (MDCK) cells or chicken embryo fibroblasts (CEF). The virus isolates causes a cytopathic effect on cells and are virtually indistinguishable from those caused by hRSV, with characteristic syncytia formation followed by rapid internal disruption of the cells and subsequent detachment from the culture plate. This cytopathic effect is visible at day's 10–14 post inoculation, slightly later than the cytopathic effects induced by hRSV-containing specimens.

#### **b. Immunofluorescence assays (IFA)**

IFA is performed using Vero cells infected with the hMPV reference strain are mixed with uninfected Vero cells at a ratio of 70% and 30%, respectively. The cells are then spotted on acetone-cleaned glass slides and left to dry at room temperature. The cells on each slide are then fixed with ice-cold acetone and stored at  $-70^{\circ}\text{C}$  until used. Patient's sera are serially diluted in phosphate-buffered saline and incubated with either hMPV-infected or uninfected (negative control) fixed Vero cells for 45 min at  $37^{\circ}\text{C}$ . The cells are then washed three times with phosphate-buffered saline and incubated with fluorescein isothiocyanate-conjugated AffiniPure goat anti-human immunoglobulin G, Fc fragment specific (Jackson ImmunoResearch Laboratories, Inc.) for 35 min at  $37^{\circ}\text{C}$  in a humidified box. Cells are then washed and dried, and mounting fluid

was added. A inverted fluorescent microscope is used to visualize the samples. Patient samples with a titer greater than 1:32 are considered positive for hMPV antibodies as described by van den Hoogen et al.

### **c. Molecular Diagnosis by One- step Reverse transcriptase – Polymerase chain reaction ( RT-PCR )**

Nowadays, RT-PCR is the mostly followed method to detect hMPV. Briefly, cDNA is synthesized from total RNA extracted from each nasopharyngeal swab sample. A forward primer and a reverse primer are used to detect the viral protein gene needed for the study. RT-PCR is to be performed on extracted RNA. Amplicons were visualized by ethidium bromide staining following electrophoresis on a 2% agarose gel.

The N and L genes codes for two internal viral proteins particularly suitable and highly sensitive for the detection of hMPV strains of both genotypes. Comparative studies for the sensitivity of real-time RT-PCR assay to amplify the viral nucleoprotein (N), Matrix (M), Fusion (F), Phosphoprotein (P), and polymerase (L) genes, showed that.

The accuracy and timing of the diagnosis is a major element in the appropriate treatment of patients with respiratory illnesses. An early diagnosis of viral infections can reduce the inappropriate use of antibiotics and provide the option of using antiviral therapy. However, if suspected, bacterial infections

should be considered and appropriately treated because certain bacterial infections can produce symptoms similar to viruses. In addition, bacterial infections can occur as a complication of viral infections.

For a more serious treatment, hMPV surveillance information and diagnostic testing are a must. And since symptoms from illness caused by other pathogens can overlap considerably with hMPV, the accuracy of clinical diagnosis of hMPV on the basis of symptoms alone is limited.

### **1.12 Treatment**

The medical care treatment is supportive: maintain hydration and provide supplemental Oxygen if necessary. In patients with respiratory failure, mechanical ventilation is necessary. It is clear that there is a need for agents that can prevent or ameliorate infections caused by the hMPV virus.

**a. Ribavirin** is a compound with known broad-spectrum antiviral activity, suggested for the treatment of hMPV infections. However clinical use of ribavirin is controversial, because it is a potential teratogen, and thus is a potential health hazard; it is also an expensive medicine. Despite these problems, ribavirin has shown to be effective in limiting the disease and mortality in immunosuppressed people infected with hRSV provided that treatment is started early.

**b. NMSO3**, a sulfated sialyl lipid that has a potent antiviral activity against hRSV in tissue culture cells, has shown antiviral activity against hMPV

too. Clinical trials of anti-human Metapneumovirus monoclonal antibodies for prevention of hMPV infections in high risk infants are being investigated.

### **1.13 Vaccination against hMPV**

Several promising vaccine candidates have been tested in animal models. A live recombinant human parainfluenza virus that contains the hMPV F gene has been shown to induce hMPV – specific antibodies and to protect experimental animals from hMPV challenge. These animals produced antibodies specific for the hMPV F protein.

## **2. Materials and Methods**

### **2.1 Study Location**

This study was performed at the Caritas Baby Hospital (CBH). CBH is located in the Southern part of Palestine. CBH is a non-profit charitable hospital that serves the children of Bethlehem and the surrounding villages and cities. The primary aim of this institution is to provide services mainly for low-income families. The hospital has capacity for 89 beds and incubators located in three wards; premature ward, and two wards for babies and toddlers.

### **2.2 Patient Demographics and study period**

Patients (N=790) admitted to CBH with acute respiratory tract infections as the main cause of illness between the period November 1<sup>st</sup> 2005 and October 11<sup>th</sup> 2006 were enrolled in this study. The majority of the patients were residents of Bethlehem district (63.2%), followed by Hebron (34.3 %), Jerusalem (1.5%), Jericho (0.63%), Ramallah (0.25%) and Nablus (0.13%). The patients' age ranged from one day old new born to 13 years old children. Stratifying the patients by age group, most of patients were less than two years old (92.66%) (Table 3) and the male to female ratio was 493 (62.4%) to 297 (37.6%), respectively.

<b>Age Group</b>	<b>Number of Children</b>	<b>Infected / Percent</b>
< 6 Months	475	60.1%
0.5-1 Y	189	23.9%
1-1.5 Y	68	8.61%
1.5-2 Y	24	3.04%
2-2.5Y	15	1.90%
2.5-3 Y	11	1.39%
3-3.5 Y	1	0.13%
3.5-4 Y	1	0.13%
4-4.5 Y	2	0.25%
4.5-5 Y	1	0.13%
5-5.5 Y	1	0.13%
5.5-6 Y	0	0.00%
6-6.5 Y	1	0.13%
>7 Y	1	0.13%
<b>Total</b>	<b>790</b>	<b>100%</b>

Table 3: Age distribution of patients infected with hMPV

### **2.3. Patients Samples Collection and Preparation**

#### **a. Collection of nasopharyngeal aspirates:**

Nasopharyngeal aspirates (NPA), the gold standard patient specimens for the diagnosis of respiratory tract infections, were collected according to the recent European Respiratory Society guidelines. Health care providers were educated on



the proper methods of specimen collection, storage, and transport. Briefly, a sterile soft polyethylene #8 French feeding suction tube was attached to a disposable aspiration trap. Using the tube, the distance from patient's nostril to the ear was measured; the distance was marked on the tube using the thumb and forefinger. The tube was then gently inserted into the nostril until using half the distance the thumb and forefinger touch the patient's nose. Intermittent suction was applied; the tube was slowly removed from the nasopharynx. The end of the tube was placed in a vial containing sterile saline and the contents were aspirated into the trap (don't leave the specimen in the tubing). The trap was closed tightly with the cover making it leak proof.

The trap was labeled with patient's information, and send to the laboratory within 30 minutes. If the specimen was not analyzed immediately, it was kept refrigerated at 2-8 °C.

**b. DFA Slide Preparation:**

Patient NPA samples were vortexed for 30 seconds to loosen the mucus and release the trapped cells. The samples were then centrifuged at 300 x g for 10 minutes followed by removing the supernatant. PBS 5 ml at (4 °C) was then added to the pellet to wash and loosen the cell pellet by gentle mixing using a clean disposable pipette. The samples were then centrifuged at 300 x g for 10 minutes followed by decanting the supernatant into the waste container. This step was repeated one more time to ensure that the cell pellete is clean and the cells are loose. The pellet was then resuspended in 0.2-0.5 ml with cold PBS. The cells

were then spotted on acetone cleaned and air dried 8 well slides. The cells were then fixed with ice cold acetone for 10 minutes. An aliquot of the patients' cells were kept in a sterile eppendorf tube and stored at -25 °C.

#### **2.4 DFA Staining:**

FITC labeled antibodies against each of the viruses (Influenza A and B, RSV A and B, Parainfluenza viruses 1, 2, 3, and Adenovirus) were added to the appropriate well and incubated at 37°C for 30 minutes. The slides were then washed for 45 sec with 0.5% Tween 20 / PBS, followed by washing with distilled water for 15 seconds. The slides were then dried on a slide warmer. The slides were then mounted with mounting oil, and examined for the presence of fluorescence using a Hund H 600 fluorescent microscope at 20X and confirmed at 40 X. Each slide was screened for the presence of ciliated cells as an indication that the sample was collected properly.

#### **2.5 Identification of Positive cells**

A positive result was indicated by the presence of two or more intact cells exhibiting specific fluorescence. The presence of only one single positive cell was considered to be positive after the evaluation of an expert. A negative result was indicated by the absence of fluorescence in a minimum of 20 ciliated epithelial cells. Samples containing fewer than 20 ciliated epithelial cells were considered inadequate. The staining pattern varied with the infecting virus and the stage of growth. Fluorescent staining pattern was nuclear and/or cytoplasmic and often

punctate; it was bright apple green for FITC-labeled antibodies and reddish gold for rhodamine-labeled antibodies.

## **2.6 Virus and Bacterial stocks:**

hMPV reference strain virus 200026583 (CAN-83) passage 3. For specificity testing, the RT-PCR assay did not cross react with influenza virus A, influenza virus B, RSV, parainfluenza viruses 1, 2, 3, and adenovirus. ATCC bacterial strains were used to measure the specificity of the RT-PCR assay. The strains used were *Haemophilus influenzae* (ATCC 49766), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Streptococcus pyogenes* (ATCC 19615), *Streptococcus pneumoniae* (ATCC 49619), and *Moraxella catarrhalis* (ATCC 25239) and one clinical bacterial isolate (*Klebsiella pneumoniae*).

## **2.7 RT-PCR sensitivity.**

The sensitivity and specificity of the assay developed in this lab was challenged with Quality Control for Molecular Diagnostics (QCMD) external quality control 2006 batch. Twelve QCMD RNA samples were analyzed with the in house hMPV RT-PCR assay.

## **2.8 Molecular Diagnosis**

### **a. Primers design:**

Two sets of primers published by Maertzdorf et al (74) were modified and used to detect all hMPV strains from all known genetic lineages. The primers targeted the most conserved sequence within the nucleoprotein gene (N gene) (Table 4).

	<b>Forward primer</b>	<b>Reverse primer</b>
<b>NL-N (73)</b>	CAT ATA AGC ATG CTA TAT TAA AAG AGT CTC	CCT ATT TCT GCA GCA TAT TTG TAA TCA G
<b>NL- N- CBH</b>	CAT AYA AGC ATG CTA TAT TAA AAG AGT CTC	CCT ATT TCT GCA GCA TAT TTG TAA TCA G
<b>ALT-N (73)</b>	CAA CAA CAT AAT GCT AGG ACA TGT ATC	CCG AGA ACA ACA CTA GCA AAG TTG
<b>ALT- N-CBH</b>	YAA CAA YAT AAT GYT AGG ACA TGT ATC	CCG AGA ACA ACA CTW GCA AAG TTG

Table 4: Alignment of sequences of primers for the NL-N and ALT-N assays with the target sequences of the hMPV. Residue Y represents a C or a T, and W represents A or T

A 162 bp fragment was amplified with NL-N- Forward, and NL-N-Reverse; while a 176 bp fragment was amplified after the utilization of the ALT-N-Forward, and ALT-N-Reverse. We utilized the NL-N set of primers for hMPV screening, since this set of primers gave us consistent, sensitive and reproducible results. Moreover, for phylogenetic analysis, we established an RT-PCR assay using the NL-N-Forward and ALT-reverse which amplified a 1019 bp fragment that was used for sequencing.

**b. Contamination control:**

All extraction procedures were performed inside a Biological Safety Hood -2. Before each extraction procedure, the hood was cleaned with 0.5% bleach, followed by 70% alcohol. The same procedure was performed after each extraction procedure. White absorbent paper was then added on the hood surface. All extraction racks and pipettes were also cleaned with 0.5% bleach, followed by 70% alcohol before and after each usage. All these extensive decontamination steps were thoroughly followed to prevent the buildup of contaminating genetic material.

**c. Nucleic acid extraction:**

Viral genomic RNA was extracted from the concentrated patient samples by using a QIAamp RNA extraction kit (Qiagen GmbH, Hilden, Germany), according to the protocol suggested by the manufacturer. Briefly, clinical samples were homogenized by vortexing for 30 sec, and 140 µl was used for the extraction of viral genomic RNA. The RNA was eluted from the columns with 50 µl of elution buffer. The RNA was immediately stored at -25°C after a 10-µl aliquot was used for the one step RT-PCR.

**d. RT-PCR amplification:**

RT-PCR was performed using QIAGEN OneStep RT-PCR kit (QIAGEN GmbH, D-40724 Hilden, Germany). Briefly, 10µl extracted RNA was added to a master mix composed of enzyme mix (mixture of heterodimeric recombinant reverse transcriptases Omniscript, Sensiscript, and HotStart Taq DNA polymerase),

400  $\mu$ M concentrations of each deoxynucleoside triphosphates and the hMPV primers sets at 20 pM final concentration (each). Amplification followed the optimized profile that was established at Caritas in the thermal cycler (PTC-100; MJ Research, Watertown,MA): 42°C for 45 min, 95°C for 7 min, followed by 35 amplification cycles (denaturation at 95°C for 1 min, annealing at 45°C for 2 min, and synthesis at 72°C for 3 min). Amplification was completed with a prolonged synthesis at 72°C for 10 min (Table 5). Amplicons were visualized by ethidium bromide staining following electrophoresis on a 2% agarose gel.

<b>Thermal cycler steps</b>	<b>Temperature</b>	<b>Time</b>
Step 1- Reverse transcription	50 °C	30 mints
Step 2- Denaturation 1	95 °C	15 mints
Step 3- Denaturation 2	95 °C	45 seconds
Step 4- Annealing	56 °C	45 seconds
Step 5- Synthesis	72 °C	1 mint
Step 6- Amplification	GO to step 3	40 times
Step 7- Prolong synthesis	72 °C	10 mints
Step 8- Storage	4 °C	For ever

Table 5: protocol used for hMPV detection and amplification

## **2.9 Phylogenetic analysis:**

RT-PCR products were purified directly from the RT-PCR mix using QIA quick PCR purification Kit (Qiagen GmbH, Hilden, Germany), following the protocol suggested by the manufacturers, and following their precautions to avoid cross-contamination between samples preparations. Sequencing using ABI PRISM Di- Deoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) was performed at Bethlehem University. Reaction mixtures were analyzed on Applied Biosystems model 373 DNA automatic sequencing systems. The Sequencer (Gencodes Corporation, Ann Arbor, MI) was used to compare nucleotide sequences. Phylogenetic trees were prepared by the nearest neighbor analysis using Clustal X with 1,000 bootstraps for each bootstrap. Trees were visualized using TreeView or NJ plot.

### **3. Results**

This study was conducted at CBH between the period November 1<sup>st</sup> 2005 and October 11<sup>th</sup> 2006. Nasopharyngeal aspirates were obtained and tested by an in-house RT-PCR assay for the presence of hMPV from 790 patients.

#### **3.1 Validation of an RT-PCR assay for the detection of hMPV RNA**

Serial dilution of hMPV RNA was used to validate an RT-PCR assay for hMPV RNA detection. Two sets of primers were evaluated for detecting the various strains from all genetic lineages of the virus. The primers targeted the most conserved sequences within the nucleoprotein gene and were designated to match all the four genetic lineages of hMPV. The sensitivity of both primers were determined by using serially diluted RNAs obtained from positive hMPV reference strain virus 200026583 (CAN-83) passage 3. Identical RNA aliquots were used in both the NL-N and the ALT-N assays. Both assays were able to detect viral RNAs from the reference hMPV strain. The NL-N primers were more sensitive since they were able to detect  $10^{-6}$  dilution (Figure 4) whereas the ALT-N primers detected up to  $10^{-5}$  hMPV RNA dilution (Figure 5). Moreover, the forward primer of the NL-N primer set was paired with the reverse ALT-N primer to amplify a 1019 bp PCR product that was used for phylogenetic analysis. Amplification of NL-N forward, ALT-N reverse was not as sensitive as the NL-N primers set since it was able to detect dilution  $10^{-5}$  consistently (Figure 6).



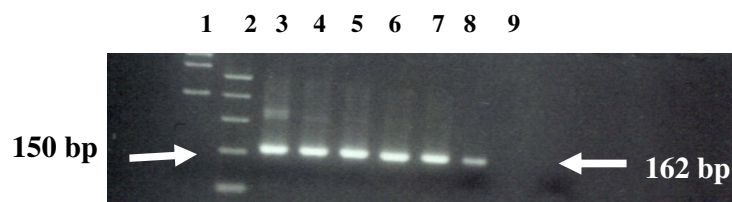


Figure 4: 2% Gel electrophoresis of hMPV RT-PCR reaction using the NL-N primers on control strain serially diluted in water. Lane 1: 1000 bp marker, lane 2: PCR marker #N3234S, lane 3: hMPV RNA dilution  $10^{-1}$ , lane 4: hMPV RNA dilution  $10^{-2}$ , lane 5: hMPV RNA dilution  $10^{-3}$ , lane 6: hMPV RNA dilution  $10^{-4}$ , lane 7: hMPV RNA dilution  $10^{-5}$ , lane 8: hMPV RNA dilution  $10^{-6}$ , and lane 9: Negative Control.

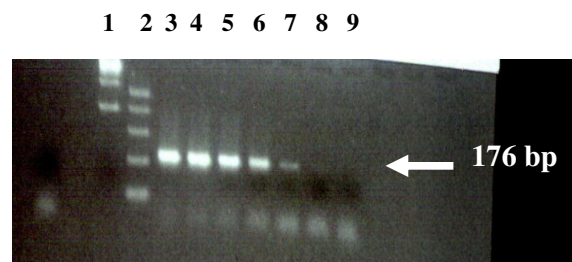


Figure 5: 2% Gel electrophoresis of hMPV RT-PCR reaction using the ALT-N primers on control strain serially diluted in water. Lane 1: 1000 bp marker, lane 2: PCR marker #N3234S, lane 3: hMPV RNA dilution  $10^{-1}$ , lane 4: hMPV RNA dilution  $10^{-2}$ , lane 5: hMPV RNA dilution  $10^{-3}$ , lane 6: hMPV RNA dilution  $10^{-4}$ , lane 7: hMPV RNA dilution  $10^{-5}$ , lane 8: hMPV RNA dilution  $10^{-6}$ , lane 9: Negative control.

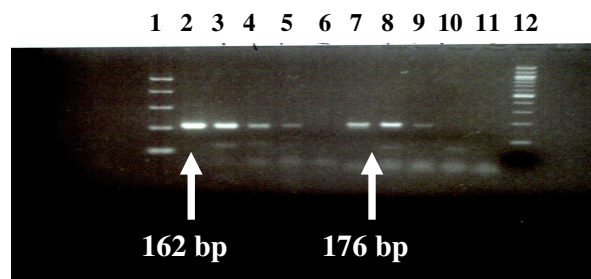


Figure 6: 2% Gel electrophoresis of hMPV RT-PCR reaction using the NL-N (from lane 2 to lane 5) and ALT-N (from lane 7 to lane 11) primers on control strain serially diluted in water. . . Lane 1: PCR marker #N3234S, , lane 2: hMPV RNA dilution  $10^{-3}$ , Lane 3: hMPV RNA dilution  $10^{-4}$ , Lane 4: hMPV RNA dilution  $10^{-5}$ , Lane 5: hMPV RNA dilution  $10^{-6}$ , Lane 6: Negative control, Lane 7: hMPV RNA dilution  $10^{-3}$ , Lane 8: hMPV RNA dilution  $10^{-4}$ , Lane 9: hMPV RNA dilution  $10^{-5}$  Lane 10: hMPV RNA dilution  $10^{-6}$ , Lane 11: Negative control , Lane 12: 1000 bp marker

### 3.2 Specificity of the hMPV NL-N RT-PCR assay

To test the specificity of the NL-N primers designed for the assay, genomes from influenza virus A, influenza virus B, RSV, parainfluenza viruses 1, 2, 3, and adenovirus was used as a template in the assay. None of the genetic material of these viruses cross reacted with the assays primers.

In addition, we used ATCC bacterial strains that are common in the respiratory tract to check for cross reactions. The strains evaluated were *Haemophilus influenzae* (ATCC 49766), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Streptococcus pyogens* (ATCC 19615), *Streptococcus pneumoniae* (ATCC 49619), and *Moraxella catarrhalis* (ATCC 25239) and one clinical bacterial isolate (*Klebsiella pneumoniae*). No cross-reaction was observed by any of the bacterial stains evaluated (Figure 7).

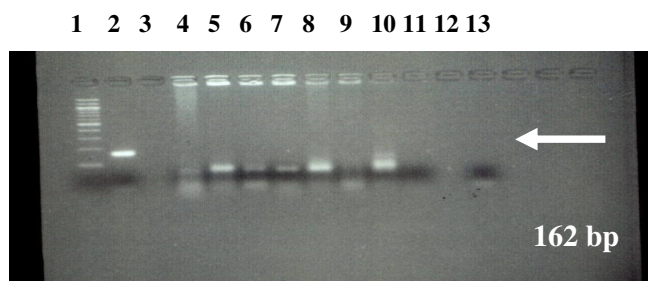


Figure 7: 2% Gel electrophoresis of hMPV RT-PCR reaction using the NL-N primers on control strain serially diluted in water. Lane 1: 1000 bp marker, Lane 2 hMPV positive control, Lane 3: Blank, Lane 4: *H. influenzae*, Lane 5: *Klebsiella*, Lane 6: *Escherichia coli*, Lane 7: *Staphylococcus aureus*, Lane 8: *Pseudomonas aeruginosa*, Lane 9: *Streptococcus pyogens*, Lane 10: *Streptococcus pneumoniae*, and Lane 11: *Moraxella catarrhalis*. Lane 12: Blank, Lane 13: Negative control.

### **3.3 Evaluation of the hMPV NL-N RT-PCR assays sensitivity.**

The sensitivity of the assay developed in this lab was challenged with Quality Control for Molecular Diagnostics (QCMD) external quality control 2006 batch. Twelve QCMD RNA samples were analyzed with the in house hMPV RT-PCR assay. All samples did show the expected results as reported by the manufacturer. (Figure 8).

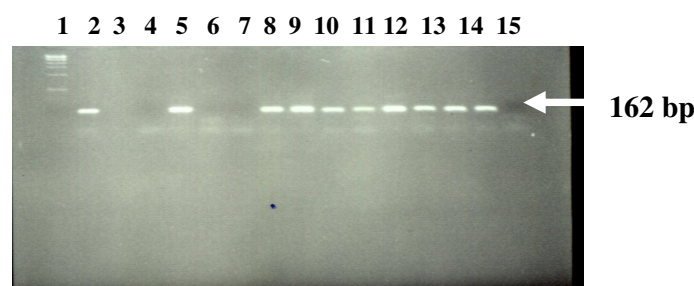


Figure 8: 2% Gel electrophoresis of hMPV RT-PCR reaction using the NL-N primers on control strain serially diluted in water. Lane 1: 1000 bp marker, lane 2: Positive control, lanes 3 up to 14 are the external quality control 2006 batch. Lane 15: Negative control.

### **3.4 Prevalence of hMPV RNA in patient samples.**

A total number of Patients (N=790) admitted to CBH with acute respiratory tract infections between the period November 1<sup>st</sup> 2005 and October 11<sup>th</sup> 2006 were enrolled in this study. NPA samples were analyzed by RT-PCR for hMPV after amplification of the N gene using the NL-N primers. hMPV RNA was detected in 251 (31.7%) of the 790 patients tested. (Figure 9) illustrate a positive and negative patient for hMPV.

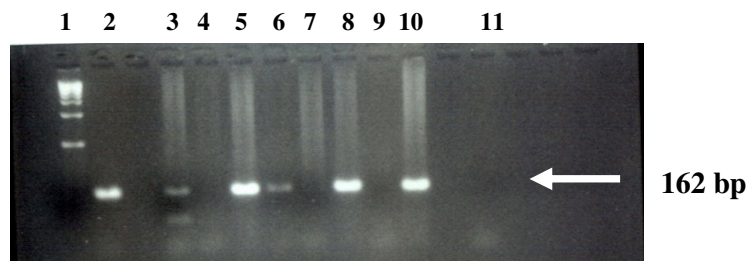


Figure 9: 2% Gel electrophoresis of hMPV RT-PCR reaction using the NL-N primers on control strain serially diluted in water. Lane 1: 1000 bp marker, lane 2: Positive control, lanes 3, 4, 5, 6, 7, 8, 10 are patients samples, Lane 9: H<sub>2</sub>O (Internal negative control), and Lane 11: Negative control.

### **3.5 Distribution of hMPV RNA in the Southern Palestinian Districts.**

The majority of the patients tested were residents of Bethlehem district (63.2%), followed by Hebron (34.3 %), Jerusalem (1.5%), Jericho (0.63%), Ramallah (0.25%) and Nablus (0.13%). Of the patients from Bethlehem district (N=499), 168 (33.66%) were positive for hMPV, while 78 (28.78%) out of 271 patients from the Hebron district, where also positive for hMPV. Few of the patients enrolled in the study were from Jerusalem district and were positive for hMPV.

The distribution of patients to the different areas of the West Bank is shown in table 6 and illustrated in (Figure 10)

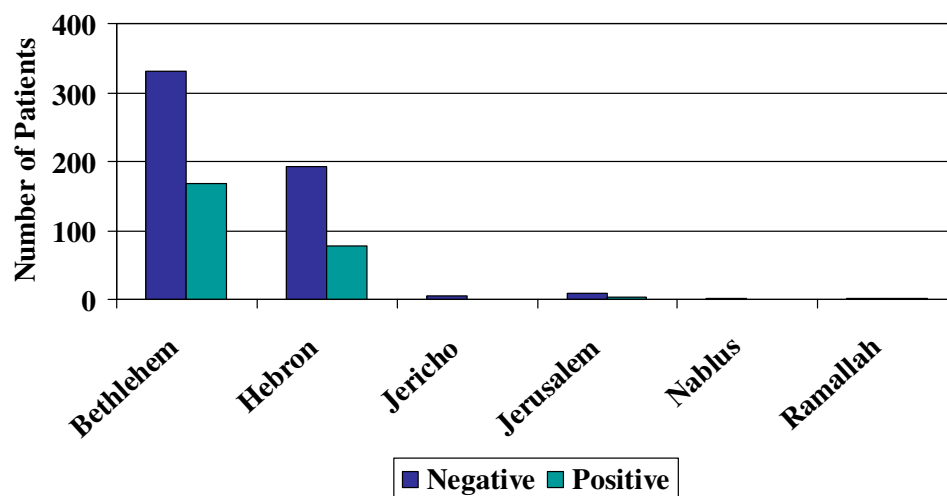


Figure 10: Distribution of hMPV RNA in the Southern Palestinian Districts. Blue columns are negative patients while green columns are hMPV positive patients.

	Negative	Positive	Total
<b>Bethlehem</b>	331	168	499
<b>Hebron</b>	193	78	271
<b>Jericho</b>	5		5
<b>Jerusalem</b>	8	4	12
<b>Nablus</b>	1		1
<b>Ramallah</b>	1	1	2
<b>Total</b>	539	251	790

Table 6: Distribution of hMPV RNA in the southern Palestinian Districts.

### 3.6 Distribution of hMPV positive patients by cities and villages.

Patients admitted to CBH came from many cities and villages from Bethlehem and Hebron districts (Figure). The main areas where hMPV circulated during the study period were Bethlehem city (11.77%), Al khader (3.42%), Tque (2.91%), Al Doha (3.29%), Beit Jala (4.30), Beit Fajjar (4.81%), and Hebron city (5.57%). The distribution of patients to the different cities and villages of the west-Bank is illustrated in (Figure 11).

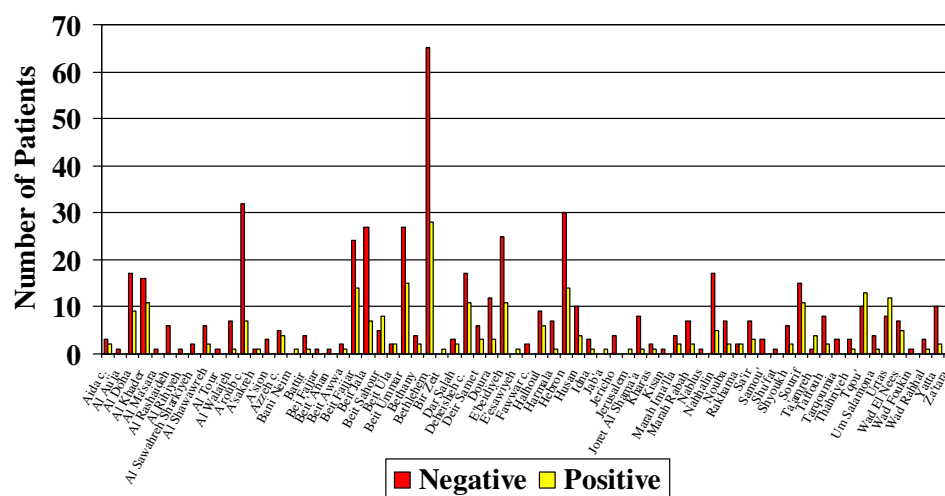


Figure 11: Distribution of patients infected with hMPV by cities and villages. Red columns are negative patients while yellow columns are positive patients.

### 3.7 Distribution of hMPV by Gender

Upon analyzing the patient population by gender type, the male to female ratio was 1.6:1. Of the male patient population 64.4% were positive for hMPV while 35.6% of the female population was positive for hMPV. The distribution of the male to female ration is shown in table 2 and illustrated in (Figure 12)

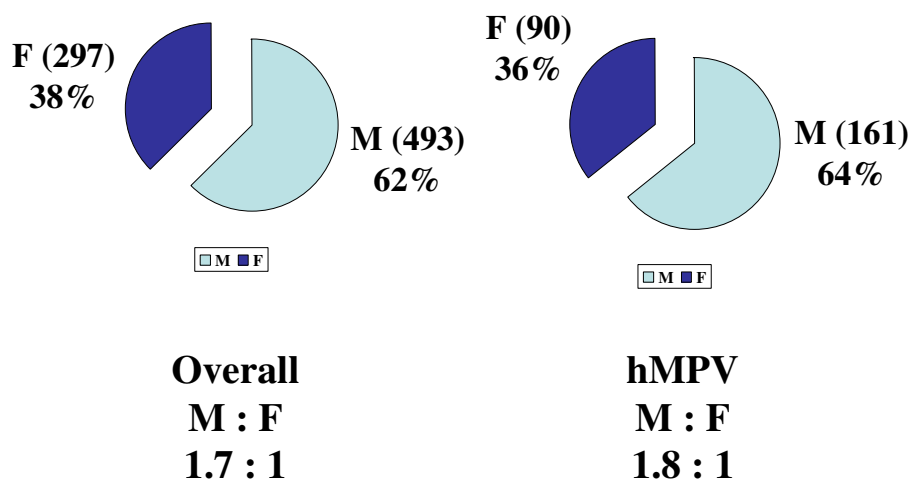


Figure 12: Distribution of hMPV by Gender. Light blue are male patients while blue are female patients.

### 3.8 Distribution of positive hMPV patients by age

The patient's age ranged from one day old new born to 13 years old children. Stratifying the patients by age group, most of patients were less than two years old (92.66%) (Table 3)

The age group for those patients is shown in (Figure 13) the main age of hMPV positive babies were less than 6 months of age followed by 6 month age group. No difference in age group between male and female patients was noted.

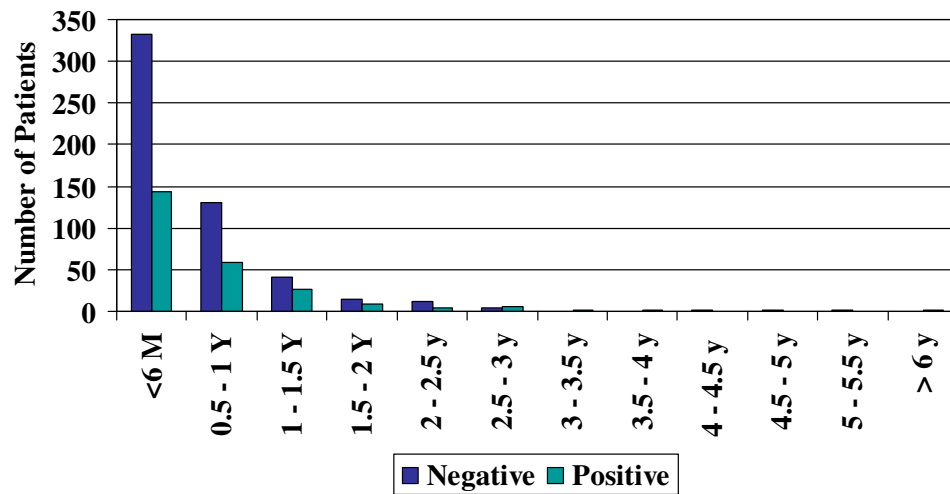


Figure 13: Distribution of positive hMPV patients by age. Blue columns are negative patients while green columns are hMPV positive patients.

### 3.9 Distribution of positive patients by month

The NPA samples were collected from November 2005 until October 2006 but mainly during the respiratory disease season. The hMPV activity was between January and July 2006 with two peak activities, one during February 2006 and a smaller peak activity during April 2006 (Figure 14).

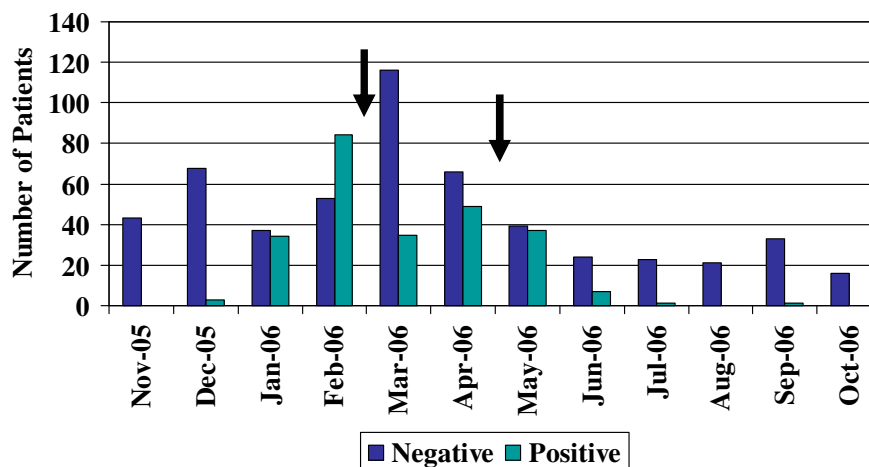


Figure 14: Distribution of positive patients by month. Blue columns are negative patients while green columns are hMPV positive patients.



Upon stratifying hMPV activity by weeks, clearly two hMPV peak activity can be noted as shown in (Figure 15). One hMPV activity was between week 4 and 14 while the other peak was between week 14 and week 23. Interestingly during the second peak an increased activity of RSV virus was noted (Figure 16). Moreover an increase in the double infection rate between RSV and hMPV were noted during the second hMPV peak.

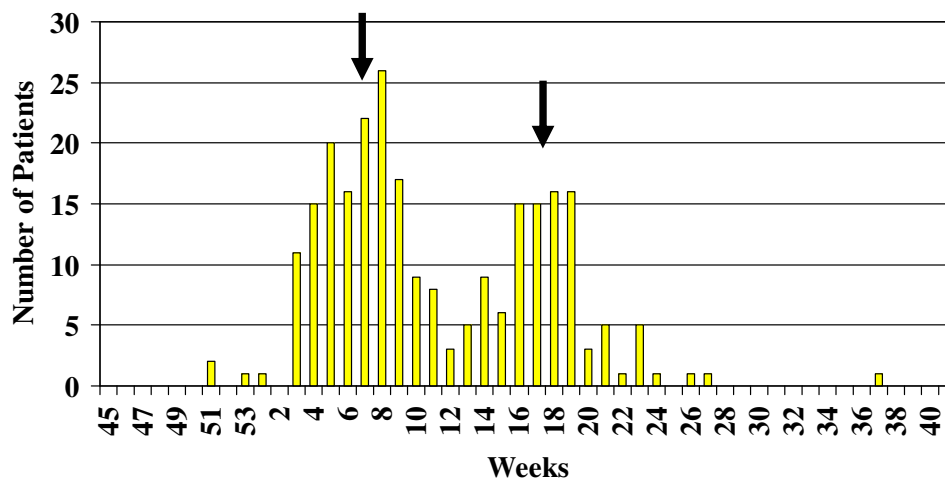


Figure 15: Distribution of hMPV positive patients by weeks. Two peaks of hMPV are indicated by the arrows.

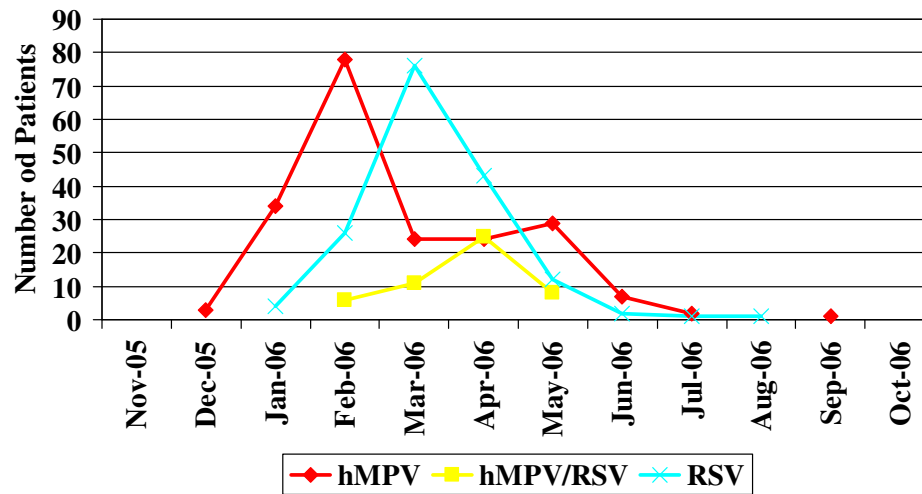


Figure 16: Distribution of hMPV positive patients in red. Distribution of RSV positive patients in blue, while the distribution of hMPV/RSV co-infected patients is shown in yellow.

### **3.10 Phylogenetic analysis of hMPV positive samples**

Of the 251 positive hMPV positive samples 34 (16%) were processed and sent to Bethlehem University for sequence analysis of the N gene. Figure 17 illustrates a gel electrophoresis of 8 hMPV patient samples PCR products that were amplified with the NL-N forward and ALT-R (1019 bp PCR product) and sent for sequencing. Initial processing of the positive patient samples involves cleaning the PCR product sample with the Qiagen PCR purification kit as shown in (figure 18). The purified PCR products were then sent to Bethlehem University for sequencing using the ALT-N-Reverse primers.

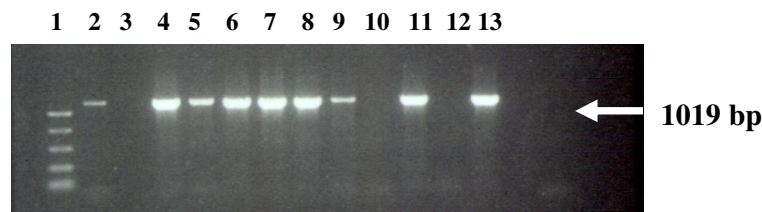


Figure 17: 1% Gel electrophoresis of hMPV RT-PCR reaction using the NL-N (Forward)/ALT-N (Reverse) primers on control strain serially diluted in water **before** gel purification. Lane 1: 100 bp marker, lane 2: positive control, Lane: 3 blank, Lanes: 4 – 9, 11 & 13 are hMPV positive samples, Lanes 10 & 12: unamplified patient sample.

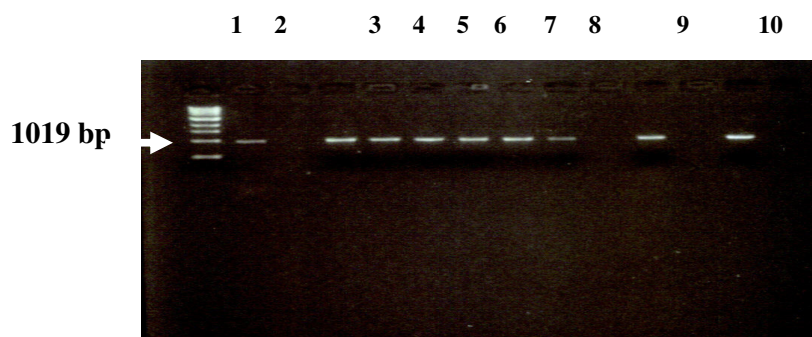
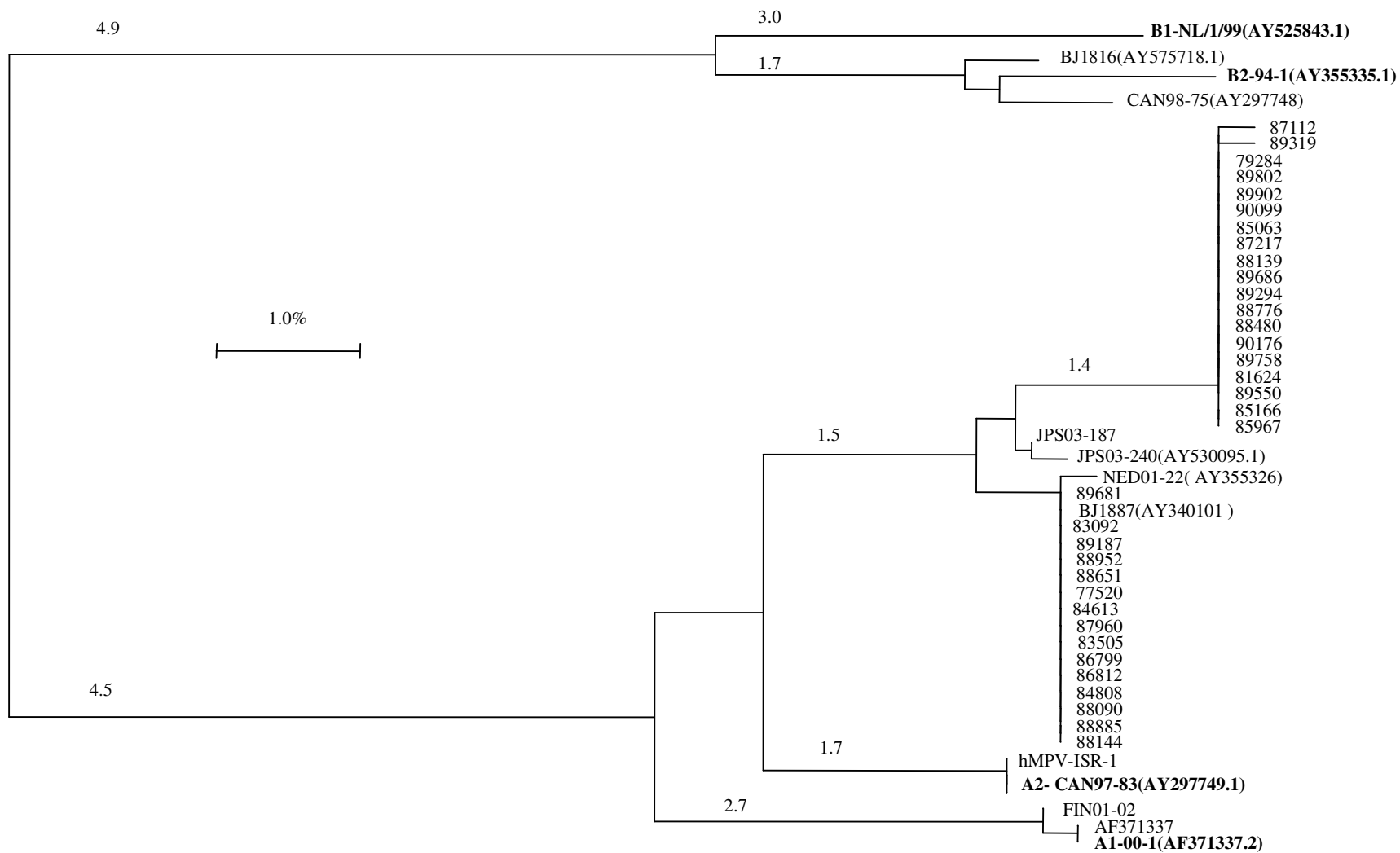


Figure 18: 1% Gel electrophoresis of hMPV RT-PCR reaction using the NL-N (Forward)/ALT-N (Reverse) primers on control strain serially diluted in water **after** gel purification. Lane 1: 100 bp marker, lane 2: positive control, Lane: 3 blank, Lanes: 4 – 9, 11 & 13 are hMPV positive samples, Lanes 10 & 12: unamplified patient sample.

Sequence analysis from the 34 isolates had high homology to the reference hMPV viral N gene (A1, A2, B1, B2) sequences published in the EMBL/GenBank/DDBJ databases. Depending on the group of hMPV sequenced from Southern Palestine, the similarity against the reference strains ranged from 95.4% and 96.8%. Two groups of hMPV appeared to have circulated in Southern Palestine during the study period (Figure 16). High homology (96.8%) was noted for one group (N=15) of hMPV from Southern Palestine when compared to the reference strain hMPV-A2 (CAN97-83; AY297749), while 100%

homology was noted when the same sequence from Palestinian children was compared to that from Britain (BJ1887; AY340101), the positive isolates (N = 19) were about 95.4% similar to the reference strain the reference strain hMPV-A2 (A2-CAN97-83(AY297749) however, they were still faraway by 1.4% from the closest published sequence from Japan (JPS03-240; AY530095). This suggests a new clade of the hMPV circulated in Southern Palestine.

Figure 19: Phylogenetic analysis of N-gene of hMPV virus detected in Palestinian children from Southern Palestine.



#### **4. Discussion**

One of the biggest challenges in diagnostic microbiology is the determination of the causative agent of respiratory tract infections (RTI). This is due to the fact that RTI in children and other age groups are caused mainly by viruses such as RSV, influenza viruses, parainfluenza viruses, rhinoviruses, and coronaviruses (75). Even with extensive diagnostic testing with highly sensitive assays such as PCR and real-time PCR, a substantial portion of RTI cannot be attributed to any known pathogen (76). Up to 40% of patients with respiratory illnesses during the winter months contain no identifiable viral pathogen (76). At Caritas Baby Hospital about 40% of hospitalized patients presenting with upper respiratory tract infections do not have a causative agent identified. In part, this may reflect the presence of human pathogens similar to the newly discovered one (hMPV) or ones that have not been discovered yet. hMPV, the newly discovered virus in Dutch children (12) 2001 was found to cause ARTI in all age groups (13, 16). Studies have shown that hMPV has circulated in the human for around 50 years (12). The reason behind this delay in the discovery is due to hMPV's slow replication kinetics and restricted cell line permissivity (12, 13).

In our study, by using hMPV RNA detection assays, we found hMPV to be associated with about 31.8% of the respiratory illness in hospitalized children residing in the Southern parts of Palestine. This high percent of positivity rate is one of the highest percents reported to date and might suggest that there was an outbreak of hMPV during the study period. Depending on the study hMPV

positivity rates ranged between 1.5% in Australia, to 7.1% in Canada and 25% in Italy (13, 19, 23).

As expected the majority of positive patients came from Bethlehem district (33.7%) followed by Hebron District (28.8%). Indeed, hMPV was detected in all cities and villages of Southern Palestine indicating that the virus was capable of spreading and one of its genes might have some alterations allowing it to spread and cause disease in children (Figure 11). The male to female (1.6:1) ratio of positive Palestinian children was similar to that reported by other groups (17).

Similar to earlier reports on hMPV, the majority of the Palestinian children infected were less than 5 years of age, indeed the majority 92% of the patients were less than 2 years of age (Figure 13). Our results are similar to that reported by McAdam et al. who showed that the majority of the infected children are less than 5 years of age (77).

Interestingly, the distribution of the patients was unique during the study period since the virus had two circulation peaks. One during February 2006 and the other two months later during April 2006 (Figure 14). The distribution of hMPV activity can be seen even more clearly once the virus activity was evaluated over weeks (Figure 15). The circulation of hMPV in Southern Palestine was similar to the hMPV circulation reported by Regev et al in Central Israel where hMPV main activity was between February and March 2003 (26).

One of the interesting features of the study, was the timing of hMPV circulation in comparison to the other most common virus, RSV. A study presented by Boivin et al. showed that in Canada, hMPV was mostly active during the month of April (17), whereas another Dutch analysis reported the active month as being that of December (78). Unlike other report where they described RSV circulation before hMPV (26), in our study, hMPV was detected before RSV (Figure 16). Moreover, we had a large number of double infections during the second wave of hMPV (Figure 16). During the study period 50 patients (6.3%) had double infection between hMPV and RSV.

Sequence analysis of the hMPV reference genome shows that it has two main genetic lineages (A and B) representing two serotypes. Each of the latter comprises two sub lineages (A1, A2, B1 and B2) (53, 79). Phylogenetic analysis of the N-gene of 14% of the positive Palestinian patients amplified hMPV PCR products showed that two serotypes of hMPV A lineage circulated in Southern Palestine, while no isolates of B lineage was detected. Indeed, they two serotypes were not the same that that reported for the reference strains. Of the samples analyzed, 43% of the sequenced hMPV samples belonged to a novel serotype of the lineage A (Figure 19). Upon determining the homology of the newly discovered serotype, 95% homology was observed. Having this 5% difference from the 377 base pair sequences allows to hypothesis that we are dealing with a new serotype and that A lineage has three serotypes and not two. Moreover, a very recent report from Germany hypothesized that there are three serotypes for



A2 hMPV lineage (80). Unfortunately, they have not published enough sequenced so we can compare the sequence from Palestine with their sequence.

One of the important outcomes that we are pursuing is the clinical presentation of the children infected with hMPV and the ones co-infected with hMPV and RSV. Our initial investigation revealed that the patients that were infected with hMPV presented with similar symptoms as RSV. Compiling the data is still in progress and it will be of interest to compare the patients that are infected with the two different serotypes of hMPV. Over the world people have reported similar clinical presentation between hMPV and RSV (13, 81). In addition, it will be of great interest to see the severity of respiratory illness in patients co-infected with hMPV and RSV.

In conclusion, this study is the first report that describes the viral genetic lineages and the prevalence of hMPV infections in Southern Palestine. The discovery of hMPV and its incorporation to routine diagnostic procedures has allowed us to identify the cause of a significant number of respiratory tract infections (31.8%). More upper RTI will be identified when sensitive diagnostic techniques are developed and routinely applied from testing common respiratory pathogens such as rhinoviruses, and coronavirus. However, there still remain a significant number of infections that have still not been associated with any known pathogen.

It is of utmost importance for our Palestinian physicians to know that hMPV is present in the Palestine. Not only that, but it can cause large outbreaks

as shown in our study. Our study clearly showed that hMPV diagnostic assays should be made available for our physicians. Home brew assays for the detection of hMPV are not expensive and hospitals in Palestine should bring these laboratory diagnostic techniques to their laboratories. This will help them in controlling the usage of antibiotics thus indirectly helping in reducing the emergence of drug resistant bacteria such as multidrug resistant *Klebsiella pneumoniae*, *Acinitobacter*, *Streptococcus pneumoniae* and methicillin resistant *Staphylococcus aureus*. Moreover, in severe hMPV infections, physicians might be able to use the antiviral drug Ribavirin as a therapy to help the infected patients get over the hMPV infection since hMPV is a close relative to RSV and RSV is well know to respond to Ribavirin. Finally, researchers should invest more time and effort in developing a vaccine against hMPV in order to protect the children less than 5 years of age.

### **Conclusions and Recommendations:**

1. hMPV circulated in Southern Palestine and preceded the RSV season.
2. hMPV frequency in Palestine during the study period was the highest rate reported worldwide to date (31.8%).
3. hMPV major activity was during January and April 2006.
4. 95% of hMPV infected children were less than 2 years of age.
5. hMPV A2 lineage circulated in Southern Palestine during the study period.
6. Our Phylogenetic analysis proposes the discovery of a new A2 sub-lineage.
7. Careful clinical evaluation of infected children should be performed to compare the clinical presentations of hMPV and RSV.
8. Role of hMPV/RSV double infection in patient hospitalization should be evaluated.
9. Evaluation of the clinical presentation of infected children with the two lineages should be determined.

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