COMPARATIVE EVALUATION OF CULTURAL AND MOLECULAR IDENTIFICATION METHODS OF *Mycoplasma pneumoniae* FROM SPUTUM SAMPLES OF SUBJECTS IN NNEWI.

BY

ADIKE, CALISTA NDIDI
REG. NO. 2008617002F.

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MEDICAL LABORATORY SCIENCE, FACULTY OF HEALTH SCIENCES AND TECHNOLOGY, NNAMDI AZIKIWE UNIVERSITY, NNEWI CAMPUS, NNEWI, NIGERIA. IN PARTIAL FULLFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph. D) IN MEDICAL MICROBIOLOGY.

JUNE, 2018

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SUPERVISORS: PROF. N.R. AGBAKOBA

PROF. I.B. ENWEANI

JUNE, 2018

CERTIFICATION

I, Adike Calista Ndidi hereby certify and declare that this study titled 'COMPARATIVE EVALUATION OF CULTURAL AND MOLECULAR IDENTIFICATION METHODS OF *Mycoplasma pneumoniae* FROM SPUTUM SAMPLES OF SUBJECTS IN NNEWI' was done by me. I accept responsibility for any shortcomings found in the dissertation.

……………………………
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Adike Calista Ndidi Date.

APPROVAL PAGE

The dissertation titled '**COMPARATIVE EVALUATION OF CULTURAL AND MOLECULAR IDENTIFICATION METHODS OF *Mycoplasma pneumoniae* FROM SPUTUM SAMPLES OF SUBJECTS IN NNEWI'** submitted by Adike Calista Ndidi (2008617002F) has been read and approved by the undersigned as adequate in scope and quality in partial fulfillment of the requirements for the award of Doctor of Philosophy (Ph.D) in Medical Microbiology.

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iv.

DEDICATION

I dedicate this work to

God Almighty

For His infinite mercy and love on me.

And

To the memory of my late father, Mr. Hycienth N. Iloetomma.

And

Also to the memory of my late beloved brother, Mr Anayochukwu B. Iloetomma.

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ABSTRACT

Mycoplasma pneumoniae is an organism that belongs to the family mycoplasmataceae. It's role as a disease causing agent continues to draw interest especially with the advent of highly sensitive detection techniques. This bacterium poses a health problem to both animals and humans resulting in serious illnesses such as community-acquired pneumonia, lung damage and death. The virulence gene of *M. pneumoniae,* glycerophosphodiesterase (GLPQ gene) has not been detected in Nigeria. This work investigated the prevalence of *M. pneumoniae* as agent of respiratory tract infections using culture and molecular methods of identification, in patients attending Pulmonary Tuberculosis Clinic at Nnamdi Azikiwe Teaching, Hospital, Nnewi as well as detecting the most virulence gene of this organism. A total of 263 sputum samples were collected: 188 test subjects and 75 control subjects. These samples were examined bacteriologically using PPLO broth and agar, MacConkey, blood and chocolate agars. *M. pneumoniae* was further identified by polymerase chain reaction (PCR) technique using *M. pneumoniae* specific primer. All *M. pneumoniae* DNA positive samples were further amplified to detect the GLPQ gene of the organism using GLPQ specific primer. The overall prevalence rates of *M. pneumoniae* among the 263 subjects were 4.9% and 8.0% by culture and PCR respectively. The prevalence rate of the organism was significantly higher among the test subjects 11(5.9%) by culture and 18(9.6%) by PCR than the control subjects 2(2.7%) by culture and 3(4.0%) by PCR. The colonization of the organism was significant among the age groups 31-40 years ($P<0.05$). The prevalence of *M. pneumoniae* most virulence gene (GLPQ) among the 18-PCR positive subjects was 27.8%. The GLPQ gene was detected only in the female gender and this showed that there was a significant difference between the occurrence of the GLPQ gene among the female gender than the male gender ($P<0.05$). The antibiotic sensitivity pattern of *M. pneumoniae* showed that the organism was susceptible to Lyntriaxone, Levofloxacin, Ciprofloxacin, Azithromycin and Doxycycline while it showed resistance to Septrin, Peflacine, Rifampicin, Erythromycin and Norbactin. Other bacteria isolated from the sputum specimens included *Staphylococcus aureus,* 35 (18.6%); *Streptococcus pneumoniae*, 31 (16.5%); *Pseudomonas aeruginosa, 11*(5.9%) and *Klebsiella pneumoniae* 12(6.4%). The presence of *M. pneumoniae* in the sputum samples of subjects as well as the presence of the virulence gene (GLPQ) only from test subjects shows that the bacterium actually contributed to the aetiology of lower respiratory tract infections. These

were better detected by PCR. It is recommended that the search for *M. pneumoniae* be included in the routine laboratory investigations for lower respiratory tract infection

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Mycoplasma pneumoniae (*M. pneumoniae*) is a common respiratory pathogen that produces diseases of varied severity ranging from mild upper respiratory tract infection to severe atypical pneumonia. *Mycoplasma pneumoniae* causes up to 40% or more of community acquired pneumonia (CAP) cases, and as many as 18% of cases requiring hospitalization in children (Ferwerda *et al*., 2001). *Mycoplasma pneumoniae* causes both upper and lower respiratory tract infections. Respiratory tract infection can be defined as any infection of the respiratory tract. It refers to the pathological state resulting from the invasion of the body by pathogenic microorganism. Respiratory diseases are major cause of mortality and morbidity world wide (WHO, 2014). In most developing countries including Nigeria, the burden of respiratory disease is largely unknown. Respiratory tract infections are divided into two parts: the upper respiratory tract infection (URTI) and the lower respiratory tract infection (LRTI).

Upper respiratory tract infections are the illnesses caused by an acute infection which involves the upper respiratory tract: nose, sinuses, pharynx or larynx. This commonly include: Tonsilitis, pharyngitis, laryngitis, sinusitis, otitis media and the common cold (Ecceles *et al*., 2007). Symptoms of URTI’s commonly include cough, sore throat, running nose, nasal congestion, headache, low grade fever, facial pressure and sneezing. Onset of the symptoms usually begins 1-3 days after the exposure to a microbial pathogen.

Lower respiratory tract is the part of the respiratory tract below the vocal cords. This includes the lungs, bronchi, bronchioles etc. Symptoms of lower respiratory tract infections are shortness of breath, weakness, high fever, coughing and fatique. LRTI are more serious than upper respiratory infections. In 2012, lower respiratory tract infections were still the leading cause of deaths among all infectious diseases and they accounted for 3.9 million deaths world wide and 6.9% of all deaths that year (Robert, 2004). *M. pneumoniae* affects the upper or lower respiratory tract or both. Symptoms commonly appear gradually, during a few days and can persist for weeks or months. Typical clinical features include an initial pharyngitis, sore throat and hoarseness; fever (Vervloet *et al.,* 2007). An intractable day and night cough characterises extension of the infection to lower airways. Initially, cough is non-productive but later may yield small to moderate amounts of non bloody sputum (Vervloet *et al*., 2007). Dyspnoea may be evident in more severe cases. Although most infections occur among out patients hence the colloquial term “walking pneumonia”, *Mycoplasma pneumoniae* is a significant cause of bacterial infections in adults requiring hospitalization in USA. Marston *et al*., (1997), reported that *M. pneumoniae* was definitively responsible for 5.4% and possible for 32.5% of 2776 cases of CAP in hospitalized adults based on complement fixation (CF) test for detection of infection.

Children under 5 years of age are less commonly affected by *M. pneumoniae* respiratory tract infection whereas older children aged 5-15years develop bronchopneumonia in one or more lobes, sometimes requiring hospitalizations and bronchopneumonia develops in 3-10% of infected adults (Cassel *et al*., 1985; Jain *et al*., 2015).

M. pneumoniae not only causes respiratory tract infections, in severe cases it disseminates to various organs of the body causing extra pulmonary complications (Koletsky and Weinsteen, 1980; Waites *et al*., 2017). Extrapulmonary manifestations of *M. pneumoniae*

infections include central nervous system manifestations which can at times be life threatening (Narita *et al*., 2005), encephalitis, meningoencephalitis, polyradiculitis and aseptic meningitis (Stamm *et al*., 2008). Among patients with *M. pneumoniae,* 25% have dermatological manifestations such as erythema multiforme and toxic epidermal necrolysis (James and Berger, 2006).

Haematological manifestations of *M. pneumoniae* infection include auto immune haemolytic anaemia, auto immune thrombocytopenia and disseminated intra vascular coagulation (Vervloet *et al*., 2007). Gastro intestinal manifestations are frequent and have been described roughly in 25% of cases, manifesting as nausea, vomiting, abdominal pain, diarrhoea and loss of appetite (Kashyap and Sarkar, 2010). *M. pneumoniae* is also associated with musculo – skeletal manifestations such as non-specific myalgias, arthtralgia and polyathropathies in approximately 14% of cases (Vervloet *et-al*., 2007). The organism also causes polyarthritis (Kashyap and Sarkar, 2010). Glomerulonephritis associated with *M. pneumoniae* is rare and a few cases have been described in children (Vervloet *et al*., 2007; Kashyap and Sarkar, 2010). *M. pneumoniae* has been linked to asthma in various ways and has been reported to exacerbate asthma and make control of asthma more difficult (Lieberman *et al*., 2003).

M. pneumoniae genome contains 687 genes that encode for protein, of which about 56.6% code for essential metabolic enzymes, notably those involve in glycolysis and organic acid fermentation (Romero-Arroyo *et al*., 1994). A variety of proteins are known to contribute to formation and functionality of attachment organelle including the accessory proteins HMW1 – HMW5, P30, P56 and P90 that confer structure and adhesin support and P1, P30 AND P116 which are involved directly in attachment (Romero-Arroyo *et al*., 1994). *M. pneumoniae* has 677 open reading frames (OFRS) corresponding to 716, 617 base pair (bp) and 39 (9998bp) other coding regions for molecular machinery

such as rRNA, tRNA etc (Romero-Anoyo *et al*., 1994). *M. pneumoniae* possesses two potential glycerophosphodiesterases, MPN420 (GLPQ) and MPN 566. Only GLPQ is an active glycerophosphodiesterase (Schmidi *et al,* 2011). MPN 566 has no enzymatic activity as glycerophosphodiesterase and inactivation of the gene did not result in any detectable phenotype (Schmidi *et al.,* 2011). However, inactivation of the GLPQ gene resulted in reduced growth in medium with glucose as the carbonsource, in loss of hydrogen peroxide production when phosphatidylchlorine was present, and in a complete loss of cytotoxicity towards Hela cells (Schmidi *et al.,* 2011). Among all the genes possessed by *M. pneumoniae*, GLPQ gene is the most active and most virulent gene (Schmidi *et al*., 2011).

Mycoplasma pneumoniae infections have been reported in parts of African and some other parts of the world. Naoyuki and Atsushi (2004) reported a prevalence rate of 4.8% in Germany and Tsutomu *et al*., (2006) reported a prevalence of 19.5% in Japan. A study in Kenya on etiology and incidence of viral and bacterial acute respiratory illness among older children and adults, *M. pneumoniae* was detected in 0.7% of patients (Daniel *et al*., 2010). Another study of hospitalized adults with community acquired pneumonias performed in Isreal showed *M. pneumoniae* to be responsible for 29.2% of pneumonias over all (Porath *et al*., 1997). Maston *et al*., (1997) reported that the organism was definitely responsible for 5.4% and possible responsible for 32.5% of 2,776 cases of community acquired pneumonia in hospitalized adults in two county region of Ohio. In a study in Nigeria on *Mycoplasma pneumoniae* and the aetiology of Lobar pneumonia in Zaria, Northern Nigeria, 12 patients out of 74 suffering from lobar pneumonia were discovered to have *Mycoplasma pneumoniae* as the causative agent of their disease (MacFarlane *et al*., 1979). In another study by Oluwa *et al*., (2015), the researchers reported the prevalence rate of *Mycoplasma pneumoniae* infection to be 5.1%.

Following the above incidences of *M. pneumoniae* infections, the role of the organism in respiratory tract infections in many parts of the world, including African will not be over emphasized. Unlike the genital tract mycoplasmas that have been studied extensively in Nigeria (Agbakoba *et al*., 2006; Agbakoba *et al*., 2007a, 2007b; Agbakoba *et al*., 2008; Chukwuka *et al*., 2013) only limited work has been done on the respiratory tract mycoplasma in Nigeria (MacFarlene *et al*., 1979; Oluwa *et al*., 2015). *Mycoplasma pneumoniae* is still not being sought for routinely in respiratory tract specimens from patients in this country. There is need for the study to be conducted to create awareness on the incidence and prevalence of this *M. pneumoniae* as a causative agent of respiratory tract infections. This will further save the lives of patients suffering from this and subsequently improve the public health.

1.2 **STATEMENT OF THE PROBLEM**

Respiratory infection is one of the most common deadly diseases and if not detected early and treated, it kills (Santos, 2001). *M. pneumoniae* among other agents of respiratory tract infections causes both upper and lower respiratory tract infections in all age groups especially in children. Commonly among diseases caused by this organism is community acquired pneumonia. Despite this, much work has not be done on it mainly due to the fastidiousness, tedious, laborious and technically challenging culture methods needed to link the organism to clinical conditions. Little information is available especially here in Nigeria regarding the prevalence of this organism in causing respiratory tract infections. This study will help to create more awareness on the role played by this organism so that the detection and isolation could be incorporated into the routine laboratory diagnosis.

In previous studies, PCR has been compared to serological diagnosis of *M. pneumoniae* infections (Abele *et al.,* 1998). In another study, applying PCR for diagnosis of *M. pneumoniae* infection, serological assay were not used at all (Leven *et al*., 1996; Thurman *et al*., 2011). None of the studies so far prospectively compared culture with PCR in both test and control subjects. Hence this study is aimed at bridging this gap.

1.3 JUSTIFICATION OF THE STUDY

Correct diagnosis of *M. pneumoniae* infection is important to allow the appropriate antibiotics treatment of patients, since it is impossible to identify *M. pneumoniae* infection solely on the basis of clinical signs and symptoms. This will decrease inappropriate use of antibiotics, influence the patient's outcome by reduction of morbidity and mortality and improve our knowledge of the prevalence of the causes of atypical pneumonia. Conventional assays for the detection of M. *pneumoniae* have their limitations resulting in the need for more accurate diagnostic methods.

1.4 **AIM.**

To comparatively evaluate cultural and molecular identification methods of *Mycoplasma pneumoniae* from sputum samples of subjects attending tuberculosis clinic at Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nnewi North LGA, Anambra State.

1.5 OBJECTIVES

- To determine the prevalence of *Mycoplasma pneumoniae* in respiratory tract infection using culture method.
- To detect *Mycoplasma pnmoniae* infection using molecular technique.
- To ascertain the presence of virulence gene of *M. pneumoniae* using polymerase chain reaction (PCR) method.
- To determine the antibiotic sensivity pattern of the organism.

- To determine the prevalence of other pathogens and their co-existence with *M. pneumoniae*.

1.6 RESEARCH QUESTIONS

1. Is *Mycoplasma pneumoniae* an agent of respiratory tract infection?

   Null hypothesis: *Mycoplasma pneumoniae* is not an agent of respiratory tract infection. Any occurrence is due to chance.

   Alternate hypothesis: *Mycoplasma pneumoniae* is an agent of respiratory tract infection. Any occurrence is not due to chance.

2. Is GLPQ gene responsible for *Mycoplasma pneumoniae* virulency?

   Null hypothesis: GLPQ gene is not responsible for *Mycoplasma pneumoniae* virulency.Any virulency is due to chance.

   Alternate hypothesis: GLPQ gene is responsible for *Mycoplasma pneumoniae* virulency. Any virulency is not due to chance.

CHAPTER TWO

LITERATURE REVIEW

2.1 History of Mycoplasma

The name mycoplasma, from the Greek Mykes (Fungus) and plasma (formed), was first used by Albert Bernhard Frank in 1889. He thought it was a fungus, due to fungus-like characteristics (Krass and Gardner, 1973; Cunha, 2010). The discovering of Mycoplasma dated back to 1898. Initially, due to their unknown nature and relationships with other organisms, while being minute in size and not being qualified as bacterial they were considered viruses for years. However, many years later with further discovery mycoplasmas were confused with L-forms, which are bacteria that have lost their cell walls either completely or partially, nevertheless, in 1950s and 1960s this confusion came to an end when first genomic analysis data through DNA hybridization were obtained. This analysis ruled out any relationship of mycoplasmas to the L-forms (Morowitz and Wallace, 1973; Gumpert and Hoischen, 1998). Currently, mycoplasmas are considered to have evolved from Gram positive, walled eubacteria by degenerative evolution, meaning their evolutionary history appears to include the loss of cell wall. Further, in 1960s and 1970s knowledge about ultrastructure, cell membrane, genome and metabolic pathways of mycoplasmas resulted in conclusion that they are smallest and simplest self replicating organism (Morowitz and Wallace, 1973; Browning and Citti, 2014).

The first isolation of Mycoplasma was done by Nocard and Roux from cattle with Contagious bovine Pleuropneumonia (CBPP) in 1898 which was at that time a grave and widespread disease in cattle herds (Nocard and Roux 1990). The causative agent of the CBPP is *Mycoplasma mycoides* SC (small – colony type), (Edward and Freundt, 1956). The fact that the original isolation of mycoplasma was from bovine plueropneumonia, the organisam was called the pleuro pneumonia organism and subsequent isolation of the

organisms with simliar colonial morphologic features from a variety of animals was designated pleuropneumonia like organisms (PPLO) (Edward and Freundt, 1956). Dienes and Edsall detected the first mycoplasma isolated from humans in a Bartholin's gland abscess in 1937 (Dienes and Edsall, 1937).

The organism eventually known to be *Mycoplasma pneumoniae* was first isolated in tissue culture from the sputum of a patient with primary atypical pneumonia by Eaton *et al*., in 1944 and thereafter it became known as the Eaton agent (Eaton *et al*., 1944). Chanock *et al*., later succeeded in culturing the Eaton agent on cell-free medium (Chanock *et al.,* 1962).He proposed the taxonomic designation *Mycoplasma pneumoniae* in 1963 (Chanock *et al.,* 1963). Methodologically, the organism has a tendency to grow down into agar that produces a dark center and light periphery the so called "Fried egg" appearance, (Baseman *et al*., 1997).

2.2 Mollicute Taxonomy and Classification.

Mycoplasma were designated members of a class named mollicutes, which derives from Latin words meaning soft ("Mollis") and skin ("Cutis"). The current taxonomic designations included in class mollicutes comprises 4 order, 5 families, 8 genera and about 200 known species that have been detected in humans, vertebrate animals, arthropods and plants as shown below. *M. pneumoniae* is a member of the family Mycoplasmataceae and order Mycoplasmatales (Waites *et al*., 2003).

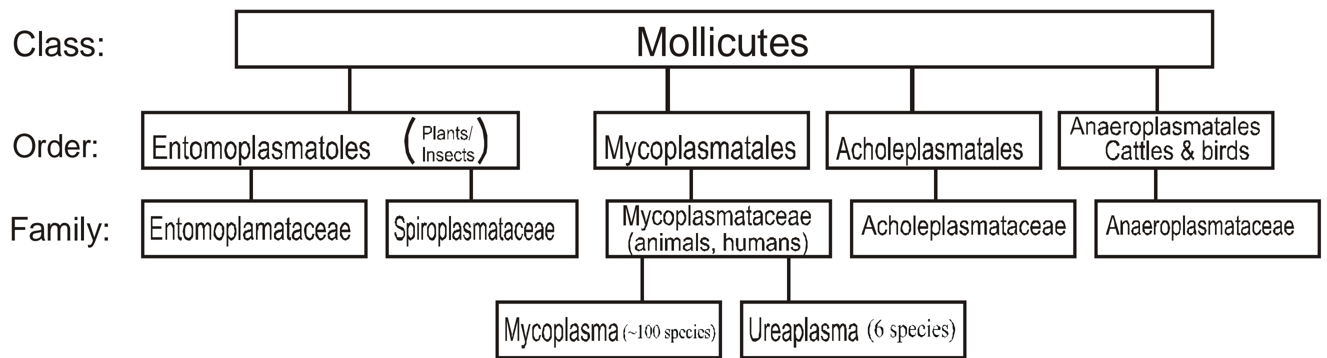

Figure 2.1: Mollicutes that have been detected in humans. (Courtesy Clinical Microbiology Laboratory, Suny Upstate Medical University, Syracuse New York).

Members of the class mollicutes are characterized by their small genomes consisting of a single circular chromosome containing 0.58 to 2.2 mbp, a low guanine – plus –cytosine (G+C) context (23 to 40 Mol%), and the permanent lack of a cell wall (Johansson and Petterson, 2002)

2.3 CELL BIOLOGY

Mycoplasma represents the smallest self, replicating organism, in both cellular dimensions and genome size that are capable of cell-free existence (Wilson and Collier, 1976). The genome of *M. pneumoniae* was completely sequenced in 1996 and shown to consist of 816, 394 bp with 687 genes (Himmelreich *et al.,* 1996). The small genome of *M. pneumoniae* and its limited biosynthetic capabilities are responsible for many of the biological characteristics and requirements for complex medium supplementation in order for this organism to be cultivated in –vitro.

Mycoplasmas are characterized by lack of a cell wall. *M. pneumoniae* cells are pleormorphic, with an attachment organelle of regular dimension at one pole and a trailing filament of variable length.

Another structural component of the *M. pneumoniae* cell that is important for extracellular survival is a protein network that provides a cytoskeleton to support the cell membrane. *M. pneumoniae* possesses very limited metabolic and biosynthetic activities for proteins, carbohydrate and lipids in comparison to conventional bacteria. Like other mollicutes, it scavenges for nucleic acid precursors and apparently does not synthesize purines or pyrimidines de novo (Dandekar, *et al*., 2002). *M. pneumoniae* reduces tetrazolium either aerobically or anaerobically and this has been one of several characteristics that have been used historically to identify the species and distinguish it from commensal mycoplasmas of the oropharynx. *M. pneumoniae* like other mollicutes has developed specialized reproductive cycles as a result of its adaptation to existence

with a limited genome and a parasitic life style that requires attachment to host cells (Dandekar *et al.,* 2002*)*. It reproduces 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 separations. (Baseman and Tully, 1997).

2.4 EPIDEMIOLOGY

Soon after the identification of *M. pneumoniae* as the etiologic agent of primary atypical pneumonia in early 1960s, considerable interest arose in elucidation and characterization of its incidence, prevalence, mode of spread and spectrum of disease. *M. pneumoniae* infection can involve both upper and the lower respiratory tracts and occur both endemically and epidemically world wide in children and adults. Climate and geography are not thought to be of major significance. Even though most available data concerning the occurrence of *M. pneumoniae* infections have come from studies performed in the United States, Japan and Europe, seroprevalence investigations using complement fixation technology in arctic and tropical zones have also indicated the presence of *M. pneumoniae* antibody, suggesting that populations in these region have had infections due to this organism (Subs and Feldman, 1966; Golubisnikov *et al*., 1975; Carrim *et al*., 2018).

Foy used CF antibody determinations and culture to show that *M. pneumoniae* was responsible for 15 to 20% of all cases of community acquired pneumonia or two cases per 1000 persons on annual basis between 1962 and 1975, in Seattle, Washington (Foy, 1993). Additional retrospective serological studies performed in Denmark showed a pattern of *M. pneumoniae* infections over a 50-year period from 1946 through 1995 with endemic disease transmission punctuated with cyclic epidemics every 3 to 5 years similar to what was observed in the United States (Foy, 1993; Lind *et al*., 1997). Additional

studies in North America and Europe performed over the past 3 decades have also reported similar trends (Dominquez *et al*., 1996; Jacobs *et al*., 1996; Hauksdottir *et al*., 1998).

Although the incidence of disease does not vary greatly by season, the proportion of patients with pneumonia due to *M. pneumoniae* is greatest during the summer in temperate climates due to the lower incidences of other respiratory pathogens at this time (Alexander *et al*., 1966; Marston *et al*., 1997; Bjarnason *et al*., 2018). Outbreaks of *M. pneumoniae* infections also tend to occur in summer of early fall (Feikin *et al.,* 1999; Talkington *et al*., 2001; Waller *et al*., 2014). The long incubation period and relatively low transmission rate have been implicated in the prolonged duration of epidemics of *M. pneumoniae* infections (Ferwerda *et al.,* 2001).

Layani – Milon *et al*., used data obtained by PCR assays on nasal swabs to define the incidence of *M. pneumoniae* and several respiratory viruses among persons with evidence of acute respiratory tract infection in a region of France over a 5-year period. The distributions of organisms varied from year to year, with *M. pneumoniae* ranking second to influenza A virus as the most frequent pathogen encountered during the surveillance period (Layani-milon *et al*., 1999).

M. pneumoniae can persist for variable periods in the respiratory tract following infections that has resolved clinically with appropriate antimicrobial therapy (Foy, 1993). The usual explanation for such persistence has been that the organism attaches strongly to and invades epithelial cells and that macrolides or tetracycline antibiotics commonly used for treating mycoplasma infections are bacteriostatic and unable to kill all the organisms. Surveilaince studies using culture and /or PCR indicate that a prolonged asymptomatic

carrier state may occur in some persons, providing a reservoir for spread of the organism to others (Gnarpe *et al*., 1992; Dorigo – Zetsma *et al.,* 2001).

Powerful molecular techniques such as PCR have extremely high sensitivity, being able to detect a single organism or a single copy of the targeted gene when purified DNA is used; this greatly exceeds the detection threshold of culture which is approximately 100 to 1000 cells under optimum condition (Buck *et al.,* 1992).

2.5 PATHOGENESIS OF DISEASE

Mycoplasmas are primarily mucosal pathogens, living a parasitic existence in close association with epithelia cells of the host, usually in the respiratory or urogenital tract. *M. pneumoniae* exclusively parasitizes humans, whereas some of the other human mycoplasmas have also been recovered from non human primates (Johansson and Petterson, 2002; Saraya *et al*., 2014).

After inhalation of respiratory aerosols, the *M. pneumoniae* attaches to host cell in the respiratory tract. The PI adhesion and other accessory proteins mediate attachments, followed by induction of ciliostasis, local inflammation that consists primarily of perivascular and peribronchial infiltration of mononuclear leukocytes, and tissue destruction that may be mediated by liberation of peroxides (Kannan *et al*., 2005; Xiao *et al*., 2014). *M. pneumoniae* possesses a complex terminal organelle that functions in cytadherence, gliding, motility and cell division (Krause and Belish, 2001). This can be discussed under the following headings.

I. Cytadherence

Evidence accumulated since the 1960s through animal models as well as in-vitro cell and organ culture systems indicates that attachment of *M. pneumoniae* to the respiratory epithelium is a prerequisite for later events that culminate in production of disease (Talkington *et al.,* 2001). This close interaction between the organism and host cells protects it from removal by the host's mucociliary clearance mechanism and allows it to produce a variety of local cytotoxic effect. Because *M. pneumoniae* is primarily an extracellular pathogen that depends on close association with host cells to survive, it has evolved a complex and specialized attachment organelle to facilitate its parasitic existence this attachement organelle consists of a specialized tip structure with a central core of a dense rod like central filament surrounded by a lucent space that is enveloped by an extension of the organism's cell membrane. The tip structures actually a network of Adhesins, interactive protein and adherence accessory proteins that cooperates structurally and functionally to mobilize and concentrate adhesions at the tip of the organism. The host cell ligand for mycoplasmal adhesions has not been characterized conclusively, although sialoglyco conjugates and sulfated glycolipids have been implicated (Krivan *et al.,* 1989). Recently data have shown that two proteins expressed on the *M. pneumoniae* cell surface, elongation factor (TU) and pyruvate dehydrogenase (EIβ), are also involved in binding the organism to fibronectin, a very common component of eukaryotic cell surfaces, basement membranes and the extracellular matrix (Dallo *et al*., 2001).

The P1 Adhesin is a 170-KDa protein concentrated in the attachment tip that is known to be the major structure responsible for interaction of *M. pneumoniae* with the host cells (Baseman *et al.,* 1996; Krause, 1998). Loss of P1 actively through spontaneous mutation or by trypsin treatment results in virulence by reduced adherence of mycoplasma to

eukaryotic cells (Baseman, 1993). Their structures produced by *M. pneumoniae* that have been studied as mediator in cytatherence in *M. pneumoniae* include proteins HMW1, HMW2, HMW3, HMW4, HMW5, P90, and P65, which in addition to P30, are believed to participate in establishment of the polar structure (Balish and Krause, 2002).

II. Motility

Balish and Krause recently suggested that P30 may be involved in gliding motility as well as coordination of cell division with biogenesis of the attachment organelle (Balish and Krause, 2002). *M. pneumoniae* exhibit gliding motility possible under chemotactic influence, which brings the organism to the base of the cilla where it is safe from ciliary clearance (Gillespie, 1994). Santos (2001) agreed that the prolonged paroxymal cough seen in this disease is thought to be due to inhibition of ciliary movement, since the organism has a filamentous end that allows it to slip between cilia within the respiratory epithelium.

After adhesion to the ciliated epithelium has occurred, ciliostasis develops. This phenomenon may be induced by secreted hydrogen peroxides which damages host membranes and interferes with superoxide dimutase and catalase.

III. Intracellular Localization

An intracellular existence that sequester *M. pneumoniae* could facilitate the establishment of latent or chronic states, circumvent mycoplasmacidal immune mechanism, facilitate its ability to cross mucosal barriers and gain access to internal tissues, and impair the efficacy of some drug therapies, accounting for difficulty in eradicating the mycoplasmas under clinical conditions, (Baseman, *et al.,* 1995; Rottem, 2002; Talkington *et al.,* 2002). Fussion of mycoplasma cell membrane with that of the host also result in release of various hydrolytic enzymes produced by the mycoplasma as well as insertion of

mycoplasmal membrane components into the host cells membrane, a process that potentially alters receptor recognition sites and affect cytokine induction and expression (Rottem, 2002).

IV. Cytotoxicity and Inflamation:

Close approximation of the *M. pneumoniae* to the host cells, facilitated by the adhesion protein appears to be important to facilitate localized tissue disruption and cytotoxicity. Hydrogen peroxide and superoxide radicals synthesized by the organism act in concert with endogenous toxic oxygen molecules generated by the host cells to induce oxidative stress in the respiratory epithelium (Tryon and Baseman, 1992). Hydrogen peroxide production in the organism occurs as a result of a flavin terminated electron transport chain and it is an important virulence factor (Tryon and Baseman, 1992). The Ultrastructural effects of peroxide on host cells such as erythrocytes includes loss of reduced glutathione, denaturation of haemoglobin, peroxidation of erythrocytes, lipids and eventually lysis of the cells (Somerson *et al.,* 1965).

Mammalian cells parasitized by *M. pneumoniae* exhibit a number of cytopathic effects that may occur as a result of the local damage mediated biochemically following cytadherence. *M. pneumoniae* infection leads to deterioration of cilia in the respiratory epithelium, both structurally and functionally cells may lose their cilia entirely, appear vacuolated and show a reduction in oxygen consumptions, glucose utilization, amino acid uptake, and macromolecular synthesis, ultimately resulting in exfoliation of all or parts of the infected cells. (Collier and Baseman, 1973; Clyde, 1979). These sub cellular events can be translated into some of the clinical manifestations of respiratory tract infection that are associated with this organism such as the persistent hacking cough that is so commonly associated with the organism. When *M. pneumonia*e reaches the lower

respiratory tract, the organism may be opsonized by complement or antibodies. Macrophages become activated, begin phagocytosis and undergo chemotactic migration to the site of infection. High percentages of neutrophils and lymphocytes are present in alveolar fluid. CD4+ T- lymphocytes, B- lymphocytes and plasma cells infiltrate the lung (Chan and Welsh, 1995; Onitz *et al.,* 1996) manifested radiologically as pulmonary infiltrates. Further amplification of the immune response in association with lymphocyte proliferation, production of immunoglobulins, and release of tumor necrosis factor alpha (TNF- α), gamma interferon (IFN- α) and various interleukins (including interleukin – Iβ [IL – Iβ], IL – 2, IL – 4, IL-5, IL-6, IL-8, IL-10, and IL-18) occurs, based on evidence from Clinical and in-vitro studies and from animal models (Lieberman *et al.,* 1997; Narita *et al*., 2000; Hsieh *et al*., 2001 and Hoek *et al*., 2002).

V. Immune Invasion.

Once *M. pneumoniae* reaches the lower respirator tract, the organism may be opsonized by complement or antibodies. Opsonized *M. pneumoniae* is readily killed by macrophages and by the activity of the complement system. The organisms are able to overcome these problems as *M. pneumoniae* can successfully be isolated from patients after recovery or treatment of infection (Gillespie, 1994). There are significant similarites between mycoplasma and host membrane glycerol phospholipids and this may inhibit host response. In addition, albumin is adsorbed by the mycoplasma cell membrane. The effect of this is that alveolar macrophages have been shown to be unable to phagocytose *M. pneumoniae* in-vitro experiments (Gillespie, 1994).

VI. Asthma and Chronic Lung Conditions

The release of proinflammatory cytokines in association with *M. pneumoniae* infection has also been implicated as a possible mechanism leading to or exacerbating underlying chronic pulmonary diseases such as bronchial asthma. Multiple lines of evidence suggest

why *M. pneumoniae* may play a role in the pathogenesis of asthma beyond simple, acute exacerbation (Gil *et al*., 1993; Kraft *et al.,* 1998; Freymuth *et al*., 1999). The organism can be detected by polymerase chain reaction (PCR) and/or culture more often from the airways of patients with chronic, stable asthma than from matched control patients. Kraft *et al*., 1998, detected *M. pneumoniae* by PCR in respiratory secretions of 10 of 18 stable adult asthmatics (56%) and in only 1 of 11 healthy controls. In another study, throat cultures for *M. pneumoniae* were positive in 24.7% of children and adults with asthma exacerbation, compared with 5.7% of healthy controls (Gilet *et al*., 1993) Mycoplasma has been detected by PCR in airways even when cultures and serological results are negative, suggesting that low numbers of organisms may evade detection by the immune system (Kraft *et al.,* 2002).

Lung abnormalities, including reduced pulmonary clearance and airway hyper responsiveness, may persist for weeks to months after an infection with *M. pneumoniae* (Kim *et al.,* 2000; Marc *et al.,* 2000). Marc *et al*., 2000, reported abnormalities in pulmonary function tests in up to 50% of children and Kim *et al*., 2000, described abnormal computerized axial tomography studies for 37% of children months to years after an episode of *M. pneumoniae* respiratory tract infection, thus establishing the ability of mycoplasma to induce chronic and possibly permanent lung damage long after resolution of respiratory tract symptoms. *M. pneumoniae* is known to induce a number of the inflammatory mediators implicated in the pathogenesis of asthma that may play a role in exacerbations which often include wheezing (Esposito *et al.,* 2002). The organism triggers the wheezing process by means of IL – 5 secretions in persons who are genetically predisposed or are otherwise susceptible. Furthermore, the organism is associated with significantly greater numbers of mast cells in patients with chronic asthmas (Martin *et al.,* 2001). Lieberman *et al*., 2001, used serology to assess the

presence of *M. pneumoniae* evaluating a group of 219 patients hospitalized with acute exacerbations of chronic obstructive pulmonary disease (COPD), a total of 34 patients (14.2%) had serological evidence of acute *M. pneumoniae* infection, making it the third most common bacterial pathogen detected (Lieberman *et al.,* 2001). *M. pneumoniae* also cause acute exacerbation in patients with cystic fibrosis (Peterson *et al.,* 1981; Kumar *et al*., 2018).

VII. Immune Response and Inflammatory Effects

M. pneumoniae possesses both protein and glycolipid antigens that elicit antibody responses in infected individuals. The P1 protein is the target of many antibodies that are produced by the host in response to the *M. pneumoniae* infection and it has also served as a target for development of serological assays. Following an initial infection, the normal immune system responds by rapidly producing antibodies that peak after 3 to 6 weeks followed by a gradual decline over months to years. Elevation of *M. pneumoniae* specific IgM alone can often be interpreted as evidence of acute infection, since this antibody typically appear within 1 week of initial infection and approximately 2 weeks of initial infections and approximately 2 weeks before IgG antibody (Sillis, 1990; Matas *et .,al* 1998).

However, the presence of IgM is considered most significant in paediatric populations, where there have been fewer opportunities for repeated exposures. Adults who have been infected repeatedly over a period of years may not respond to mycoplasma antigens with a brisk IgM response (Waites *et al.,* 2001). IgA, while often overlooked as a diagnostic antibody class, may actually be a better indicator of recent infections in all age groups (Sillis, 1990).

Mycoplasma adhesions also exhibit amino acid sequence homologies with human CD4 and class II major histocompatibility complex lymphocyte proteins, which could generate auto reactive antibodies and trigger cell killing and immunosuppression (Boot-Bernstein and Hobbs, 1991). Circulating immune complexes also occur during acute phases of *M. pneumoniae* diseases (Clyde, 1979).

Specific T- cell- mediated immunity is also involved in the host reaction to infection by *M. pneumoniae*. Lymphocytes from persons known to have had a prior mycoplasma infection will undergo blast transformation when cultured in the presence of *M. pneumoniae* (Fernald, 1972) Leukocytes from individuals with *M. pneumoniae* infections will show evidence of chemotaxis in the presence of the organism and these individuals will respond with IFN- α in their blood (Martin *et al*., 1973); Nakayama *et al*., 1986). A property of many species of mycoplasmas that affects the immune responsiveness of the host is their prospensity for mitogenic stimulation of B and T Lymphocytes, thereby inducing autoimmune disease through the activation of anti-self T cells or Polyclonal B Lymphocytes (Talkington *et al*., 2001). This property is associated with the ability of *M. pneumoniae* to stimulate production of cytokines in the initiation of the acute inflammation response.

VIII. Antigenic Variation

Many Mycoplasma species that infect animals or humans are known for their ability to induce chronic disease states in which clearing of the organism is extremely difficult. Therefore, these organisms must have evolved means by which they can successfully evade or modulate the host immune response. Intracellular localization and immunomodulatory activities are possible means to this end. Another mechanism that has been extensively studied in many other bacterial is variation in surface antigens. Recombinational events among the repetitive elements themselves and with regions of the three-gene P1 adhesin operon promote diversity and altered specificites and affinities and

maximize the coding potential of the limited mycoplasma genome (Baseman and Tully, 1997: Yogey *et al*., 2002).

2.6.0 DISEASE TRANSMISSION

M. pneumoniae can be transmitted through aerosols from person to person, and disease has been produced experimentally by aerosol inoculation (Clyde, 1979). Persons with active mycoplasma infection will carry the organism in the nose, throat, trachea and sputum, indicating diffuse involvement. Spread of organism is greatly facilitated by the ubiquitous cough. Since the organisms tend to be associated with desquamated cells, relatively large droplets may be required for transmission, as evidenced by the close personal contact typical of outbreak settings example, schools, military barracks and institutions. *M. pneumoniae* infections commonly spread gradually among family members within a household (Dorigo-Zetsma *et al.,* 2001; Ferwerda *et al,* 2001; Talkington *et al*., 2001). In view of the intimate contact needed for droplet transmission and the slow (6 hours) generation time of *M. pneumoniae*, 1 to 3 weeks of incubation for each case is typical and several cycles may be necessary before intrafamily transmission is complete. Some studies have reported incubation periods from common-source outbreaks of as short as 4 days (Sande *et al*., 1975) whereas others have reported longer incubations with a median of 23 days with intrafamilial spread, where smaller inocula may be involved and transmission may be less effective until index case has exhibited symptoms for several days (Foy, 1993). Foy *et al*., reported that 39% of family contacts may eventually become infected with the organism, many asymptomatically (Foy *et al.,* 1996). Dorigo-Zetmal and co-workers found that among 79 asymptomatic household contacts of 30 index cases with acute respiratory tract infection due to *M. pneumoniae* 15% harbored the organism, with a significantly greater number being children under 15 years of age (Dorigo-Zetmal *et al*., 2001).

2.6.1. DISEASE OUT BREAKS

Numerous out breaks of *M. pneumoniae* infections have been documented in the community or in closed or semi closed settings such as military bases (Gray *et al*., 1997; Feikin *et al*., 1999; Gray *et al*., 1999), hospitals (Kleemola and Jokine, 1992), religious communities (Muldoon *et al*., 1982; Leibowitz *et al*., 1988) and facilities for the mentally or developmentally disabled (Klausner *et al.,* 1998; Hyde *et al*., 2001). In outbreaks where many more persons usually living close together in military barracks or similar situations, are exposed to *M. pneumoniae* aerosols simultaneously, the rate of spread within a facility appears to be higher than in single-family households. Attack rates of *M. pneumoniae* among military recruits and other closed or semi closed populations can be quite high with reports ranging from 25 to 71% in some settings (Klausner *et al.,* 1998; Feikin *et al*., 1999; Waller *et al*., 2014). Some studies have shown *M. pneumoniae* to be the leading cause of bacterial pneumonia among hospital and non hospitalized military personnel (Gray *et al.,* 1994; Gray *et al.,* 1997). Although long-term morbidity is uncommon, these out breaks can be very disruptive and can consume significant resources. Strategies to control these out breaks have included cohorting and use of antibiotics for symptomatic persons and for prophylaxis.

2.6.2 DEMOGRAPHICS AND SPECTRUM OF DISEASE.

Serological studies performed in the 1960s and 1970s, evaluating the attack rates of *M. pneumoniae* according to sex and broken down into various age groups have yielded mixed results, with slight gender differences apparent between some age groups. Overall, there appears to be little reason to suspect that males and females have greatly differing susceptibilities to *M. pneumoniae* infection (Foy *et al*., 1966; Noah, 1974; Markham, 1979).

Mycoplasma pneumoniae caused up to 40% or more of cases of community- acquired pneumonias and as many as 18% of cases requiring hospitalization in children (Block *et al.*, 1995; Gendrel, 1997; Harris *et al.*, 1998 and Ferwerda *et al.,* 2001). Older studies relying upon serology and culture reported *M. pneumoniae* to be somewhat uncommon in children aged less than 5 years and greatest among school aged children 5 to 15 years of age with a decline after adolescence and on into adulthood (Alexander *et al.,* 1966, Foy *et al.*, 1966, Foy *et al.*, 1993). However, *M. pneumoniae* may occur endemically and occasional epidemically in older individual as well as in children under 5 years of age (Leven *et al.,* 1996; Harris *et al.,* 1998).

Whereas pneumonia may be the most severe type of *M. pneumoniae* infection, the most typical syndrome, especially in children is of tracheo-bronchitis, often accompanied by a variety of upper respiratory tract manifestations. Esposito *et al.,* demonstrated acute *M. pneumoniae* infection in 44 of 184 children with non streptococcal pharyngitis (23%) using the criteria of an elevated IgM antibody titer or a fourfold increase in IgG antibody titer and / or a positive PCR assay on the nasopharyngeal aspirate (Esposito *et al.,* 2002). Although most mycoplasma infections occur among out patients (hence the colloquial term “walking pneumonia”), *M. pneumoniae* is a significant cause of bacterial pneumonia in adults requiring hospitalization in the United States. Marston *et al.,* reported that the organism was definitely responsible for 5.4% and possible responsible for 32.5% of 2, 776, cases of community acquired pneumonia in hospitalized adults in a two county region of Ohio, using CF antibody determinations for detection (Marston *et al.,* 1997). Extrapolation of these data nationally provides an estimated 18, 700 to 108,000 cases of pneumonia in hospital adults due to *M. pneumoniae* annually in the United States. Since the majority of patients with pneumonia in the United States are treated as out patients, the total number of pneumonias due to *M. pneumoniae* is almost certainly many times

greater and as many as half of all infections in adults may even be asymptomatic. An additional striking finding of the study by Marson *et al.,* 1997, was their observation that the incidence of pneumonias due to *M. pneumoniae* in hospitalized adults increased with age, and it was second only to *Streptococcus Pneumoniae* (*S. pneumoniae*) in elderly persons.

Another study of hospitalized adults with community acquired pneumonias performed in Israel which used commercial serological kits to detect antibodies, showed *M. pneumoniae* to be second only to *S. pneumoniae* and it was responsible for 29.2% of pneumonias over all (Porath *et al*., 1997).

In a study Nigeria, on *Mycoplasma pneumoniae* and the aetiology of lobar pneumonia in Zaria, Northern Nigeria, 12 patients out of 74 suffering from lobar pneumonia were discovered to have *M. pneumoniae* as the causative agent of their disease (MacFarlane *et al.,* 1979). Pneumonia accounted for approximately one– fifth (19%) of 2 million deaths, with 90% of these occurring in developing world with Nigeria inclusive (WHO, 2014)

A study in Kenya on etiology and incidence of viral and bacterial acute respiratory illness among older children and adults, *M. pneumoniae* was detected in 0.7% of patients (Daniel *et al*., 2010). In another study in Kenya on factors associated with increase risk of progression to respiratory syncytial virus (RVS) associated with pneumonia in young Kenyan children, *M. pneumoniae* was also detected as one of the aetiologic agent isolated (Okoro *et al*., 2008).

2.7 CLINICAL SYNDROMES

I. Respiratory Tract Infections

The clinical entity of pneumonia eventually proven to be caused by *M. pneumoniae* was recognized many years before the actual identity and nature of the etiological agent were established. The first clues to differentiate pneumonia eventually proven to be due to mycoplasma from classical pneumococcal pneumonia came from the observations that some cases failed to respond to treatment with sulfonamides or penicillin. The lack of response to antimicrobial therapy was deemed "atypical", and the condition was though likely to be a primary form of lung disease of uncertain etiology, hence the term "primary atypical pneumonia" was coined. This term, along with "walking pneumonia", has been used widely by physicians and the lay public to denote mycoplasmal respiratory disease.

M. pneumoniae infections may be manifested in the upper respiratory tract, the lower respiratory tract or both. The frequency of non-specific upper respiratory tract infection manifestations has varied among numerous studies published since mid-1960s, with some reports indicating that as many as 50% patients with *M. pneumoniae* infection present with upper respiratory tract illness, (Feizi *et al*., 1967). Symptomatic disease typically develops gradually over a period of several days, often persisting for weeks to months. The most common manifestations include sore throat, hoarseness, fever, cough which is initially non productive but later may yield small to moderate amounts of non bloody sputum, headache, chills, coryza, myalgias, ear ache and general malaise (Luby, 1991; Ferwerda *et al*., 2001; Talkington *et al*., 2001). Dyspnea may be evident in more severe cases and the cough may take on a pertussis-like character, causing patients to complain

of chest soreness from protracted coughing (Clyde, 1979; Dumke *et al*., 2015). Inflammation of the throat may be present, especially in children with or without cervical adenopathy and conjunctivitis and myringitis sometimes occur (Esposito *et al*., 2002). Children under 5 years of age are more likely to develop bronchopneumonia, involving one or more lobes, sometimes requiring hospitalization (Luby, 1991; Ferwerda *et al.,* 2001; Izadneghdar *et al*., 2013). Mild infections and asymptomatic conditions are particularly common in adults and bronchopneumonia involving one or more lobes develops in 3 to 10% of infected persons (Cassell *et al*., 1985; Kashyap and Sarkar, 2010). As mentioned above, *M. pneumoniae* is an important cause of pneumonia sufficiently severe to require hospitalization especially in elderly ones (Marston *et al.,* 1997). Chest auscultation may show scattered or localized rhonchi and expiratory wheezes. Since alveoli are usually spared, Rale and Frank consolidation are firmly uncommon unless atlelectasis is widespread in complicated cases; the acute febrile period lasts about a week, while the cough and lassitude may persist for 2 weeks or even longer. The duration of symptoms and signs will generally be shorter if antimicrobial treatment is initiated early in the course of illness (Clyde, 1979). Children with functional asplenia and immune systems impairment due to sickle cell disease, other conditions such as Down syndrome, and various immunosuppressive states are at risk of developing more fulminant pneumonia due to *M. pneumoniae* (Ferwerda *et al.,* 2001; Talkington *et al*., 2001).

Children with hypogammaglobulinemia are also known to be at greater risk for development of respiratory and joint infections due to the organism demonstrating the importance of functional humoral immunity in protection against infections due to this organism (Ferwerda *et al*., 2001; Talkington *et al.,* 2001). *M.*

pneumoniae was isolated from the joint of a patient with arthritis and from six patients with chronic lung disease. There are few case reports of *M. pneumoniae* infections in pediatric AIDS patients (Jensen *et al.,* 1994) but is not known whether the incidence or severity of pulmonary or extrapulmonary *M. pneumoniae* infections in AIDS patients is increased significantly or how any immunosuppresed state specifically affects, host resistance to *M. pneumoniae* infection. Fulminant infections with multiple organ involvement and deaths due to *M. pneumoniae* usually in otherwise healthy adults and children have been reported but are uncommon (Scully *et al.,* 1992; Leven *et al.,* 1998; Dayboeck *et al.,* 2002; Liu *et al*., 2015).

II. Extrapulmonary Manifestations

As many as 25% of people infected with *M. pneumoniae* may experience extrapulmonary complications at variable time periods after onset of or even in the absence of respiratory illness, autoimmune reactions have been suggested to be responsible for many of the extrapulmonary complications associated with mycoplasmal infection (Talkington *et al.,* 2001). However, the availability of PCR has greatly enhanced understanding of how the organism can disseminate through the body. The presence of *M. pneumoniae* in extrapulmonary sites such as blood, synovial fluid and cerebrospinal fluid, pericardial fluid and skin lesions has been documented by PCR, so direct invasion must always be considered (Said *et al.,* 1999; Bar-meir *et al.,* 2000).

Central nervous system (CNS) complications are recognized as among the most common of extrapulmonary manifestation of *M. pneumoniae* infection (Smith and Eviatar, 2000). Approximately 6 to 7 of hospitalized patients with serologically

confirmed cases of *M. pneumoniae* pneumonia may experience neurological complications of varying severity (Koskiniem, 1993). Such complications have included encephalitis, cerebella syndrome and polyradiculitis, cranial nerve palsies, aseptic meningitis or meningoencephalitis, coma, optic neuritis, diplopia, mental confusion and acute psychosis secondary to encephalitis (Decaux, 1980; Gilberg, 1980; Yimenicioglu *et al*., 2014). Encephalitis has been the most common neurological manifestation in children (Koskiniemi, 1993). Antibodies against galactocerebroside, a component of CNS myelin, have been detected in 100% of patients with *M. pneumoniae* and CNS involvement and in only 25% of those without CNS involvement (Nishimura *et al.,* 1996). Post infectious leukoencephalopathy due to *M. pneumoniae* also suggests a role for autoimmunity in some cases (Pfausler *et al*., 2002) Rautonen *et al*., reported that children *M. pneumoniae* were seven times more likely to die or have severe neurological sequelae than other children, second only to cases of herpes simplex virus infection (Rautonen *et al*., 1991).

Whereas neurological disorders may be the most extrapulmonary manifestations of *M. pneumoniae* infections, dermatological disorders, including erythematous maculopapular and vesicular rashes, are perhaps the most common clinically significant complications, occurring in up to 25% of patients. Although the disorders are usually self-limited, severe forms of Stevens – Johnson syndrome, conjunctivitis, ulcerative stomatitis and bullous exanthems have been reported and the organism has bee detected directly in the cutaneous lesions (MacFarlane, 1980, Cherry, 1993).

Non specific myalgias, arthralgias and polyathropathies occur in approximately 14% of patients with acute *M. pneumoniae* infection and may sometimes persist

for long periods (Ali *et al.,* 1986). The organism has also been reported to cause osteomyelitis in a splenectomized patient with hypogammaglobulinemia (La Scola *et al.,* 1997). Cardiac complication associated with *M. pneumoniae* are relatively uncommon but involvement has been reported at rates of from 1 to 8.5% in persons with serological evidence of infection, somewhat more commonly in adults than in children (Mesequer *et al*., 1996). Pericarditis, myocarditis and pericardial effusion with and without cardiac tamponade have been described and the organism has been detected in pericardial fluid (Karialainen, 1990; Smith and Eviatar, 2000; Szymanski *et al.,* 2002). Haemolytic anemia is recognized as a rare but severe complication of mycoplasmal pneumonia, occuring more often in children than in adults (Simonian and Lanner, 1998) the mechanism by which the organism causes this complication has been attributed to cross-reacting cold agglutinins (Simonian and Lanner, 1998; Talkington *et al*., 2001). Two cases of aplastic anaemia associated with *M. pneumoniae* have also been reported (Stephan *et al*., 1999).

Acute glomerulonephritis, renal failure, tubulointerstitial nephritis and IgA nephropathy as well as other conditions have been sporadically reported in association, with *M. pneumoniae* infections (Kanayama *et al.,* 1982; Koletsky and Weinstein, 1980). Up to one third of patients with *M. pneumoniae* infection may have nonspecific ear symptoms, including otitis externa, otitis media and myringitis (Murray *et al*., 1975; Steven *et al*., 1978).

Acute Rhabdomyolysis was recently reported in association with *M. pneumoniae* infection in a 15 - years – old patient (Berger and Wadowksy, 2000). Ocular manifestations have been reported in children occasionally and include conjunctivitis, and retinal hemorrhages iritis and optic disk swelling with or without permanent degradation of vision (Salzma*, et al*., 1992; Milla *et al.* 1998).

M. pneumoniae has been isolated from the urogenital tracts of males and females and have been cultured from a tubo-ovarian abscess (Goulet *et al.,* 1995) .Given the apparent ability of the organism to invade the blood stream; infections in almost any organ system are possible.

2.8.0 DIAGNOSIS

2.8.1 General Laboratory Features.

Clinical laboratory findings are seldom diagnostic for *Mycoplasma pneumoniae* infection. About one third of persons with lower respiratory tract infections may have leukocytosis (Stevens *et al*., 1978; Waites, 2011). An elevated erythrocyte sedimentation rate may also be observed (Riberfield *et al*., 1965). Gram Staining of sputum may show monocular cells or neutrophils and normal flora. There are no hepatic or renal abnormalities typical of *M. pneumoniae* infection, although the hemolytic anemia that develops in some patients may be reflected in the hemogram. Prior to widespread availability of commercialized antibody assays, and even before the precise etiological bacteriological characterization of the etiological agent was known, clinicians sometimes used the presence of cold agglutinin to confirm their clinical suspicious of primary atypical pneumonia, which was also known as cold-agglutinin disease. Cold agglutinins are actually IgM antibodies in about out 50% of *M. pneumoniae* infections and may persist for several weeks. One theory is that cold agglutinins are the result of cross reactive autoantibodies developed against the antigen of human erythrocytes during acute mycoplasmal infection. Another theory is that they develop directly as a result of antigenic alteration of the erythrocytes caused by *M. pneumoniae*. The latter theory is supported by the facts that *M. pneumoniae* is known to interact with and adsorb to human erythrocytes and to produce haemolysin and because cold agglutinins can be induced in rabbits inoculated with human

erythrocytes that were preincubated with *M. pneumoniae* but not in rabbits inoculated with either *M. pneumoniae* or erythrocytes alone (Barile, 1979).

To varieties of cold agglutinin determination have been used. The simplest version of the test was what is referred to a “bedside cold agglutinins’’. This consists of collecting blood from a patients suspected of having mycoplasma pneumonia into a tube containing citrate or another suitable anticoagulant and placing it in ice water in freezer for approximately 30s. The tube is then examined for coarse agglutination by being tilted on its side. On warming, the agglutination should resolve, but it can be reproduced by repeating the cooling procedure of more precise test to determine the cold agglutinin titer by reacting doubling dilutions of patient sera suspected to contain the cold agglutinins with human type O erythrocytes in the cold and determining the highest dilution at which agglutination occurs. The cold agglutinin response often correlates directly with the severity of pulmonary involvement, patients with extensive lobar involvement will usually have a titter of ≥ 1:32, whereas those with one minimal illness may not develop could agglutinins. Due to the availability of antibody assays that are based on immunological reagents specific for *M. pneumoniae*, the popularity of testing serum for the presence of cold agglutinins has declined, If used, this test should be limited to persons in whom mycoplasmal pneumonia is a strong possibility. Under these circumstances, a positive test provides a reasonable supportive evidence of a mycoplasmal etiology, on the basis of which clinical management decisions can be made.

2.8.2 RADIOGRAPHIC FINDINGS

Primary atypical pneumonia due to *M. pneumoniae* can be extremely variable and mimic a wide variety of lung diseases. *M. pneumoniae* causes interstitial mononuclear inflammation in the lungs that may be manifested radiographically as diffuse reticular

infiltrates of bronchopneumonia in the perihilar regions or lower lobes, usually with a unilateral distribution and hilar adenopathy. Bilateral involvement may occur in about 20% of cases (Ferwerda, 2001). However, lobar consolidation with bilateral alveolar involvement has bee described and the degree of consolidation may exceed what would be expected based on the severity of clinical manifestation (Decang *et al*., 1965). Among patients with *M. pneumoniae* pneumonia who require hospitalization, up to 10.9% require mechanical ventilation (Marrie, 1993).

2.8.3 PATHOLOGICAL FINDINGS

Histopathological examinations from fatal case of *M. pneumoniae* pneumonia, biopsy material, examination of tissues from animal models, the tracheal organ cultures have shown lesions of the epithelium, lining of the mucosal surfaces with ulceration and destruction of ciliated epithelium of bronchi and bronchiole. Edema of bronchial and bronchiolar walls; bronchiolar and alveolar infiltrate of macrophages, lymphocytes, neutrophils, plasma cells, fibrin and brochiolities obliterans have been described (Cimolai *et al*., 1992; Chan and Welsh, 1995; Libya *et al.,* 1997; Dayboeck *et al.,* 2002). Type II pneumocyte hyperplasia and diffuse alveolar damage have also been reported. Pleura may contain patches of fibrinous exudates. Pleural effusions and diffuse alveolar damage sometimes occur in association with more severe cases and long-term sequelae such as pleural scarring, bronchioctasis, and pulmonary fibrosis have been reported(Scully *et al,* 1992; Radisic *et al.,* 2000). Lung abscesses may also occur (Siegler, 1973). Recent studies utilizes animal model developed to investigate potential role of *M. pneumoniae* in chronic lung conditions such as asthma have also provided insights into the histopathological aspects of *M. pneumoniae* lung disease(Hardy *et al.,* 2001).

2.8.4 CULTURE

Culture of *M. pneumoniae* from the respiratory tract and other body sites is laborious and expensive requiring serial blind passage, specialized and expensive growth media and incubation periods of up to several weeks. Compared to Molecular techniques such as PCR, its analytical sensitivity may be no more than 60% in experienced laboratory even when rigorous adherence to procedures known to enhance cultural isolation are used (Leven *et al.,* 1996; Ratliff *et al.*, 2014). However, when positive, culture has the advantage of being 100% specific, provided that appropriate additional procedures are used to identify the organism isolated to specie level. Due to the organism sensitivity to adverse environmental conditions, proper specimen collection storage, and transport are critical for maintaining viability for culture processing and DNA extraction. Specimens needed for diagnosis of respiratory mycoplasmal infections include: Broncho alveolar lavage (BAL) sputum, plural fluid, nasopharyngeal swab, throat swab, endotracheal aspirates (ETA) and lung biopsies. When extrapulmonary disease is suspected specimens such as blood, cerebrospinal fluid, pericardial fluid and synovial fluid should be collected.

Isolation of *M. pneumoniae* from essentially any type of clinical specimen from the respiratory tract as well as from other body fluids and tissues that are suitable for cultivation of conventional bacteria is possible. SP4 medium developed over 20 years ago by Tully *et al.,* (Tully *et al.,* 1979), and pleuropneumonia-like organism medium (PPLO Medium) are successful and widely used broth and agar medium for cultivating *M. pneumoniae.* Detection of *M. pneumoniae* by culture is predicated on its characteristic hydrolysis of glucose with a resultant acidic shift after 4 days or more of incubation in broths containing phenol red PH indicator. Addition of a PH indicator such as phenol red is important for detection because mycoplasma usually do not produce turbidity in broth culture owing to their small size, (Waites and Taylor-Robinson, 2007).

Growth of mycoplasma pathogenic to humans requires the presence of serum, growth factors such yeast extract and metabolic substrates. Penicillin G or broad spectrum semi-synthetic penicillin should be added to minimize bacterial overgrowth. Initial specimens, as well as broths with colour change and blind subcultures should be transferred to SP4 agar or PPLO Agar, incubated and examined under a stereomicroscope at regular intervals to look for development of spherical colonies of up to 100 µm in diameter. According to Agbakoba *et al*., the specimen could be inoculated mycoplasma broth and incubated at 37°c up to 3 days. After incubation, subculture is made from mycoplama broth onto mycoplasma agar. The agar plate then incubated under increase carbon dioxide atmosphere at 37°c up to 10 days. The plate is then examined daily with the use of dissecting microscope for the presence of "fried egg" colonies, which if present, indicate suspected mycoplasma specie (Agbakoba *et al*., 2007).Incubation of *M. pneumoniae* broth culture under atmospheric conditions at 37°c is satisfactory but agar plates will yield the best colonial growth if 5% CO2 is provided and plates sealed to prevent loss of moisture during prolonged incubations.

Although the common commensal mycoplamal species from the Oropharynx have different metabolic properties, growth rates and requirements that distinguish them from *M. pneumoniae*, it is necessary to perform additional tests to conclusively prove the identification of a mycoplasma from the respiratory tract or another body sites as *Mycoplasma pneumoniae*. Such procedures for species identification include:

i. **Haemolysis Test:**

Isolates of mycoplasmas are inoculated onto standard medium to give well disperse colonies and incubated until colonies are well grown (5-8 days). The plate is then overlayed with a thin layer of saline-agar containing 1 percent v/v sheep or guinea pig erythrocytes and re-incubated aerobically overnight. *M.*

pneumoniae produces a maximum clearing resembling β- haemolysis. T-strains also produce β-haemolysis of guinea pig erythrocytes but test conditions are critical.

Other mycoplasmas may produce a greenish clearing of the overlay. Haemolysis depends on production of hydrogen peroxide.

ii. **Haemadsorption:**

This may be tested by flooding the culture plate or a block excised from it with a 1 percent v/v suspension of sheep erythrocytes in saline and leaving them in contact for 30 min. The erythrocyte suspension is then aspirated and the colonies gently washed with saline and then inspected under the microscope. Positive colonies are seen to be plastered with erythrocytes.

iii. **Fermentation of Carbohydrate:**

A 1 percent w/v concentration of the carbohydrates under test is incorporated into agar slope or in semisolid or fluid preparation of the standard medium together with phenol red (0.002 percent w/v). The range of sugars fermented is limited and is not of great differential important. For most purposes, it is sufficient to test for acid production from glucose.

iv. **Tetrazolium Reduction:**

Plates of standard mycoplasma agar are prepared with addition the addition of 2.0ml of 1 percent w/v stock solution of 2-3-5 triphenyltetrazolium chloride per 100ml of medium. The stock solution is sterilized in the autoclave. A block of agar containing numerous colonies is place colony-side down, on the tetrazolium plate and the plate is re-incubated aerobically. In 3-6 days the colony containing block becomes pink in colour if *Mycoplasma pneumoniae* is present. Other procedures for species identification include agar growth inhibition with homologous antisera, epi-immunofluorescence or immunoperoxidase staining,

immunoblotting with monoclonal antibodies, metabolism inhibition tests and PCR assays. Unfortunately, none of these methods has been adapted for development of a commercial product that can be easily purchased and used in a diagnostic laboratory. Haemadsorption assays and tetrazolium reduction can theoretically be accomplished in most clinical laboratories that do not have capabilities for molecular diagnostic testing. However, the cost, the technical expertise required and the very limited availability of the reagents for the immunologically based methods of species identification effectively preclude their use outside specialized reference or research laboratories.

2.8.5 ANTIGEN DETECTION TECHNIQUES.

Rapid assays for direct antigenic detection of *M. pneumoniae* in respiratory tract specimen have included direct immunofluorescence, counter immunoelectrophoresis; immunoblotting and antigen capture enzyme immunoassay (Hirschberg and Holme, 1991; Hirai *et al.,* 1991; Gerstenecker and Jacob, 1993). The utility and general acceptance of these technique have been reduced by low sensitivity and cross-reactivity with other mycoplasmas found in sputum specimens from detection techniques maybe able to detect about 10^3 to 10^4 CFU/100µl of specimen, non amplified antigen detection assay are at the limit of sensitivity for detection and are not recommended for diagnostic purposes when superior technology utilizing nucleic acid amplification is possible.

2.8.6 DNA PROBES

DNA hybridization techniques for the diagnosis of *M. pneumoniae* infection were developed in the early 1980s and had about the same diagnostic sensitivity as antigen detection techniques. These methods have been reviewed by Razin (Razin, 1994). The 16s rRNA genes have been widely used as targets, as have probes consisting of rDNA.

Genprobe previously sold a ^{125}1-labeled DNA probe for an rRNA sequence specific for *M. pneumoniae* but the more recently available amplification techniques, such as the PCR assay that do not involve radioisotopes and have greater sensitivity led to its discontinuation.

2.8.7 PCR

Development of molecular base testing such as the PCR assay has lessened the importance of culture as means of detecting *M. pneumoniae* Studies since 1980s using simulated clinical specimens, animal models, and later clinical trials have validated the ability of PCR to detect *M. pneumoniae* often in conjunction with serology and / or (Buck *et al*., 1992; de-Barbeyrac *et al*., 1993; Blackmore *et al*., 1995; Block *et al.;* 1995). The same types of clinical specimens that can undergo culture can also be tested by PCR. The use of two difference targets can maximize the ability to detect the organism. The conventional PCR procedure currently used at the Centers for Disease Control and Prevention has been adopted from the procedure originally described by Bernet *et al*., using primers derived from the *M. pneumoniae* ATPase gene (Bernet *et al*., 1989). They have also developed a real- time PCR which targets this gene. Other sequences primarily the P1 adhension and conserved regions of 16s rRNA have also been utilized (de-Barbeyrac *et al*., 1993; Luneberg. *et al.;* 1993; Kong *et al.;* 2000). Among the other advantages of PCR are that it can be used to detect mycoplasma in tissue that has already been processed for historical or in cultures that are contaminated where culture is impossible, it requires only one specimen, it can be completed in 1 day, it maybe positive earlier in infection than serology and it does not require viable organism (Williamson *et al.,* 1994., Talkington *et al.;* 1998; Waites *et al.,* 2001). Specific advantages of RNA – based amplification techniques are the high sensitivity that can be achieved due to the

large number of rRNA copies per mycoplasmal cell and the fact that its detection is more indicative of viable mycoplasmas in a clinical sample (Dayboeck *et al.;* 2003).

It is difficult to compare results of one study utilizing PCR for epidemiological or diagnostic purposes directly with another because of the varied specimen types, DNA extraction and amplification techniques, primer selection and reference standards used for comparison. Most techniques are basically similar, but they may differ in targeted sequences and Primers. However, comparison of the PCR technique with culture and / or serology has yielded varied results and large-scale experience with this procedure is still limited for *M. pneumoniae*

In view of enhanced analytical sensitivity of the PCR assay over culture, a positive PCR result and negative culture can be easily explained. However, in a case with a negative PCR assay and a positive culture (or serology), the presence of inhibitors or some other technical problem with the PCR assay must be considered (Reznikov *et al.,* 1995; Leven *et al.,* 1996; Whistler *et al*., 2017). Dorigo – Zetsmal *et al*., performed a comprehensive examination with 18 patients with *M. pneumoniae* respiratory tract infection detection by PCR or serology and showed that sputum was the specimen that was most likely to be PCR positive (62.5%, versus 41% for nasopharynx, 28% for throat swabs ad 44% for throat washes (Dorigo-zetsma *et al*., 2001). Dilution of samples may sometimes overcome inhibition of PCR, but this may also diminish the sensitivity because the nucleic acid is diluted along with any inhibitors that may be present. There are also commercial reagents for nucleic acid purification that are effective in removing most inhibitors of amplification in PCR assays.

Dorigo-Zetma *et al.,* suggested that elderly adults with pneumonia who are PCR positive and serologically negative for *M. pneumoniae* might be deficient in antibody response

due to a natural decline in the humoral immune system as part of the aging process (Ginaldi *et al.,* 1999). This was also observed in other seroepidemiological studies (Hauksdottir *et al.,* 1998; Dayboeck *et al.,* 2002). A positive PCR test and negative serology could also mean that the specimen was collected too early in the course of illness to allow sufficient time for antibody to develop. In view of the difficulty in the interpretation of serological data because of high background IgG level and sometimes prolonged persistence of IgM, there is also the possibility of false-positive serological data when concomitant PCR testing is negative depending on the test methods and interpretive criteria.

Further refinements to traditional PCR assays, such as combining PCR with a hybridization step, employment of a two step (nested) procedure in which there is reamplification of a PCR product with a second primer set, real time detection using specific problems with matched internal controls to evaluate polymerase inhibition and quantification of *M. pneumoniae* in acute and sub clinical infection will be very important. (Hardegger *et al.,* 2000; Talkington *et al.,* 2001). Further development multiplex PCR tests to detect other atypical pathogens-such as *C. pneumoniae* simultaneously with *M. pneumoniae* may make this type of test more attractive and practical for routine use in diagnostic laboratories. Such endeavors have been undertaken already, but no diagnostic products are being sold commercially (Corsaro *et al.,* 1999; Grondahl *et al*., 1999; Top *et al.,* 1999). Hardegger *et al*., found that a real-time PCR assay was equal to a conventional nested PCR with regard to sensitivity in detection of *M. pneumoniae* in clinical samples, allowing for hands-on time (Hardgger *et al*., 2000). Development of quantitative PCR assays will be beneficial in facilitating better understanding of the carrier state associated with *M. pneumoniae*. Other modifications of

the PCR assay for detection of *M. pneumoniae,* in which amplification is carried out in sealed glass capillary tubes have been described by (Honda *et al*., 2000).

Mycoplasma pneumoniae **virulent genes**

Mycoplasma pneumoniae is a causative agent of atypical pneumonia. The formation of hydrogen peroxide, a product of glycerol metabolism, is essential for host cell cytotoxicity. Phosphatidycholine is the major carbon source available on lung epithelia, and its utilization requires the cleavage deacylated phospholipids to glycerol -3-phosphate and choline. *M. pneumoniae* possesses two potential glycerophosphodiesterases, MPN420 (GLPQ) and MPN 566. Only GLPQ is an active glycerophosphodiesterase (Schmidi *et al.,* 2011). MPN 566 has no enzymatic activity as glycerophosphodiesterase and inactivation of the gene did not result in any detectable phenotype (Schmidi *et al.,* 2011). However, inactivation of the GLPQ gene resulted in reduced growth in medium with glucose as the carbonsource, in loss of hydrogen peroxide production when phosphatidylchlorine was present, and in a complete loss of cytotoxicity towards Hela cells (Schmidi *et al.,* 2011). GLPQ acts as a trigger enzyme that measures the availability of its product glycerol-3- phosphate and uses this information to differentially control gene expression. The major virulence determinant of *M. pneumoniae* is hydrogen peroxide that is generated during the utilization of glycerol – 3- phosphate which might be derived from free glycerol or from the degredation of phospholipids.

2.8.8 SEROLOGY

Despite its drawbacks for use with immunosuppressed persons who are unable to mount an antibody response, serological diagnosis of *M. pneumoniae* respiratory infections has long been the corner stone of *Mycoplasma pneumoniae* diagnosis and for epidemiological studies because of the relative lack of sensitivity and time - consuming nature of culture.

Also, the carrier state that may occur in an unknown percentage of persons in the absence of acute infection can potentially confound interpretation of PCR test results. Serum is easy to collect, store and ship, but the need for acute and convalescent phase specimens and the complex and time consuming nature of many of the serological assays limited acceptance of serology for routine diagnostic testing. Some of the newer and improved commercial assays have overcome some of these limitations. In view of these considerations, it is advisable to test simultaneously for both IgM and IgG in paired specimens collected 2 to 3 weeks apart for the most accurate diagnosis of recent or current *M. pneumoniae* infection, especially in adults (Thacker and Talkington, 2000). A fourfold or greater rise in antibody titer indicates a current or recent infection. The late elevation of IgG that sometimes occurs, the high seroprevalence of IgG antibodies that persist for long periods in persons with a history of *M. pneumoniae* infection and the lack of an IgM response in adults complicate and impose serious limitations on the use of serology as a sole means for diagnosis of *M. pneumoniae* infections (Razin, 2002). Thus, a logical approach would be to incorporate PCR and serological studies for IgG and IgM for optimum diagnosis of *M. pneumoniae* infections (Herrara *et al*., 2016).

Historically, the (CF) test gained popularity among laboratories that routinely ran CF tests for viral agents, and it was the sole means of detecting antibodies to *M. pneumoniae* for many years. However, mycoplasmas are much more antigenically complex than viruses leading to nonspecific reactions. The glycolepids of *Mycoplasma genitalium* are highly cross-reactive with *M. pneumoniae* due to shared lipid antigens (Lind, 1982), and this also causes problems for CF tests. Sera from patients with bacterial meningitis also tend to have high mycoplasmal CF titers. Cross reactions of CF antigens with other organ-specific antigens unrelated to micro organisms may also occur.

Kenny *et al.,* reported that among patients with *M. pneumoniae* culture-positive, X –ray-proven pneumonia, 53% showed a four fold titer increase and 36% showed antibody titer of ≥ 32 with both high titers and high stationary – phase titers as criteria, the sensitivity of the CF test was 90% and specificity was 88% (Kenny *et al*., 1990). Single titers of >32 are sometimes considered to be indicative of recent infection. However, this end point varies greatly among laboratories and antibodies to glycolipid antigens may persist for at least a year after infection (Razin, 2002). Confirmation of CF test results with Western immunoblots can aid in interpretation but greatly adds to the time and expense of testing. Since CF measures mainly IgM and to a minor extent, IgG, its diagnostic value may be limited to initial *M. pneumoniae* infections (Jacobs, 1993).

Some recent studies evaluating commercial kits have merely compared one method of product with another without consideration of CF data, but since results and conclusion from some studies are based upon assay of a single acute-phase serum sample while others have used paired specimens, direct comparisons and extrapolations from studies become rather complex and are not always feasible. Assay formats adopted for commercial distribution include indirect immuno florescence assay (IFA), particle agglutination (PA) assay, and enzyme linked immunoassay (EIA).

IFAs for *M. Pneumoniae* consist of antigen affixed to glass Slide. Specific antibody is detected after staining with anti-human IgM or IgG fluorochrome conjugate. These kits provide accurate, quantitative serology data, but their interpretation is subjective and a fluorescence microscope is necessary. Results can be affected by the presence of rheumatoid factor and high *M. pneumoniae* specific IgG antibody levels and additional procedures are required to validate IgM results in these settings (Talkington *et al*., 1998)

The PA tests in use today mainly utilize latex or gelatin as the carrier particles that are incubated with test serum. If the serum contains specific antibodies, the particles agglutinate resulting to a visible reaction. PA test use a mixture of *M. pneumoniae* antigens to detect both IgG and IgM simultaneously. PA does not offer any advantage over other techniques such as EIAs except possibly their ease and simplicity of performance.

EIAs are the most widely used commercial mycoplasma serology tests. They are amenable to a variety of assay conditions suitable for testing large or small numbers of sera, detect very small amounts of antibody, require serum volumes of ≤ 100µl can be made isotype specific and are generally more sensitive than CF (Waites *et al.,* 2001). EIAs are more sensitive for detecting acute infection than culture and can be comparable in sensitivity to PCR provided that a sufficient time has elapsed since infection for antibody to develop and that the patient has a functional immune system. Crude multiantigen preparations, purified proteins (including the P1 Adhesin), µ-capture approaches, purified glycolipids and synthetic peptides have all been used as targets (Hirsch berg *et al*., 1991; Jacobs, 1993). Patient sera are incubated with the solid – phase antigen and bound antibodies are visualize by using substrate and enzyme – labeled conjugates directed against the primary antibody. The amount of conjugate reacting is proportional to the levels of antibody present in the patient's serum, measured quantitatively with a spectrophotometer.

2.9.0 ANTIMICROBIAL SUSCEPTIBILITIES AND CHEMOTHERAPY

2.9.1 ANTIMICROBIAL SUSCEPTIBILITY PROFILES

M. pneumoniae is inhibited by tetracyclines, macrolides, ketolides and fluoroquinolones, with little variation in MICs among clinical isolates (Waites *et al*., 2003; Bebear *et al*.,

2011). Thus, in vitro susceptibility testing is not indicated for routine patient management purposes for infections of the respiratory tract. Other agents that are active at the bacterial ribosome such as streptogramins, chloraphenicol and aminoglycosides, may also show in vitro inhibitory activity against *M. pneumoniae* but are not widely used for therapeutic purpose against this organism. Clindamycin may be effective in- vitro, but limited reports suggest that it may not be active in-vivo, and it has not been considered first line treatment (Clyde, 1979). Due to the lack of a cell wall, mycoplasmas are innately resistant to all beta-lactams and glycopeptides. Sulfonamides, trimethoprim, polymixins, nalidixic acid, and rifampin are also inactive. Linezolid is the prototype agent of the oxozolidinone class. These agents are much less active against *M. pneumoniae* than the other agents that inhibit protein synthesis (Kenny and Cartwright, 2001). New quinolones such as moxifloxacin, gatifloxacin, garenoxacin, gemifloxacin, and sparfloxacin tend to have somewhat greater in vitro activity than other agents such as ciprofloxacin, ofloxacin and levofloxacin, although MICs of all fluoroquinolones are several folds higher than those of macrolides (Kenny and Cartwright, 2001; Waites *et al.,* 2003). Fluoroquinolones have been shown to be bactericidal for *M. pneumoniae* whereas macrolides and tetracycline are primary bacteriostatic (Duffy *et al*., 2003; Waites *et al.,* 2003).

The extent of naturally occurring clinically significant acquired antimicrobial resistance of *M. pneumoniae* to any drug class is not uncommon. Treatment failures have been rarely reported for microbiological proven cases of *M. pneumoniae* infection and recent studies evaluating macrolides, tetracyclines or fluoroquinolones indicate comparable in vitro activities against isolates obtained from North American and Europe, with no change from data reported earlier other than some minor variations that may be due to differences in testing methods, media and inocula (Waite *et al.,* 2003). Organisms can be shed in respiratory secretions for long period after acute infection and high-level-

macrolide-resistant strains have been isolated following erythromycin treatment but resistance did not appear to affect clinical outcome (Niitu *et al.,* 1970; Stopler *et al.,* 1980; Zheng *et al*., 2015). Macrolide-resistant *M. pneumoniae* mutants can easily be selected in-vitro (Stopler and Branski, 1986; Chirronna *et al*., 2011; Diaz *et al*., 2015a). Such mutants typically exhibit the macrolide lincosamide-streptogramin B-type resistance, rendering Lincosamides and streptogramin B inactive in addition to the macrolides.

2.9.2 IN VITRO SUSCEPTIBILITY TESTING

Even though susceptibility testing is not necessary to guide treatment of *M. pneumoniae* Infection, such procedures are needed in order to evaluate new or investigational antimicrobial agents in comparison to existing drugs, occasionally for systemic infections (especially if the host is immunosuppresed), if treatment is not successful clinically, and / or for eradication of the organism from a normally sterile site. There are no official guidelines for performance, interpretation or quality control of in vitro susceptibility tests for human mycoplasmas published by the National Committee for Clinical Laboratory Standard (NCCLS) now known as Clinical and Laboratory Standard Institute (CLSI). However, a subcommittee of the NCCLS was established in 2001 to address this, work is currently under way to develop in vitro. Susceptibility protocols and quality control ranges for representative drug classes for *M. pneumoniae*, *M. hominis* and Ureaplasma spp. In the absence of NCCLS sanctioned methods, the mycoplasma chemotherapy working Team of the international research program on comparative mycoplasmology has formulated methods, for determination of MICs by broth dilution and agar dilution methods. Many aspects of susceptibility testing of mycoplasmas are identical to the procedures that have been developed and standardized for conventional bacteria. However, the fastidious nature, complex growth requirements and incubation lengths and

conditions for mycoplasma growth have to be considered in optimizing testing for these organisms. Broth and agar based methods for determining MICs have been adapted for testing mycoplasmas, and some general comments regarding the procedures to be performed are relevant for both techniques. As with all bacteria, the inoculum of organisms is important. Since mycoplasmas do not produce turbidity in liquid medium, it is somewhat difficult and complex to determine the number of viable cells present in an active culture. Experience has shown that MICs are not greatly affected when *M. pneumoniae* taken from frozen stocks is used. Thus, some laboratories, prefer to test a frozen stock culture that was serially diluted previously to determine the number of viable cells and then dilute it to an inoculum containing 10^4 to 10^5 color changing units/ml for use directly after a 2-h prewarming period (Waites *et al.,* 2003) MIC test mixture should be incubated at 37°C. Broth –base test mixture can be incubated under atmospheric conditions, whereas agar plates should be supplemented with 5% CO2 for optimum colony development. Mandatory controls that must be used for all types of MIC assays include a sterility control, a growth control and solvent control. Simultaneous testing of an isolate of *M. pneumoniae,* for which MICs of the drugs of interest are known and consistent, is also advisable. Due to the slow growth of the organism, MIC test mixture has to be incubated for 5 days or more and examined daily. No break points to designate susceptibility or resistance have been designated for mycoplasmas. However, some inference about the meaning of the MICS obtained can be made based on breakpoint established for the other bacteria and the achievable concentrations within the body for the drugs being tested. Generally speaking, if MIC is ≤ Iµg/ml, the drug may potentially be active against *M. pneumoniae.*

Agar dilution method of susceptibility testing has the advantages of have a stable endpoint over time, unlike broth dilution, where color change endpoints tend to shift over

time. Agar dilution is suitable for testing large numbers of organism at once. Testing small numbers of isolates by agar-dilution is not very practical, since several individual agar plates must be prepared for each drug to encompass all of the dilutions that may be needed and media must be used when fresh. This method of testing is ideally performed by using a steer replicator to deliver the inoculum to the agar plate, with a goal of generating 30 to 300 colonies in the growth control (Waites, *et al.,* 2001). The MIC is read as the lowest concentration of drug that prevents colony formation when colonies are evident on the growth control plate.

The broth micro dilution technique is based on the principle that a constant number of organism are added to serial doubling concentrations of antimicrobial agents diluted in broth in a 96-well microtiter plate. Broth micro dilution is the most practical and widely used method of susceptibility testing for *M. pneumoniae*. Multiple antimicrobials can be tested in the same microtiter plate at several different dilutions. The inoculated microtiter plates are sealed with an adhesive cover to prevent drying out and are incubated until the growth control shows a colour change. The MIC is read as the lowest concentration of drug that inhibits growth at the time the growth control first shows evidence of growth. Minimal bactericidal concentrations can be tested directly by diluting and subculturing wells in the microtiter plate that show no evidence of growth (Color change) into fresh medium. When the minimal bactericidal concentration is within dilutions of the MIC (Fourfold), the agent may be considered bactericidal (Waites *et al*., 2003).

Waites *et al*., have described methods for performing time-kill assays which multiple dilutions of antimicrobials are incubated in broth with inocula of *M. pneumoniae*. Bactericidal activity over time is assessed by removal of small volumes of broth daily for up to a week and subculturing to agar plates. Colony counts performed on the subcultures

will demonstrate the extent of bacterial killing and the time of exposure to the antimicrobial that is necessary to achieve this effect. Traditionally, a reduction of 99.9% or 3 $\log_{10}$ dilution of the original inoculum has been used to define bactericidal as opposed to bacteriostatic activity (Waites e*t al*., 2003).

2.9.3 TREATMENT OF INFECTIONS DUE TO *MYCOPLASMA PNEUMONIAE*

When *M. pneumoniae* infections were first described soon after the organism was characterized and isolated in culture in the 1960s, there was some sentiment that antibiotic therapy was unnecessary since the disease is self-limiting most of the time. We now know that administration of antimicrobials will generally produce satisfactory results, with a marked reduction in duration of respiratory symptoms compared to non treatment. Treatment of *M. pneumoniae* infections on a case- by case basis has been guided primarily by its well-known and consistent susceptibilities to drugs in the macrolide, tetracycline and flouroquinolone classes that are available for oral administration. Appreciation of *M. pneumoniae* as a significant respiratory tract pathogen and the numerous new drugs in the macrolide and fluoroquinolone classes that have become available since 1990 have led to the performance of several trials involving adults and children with community acquired pneumonia in which the clinical efficacies of various antimicrobials were evaluated. Many published clinical trials have been able to identify relatively small numbers of community acquired pneumonia proven to be caused by *M. pneumoniae* pneumonia and have usually relied upon serological diagnosis, although some recent studies have incorporated culture and/or PCR and thereby improved the numbers of microbiologically proven cases. The use of serology alone as a diagnostic measure precludes determination of whether a treatment regimen actually eradicates the organism and thus data regarding the microbiology efficacy of any antimicrobial regimen are relatively sparse.

Newer fluoroquinolones are being used extensively for treatment of respiratory tract infections in adults since they can be used empirically to treat infections due to mycoplasmas, chlamydiae legionellae, *M. catarrhalis,* and *S. pneumoniae* (including penicillin- and macrolide -resistant organisms); they have an acceptable safety profile; and they can be given orally once daily with favourable results. However, fluoroquinolones are not recommended for use in children due to possible toxicity to developing cartilage. Likewise, tetracyclines are not approved for use in children younger than 8 years of age. Macrolides are generally considered the treatments of choice for *M. pneumoniae* in both adults and children.

Newer macrolides are generally preferred over erythromycin due to their greater tolerability, once- or - twice daily dosing requirements and shorter treatment duration in the case of azithromycin, even though their costs are considerably greater. The extremely high potency of azithromycin against *M. pneumoniae* infections with very short treatment courses and possibly even with a single 1.5g dose, despite the relatively slow growth of the organism (Schonwald *et al*., 1991; Schonwald *et al*., 1999). Overall, azithromycin and the investigational Ketolide Cethromycin (ABT-773) are the most potent drugs against *M. pneumoniae* in terms of MICS (Waites *et al.,* 2003). Recently published clinical trials evaluating the investigational Ketolide Telithromycin alone (Carbon *et al*., 2003) or in comparison with the fluoroquinolone travofloxacin (Pullman *et al*., 2003) for treatment of pneumonia in adults demonstrated that this Ketolide produced clinical cure rates of 100 and 93.3% respectively, from persons whose infection was due to *M. pneumoniae*.

Some studies evaluating fluoroquinolone for treatment of community acquired pneumonias in adults have included diagnostic tests, primarily serology, for *M. pneumoniae* (File *et al*., 2001; Finch *et al.,* 2002; Bionde *et al*., 2014). The general

conclusions of these studies are that newer fluoroquinolone such as levofloxacin and moxifloxacin are effective clinical against *M. pneumoniae* infections, as one might expect from their in vitro potencies. Specific treatment dosage recommendations and the therapeutic regiments for *M. pneumoniae* infections in adults and children are provided in reference texts (Waites, 2001).

Relatively little data are available regarding outcomes of antimicrobial treatment of severely ill children or adults requiring hospitalization for *M. pneumoniae* pneumonia or treatment of immunosuppressed persons with *M. pneumoniae* infection. Eradication of *M. pneumoniae* from persons with immunosuppression can be extremely difficult, requiring prolonged therapy even when the organisms are susceptible to the expected agents. This difficulty highlights the fact that mycoplasma are inhibited but not killed by most commonly used antimicrobial agents in concentrations achievable in vivo.

Limited information from case reports suggest that high close steroid therapy may be effective in reversing neurological symptoms in children with complicated *M. pneumoniae* infections, (Guluvener *et al.*, 2000; Narita, 2009) and some clinicians recommend use of steroids in combination with an antibiotic that can penetrate the CNS such as doxycycline or chloramphenicol (Dionisio *et al.*, 1999, Jafri and McCracken Ir, 1999). Azithromycin and Clarithromycin are commonly used for *M. pneumoniae* respiratory infections, and both have been used successfully in *M. pneumoniae* CNS infections, even though macrolides penetrate poorly into the CNS (Gucuvener *et al.,* 2000; Smith and Eviatar, 2000).

Both plasmaphoresis and intravenous immunoglobulin therapy might be considered if steroid therapy is ineffective for cases of acute disseminated encephalomyelitis, which is

considered to have an important immune component (Smith and Eviator, 2000).A trial of intravenous immunoglobulin in a critically ill patient with encephalitis and *M. pneumoniae* pneumonia was associated with neurological improvement within 48 hrs of treatment (Sakoulas, 2001). A patient with *M. pneumoniae* infection suffering from bilateral optic neuritis as well as acute Gulluin – Barre syndrome recovered after plasmapheresis (Pfausler *et al.,* 2002). A survey of commercial intravenous immunoglobulin therapy preparations found that most preparations had significant activity against *M. pneumoniae* (Krause *et al.,* 2002).

Antibiotic therapy for person with asthma and documented infection with *M. pneumoniae* or other organisms that are amenable to such treatment may prove useful. However, it should be emphasized that antibiotic administration should ideally be reserved for those asthmatic persons who are actually shown to harbour the organism.

A number of trial of prophylactic antibiotics to controls outbreaks of *M. pneumoniae* disease have been performed, with varied success while some earlier studies used tetracycline (Jensen *et al.,* 1969), recent investigations used the newer macrolide. Azithrromycin has been used effectively for prophylaxis against clinical infection with *M. pneumoniae* during out breaks (Klausner *et al.,* 1998; Hyde *et al.,* 2001) with a 75% protective efficacy against illness resulting in physician visits among hospital employee according to one study (Hyde *et al.,* 2000). Weekly oral azithromycin (500mg) had a 64% protective efficacy based on data from serological tests against *M. pneumoniae* infections in U.S. Marine (Gray *et al.,* 1998). These findings support the use of prophylactic antibiotics that are highly active against *M. pneumoniae* in outbreak settings.

2.9.4 VACCINES

Interest in the development of a Vaccine for *M. pneumoniae* has been evident since the early 1960's, soon after the organism was successfully identified and isolated in culture and much of its epidemiology had become apparent, interest was fostered by the lack of natural protective immunity following primary infection, prolonged carriage and propensity for out breaks of infections in military camps, schools and hospitals. Development of a vaccine also seemed promising in view of the facts that the organism is rather homogeneous antigenically and there appears to be some protection against reinfection (Fernald and Clyde, 1970; Couch, 1994). Animal models have been used extensively to improve understanding of natural immune response to *M. pneumoniae* infection and how it might be modified by immunization (Cimolai *et al*., 1992; Cimolai *et al*., 1995; Cimolai *et al.,* 1996). Ellison *et al*. provided an in-depth discussion of protective antigens, protective immune mechanism, and prior vaccine strategies for *M. pneumoniae*, summarizing the work performed in this area though in the early 1990s (Ellison *et al*., 1992).

Considering that attachment and initiation of local damage at the cellular and sub cellular levels are responsible for *M. pneumoniae* disease, the logical vaccine strategy is to prevent attachment and thereby prevent initiation of disease. The initial studies with human volunteers used formalin – inactivated vaccine, but their protective efficacy result were generally disappointing for a variety of reasons that have been explored in depth by Ellison *et al.,* . Perhaps the most significant event related to volunteer immunization was the experience of some immunized volunteers who developed more severe illness after experimental challenge with live mycoplasmas. This occurrence has also been documented in animal models after administration of inactivated as well as component vaccines (Cimolai *et al*., 1992), Signifying that immunization sentisizes the host in some

way through a cell-mediated immune response. Development of live attenuated vaccines administered intranasally or by aerosol inhalation in animal models showed some protective efficacy against disease upon rechallenge with virulent strains, suggesting that simulation of local mucosal defense mechanism is important (Yavoshi *et al*., 1992). Unfortunately, the live attenuated vaccines never made it to human use due to concern over residual virulence of the vaccine strain of *M. pneumoniae*. Similar problem were encountered with temperature- sensitive mutant vaccines (Greenberg *et al*., 1974; Steinberg *et al.,* 1991). Other vaccine candidates have included acellular protein and polysaccharide components and recombinant DNA (Jacobs *et al*., 1988; Ellison *et al.,* 1992). While the importance of the PI adhension in mediating *M. pneumoniae* cytadherence and initiation of disease cannot be denied, animal studies using PI as a vaccine antigen have not demonstrated protective efficacy (Jacobs *et al*., 1988; Cimolai *et al.,* 1995). Experimental animal studies involving mice (Yavoshi *et al*., 1992) hamsters, Cimolai *et al.,* 1992), guinea pigs (Jacobs *et al*., 1988), and Chimpanzees (Franzoso *et al*., 1994) have continued through the 1980s and 1990s, but to our knowledge there have been no recent clinical trials in human with any newer version of *M. pneumoniae* vaccines, and there is no indication that any type of vaccine will be approved for use against *M. pneumoniae* any time soon.

2.9.5 PREVENTION AND CONTROL

Hands should be cleaned with either alcohol rub or soap and water. This is especially important after, contact with another person or a contaminated environment or after wiping nose or covering mouth with hand. The nose and mouth should be covered while coughing or sneezing. This helps to control the spread of respiratory droplets. Commonly touched surfaces should be regularly cleaned and disinfected with disinfectant wipes or spray. Such areas include phones, keyboards and counters. Keep potentially contaminated

hands away from eyes, nose and mouth, the entry points of this bacterium to the body. Do not share cups or eating utensils as this allows the exchange of saliva that can carry *Mycoplasma pneumoniae.* Limit eating “community” food that has the potential of being contaminated by someone’s hand. Be sure to provide and use utensils for serving any food.

CHAPTER THREE

MATERIALS AND METHOD

3.1 STUDY DESIGN:

In an attempt to evaluate cultural and molecular identification methods of *Mycoplasma pneumomae*, from sputum samples of subjects attending a Tuberculosis Clinic, this case controlled study was carried out. The patients were recruited from the Directly Observed Treatment Short Course Clinic (DOTS Clinic) of the Nnamdi Azikiwe University Teaching Hospital, Nnewi. Informed consent was obtained and questionnaire administered. Sputum samples were collected and part of each sample was cultured using appropriate media while the other part transported in an ice-park to Safety Molecular Laboratory, University of Nigeria, Enugu Campus where molecular diagnosis was performed.

3.1.1 Study Setting: This study was carried out at the Tuberculosis Clinic now known as Directly Observed Treatment Short Course Clinic (DOTS Clinic), a subunit of Microbiology Department of Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nnewi North LGA, Anambra State.

3.1.2 Ethical Approval:

This was obtained from Nnamdi Azikiwe University Teaching Hospital Ethics Committee (Appendix I).

3.1.3 Questionnaire and Informed Consent: A written **consent** (Appendix II) was obtained from the adult test subjects and controls and from the parents of the children who were under-aged and could not fill the consent form. Also, questionnaire (Appendix III) was given to every participant which assisted in providing the demographic data of the subjects as well as the selection and exclusion of the subjects.

3.1.4 Selection Criteria: Entry criteria were

Age between 1 year and above and those having one or more of the following symptoms:

a productive cough associated with (a) signs of upper or lower respiratory tract infection for at least 3 days or (b) a sore throat or chest pain.

3.1.5 Exclusion Criteria: Patients treated with antibiotics especially non beta-lactam antibiotics in the preceding 7 days were excluded, likewise those who objected to the screening.

3.1.6 Study Population: This comprised two hundred and sixty three male and female subjects aged between 1 - 70 years attending Directly Observed Treatment Short Course (DOTS) in Nnamdi Azikiwe University Teaching Hospital, Nnewi in Anambra State for a 17 month period. May 2013 through September, 2014. The 263 subjects selected for this study were distributed as follows: 188 having signs and symptoms of respiratory tract infection served as test subjects and 75 apparently healthy individuals without any signs and symptoms of respiratory tract infection served as controls. Of these, females were 139; 99 test and 40 control subjects. Males were 124; 89 test and 35 control subjects.

3.1.7 Sample Size:

Sample size was determined using Naing *et al.,* (2006) formular. The prevalence of *Mycoplasma pneumoniae* respiratory tract infection in all age groups carried out in Nigeria was 9% (WHO, 2013). Using the formular by Naing and co-workers, the sample size was calculated thus.

$$n = \frac{z^2 pq}{d^2}$$

Where n = minimum sample size required.

P = expected prevalence rate in % = (9.0)

q = 1 – p

d - Degree of accuracy desired set at 0.05

Z = the standard normal deviate set at 1.96 which corresponds to the 95% confidence level.

$$N = \frac{(1.96)^2 \times 0.09 \times (1 - 0.09)}{(0.05)^2}$$

$$= \frac{3.8416 \times 0.09 \times 0.91}{0.0025}$$

$$= 125.8508$$

$$= 126$$

Computation with the values above gave a sample size of 126. However, the minimum sample size was increased to 263 to increase the chances of isolating this aetiologic agent from the study population.

Therefore, 188 subjects having symptoms of respiratory tract infection were selected with 75 other individuals not having symptoms of respiratory tract infection as controls.

3.1.8 Controls: These were patients who visited the hospital for diseases other than respiratory tract infection and students on laboratory posting in all the laboratory units of the Nnamdi Azikiwe Teaching Hospital, Nnewi and they were seventy five (75) in number. The sputum production was induced in the control subjects using 5% saline mist (Appendix IV) as a stimulant. Each of the control subjects inhaled the 5% saline mist which stimulated coughing up of alveolar mucus material. Entry criteria for them were:

i. No antibiotic usage in the 7 days before entry in the study.

ii. No signs of upper or lower respiratory tract infec

3.2 Specimen Collection: A wide mouth sterile universal container was given to each subject to produce sputum. The container was labeled assigned identification number and each subject was also given a questionnaire to fill.

3.2.1 Sample Preparation for Molucular Studies: Part of the sputum specimens were stored at a refrigerator temperature of 4^oC before transporting in an ice-pack to Safety molecular laboratory, University of Nigeria Enugu Campus (UNEC).

3.2.2 **Laboratory Analysis:**

3.2.3 Media: The media used were Pleuro pneumonia like organism (PPLO) Broth, PPLO Agar, mycoplasma supplement, for the isolation of the *Mycoplasma pneumoniae* and Tryptose soy broth, blood agar, MacConkey agar and chocolate agar for the isolation of other pathogens.

3.2.4 Methods

- **Inoculation and incubation of media:**

The sputum specimen from each subject was inoculated into PPLO broth (Biotech, USA) (Appendix V) and incubated at 37^0C for up to 4 days. After incubation, subcultures were made from the PPLO broth onto PPLO Agar (Biotech, USA) (Appendix V). The Agar plates were incubated under increased carbon dioxide atmosphere for up to 2 weeks. These were examined daily for growth and the sterile plates were re-incubated and any plate that did not show any growth after 14 days was discarded. The plates that showed growth were examined with the use of dissecting microscope for the presence of “fried egg” colonies, which if present, indicates suspected *Mycoplasma pneumoniae.* Isolates were identified as described by Cruickshank *et al.,* (1975) and Waites *et al.,* (2003). The sputum from each subject was also inoculated into Tryptose soy broth (Appendix V) and incubated aerobically at 37^0C for 24 hours and subcultures were made onto blood agar (Appendix V), chocolate agar (Appendix V) and MacConkey agar plates (Appendix V) for isolation of other respiratory pathogens that might have been in the sputum co-existing with *Mycoplasma pneumoniae* or which might have singly colonized the respiratory tract. The Blood agar and MacConkey agar plates were incubated in air at 37^oC for 24-48hours while the chocolate agar plates were incubated under increased

carbon dioxide atmosphere at 37^oC for 24-48 hours. The isolates were identified according to the methods described by Cheesbrough (2000).

3.2.5 Identification of *Mycoplasma pneumoniae*

3.2.5.1 Haemolysis Test:

Isolates of mycoplasma were inoculated onto PPLO medium to give well dispersed colonies and incubated until colonies are grown, about 5-8 days. The plate was then overlayed with a thin layer of saline-agar containing 1 percent V/V sheep erythrocytes (Appendix VI) and re-incubated aerobically overnight. *M. pneumoniae* produces a maximum clearing resembling β-haemolysis.

3.2.5.2 Tetrazoluim Reduction Test:

Plates of PPLO agar were prepared with the addition of 2.0ml of 1 percent W/V stock solution of 2-3-5 triphenyltetrazolium chloride per 100ml of medium. The stock solution was sterilized in the autoclave.

A block of agar containing numerous colonies was placed, colony-side down, on the tetrazolium plate and the plate reincubated aerobically. The plates were examined after 3 days and the colony-containing block became pink in colour indicating the presence of *Mycoplasma pneumoniae*.

3.2.6 Molecular Detection (PCR) of *Mycoplasma pneumoniae.*

The method employed in this research was the standard polymerase chain Reaction (PCR) method and it comprised many stages as follows:

- Genomic DNA extraction Stage (Extraction of DNA samples)
- Preparation of Master mixes
- Preparation of primer mixes
- PCR protocol optimization
- PCR set up proper
- Running the PCR product on the gel electrophoresis

- Visualization with ultra Violet (UV) light.

3.2.7 Genomic DNA Extraction

This involved three stages which are:

- Sputum lysis stage
- DNA Extraction Stage
- Testing Stage (Final Stage)

- **Sputum Lysis Stage**

Principle: Using a lysis buffer containing N-acetylcystein and NaoH, the sputum was lysed to release nucleated cells with which were further lysed with tissue lysis buffer (ATL) and proteinase K treatment for nucleic acid extraction.

Sample: 500µl of sputum.

Reagents, Materials and equipment (Appendix VII)

The sputum specimens were arranged serially with 10 tubes labeled 1-10. Appropriate numbers of 10ml tubes were arranged and labeled to correspond with the number on sputum samples. Five hundred microlitres (500 µl) of each of the sputum was transferred into a clean 10ml tube. About 500µl of sputum lysis buffer (ATL buffer) was added and mixed gently by vortexing using vortex machine. The mixture was incubated at room temperature for 25 minutes, with shaking using a rocking platform. The volume was then adjusted to 10ml with sterile distilled water after the incubation. This content was centrifuged at 6000 rpm for 30 minutes. At this stage, the sample was separated into two layers. The aqueous supernatant layer and the pellet (deposit or lysate).The pellet were resuspended in 500µl of ATL tissue lysis buffer and vortexed gently. This was transferred to 2ml Eppendoff tube for onward procedure to DNA extraction.

3.2.8 DNA Extraction Proper

The DNA Extraction was done in three (3) stages

i. DNA precipitation

ii. DNA purification and

iii. DNA Elution (Redissolving DNA)

i. DNA precipitation

A 400µl volume of Lysis solution and 20µl of proteinase K. solution was added to the sputum lysate.This was thoroughly mixed by vortexing to obtain a uniform suspension .Each of the samples was incubated at 56°C for 10ml while vortexing occasionally until the cells were completely lysed. A 200µl volume of 96% ethanol was added to the mixture and vortexed to mix properly. This gave us our prepared lysate

ii**. DNA Purification**

The prepared Lysate was transferred to a Gene JET Genomic DNA purification column inserted in a collection tube. Column was centrifuged for 1 min at 6000 x g. The collection tube containing the flow – through solution was discarded. The Gene JET Genomic DNA purification column was placed into a new 2ml collection tube. About 500µl of wash buffer I was added together with 200µl of 96% Ethanol. The content was centrifuged for 1min at 8000 x g. The flow-through was again discarded and the purification column placed back into the collection tube. Again, 500µl of wash buffer II and 200µl of ethanol was added to the Gene-Jet Genomic DNA purification column. The mixture was centrifuge for 3 minutes at maximum speed of 12000 x g. The collection tube containing the flow-through solution was discarded. The Gene-Jet Genomic purification column was then transferred to a sterile 1.5ml microcentrifuge tube for onward procedure to DNA Elution / redissolving.

iii. **DNA Elution (Redissolving DNA)**

A 60μl volume of the Elution buffer was added to the center of the gene Jet Genomic DNA purification column membrane to elute the genomic DNA. This was incubated at room temperature for 2 minutes. It was then centrifuged for 1 minute at 8000xg. The purification column was then discarded. The purified DNA was stored at -20°C for testing

3.2.9 Polymerase Chain Reaction Procedure

Equipment / Reagents and Materials used (Appendix VII).

3.3 Preparation of Agarose Gel

To prepare a 1.5% Agarose gels, 1.5g of Agarose DNA grade was weighed and dissolved in 100ml of 0.5x Tris Boric EDTA buffer (TBE buffer).This was swirled to dissolve and molten in microwave (Mildea Microwave) for 3 minutes. This was brought out and allowed to cool. While cooling outside the wave, 10μl of Ethidium Bromide was added. Ethidium bromide acts as a dye that helps the band or electrophoretic movement to be visualized on UV transilluminator. The gel was poured into the casting trough containing the casting comb at one end (This provides the holes into which the PCR products were added). It was allowed to set for 30mins. When set, the gel block was transferred to the electrophoresis gel tank containing I X Tris Acetate EDTA buffer (TAE buffer).

3.3.1 Samples and Control

Sample constituted DNA elutes extracted from the sputum of the study subjects. Control: Negative control constituted DNA – free distilled water. Positive control constituted the ATCC 29342D *Mycoplasma pneumoniae* genomic DNA. This was loaded in the DNA ladder and was included in each run.

3.3.2 Preparation of PCR Primer Mix

Primers used were 10µl of MP88F, MP88R and MP178F.

Primer mix for each tube was set as follows:

F-primer (MP88F) 300nanometer	10µl
R-primer (MP88R) 600 nanometer	5µl
F-primer (178F) 300nanometer	5µl
Water	56µl

As shown above, the primer mixes were prepared with 10µl and 5µl of forward primer at a concetentration of 300nm each and 5µl of reverse primer at a concentration of 600nm in 56µ of water, giving a final volume of 76µl.

The primer sequence is as follows:

- **Forward primer:**5^1 CAAGCCAAACACGACCTCCGGCC3^1
- **Reverse primer:** 5^1AGTGTCAGCTGGTTTGTCCTTCCCC3^1

3.3.3 Preparation of Master Mix

The master mix used was 2x hot start PCR master mixes (Promega UK).The PCR master mix contains hot start DNA polymerase and a unique PCR- buffer containing factor MP and optimized salt concentrations. The exact concentrations and compositions of these were not given as they are proprietary. This factor stabilizes specifically bound primer and enables efficient extension of all primers in the reaction without optimization. This master mix enables reliable quantification of up to 5 genomic DNA target in a single tube.

3.3.4 PCR – Proper

The amplification reaction mixture for one sample against the primer is shown below:

Primer mix - 8 microlitres

PCR master mix - 10 microlitres

Genomic DNA - 2 microliter

Final Volume - 20 Microlitres

The PCR amplification of the extracted DNA was performed using 20 microlitre of the PCR mix. The PCR machine used was applied biosystem 2720 thermal cycler. These were put into 1.5ml microcentrifuge tubes and spun briefly using the mini centrifuge, the tubes being arranged row by row. All tubes were then transferred into the thermal cycler (2720 Applied Biosystems). Initial denaturation was at 95°C for 5minutes. This was followed by:

- Denaturation at 95°C for 30 seconds
- Annealing at 62°C for 45 seconds
- Extension (elongation) at 72°C for 45 seconds.

These three steps were repeated for 35 cycles .This was followed by Final extension at 72°C for 7 minutes.The content of the tubes at this stage is referred to as an Amplicon. It was kept at a holding temperature of 20°C until ready to use.

3.3.5 Analysis of Amplified Samples

PCR products were analyzed electrophoretically on 1.5% Agarose gel stained with 10µl Ethidium bromide.

3.3.6 Electrophoresis

Two microlitre (2µl) PCR products (already containing dye) were loaded into the wells on one end of the gel block. The order of loaded samples and the controls were noted down as follows: The DNA ladder (L) that is size marker of 1.5 kilobites (kb) (Promega UK) was put on the first lane of the gel, while the test

samples were put on lanes 2 to 15 and labeled 1 to 14. An 87bp positive (P) control ATCC 29342D *Mycoplasma pneumonaie* genomic DNA ladder was loaded on lane 16 and labeled P. This was the positive control while the negative control was loaded on the last lane and labeled N. The second batch of the test samples were loaded on the second gel with the DNA ladder (L) put on the first lane while the test samples were put on lanes 2 to 15 and labeled 1 to 14. The positive control was put on lane 16 and labeled P and the negative control on lane 17 and labeled N. The power pack of the electropheresis was switched on and ran at 100v for 30 minutes, checking quality of separation with the UV transilluminator.

3.3.7 Detection from UV light/interpretation of result

At the end of the electropheresis, the gel block was taken from the tank and placed on large UV transilluminator, where the amplified product was visualized and the result of the electropheresis was photographed under the UV light using digital camera. Using the primer pair, the length of the PCR products were as follows and were controlled with the 87bp (base pair) DNA ladder.

Interpretation:

87bp only – neg ative (No *Mycoplasma pneumoniae* DNA detected)

172bp - positive (*Mycoplasma pneumoniae* DNA detected)

3.3.8 Detection of *Mycoplasma pneumoniae* virulence gene (GLPQ gene or MPN 420).

The GLPQ gene formerly called MPN 420 is the most virulent enzyme in *Mycoplasma pneumoniae* (Schmidi *et al.,* 2011). The GLPQ gene acts as a trigger enzyme that measures the availability of its product glycerol- 3- phosphate and uses this information to differentially control gene expression.

Further, PCR test was performed on the 18 positive *Mycoplasma pneumoniae* DNA samples to detect whether they contain GLPQ gene which is the most virulent gene in *M. pneumoniae.*

3.3.9 PCR procedures for the detection of GLPQ gene (MPN 420)

Equipment, Reagents and Materials used, (Appendix VIII)

3.4 Preparation of Agarose Gel.

To prepare a 2% Agarose gel, 2g of Agarose powder was weighed and dissolved in 10ml of 0.5x TBE buffer (TRI Boric EDTA buffer). This was swirled to dissolve and melted in microwaves (Mildea Microwave) for 3 minutes. This was brought out and allowed to cool. While cooling outside the wave, 10µl of syber safe stain was added. Syber safe stain acts as a dye that helps the band or electrophoretic movement to be visualized on U.V transilluminator. The gel was poured into the casting trough containing the casting comb at one end (This provides the holes into which the PCR products were added). It was allowed to set for 30 minutes. When set, the gel was transferred to the electrophoretic gel tank containing I x TAE buffer.

3.4.1 Samples and Control

- Sample constituted all the 18 positive *Mycoplasma pneumoniae* DNA samples already detected from the 188 sputum samples of the subjects from the first PCR performed.
- **Control**: Negative control constituted DNA free distilled water while positive control constituted the ATCC 29342D *mycoplasma pneumoniae* DNA. This was loaded in the DNA ladder and was included in each run.

3.4.2 Preparation of PCR Primer

Tubes were set a follows for the primer mixes

MPN – F (200 nanomolar)   6µL

MPN – R (250 nanomolar) 6µL

Ultra pure water (Promega, UK) 68µL

Final volume 80µL

As shown above the primer mixes were prepared with 6µl of MPN forward primer at a concentration of 200nm and 6µl of reverse primer at a concentration of 250nm in 68µl of water giving a final volume of 80µl.

3.4.3 Preparation of Master Mix

The master mix used was 2 x hot start PCR mixes (Promega, UK). The PCR master mix contains Hot start DNA polymerase and a unique buffer containing factor MPN 20 and optimized salt concentration and composition is proprietary.

3.4.4 PCR

The amplification reaction mixture for one sample against the primer is shown below:

Primer mix - 8 microlitres

PCR master mix - 10 microlitres

Genomic DNA - 2 microlitres

Final Volume - 20 microlitres.

The PCR amplification of the extracted 18 positive *Mycoplasma pneumoniae* DNA was performed using 20 microlitre of the PCR mix. The PCR machine used was Eppendorf Nexus Themal cycler (Eppendorf, Germany). These were put into 1.5ml and spun briefly using the mini centrifuge, the tubes being arranged row by row. All tubes were then transferred into the thermal cycler. Initial denaturation was at 94°C for 3 minutes. This was followed by:

- Denaturation at 94°C for 30 seconds.
- Annealing at 62°C for 30 seconds.

- Extension (elongation) at 72°C for 30 seconds.

These were followed by final extension at 72°C for 5 minutes. The content is referred to as an Amplicon. It was kept at a holding temperature of 20°C until ready to use.

3.4.5 Analysis of Amplified Samples

The PCR product were analysed electrophoretically on 2% Agarose gel stained with 10µl syber stains.

3.4.6 Electrophoresis

The PCR products (2µl) already mixed with the loading dye were put in separate wells in the agar block already inside the tank. The loading dye enabled the PCR products to sink into their respective wells and coloured. It also enabled one to observe the migration of the products during electrophoresis. The DNA ladder (L) that is, size marker was put on the first lane of the gel and the positive *Mycoplasma pneumoniae* DNA sample to be tested for the presence of GLPQ gene (MPN420) were put on lanes 2 to 14 and labeled 1 to 13. The positive and negative controls were put on lanes 15 and 16 and labeled P and N respectively. The second batch of the remaining test samples were loaded on the second gel with the DNA ladder (L) put on the first lane while the test samples were put on lanes 2 to 6 and labeled 14 to 18. The positive and negative controls were put on lanes 7 and 8 and labeled P and N respectively. The power pack of the electropheresis tank was switched on and ran at 100V for 45 minutes. The expected band of positivity was 90bp (base pair)

3.4.7 Detection from the UV light

At the end of the electrophoresis, gel block was taken from the tank and placed on large UV transilliuminator, where the amplified product was visualized and the result of the electrophoresis was photographed under the UV light using digital

camera (Victoria, USA). Using the primer pair, the length of the PCR products were controlled with the 87bp (base pair).

Interpretation

87bp only - negative (No GLPQ gene detected)

90bp - positive (GLPQ gene detected)

3.4.8 Antibiogram of *M. pneumoniae*

Disc diffusion antibiotic sensitivity test method as described by Cheesbrough, (2000) was used to detect the antibiotic sensitivity pattern of *M. pneumoniae* isolated by cultural method.

3.4.8.1 Preparation of McFarland Standard

Method

First, turbidity standard was prepared as follows:

One percent (1%) V/V solution of sulphuric acid was added to 99ml of water. This was well mixed. Then, 1% W/V solution of barium chloride was also prepared by dissolving 0.5g of dihydrate barium chloride (BaCl2. 2H20) in 50ml of distilled water. A small volume (0.6ml) of the barium chloride solution was added to 99.4ml of the sulphuric acid solution and mixed. A small volume of the turbid solution was transferred to capped tube for use and the rest was stored in a well-sealed container in the dark at room temperature for future use.

3.4.8.2 Susceptibility Testing

About 5 colonies of the test organism were touched with a sterile wire loop and emulsified in 3ml of sterile physiological saline.

The turbidity of the suspension was matched with the turbidity of the standard in a good light. Using a sterile swab, a plate of PPLO agar was inoculated making sure that the excess fluid was removed by pressing and rotating the swab against the

side of the tube above the level of the suspension. The swab was streaked evenly over the surface of medium in the directions rotating the plate approximately 60° to ensure even distribution

The Petri-dish lid was placed back and the surface of the agar was allowed to dry for 3 minutes. Using a sterile forceps, the appropriate antimicrobial discs were evenly distributed on the inoculated plate. These antibiotics tested were Erythromycin, Azithromycin, Doxycycline, Ciprofloxacin, Levofloxacin, Rifampicin, Lyntriaxone, Septrin, Norbactin and Peflacine (Abtek Biological limited, UK). The plates were incubated and examined after 24hrs. The zone of inhibition was measured in millimeter (mm) and was compared against a standard interpretation chart used to categorise the isolate as susceptible, intermediately susceptible or resistant using the criteria published by the Clinical and Laboratory Standard Institute (CLSI). Zones of inhibition less than 14mm in diameter were considered resistant. Zones of inhibition between 14 -16mm were considered intermediately sensitive while zones of inhibition from 17mm or more were considerd sensitive.

3.4.9 Identification Of Other Pathogens

The blood, chocolate and MacConkey agars were examined for growth and identification of isolates made as follows:

Smear of each of the organisms was made on a grease free slide and stained by Gram technique (Appendix IX). *Staphylococcus aureus* appeared as Gram – positive cocci in singles, doubles and in clusters while *Streptococcus pneumoniae* appeared as Gram positive enlongated (Lancet shaped) capsulated diplococci while some appeared as short chains. *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* appeared as Gram negative rods. Further identification tests were

carried out to identity each of the organisms. Catalase test and Coagulase test were used to further identify *Staphylococcus aureus* according to Cheesbrough (2000).

Bile solubility test according to Cheesbrough (2000) was used to identify *Streptococcus pneumoniae* using sodium deoxycholate reagent (Appendix X) as it is the only streptococcus specie that dissolves in bile salt.

Urease test using Motility Indole Urease (MIU) medium (Appendix XI) was used to further identify Kebsiella pneumoniae.

Oxidase test was done up described by Cheesbrough (2000) using Oxidase reagent. (Appendix XII) to identify *Pseudomonas aeruginosa.*

3.5 STATISTICAL ANALYSIS

The Data Obtained was described as numbers and percentages when appropriate. The calculations between variables were explored with the chi-square (χ^2) test using fisher's exact test. P-values less than 0.05 ($P<0.05$) were considered significant. Cross tabulations were equally used to establish the prevalence of the above infection.

All calculations were performed using statistical package for social sciences (SPSS) version 21. The signifcant level was set at 95% confidence interval and P-value less than 0.05 ($P<0.05$) were considered siginificant.

CHAPTER FOUR

RESULTS

The macroscopic appearance of *Mycoplasma pneumoniae* on PPLO agar is shown in plate1. The organism grew as tiny ball shaped colony that grew up to the surface of the agar and spread along giving a halo of delicate growth while plate 2 shows the microscopic appearance of the organism. The colony of the organism when viewed from the above using dissecting microscope, presented a "fried egg" appearance. Plate 3 is a sample of the tetrazolium reduction test carried out to identify *M. pneumoniae.* The colony-containing block became pink in colour indicating presence of *Mycoplasma pneumoniae.*

The electrophoresis analysis of the products of some of the *Mycoplasma pneumoniae* from the sputum samples are shown in Figure 4.1. Lane 1 was the DNA Ladder (Size marker). Lanes 7,10,12, and 14 were patients samples showing bands at 172bp, lane16 was positive control(P) also showing band at 172bp and lane 17 was a negative control (N).

The overall prevalence of *M. pneumoniae* detected from the 263 subjects showed 13(4.9%) and 21(8.0%) rates for culture and PCR methods respectively. The break down showed the test subjects having 5.9% and 9.6% for culture and PCR methods respectively while the control subjects gave rates of 2.7% and 4.0% for culture and PCR respectively (Table 4.1).

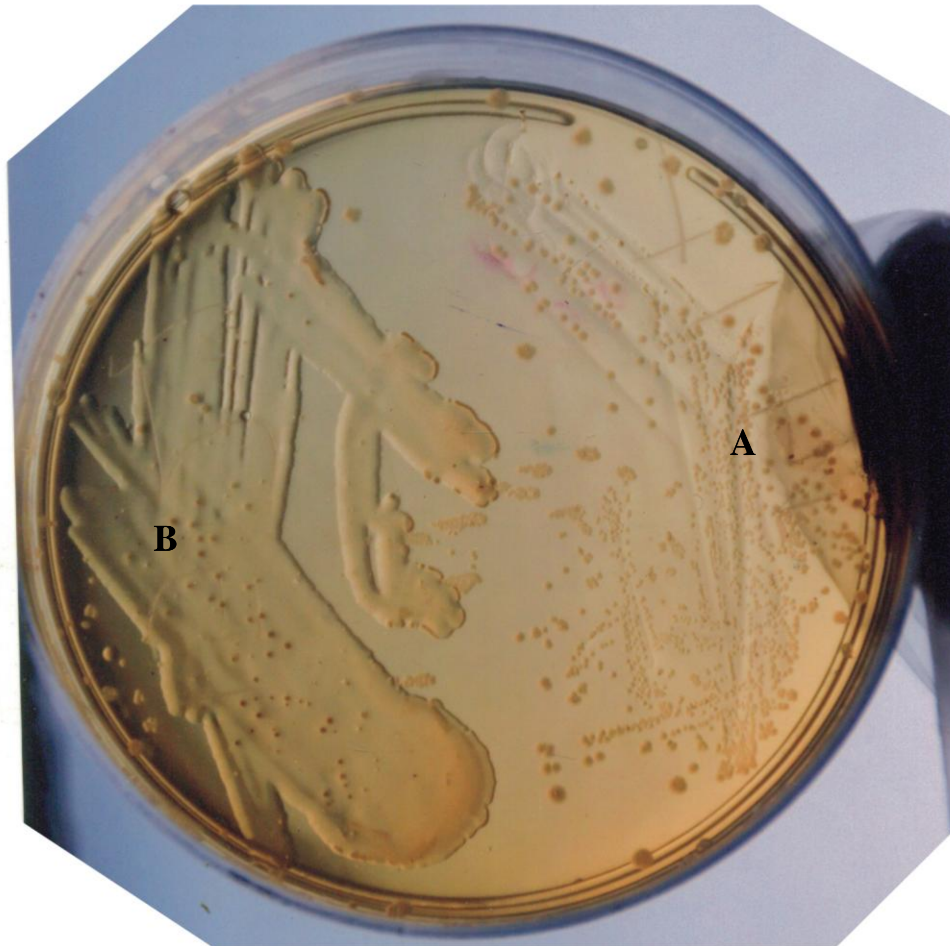

Plate 1: Section “A” shows the macroscopic appearance of *Mycoplasma pneumoniae* on PPLO Agar plate.

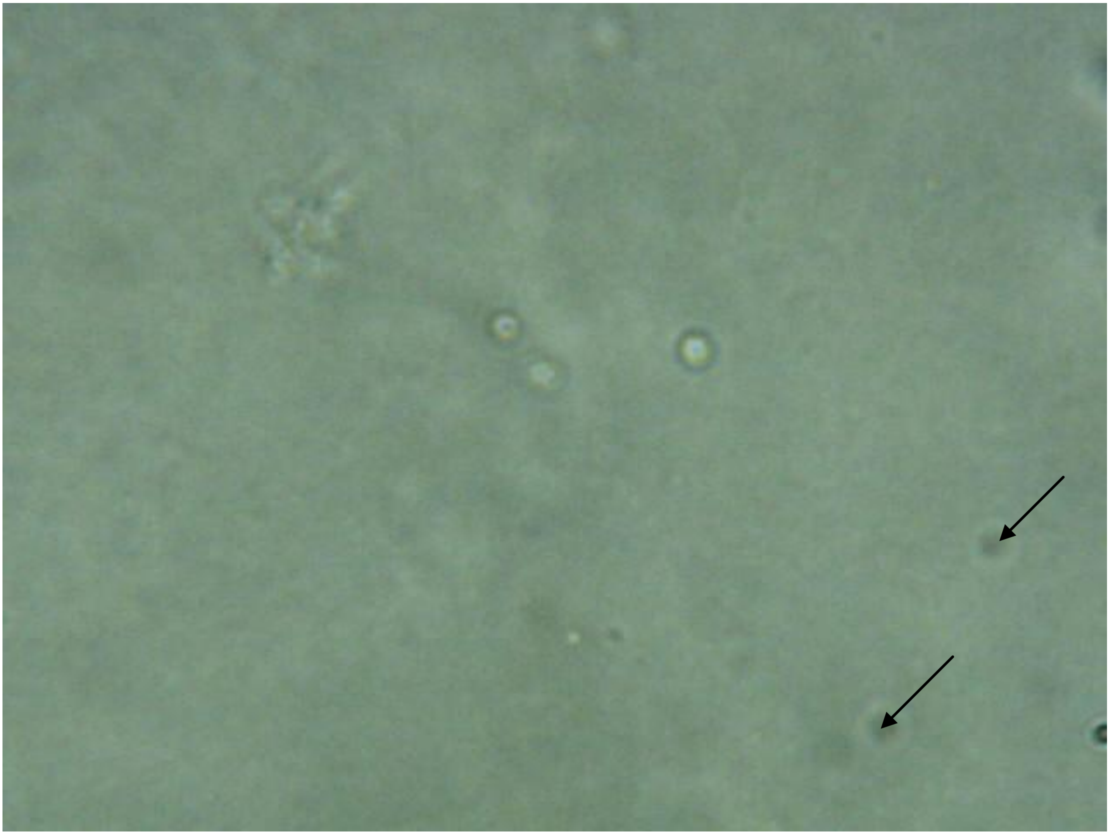

Plate 2: A typical Mycoplasma colony with “fried egg” appearance. Arrows show dark centre and smooth edges (x40).

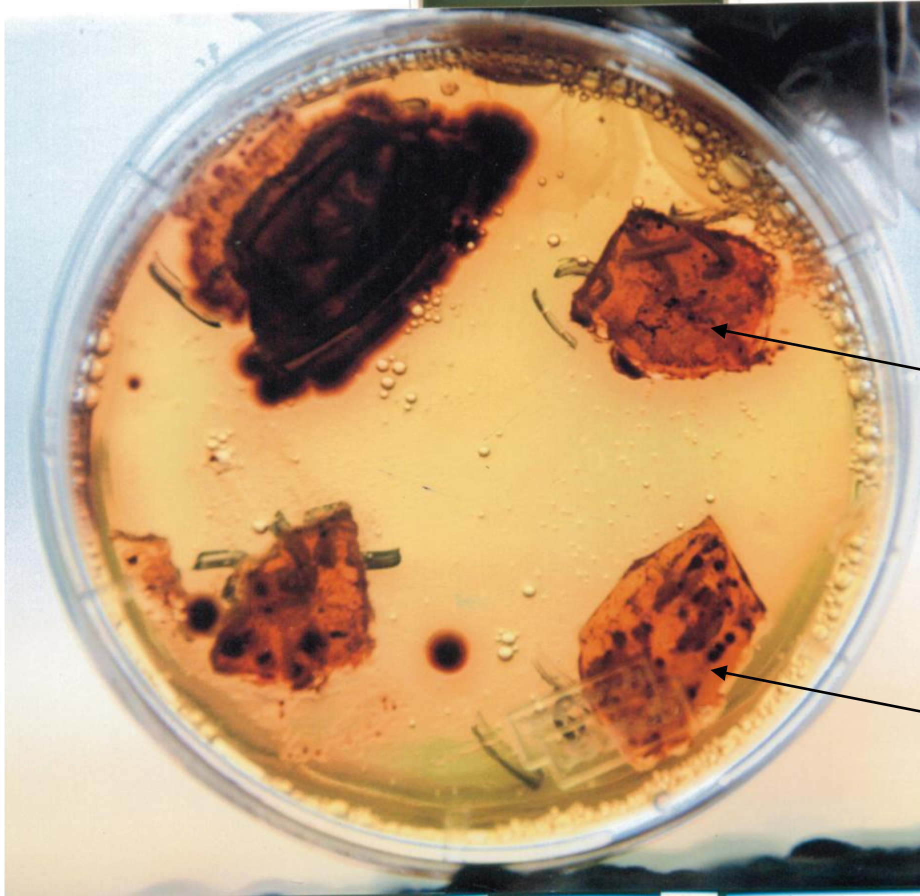

Plate 3: Pink coloured colony containing blocks indicating presence of *Mycoplasma pneumoniae* arrowed.

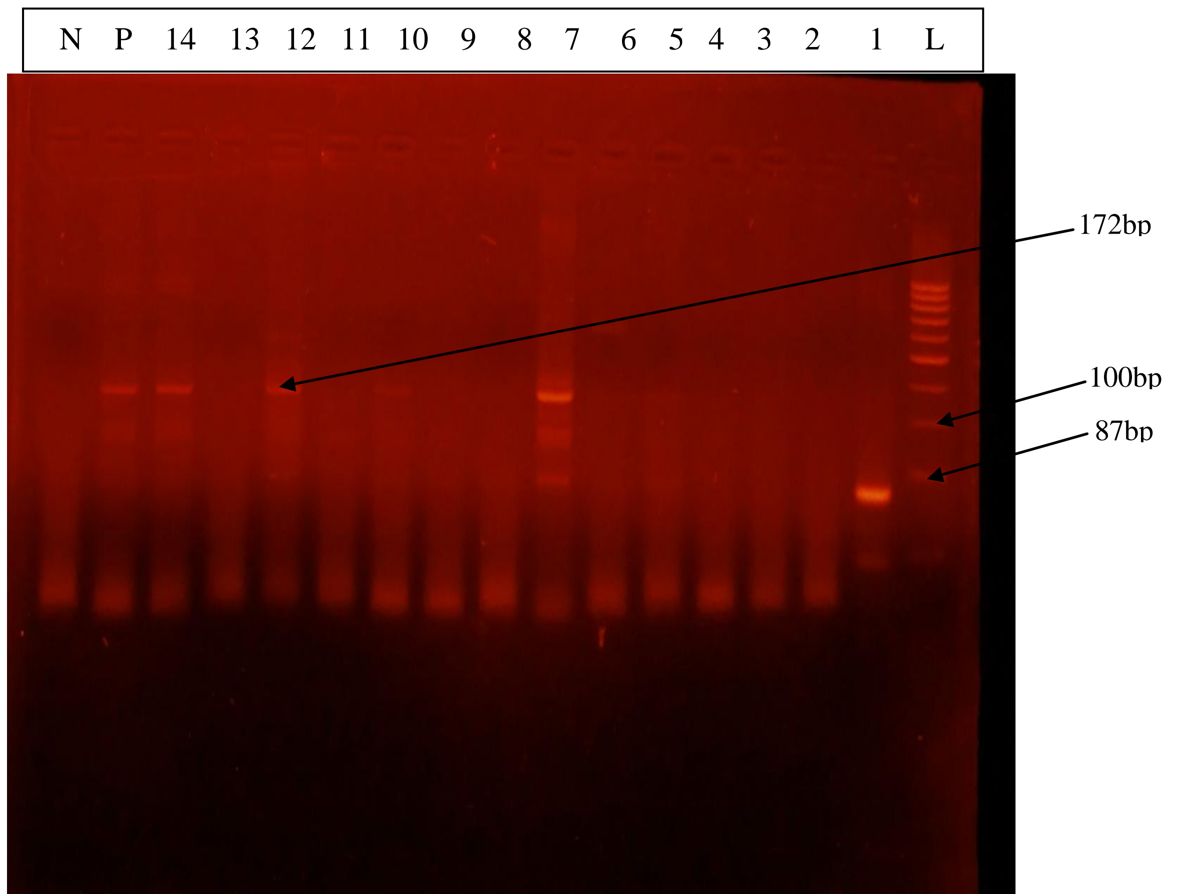

Figure 4.1: Result of PCR diagnostic test for *Mycoplasma pneumoniae* from sputum specimens

Key:

Expected band for positivity result = 172bp

L = DNA ladder (marker lane)

1 – 14 = Test samples lanes

Samples in lanes 7, 10, 12 and 14 are positive for *Mycoplasma pneumoniae*

P = Positive control lane

N = Negative control lane

Table 4.1: Overall prevalence of *Mycoplasma pneumoniae* among the subjects

	Study subjects n = 188	Control subjects n = 75	Total n = 263
Method	n (%)	n (%)	n (%)
By culture	11 (5.9)	2 (2.7)	13 (4.9)
By PCR	18 (9.6)	3 (4.0)	21 (8.0)

The highest prevalence 12 (66.7%) and 8 (72.7%) for PCR and culture respectively were observed among females while prevalence 6 (33.3%) and 3(27.3%) respectively for PCR and culture were observed among males (Tables 4.2 and 4.3) respectively.

However, among the controls, the highest prevalence rates of 2(66.7%) and 2 (100%) were observed among males in both PCR and culture methods (Tables 4.2 and 4.3) while the prevalence rate of 1 (33.3%) was observed in female by PCR method (Table 4.2). There was no *M. pneumoniae* isolated in the female control by culture method (Table 4.3). There was no significant relationship between *M. pneumoniae* infection and gender ($P>0.05$).

Table 4.2: Prevalence of *Mycoplasma pneumoniae* detected by PCR method in the test and control groups in relation to gender.

Group	**PCR Results**	**Female n (%)**	**Male n (%)**	χ^2	**P-value**
Test	Positive	12(66.7)	6(33.3)	1.567	0.211
	Negative	87 (51.2)	83(48.8)		
Control	Positive	1 (33.3)	2 (66.7)	0.502	0.479
	Negative	39(54.2)	33(45.8)		

Table 4.3: Prevalence of *Mycoplasma pneumoniae* isolated by culture method in the test and control groups in relation to gender.

Group	**Culture Results**	**Female n (%)**	**Male n (%)**	χ^2	**P-value**
Test	Growth	8(72.7)	3(27.3)	1.887	0.169
	No Growth	91(51.4)	86(48.6)		
Control	Growth	0(0.0)	2(100.0)	2.348	0.125
	No Growth	40(54.8)	33(45.2)		

Prevalence of *Mycoplasma pneumoniae* according to ages were found to be 1(9.1%) in age group 1-10 years, 1(9.1%) in age group 11- 20 years, 3(27.3%) in age group 21-30 years, 4 (36.4%) in age group 31-40 years, 1(9.1%) in age group 41-50 years and 1(9.1%) in age group above 50 years by culture method while prevalence of *Mycoplasma pneumoniae* in various age groups by PCR method were as follows: 2 (11.1%) in age group 1-10 years, 2(11.1%) in age group 11-20 years, 4(22.2%) in age group 21-30 years, 5(27.8%) in age group 31-40 years, 2(11.1%) in age group 41-50 years and 3(16.7%) in age group above 50 years (Table 4.4). In both culture and PCR methods, the highest prevalence 36.4% and 27.8% respectively were observed in age group 31-40 years followed by age group 21-30 years with prevalence of 30.8% and 23.8% by culture and PCR respectively (Table 4.4).

Mycoplasma pneumoniae were also found among the different age groups of the control. Table 4.4 shows that *Mycoplasma pneumoniae* was obtained among the age groups 21-30 years and 31-40 years with a rate of 66.7% and 33.3% respectively by PCR method while a rate of 50% each was obtained by culture in age groups 21-30 and 31-40 years. The result is statistically significant showing that there is a significant relationship between *Mycoplasma pneumoniae* infection and age ($P<0.05$).

Table 4.4: Distribution of Mycoplasma pneumoniae among the age groups investigated

Mycoplasma pneumoniae n (%)

Age range (Years)	Test group by culture	Control group by culture	Test group by PCR	Control group by PCR
1-10	1 (9.1)	0 (0)	2 (11.1)	0(0)
11-20	1 (9.1)	0(0)	2 (11.1)	0(0)
21-30	3 (27.3)	1 (50)	4 (22.2)	2(66.7)
31-40	4 (36.4)	1 (50)	5 (27.8)	1(33.3)
41-50	1 (9.1)	0(0)	2 (11.1)	0(0)
> 50	1 (9.1)	0(0)	3 (16.7)	0(0)
Total	**11 (100.0)**	**2(100)**	**18 (100.0)**	**3(100)**
χ^2	4.58	6.483		
P-value	0.03*	0.011*		

Key:

* = Significant at $P < 0.05$

Mycoplasma pneumoniae isolated from the test subjects were predominatly from patients who complained of symptoms of cough, 4(36.4%) by culture and 7(38.9%) by PCR. Those who complained of chest pain had prevalence of 1(9.19%). Those with sorethroat 1(5.6%) was only observed by PCR method (Figure 4.2). Among those who had more than one symptoms, *M. pneumoniae* were predominant in those with chest pain and cough 3(27.3%) and 4(22.2%), sorethroat and cough 2(18.2%) and 4(22.2%) by culture and PCR respectively while the least were isolated from patients who had chest pain, sorethroat and cough concurrently 1(9.1%) and 2(11.1%) by culture and PCR respectively (Figure 4.2). There was a significant relationship between *M. pneumoniae* infection and symptoms, P–value = 0.000($P<0.05$). None of the control group had any symptoms/complaints.

GLPQ gene formerly called MPN 420 is the most virulent and active gene among all the genes (enzymes) produced by *M. pneumoniae.* This gene acts as a trigger enzyme that measures the availability of its product glycerol-3-phosphate and uses this information to differentially control gene expression. The electrophoresis analysis of 18 PCR positive *Mycoplasma pneumoniae* from Sputum specimens are shown in figure 4.3. Lanes 1 and 17 were the DNA ladder (size markers) while lanes 15 and 23 were positive controls used and they showed their bands at 90bp.

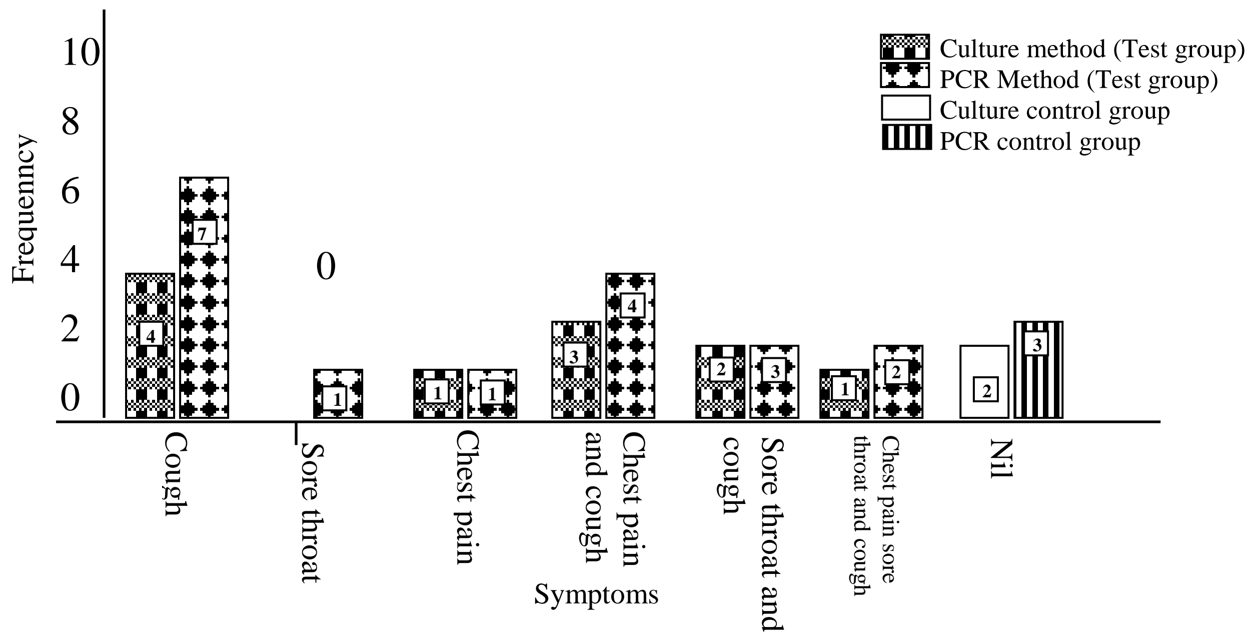

Figure 4.2: Distribution of *Mycoplasma pneumoniae* among the subjects according to complaints of patients.

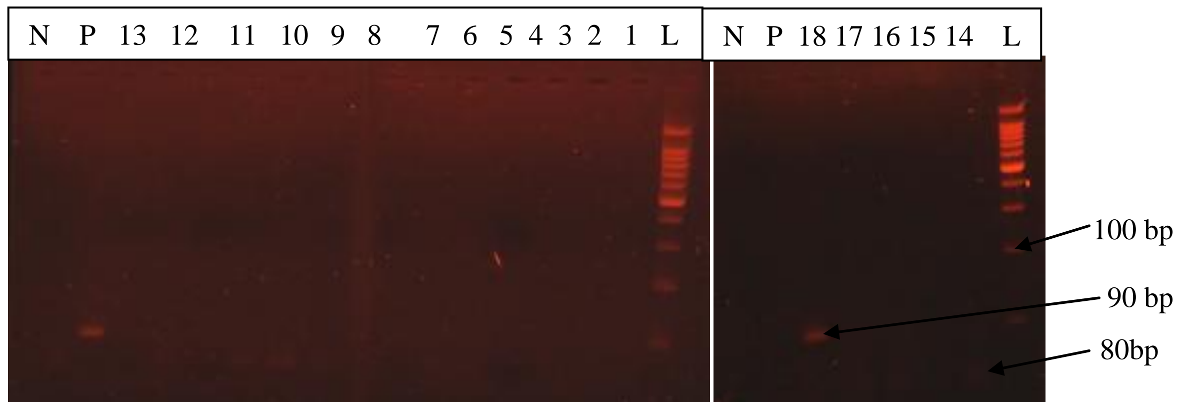

Figure 4.3: Result of PCR diagnostic test for the GLPQ gene (MPN 420 virulent gene) from the 18 PCR *Mycoplasma pneumoniae* positive samples.

Key:

L = DNA ladder (marker lane)

Expected band for positivity = 90bp

1 – 18 = Test samples lanes

Sample in lane 18 is positive for GLPQ gene

P = Positive control lane

GLPQ gene was detected in 5 samples out of the 18 PCR-positive *Mycoplasma pneumoniae* samples from the test subjects and they all showed their bands at 90bp. Lanes 15 and 23 were positive controls used and they also showed their bands at 90bp. Lanes 16 and 24 were the negative controls used. Among the 18 PCR positive *Mycoplasma pneumoniae* detected from the test subjects, 5(27.8%) contained the GLPQ gene while the other 13(72.2%) PCR positive *Mycoplasma pneumoniae* had no GLPQ gene (Figure 4.4).

There was no GLPQ gene detected from the three PCR positive *M. pneumoniae* obtained from the control subjects.

Figure 4.5 shows the distribution of the GLPQ, gene among the age groups. The virulent gene occurred highly between the ages of 11 and 30 years with the rate of 40% each among age groups 11-20 years and 21-30 years followed by age range 1-10 years with 20% rate. The virulent gene GLPQ was not found in age groups 31-40 years and above 50 years. The statistical analysis showed the result was not significant ($P > 0.05$).

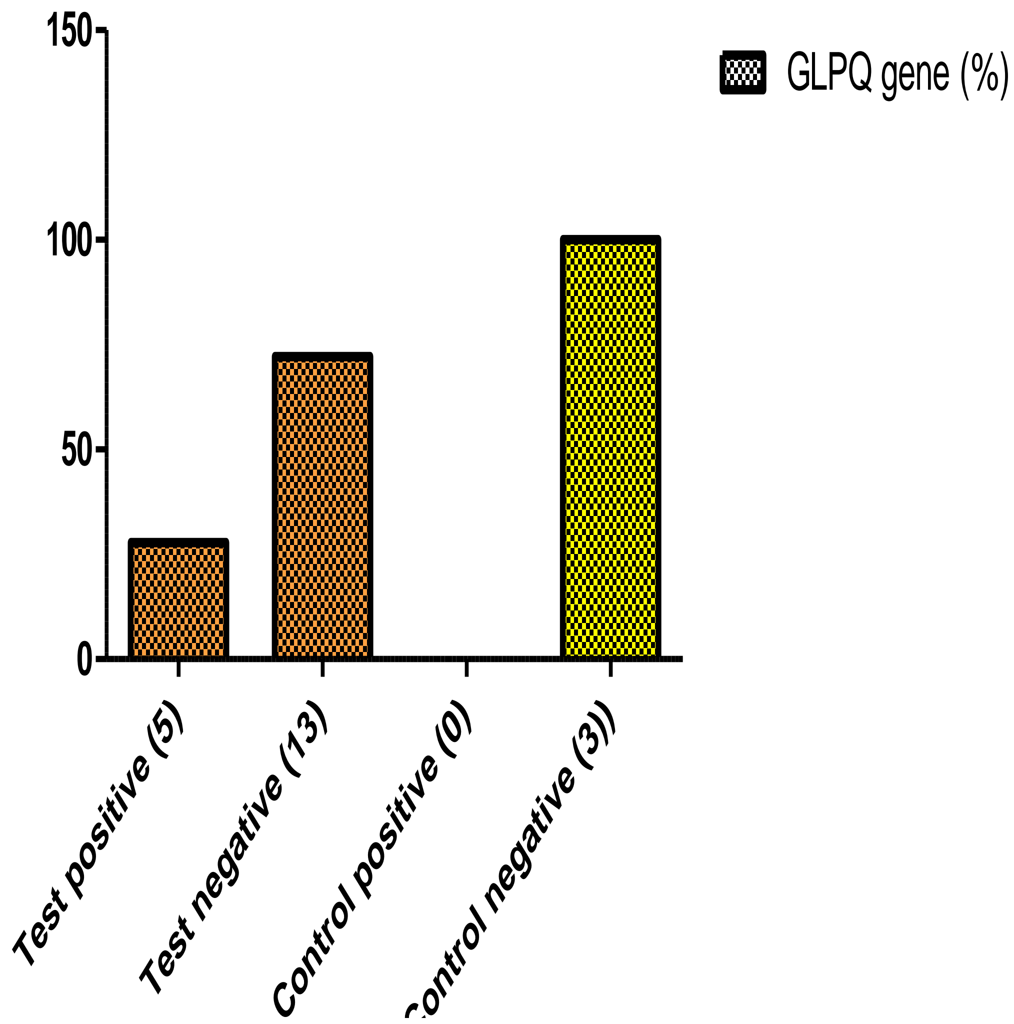

Figure 4.4: Percentage Occurrence of GLPQ Virulence gene among the PCR Positive *Mycoplasma pneumoniae.*

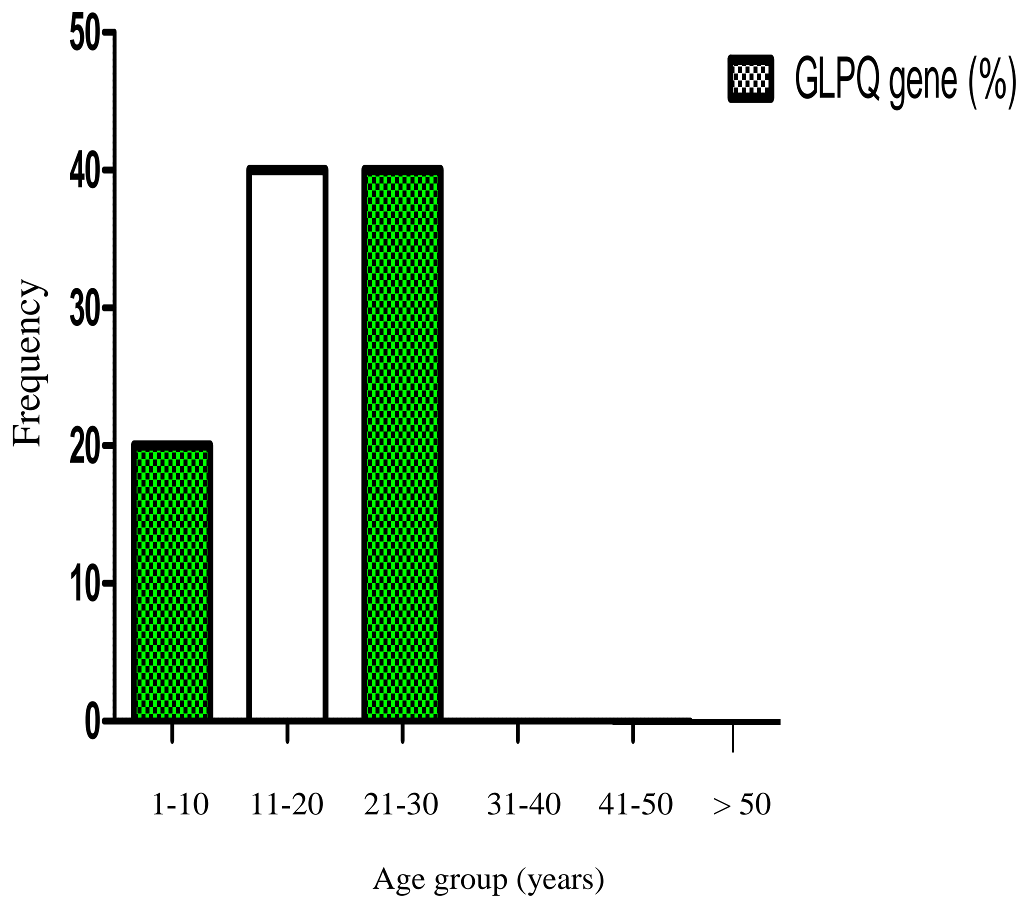

Figure 4.5: Percentage distribution of the GLPQ gene among the age groups

Figure 4.6 shows the distribution of the five GLPQ genes according to patients' complaints. The virulent gene was predominantly found in patients with symptoms of cough with a rate of 2(40%) followed by one patient who complained of Chest pain alone with a rate of 1(20%). There were no symptoms of sorethroat only among the five patients. However, 1(20%) each had symptoms of chest pain and cough and sore throat and cough respectively. None of the five patients had chest pain, Sorethroat and cough symptoms concurrently.

The antibiogram of *M. pneumoniae* isolates showed that Ciprofloxacin, levofloxacin, Lyntriaxone and Azithromycin showed excellent clearance of *M. pneumoniae* isolates indicating that *M. pneumoniae* was highly susceptible to them while Doxycycline showed intermediate clearance (Table 4.5). Other antibiotics like Rifampicin, Erythromycin, Septrin, peflacine and Norbactin were not effective against *M. pneumoniae* (Table 4.5).

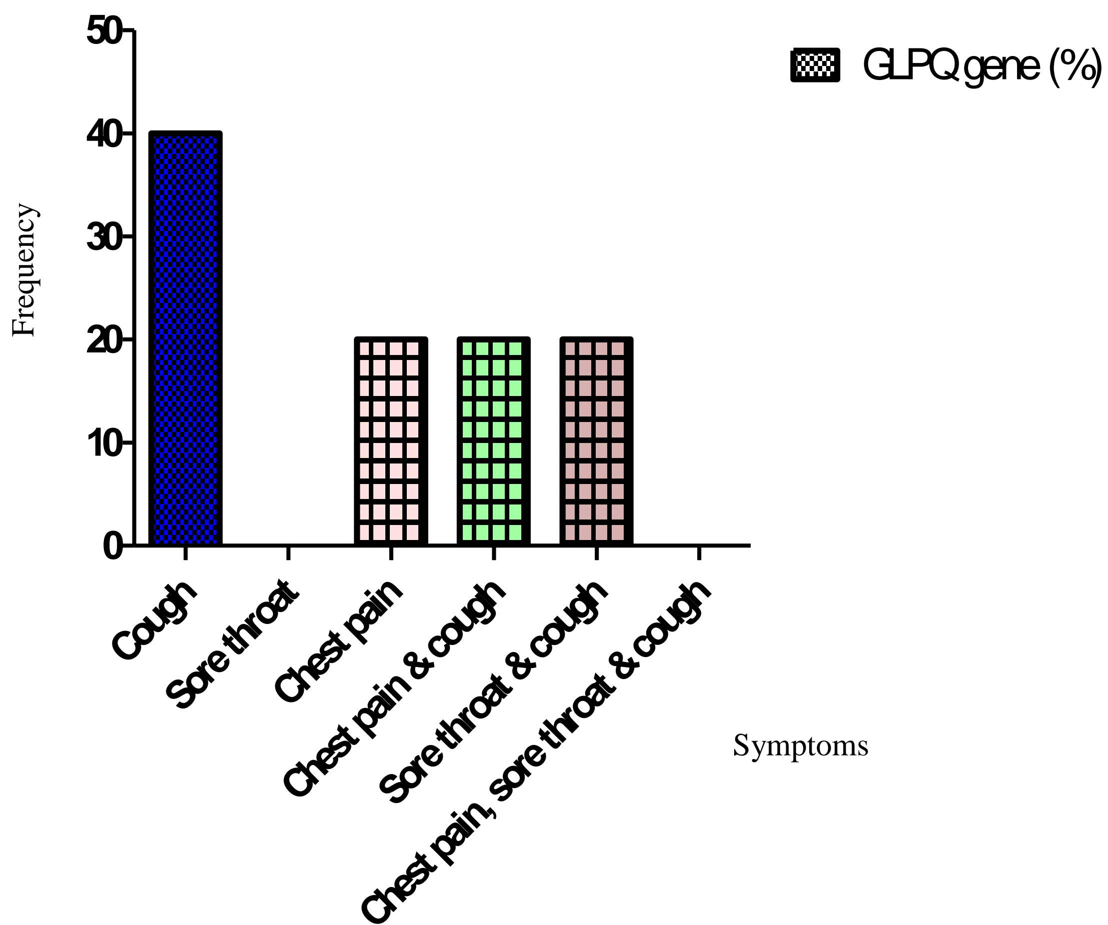

Figure 4.6: Percentage Distribution of the GLPQ gene according to the patients' symptoms.

Table 4.5: Antibiogram of *Mycoplasma pneumoniae*

Antibiotic	**Disc Potency**	**Average Diameter of zone of inhibition (mm)**	**Sensitivity**
Azithromycin	15µg	19	S
Doxycycline	5µg	15	I
Ciprofloxacin	10µg	18	S
Lyntriaxone	30µg	25	S
Levofloxacin	5µg	21	S
Erythromycin	10µg	13	R
Peflacine	10µg	10	R
Septrin	30µg	10	R
Rifampicin	5µg	11	R
Norbactin	30µg	9	R

Key:

S - Sensitivity

I - Intermediate

R - Resistance

Figure 4.7, shows other pathogens isolated from the various sputum specimens of the study subjects. *Staphylococcus aureus* had highest prevalence (18.6%) followed by *Streptococcus pneumoniae* (16.5%), then, *Klebsiella pneumoniae* (6.4%), the least prevalence was observed in *Pseudomonas aeruginosa* (5.9%).There was no co-existence of *M. pneumonia*e with other isolates in the control subjects. Also figure 4.7 shows other pathogens isolated from the sputum specimens of the control subjects. *Staphylococcus aureus* again had the highest prevalence (5.3%) followed by *Streptococcus pneumoniae* (1.3%), however, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were not isolated from the controls.

Relationship of *M. pneumoniae* with other microbial isolates was shown in Figure 4.8. *M. pneumoniae* co-existed with *Staphylococcus aureus* in one subject and co-existed with *Streptococcus pneumoniae (S. pneumoniae)* in two subjects (Figure 4.8).

However, *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Klebsiella pneumoniae* (*K. pneumoniae)* showed no level of co-existence with *M. pneumoniae* (Figure 4.8). There was no significant relationship between *M. pneumoniae* and other isolates (P>0.05).

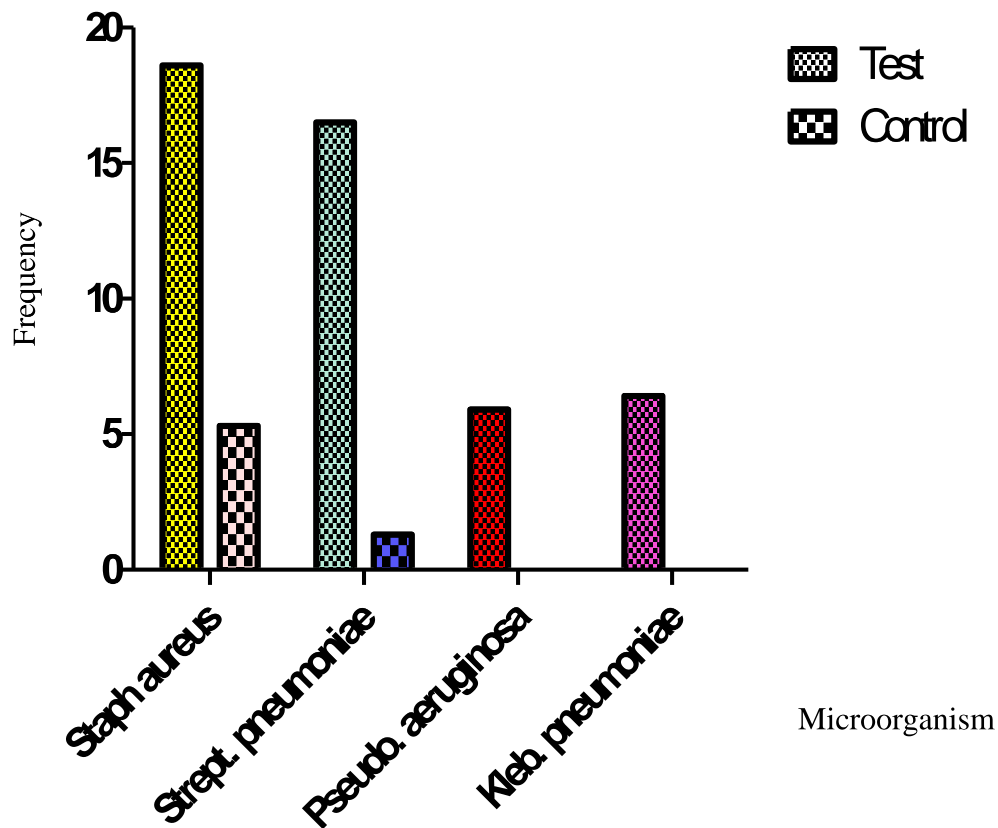

Figure 4.7: Prevalence of other microorganisms isolated from the sputum samples of the subjects.

Key:

Staph. aureus = *Staphylococcus aureus*

Strep. pneumoniae = *Streptococcus pneumoniae*

Pseudo. aeruginosa = *Pseudomonas aeruginosa*

Kleb. pneumoniae = *Klebsiella pneumoniae*

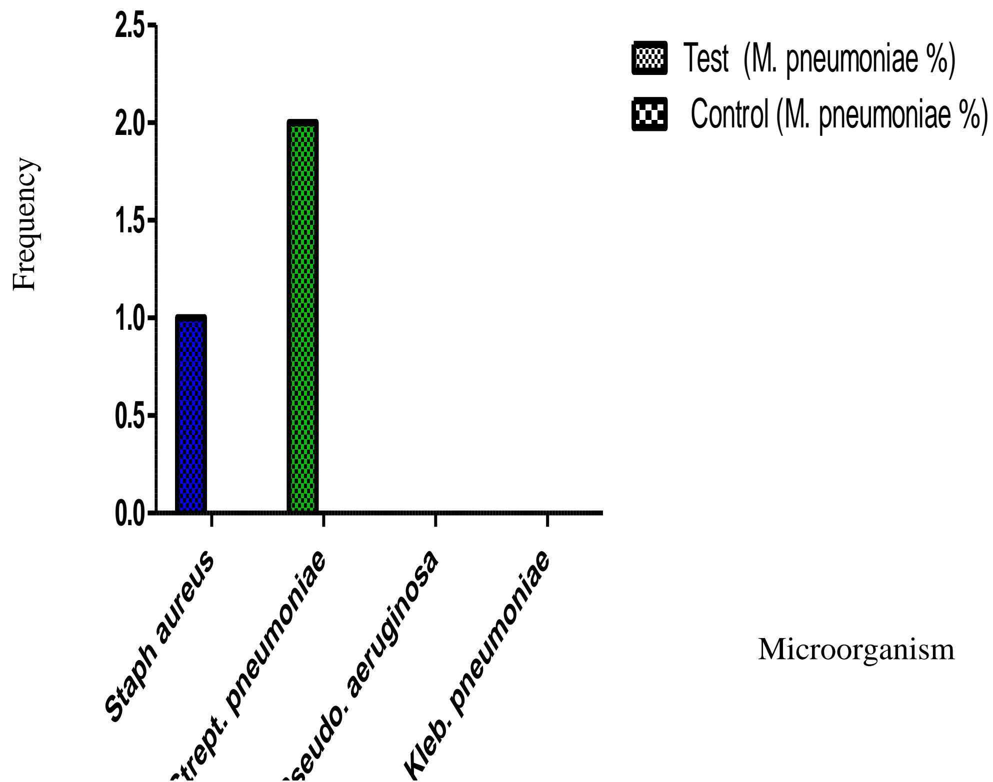

Figure 4.8: Co-existence of other bacteria isolates with *M. pneumoniae.*

Key:

Staph. aureus = *Staphylococcus aureus*

Strep. pneumoniae = *Streptococcus pneumoniae*

Pseudo. aeruginosa = *Pseudomonas aeruginosa*

Kleb. pneumoniae = *Klebsiella pneumoniae*

In Table 4.6, there was no significant relationship between *Staphylococcus aureus* infection and gender though the prevalence appeared to be higher in females than in

males. Table 4.6 also shows that *Staphylococcus aureus* was higher in male than in female among the control group. The prevalence of *Staph aureus* appeared to be higher in age group 21-30 years (Table 4.7). However, it showed equal prevalence in age groups 11-20 years and 31-40 years (Table 4.7). *Staphylococcus aureus* was not found in age group 1-10 years and above 50 years of the control; however, it shows equal prevalence in all other age groups of control (Table 4.7).

The incidence of *Streptococcus pneumoniae* (*S. pneumoniae*) appeared to be higher in female 18 (58.1%) than in male 13 (41.9%). However, *S. pneumoniae* was not significantly related with gender (P>0.05) (Table 4.6). In control group, only one male subject had *S. pneumoniae* whereas no female had *S. pneumoniae.* The frequency of *S. pneumoniae* infection appeared to be more in younger age groups 1-10 years and 11 -20 years (Table 4.7). However, there was a little high tendency towards age group above 50 years (Table 4.7).

The prevalence of *Pseudomonas aeruginosa* (*P. aeruginosa)* in male was 6 (54.5%) while the prevalence in female was 5 (45.5%) (Table 4.6). Statistically, there was no significant different between *P. aeruginosa* infection and gender (P>0.05). *Pseudomonas aeruginosa* was not isolated from the control group. *Pseudomonas aeruginosa* appeared to have occurrence in active age groups 21 -30 years and 31-40 years, Middle Ages 41-50 years and in older ages 50years and above (Table 4.7). There was no incidence observed in younger age groups 1-10 years and 11 – 20 years (Table 4.7). There was no relationship between *P. aeruginosa* infection and age (*P*>0.05).

The prevalence of *Klebsiella pneumoniae (K. pneumoniae)* in male was 7(58.3%) and in female 5(41.7%) (Table 4.6). There was no significant difference between *Klebsiella pneumoniae* infection and gender (P>0.05). However, no *Klebsiella pneumoniae* was isolated from the control group. *Klebsiella pneumoniae* appeared to be evenly distributed among the various age groups (Table 4.7). Statistically, there was no relationship between *K. pneumoniae* and age range (Table 4.7).

Table 4.6: Prevalence of other bacteria in relation to gender

Organism	Male N (5)	Female N(5)	Total	χ^2	P value
S. aureus	16(45.7)	19(54.3)	35	1.24	7.815
S. pneumoniae	13(41.9)	18(58.1)	31		
P. aeruginosa	6(54.5)	5(45.5)	11		
K. pneumoniae	7(53.3)	5(41.7)	12		
Control					
S. aureus	3 (75)	1 (25)	4	10.000	12.592
S. pneumoniae	1 (100)	0 (0)	1		
P .aeruginosa	0 (0)	0 (0)	0		
K .pneumoniae	0 (0)	0 (0)	0		

P > 0.05

Table 4.7: Prevalence of other bacterial isolates among the subjects in relation to age.

Age group (Years)	Staph. aureus		Strep. pneumoniae		Pseudo. aeruginosa		Kleb. pneumoniae	
	Test	Control	Test	Control	Test	Control	Test	Control
1-10	1	0	8	0	0	0	1	0
11-20	8	1	9	0	0	0	2	0
21-30	12	1	3	1	4	0	3	0
31-40	8	1	3	0	3	0	3	0
41-50	4	1	2	0	3	0	2	0
> 50	2	0	6	0	1	0	1	0
Total	**35**	**4**	**31**	**1**	**11**	**0**	**12**	**0**

Staphylococcus aureus co-existed with *Streptococcus pneumoniae* in six subjects. There was also co-existence between *S. aureus* and *Pseudomonas aeruginosa* in one subject while it co-existed with *Klebsiella pneumoniae* in two subjects (Table 4.8). *S. aureus* showed no co-existence with other bacteria in the control group.

The specificity of *M. pneumoniae* using culture method in this study is 96.7% while the sensitivity is 23.8% (Table 4.9).

Table 4.8: Co-existence of *Staphylococcus aureus* with other bacterial isolates.

Staphylococcus aureus		
Other organisms	**Test subjects**	**Control subjects**
Streptococcus pneumoniae	6	0
Pseudomonas aeruginosa	1	0
Klebsiella pneumoniae	2	0
Total	8	0

Table 4.9: Sensitivity and specificity of culture and PCR

		PCR RESULT	
		Positive n (%)	Negative n (%)
Culture Result	Growth	5 (23.8)	8 (3.3)
	No Growth	16 (76.2)	234 (96.7)

The specificity of culture method = 96.7%.

The sensitivity of culture method = 23.8%.

CHAPTER FIVE

DISCUSSION

In this study, it was observed that 13 out of 263 and 21 out of 263 patients visiting the DOTS' clinic (Directly Observed Treatment, Short Course clinic) at the Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State were positive for *Mycoplasma pneumoniae* by culture and PCR methods respectively, thus giving overall prevalence rates of 5.9% and 9.6% respectively. The result is higher than that obtained by Naoyuki and Atsushi (2004), who reported a prevalence rate of 4.8% for *M. pneumoniae* and 5.1% obtained in a study in Nigeria by Oluwa *et al.,* (2015), also 1.6% reported in a study in South African by Carrim *et al*., (2018) but is lower than that obtained in a study done in Zaria, Northern Nigeria which showed an overall prevalence of 16.2% (Macfarlens *et al.,* 1979). This result is also lower than the 19.5% reported by Tsutomu *et al.,* (2006), 27% by Anna *et al*., (2008), 32% by PCR reported by Fang-chang *et al.,* (2007), 52% by Maczynska *et al.,* (2002) and 12% by Bjarnason *et al*., (2018).

However, our result is comparable with the findings of some other workers: Dorigo-Zetsma *et al.,* (1999) who reported 8% prevalence rate for *M. pneumoniae* by PCR and 7% by culture, Csango *et al*., (2004) with overall prevalence of 9.8% and Fang-Chang *et al,* (2008) who reported an overall prevalence rate of 6.9% for *M. pneumoniae* infection in children in Taiwan Republic of China.

This not withstanding, work done by different researchers on different categories of subjects showed varying rate of isolation/detection which may be attributed to different detection methods, cultural techniques, sensitivities of isolation media and categories of subjects used. Considering the prolong period of incubation and fastidious nature of Mycoplasma, Krause and Taylor-Robinson (1992) reported that failure to meet their nutritional needs could limit successful culture.

The finding that more *M. pneumoniae* was detected from test subjects is in total disagreement with the work done by Dorigo –Zetsma *et al*., (1999), who reported that all the 74 controls they sampled were both culture and PCR negative, although they worked on a different sample type (Throat swab).

Sex distribution of *M. pneumoniae* among the subjects showed that the overall prevalence rate of the organism was more among the females 13 (9.4%) than males 8(6.5%) by PCR and 8(5.8%) and 5(4.0%) respectively for females and males by culture. Statistically, there is no significant relationship between *M. pneumoniae* infection and gender, (P>0.05). This result agrees with the findings of some other workers: Foy *et al*., (1966), Noah, (1974) and Markham (1979) who reported that there was little or no reason to suspect that males and females have greatly differing susceptibilities to *M. pneumoniae* infections. Our result is also in agreement with the findings of these researchers: Chung *et al*., (2015) and Deng *et al*., (2018) who reported that there was no significant difference between *Mycoplasma pneumoniae* infection and gender.

Age distribution of *Mycoplasma pneumoniae* in this study showed that *M. pneumoniae* was isolated more in adults and elderly than in children. The result is statistically significant (P <0.05). This shows that there is a significant relationship between *Mycoplasma pneumoniae* infection and age. This result is in agreement with the findings of Marston *et al;* (1997) who reported that *M. pneumoniae* was definitely responsible for 32.5% of 2,776 cases of community-acquired pneumonia in hospitalized adults in a two-county region of Ohio, USA. They also had an additional striking finding that the incidence of pneumonias due to *M. pneumoniae* in hospitalized adults increased with age and it was second only to *Streptococcus pneumoniae* in elderly people. Our result is also in agreement with another study of hospitalized adults with community-acquired pneumonias performed in Isreal (Porath *et al*, 1997) and that performed in South Africa

(Carrim *et al*., 2018). On the contrary, this result is in total disagreement with older studies which relied upon serology and culture and reported *M. pneumoniae* pneumonia to be somewhat uncommon in children aged less than 5 years and greatest among school-aged children 5 to 15 years of age with a decline after adolescence and on into adult hood (Foy *et al*., 1966; Foy *et al*., 1979; Alexander *et al*., 1996). Our result is also in disagreement with the work of Kim *et al*., (2015) who reported that *M. pneumoniae* pneumonia had been more in older children including adolescents.

The virulence gene GLPQ gene formerly called MPN 420 was detected in 5 out of the 18 PCR-positive patients giving a rate of 27.8%, the finding is supported by the work of Schmidi *et al*, (2011) who stated that GLPQ gene is the only active glycerophosphodiesterase in *M. pneumoniae* and the active glycerophosphodiesterase is required for full gliding velocity of the bacteria. They finally concluded that the active GLPQ gene is required for maximal growth in the presence of glucose, whereas its absence does not interfere with the slow growth in the presence of glycerol.

The *M. pneumoniae* isolated and detected from this study were observed to be more predominant from symptomatic subjects with various complaints (86%) than asymptomatic (14%). Infact, all the test subjects had different types of symptoms. This result is in agreement with the findings of some other workers who reported that those infected with *M. pneumoniae* develop symptoms gradually over a period of several days, often persisting weeks to months (Luby, 1991 and Dorigo-Zetsma *et al.,* 2001). On the contrary, *M. pneumoniae* from the control subjects were observed from those without any complaints that is to say they were asymptomatic. This is in agreement with the work of Dorigo-Zetsma *et al*., (2001) who found that among the 79 asymptomatic house hold

contacts of 30 index cases with acute respiratory tract infection due to *M. pneumoniae,* 15% harboured the organism.

The most common complaints (symptoms) observed among the patients who had respiratory tract infection due to *M. pneumoniae* in this study were cough followed by chestpain and cough. Other complaints included sorethroat, sorethroat and cough, and chestpain, sorethroat and cough concurrently. It was observed that *M. pneumoniae* respiratory tract infection in this study had relationship with symptoms as almost all those who were positive to *M. pneumoniae* had one symptom or more. This is statitistically significant, P-value = 0.00, ($P < 0.05$). This shows that there is a relationship between *M. pneumoniae* infection and symptoms. This is in agreement with the work of the following: Steven *et al*., (1978); Luby, (1991); Ferwerda *et al*., (2001) and Talkington *et al.,* (2001) who reported that most common manifestation of patients with *M. pneumoniae* respiratory tract infection include sorethroat, hoarseness, fever, cough which is initially nonproductive but later may yield small to moderate amounts of nonbloody sputum, headache, chills, coryza, Myalgias, earache and general malaise.

M. pneumoniae isolated from this study were predominantly from patients that presented with cough and chest pain which may likely lead to development of bronchopneumonia. This is supported by the work of Luby, (1991) and Ferwerda *et al*., (2001) who reported that older children aged 5 to 15 years with respiratory tract infection due to *M. pneumoniae* are more likely to develop bronchopneumonia involving one or more lobes, sometimes requiring hospitalization. Another work by Cassel *et al.,* (1985) stated that about 3 to 10% of infected adults develop bronchopneumonia involving one or two lobes. *M. pneumoniae* respiratory tract infection, also causes up to 5% of cases of bronchiolities in young children (Dowdle *et al.,* 1967 and Denny *et al.,* 1971). Sorethroat was also

among the complaints of the patients studied in this work and this is supported by the work of Esposito *et al*., (2002), who reported that inflammation of throat was observed in patients having respiratory tract infection due to *M. pneumoniae* especially in children. Respiratory tract infection due to *M. pneumoniae* should not be treated lightly but should be taken serious because negligence could lead to death if the organism is allowed to disseminate throughout body (Talkington *et al*., 2001).

The 27.8% prevalence rate of the virulent gene, GLPQ, detected from *M. pneumoniae* positive samples in this study though not statistically significant (P>0.05), indicates that not all the positive samples possess this enzyme. This enzyme was totally lacking in the control subjects that were *M. pneumoniae* positive. This fact probably shows the importance of the enzyme in disease causation. This finding is supported by the work of Schmidi *et al.,* (2011) who stated that GLPQ gene is the only active glycerophosphodiesterase in *M. pneumoniae* and the active glycerophosphodiesterase is required for full gliding velocity of the bacteria. These investigators also reported that *M. pneumoniae* is unable to cause any detectable damage to the host cells in the absence of GLPQ. They further observed that in addition to the enzymatic activity of GLPQ, it is also involved in the control of expression of several genes, among them the glycerol transporter. They thus concluded that GLPQ is central of the normal physiology and pathogenicity of the pathogen, *M. pneumoniae.*

The antibiotic sensitivity test conducted on the isolates of *M. pneumoniae* in this study revealed that *M. pneumoniae* was highly susceptible to Lyntriaxone, Ciprofloxacin, Levofloxacin and Azithromycin. The organism showed moderate susceptibility to Doxycycline. However, *M. pneumoniae* showed resistance to Rifampicin, Septrin, Peflacine, Erythromycin and Norbactin. This is in agreement with the finding of Waites *et*

al., (2003a) and (2003b) who reported that *M. pneumoniae* is inhibited by tetracyclines, macrolides, ketolides and fluoroquinolones. Macrolides include Azithromycin, Clarythromycin etc. Fluoroquinolones include Levofloxacin, Ciprofloxacin, Ofloxacin, gatifloxacin, gemifloxacin, garenoxacin, ketolides eg cethromycin. Our discovering is in line with the work of recent studies that evaluated macrolides and reported that tetracyclines and fluoroquinolones indicated comparable in-vitro activities against isolates of *M. pneumoniae* (Niitu *et al*., 1974; Bebear *et al.,* 1993 and Bebear, 2002).

Other microorganisms isolated from the patients in this study include *Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* These organisms were isolated more in the test subjects than in the controls. One isolate of *Staphylococcus aureus* and two isolates of *Streptococcus pneumoniae* were found co-existing with *M. pneumoniae.* None of the few bacterial isolates from the control subjects co-existed with *M. pneumoniae.* It was observed that those subjects having co-infection of *M. pneumoniae* with other bacteria had severe pneumonia than those having *M. pneumoniae* infection singly. This notwithstanding was indicated in the symptoms presented by the subjects as those with co-infection of *M. pneumoniae* and other bacteria presented with two or more severe symptoms/complaints than those without co-infection. Bezerra *et al.,* (2011) reported that 19.3% of the total infection in children less than 5 years old presented with two or more pathogens. Children with co-infection were also observed to have relatively more severe clinical symptoms and are more prone to suffer wheezing and shortness of breathe which also puts them at risk for developing severe pneumonia (Berkley *et al*., 2010). Bisno, (2001), stated that up to 15% of acute pneumonia may be caused by *Streptococcus pneumoniae, Haemophilus influenza* and *Corynebacteria diphthera.*

In community- acquired pneumonias, the most common bacterial agent is *Streptococcus pneumoniae (*Bisno, 2000*).* Others that cause atypical pneumonia are caused by such agents as *M. pneumoniae*, Chlamydia spp, Leginonella, *Coxiella burnetti* and viruses. These organisms cause broncholitis and bronchitis (Dasaraju and Liu, 1996).

Nosocomial pneumonias and pneumonias in immnosuppressed patients have Gram negative organism and *Staphylococcus aureaus* as predominant organisms (Dasaraju and Liu, 1996).

Staphylococcus aureus had the highest prevalence among the bacterial isolates followed by *Streptococcus pneumoniae* in both test and control groups. There was no significant relationship between *Staphylococcus aureus* infection and gender (P > 0.05) though it occurred higher in females than in males. This is also applicable in *Streptococcus pneumoniae* infection. However, opposite is the case with *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* respiratory tract infection as they were isolated more in males than in females. The result is not also statistically significant (P>0.05). There was no *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* isolated from the control group. This is in agreement with the findings of Guilde lines, 2011 that the overall incidence rates of 14.4% per 10,000 in children aged 0-16 years per annum and 33.8% those aged ≤ 5 years but disagrees with their report that boys were more affected than girls as were children born between 24-28 weeks compared to those born at terms. This is comparable to the work of Oosterheert and Andy, (2013) who reported that *Staphylococcus aureus* causes community -acquired pneumonia in all age groups with 60% occurrence in higher age groups. This could be attributed to the fact that these are active ages when people interact and mingle with a lot of people. The organism also occurred moderately among the age group above 50 years. This could be attributed to low immunity level as immunity

decreases as age increases. There was no significant relationship between *Staphylococcus aureus* respiratory tract infection and age (P>0.05).

The reverse is the case of *Streptococcus pneumoniae* respiratory tract infection as the organism was isolated more in younger ages (in children and adolescents) between age group 1-20 years than in adults though it also showed a moderately high prevalence in the elderly (age group above 50 years). Statistically, the result is not significant (P>0.05). This is in agreement with the work of Korppi *et al*., (1993) and Juven *et al*., (2000) who reported that *Streptococcus pneumoniae* is the primary cause of community-acquired lower respiratory infections among children accounting for 12%-45% cases. The most common bacteria responsible for acute sinusitis are *Streptococcus pneumoniae, Haemophilus influenza, Moraxella catarrhalis* and *Stapholoccus aureus (*Dasaraju and Liu, 1996). *M. pneumoniae* and *M.* hominis have been associated with acute pharyngitis (Dasaraju and Liu, 1996).

Pseudomonas aerugnosa was not isolated from children and adolescent, that is from age group 1-20 years. However, the organism occurred almost equally in age groups 21-50 years and above 50 years. Klebsiella on the other hand, occurred equally among all ages except ages 5-20 years. It has been discovered that respiratory pathogens such as *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Haemophilus influenza* can also cause Otitis media (Dasaraju and Liu, 1996). This is in agreement with the work of Dasaraju and Liu, (1996) who reported that *Klebsiella pneumoniae* are more common among patients over 50 years old who have chronic obstructive lung disease or alcoholism. *Pseudomonas aeruginosa* infections are increasingly associated with acute exacerbations in chronic obstructive pulmonary disease (COPD), (Muphy *et al*., 2008).

Klebsiella pneumoniae has also been implicated to cause acute exacerbation of chronic obstructive pulmonary disease (Lin *et al.,* 2007).

Implication

Detection of *M. pneumoniae* in the respiratory tract has been associated with lung abnormalities including reduced pulmonary clearance and air way hyper responsiveness (Marc *et al.,* 2000). Marc *et al*., (2000) also reported abnormality in pulmonary function tests in up to 50% of children and Kim *et al.,* (2000) described abnormal computerized axial tomography studies for 37% of children months to years after an episode of *M. pneumoniae* respiratory tract infection thus establishing the ability of Mycoplasma to induce Chronic and possibly permanent lung damage long after resolution of respiratory tract symptoms. *M. pneumoniae* is known to induce a number of inflammation mediators implicated in the pathogenesis of asthma that may play a role in exacerbation and the complications and consequences associated with neglected or undetected *M. pneumoniae* in respiratory tract have been documented (Seggey *et al.,* 1986 and Shimozu *et al*., 1991).

Limitations

The weaknesses of our study were difficulty in obtaining sputum specimens from children and also small sample size of the control group. The control group did not have any symptom (s) of respiratory tract infection like cough, sorethroat etc. This made it difficult for some of them to produce sputum until they were induced with saline-mist. Some of the control subjects were not able to produce sputum even after induction with saline-mist.

Conclusion

M. pneumoniae was successfully isolated from the respiratory tract of the test and control subjects. The sensitivity and specificity of culture method to PCR method in this study were 23.8% and 96.7% respectively. PCR is recommended for *M. pneumoniae* as the best

method for detecting the organism as it detects the DNA of the organism overcoming so many limitations of culture and serology methods. *M. pneumoniae* is very fastiditious and has a long incubation period. These characteristics make it difficult and laborious to be isolated by culture as the organism at times lost its viability before growth is observed on culture media. PCR, however, overcomes this problem.

In view of the various roles *M. pneumoniae* plays in the outcome of several respiratory tract diseases and extrapulmonary complications, there is need to widen the scope of the laboratory investigation. Most Mycoplasma infections in clinical settings never have a microbiological diagnosis because rapid, sensitive, specific and reasonably price methods for its detection are not readily available in many hospitals and laboratories. Respiratory tract samples should not be spared the right of isolation of *M. pneumoniae* by putting in place all the necessary laboratory procedures required for its isolation, characterization and identification.

In conclusion, *M. pneumoniae* is an additional bacterium that might contribute to respiratory tract infections and consequently to death when it disseminates to various organs of the body, hence their presence in the respiratory tract of children, adolescent and adults should not be treated with levity.

Recommendation

PCR is recommended as the best method of detection of *M. pneumoniae* from respiratory tract as it offers improvements in sensitivity, specificity and rapidity over culture. However, there is need for development of cheap, widely available and reproducible diagnostic techniques suitable for our country.

It is also recommended that a reliable and user-friendly amplified or non-amplified method for detection of this *M. pneumoniae* or its nucleic acid in clinical specimens

would be of immense importance for patients' diagnosis and management for furthering knowledge of a potential role of this organism in chronic lung diseases.

Further studies should also cover aspects of extrapulmonary human specimens such as blood, synovial fluid, cerebrospinal fluid, pericardial fluid and skin lesions to enhance understanding of how *M. pneumoniae* can disseminate throughout the body and cause serious and at time fatal extrapulmonary complications. This will strengthen knowledge of the epidemiology trend of *M. pneumoniae* as a disease-causing agent.

Contribution to knowledge

This study has succeeded in detecting *M. pneumoniae* from sputum samples in the study population; an organism that is very fastidious and difficult to grow culturally.

This is the first report of the detection of the virulence gene (GLPQ gene) of *M. pneumoniae* in this country, Nigeria. Other studies done identified *M. pneumoniae* in various specimens using culture and PCR, or serology or PCR alone, without further discovering the virulence factors responsible for its pathogenicity. It is furthermore recommended a wider study of *M. pneumoniae* as agent of respiratory tract infections be carried out to cover other aspects of human specimens like nasopharngeal washing, throat swab, bronchial washing and lung biopsy. Sputum though, is the best specimen.

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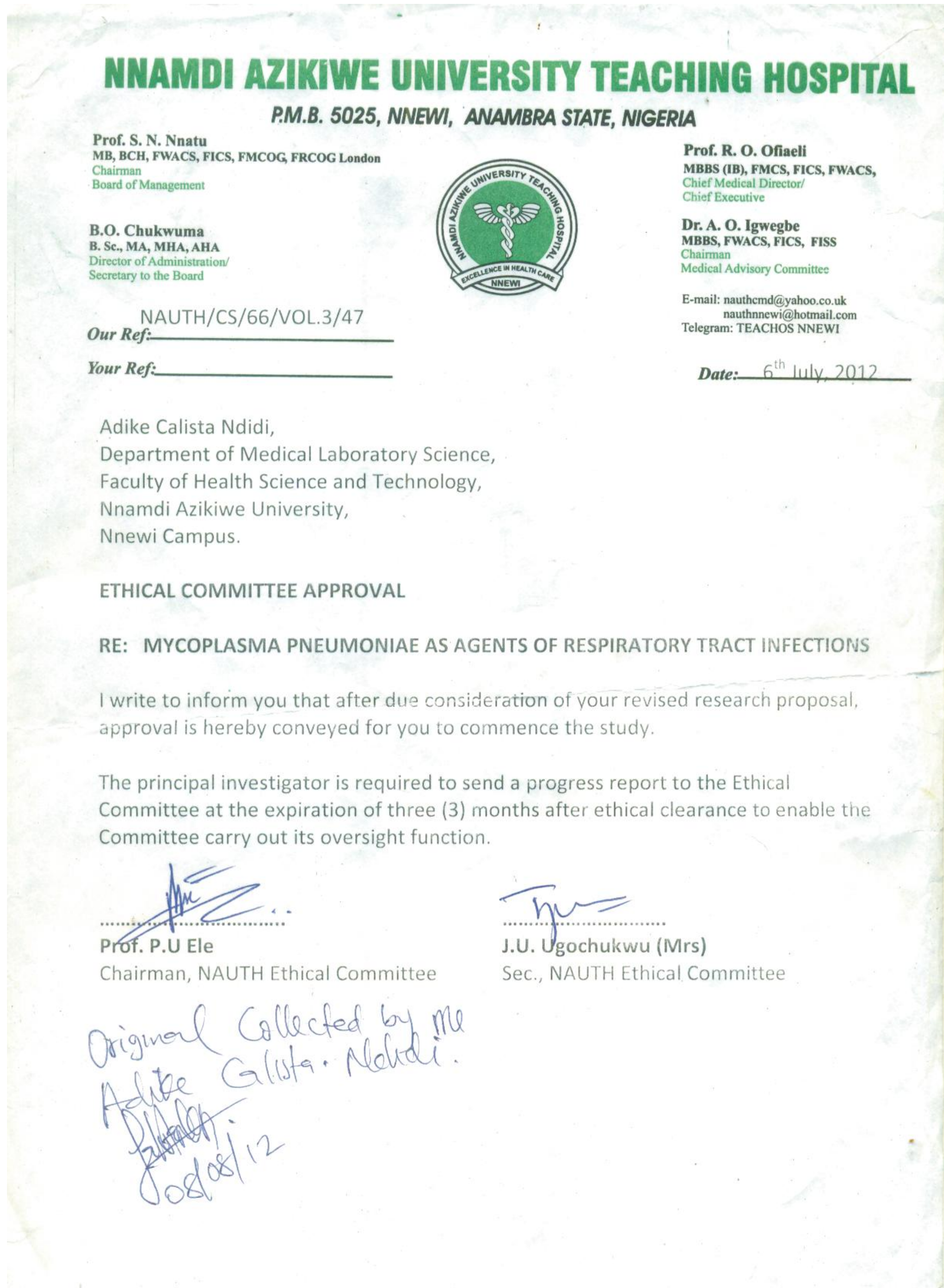
APPENDICES

APPENDIX I: ETHICAL APPROVAL



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Date: 6th July, 2012

Adike Calista Ndidi,
Department of Medical Laboratory Science,
Faculty of Health Science and Technology,
Nnamdi Azikiwe University,
Nnewi Campus.

ETHICAL COMMITTEE APPROVAL

RE: MYCOPLASMA PNEUMONIAE AS AGENTS OF RESPIRATORY TRACT INFECTIONS

I write to inform you that after due consideration of your revised research proposal, approval is hereby conveyed for you to commence the study.

The principal investigator is required to send a progress report to the Ethical Committee at the expiration of three (3) months after ethical clearance to enable the Committee carry out its oversight function.

Prof. P.U Ele
Chairman, NAUTH Ethical Committee

J.U. Ugochukwu (Mrs)
Sec., NAUTH Ethical Committee

Original Collected by me
Adike Calista. Ndidi.
08/08/12

APPENDIX II: INFORMED CONSENT FORM

Dear respondent,

My name is Adike Calista Ndidi (Mrs), a post graduate student of the Dept. of Medical laboratory science, faculty of Health Science and Technology, Nnamdi Azikiwe University, Nnewi Campus. I am carrying out a study on the topic: *Mycoplasma pneumoniae* as agents respiratory tract infections.

Purpose of Study: Little information is available especially here in Nigeria regarding the prevalence of *Mycoplasma Pneumoniae* in causing respiratory tract infections in all ages and sexes. This study will help to create more awareness on the role played by the organisms so that the detection and isolation could be incorporated into routing laboratory diagnosis. This in effect, will allow appropriate antibiotics treatment of patients since it is impossible to identity *M. pneumoniae* infection solely on the basis of clinical signs and symptoms. Subsequently, this will influence the patient's outcome by reduction of morbidity and mortality.

Procedure: This will include asking the respondents (adult subjects) and the parents of the children some questions via questionnaires. The pediatrician will get the information needed from the parents of the children and also sputum will be collected from each respondent using sterile universal container.

Participation: Participation is not compulsory. You are free either to participate or not in this research. You are also free to withdraw anytime you choose to. Participation is voluntary and no payment will be made to the participants as this project is not paid for. It is strictly for research / academic purpose.

Confidentiality: I wish to assure you that every information in your respondent form will be kept confidential. Again your name is not needed in this study.

Risk/Benefit: There are no known risks in participating in this study.

Consent:

Now that this research has been well explained to me and I fully understand the purpose involved, I shall be willing to volunteer myself or my child or ward to take part in the program.

Signature of subject……………………………………….

Date:………………………………………………………

Researchers Signature……………………………………

Date:…………………………………………………………

Phone No:…………………………………………………..

APPENDIX III: QUESTIONNAIRE

Questionaire for the Research on the Comparative Evaluation of Cultural and Molecular Identification Methods of *Mycoplasma pneumoniae* from Sputum Samples of Subjects Attending Pulmonary Clinic in Nnewi.

1. What is your age?

   (a) 1 – 10

   (b) 11 – 20

   (c) 21 – 30

   (d) 31 – 40

   (e) 41 – 50

   (f) Above 50

2. Indicate your gender (sex)

   (a) Male

   (b) Female

3. Do you have cough?

   Yes / No

4. If yes, how long?

   a) 3 – 6 day

   b) 1 – 2 weeks

   c) More than 2 weeks

5. Are you breathing fast?

   Yes / No

6. Do you sleep under;

   a. Fan

   b. Air conditioner (AC)

   c. Both

   d. None.

7. Do you have;

a) Sore throat

b) Chest pain

c) Sore throat and Chest pain

d) None.

8. Are you on antibiotic therapy?

Yes / No

9. If yes, how long?

a) 1 – 6 days

b) 1 – 2 weeks

c) More than 2 weeks

Thanks for answering truthfully.

APPENDIX IV: PREPARATION OF SALINE MIST

Sodium chloride	5g
Distilled water	100ml

Dissolve 5g of sodium chloride in 100ml of distilled water. Labeled and store in a cool place at room temperature.

APPENDIX V: MEDIA COMPOSITION AND PREPARATION

PREPARATION OF PPLO BROTH

Beef heart, infusion from	250.ogm
Peptic digest of animal tissue	10.ogm
Sodium chloride	5.0gm
Crystal violet	0.001
PH	7.8
Distilled water	700.0ml

21g of the PPLO broth was dissolved in 700ml of distilled water. This was gently heated to dissolve the medium completely. This was sterilized by autoclaving at 121°C for 15minutes after all the trapped air had been expelled through the air cock. It was then allowed to cool to 45°C and 300ml of mycoplasma enrichment supplement was aseptically added. This was mixed and dispensed into aliquots of 5.0ml into sterile screw capped bijou bottles.

APPENDIX V CONTINUED

PREPARATION OF PPLO AGAR

Beef heart, infusion from	250.ogm
Agar	15.ogm
Peptic digest animal tissue	10.ogm
Sodium chloride	5.ogm
PH	7.8
Distilled water	1000 ml

36g of the agar was dissolved in 1000ml of distilled water. This was gently heated to dissolve the medium completely. This was then autoclave at 121°C for 15 minutes. It was allowed to cool to 50°C. Finally 10 vials of mycoplasma enrichment supplement was aseptically added to the medium and the medium was properly mixed and about 10ml was poured into mini-Petri dishes. The agar plates were allowed to set.

APPENDIX V CONTINUED

PREPARATION OF TRYPTOSE SOY BROTH

Tryptose soy broth	4.ogm
Distilled water	100.0ml

The broth was dissolved in water and dispensed in aliquots of 5.0ml into screw-capped bijou bottles. These were autoclaved at 121°C for 15 minutes.

APPENDIX V CONTINUED

PREPARATION OF BLOOD AGAR

Nutrient agar	14.0g
Distilled water	500.0ml

The agar was dissolved in the distilled water and sterilized by autoclaving at 121° C for 15 minutes after all the trapped air had been expelled through the aircock was allowed to cool to 50°C and about 50.0ml of sterile human blood was aseptically added. This was well mixed and poured into 8.5cm sterile Petri dishes. The agar plates were allowed to set.

APPENDIX V CONTINUED

PREPARATION OF CHOCOLATE AGAR.

Nutrient agar	14.0g
Distilled water	500.0ml

The agar was dissolved in the distilled water and sterilized by autoclaving at 121°C for 15 minutes. It was allowed to cool to 50°C and about 50.0ml of sterile human blood was aseptically added. This was well mixed and the medium was heated in a 70°C water bath for 10 minutes until it turned brown in colour (chocolate colour). The medium was cooled to 50°C and remixed and dispensed in 8.5cm sterile petri dishes and allowed to set.

APPENDIX V CONTINUED

PREPARATION OF MACCONKEY AGAR.

Peptic digest animal tissues	20.0g
Agar	15.0g
Lactose	10.0g
Sodium chloride	5.0g
Bile salts	1.50g
Neutral red	0.05g
Crystal violet	0.001
PH	7.2
Distilled water	100.oml

51.6g of the medium was dissolved in 1000.oml distilled water. This was gently mixed to dissolve completely. It was then autoclaved at 121°C for 15 minutes. This was cooled to 50°C and was aseptically poured into 8.5cm sterile Petri dishes and allowed to set.

APPENDIX VI: SALINE AGAR CONTAINING 1% SHEEP ERYTHROCYTES (HAEMOLYSIS TEST).

Composition of saline Agar base medium

Sodium Chloride	0.85g
Agar	1.50g
PH	7.2
Distilled water	100ml

2.35g of the medium was dissolved in 100ml of distilled water. This was autoclaved at 121°C for 15 minutes. After cooling to 50°C, about 10ml of 1% sheep erythrocytes was added to it and aseptically poured into Petri dishes and allowed to set.

APPENDIX VII: REAGENTS, MATERIAL AND EQUIPMENT FOR PCR OF *M. pneumoniae* DNA DETECTION.

1. Sputum lysis buffer

1. Sterile Distilled water
2. ATL bufferAE buffer (TE buffer)
3. 10ml tubes
4. 200 ml pipettes
5. pulse vortex machine
6. stop watch
7. Proteinase K (PH.7.2, Conc. 20µg/µL)
8. 96% Ethanol
9. 2ml Eppendorf – tubes
10. Sterile 1.5 ml microcentrifuge tube
11. Thermoscientific Gene – Jet Genomic DNA purification Kit.
12. Vortex Machine

1.5ml Microcentrifuge tube

APPENDIX VIII: EQUIPMENT/REAGENTS AND MATERIAL USED FOR PCR OF GLPQ GENE.

1. Electrophoresis machine / power supply consort (300 – 500MA) E835.
2. Electrophoresis Tank
3. UV rays machine (Ultra Violet ray)
4. Agarose Plate with comb
5. Camera (Victoria) Connected / Installed
6. PCR machine (applied Biosystem 2720 thermal cycler)
7. Eppendorf Tubes connected to the computer to trap or capture bands / base pair to be visualized.
8. TBE buffer (Tris Boric EDTA buffer) (Alpha Lab, UK)
9. ATCC 29342D *Mycoplasms pneumoniae* "DNA CONTROL STANDARD" from American Type Culture Collection (ATCC)

10 Agarose powder (Alpha Lab, UK)

APPENDIX IX: GRAM STAIN

Commercially prepared stains were used and they include: Crystal violet, lugol's iodine, Acetone and Neutral red.

Method

A smear of the isolate was made on a slide .The smear was fixed by passing through the flame 3 times. The primary stain, Crystal violet was poured to cover the smear and this was allowed to stay for 60 seconds. This was washed with water. The smear was then covered with Lugol's iodine for 60 seconds and washed with water.

The smear was rapidly decolorized with acetone and washed off with water. The smear was covered with Neutral red stain for 2 minutes. This was then washed off with water and the slide allowed to drain. It was examined with oil immersion objective.

APPENDIX X: PREPARATION OF SODIUM DEOXYCHOLATE REAGENT (BILE SALT)

To make 20ml

Sodium deoxychocolate	2g
Sodium Chloride, 8.5g/l	20ml

The chemical was dissolved in the physiological saline. This was sterilized by autoclaving at 121°C for 15 minutes. This was allowed to cool and the bottle labeled and stored at 2-8°C. The reagent is stable for several weeks.

APPENDIX XI: PREPARATION OF MIU MEDIUM (MOTILITY INDOLE UREA MEDIUM).

Trytone	30g
Potassium dihydrogen phosphate (KH_2PO_4)	1g
Sodium Chloride	5g
Agar	4g
Phenol red 2.5g/l (0.25%) w/v	2ml
Distilled water	1 litre

The dry ingredient was mixed in 1litre (1000ml) of distilled water and heated to 100^oC to dissolve the chemicals. This was allowed to cool to 50^oC and then phenol red solution was added. This was well mixed. The mixture was dispensed in 95ml amount in screw-cap bottles. The content was sterilized with the cap loosened by autoclaving at 121^oC for 15 minutes. Then the medium was allowed to cool and the bottle caps tightened. This was labeled and stored in a cool dark place. The base medium can be stored for 6 months or longer providing there is no change in the appearance to suggest contamination or alteration of PH.

To make 20 tubes of MIU medium, 95ml of the base medium was melted by heating to 100^oC in a container of water.

This was allowed to cool to 50^oC and 5ml of sterile 40% W/V urea solution added and mixed well. This was dispensed aseptically in 5ml amounts in sterile screw-cap tubes. This was allowed to cool in an upright position. For use, an indole detection paper strip was placed in the neck of the tube and the bottle cap used to hold the paper in placed.

APPENDIX XII: PREPARATION OF OXIDASE REAGENT.

The reagent was prepared fresh before used and to make 10ml. Tetramethyl-p-phenylenediamine 0.1g.

Dihydro chloride.

Distilled water 10ml

The chemical was dissolved in the water and used immediately.

Appendix XIII: Cross tabulation of the Distribution of *M. pneumoniae* among the subjects according to symptoms/complaints.

SYMPTOMS * GROUP

Crosstab

			GROUP		Total
			TEST	CONTROL	
SYMPTOMS	Cough	Count	106	0	106
		% within SYMPTOMS	100.0%	0.0%	100.0%
		% within GROUP	56.4%	0.0%	40.3%
	Sore throat	Count	19	0	19
		% within SYMPTOMS	100.0%	0.0%	100.0%
		% within GROUP	10.1%	0.0%	7.2%
	Chest pain	Count	17	0	17
		% within SYMPTOMS	100.0%	0.0%	100.0%
		% within GROUP	9.0%	0.0%	6.5%
	Chest pain and cough	Count	18	0	18
		% within SYMPTOMS	100.0%	0.0%	100.0%
		% within GROUP	9.6%	0.0%	6.8%
	Sore throat and cough	Count	21	0	21
		% within SYMPTOMS	100.0%	0.0%	100.0%
		% within GROUP	11.2%	0.0%	8.0%
	Chest pain, sore throat and cough	Count	7	0	7
		% within SYMPTOMS	100.0%	0.0%	100.0%
		% within GROUP	3.7%	0.0%	2.7%
	Nil	Count	0	75	75
		% within SYMPTOMS	0.0%	100.0%	100.0%
		% within GROUP	0.0%	100.0%	28.5%
Total		Count	188	75	263
		% within SYMPTOMS	71.5%	28.5%	100.0%
		% within GROUP	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	263.000[a]	6	.000	.000		
Likelihood Ratio	314.428	6	.000	.000		
Fisher's Exact Test	290.053			.000		
Linear-by-Linear Association	187.722[b]	1	.000	.000	.000	.000
N of Valid Cases	263					

a. 2 cells (14.3%) have expected count less than 5. The minimum expected count is 2.00.

b. The standardized statistic is 13.701.

Appendix XIV: Cross tabulation of relationship between occurrence of GLPQ gene and gender.

GLPQ RESULT * Sex

Crosstab					
			Sex		Total
			FEMALE	MALE	
GLPQ RESULT	POSITIVE	Count	5	0	5
		% within GLPQ RESULT	100.0%	0.0%	100.0%
		% within Sex	3.6%	0.0%	1.9%
	NEGATIVE	Count	134	124	258
		% within GLPQ RESULT	51.9%	48.1%	100.0%
		% within Sex	96.4%	100.0%	98.1%
Total		Count	139	124	263
		% within GLPQ RESULT	52.9%	47.1%	100.0%
		% within Sex	100.0%	100.0%	100.0%

Chi-Square Tests						
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	4.547[a]	1	.033	.062	.040	
Continuity Correction[b]	2.823	1	.093			
Likelihood Ratio	6.463	1	.011	.062	.040	
Fisher's Exact Test				.062	.040	
Linear-by-Linear Association	4.530[c]	1	.033	.062	.040	.040
N of Valid Cases	263					

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.36.

b. Computed only for a 2x2 table

c. The standardized statistic is 2.128.

P – Value = 0.03

:. $P < 0.05$

APPENDIX XV: RAW RESULTS OF THE TEST SUBJECTS

S/N	Age	Gender	Sympto-ms	PPLO Agar culture results	Antibiogram of *M. pneumoniae* isolates	PCR result	Tetrazolium reduction test	Gram reaction	Other isolates	GLP Q gene result
1	Above 50	F	Cough	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
2	31-40	F	Cough	NMI	-	Neg	-	GNB	*Pseudo. aeruginosa*	-
3	41-50	F	Cough	NMI	-	Neg	-	GPC**	*Strep. pneumoniae*	-
4	21-30	M	Cough, sore throat	NMI	-	Neg	-	-	NSG	-
5	21-30	M	Cough	NMI	-	Neg	-	-	NSG	-
6	11-20	M	Cough	MNI	-	Neg	-	-	NG	-
7	40-50	F	Sore throat	NMI	-	Neg	-	GPC**	*Strep. pneumoniae*	-
8	1-10	M	Cough	NMI	-	Neg	-	GPC**	*Strep. pneumoniae*	-
9	31-40	F	Cough, sore throat	NMI	-	Neg	-	-	NSG	-
10	21-30	F	Cough	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
11	21-30	M	Cough, sore throat	NMI	-	Neg	-	-	NG	-
12	21-30	M	Chest pain, cough	NMI	-	Neg	-	-	NSG	-
13	21-30	M	Sore throat, cough	NMI	-	Neg	-	GNB	Pseudo. aeruginosa	-
14	21-30	F	Cough	NMI	-	Neg	-	-	NG	-
15	31-40	M	Cough, sore throat	NMI	-	Neg	-	-	NSG	-
16	1-10	F	Cough	NMI	-	Neg	-	GPC**	Strep. pneumoniae	-
17	11-20	M	Sore throat, cough	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
18	1-10	F	Cough	NMI	-	Neg	-	-	NSG	-
19	21-30	M	Cough, sore throat	NMI	-	Neg	-	-	NSG	-
20	31-40	M	Cough, chest pain, & sore throat	NMI	-	Neg	-	GNB	*Kleb. pneumoniae*	-
21	21-30	M	Cough	NMI	-	Neg	-	GPC*	*Staph aureus*	-
22	1-10	M	Cough	NMI	-	Neg	-	GNB	*Kleb. pneumoniae*	-
23	11-20	F	Cough	NMI	-	Neg	-	GPC**	*Strep. pneumoniae*	-
24	21-40	F	Sore throat	NMI		Neg	-	GPC*	*Staph. aureus*	-
25	41-50	F	Cough	NMI	-	Neg	-	-	NG	-
26	21-30	F	Sore	NMI	-	Neg	-	-	NSG	-

APPENDIX-XV CONTINUED

			Throat							
27	41-50	F	Cough	NMI	-	Neg	-	-	NSG	-
28	31-40	F	Cough	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
29	21-30	F	Sore throat	NMI	-	Neg	-	GNB	*Pseudo. aeruginosa*	-
30	11-20	M	Cough	NMI	-	Neg	-	GPC**	*Strep. pneumoniae*	-
31	41-50	F	Cough	NMI	-	Neg	-	-	NSG	-
32	Above 50	F	Chest pain	NMI	-	Neg	-	GNB	*Pseudo. aeruginosa*	-
33	21-30	M	Sore throat, cough	NMI	-	Neg	-	GPC**	*Strep. pneumoniae*	-
34	Above 50	M	Cough, sore throat	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
35	21-30	M	Cough, sore throat	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
36	21-30	F	Cough	NMI	-	Neg	-	-	NSG	-
37	31-40	F	Cough	NMI	-	Neg	-	-	NSG	-
38	21-30	F	Cough	*Mycoplasma pneumoniae*	AZ^{+++},CIP^{+++}, LYN^{+++}, LEV^{+}, RF, PEF, NB, DOX, SXN, E	Neg	POS	-	NG	-
39	31-40	F	Chest Pain	NMI	-	Neg	-	-	NG	-
40	11-20	M	Cough	NMI	-	POS	-	-	NG	Neg
41	21-30	M	Cough	NMI	-	Neg	-	GPC* GPC**	*Staph. aureus & Strep. pneumoniae*	-
42	1-10	M	Cough	NMI	-	Neg	-	GPC**	*Strep. pneumoniae*	-
43	Above 50	M	Cough	NMI	-	Neg	-	GNB	*Pseudo. aeruginosa*	-
44	11-20	M	Cough	NMI	-	Neg	-	-	NSG	-
45	31-40	M	Cough	NMI	-	Neg	-	-	NSG	-
46	41-50	M	Cough	NMI	-	Neg	-	GPC**	*Strep. pneumoniae*	-
47	31-40	F	Cough	NMI	-	Neg	-	-	NSG	-
48	31-40	F	Cough	NMI	-	Neg	-	-	NSG	-
49	41-50	M	Cough	NMI	-	Neg	-	-	NG	-
50	11-20	M	Cough	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
51	41-50	M	Chest pain, sore throat	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
52	11-20	F	Cough	NMI	-	Neg	-	GPC**	*Strep. pneumoniae*	-
53	31-40	M	Cough	*Mycoplasma pneumoniae*	AZ^{+++}, LYN^{++}, CIP^{++}, LEV^{+++}, NB, RF, PEF, E, SXN, DOX	Neg	POS	-	NG	-
54	31-40	M	Cough	NMI	-	Neg	-	-	NG	-
55	31-40	F	Cough	*Mycopl*	CIP^{+}, LEV^{++},	Neg	POS	-	NG	-

				asma pneumoniae	AZ^{++}, LYN^{++}, NB, RF, DOX, PEF, SXN, E					
56	31-40	F	Cough	NMI	-	Neg	-	-	NSG	-
57	31-40	F	Sore throat, cough	*Mycoplasma pneumoniae*	LYN^{+++}, LEV^{++},CIP^{++}, AZ^{++}, DOX, PEF, E, NB, RF, SXN	Neg	POS	-	NG	-
58	41-50	F	Cough	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
59	Above 50	M	Sore throat, Cough	NMI	-	POS	-	-	NG	Neg
60	21-30	F	Chest pain, cough	NMI	-	Neg	-	GPC*	*Staph. Aureus*	-
61	41-50	M	Sore throat	NMI	-	Neg	-	-	NG	-
62	41-50	F	Cough, chest pain	NMI	-	POS	-	-	NG	Neg
63	11-20	F	Cough	NMI	-	POS	-	-	NG	Neg
64	31-40	F	Cough	NMI	-	Neg	-	GNB	*Kleb. pneumoniae*	-
65	21-30	M	Cough	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
66	11-20	F	Cough	NMI	-	Neg	-	-	NG	-
67	21-30	M	Cough	NMI	-	Neg	-	-	NSG	-
68	11-20	F	Cough	NMI	-	POS	-	-	NG	Neg
69	Above 50	F	Chest pain, cough	*Mycoplasma pneumoniae*	DOX^{+}, AZ^{++}, CIP^{+++},LYN^{+++}, LEV^{++}, E, SXN, NB, PEF, RF	POS	POS	GPC*, GPC**	*Strep. pneumoniae & Staph. aureus*	Neg
70	21-30	M	Sore throat, chest pain, cough	NMI	-	Neg	-	-	NSG	-
71	11-20	M	Cough,	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
72	21-30	F	Cough,	NMI	-	Neg	-	GNB	*Kleb. pneumoniae*	-
73	21-30	F	Sore throat, cough,	NMI	-	POS	-	GPC*	*Staph. aureus*	POS
74	11-20	F	Cough,	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
75	1-10	F	Sore throat, cough, chest pain	*Mycoplasma pneumoniae*	AZ^{+++} DOX^{+}, CIP^{+++}, LYN^{+++}, LEV^{+++}, E, SXN, NB, RF, PEF.	POS	POS	-	NG	POS
76	21-30	M	Sore throat , cough	NMI	-	Neg	-	GNB	*Pseudo. aeruginosa*	-
77	21-30	F	Cough, chest	NMI	-	POS	-	GPC*	*Staph. aureus*	POS

APPENDIX XV CONTINUED

			pain							
78	11-20	M	Cough	NMI	-	Neg	-	GNB	*Kleb. pneumoniae*	-
79	1-10	F	Cough	NMI	-	Neg	-	-	NG	-
80	41-50	M	Cough	NMI	-	Neg	-	-	NG	-
81	11-20	F	Chest pain	*Mycoplasma pneumoniae*	CIP $^{+++}$,LYN^{+++}, LEV^{+++}, AZ^{++}, DOX, E, RF, SXN, NB, PEF	POS	POS	GPC**	*Strep. pneumoniae*	POS
82	31-40	M	Chest pain	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
83	Above 50	M	Cough	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
84	11-20	F	Cough	NMI	-	Neg	-	-	NG	-
85	21-30	M	Chest pain	NMI	-	Neg	-	-	NSG	-
86	21-30	F	Chest Pain	NMI	-	Neg	-	GNB	*Kleb. pneumoniae*	-
87	11-20	F	Cough	NMI	-	Neg	-	GPC ** GPC ***	*Staph. aureus & Strep. pneumoniae*	-
88	31-40	F	Cough	NMI	-	Neg	-	GNB	*Pseudo. aeruginosa*	-
89	11-20	F	Sore throat, cough	NMI	-	POS	-	-	NG	POS
90	21-30	F	Cough	NMI	-	Neg	-	-	NSG	-
91	11-20	F	Cough	NMI	-	Neg	-	-	NSG	-
92	1-10	M	Chest pain	NMI	-	Neg	-	-	NG	-
93	21-30	F	Cough	NMI	-	Neg	-	GNB	*Kleb. pneumoniae*	-
94	41-50	M	Cough	NMI	-	Neg	-	GPC *	*Staph. aureus*	-
95	11-20	M	Chest pain	MNI	-	Neg	-	GPC **	*Strep. pneumoniae*	-
96	41-50	M	Sore throat	NMI	-	Neg	-	GPC *	*Staph. aureus*	-
97	Above 50	M	Chest pain	NMI	-	Neg	-	GPC **	*Strep. pneumoniae*	-
98	11-20	M	Cough	NMI	-	POS	-	-	NG	Neg
99	21-30	F	Cough, sore throat	*Mycoplasma pneumoniae*	AZ $^{+++,}$ DOX $^{+}$, LYN $^{++}$, LEV $^{++}$ CIP^{+}, RF, E, SXN, PEF, NB	Neg	POS	-	NG	-
100	31-40	M	Cough	NMI	-	Neg	-	-	NSG	-
101	31-40	F	Cough	NMI	-	Neg	-	-	NSG	-
102	Above 50	F	Chest pain, cough	*Mycoplasma pneumoniae*	CIP^{+}, LEV $^{++}$, AZ^{++}, DOX, PEF, NB, SXN, RE, E	Neg	POS	GPC **	*Strep. pneumoniae*	-
103	Above 50	M	Sore throat, chest pain	NMI	-	Neg	-	GPC *	*Staph. aureus*	-
104	31-40	M	Cough	NMI	-	Neg	-	-	NG	-
105	21-30	F	Chest	NMI	-	POS	-	-	NG	Neg

APPENDIX XV CONTINUED

			pain, cough							
106	Above 50	F	Cough	NMI	-	Neg	-	GPC **	*Strep. pneumoniae*	-
107	Above 50	F	Cough, sore throat	NMI	-	Neg	-	-	NSG	-
108	1-10	F	Cough	NMI	-	Neg	-	GPC **	*Strep. pneumoniae*	-
109	21-30	F	Cough	NMI	-	Neg	-	GNB	*Pseudo. aeruginosa*	-
110	Above 50	M	Cough	NMI	-	Neg	-	GNB	*Kleb. pneumoniae*	-
111	21-30	M	Sore throat, Cough	NMI	-	Neg	-	GPC **	*Strep. pneumoniae*	-
112	21-30	F	Chest pain, cough	NMI	-	Neg	-	GPC *	*Staph. aureus*	-
113	31-40	F	Cough	NMI	-	Neg	-	-	NG	-
114	11-20	M	Cough	NMI	-	Neg	-	-	NG	-
115	41-50	F	Cough	NMI	-	Neg	-	-	NSG	-
116	31-40	F	Chest pain, cough	*Mycoplasma pneumoniae*	CIP $^{+++}$, LYN $^{++}$, AZ, LEV, DOX, PEF, NB, SXN, RF, E	Neg	POS	-	NG	-
117	31-40	M	Cough	NMI	-	Neg	-	-	NG	-
118	21-30	F	Sore throat, cough	NMI	-	Neg	-	-	NG	-
119	41-50	M	Cough	NMI	-	Neg	-	-	NSG	-
120	21-30	M	Sore throat	NMI	-	Neg	-	GNB	*Pseudo. aeruginosa*	-
121	Above 50	F	Cough	NMI	-	Neg	-	GPC **	*Strep. pneumoniae*	-
122	21-30	M	Cough sore, throat	NMI	-	Neg	-	-	NG	-
123	41-50	F	Cough, chest pain	NMI	-	Neg	-	-	NG	-
124	21-30	F	Sore throat, Cough	NMI	-	Neg	-	-	NG	-
125	31-40	F	Cough,Chest pain	NMI	-	Neg	-	-	NG	-
126	21-30	M	Sore throat, Cough	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
127	41-50	M	Cough	NMI	-	Neg	-	-	NSG	-
128	31-40	M	Cough, Chest pain	*Mycoplasma pneumoniae*	LYN $^{+++}$, LEV^{++}, CIP^{++}, AZ^{++}, DOX, SXN, RF, NB, PEF, E	Neg	POS	-	NG	-
129	1-10	M	Cough	NMI	-	Neg	-	GPC **	*Strep. pneumoniae*	-
130	11-20	F	Cough	NMI	-	Neg	-	GPC * GPC**	*Staph. aureus & Strep*	-

									pneumoniae	
131	1-10	F	Cough	NMI	-	Neg	-	GPC **	*Strep. pneumoniae*	-
132	1-10	F	Cough	NMI	-	Neg	-	GPC**	*Strep. pneumoniae*	-
133	21-30	M	Chest pain	NMI	-	Neg	-	-	NSG	-
134	21-30	M	Chest pain	NMI	-	Neg	-	-	NG	-
135	21-30	F	Cough	NMI	-	Neg	-	-	NG	-
136	41-50	M	Cough	NMI	-	Neg	-	-	NG	-
137	41-50	F	Cough	NMI	-	Neg	-	-	NG	-
138	31-40	M	Cough	NMI	-	Neg	-	GPC **	*Strep pneumoniae*	-
139	21-30	F	Cough	NMI	-	Neg	-	-	NG	-
140	31-40	M	Cough	NMI	-	Neg	-	-	NSG	
141	11-20	M	Cough	NMI	-	Neg	-	GNB	*Kleb. pneumoniae*	-
142	21-30	M	Cough	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
143	1-10	F	Cough	NMI	-	Neg	-	GPC * GPC **	*Staph. aureus & Strep. pneumoniae*	-
144	31-40	F	Chest pain	NMI	-	Neg	-	-	NSG	-
145	11-20	F	Cough	NMI	-	Neg	-	GPC *	*Staph. aureus*	-
146	21-30	M	Cough, Chest pain	NMI	-	Neg	-	GPC **	*Strep. pneumoniae*	-
147	21-30	M	Cough, Chest pain	NMI	-	Neg	-	-	NG	-
148	Above 50	M	Cough	NMI	-	Neg	-	GNB GPC *	*Pseudo. aeruginosa & Staph. aureus*	-
149	31-40	M	Cough	NMI	-	Neg	-	GNB GPC *	*Kleb. pneumoniae & Staph. aureus*	-
150	Above 50	F	Chest pain, sore throat	NMI	-	Neg	-	GPC **	*Strep. pneumoniae*	-
151	31-40	M	Cough, sore throat	NMI	-	Neg	-	GNB	*Pseudo. pneumoniae*	-
152	Above 50	F	Cough	NMI	-	Neg	-	GPC* GNB	*Staph. aureus & Kleb. pneumoniae*	-
153	11-20	M	Sore throat	NMI	-	Neg	-	GPC **	*Strep. pneumoniae*	-
154	11-20	F	Cough, chest pain	NMI	-	Neg	-	GPC * GPC **	*Staph. aureus & Strep. pneumoniae*	-
155	Above 50	M	Cough	NMI	-	Neg	-	GNB	*Kleb. pneumoniae*	-
156	41-50	F	Cough, sore throat	NMI	-	Neg	-	-	NSG	--
157	Above 50	M	Chest pain,	NMI	-	Neg	-	-	NG	-

APPENDIX XV CONTINUED

			sore throat							
158	Above 50	F	Cough	NMI	-	Neg	-	-	NG	-
159	Above 50	F	Cough	NMI	-	Neg	-	-	NG	-
160	41-50	M	Chest pain	NMI	-	Neg	-	-	NG	-
161	31-40	F	Sore throat	NMI	-	Neg	-	-	NSG	-
162	11-20	F	Chest pain	NMI	-	Neg	-	-	NSG	-
163	31-40	M	Cough	NMI	-	Neg	-	-	NSG	-
164	11-20	M	Chest pain, cough	NMI	-	POS	-	-	NSG	Neg
165	21-30	F	Cough	NMI	-	POS	-	-	NG	Neg
166	21-30	M	Sore throat	NMI	-	Neg	-	-	NG	-
167	Above 50	M	Cough, sore throat	NMI	-	POS	-	-	NSG	Neg
168	41-50	F	Sore throat	NMI	-	Neg	-	-	NG	-
169	21-30	F	Chest pain, cough	NMI	-	Neg	-	-	NSG	-
170	Above 50	F	Sore throat	NMI	-	Neg	-	-	NSG	-
171	21-30	F	Cough	NMI	-	Neg	-	-	NSG	-
172	41-50	M	Chest pain	NMI	-	Neg	-	-	NSG	-
173	1-10	F	Cough	NMI	-	Neg	-	-	NSG	-
174	31-40	M	Cough	NMI	-	POS	-	-	NG	Neg
175	Above 50	F	Sore throat	NMI	-	Neg	-	-	NSG	-
176	31-40	M	Cough	NMI	-	Neg	-	-	NSG	-
177	Above 50	M	Cough	NMI	-	Neg	-	-	NG	-
178	21-30	F	Chest pain	NMI	-	Neg	-	-	NG	-
179	Above 50	M	Sore throat	NMI	-	Neg	-	-	NSG	-
180	Above 50	F	Cough	NMI	-	Neg	-	-	NG	-
181	31-40	F	Cough	NMI	-	Neg	-	-	NG	-
182	Above 50	M	Chest pain, cough sore throat	NMI	-	Neg	-	-	NSG	-
183	Above 50	F	Chest pain, cough , sore throat	NMI	-	POS	-	-	NG	Neg
184	21-30	F	Sore throat, cough	NMI	-	Neg	--	-	NSG	-
185	41-50	F	Chest pain, cough, sore	NMI	-	Neg	-	-	NG	-

APPENDIX XV CONTINUED

			throat							
186	41- 50	M	Cough	NMI	-	Neg	-	-	NSG	-
187	Above 50	M	Sore throat	NMI	-	Neg	-	-	NG	-
188	11-20	F	Cough	NMI	-	Neg	-	-	NG	-

KEY:

NMI	=	No Mycoplasma Isolated
F	=	Female
M	=	Male
POS	=	Positive
Neg	=	Negative
NG	=	No growth
NSG	=	No significant growth
GPC*	=	Gram positive Cocci in Clusters
GPC**	=	Gram positive Cocci in chains
GNB	=	Gram negative bacilli
Kleb.	=	Klebsiella
Strep.	=	Streptococcus
Staph.	=	Staphylococcus
Pseudo.	=	Pseudomonas
LYN	=	Lyntriaxone
CIP	=	Ciprofloxacin
AZ	=	Azithromycin
LEV	=	Levofloxacin
DOX	=	Doxycycline
E	=	Erythromycin
SXN	=	Septrin
RF	=	Rifampicin
NB	=	Norbactin
PEF	=	Peflacine
+++	=	Highly sensitive
++	=	Intermediate
+	=	Slightly sensitive
No plus sign	=	Resistant

APPENDIX XVI: RAW RESULT OF THE CONTROL SUBJECTS

S/N	Age	Gender	Symptoms	PPLO Agar culture results	Antibiogram of *M. pneumoniae* isolates	PCR result	Tetrazolium reduction test	Gram reaction	Other isolates	GLP Q gene result
1	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
2	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
3	11-20	F	Nil	NMI	-	Neg	-	-	NSG	-
4	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
5	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
6	21-30	F	Nil	NMI	-	Neg	-	-	NSG	-
7	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
8	31-40	M	Nil	NMI	-	Neg	-	-	NG	-
9	11-20	F	Nil	NMI	-	Neg	-	-	NG	-
10	1-10	F	Nil	NMI		Neg	-	-	NG	-
11	41-50	F	Nil	NMI	-	Neg	-	-	NG	-
12	11-20	M	Nil	NMI	-	Neg	-	-	NG	-
13	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
14	21-30	F	Nil	NMI	-	POS	-	-	NSG	Neg
15	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
16	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
17	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
18	41-50	F	Nil	NMI	-	Neg	-	-	NG	-
19	31-40	M	Nil	NMI	-	Neg	-	-	NG	-
20	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
21	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
22	1-10	F	Nil	NMI	-	Neg	-	-	NG	-
23	11-20	F	Nil	NMI	-	Neg	-	-	NG	-
24	1-10	M	Nil	NMI	-	Neg	-	-	NG	-
25	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
26	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
27	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
28	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
29	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
30	11-20	M	Nil	NMI	-	Neg	-	-	NG	-

APPENDIX XVI CONTINUED

31	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
32	11-20	F	Nil	NMI	-	Neg	-	-	NG	-
33	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
34	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
35	11-20	M	Nil	NMI	-	Neg	-	-	NG	-
36	41-50	F	Nil	NMI	-	Neg	-	-	NG	-
37	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
38	Above 50	M	Nil	NMI	-	Neg	-	-	NG	-
39	41-50	F	Nil	NMI	-	Neg	-	-	NG	-
40	31-40	F	Nil	NMI	-	Neg	-	-	NG	-
41	Above 50	M	Nil	NMI	-	Neg	-	-	NG	-
42	Above 50	M	Nil	NMI	-	Neg	-	GPC*	Staph. aureus	-
43	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
44	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
45	11-20	M	Nil	NMI	-	Neg	-	GPC*	Staph. aureus	-
46	1-10	M	Nil	NMI	-	Neg	-	-	NG	-
47	21-30	F	Nil	NMI	-	Neg	-	GPC*	Staph. aureus	-
48	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
49	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
50	41-50	M	Nil	*Mycoplasma pneumoniae*	LYN^{+}, LEV^{++}, CIP^{++}, AZ^{+}, SXT, PEF, E, NB, RF, DOX	POS	POS	-	NG	Neg
51	11-20	F	Nil	NMI	-	Neg	-	-	NG	-
52	11-20	F	Nil	NMI	-	Neg	-	-	NG	-
53	21-30	M	Nil	*Mycoplasma pneumoniae*	CIP^{++}, LYN^{++}, AZ, LEV, DOX,	POS	POS	-	NG	Neg

					PEF, NB, SXT, RF,E					
54	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
55	21-30	F	Nil	NMI	-	Neg	-	GPC**	*Strep. pneumoniae*	-
56	11-20	F	Nil	NMI	-	Neg	-	-	NG	-
57	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
58	41-50	F	Nil	NMI	-	Neg	-	-	NG	-
59	Above 50	M	Nil	NMI	-	Neg	-	-	NG	-
60	41-50	M	Nil	NMI	-	Neg	-	GPC*	*Staph. aureus*	-
61	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
62	11-20	F	Nil	NMI	-	Neg	-	-	NG	-
63	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
64	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
65	11-20	M	Nil	NMI	-	Neg	-	-	NG	-
66	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
67	21-30	M	Nil	NMI	-	Neg	-	-	NG	-
68	1-10	F	Nil	NMI	-	Neg	-	-	NG	-
69	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
70	21-30	F	Nil	NMI	-	Neg	-	-	NG	-
71	31-40	M	Nil	NMI	-	Neg	-	-	NG	-
72	11-20	M	Nil	NMI	-	Neg	-	-	NG	-
73	41-50	F	Nil	NMI	-	Neg	-	-	NG	-
74	1-10	M	Nil	NMI	-	Neg	-	-	NG	-
75	Above 50	M	Nil	NMI	-	Neg	-	-	NG	-

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RF	=	Rifampicin
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PEF	=	Peflacine
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++	=	Intermediate
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No plus sign	=	Resistant