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Molecular Detection and Characterization of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* in Southern Palestine (2015- 2017)

By

Jane Issam Manauel Jeris

In Partial Fulfillment of the Requirements for the Degree

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

Molecular Detection and Characterization of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae in* Southern Palestine 2015- 2017

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In partial fulfillment of the requirements for the degree of Master of Science in biotechnology

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Molecular Detection and Characterization of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* in Southern Palestine 2015-2017

By: Jane Issam Manauel Jeris

Abstract

Background: *Mycoplasma pneumoniae;* is the smallest self-replicating organism that belongs to a special class of bacteria called the "Mollicutes". *M. pneumoniae* is a common etiological agent that causes community acquired pneumoniae (CAP). On the other hand, *Chlamydophila pneumoniae*, an intracellular bacterium and a causative agent of pneumonia. Worldwide, *M. pneumoniae* and *C. pneumoniae* together may be responsible for 3% to 43% of CAP infections.

Objectives: The study aimed at developing an internally controlled multiplex qRT-PCR for the detection of *M. pneumoniae* and *C. pneumoniae* from nasopharyngeal samples collected from hospitalized patients (≥ 5 years old), in Southern Palestine. In addition, the study assessed macrolide resistance pattern of *M. pneumoniae.*

Methods: A retrospective study was conducted using respiratory samples of 350 hospitalized patients with suspected pneumonia and hospitalized at Caritas Baby hospital between 2015-2017. All samples were subjected to multiplex qRT-PCR to determine the presence of *M. pneumoniae* and *C. pneumoniae*, moreover all positive *M. pneumoniae* samples were screened for macrolides resistance in the 23S rRNA.

Results: Multiplex qRT-PCR showed that between 2015 and 2017 in Southern Palestine, out of 350 NPA samples, 23 (6.6%) samples were positive for both *M. pneumoniae* (n=17, 4.9%) and *C. pneumoniae* (n=6, 1.7%). Nested PCR and sequencing showed that there are 3 isolates (17.6%) that have macrolide resistance (erythromycin) in domain V of 23S rRNA in region 1 at position 2063.

Conclusion: qRT-PCR for the detection of *M. pneumoniae* and *C. pneumoniae* is a sensitive test that should be used by medical laboratories to promote an accurate and

rapid laboratory diagnostic method that will lead to improved patient care, appropriate use of antimicrobial therapy and a better understanding of the epidemiology of these two pathogens.

وصف وكشف جزئي للمفطورة الرئوية والكلاميدوفيليا الرئوية في جنوب فلسطين 2015-2017

جين عصام مناويل جريس

ملخص

مقدمة: المفطورة الرئوية هي نوع من انواع البكتريا و مسبب شائع لالتهابات الرئة المكتسبة من المجتمع والتي ايضاً تعرف ب التهاب الرئة اللانمطية. الكلاميدوفيليا الرئوية هي طفيلي داخل الخلية و مسبب للالتهابات الرئوية المكتسبة من المجتمع. في جميع أنحاء العالم, المفطورة الرئوية و الكلاميدوفيليا الرئوية هما مسؤولتان عن التهابات الرئة المكتسبة من المجتمع بنسبة بين 3% و 43%.

الهدف:هدفت هذه الدراسة إلى تطوير فحص تفاعل البوليميريز المتسلسل للكشف عن المفطورة الرئوية و الكلاميدوفيليا الرئوية من عينات البلعوم الأنفي للأطفال المقيمين بل المستشفى و التي أعمارهم خمسة سنين و أكثر من جنوب فلسطين. و أيضاً هدفت إلى فحص انماط المقاومة للمايكروليدات من قبل المفطورة الرئوية.

المنهجية: أجريت دراسة بأثر رجعي باستخدام عينات من الجهاز التنفسي من 350 مريضاً بين عامي 2015 و 2017. جميع العينات اخضعت لفحص تفاعل البوليميريز المتسلسل للكشف عن المفطورة الرئوية و الكلاميدوفيليا الرئوية، بلإضافة إلى فحص عينات المفطورة الرئوية الموجبة لمقاومة المايكروليدات في الحمض النووي الرايبوزي الريبوسومي.

النتائج: كشفت هذه الدراسه باستخدام تفاعل البوليميريز المتسلسل أنه في جنوب فلسطين بين 2015 و 2017 هناك نسبة 6.6% من المفطورة الرئوية والكلاميدوفيليا الرئوية . وأيضاً كشفت أنه هناك نسبة 17.6% مقاومة للمايكروليدات في عينات المفطورة الرئوية الموجبة.

الاستنتاج: تفاعل البوليميريز المتسلسل هو فحص فعال و حساس للكشف عن المفطورة الرئوية و الكلاميدوفيليا الرئوية و يجب استعماله من قبل المختبرات الطبية لضمان تشخيص سريع و فعال لضمان رعاية مناسبة للمريض و استعمال مناسب للمضادات الحيوية و فهم أكثر لانتشار هذه البكتريا في المجتمع.

Declaration

I declare that the Master Thesis entitled " Molecular Detection and Characterization of *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* in Southern Palestine 2015-2018" 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: Jane Issam Manauel Jeris

Date: March, 2019

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Date: March, 2019

Ddedication

This thesis is dedicated to my loving parents.

Issam and Ibtissam Jarayseh

Acknowledgement

"Success is not an accident; it is hard work, perseverance, learning, studying, sacrifice and most of all love of what you are doing or learning to do"

During the last four years, things were changing rapidly except for this research which was growing with me day by day and was the only constant part of my life. This work wouldn't be completed without support and help from various people I would like to thank in person.

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For sure I can't forget my colleagues whom helped me a lot to build up this thesis. Also this project would have been impossible without the generosity of Caritas Baby Hospital. And here I would like to thank specially Dr. Nader Handal and Dr. Ahmad Nairoukh for providing me the necessary data concerning the patients to complete this research.

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At last I would like to thank my fiancé khader, though he came up when i was about finishing this research but he never failed to give me the courage and support i needed.

List of Abbreviations

16 S rRNA	16S ribosomal RNA
ADP	Adenosine diphosphate
ATPase	Adenosine triphosphatease
CRP	C - Reactive Protein
CBH	Caritas Baby Hospital
C^{o}	Celsius
CDC	Centers for disease control and prevention
CP, *C. pneumoniae*	*Chlamydophila pneumoniae*
COPD	Chronic obstructive pulmonary disease
Cfu	Colony forming unit
CAP	Community Acquired Pneumonia
CARDS	Community Acquired Respiratory Distress Syndrome
CFA	Complement Fixation Assay
Ct	Cycle threshold
C	Cytosine
DNA	Deoxyribonucleic acid
DFA	Direct fluorescent antibody
EB	Elementary body
ESR	Erythrocytes Sedimentation Rate
F	Forward
G	Guanine
HRM	High-Resolution Melt
Hmpv	Human metapneumovirus
RNase P	Human Ribonuclease P
IFA	Immunofluorescence Assay
IgE	Immunoglobulin E
IL	Interleukin
LOD	limit of detection
MLS	Macrolide-lincosamide-streptogramin B
MOMP	Major Outer Membrane Porin
µL	Micro liter
µm	Micro meter
MIF	Microimmuno-fluorescence
MP, *M. pneumoniae*	*Mycoplasma pneumoniae*
nM	Nano Molar
NPAs	Nasopharyngeal aspirates
PA	Particle Agglutination Assay
PMP4	Peripheral myelin proteins 4
PPLO	pleuropneumonia-like organisms
PCR	Polymerase Chain Reaction
P	Probe
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
Rxn	Reaction
Rep	Repetitive regions
RSV	Respiratory Syncytial Virus
RFLP	Restriction Fragment Length Polymorphism
RB	Reticulate body
R	Reverse

RNA	Ribonucleic Acid
SNPs	Single Nucleotide Polymorphisms
SP	Soy Peptone
TBE	Tris-Borate-Ethyldiaminetetraacetic acid
UV	Ultra violet
V	Variant
WHO	World Health Organization
Yrs	Years

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Chapter One : Introduction

1.1 Introduction

Pneumonia is an acute respiratory illness of one or both lungs affecting primarily the small air sacs (Alveoli) in which these alveoli become filled with fluid or pus making it difficult to breath. Pneumonia is considered the leading infectious cause of death worldwide, in particular in the developing countries and among children's population. In fact, pneumonia was responsible for 16% of all deaths of children under 5 years old Worldwide, killing 920,136 children in 2015 (WHO, 2016). Pneumonia classification relies on the causative agent or the area of lung infected, but most commonly it is classified on where it was acquired; hospital or community (Dunn , 2005).

Pneumonia is mainly caused by infections with viruses or bacteria and less commonly by other microorganisms like fungi. Though, in some cases, pneumonia can develop as a result of body response to ingestion of certain medications or due to conditions like autoimmune diseases (Leach, 2009). Common respiratory viruses that may progress viral pneumonia among children are respiratory syncytial virus (RSV), influenza types A and B, human metapneumovirus (HMPV), adenovirus and others (Ruuskanen, Lahti, Jennings, & Mardoch, 2011). Less commonly; fungal pneumonia as a result of fungus entering the lungs after inhalation of the spores or through the bloodstream if other parts of the body were infected. *Histoplasma capsulatum*, *Coccidioides immitis* and other fungus species can develop fungal pneumonia (Mandanas, 2017). Additional type of pneumonia is aspiration pneumonia; in this case the person breathes food, liquids, or stomach contents into the lungs (Johnson J. , 2018).

Bacterial pneumonia is a broad field to study; furthermost common species that causes it is *Streptococcus pneumoniae* (CDC: Pneumonia, 2017). Other species of bacteria that can cause pneumonia include *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Legionella pneumophilae*, these species are sometimes referred to "Atypical pneumonia" because pneumonia caused by these organisms might have slightly different symptoms, appear different on a chest X-ray or respond to different antibiotics than the typical

bacteria do. Statistically; *M. pneumoniae* and *C. pneumoniae* together may be responsible up to 43% of Community Acquired Pneumonia (CAP) infections. Table 1.1 lists commonly and less commonly causes of CAP by age group (Ostapchuk & et al., 2004).

M. pneumoniae is an obligate parasitic bacterium of the human respiratory tract which causes tracheobronchitis or atypical pneumonia. *M. pneumoniae* is a member of the class Mollicutes, which includes bacterial pathogens and commensals found in many animals and plants. Though *M. pneumoniae* grows exclusively by parasitizing mammals, humans are the only known natural host for *M. pneumoniae* (Shah, 2012). Class Mollicutes includes bacteria with a unique life form comprising of a small genome around 800,000 base pair and characterized by the absence of a peptidoglycan cell wall. In addition, *M. pneumoniae* infections persistence is associated with its ability to mimic host cell surface composition even after treatment leading to many complications. Typically, the infection occurs during the summer and fall months (Waites & et al., 2017). *C. pneumoniae* is also characterized by lacking the cell wall, aerobic and obligate intracellular pathogen. *C. pneumoniae* is typically coccoid or rod-shaped and considered one of the main causative agents of pneumonia. Moreover, it has also been linked with atherosclerosis and Cornery Heart Disease (Kern, Maass, & Maass , 2009). Many studies lately showed that humans are not the only natural hosts with *C. pneumoniae*; strain isolated from various animals like horses, koala bears, Australian and African frogs have showed high similarity with the human *C. pneumoniae* (Pospissi & Canderle, 2004).

On a regular basis, pediatricians physically and clinically diagnose atypical pneumoniae as the laboratory diagnosis of *M. pneumoniae* and *C. pneumoniae* is challenging due to the demanding nature of the pathogens, the variation 46of the serotypes and the possibility of transient asymptomatic carriage. Although culture is highly specific but is technically demanding, expensive, has a long turnaround time, contamination is common and its sensitivity is highly dependent on specimen transport conditions (Daxboeck, Krause, & Wenisch, 2003), thus the direction of diagnosis is towards molecular approaches like PCR to establish an accurate and rapid laboratory diagnostic method that

will lead to improved patient care, appropriate use of antimicrobial therapy and a better understanding of the epidemiology of these pathogens.

Palestine in general and Southern Palestine in particular lacks real statistics concerning *M. pneumoniae* and *C. pneumoniae* and their incidence among pediatric population. In this thesis we developed an assay to detect these two types of bacteria in an internally controlled Multiplex Real-Time Polymerase Chain Reaction (RT-PCR), revealed any co-infections between the two bacterial pathogens and the common human respiratory viruses and evaluated *M. pneumoniae* resistance patterns to the macrolide's antibiotics. Finally, we evaluated the clinical presentations of the patients infected with either of these two pathogens. The samples that were used were nasopharyngeal aspirates (NPAs) which were used mainly to detect common respiratory viruses using direct fluorescent antibody technique in Caritas Baby Hospital.

Table 1.1: List of commonly and less commonly causes of CAP by age group (Ostapchuk & et al., 2004).

Age	Common causes	Less common causes
Up to 20 days	Bacteria - *Escherichia coli* - Group B streptococci - *Listeria monocytogenes*	Bacteria - Anaerobic organisms - Group D *Streptococci* - *Haemophilus influenzae* - *Streptococcus pneumoniae* - *Ureoplasma urealyticum* Viruses - Cytomegalovirus - Herpes simplex virus
3 weeks-3 months	Bacteria - *Chlamydia trachomatis* - *Streptococcus pneumoniae* Viruses - Adenovirus - Influenza Virus - Parainfluenza virus (1,2&3)	Bacteria - *Bordetella pertussis* - *Haemophilus influenzae B* - *Moraxella catarrhalis* - *Staphylococcus aureus* - *Ureoplasma urealyticum* Viruses

	- Respiratory syncytial virus	- Cytomegalovirus
4 months- 5 years	Bacteria - *C. pneumoniae* - *M. pneumoniae* - *Streptococcus pneumoniae* Viruses - Adenovirus - Influenza Virus - Parainfluenza virus - Rhinovirus - Respiratory syncytial virus	Bacteria: - *Haemophilus influenzae B* - *Moraxella catarrhalis* - *Mycobacterium tuberculosis* - *Neisseria meningitidis* - *Staphylococcus aureus* Viruses - Varicella-zoster virus
More than 5 years	Bacteria - *C. pneumoniae* - *M. pneumoniae* - *Streptococcus pneumoniae*	Bacteria - *Haemophilus influenzae B* - *Legionella species* - *Mycobacterium tuberculosis* - *Staphylococcus aureus* Viruses - Adenovirus - Epstein Bar virus - Influenza virus - Parainfluenza virus - Rhinovirus - Respiratory syncytial virus - Varicella-zoster virus

Chapter Two : Literature Review

2.1 Mycoplasma pneumoniae

2.1.1 History

Early in the 1930s, one type of pneumonia "Atypical pneumonia" was able to be clinically distinguished from typical pneumonia by its unfamiliar clinical signs and symptoms, in addition to its resistance to sulfonamides (bacteriostatic antibiotics that inhibit growth and multiplication of bacteria) (Saraya, 2016). Years later these infectious agents were known as filterable viruses that could escape Seitz filter and were reported to be a psittacosis-like or new virus. Several years before 1950s, Eaton et al (1942-1944) identified and isolated an agent that was the primary cause of atypical pneumoniae using animal models (Eaton, Meiklejohn, & Herick, 1944). During 1950s, Fluorescent Stainable Antibodies were brand new and helped in the acceptance of Eaton agents as *M. pneumoniae* after several serological approaches (Saraya, 2016).

Historically, the first isolated *Mycoplasma* was reported by Nocard and Roux in 1898, at that time and through the next 50 years *Mycoplasma* was the bovine pleuropneumonia agent and known as pleuropneumonia-like organisms (PPLO) in various animals (Pierre, 1898). In 1937 the first human Mycoplasma was isolated by Dienes and Edsall from Bartholin's gland abscess, now it's known as *Mycoplasma hominis* (Dienes & Edsall, 1937). On the other hand, Eaton et al. in 1944 succeeded to isolate the first *M. pneumoniae* from sputum tissue culture from a patient suspected to have atypical pneumoniae (Eaton, Meiklejohn, & Herick, 1944). Yet, until the 1960s *M. pneumoniae* taxonomy and structure were still challenging to study but researchers were able to identify it as a bacterium, don't exceed 100 micrometer (μm) in diameter, less 5% in volume compared to bacillus and lacks cell wall (Saraya, 2016). Regarding the term *M. pneumoniae*; it was suggested by Chanock et al. in 1962, In Greek, *Myco* means "Mykes" (Fungus) and *Plasma* "Formed" and "pneumoniae": relating it to the disease it causes (Chanock & et al., 1963).

2.1.2 Taxonomy and Classification

M. pneumoniae belongs to the class Mollicutes. Under this class, branches 4 orders, 5 families and about 200 species that have been spotted in humans, vertebrates, arthropods and plants. Currently, 16 species isolated from humans are known. Out of these species, six are recognized to cause diseases*: Mycoplasma fermentans, Mycoplasma hominis, Mycoplasma genitalium, Mycoplasma pneumoniae, Ureoplasma urealyticum*, and *Ureoplasma parvum* (Waites & Talkington, 2004). 16S ribosomal RNA (16S rRNA) analysis have showed that Mollicutes are closely related to and diverged from the gram-positive eubacterial subgroup that includes the bacilli, streptococci, and lactobacilli about 605 million years ago (Maniloff, 1992). Figure 2.1 shows *Mycoplasma* phylogeny reconstructed from 16S rRNA sequence comparisons (Waites & Talkington, 2004). Referring back to *M. pneumoniae*, Figure 2.2 shows the Hierarchical order of *M. pneumoniae* (Waites & Talkington, 2004).

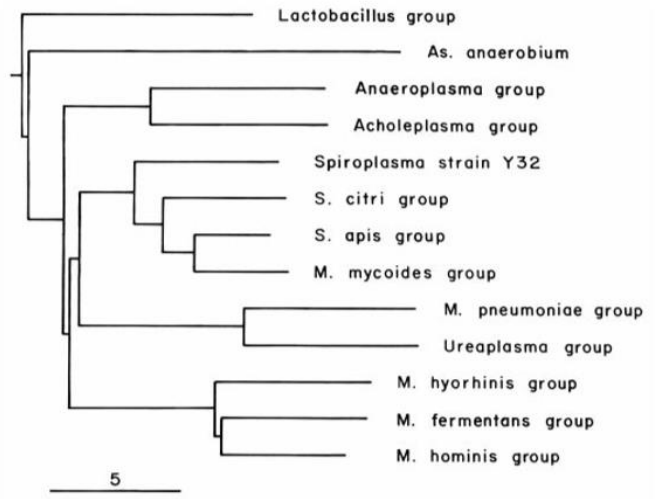


Figure 2.1: Phylogeny of Mycoplasmas reconstructed from 16S rRNA sequence comparisons. [1]

[1] Branch lengths are proportional to evolutionary distance (the number of base changes per 1,000 nucleotides). The scale at the bottom denotes the branch distance corresponding to five base changes per 100 nucleotides.

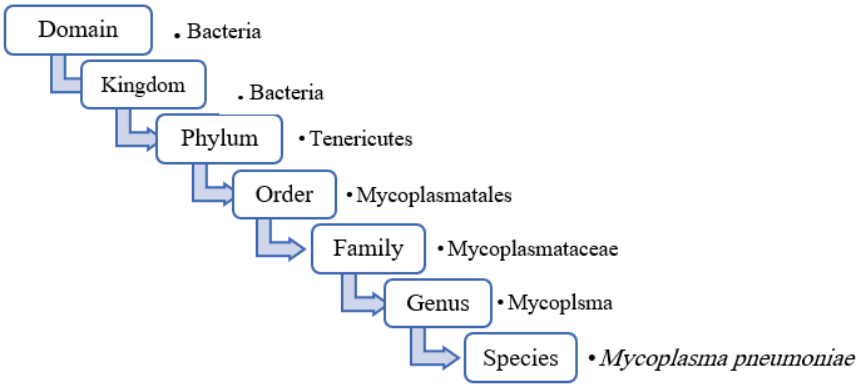


Figure 2.2: Scheme of the classification series of Mycoplasma pneumoniae

2.1.3 *Mycoplasma* species

Mycoplasma species are the smallest known free-living self-replicating organisms, from these *M. pneumoniae*; the most important and the most well-known. Generally, members of *Mycoplasma* are characterized by their absence of cell wall, relatively small genomes consisting of a single circular chromosome around 0.58 to 2.2 Mega base pairs and low guanine (G) and cytosine (C) content (23% - 40%) (Johansson & Pettersson, 2002). Lack of cell wall makes *Mycoplasmas* susceptible to lysis by hypotonic solutions; resistant to Gram staining thus prevents direct detection from patient samples, adding to these, resistant to antibiotics that act on the cell wall (i.e. beta lactams). *M. pneumoniae* has extra features other than *Mycoplasma* species that may play a role in diagnosis (Ferwerda, Moll, & De Groot, 2001); it has slower growth, ferments glucose, absorbs erythrocytes in the growing colonies and reduces tetrazolium when grown aerobically or anaerobically (Waites & Talkington, 2004).

2.1.4 Structure

Outer membrane of *M. pneumoniae* has an elongated shape with adhesive extremes that consist of an electron dense core with a trilaminar membrane. *M. pneumoniae* is about 1 to 2µm long and 0.1 to 0.2 µm wide. Figure 2.3 shows the longitudinal schematic depicting the cellular architecture of *M. pneumoniae* (Parrott, Takeshi , & Fujita, 2016). Since *M. pneumoniae* characterized by small size and volume, cells can pass through

0.45-µm-pore filter sterilize media, cannot be detected by light microscopy and do not produce visible turbidity in liquid growth media.

Typically, colonies of *M. pneumoniae* don't exceed 100 µm in diameter when cultured on high rich medium like Soy Peptone (SP4) and observing the colony's features require stereo microscope (Waites & Talkington, 2004). Figure 2.4 shows the "fried egg" appearance of *M. pneumoniae* colonies (Saraya, 2016).

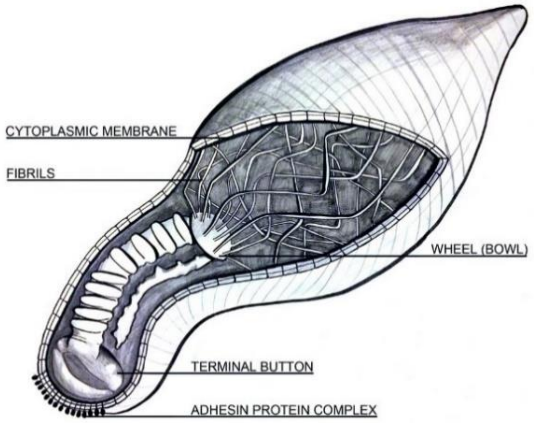


Figure 2.3: A longitudinal schematic depicting the cellular architecture of M. pneumoniae.

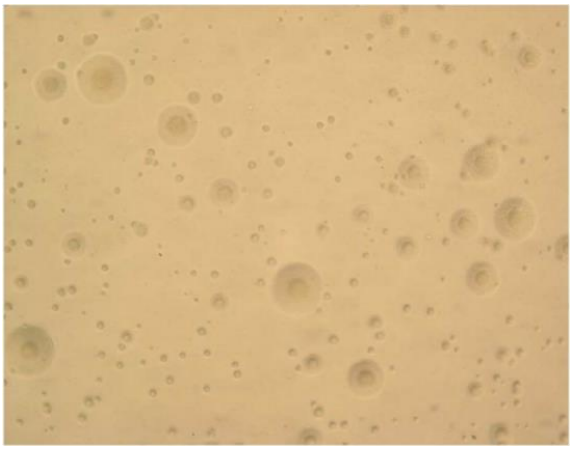


Figure 2.4: Appearance of colonies of M. pneumoniae. Colonies of M. pneumoniae on an agar plate typically have a unique "fried egg" appearance

2.1.5 Genomics

Complete genome sequencing of *M. pneumoniae* (strain M129) was accomplished in 1996. Genomic annotations have exposed that *M. pneumoniae* consists of 816,394 base pairs with 677 open reading frames, 39 coding genes for various RNA and an average G+C content of 40.0 mol%. Furthermore, phylogenetic analysis of *M. pneumoniae* pointed that there was a reduction of genome size during its reductive evolution from ancestral bacteria which can be explained by the loss of complete anabolic (e.g. no amino acid synthesis) and metabolic pathways, therefore *M. pneumoniae* signifies an obligate bacterium in nature (Himmelreich, et al., 1996). Although, *M. pneumoniae* compared to conventional bacteria owns limited metabolic and biosynthetic activities for proteins, carbohydrates and lipids, some unexpected findings were observed since sequencing followed by annotation of the genome. For example, there is genomic evidence for enzymes such as arginine deiminase for ammonia production (Pollack, Williams, & McElhaney, 1997). Extra unique property of *Mycoplasma* spp. in general, is the use of the universal stop codon (UGA) as a codon for tryptophan (Inamine , Ho, Loechel, & Hu , 1990).

2.1.6 Genotypes and P1 protein

Protein 1 (P1), adhesion surface protein is considered a significant immunogenic and virulent factor due to its interaction with other proteins to form complexes that localize at ciliated respiratory epithelium to initiate an infection. Researches have agreed that *M. pneumoniae* isolates is preferable to be classified into reference strains M129 (type 1) and FH (type 2) based on sequence variation of the repetitive regions RepMP2/3 and RepMP4 of P1(MPN141) gene (Dorigo-Zetsma, Wilbrink, Dankert, & Zaat, 2001)(Cousin-Allery , et al., 2000). At the same time, additional recombination could take place outside P1 gene creating V1, V2a, V2b, V2c and V2d variants. It is proposed that recombination within and outside P1 gene is associated with antigenic variation that triggers surface of *M. pneumoniae*, furthermore these recombinants may favor evasion from the immune response thus generating special pattern of the infection in particular in outbreaks (Razin, Yogev , & Naot , 1998) (Kenri, et al., 1999). Figure 2.5 represents a

schematic of the p1 gene structure of *M. pneumoniae* (Zhao & et al., 2011). Genotyping for *M. pneumoniae* isolates focus mainly on standard methods including PCR-restriction fragment length polymorphism (PCR-RFLP), nested-PCR followed by sequencing, Real-time PCR followed by High-Resolution Melt (HRM) analysis and Single Nucleotide Polymorphisms (SNPs) (Schwartz, Thurman, Mitchell, Wolff, & Winchell, 2009).

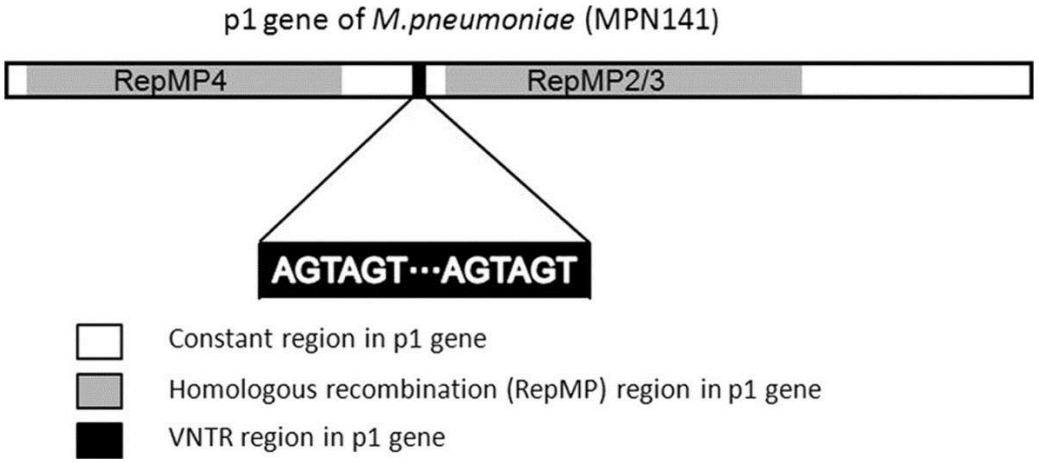


Figure 2.5: Schematic of the p1 gene structure of M. pneumoniae.[2]

2.1.7 Reproduction

It is believed that *M. pneumoniae* has developed specialized reproductive cycle to adapt small genomic size and nature of parasitic life style that requires attachment to host cell. According to Waite and Talkington, specialized reproduction occurs by "binary fission, temporally linked with duplication of its attachment organelle, which migrates to the opposite pole of the cell during replication and before nucleoid separation" (Waites & Talkington, 2004). However mutations that affect the formation of the attachment organelle not only hinder motility and cell division, but also reduce the ability of *M. pneumoniae* cells to adhere to the host cell

2.1.8 Epidemiology

It is well known that *M. pneumoniae* is a major cause of CAP worldwide, accounting for 11-15% of CAP throughout the world (Nir-Paz, Saraya, Shimizu, Van-Rossum, &

[2] The VNTR, which is composed of a different AGT repeat, is located in the region between the RepMP4 and RepMP2/3 elements of the p1 gene.

Bebear, 2017), but the exact epidemiology of pediatric pneumonia is still poorly defined in the majority of the countries. An important study by Chen et al. (2013) investigated the epidemiology of *M. pneumoniae* infections in hospitalized children with respiratory tract infections and its association with meteorological factors in China. The researchers have concluded that *M. pneumoniae* is one of the most commonly held pathogens and *M. pneumoniae* infection has its own epidemic season, especially in the summer. In fact, mean temperature is the main meteorological factor affecting the epidemiology of *M. pneumoniae* infections (Chen, et al., 2013). However, although, outbreaks of *M. pneumoniae* infection commonly occur in closed or semi-closed communities and settings, causing mild upper respiratory tract infection; the control of such outbreaks is challenging, owing to delayed detection, long incubation period and paucity of infection control guidelines.

2.1.9 Pathogenesis

M. pneumoniae spreads from person to person through droplet nuclei. It has special attachment organelle to reach epithelium of the respiratory tract in order to survive, reproduce and infect. Likewise, tight adhesion between *M. pneumoniae* and epithelium depresses muco-ciliary clearance from removing foreign bodies (Chaudhry, Ghosh, & Chandolia, 2016). Tight adhesion also mediates the damage of cilia and recruits innate immune system cells as an immune response where local cytotoxic effects take place. A virulent factor that *M. pneumoniae* produces is Community Acquired Respiratory Distress Syndrome (CARDS) toxin; an adenosine diphosphate (ADP) ribosylating and vacuolating protein that leads to Th1/Th2 imbalance thus leading to inflammation and airway dysfunction (Segovia & et al., 2017). However, extra pulmonary complication also could take place with 25% of cases involving: nervous system, cardiovascular system, bones and joints, kidney, skin and mucosa. From these, the most common one is neurological complications where encephalitis occurs in children less than 10 years old (Narita, 2009).

2.1.10 Signs and Symptoms

Pneumonia caused by *M. pneumoniae* is referred to walking pneumonia due to minor respiratory illness and self-limiting infection where only 3%-10% of infected individuals develop major pneumonia (Ferwerda, Moll, & De Groot, 2001). *M. pneumoniae* affects either upper or lower respiratory tracts where symptoms commonly appear gradually. Typically, incubation period ranges between 2 to 4 weeks. Clinical signs and symptoms vary from person to person depending on age and health status but the general picture includes: onset of fever, chills, malaise, sore throat, headache and dry cough, whereas persistent cough is a clinical feature of *M. pneumoniae* infection. Table 1 lists the various clinical manifestations of *M. pneumoniae* infection (Waites K. , 2003). Mostly; *M. pneumoniae* infection is hard to discriminate in a way that influences antibiotic administration by pediatricians. Though, it may differ from those viral infections or pneumococcal pneumoniae that it has a more gradual onset of symptoms, besides this diarrhea, nausea and vomiting are infrequent. Concerning the radiological findings of chest x-ray for patients, actually *M. pneumoniae* has variable pattern but most commonly bilateral lower lobe consolidation with small pleural effusions are observed. Bilateral reticulonodular densities and areas of atelectasis may also be seen (Vervloet, Marguet, & Camargos, 2007). Hallander et al. (1999) concluded that *M. pneumoniae* was one of the main causes of persistent cough in patients with *Bordetella pertussis* with 56% as double infection (Hallander, Gnarpe , Gnarpe, & Olin, 1999). Furthermore, some studies found that *M. pneumoniae* was the cause of asthma attacks in 50% of 119 hospitalized children (Biscardi & et al., 2004). Though, some patients are susceptible to develop sever pneumonia from *M. pneumoniae* more than others, such as hypogammaglobulinemia and sickle cell anemia patients (Saraya, 2017).

Table 2.1: Clinical manifestations associated with infection by M. pneumoniae.

Manifestation	Frequency observed
Fever	++++
Cough	++++

Rales on chest auscultation	+++
Malaise	+++
Headache	++
Sputum production	++
Sore throat/ pharyngitis	++
Chills	+
Hoarseness	+
Earache	+
Coryza	+
Diarrhea	+
Nausea/Vomiting	+
Chest Pain	+
Lymphadenopathy	+
Skin rash	+
Conjunctivitis	+/-
Otitis media/ myringitis	+/-

2.1.11 Diagnosis

2.1.11.1 General Laboratory Findings

Generally, leukocytes elevation is the first sign to have an infection coupled with elevated Erythrocytes Sedimentation Rate (ESR) and C - reactive protein (CRP) as well, these findings are considered nonspecific for *M. pneumoniae* infection. Concerning the hepatic and renal abnormalities they are absent. On the other hand, cold agglutinins are considered the first humoral immune response to *M. pneumoniae*, these agglutinins are formed at the end of 1st week and the beginning of the 2nd week then vanish after 2-3 months. Although, cold agglutinins can be present in 50%-60% of the cases, still they are considered nonspecific findings due to their presence in various illnesses like Epstein Barr Virus, adenovirus and *Legionella pneumoniae* infections (Kashyap & Sarkar, 2010).

2.1.11.2 Serology

During recent years, various serological techniques were adopted regarding the detection of *M. pneumoniae* in which they focused on the critical etiological factors of this microorganism for instance P1 adhesion protein. An important study revealed that by using Enzyme Immunoassay (ELISA) with various epitopes like glycolipids, purified proteins (i.e. Parts of P1 protein) and combinations of synthetic peptides can reach 100% sensitivity, 96.5% specificity (Suni , Vainiops, & Tuuminen, 2001). Cross reactivity is common in particular with *Mycoplasma genitalium* since it's closely related to *M. pneumoniae* (Daxboeck, Krause, & Wenisch, 2003). However, other serological techniques are also available including: complement fixation assay (CFA), particle agglutination assay (PA) and indirect immunofluorescence assay (IFA) (Andreu, Molions, Fernandez, & et al., 2006).

2.1.11.3 Culture

Since *M. pneumoniae* is a fastidious bacterium in nature, the preferable culture medium for it is SP4 medium (Daxboeck, Krause, & Wenisch, 2003). Even though culture and isolation are the ideal microbiological technique for bacterial identification, isolation of *M. pneumoniae* is time consuming (1-4 weeks), require special handling, contamination is common and has low sensitivity thus not highly recommended.

2.1.11.4 Molecular diagnosis

Going back to 1989, the first PCR applied to detect *M. pneumoniae* was by Bernet et al. (Bernet, Garret, De Barbeyrac, Bebear, & Bonnet, 1989) and it's interesting to know that the first set of primers used were triggering the Adenosine triphosphatase (ATPase) operon gene (144bp). Years later, advanced molecular diagnosis opened the door for researchers to understand more the genomics of microorganisms and to look for more conserved regions to trigger for detection. For *M. pneumoniae* P1 adhesion gene, 16S ribosomal ribonucleic acid (16 S rRNA) and 23s rRNA are the appropriate areas to look within and the most conserved ones. In one hand, DNA probes were used against 16S rRNA but they had short lifespan (6weeks), high cost and require appropriate handling of

radioisotopes (Andreu, Molions, Fernandez, & et al., 2006). On the other hand, PCR has high sensitivity and specificity when compared to other methods (Mehrota, Mehra , Siddque , & Suri, 2015). Moreover, PCR is rapid and very effective for early diagnosis and nowadays, PCR is considered the ideal method for detecting *M. pneumoniae*. PCR related methods enrich the identification and characterization of *M. pneumoniae* including: Nested PCR which is able to detect small quantities/ copy number of the pathogen, Multiplex PCR in which *M. pneumoniae* can be combined with other respiratory pathogens like *C. pneumoniae* and *L. pneumophilae.* In addition to Real-Time PCR that quantifies the existence of *M. pneumoniae.*

2.1.12 Treatment and Prevention

Although, *M. pneumoniae* is naturally resistant to antibiotics that act on cell wall; still it is sensitive to macrolides, cyclines, and quinolones. According to the international guidelines; it is recommended to use macrolides (erythromycin, azithromycin and clarithromycin) and cyclines (doxycycline) as first line antibiotics while quinolones (Fluoroquinolones) as second line antibiotics for pediatrics (Yin Yu & et al. , 2017). Unfortunately, extreme usage of macrolide antibiotics to treat respiratory infections results in the emergence of resistant strains of *M. pneumoniae* in 23S rRNA (Copete & et al. , 2018).

2.1.12.1 *M. pneumoniae* Macrolide Resistance

Macrolides are natural products that contain macrocyclic lactone ring that attach to one or more deoxy sugars in which it includes: erythromycin, roxithromycin, azithromycin and clarithromycin. Macrolide-lincosamide-streptogramin B (MLS) antibiotics target protein synthesis through binding to domain II or domain V of 23S rRNA (Dowthwaites , Hansen , & Mauvais, 2000). Depending on bacterial concentrations there are two options; if the concentration is relatively high macrolides works as bacteriostatic (inhibiting the growth), in the opposite scenario macrolides work as bactericidal (killing the bacteria) (Ikram, 2012). In addition, these classes of antibiotics are preferable because of lower

chance to develop gastroenteritis, lower possibility of drug interaction and do not affect normal flora (Fernandes , Martens, & Pereira, 2017).

Macrolides are effective against Gram positive bacteria and some Gram-negative bacteria, in addition to some special classes of atypical bacterial such as *M. pneumoniae*, *C. pneumoniae*, *Bordetella pertussis*, *Legionella* spp. and *Campylobacter* spp. Furthermore, macrolides may also have immunomodulatory and anti-inflammatory effects for instance; they are used in treating cystic fibrosis (Kanoh & Rubin, 2010). Nevertheless, macrolides are considered as an analog to benzylpenicillin in terms of effectiveness so they could be used as a substitute for patients with allergy to penicillin and or cephalosporins (Ikram, 2012).

Macrolides are recognized as first choice treatment for *M. pneumoniae* in particular Erythromycin. Yet, macrolides are subject to point mutations in 23S rRNA in domain V of the peptidyl-transferase loop in addition to mutations in the binding site of macrolide to the 50 S ribosome subunit that leads to reduction of the affinity to the antibiotic (Yin Yu & et al. , 2017). Some valuable studies have identified point mutations and their positions; A→ C/G at positions 2063, 2064 and C→ A/G at position 2617, these mutations were found in domain V. Regarding domain II no mutations were detected (Lucier & et al., 1995) (Okazaki & et al. , 2001). Figure 2.6 shows the secondary structure of the peptidyl-transferase loop in domain V of *M. pneumoniae* 23S rRNA (Matsuoka & et al., 2004).

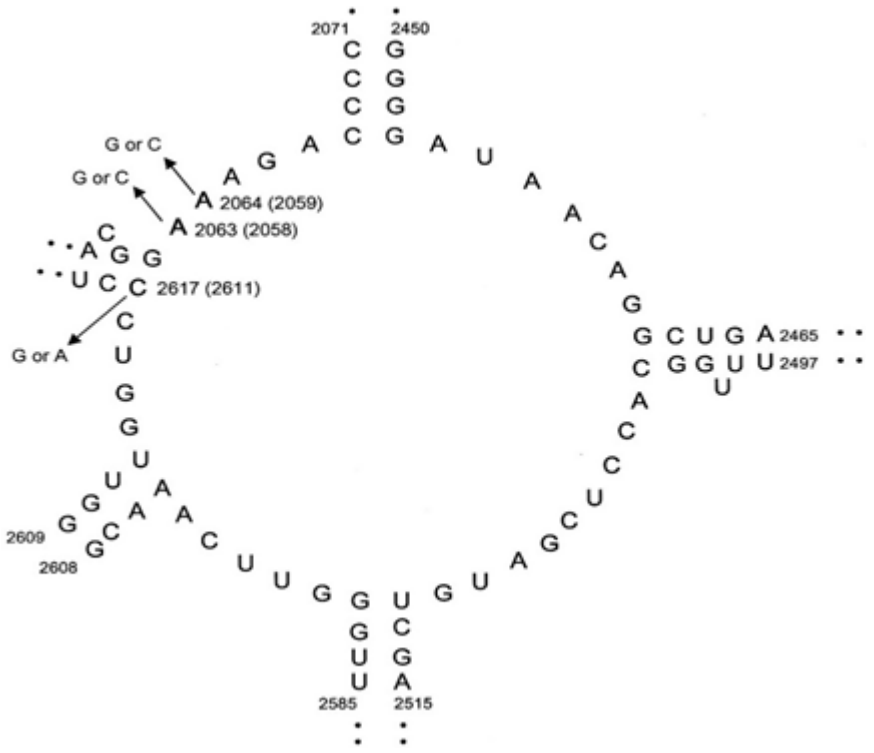


Figure 2.6: Secondary structure of the peptidyl-transferase loop in domain V of M. pneumoniae 23S rRNA.

Positions of the newly found mutations (A2063C and C2617G), as well as previously reported in vitro mutations (A2063G, A2064G, and A2064C), in clinical isolates are indicated by using the numbering for *M. pneumoniae* 23S rRNA (accession no. X68422). The numbers in parentheses indicate *E. coli* numbering.

2.1.12.2 Vaccination

Although *M. pneumoniae* is a self-limiting pathogen; but immunity is required sometimes due to prolonged carriage, possibility of outbreaks in crowded places such as hospitals and schools besides the lack of natural immunity after primary infection. Virulent factors in *M. pneumoniae* are various but studies have focused on attachment and/or adhesion epitopes including P1/ P30 adhesion genes (Dunke & Jacobs , 2016). Another optional virulent factor is toxins like CARDS toxin. In fact, a study by Medina et al. (2017) on Balb/c mice has showed that a single mucosal exposure to CARDS toxin was sufficient to increase total serum Immunoglobulin E (IgE) and CARDS toxin-specific IgE in mice,

where CARDS was used as an adjuvant that lead to increase the interplay between Interleukin (IL4) and IgE (Medina & et al., 2017). However, live and attenuated agents' vaccines of *M. pneumoniae* still under processing but yet not recommended for children.

2.2 Chlamydophila pneumoniae

2.2.1 History

C. pneumoniae was first isolated in 1965 in Taiwan from the conjunctiva of a child using yolk sac of an embryonated chicken egg during trachoma vaccination trial. At that time egg yolk was used as a growth and/or culture medium for *Chlamydia* (Kuo & et al., 1986). Years later the isolated pathogen (TW-183) role was not fully known but it was distinguished that it is not related to eye disease and in morphology it is close to *Chlamydia psittaci* than *Chlamydia trachomatis.* In 1983 TW-183 was confirmed and related to pneumonia after the isolation of respiratory pathogen (AR-39) from a university student with pharyngitis in Seattle, Washington, where TW-183 and AR-39 were related to each other using serological evidence and named (TWAR) (Grayston & et al., 1986). Since 1989 TWAR/ *C. pneumoniae* is known as the third species of genus *Chlamydia* that cause acute respiratory infection (Kuo, Jackson, Campbelle, & Grayston, 1995).

2.2.2 Taxonomy and Classification

C. pneumoniae belongs to the order *Chlamydiales*, Family *Chlamydiaceae* and Genus Chlamydia (figure 2.7). Chlamydiae phylum branches Chlamydiasceae family that includes 11 species, causes pathogenicity to humans or animals main species (Bachman, Polkinghorne, & Timms, 2014), of these that are related and/or infect humans: *Chlamydia psittaci*; causes the zoonotic infectious disease (Parrot Fever), *Chlamydia trachomatis*; sexually transmitted pathogen that causes trachoma, *Chlamydia abortus*; that causes abortion and fetal death in mammals (newly added) and *C. pneumoniae* ; causes acute respiratory infection (Johnson, 2017) . However, classification was based on cumulative immunological, ultrastructural and DNA analysis.

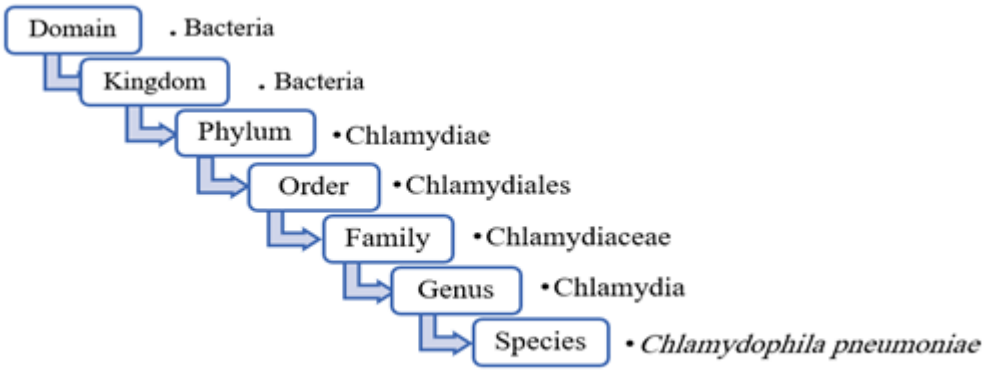


Figure 2.7: Scheme of the classification series of Chlamydophila pneumoniae.

2.2.3 Genomics

Generally, members of the Family *Chlamydiaceae* are composed of compact genomes and highly conserved regions; over 500 genes in spite of the wide range of hosts and diseases (Collingro, et al., 2011). From *Chlamydia* spp., the human *C. pneumoniae* was highlighted to have the most conserved nucleotide sequence, with the greatest coding capacity and about 300 SNPs only, its circular chromosome consists of 1,226, 565 nt (40.7% G+C) with 1072 likely protein-coding genes (Roulis E. , et al., 2015). Additional coding capacity of *C. pneumoniae* could be explained by the expansion of polymorphic membrane protein (pmp) and inclusion membrane protein (inc) genes, these genes are important in the formation and maintenance of the chlamydial inclusion body, in addition they play a role in the modulation of the host cell response (Dehoux, Flores, Dauga, Zhong, & Subitil, 2011).

Till this date, based on Roulis et al. (2015) there are 30 different strains of *C. pneumoniae*, the study concluded that there is great variation among the strains when sequenced, furthermore there is a possibility of various *C. pneumoniae* strains circulating in the human population still not detected yet (Roulis E. , et al., 2015). Other studies have demonstrated through whole genome sequencing that out of the 1073 genes in *C. pneumoniae*, 186 genes are specific with no other homology with other organisms nor

chlamydial spp. (Roulis, Polkinghrone, & Timms, 2013). Furthermore, some studies revealed when sequencing *C. pneumoniae* from isolates found in atherosclerotic carotid plaque that the variation among strains are found in domain IV major outer membrane protein A gene (MOMPA) (Roulis, Polkinghrone, & Timms, 2013). However, further analysis on genomic sequence of *C. pneumoniae* has showed that some strains may comprise extrachromosomal elements (Plasmids) that historically suggest that Human *C. pneumoniae* was zoonotically acquired (Myers, et al., 2009).

2.2.3.1 MOMP gene

Major outer membrane porin (MOMP) has three main roles: regulation of the cell shape, structural molecule activity and porin activity. In other words, MOMP provides structural integrity of outer cell envelope through disulfide cross-links with the small cysteine-rich protein and the large cysteine-rich periplasmic protein and permits diffusion of specific solutes and ions through the outer membrane (UniprotKB, 2018).

2.2.4 Structure

C. pneumoniae is a Gram negative, coccoid, nonmotile bacterium and 0.2–1.5 μm in diameter. Characterized by cell wall containing an outer lipopolysaccharide membrane but missing peptidoglycan layer, furthermore the cell wall is rich with cysteine proteins that play a role in intracellular division and extracellular survival (Johnson, 2017). *C. pneumoniae* lacks several metabolic and biosynthetic pathways thus make it an obligate intracellular organism that reproduce in a vacuolar compartment; the inclusion (Dehoux, Flores, Dauga, Zhong, & Subtil, 2011).

2.2.5 Life Cycle

Chlamydiae members are characterized by their distinct life cycle, where there is a switching between extracellular infectious elementary body (EB) and intracellular non-infectious reticulate body (RB). Changes in the outer membrane and nucleoid structures take place as well to complete the switching (Roulis, Polkinghrone, & Timms, 2013). EB protects against environmental stress, once it reaches a new host (lungs) the EB is phagocytized to an endosome and transformed into large intracellular form RB and starts

multiplying within the endosome. After several multiplications the RBs are switched into new EBs and released into the cytoplasm to reinitiate new cycles and infect new host (Kuo, Stephens, Bavoil, & Kaltenboeck, 2015). Figure 2.8 shows the morphology of *C. pneumoniae* through electron micrograph (Kuo, Jackson, Campbelle, & Grayston, 1995) while figure 2.9 shows *C. pneumoniae* life cycle (Carmen & et al., 2012).

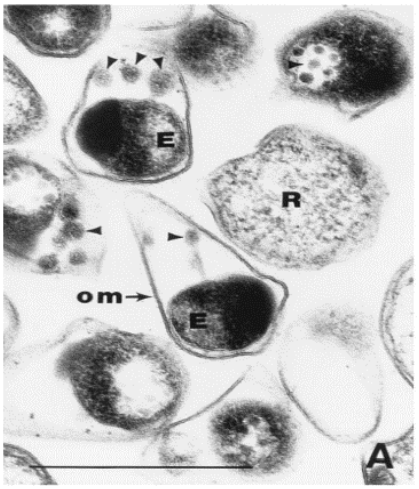


Figure 2.8: Electron micrographs of C. pneumoniae: E, elementary body; R, reticulate body; om, outer membrane. Arrowheads indicate small electron-dense bodies (mini bodies). Bar = 0.5 µm.

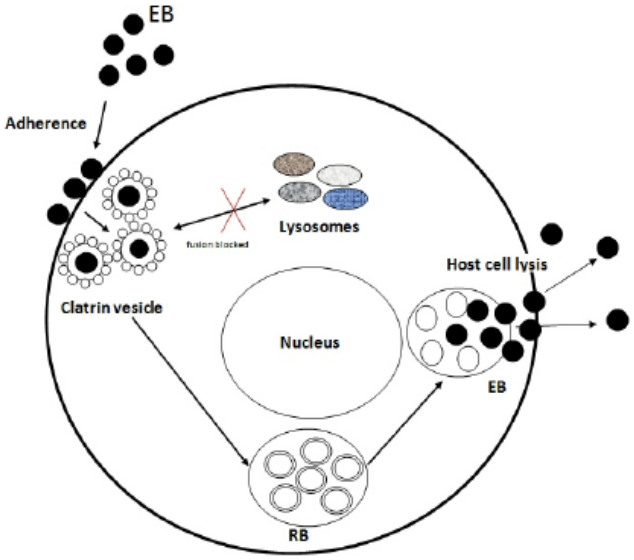


Figure 2.9: An illustration of C. pneumoniae life cycle.

2.2.6 Epidemiology

Each year, about 300,000 cases of pneumonia is caused by *C. pneumoniae*, in which it accounts for 10-20% of CAP (CDC, 2016). *C. pneumoniae* may infect any age worldwide, but it is focused on age group between 5-14 years old and reinfection is very common. Regarding region distribution of *C. pneumoniae*, tropical and less developed countries tend to have higher incidence (Casadevall & Pirofski, 2003).

2.2.7 Pathogenesis

C. pneumoniae is recognized as an important respiratory pathogen that causes CAP in humans, at the same time many studies have also focused on *C. pneumoniae* role as an etiological factor of persistent infections in chronic diseases. Owing to its unique developmental life cycle and genetic ability to switch between phases, *C. pneumoniae* has been related to acute as well as chronic diseases (Kern, Maass, & Maass , 2009).

Generally, *C. pneumoniae* cause sinusitis, pharyngitis and bronchitis. Repetitive or chronic persistent infections could also increase the risk of asthma, chronic obstructive pulmonary disease (COPD) and vascular lesions (Krull & Subttorp, 2007). Beyond respiratory illness; *C. pneumoniae* is associated with cardio vascular disease, according to Hasan (2011) there is a three-fold increased risk to develop ischemic stroke with patients previously infected with *C. pneumoniae* compared to patients who didn't (Hasan, 2011). *C. pneumoniae* is also linked to Alzaheimer's disease (Itzhaki & et al., 2016), arthritis (Rizzo, Domenico, Carratelli, & Paolillo, 2012), cancer (Hua-Feng, Yue-Ming, & Junyi, 2015) and other inflammatory and chronic diseases. However, *C. pneumoniae* is not restricted to humans, but also infect both domesticated and wild animals (Mitchell, Hutton, Myers, Brunham, & Timms, 2010).

2.2.8 Signs and Symptoms

Generally, infection with *C. pneumoniae* is mild or moderate with minor symptoms. Bronchitis is the most common symptom of *C. pneumoniae* since the initial infection site happens in the lungs and ciliated cells. Other symptoms include: onset of cough, sore throat fever, pharyngitis, laryngitis and sinusitis (CDC, 2016). In addition, incubation

period for *C. pneumoniae* is about 21 days, whereas infection may persist several weeks in particular the cough.

2.2.9 Diagnosis

2.2.9.1 Culture

The appropriate cell lines used to isolate *C. pneumoniae* are HeLa and Hep-2 cells (Campbell & Kuo, 2009). Limitations of culture concerning *C. pneumoniae* includes: complexity of the technique, limited availability, variable outcome, contamination and many others. She et al. (2010) have concluded in their study, compared to molecular and serological testing, culture was less sensitive and the outcome is extremely low so it should not be recommended as a diagnostic tool for *C. pneumoniae* and should be eliminated from routine practice (She, et al., 2010).

2.2.9.2 Serology

Serological diagnosis for *C. pneumoniae* in the acute phase among other methods is the most commonly used. But the kinetics of antibody response may take time, for instance IgM antibodies appears after 2-3 weeks from the onset of infection while IgG antibodies need 6-8 weeks to appear. In case of reinfection IgM antibodies usually do not appear but IgG antibodies appear within 1-2 weeks (Dowel & et al, 2001). Serological assays for the detection of *C. pneumoniae* are numerous, including: CFA, Microimmuno-fluorescence (MIF), ELISA and EIA. Unfortunately, these serological methods have limitations for instance: cross reactivity and disability to discriminate previous and current infection (Villegas, Sorlozano, & Gutierrez, 2010). However, Microimmuno-fluorescence (MIF) represents the gold standard to detect *C. pneumoniae* specific antibodies and has been recommended by CDC to detect acute *C. pneumoniae* infections (Dowel & et al, 2001).

2.2.9.3 Molecular Methods

16S rRNA, peripheral myelin proteins 4 (PMP4) and MOMP are the most targeted genes (Abdallah, Salih, & Al-Azawi, 2017). But still no molecular method is standardized and approved (Dowel & et al, 2001). Maybe this could be related to the variation of methods used from study to study and from lab to lab, in other words; variation of targeted genes

(16 S rRNA, MOMP …), variation of nucleic acid amplification (Conventional PCR, nested PCR …) and the detection method used (SYBR green, Molecular beacons…) (Dowel & et al, 2001).

2.2.10 Treatment and Prevention

The drug of choice for treating *C. pneumoniae* is macrolides, in particular azithromycin, tetracyclines could be also used but they are recommended for adults only. However, *C. pneumoniae* is resistant to penicillin, ampicillin and sulfa drugs (CDC, 2016). Despite the fact that *C. pneumoniae* infection sometimes is mild and self-limiting, it should be treated for two main reasons: 1. reinfection is very common, even a second dose of treatment is recommended sometimes. 2. *C. pneumoniae* persistent infection is associated with the development of chronic and inflammatory diseases like atherosclerosis and asthma (Kutlin, Roblin, & Hammrschlag, 2002). Furthermore, many studies have focused on using prolonged courses of treatment as a prevention and/or reduction of secondary cardiovascular events (Cannon & et al, 2005). Yet, no proven potential resistance of macrolides has been found in *C. pneumoniae.*

2.2.10.1 Vaccination

No vaccines against *C. pneumoniae* are available. From researches point of view, the development of safe and effective vaccines against *C. pneumoniae* is cost effective approach that play a role in the prevalence and prevention of chlamydial infections. Pinchuk et al. (2005) concluded in their study by: immunization with a CD8+ T cell epitope-based DNA has given a significant protection against *C. pneumoniae* in mice model thus provides the basis for optimal design of multicomponent anti *C. pneumoniae* vaccines for humans (Pinchuk, et al., 2005).

Chapter Three : Thesis Objectives

3.1 Research goal

Epidemiological and molecular characterization of *M. pneumoniae* and *C. pneumoniae* circulating in Southern Palestine will enhance our understanding of the role of these two bacteria in respiratory illnesses and their local distribution.

3.2 Overall aim

To describe epidemiological and molecular characterization of *M. pneumoniae* and *C. pneumoniae* identified from NPA's from hospitalized patients in Caritas Baby Hospital between January 2015 and December 2017.

3.3 Specific aims

1. To screen for *M. pneumoniae* and *C. pneumoniae* in NPA samples collected from patients 5 years of age and older.
2. To reveal any double infections with respiratory viruses in the clinical samples.
3. To characterize *M. pneumoniae* and *C. pneumoniae* clinical presentation.
4. To screen for the macrolide's resistance pattern of *M. pneumoniae*.

Chapter Four : Materials and Methods

In conducting this research, several materials and methods were used. Below is a brief description of each.

Figure 4.1: Mucus Extractors used at CBH

4.1 Clinical Samples

Respiratory samples represented by nasopharyngeal aspirates (NPA) were collected by well-trained CBH nurses using mucus extractors (figure 4.1) from patients admitted to the hospital with respiratory tract infection or pneumonia. NPA samples later were processed by qualified laboratory technologists to be screened for the existence of 8 common human respiratory viruses under fluorescent microscope: adenovirus, influenza Virus (A& B), parainfluenza virus (1, 2 &3), RSV and hMPV. Later, processed samples were labeled and stored at -70^{o} Celsius (C) pending for further analysis. The main criteria while choosing the samples was to depend on age. In fact, age is a crucial variable in the existence of these two pathogens; age group 5 years old and older was chosen to investigate. In this study, NPA database was checked (N= 7673); all tested samples for viruses belonged to patients older than 5 years were spotted and retrieved from their boxes to proceed with the molecular testing as to achieve the goals of this research covering the period from January 1st 2015 to December 31st 2017 (N=350), taking into

consideration that duplicate samples tested in a time period less than one month were removed from the study.

4.2 Bacterial molecular analysis

4.2.1 Nucleic Acid Extraction

Nucleic acid from all NPA samples was extracted using High Pure Viral Nucleic Acid Extraction Kit (Roche, Germany, Catalogue number 11858882001) according to manufacturer's instructions. The kit guarantees Nucleic Acid recovery of at least 2 x 10^5 nucleic acid molecules /200 micro liter (µL) sample in 10 minutes time interval. Extraction was performed at CBH in the extraction room (Appendix 1). Later, extracted nucleic acids from samples were labeled and stored using sterile Eppendorf tubes at -30^o C.

4.2.2 Detection of *M. pneumoniae* and *C. pneumoniae* using Multiplex qRT-PCR

RT-PCR assay was applied using *Taq*Man chemistry on the ABI 7500 instrument (Life Technologies, Foster City, CA). Briefly, amplification was performed in 25 µl reaction volumes as shown in table 4.1, the materials needed for the reactions are described in table 4.2. As for the primers; for *M. pneumoniae* P1 gene (Malhotra & et al., 2013) was targeted meanwhile for *C. pneumoniae* MOMP gene (Lahesmaa & et al., 2012) was the target, the nucleotide sequences for each primer is shown in table 4.3(a) and PCR program is described in table 4.3(b). Human Ribonuclease P (RNase P) (Pace & et al., 1994) was used as an internal control since its presence was necessary to monitor the presence of PCR reaction inhibitors, confirms a successful extraction of the samples and shows that the PCR conditions were suitable.

4.2.2.1 Validation of the Multiplex qRT-PCR

Known amount of each of the two bacteria: *M. pneumoniae* (ATCC 15293, Virginia USA) (stock = 3.52 *10^5 colony forming unit/ reaction (cfu/rxn)/ colony forming unit / µl) and *C. pneumoniae* (TWAR, quantitated clinical isolate) (stock = 4.09 *10^7 cfu /µl (cfu/rxn))) extracted nucleic acid were diluted in molecular grade water and subjected to RT-PCR in triplicates to determine the limit of detection (LOD) of the assay.

Both singleplexes and multiplexes were applied for both strains. In addition, the analytical sensitivity for the two species was checked by comparing the outcome from singleplex and multiplex assays. However patient samples with cycle threshold (Ct) values more than 40 were considered negative.

4.2.2.2 Testing the samples using Multiplex qRT-PCR

Extracted samples were subjected to multiplex qRT-PCR to determine the presence of *M. pneumoniae* and *C. pneumoniae*. Positive controls set at Ct ~ 28 for *M. pneumoniae* and Ct ~ 27 for *C. pneumoniae* were used for each run in which they were prepared from serial dilutions, in addition to the internal control RNase P as previously reported.

Table 4.1: RT-PCR recipe for the Singleplex / Multiplex qRT-PCR viewing the materials and quantities used.

Singleplex qRT-PCR			
Reagents	**1 rxn (µl)**	**X rxns (µl)**	**X value**
Master Mix	12.5	625	50
F- CP/F-MP (300 nM)	1	50	
R- CP/R-MP (300 nM)	1	50	
P-CP/P-MP (300 nM)	1	50	
RNase P- F (150 nM)	0.5	25	
RNase P- R (150 nM)	0.5	25	
RNase P- P (150 nM)	0.5	25	
H_2O	3	150	
DNA	5		
Total	25		

Multiplex qRT-PCR			
Reagents	**1 rxn (µl)**	**X rxns (µl)**	**X value**
Master Mix	12.5	625	50
F- CP + F-MP (300 nM)	1	25	
R- CP +R-MP 300 nM)	1	25	
P- CP + P-MP (300 nM)	1	25	
RNase P- F (150 nM)	0.5	25	
RNase P- R (150 nM)	0.5	25	
RNase P- P (150 nM)	0.5	25	
H_2O	3	150	
DNA	5		
Total	25		

Table 4.2: List of Materials needed for RT-PCR reaction and brief description of each.

Component	Catalogue no.	Description
Master Mix	ABsolute Blue qPCR Low ROX Mix (Cat# 00529434)	Contains the deoxy nucleoside triphosphate (dNTPs) which will build up the new sequence and provide energy to the reaction, DNA will build up the bases and $MgCl_2$ working as polymerase stabilizer.
~ *M. pneumoniae* primer and probe (P1 gene) **~ *C. pneumoniae* primers and probe (MOMP gene)** **~ RNase P primers and probe**		As the reaction starts, during the annealing stage of the PCR both probe and primers anneal to the DNA target. Polymerization of a new DNA strand is initiated from the primers, and once the polymerase reaches the probe, its 5'-3'-exonuclease degrades the probe, physically separating the fluorescent reporter from the quencher, resulting in an increase in fluorescence. Fluorescence then is detected and measured in a real-time PCR machine, and its geometric increase corresponding to exponential increase of the product is used to determine the quantification cycle (Cq) in each reaction.
Water	Nuclease free water	To complete the reaction recipe
Nucleic Acid		Extracted from Samples

Table 4.3(a): list of primers and probes used in the Singleplex and Multiplex qRT-PCR.

Target Gene	Sequences (5' to 3')	No. of bases	GC%
P1 ***M. pneumoniae***	F: AACCTCGCGCCTAATACTAATACG	24	45.8%
	R: TTGCGGCGTTGCTTTCAG	18	55.6%
	P: Fam-AAAGTCGACCAACCCC-NFQ	16	56.3%
MOMP ***C. pneumoniae***	F: AAGGGCTATAAAGGCGTTGCT	21	47.6%
	R: TGGTCGCAGACTTTGTTCCA	20	55.0%
	P: FAM-TCCCCTTGCCAACAGACGCTGG-TAMRA	22	63.6%
RNase P	F: AGATTTGGACCTGCGAGCG	19	57.9%
	R: GAGCGGCTGTCTCCACAAGT	20	60.0%
	P: CY5-TTCTGACCTGAAGGCTCTGCGCG-BHQ2	23	60.9%

Table 4.4(b): RT-PCR amplification program

Step	Temperature C^{o}	Time	Number of cycles
Denature	95	5 minutes	1
Denature	94	15 seconds	50
Annealing	56	30 seconds	
Extension	72	1.5 minutes	
Final extension	72	7 minutes	1
Hold	4	Timeless	1

4.2.3 Detection of Macrolide Resistant in *M. pneumoniae* using Nested PCR

Nested PCR was used for the detection of macrolide resistant in *M. pneumoniae*. Nested PCR targeted first domain V in 23S rRNA then PCR product was nested as a template for the amplification of Region 1 (R1) at positions 2063, 2064 and Region 2 (R2) at position 2617 (Matsuoka & et al., 2004). Figure 4.3 illustrates sets of primers used in Nested PCR amplification meanwhile table 4.4 shows these primer's sequences, their positions and expected amplicon size. Amplified PCR products were separated on 1.5 % agarose gel and visualized with ethidium bromide staining. Furthermore, amplified PCR products were purified then sequenced in both directions with the primers for region 1 and 2. Regarding the PCR recipe it is described in table 4.5(a). Red mix (2x PCRBIO HS *Taq* Mix Red, Cat. No. PB 10.23.02) was used; which contains *Taq* DNA Polymerase, $MgCl_2$, dNTPs, enhancers, stabilizers and red dye for tracking the bands during agarose electrophoresis. Regarding nested PCR program it is illustrated in table 4.5(b).

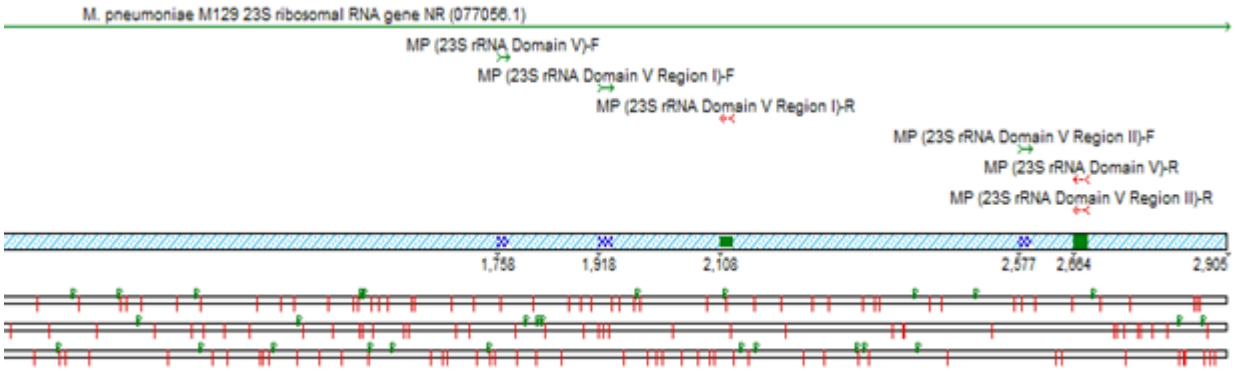


Figure 4.2: Primers Sets in Nested PCR

Table 4.5: Primers used for Nested PCR amplification of domain V of 23S rRNA in M. pneumoniae (Matsuoka & et al., 2004).

Target	Sequence (5' to 3')	Position	Amplicon Size (bp)
Domain V of 23S rRNA	F – GCAGTGAAGAACGAGGGG	1758-1775	927
	R – GTCCTCGCTTCGGTCCTCTCG	2664-2684	
R1: 23S rRNA of 2063,2064	F – ACTATAACGGTCCTAAGGTA	1918-1937	210
	R – ACCTATTCTCTACATGATAA	2108-2177	
R2: 23S rRNA of 2617	F – TACGTGAGTTGGGTTCAAA	2577-2595	108
	R – GTCCTCGCTTCGGTCCTCTCG	2664-2684	

4.2.3.1 Validation of Nested PCR

Serial dilution -1 to -7 of known concentrations of *M. pneumoniae* used in the validation of the multiplex qRT-PCR step was used again in the validation of Nested PCR to ensure the functioning of the assay and the LOD for both assays is the same. First amplification of 23s rRNA was done then the product was nested two times in the amplification for the two regions. Later PCR products were visualized using ultra violet light (UV).

4.2.3.2 Testing the samples using nested PCR

Extracted samples which are *M. pneumoniae* positive detected by multiplex qRT-PCR were assessed for macrolides resistance using nested PCR.

Table 4.6(a): Nested PCR recipe and quantities used for nested PCR including domain V, R1 and R2

Nested PCR			
23s rRNA domain V/ R1/R2			
Reagents	**1 rxn (µl)**	**X rxns (µl)**	**X value**
Master Mix (RED)	25	250	10
F-MP100 nM	3	30	
R-MP 100 nM	3	30	
H2O	14	140	
DNA	5		
Total	50		

Table 4.7(b): Nested PCR amplification program

Step	**Temperature C^{O}**	**Time**	**Number of cycles**
Denature	95	1 minute	35
Annealing	45	1 minute	
Extension	72	1.5 minutes	
Hold	4	Timeless	1

4.2.3.3 Gel Electrophoresis for Nested PCR products

This step was necessary to insure the amplification of Domain V and the two regions within R1 and R2 before purification and sequencing. First gel was prepared in the try: 1.5 grams (g) of Invitrogen Ultrapure ^{TM} with 100 ml Tris-Borate-Ethyldiaminetetraacetic acid (TBE) buffer + 50 µL Ethidium Bromide to visualize the bands. After that PCR

products were loaded in the wells with 100bp deoxyribonucleic acid (DNA) ladder in the first well to compare size of the bands, then electric current was applied ~ 85-90 Volt for 45 minutes to enhance the bands to move towards the positive anode. Finally, bands were able to be visualized using UV light.

4.2.3.3 PCR Product Purification

This step comes before sequencing the product where all the amplifications must be first cleaned. The purpose behind this is to avoid any impurities from interfering with the sequencing process. Roche High Pure PCR Product Purification Kit (lot number: 11732668001) was used where purification was applied in the extraction room at CBH (Appendix 2). After purification another gel electrophoresis run was done for all samples to make sure bands ready for sequencing plus to visualize the thickness of these bands.

4.2.3.4 Sequencing

Sequencing of the purified product was done using both forward and reverse sets of primers used in the amplification of nested PCR (Domain V, R1 and R2). Sequencing was conducted in Augusta Victoria Hospital, Molecular Genetics Laboratory and was performed using Applied Biosystems, 3500-Gene analyzer according the manufacturer's instructions.

4.2.3.5 Sequence assembly and analysis

Sequences were aligned using Sequencer program (Gencodes Corporation, Ann Arbor, MI) to clean and align Domain V (R1 and R2) nucleotides sequences with the *M. pneumoniae* reference sequence to catch any point mutations that emphasizes the presence of Macrolide resistant *M. pneumoniae*.

4.3 Statistical Analysis

IBM SPSS software (version 21) was used along with Microsoft Excel 2018 in this research in a way to facilitate the description and the analysis of the data. Many statistical tests were used to organize, summarize the research results and to support the research hypothesis. Brief description of each as follows:

1. Average (Mean ($\bar{x}$))

$$\bar{x} = \frac{x1 + x2 + x3}{3}$$

2. Standard Deviation (σ)

$$SD = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})}{N-1}}$$

3. Coefficient of Variation (CV)

$$CV = \frac{SD}{\bar{X}} * 100\%$$

4. Slope

$$m = \frac{y_2 - y_1}{x_2 - x_1}$$

5. Correlation Factor

$$r = \frac{1}{n-1}\left(\frac{\sum_x \sum_y (x - \bar{x})(y - \bar{y})}{x^{SD} y^{SD}}\right)$$

6. Efficiency

$$E = 10^{1-/slope} \text{ -1}$$

7. Minimum and Maximum Values

8. Frequency (f)

Chapter Five : Results

This retrospective study was conducted at Caritas Baby Hospital (CBH) in Bethlehem. CBH is a pediatric hospital where most of its patients are inhabitants from Southern Palestine. Upon conducting this research, NPA samples were collected by nurses during study period between January 1st 2015 and December 31st 2017. Population study had included all months during these three years, negative and positive samples of viral infection as well for all patients aged 5 years and older (N=350).

5.1 Population study

This study relied on 5 main categories to investigate: age, study years, season, district and gender as descried in table 5.1, where the number of tested samples and their percentage from each category were calculated from total population (N=350). In addition, one extra category was included in this study to investigate any double infection which is the direct fluorescent antibody (DFA) viral infections testing, and the distribution is illustrated in figure 5.1. Most of the samples used in this study were negative for viral infections (86.86%) while 13.14% of the samples were positive.

Table 5.1: Description of the population study. The number of tested samples and the percent out of total samples (N=350) are presented.

Variable	Categories	Number tested (%)
Age (years)	Minimum	5 yrs (N= 55, 15.7 %)
	Maximum	19 yrs (N= 1, 0.3 %)
	Mean	8.1 yrs (N= 43, 12.3 %)
Study years	2015	110 (31.4%)
	2016	132 (37.7%)
	2017	108 (30.8%)
Season	Winter (December through February)	110 (31.4%)
	Spring (March through May	125 (35.7%)
	Summer (June through August)	40 (11.4%)
	Fall (September through November)	75 (21.4%)
District	Bethlehem	152 (43.3%)
	Hebron	174 (49.6%)
	Jerusalem	19 (5.5 %)
	Miscellaneous	5 (1.5 %)
Gender	Males	168 (48.0%)
	Females	182 (52.0%)

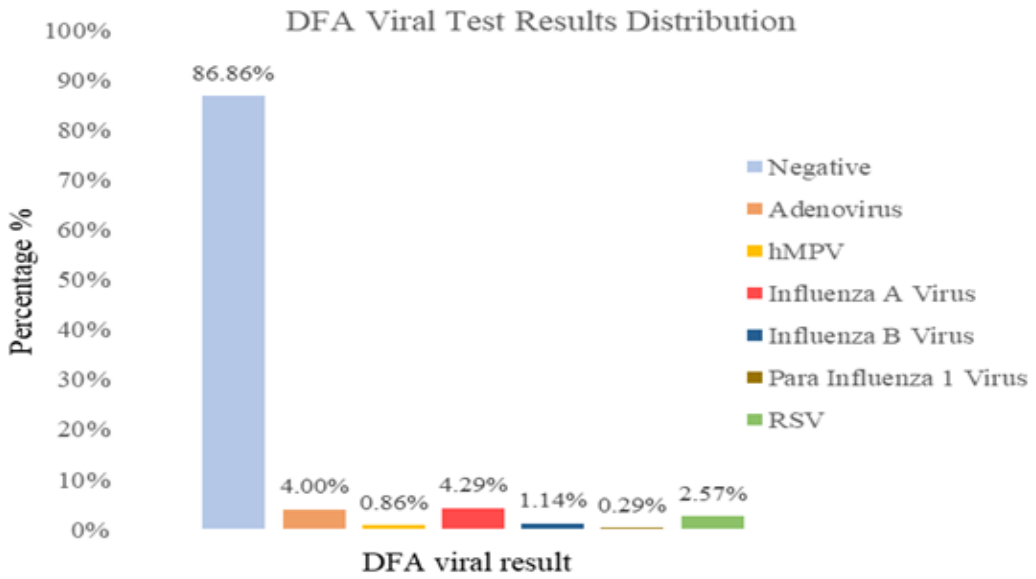


Figure 5.1: DFA Viral Test Result Distribution shown in percentage.

5.2 Bacterial molecular analysis results

5.2.1 Validation of Multiplex qRT-PCR results

Tables 5. 2 (a, b, c and d) and figure 5. 2 (a, b, c and d) show validation parameters and results obtained from singleplexes and multiplexes of qRT-PCR while using control's serial dilution to validate the testing of each of *M. pneumoniae* and *C. pneumoniae* species. For both pathogens; Average, Standard Deviation and Coefficient of Variation were calculated first for every reaction from Ct values in order to calculate Slope, Correlation Factor and Efficiency. Results have showed that Slopes have arranged between $-3.6 \leq m \leq -3.1$ while Correlation Factors were between $0.99 \leq r2 \leq 1.000$ and Efficiencies were between $0.9 \leq E \leq 1.1$ for all singleplexes and multiplexes. In other words, these validation results are considered or associated with successful PCR reactions. LOD for *M. pneumoniae* was considered -5 (3.52 cfu/rxn) while for *C. pneumoniae* it was -7 (4.09 cfu/rxn). Figures 5.3 a and b show multiplex qRT-PCR amplification plots for serial dilution curves for both organisms.

Table 5.2 (a): Singleplex qRT-PCR results for M. pneumoniae

Singleplex qRT-PCR - *M. pneumoniae*							
Log Concentration	**cfu/RXN**	**CT Values**			**AVG**	**SD**	**CV**
		1	2	3			
-1	$3.52*10^{4}$	21.000	20.300	20.300	20.533	0.404	1.968
-2	$3.52*10^{3}$	23.400	23.300	23.600	23.433	0.153	0.652
-3	$3.52*10^{2}$	26.600	26.500	26.500	26.533	0.058	0.218
-4	$3.52*10^{1}$	30.000	29.600	29.800	29.800	0.200	0.671
-5	$3.52*10^{0}$	33.100	33.300	33.300	33.233	0.115	0.347
-6	$3.52*10^{-1}$	36.300	Negative	36.100	-	-	-
-7	$3.52*10^{-2}$	Negative	Negative	Negative	-	-	-
Slope		-3.080	-3.230	-3.220	-3.907		
Correlation Factor		-0.999	-0.999	-1.000	-0.999		
Efficiency		1.095	1.040	1.044	1.064		

Table 5.2 (b): Multiplex qRT-PCR results for M. pneumoniae.

Multiplex qRT-PCR - *M. pneumoniae*							
Log Concentration	**cfu/RXN**	**CT Values**			**AVG**	**SD**	**CV**
		1	2	3			
-1	$3.52*10^{4}$	21.100	21.080	20.410	20.863	0.393	1.882
-2	$3.52*10^{3}$	23.700	23.660	23.880	23.747	0.117	0.493
-3	$3.52*10^{2}$	26.180	27.090	26.510	26.593	0.461	1.732
-4	$3.52*10^{1}$	30.900	29.500	29.380	29.927	0.845	2.824
-5	$3.52*10^{0}$	33.300	33.600	34.040	33.647	0.372	1.106
-6	$3.52*10^{-1}$	Negative	36.600	Negative	-	-	-
-7	$3.52*10^{-2}$	Negative	Negative	Negative	-	-	-
Slope		-3.160	-3.088	-3.276	-3.177		
Correlation Factor		-0.993	-0.997	-0.995	0.999		
PCR Efficiency		1.072	1.108	1.020	1.065		

Table 5.2 (c): Singleplex qRT-PCR results for C. pneumoniae.

Singleplex qRT-PCR - *C. pneumoniae*							
Log Concentration	**cfu/RXN**	**CT Values**			**AVG**	**SD**	**CV**
		1	2	3			
-1	$4.09*10^{6}$	16.010	16.249	16.200	16.153	0.126	0.782
-2	$4.09*10^{5}$	19.010	19.010	19.290	19.103	0.162	0.846
-3	$4.09*10^{4}$	22.350	22.370	22.210	22.310	0.087	0.391
-4	$4.09*10^{3}$	25.760	25.680	25.590	25.677	0.085	0.331
-5	$4.09*10^{2}$	29.150	28.560	29.120	28.943	0.332	1.148
-6	$4.09*10^{1}$	33.880	31.200	32.360	32.480	1.344	4.138
-7	$4.09*10^{0}$	34.870	36.270	36.540	35.893	0.240	0.664
-8	$4.09*10^{-1}$	Negative	Negative	Negative	-	-	-
Slope		-3.519	-3.129	-3.214	-3.452		
Correlation Factor		-0.999	-0.998	-0.1000	-0.999		
Efficiency		0.924	1.087	1.047	1.006		

Table 5.2 (d): Multiplex qRT-PCR results for C. pneumoniae.

Multiplex qRT-PCR - *C. pneumoniae*							
Log Concentration	**cfu/RXN**	**CT Values**			**AVG**	**SD**	**CV**
		1	2	3			
-1	$4.09*10^{6}$	16.230	15.970	16.130	16.110	0.131	0.814
-2	$4.09*10^{5}$	19.130	19.190	19.020	19.113	0.086	0.451
-3	$4.09*10^{4}$	22.590	22.690	22.690	22.657	0.058	0.255
-4	$4.09*10^{3}$	25.970	26.070	24.960	25.667	0.614	2.392
-5	$4.09*10^{2}$	29.380	29.230	29.260	29.290	0.079	0.271
-6	$4.09*10^{1}$	34.280	32.490	32.600	33.123	1.003	3.029
-7	$4.09*10^{0}$	36.850	35.380	38.100	36.777	1.361	3.702
-8	$4.09*10^{-1}$	Negative	Negative	Negative	-	-	-
Slope		-3.554	-3.340	-3.220	-3.1748		
Correlation Factor		-0.997	0.1000	0.999	-0.997		
Efficiency		0.912	0.993	1.044	0.948		

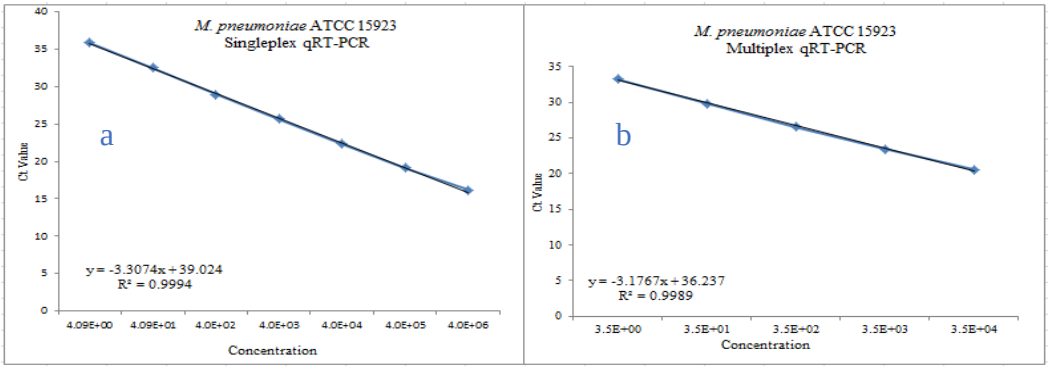


Figure 5.2: (a and b) Validation of Singleplexes and Multiplexes for Detection of M. pneumoniae showing Straight line equation, Slope and Efficiency.

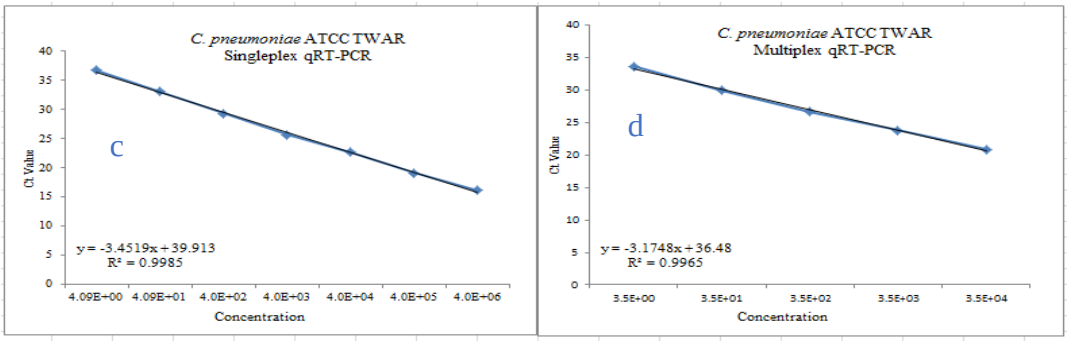
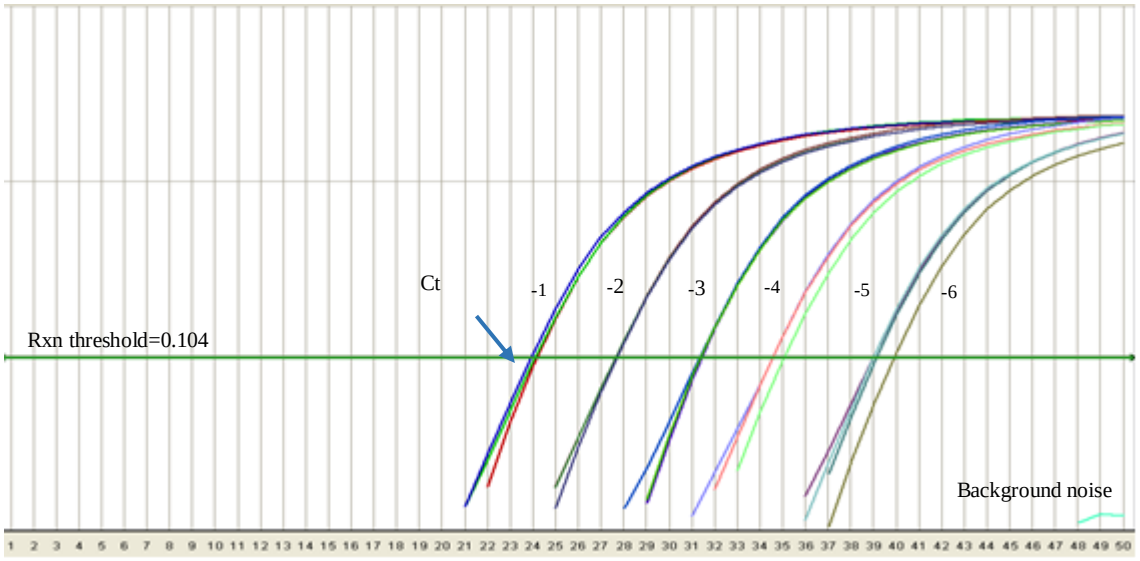


Figure 5.2: (c and d) Validation of Singleplexes and Multiplexes for Detection of C. pneumoniae showing Straight line equation, Slope and Efficiency.



qPCR Cycle Number

Figure 5.3 (a): M. pneumoniae Multiplex qRT-PCR amplification plot.

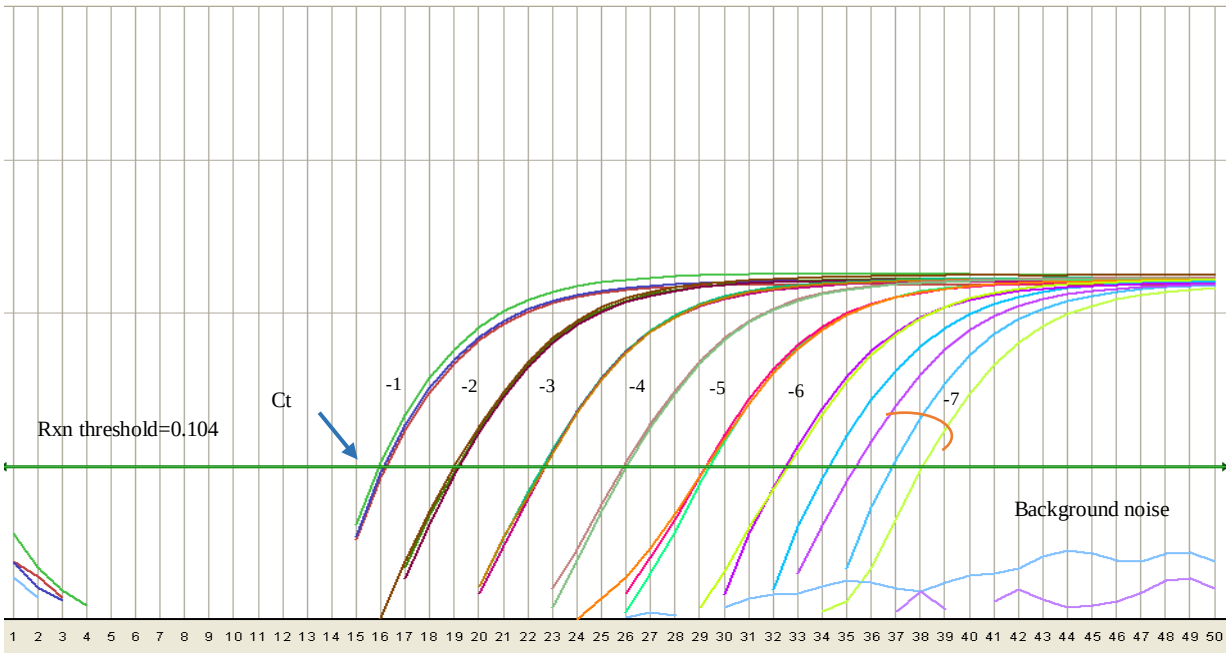


Figure 5.3 (b): C. pneumoniae Multiplex qRT-PCR amplification plot.

5.2.2 Prevalence of *M. pneumoniae* and *C. pneumoniae* positivity

Overall, 23 samples were found to be *M. pneumoniae* and *C. pneumoniae* positive, yielding a positivity rate of 6.6%. As for the individual occurrence for each species; from the total population (N=350) *M. pneumoniae* represented 4.9% whereas *C. pneumoniae* 1.7%, thus ratio of *M. pneumoniae* to *C. pneumoniae* is 2.88:1 as demonstrated in figure 5.4.

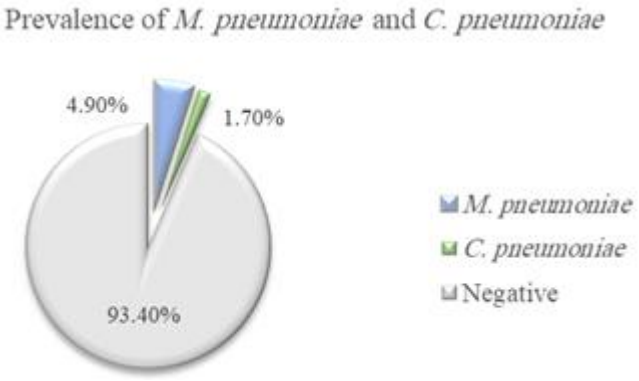


Figure 5.4: Multiplex qRT-PCR results showing percentage of negatives, M. pneumoniae and C. pneumoniae.

Ct values obtained from multiplex qRT-PCR for *M. pneumoniae* ranged between 20.15 and 39.44 and the average was 32.82 while for *C. pneumoniae* Cts were between 22.07 and 30.65 and average equaled 36.05 as clarified in tables 5.3 (a, b) and 5.4.

Table 5.2 (a): Multiplex qRT-PCR results with Ct values for C. pneumoniae.

Species	Ct values
C. pneumoniae	38.67
C. pneumoniae	37.78
C. pneumoniae	39.5
C. pneumoniae	39.75
C. pneumoniae	22.07
C. pneumoniae	38.52

Table 5.3 (b): Multiplex qRT-PCR results with Ct values for M. pneumoniae.

Species	Ct values
M. pneumoniae	30.04
M. pneumoniae	34.97
M. pneumoniae	20.15
M. pneumoniae	34.19
M. pneumoniae	36.48
M. pneumoniae	33.14
M. pneumoniae	33.04
M. pneumoniae	33.22
M. pneumoniae	39.44
M. pneumoniae	38.26
M. pneumoniae	34.8
M. pneumoniae	32.1
M. pneumoniae	28.74
M. pneumoniae	29.12
M. pneumoniae	32.04
M. pneumoniae	37.81
M. pneumoniae	30.36

Table 5.3: Average, minimum and maximum values for Ct values.

	Average	Minimum	Maximum
M. pneumoniae	32.82	20.15	39.44
C. pneumoniae	36.05	22.07	39.22

5.2.3 Epidemiological and Demographic Characteristics

5.2.3.1 *M. pneumoniae* and *C. pneumoniae* positivity by age group

The presence of *M. pneumoniae* and *C. pneumoniae* according to age groups was strongly related to children aged 7 and 10 years old for both pathogens (table 5.5). *M. pneumoniae* isolates (9.5%) correlated with patients aged 7 and 10, 9.3% of isolates were found in patients aged 8 and 9.1% of positives belonged to age group 15 years and older. On the other hand, *C. pneumoniae*; most isolates belonged to patients aged 8 and 10 years old as well (4.7% and 4.8%) and less prevalence was observed in 6 years old (1.2%), 7 years old (2.4%) and 9 years old patients (2.9%). Number of isolates found is illustrated in figure 5.5 for both bacteria.

Table 5.4: pneumoniae and C. pneumoniae cases by age groups from total population.

Age group (years)	No. of tested	*M. pneumoniae*		*C. pneumoniae*	
		No. of positives	Positive (%)	No. of positives	Positive (%)
5	**55**	0	0.0%	0	0.0%
6	**84**	1	1.2%	1	1.2%
7	**42**	4	9.5%	1	2.4%
8	**43**	4	9.3%	2	4.7%
9	**35**	2	5.7%	1	2.9%
10	**21**	2	9.5%	1	4.8%
11	**23**	2	8.7%	0	0.0%
12	**17**	1	5.9%	0	0.0%
13	**13**	0	0.0%	0	0.0%
14	**6**	0	0.0%	0	0.0%
≥15	**11**	1	9.1%	0	0.0%

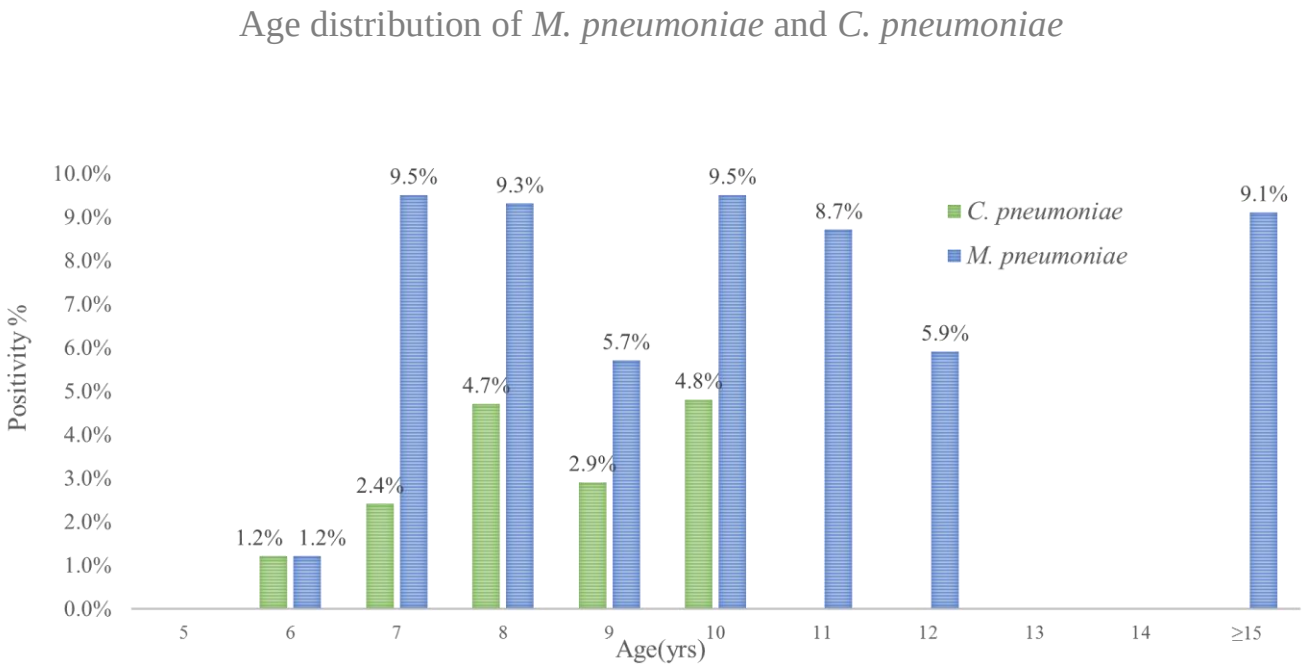


Figure 5.5: Age distribution of M. pneumoniae and C. pneumoniae shown in frequency.

5.2.3.2 *M. pneumoniae* and *C. pneumoniae* positivity by district

Hospitalized patients in CBH were inhabitants from three main regions in Southern Palestine: Bethlehem, Hebron and Jerusalem. No statistical variation was observed in *M. pneumoniae* patients; Bethlehem (5.9%), Hebron (4.0%) and Jerusalem (5.3%). As for *C. pneumoniae*, most of the isolates were concentrated in Bethlehem area (3.3%) followed by (0.6%) in Hebron and no isolates were observed in Jerusalem (table 5.6).

Table 5.5: M. pneumoniae and C. pneumoniae cases by district from total population.

District	No. of tested	*M. pneumoniae*		*C. pneumoniae*	
		No. of positives	Positive/ (%)	No. of positives	Positive (%)
Bethlehem	152	9	5.9%	5	3.3%
Hebron	174	7	4.0%	1	0.6%
Jerusalem	19	1	5.3%	0	0.0%
Miscellaneous	5	0	0.0%	0	0.0%

5.2.3.3 *M. pneumoniae* and *C. pneumoniae* positivity by year

M. pneumoniae positivity according to year distribution was the highest in 2015 (9.1%) followed by 2016 (3.0%) and 2017 (2.8%). Concerning *C. pneumoniae* positivity through study period, almost the organism was observed in three years with equal percentages (table 5.7).

Table 5.6: M. pneumoniae and C. pneumoniae cases by year from total population.

Test year	No. of tested	*M. pneumoniae*		*C. pneumoniae*	
		No. of positives	Positives (%)	No. of positives	Positives (%)
2015	110	10	9.1%	2	1.8%
2016	132	4	3.0%	2	1.5%
2017	108	3	2.8%	2	1.9%

5.2.3.4 *M. pneumoniae* and *C. pneumoniae* positivity by month

M. pneumoniae and *C. pneumoniae* positivity rate showed a variation throughout the year. April was correlated with the highest percentage 10.6% for *M. pneumoniae* followed by July (9.1%), February (8.6%), May (7.1%), June (6.3%), March (6.0%), October (4.2%) and January (2.9%). *C. pneumoniae* isolates were found mostly in July (9.1%) compared to October (4.2%), May (3.6%), January (2.9%) and February (2.9%) (table 5.8). That means seasonality of *M. pneumoniae* is higher in Spring (23.1%), medium in Summer (15.4%), lower in Winter (11.5%) and minimal in Autumn (4.2%) as shown in table 5.8. On the other hand, seasonality of *C. pneumoniae* is highest in Summer (9.1%), medium and almost the same in both Spring and Winter (5.6%, 5.8%) but it is lower in Autumn (4.2%) (table 5.8). Monthly distribution of positive isolates is shown in figure 5.6.

Table 5.7:M. pneumoniae and C. pneumoniae cases by months from total population.

Test Month	No. of Samples Tested	*M. pneumoniae*		*C. pneumoniae*	
		No. of positives	Positive (%)	No. of positives	Positive (%)
January	34	1	2.9%	1	2.9%
February	35	3	8.6%	1	2.9%
March	50	3	6.0%	1	2.0%
April	47	5	10.6%	0	0.0%
May	28	2	7.1%	1	3.6%
June	16	1	6.3%	0	0.0%
July	11	1	9.1%	1	9.1%
August	13	0	0.0%	0	0.0%
September	19	0	0.0%	0	0.0%
October	24	1	4.2%	1	4.2%
November	32	0	0.0%	0	0.0%
December	41	0	0.0%	0	0.0%

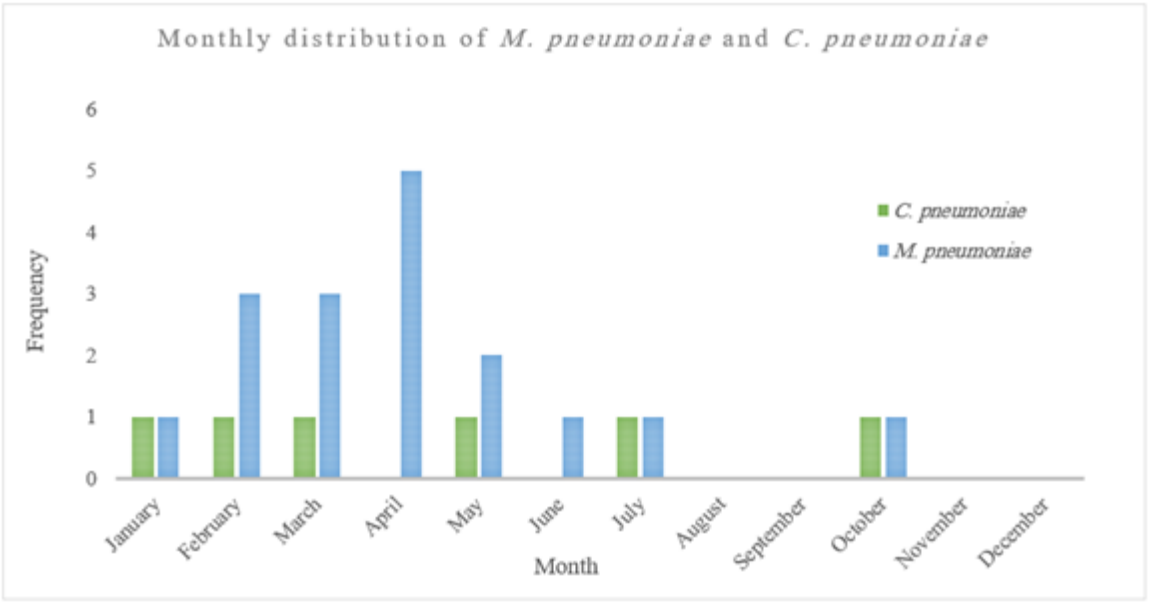


Figure 5.6: Monthly distribution of M. pneumoniae and C. pneumoniae presented in frequency.

Table 5.8: Seasonality of M. pneumoniae and C. pneumoniae.

Season	*M. pneumoniae*		*C. pneumoniae*	
	Frequency	**Percentage**	**Frequency**	**Percentage**
Spring	10	23.7%	2	5.6%
Winter	4	11.5%	2	5.8%
Summer	2	15.4%	1	9.1%
Autumn	1	4.2%	1	4.2%

By comparing year with month distribution; the incidence of both of *M. pneumoniae* is highest in July 2015 (50.0%), in contrast *C. pneumoniae* incidence is the highest in July 2016 (25.0%) table (5.10). The same story by comparing year with age distribution, *M. pneumoniae* positivity is the highest for age 15 and older in 2015 (33.33%) while *C. pneumoniae* presence was correlated with age 10 years old in 2016 (12.50%) (table 5.11).

Table 5.9: M. pneumoniae and C. pneumoniae cases by months and year from total population.

Month	*M. pneumoniae*			*C. pneumoniae*		
	Positives (%)			**Positives (%)**		
	2015	**2016**	**2017**	**2015**	**2016**	**2017**
January	(1/4) 25.00%	0.00%	0.00%	0.00%	0.00%	(1/18) 5.56%
February	0.00%	0.00%	(3/12) 25.00%	0.00%	(1/15) 6.67%	0.00%
March	(3/30) 10.00%	0.00%	0.00%	(1/3) 3.33%	0.00%	0.00%
April	(3/14) 21.43%	(2/14) 14.29%	0.00%	0.00%	0.00%	0.00%
May	(1/17) 5.88%	(1/8) 12.50%	0.00%	(1/17) 5.88%	0.00%	0.00%
June	(1/7)14.29%	0.00%	0.00%	0.00%	0.00%	0.00%
July	(1/2) 50.00%	0.00%	0.00%	0.00%	(1/4) 25.00%	0.00%
August	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
September	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
October	0.00%	(1/12) 8.33%	0.00%	0.00%	(1/12) 8.33%	0.00%
November	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
December	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%

Table 5.10: M. pneumoniae and C. pneumoniae cases by age groups and years from total population.

Age	*M. pneumoniae*			*C. pneumoniae*		
	Positives (%)			Positives (%)		
	2015	2016	2017	2015	2016	2017
5	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
6	0.00%	(1/32) 3.13%	0.00%	0.00%	0.00%	(1/24) 4.17%
7	(2/9) 22.22%	(1/17) 5.88%	(1/16) 6.25%	0.00%	0.00%	(1/16) 6.25%
8	(3/13) 23.08%	(1/21) 4.76%	0.00%	(1/13) 7.69%	(1/21) 4.76%	0.00%
9	(1/12) 8.33%	0.00%	(1/15) 6.67%	(1/12) 8.33%	0.00%	0.00%
10	(1/11) 9.09%	(1/8) 12.50%	0.00%	0.00%	(1/8) 12.50%	0.00%
11	(1/10) 10.00%	0.00%	(1/5) 20.00%	0.00%	0.00%	0.00%
12	(1/4) 25.00%	0.00%	0.00%	0.00%	0.00%	0.00%
13	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
14	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
≥15	(1/3) 33.33%	0.00%	0.00%	0.00%	0.00%	0.00%

5.2.3.5 *M. pneumoniae* and *C. pneumoniae* positivity by gender

The incidence of *M. pneumoniae* is higher in males (6.0%) than females (3.85%) meanwhile for *C. pneumoniae* the incidence is almost the same: males (1.8%) and females (1.6%).

Table 5.11: M. pneumoniae and C. pneumoniae cases by gender from total population.

Gender	No. of tested	*M. pneumoniae*		*C. pneumoniae*	
		No. of positives	Positive (%)	No. of positives	Positive (%)
Male	168	10	6.0%	3	1.8%
Female	182	7	3.8%	3	1.6%

5.2.4 Clinical manifestations for infected patients with *M. pneumoniae* and *C. pneumoniae*

During 2015, 2016 and 2017, 6.6% of hospitalized patients in CBH, aged 5 years old and more were infected with *M. pneumoniae* and *C. pneumoniae* but misdiagnosed. Because those patients were admitted to CBH it was easy to track back their files and to track doctor's observations during study period to analyze any clinical manifestation that might be correlated with the incidence of *M. pneumoniae* or *C. pneumoniae*. In general *M. pneumoniae* and *C. pneumoniae* patients were associated with moderate CRP levels between 11 and 50 mg/dl (table 5.13). Other general infection indicators were analyzed, minimum and maximum values were calculated in addition to their mean values as presented in table 5.14.

Table 5.12: M. pneumoniae and C. pneumoniae cases by CRP levels.

CRP mg/dl	*M. pneumoniae*		*C. pneumoniae*	
	Frequency	**Percentage**	**Frequency**	**Percentage**
0—10	2	11.8%	0	0.0%
11—50	9	52.9%	4	66.7%
51—100	4	23.5%	2	33.3%
>100	2	11.8%	0	0.0%

Table 5.13: Minimum, maximum and mean values for infection indicators:

Infection indicators	Minimum		Maximum		Mean	
	MP	CP	MP	CP	MP	CP
Temperature (C^{o})	36.6	36.9	40	38.5	38.1	37.5
WBC's	5.4	8.2	30.6	21	12.7	14
Neutrophils	19	25	92	90	61.9	61.8
Lymphocytes	4.5	5	56	54	24.7	28.7
Hospitalization (Days)	3	3	21	7	7	5

Signs and symptoms were analyzed for the patients. All of *C. pneumoniae* patients suffered from febrile illness and 33.3% of them have developed febrile convulsions. In contrast 80% of *M. pneumoniae* patients had febrile illness and 26.7% had febrile convulsions.

53% of the patients were reported to have hyperemic throat associated with the incidence of *M. pneumoniae.* As for *C. pneumoniae* 50% of the patients tended to have stuffy nose as a symptom (figure 5.7).

Right and left lower lobe pneumonia plus Bronchopneumonia were obvious with patients with *M. pneumoniae.* Only left lower lobe pneumonia and bronchopneumonia were reported *for C. pneumoniae* infection (figure 5.8).

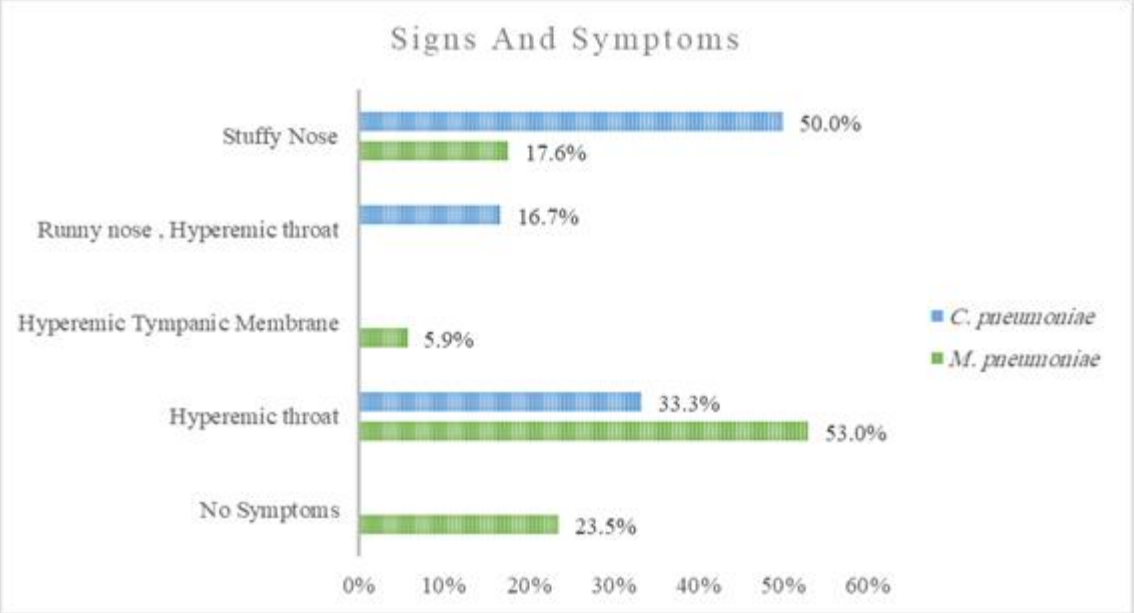


Figure 5.7: Signs and Symptoms for patients infected with M. pneumoniae and C. pneumoniae shown in percentage.

Chest examination reports revealed that most of the patients with *C. pneumoniae* suffered from chest crackles (50%) and wheeziness (33.4%). Wheeziness was reported for 29.4%

of the patients with *M. pneumoniae* infection, 29.4% of the patients have also suffered from decreased air entry (bilateral), however 23.6% had clear chest (figure 5.9).

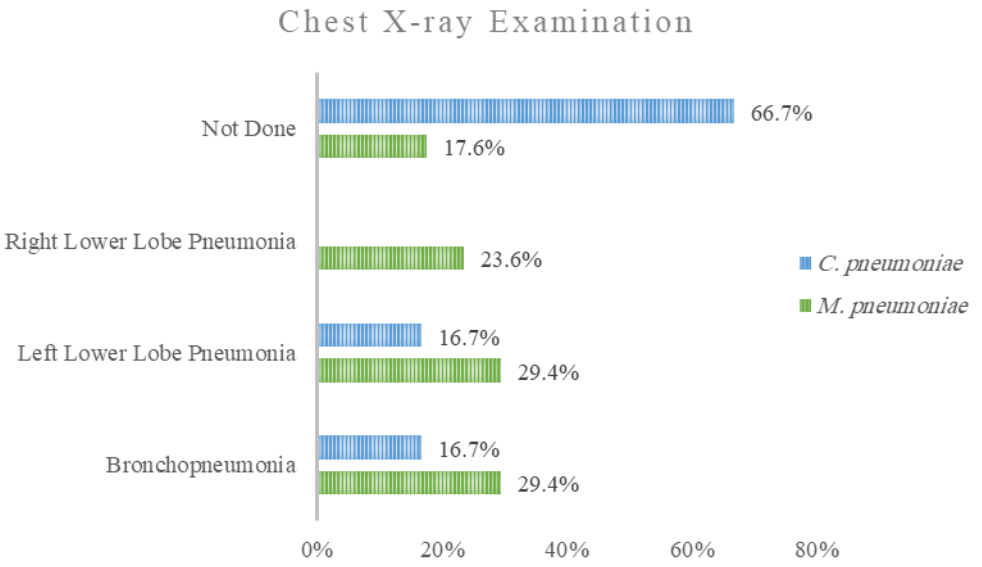


Figure 5.8: Chest X-ray examination for patients infected with M. pneumoniae and C. pneumoniae shown in percentage.

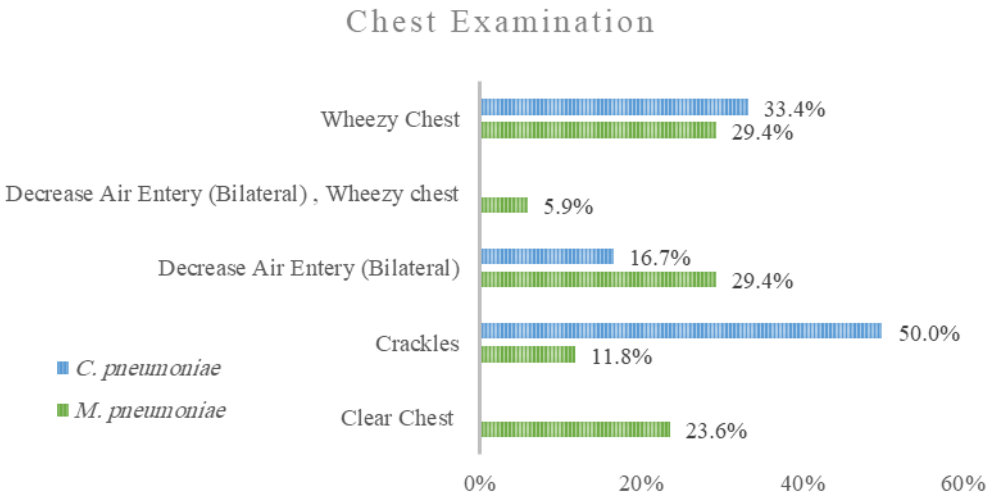


Figure 5.9: Chest examination for patients infected with M. pneumoniae and C. pneumoniae shown in percentage.

Respiratory tract statues for patients was studied as well; Figure 5.10 demonstrates breathing statues reports in patients' files, only few patients with both *M. pneumoniae* and *C. pneumoniae* faced difficulty in breathing, dropped O_2 saturation, needed O_2 and mechanical ventilation or even nasal drops

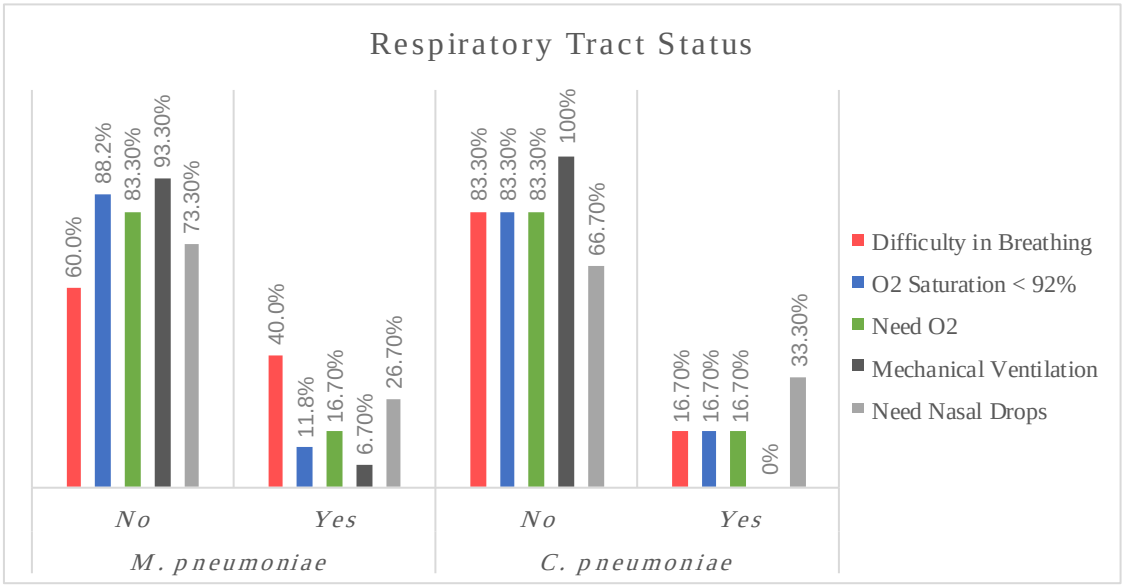


Figure 5.10: Respiratory Tract Status for patients infected with M. pneumoniae and C. pneumoniae shown in percentage.

No double infections with common respiratory viruses were observed in patients with *M. pneumoniae* and *C. pneumoniae.* Moreover, no meningitis, no conjunctivitis or gastroenteritis was observed in patients as well.

While reviewing the patients' records, most of them were under antibiotics administration. For *M. pneumoniae* 53% of the patients were under effective azithromycin coupled with ineffective cefuroxime or ceftriaxone. While 34% were taking other classes of ineffective antibiotics, for example: ceftazidime, ceftriaxone and cefuroxime. The rest didn't receive any therapy (13%) (figure 5.12). Half of *C. pneumoniae* patients didn't take any antibiotics meanwhile the other half have taken ineffective cefuroxime alone (16%), Azithromycin coupled with cefuroxime (17%) and ineffective ceftriaxone (17%) (figure 5.13).

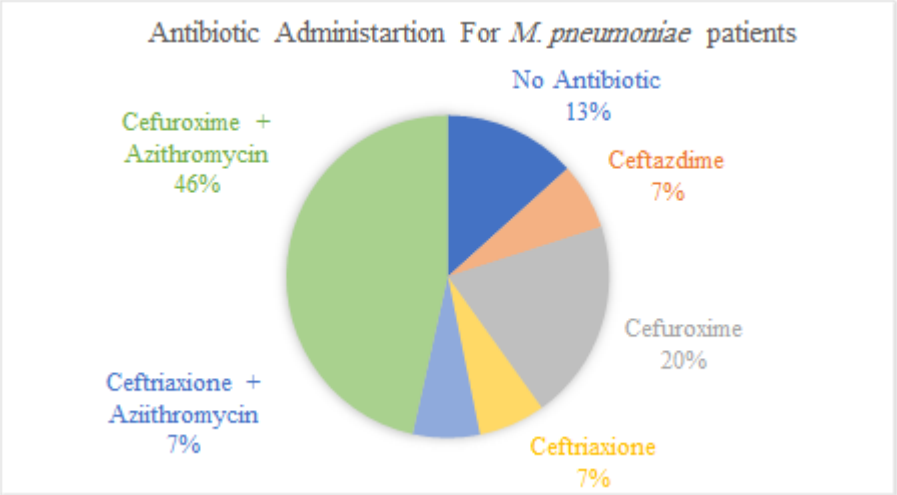


Figure 5.11: Antibiotics Administration for M. pneumoniae infected patients shown in percentage.

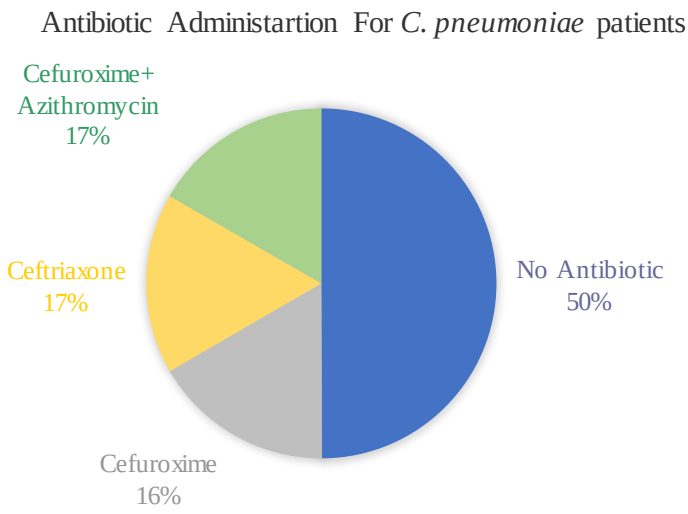


Figure 5.12: Antibiotics Administration for C. pneumoniae patients shown in percentage

5.2.5 Detection of Macrolide resistance *in M. pneumoniae* using nested PCR and sequencing

5.2.5.1 Validation of nested PCR

Serial dilution was used to validate the nested PCR region 1 and 2 before amplifying positive *M. pneumoniae* isolates detected earlier in this study. Figure 5.14 displays Gel electrophoresis for PCR products for region 1 where the LOD equals -5 same in the multiplex qRT-PCR and all bands share the same size of 210 bp. As for region 2 validation non -specific bands (108 bp) were observed as shown in figure 5.15 thus validation was done for region 3; which is the region between region 1 F primer and region 2 reverse primer which yields bands sized 746 bp as in figure 5.16.

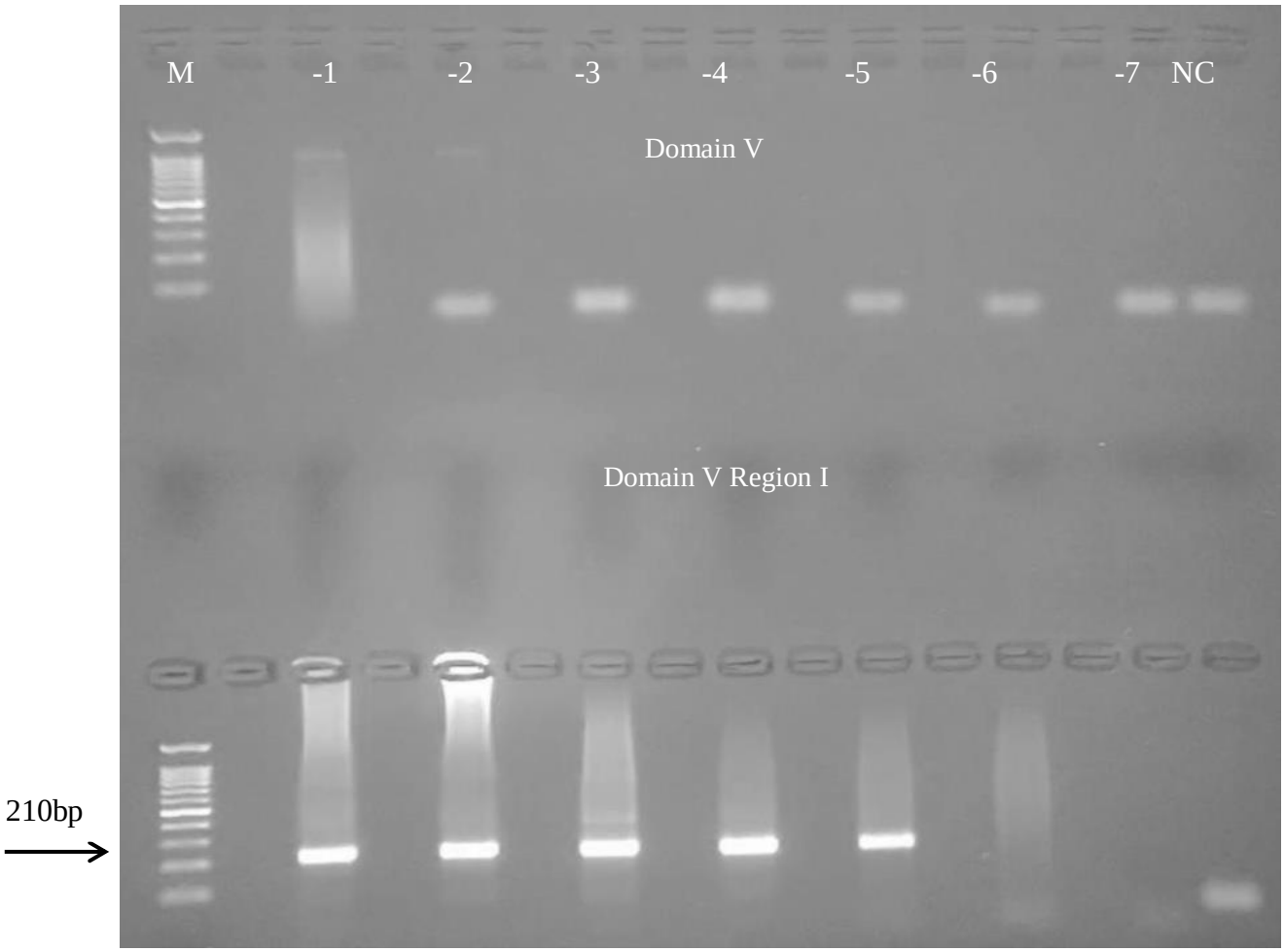


Figure 5.13: 1.5% agarose gel electrophoresis for validation of region 1.

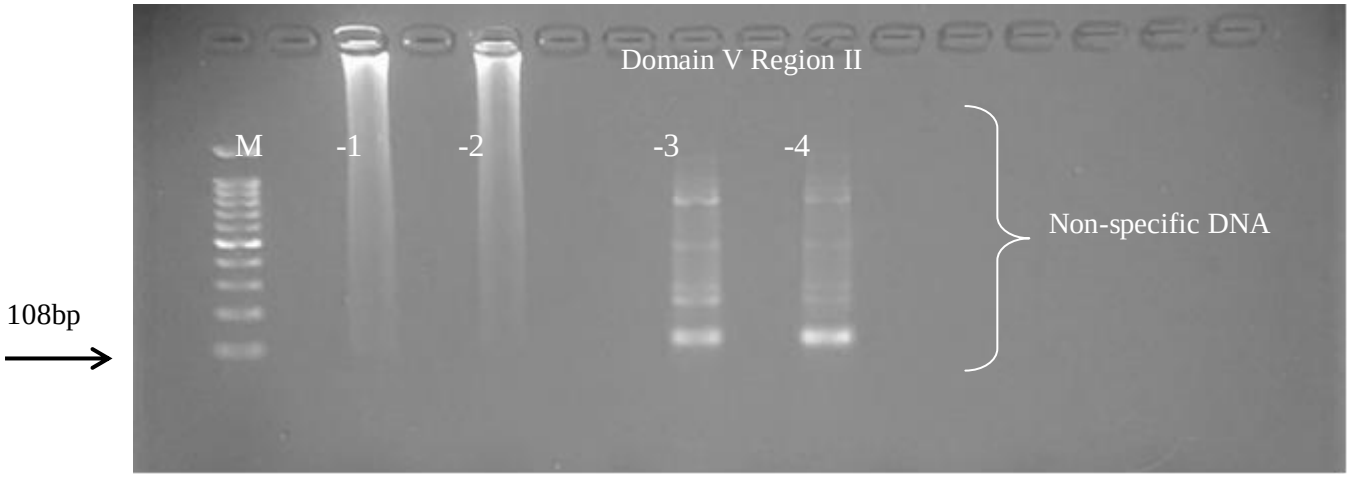


Figure 5.13: 1.5% agarose gel electrophoresis for validation of region 2.

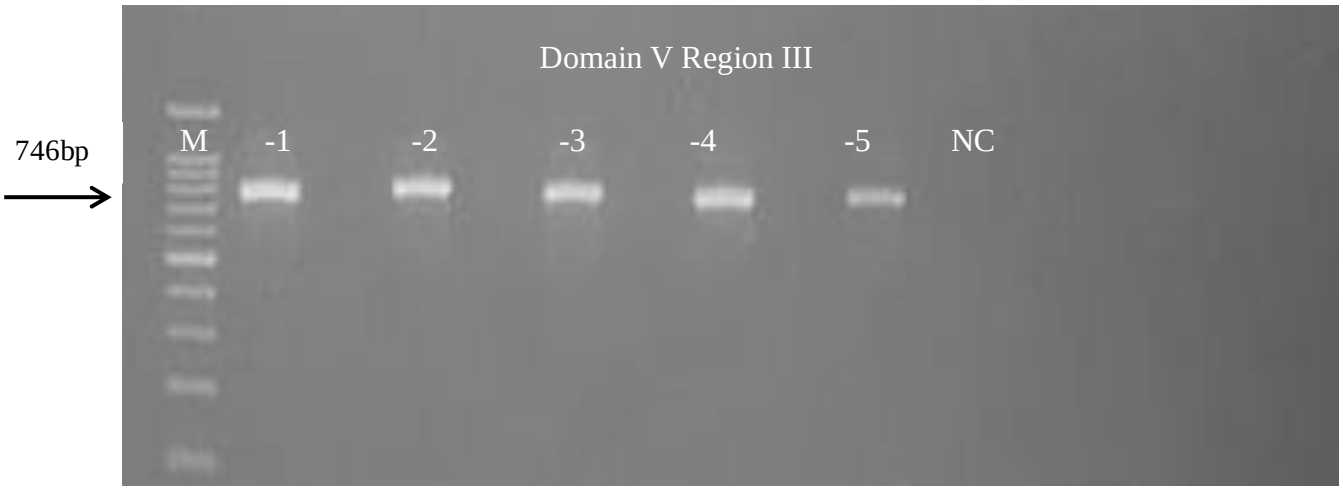


Figure 5.14: 1.5% agarose gel electrophoresis for validation of region 3.

5.2.5.2 Testing the samples using nested PCR

Seventeen *M. pneumoniae* isolates were tested for region 1 and 3 using nested PCR. Samples were loaded into gel electrophoresis before and after purification in order to be sequenced and screened for macrolides resistance. Examples of samples tested for the two regions after purification are shown in figures 5.17 and 5.18.

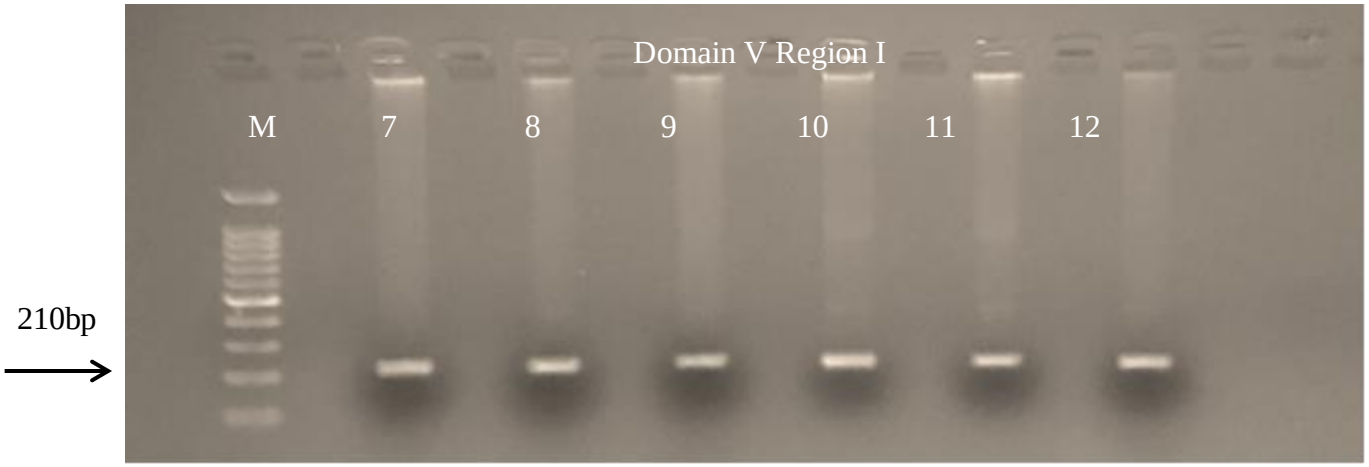


Figure 5.15: 1.5% agarose gel electrophoresis for samples 7-12 of region 1.

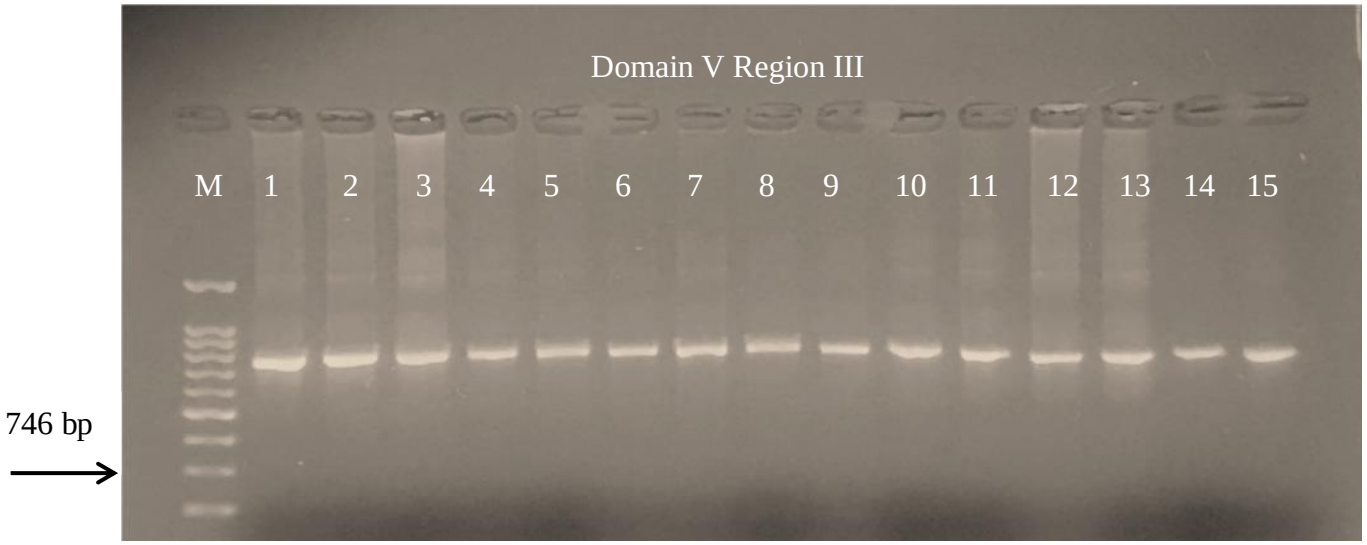


Figure 5.16: 1.5% agarose gel electrophoresis for samples 1-15 of region 3.

5.2.5.3 Sequencing

Seventeen sequences were obtained for each of region 1 and region 3 then aligned using Sequencher program (GenCode, USA). On one hand, all sequences from region1 have shown 100% resemblance to each other except for sample 1,3 and 18, these samples shown point mutation at position 2063 (A→ G) and this mutation is linked with Erythromycin resistance in *M. pneumoniae* (figure 5.20), Therefore 17.6% of samples

were resistant to Erythromycin (figure 5.19). No mutation was observed at region 3 position 2617.

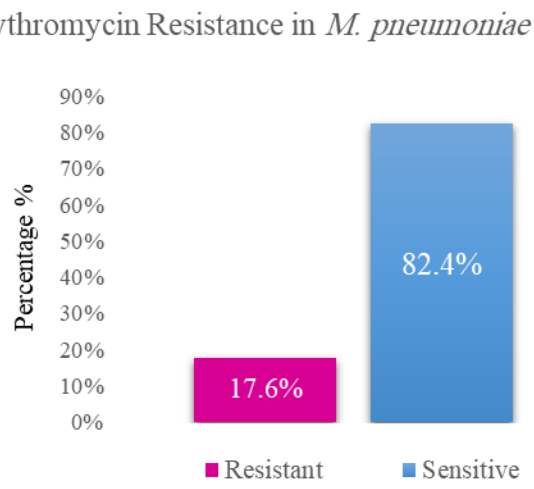


Figure 5.17: Erythromycin Resistance in M. pneumoniae detected in Region 1, Domain V 23S rRNA position 2063 shown in percentage.

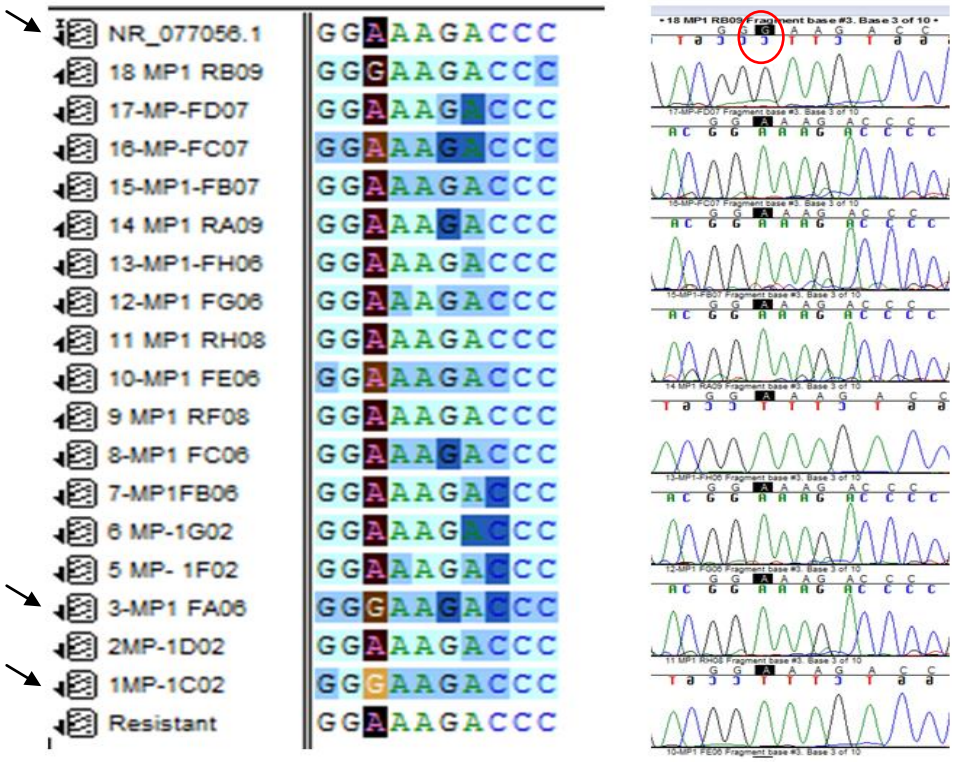


Figure 5.18: Chromatogram of domain V 23 rRNA, region 1, area shown is around 210 bp to expose the presence of point mutation at position 2063 (A→G) in isolates 1,3 and 18.

Chapter Six : Discussion

The motive behind this research was to initiate a base to study the prevalence of *M. pneumoniae* and *C. pneumoniae* in Southern Palestine. These two genera of bacteria are exclusively human pathogens that cause atypical pneumonia remarkably among children. Recently, *M. pneumoniae* has come to be recognized as the world-wide cause pneumonia, accounting for 11-15% of CAP throughout the world (Parrott, Takeshi , & Fujita, 2016). On the other hand, the incidence of *C. pneumoniae is* 6%–20% of CAP cases (Dumke & et al. , 2015). *M. pneumoniae* and *C. pneumoniae* are often misdiagnosed or their diagnosis is highly dependent on the clinical picture of the patients. Because of the fastidious nature of the two pathogens; routinely diagnostic protocols including culture and serology are not recommended thus diagnosis is shifting toward molecular approaches. In this study it was hypothesized that NPA samples might contain *M. pneumoniae* and *C. pneumoniae* pathogens that cause pneumonia for children. This research provided a general epidemiological and molecular characterization of these two bacteria in the pediatric population for the first time in Southern Palestine.

Detection relied on using multiplex qRT-PCR for screening the prevalence of these two organisms. Validation was necessary to assess the analytical sensitivity before testing the samples and to ensure that the multiplex meets well with acceptable PCR characteristic. LOD for *M. pneumoniae* was determined to be -5 (3.52 cfu/rxn) while for *C. pneumoniae* it was -7 (4.09 cfu/rxn).

Results from the study revealed that out of 350 NPA samples screened by the multiplex qRT-PCR, 23 samples were positive for *M. pneumoniae* and *C. pneumoniae* in which they compromise 6.6% of total population and this percentage is considered a significant one. As for individual frequency of each genus, *M. pneumoniae* constituted a proportion of 4.9% from total population, while *C. pneumoniae* constituted 1.7%. For every child infected with *C. pneumoniae* there are 3 children infected with *M. pneumoniae*. In Peru for example (2017) in children with acute respiratory infection; the incidence of *M.*

pneumoniae was 25.19% while *C. pneumoniae* positivity rate was 10.52% (Del Valle & et al., 2017).

Concerning Ct values; for *M. pneumoniae* isolates, Ct's ranged from 20.15 to 39.44 whereas for *C. pneumoniae,* Ct's ranged from 22.07 to 39.75, hence Ct values are associated with infectious organisms present in the sample; lower Ct value indicates higher bacterial concentration or existence.

Age was a dependent variable in this study, for each pathogen age distribution was statistically analyzed. By dividing *M. pneumoniae* infected children into three groups, we found that school aged children (5-10 yrs) have 35.2% positivity rate, young adolescent (11 to 14 yrs) tended to have 4.3% positivity rate while 15 years and older have a positivity of 9.1%. Rossi et al. in their study also showed that children aged between 5 and 10 years old are more susceptible than others to *M. pneumoniae* with a percentage of 41.2% and this supports the results of this research too (Rossi & et al., 2008). The pattern of infection varies in different countries concerning the age; In Nigeria, for example, 31% of children with pneumonia had a *M. pneumoniae* infection, with the highest prevalence in the 6–10 years old whereas in Turkey, the overall *M. pneumoniae* positivity was found to be 27% for children aged 0–14 years and the highest positivity rate was encountered at 10 years of age (65%) (Somer & et al., 2006) .

Also, by dividing positivity rate of *C. pneumoniae* in infected children, it is noticed that all positive samples belonged to patients from school aged children (5-10 yrs) with a positivity rate of 16.0%. Burillo and Brouza also concluded that *C. pneumoniae* is frequent among school aged children. (Burillo & Brouza, 2010)

All of positive samples for *M. pneumoniae* and *C. pneumoniae* belonged to patients living in Bethlehem, Hebron and Jerusalem only, thus miscellaneous districts from study population were ruled out from this investigation. *M. pneumoniae* was found in the three area with relatively close percentages: Bethlehem (5.9%), Hebron (4.0%) and Jerusalem (5.3%). By contrast, most of *C. pneumoniae* isolates were clustered in Bethlehem (3.3%)

and less isolates were spotted in Hebron (0.6%) and no positivity were observed in Jerusalem.

It was obvious that there was a variation concerning *M. pneumoniae* positivity according to year distribution; being the highest in 2015 (9.1%) followed by 2016 (3.0%) and 2017 (2.8%). No variation was observed through study period concerning *C. pneumoniae* positivity.

M. pneumoniae and *C. pneumoniae* positivity rate showed a variation throughout the year. One study proposed that the incidence of *M. pneumoniae* infection was seasonal with a peak in summer and minimum in winter (Chen & Et al., 2013), this study suggested that the peak of *M. pneumoniae* in Southern Palestine is the higher in Spring (23.1%), medium in Summer (15.4%), lower in Winter (11.5%) and minimal in Autumn (4.2%). On the other hand, seasonality of *C. pneumoniae* is the highest in Summer (9.1%), medium and almost the same in both Spring and Winter (5.6%, 5.8%) but it is lower in Autumn (4.2%). Also, Chen et al. proposed that *C. pneumoniae* prevalence is distributed in all seasons but relatively low in Autumn (Chen & Et al., 2013). By comparing the results to regional area for instance Jordan; one study concluded that *M. pneumoniae* infection occurs rarely in Jordanian adults and may be attributed to the prevalence of dry weather for most of the year in Jordan since *M. pneumoniae* infections were more commonly recognized intemperate zones and moist regions (Shehabi & et al., 2015).

Regarding gender distribution, this study revealed that the incidence of *M. pneumoniae* is higher in males (6.0%) than females (3.85%) meanwhile for *C. pneumoniae* the incidence is almost the same: males (1.8%) and females (1.6%). One study in Britain proposed an opposite scenario, the study has focused on studying gender as an influence factor that affect the incidence of CAP etiological factors like *M. pneumoniae*, *C. pneumoniae* and *L. pneumophilae*, and the conclusion was that the incidence of pneumonia caused by *M. pneumoniae* was unrelated to gender in contrast to *C. pneumoniae*; males increases

greatly the incidence of *L. pneumophilae and C. pneumoniae* (Martin-Hidalgo & et al., 2006).

Hospitalized children aged 5 years and more in CBH have been misdiagnosed for *M. pneumoniae* and *C. pneumoniae* infection during study period with an incidence of 6.6%. To enrich this research, patients' files with *M. pneumoniae* and *C. pneumoniae* during that period were tracked back and analyzed to investigate any parameter that might have contributed or increased the possibility for infection with these two organisms. First, general infection indicators we analyzed to conclude that there is a variation in infection indicators, but if the means for each species were compared, we can say that infection indicators are relatively close for both *M. pneumoniae* and *C. pneumoniae*. For instance, the temperature for both is moderately high, the same for neutrophils and CRP levels. However, hospitalization days for both ranged between 5 to 7 days.

M. pneumoniae and *C. pneumoniae* infection are considered mild infections, most frequent signs and symptoms observed with the patients were hyperemic throat, stuffy nose and fever, these signs and symptoms are from the most common ones according to the literature (Waites K. , 2003).

Generally, pediatricians diagnose atypical pneumoniae depending on chest examination and x-ray. Chest wheeziness was observed in patients with both pathogens, crackles was recorded in half of *C. pneumoniae* patients while some of *M. pneumoniae* patients have progress crackles (11.8%). *M. pneumoniae* patients (29.4%) suffered from bilateral decreased air entry that lead to dropped O_2 levels in some patients. As for chest x-ray, there were various clinical pictures; have bronchopneumonia and others have left lower lobe pneumoniae. Right lower lobe pneumoniae was noticed only in *M. pneumoniae* patients. Findings from this part concerning *M. pneumoniae* have been approved before by various studies; Kishaba also conclude that late inspiratory crackles may happen in addition to bilateral wheezes because of bronchiolitis, also bilateral reticulonodular or patchy consolidation in both lower lobes are noticed (Kishaba, 2016).

One of the aims of this study was to investigate any double infections with the respiratory viruses but no double infection was observed in *M. pneumoniae* patients neither *C. pneumoniae* patients.

As mentioned in the literature review the first line of antibiotics for both *M. pneumoniae* and *C. pneumoniae* are macrolides. Some patients were administered under azithromycin (*M. pneumoniae*: 53%, *C. pneumoniae*: 17%) and certainly that reduced the infection.

Recently, macrolide resistance in *M. pneumoniae* has emerged worldwide and has been associated with longer duration of fever, cough, hospitalization and the need to switch to alternative antimicrobial agents. Concerning Southern Palestine yet no statistics have been published. Domain V in 23s rRNA is linked to Erythromycin resistance in three main positions 2063,2064 and 2617. Investigating of resistance pattern in *M. pneumoniae* positive isolates made this research even a more valuable one.

While using nested PCR for the 17 samples, Domain V was divided into two main regions; region 1 and region 2 (Okazaki & et al. , 2001). Validation of nested PCR was performed in the first place for both Region 1 and 2 using same serial dilution prepared in the multiplex qRT-PCR, this way nested PCR assay was confirmed.

Nested PCR yielded bands sized 924 bp. First, Region 1 amplification results in bands equal 210 bp, then samples were purified and sequenced. Out of 17 *M. pneumoniae* isolates, 3 were resistant to erythromycin where there was a nucleotide substitution at position 2063 from A→ G and this constitutes 17.6% of samples and this is considered a relatively high percentage. However, no mutations were observed at position 2064m neither nucleotide substitution from A→C. Current knowledge on macrolide resistance among for last 15 years have showed that the resistance is ranging between 0 and 15% in Europe and the USA, approximately 30% in Israel and up to 90–100% in Asia (Bebear, Goret, & Pereyre, 2016).

For region 2, since the reverse primer was the same used to amplify domain V; samples were amplified directly from extracted samples without the need for domain V

amplification first, in fact band size was 108 bp and when loaded on 1.5 % agarose gel the bands were able to be visualized under UV light. But unfortunately, sequencing results were not good, samples were again tested by nested PCR, first by amplification for domain V then region 2 but still sequencing was also the same, that is maybe because the bands are relatively small. Thinking outside the box suggested that instead of using region 2 forward primer, region 1 forward primer could be used thus increasing the band's size to be 746 bp instead of 108 bp. On 1.5 % agarose gel bands were able to be beautifully visualized and they all share same size. Sequencing results confirmed that no mutation is found at position 2617 and all isolates are erythromycin sensitive.

However, the 3 isolates found to be erythromycin resistant have no significant pattern associated with the clinical presentation of the patients while tracking back patient's reports.

Chapter Seven : Conclusion

Nowadays most of microbiological diagnosis is highly dependent on molecular identification; multiplex qRT-PCR is a very sensitive test and now is available to screen samples for different fastidious pathogens like *M. pneumoniae* and *C. pneumoniae*, results arose from this study encourage pediatricians to take the advantage and diagnose atypical pneumonia using qRT-PCR since locally (Southern Palestine) we have a relatively high incidence (6.6%). The last part of this study focused on macrolide resistance and results also showed that we have a high rate of erythromycin resistance in the 23S rRNA (17.6%). The prevalence of macrolides resistance is the highest in Asia which may develop during treatment due to abuse of antibiotics administration and this emphasizes and focuses on the first point; appropriate diagnosis leads to appropriate treatment.

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Chapter Nine Appendices

Appendix 1:

Nucleic Acid Extraction protocol:

Kit used: High Pure Viral Nucleic Acid Extraction Kit (Cat#: 11858882001; Roche Applied Science)

Usage: The High Pure Viral Nucleic Acid Extraction Kit is used to purify viral Nucleic Acid form blood, plasma, NPA and other body fluid samples.

Kit contents:

A. Ready to use contents are as the following:
   I. Binding Buffer (green cap)
   II. Elution Buffer (white cap)
   III. High Pure Spin Filter Tubes
   IV. Collection Tubes

B. Preparation of working solutions; preparation of working solutions is needed beside the ready to use solutions supplied with this kit as following:
   I. Poly (A) carrier nucleic acid (Vial 2):
      - Dissolve poly A nucleic acid carries in 0.4 ml Elution Buffer (Vial 4).
      - Prepare aliquots of 50 µl into 1.5 ml Eppendorf tubes for running 8x12 purifications.
      - Store at -15 to -25^{o} C.
      - For the preparation of working solution.
   II. Working solution:
      - For 12 purifications; thaw one vial with 50 µl poly A carrier nucleic acid and mix thoroughly with 5 ml Binding Buffer (Vial 1).
      - Prepare always fresh before use; Do not store.
      - Protocol step 1.
   III. 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^{o} C; stable through the expiration date printed on kit label.
- Protocol step 5: to remove PCR inhibitors.

IV. Wash Buffer: (Vial 4, blue cap)

- Add 40 ml absolute ethanol to each of Wash Buffer vials before use and mix well.
- Label and date bottles accordingly after adding ethanol.
- Store at Store at 15 to 25^{o} C; stable through the expiration date printed on kit label.
- Protocol step 6 and 7: Removal of residual impurities.

Procedure:

1. Thaw one vial of 50 µl poly A nucleic acid carries for 12 preparations.
2. Mix thoroughly with 5 ml Binding buffer (vial 1).
3. Aliquot 400 µl Binding buffer supplemented with poly A in separate Eppendorf tubes.
4. Add 200 µl from each NPA sample to mixture; mixed gently to avoid aerosols.
5. Add to the upper reservoir of High Pure Filter Tubes.
6. Centrifuge at 8000g (10,000 rpm/ min) for 1 minute.
7. Transfer the filter to another clean collection tube.
8. Add 500 µl of Inhibitor removal buffer to the filter.
9. Centrifuge at 8000g for 1 minute.
10. Transfer the filter to another clean collection tube.
11. Add 450 µl of Wash buffer.
12. Centrifuge at 8000g for 1 minute.
13. Repeat the wash buffer step one more time; at the end of this step the filter was centrifuges at the maximum speed at 13,000g for 10 seconds to dry the filter.

14. In the last step, transfer the filter to an Eppendorf tube.
15. Add 50 µl of Elution buffer.
16. Soak for 1 minute and centrifuge at 8000g for 1 minute.
17. Nucleic Acid is now collected is in the Eppendorf tube.

Appendix 2

PCR product clean up

Kit used: High Pure PCR Cleaning Micro Kit

Kit contents: A. Ready to use contents are as the following:

I. Binding Buffer (green cap)
II. Elution Buffer (white cap)
III. High Pure Spin Filter Tubes
IV. Collection Tubes

B. Preparation of working solutions; preparation of working solutions is needed beside the ready to use solutions supplied with this kit as following:

V. Wash Buffer: (Vial 4, blue cap)

- Add 20 ml absolute ethanol to each of Wash Buffer vials before use and mix well.
- Label and date bottles accordingly after adding ethanol.
- Store at Store at 15 to 25^{o} C; stable through the expiration date printed on kit label.

Procedure:

The PCR product is transferred to an Eppendorf 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 8000g for 30 seconds followed by discarding the flow through and changing the collection tubes. The second wash step if 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 the purified PCR product is collected in the Eppendorf tube saved at – 30 ^{o} C with an appropriate labeling.