# CHAPTER ONE

# INTRODUCTION AND OBJECTIVES

## 1.1. Introduction

Community-acquired pneumonia (CAP) is defined as an acute infection of the pulmonary parenchyma in a patient who has acquired the infection in the community. This definition is distinguished from hospital-acquired (nosocomial) pneumonia (HAP), and healthcare-associated pneumonia (HCAP). The later is acquired in other healthcare facilities such as nursing homes, hem dialysis centers, and outpatient clinics (Kamizono *et al*., 2010). The disease is a common and sometimes severe disease with an annual incidence of about 1%, and a mortality rate of 0–30%. Thus, selection of antibiotic therapy in CAP is important for the emergence of antibiotic resistance in community and for the outcome in the patients. Ideally, antibiotic therapy should be directed against the pathogen that is causing the pneumonia. However, as the etiology is often not known at presentation, patients must initially receive empirical antibiotic treatment (Geneva *et al*., 2013). Young age is perhaps the single most important risk factor for the development of pneumonia, and age is also an important predictor of etiology. Most affected children in epidemiologic studies of pneumonia are less than 5 years of age. The disease incidence among this age group is higher than at any other age, including adults over 65 years (Grant *et al*., 2012). Among older children, disease burden is substantially decreased and continues to decline through young adulthood. Other risk factors include poor nutritional status, low housing quality second hand smoke exposure, co morbidities such as asthma, and preceding upper respiratory tract infection (Camargo *et al*., 2012). CAP affects approximately 4.5 million adults in the United States annually. About one third of these adults require

hospitalization. The mortality rate among hospitalized patients with CAP varies each year and can reach 35 percent (Paul *et al*., 2002). Current approaches to the empirical management of CAP emphasise the type of patient ("community" or "hospital"), rather than the type of symptoms ("typical" or "atypical (Paul *et al*., 2002, Kristopher *et al*., 2004).

The term atypical bacterial pneumonia (ABP) has been recognized as a clinical syndrome characterized by a less severe clinical course than typical bacterial pneumonia which represents approximately 15% of all cases of CAP (Cunha, 2006). The atypical pathogens do not respond to β-lactam antimicrobial therapy (Lim *et al*., 2009). Therefore, appropriate treatment of CAP requires the identification of the infecting pathogens (Blasi. 2004; Mandell *et al*., 2007). The term was originally used to describe an unusual presentation of pneumonia. The ABP affects nearly one in five people who contract pneumonia and is a significant source of morbidity for patients and costs to healthcare providers (Gupta and Sarosi, 2001). The causative agents include *Mycoplasma pneumoniae* (*M. pneumoniae*), *Chlamydia pneumoniae* (*C. pneumonia*) and *Legionella pneumophila* (*L. pneumophila*). The incidence of ABP caused by these pathogens has increased with the development of specific diagnostic techniques (Surender and Malay, 2010). Unfortunately because many of these pathogens are intracellular, culture systems are either not available or the techniques employed are costly, time consuming or unsafe (Gupta and Sarosi, 2001). *C. pneumoniae* and *M. pneumoniae* cause nearly 80% of all cases of atypical pneumonia and approximately 17% of all pneumonia among adults and young children, accounting for an estimated two to three million cases of pneumonia and 200,000 pneumonia-related hospitalizations in the United States each year (Schneeberger *et al*., 2004). In addition to ABP, *M. pneumoniae* is second only to *S. pneumoniae* as the most common bacterial agent of CAP (Chong *et al*., 2010). The organisms *M.*

*pneumoniae* causes a wide range of respiratory infections, including pneumonia, tracheobronchitis, and upper respiratory tract infection. Only 3 to 10 percent of persons infected with *M. pneumoniae* develop pneumonia (Thomas *et al*., 2008). Because *M. pneumoniae* infection becomes more common with increasing age, it is particularly important to consider this agent in elderly patients (Lauri *et al*., 2007). The bacterium infection occurs throughout the year but can cause periodic outbreaks within small communities. *M. pneumoniae* is the pathogen most often associated with atypical pneumonia. Over several days to a week constitutional symptoms, which usually are present, include headache exacerbated by a cough, malaise, myalgias, and sore throat. The cough is usually dry, paroxysmal, and worse at night. The clinical course of pneumonia caused by *M. pneumoniae* is usually mild and self-limited. The mortality rate is approximately 1.4 percent (Lauri *et al*., 2007). However, pulmonary complications can be significant and include effusion, empyema, pneumothorax, and respiratory distress syndrome. Infection can lead to prolonged carriage and therefore serve as a reservoir for the spread of the pathogen to others (Ebrahim and Gholam, 2008). It is transmitted from person to-person by respiratory droplets. Its incubation period varies from one to three weeks, although it can be as short as four days (Sánchez and Gomez, 2008). *M. pneumoniae* infections tend to be endemic, punctuated by epidemics at four to- seven-year intervals (Rasmussen *et al*., 2010). *Mycoplasma* is found more often in younger than in older people (Schneeberger *et al*., 2004).

The second causative agent of ABP is *C. pneumoniae*, an obligate intracellular organism capable of persistent latent infection. Humans are the only known reservoir. Transmission results from contact with respiratory secretions, with an incubation period of several weeks. By the age of 20 years, one half of persons have detectable levels of antibodies to *C. pneumoniae*. This antibody its present in 75 percent of elderly persons (Kristopher *et al*., 2004). *C. pneumoniae* infection is

more likely to occur in older patients with co morbid diseases than in those who are otherwise healthy (Kristopher *et al*., 2004). Patients with *C. pneumoniae* infection often present with sore throat, headache, and a cough that can persist for months if treatment is not initiated early (Ken and Deborah, 2004). Sputum is usually scant or nonexistent, and a low-grade fever is usually present. Most cases of *C. pneumoniae* infection are mild, but severe disease can occur, necessitating admission to an intensive care unit. The mortality rate has been estimated to be 9 percent, and death usually is associated with secondary infection and underlying co morbid disease (Hassan *et al*., 2006).

The third causative agent of CAP is *Legionella* species, an intracellular pathogen. The most medically important species is *L. pneumophila*, and several serotypes have been identified. Serotype 1 has been associated with most reported human cases of pneumonia caused by *L. pneumonphila* (Kazhila *et al*., 2010). Infection occurs from exposure to *Legionella* organisms in the environment. Person-to-person spread has not been reported, the organisms are found most commonly in fresh water and man-made water systems. The pathogens also can be found in moist soil, especially near streams and ponds. Man-made systems for heating and cooling water can be prime environments for the proliferation of *Legionella* because of conditions such as temperatures between 32°C (89.6°F) and 45°C (113°F), stagnation of water, and the presence of scale sediment and amebas. *L. pneumophila*, which ranks as a first or second pathogen, is sufficiently severe to require admission to an ICU (Vergis *et al*., 2000). The epidemiologic risk factors include recent travel with an overnight stay outside of the home, recent changes in domestic plumbing, renal or hepatic failure, diabetes and systemic malignancy. In Japan, hot spring bathing and bathing in a circulating bath are considered the most important environmental risk factors (Furuhata *et al*., 2004; Sasahara *et al*., 2004).

Older people are more often infected by *Legionella* and many outbreaks of Legionnaires' disease at hot springs have been reported (Okada *et al*., 2005).

## 1.2. Rationale

Respiratory infections include a broad range of diseases, such as acute respiratory infections, pneumonia, obstructive lung disease, pleural disease and pneumococosis, as well as malignancies of the respiratory tract. These diseases constitute a major cause of morbidity and mortality worldwide and accounting for 17.4% of all deaths and 13.3% of all disability-adjusted life years in the year 2000 (WHO, 2002).

Community-acquired pneumonia (CAP) is one of the most common life-threatening infections, with most deaths occurring in developing countries (Lozano *et al*., 2013). According to population based studies, the annual incidence rate of radio -logically proven CAP in adults varies between 2.6 to 13.4 per 1000 people, with an increased incidence in males and at the extreme ages of life (Almirall *et al*., 2000). In view of the changing demographics, improvements in health care and increasing incomes, the burden of communicable diseases is likely to lessen, whereas the burden of chronic respiratory diseases will worsen due to smoking and the aging of the population (WHO, 2002). Pneumonia is the sixth leading cause of death and number one cause of death from infectious disease (Anthony *et al*., 2008).

In Sudan CAP routed the second leading cause of both hospital admission and mortality (Federal Ministry of health, 2001). The primary risk factors for CAP are age, co-morbidities and smoking history. Occupational dust exposure and history of childhood pneumonia have also been associated with an increased risk, as has male gender, unemployment and single marital status (Farr *et al*., 2000; Jackson *et al*., 2004).

Delay in microbiology culture diagnostics for pneumonia-causing pathogens refractory to cultivation is a clinical problem which often results in empirical, sometimes inefficient, broad-spectrum antibiotic therapy until final microbiology results become available (Wang *et al*., 2012).

Alcoholism and chronic diseases, such as respiratory disease, cardiovascular disease, or kidney disease, also increase the risk for pneumonia, especially in the older population (Marrie, 2000; Müllerova *et al*., 2012; Haessler and Schimmel, 2012). In the pediatric population, very young children are at increased risk because their immune systems have not fully developed. Diseases or medications that suppress the immune system increase the risk among all ages (Farr *et al*., 2000).

Recently, several prospective studies on patients with CAP who required hospital admission have been published in Western countries and guidelines for the management of CAP have been prompted (Leon *et al*., 2014). The etiologic diagnosis is optimal in the management of CAP, no single test is presently available that can identify all potential pathogens, and each diagnostic test has limitations. Atypical pathogen is a common and important pathogen of CAP because it is difficult to differentiate among *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, and other pathogens that cause CAP when using clinical and conventional laboratory tests. Compared with bacteria, the clinical treatment of atypical pathogens is different, as beta-lactam antibiotics are not effective for atypical pneumonia. Therefore, laboratory diagnostic methods are particularly important for the diagnosis and treatment of atypical pneumonia (Qu and Cao, 2015).

Although of high rate of prescription of antibiotics to treat cases of pneumoniae among Sudanese, there are limited or absence studies of atypical pneumonia in Sudan. To our knowledge, there are no published studies on atypical pneumoniae.

This study is expected to domesticate molecular technique as well as serological technique as tools for accurate diagnosis of atypical pneumoniae.

## 1.3. Objectives

### 1.3.1. General objective

To study atypical community-acquired bacterial pneumonia among Sudanese patients.

### 1.3.2. Specific objectives

A) To detect agents causing atypical pneumonia in clinical specimens, using serological and molecular techniques.

B) To compare between serological and molecular techniques used for detection of agents of atypical pneumonia.

C) To characterize the causative agents of atypical pneumonia.

D) To determine prevalence of organisms that cause atypical pneumonia in Sudanese patients.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1. Pneumonia

Pneumonia is an infection of the lungs that can be caused by nearly any class of organism known to cause human infections, including bacteria, viruses, fungi, and parasites. It results in an inflammatory response within the small air spaces of the lung (alveoli) (Kyung *et al*., 2010). The difference of clinical course is associated with the virulence of etiologic agents and/or the host immune status. Antibiotics for bacterial pathogens and anti viral, if possible, for viral pathogens may help induce early recovery from pneumonia by reducing the number of pathogens and the host immune response to etiologic agents (Dallaire *et al*., 2001). The circulating immune cells including neutrophil, lymphocytes, and monocytes may be involved in the pathogenesis of pneumonia. Thus, change of these parameters may reflect the severity of pulmonary lesions. The pathogenesis of pneumonia in each etiologic agent may be different; in general, patients with typical bacterial pneumonia manifest more toxic clinical symptoms with leukocytosis, neutrophilia with band form neutrophil, and bacteremia. In initial pneumonia lesions, mainly activated neutrophil and mononuclear phagocytes are predominantly observed, and mediators such as proteolytic enzymes, oxygen radicals, and cytokines from these cells may be associated with host lung injury (Dallaire *et al*., 2001). As for the diagnosis of pneumonia, there are some difficulties in the detection of etiologic agents for lower respiratory tract infections in children (especially younger children) due to the inconsistency of adequate sampling of respiratory materials for pathogen culture and polymerase chain reaction (PCR) and the need for paired blood sampling for serologic tests. In addition, the higher rates of nasopharyngeal

carriage of bacterial pathogens, including *S. pneumoniae* in healthy children (10-50%), make it more difficult (Jain *et al*., 2005).

## 2.2. Community-acquired pneumoniae

Community-acquired pneumonia (CAP) is an acute infection of the lower respiratory tract occurring in a patient who has not resided in a hospital or healthcare facility in the previous 14 days (Bartlett *et al*., 2000). Current approaches to the empirical management of CAP emphasise the type of patient ("community" or "hospital"), rather than the type of symptoms ("typical" or "atypical"). The incidence of CAP in United States is about 258 per 100 000 population per year, rising to 962 per 100 000 among those aged 65 years or over. Mortality rates in recent years appear to have increased. Mortality averages 14%, but is less than 1% for those not requiring admission to hospital, most etiologic studies identified *S. pneumoniae* as the primary cause of CAP (Bartlett *et al*., 2000).

### 2.2.1 Atypical pneumonia

Atypical pneumonia can be defined as pneumonia that is caused by bacteria lacking a typical cell wall, and those resistant to β-lactam antibiotics**.** It was introduced to medical literatures in 1970, indicating the pneumonia caused by *M. pneumoniae*, *C. pneumonia, L.pneumophila*, psittacosis and Racket's organisms. Currently, pathogens of atypical pneumonia are still not clearly defined (Van *et al*., 2001). Generally referring to *Mycoplasma*, *Chlamydia* and *Legionella*, some researchers also included other non pneumococcal pathogens such as viruses and Racket's organisms which may also cause pneumonia. *Mycoplasma, Chlamydia* and *Legionella* are considered as the important pathogens of community-acquired pneumonia (CAP) all over the world (Plouffe, 2000; Arnold *et al*., 2007).

Atypical pathogens are an important cause of community-acquired pneumonia (CAP). *C. pneumoniae* and *M. pneumoniae* are the most common pathogens in the outpatient setting and high incidences have been recorded in hospitalized patients with CAP (Wattanathum *et al*., 2003). *L. peumophila* has also been seen in the outpatient setting, but this pathogen has been identified with the common organism in patients with CAP requiring the intensive care unit. The incidence of infection with these atypical pathogens has been as high as 40–60% of all admitted patients, often as part of a mixed infection in Western countries. In Japan, there have been some reports on the etiology of CAP among the Japanese population. All these studies demonstrated that *C. pneumoniae* and *M. pneumoniae* are common pathogens, the third or fourth leading pathogens, and that the etiology of CAP in Japan does not differ significant from that of Western countries (Ishida *et al*., 2002). During the last decade with diagnostic improvements, agents of atypical pneumonias, such as *C. pneumoniae*, *L. pneumophila* and *M. pneumoniae* have emerged as an important cause of respiratory tract infections, accounting for between 15 to 50% of CAP (Catia *et al*., 2012).

### 2.2.1.1. Historical background

In 1920s when antibiotics were initially used, a new type of pneumonia was discovered in Europe. It is manifested with mild onset symptoms, without sputa, progressively developing into different degrees of pneumonia involving organs out of the lung and without responses to antibiotics, which is different from the typical pneumococcal pneumonia characterized by acute onset, fever and vomiting (Basarab *et al* ., 2014). Hobart Reiman first referred to certain pneumonias as being ‘atypical’ in 1938. His definition of atypical pneumonia was a mild respiratory illness that progressed to cough without sputum production. Since then, this complex has come to be associated with a variety of agents including *M.*

*pneumoniae*, *Coxiella burnetii* (Q fever), *C. psittaci* (psittacosis), *C. trachomutis, C. pneumoniae* and *Legionella*. spp (Scott, 2005).

### 2.2.1.2. Epidemiology

Among 302 patients, potentially relevant fastidious pathogens were detected in only 4 patients by molecular analysis*: L. pneumophila* (n = 3), *C. pneumoniae* (n = 1), *M. catarrhalis* (n = 1). Of these, only the signal for *M. catarrhalis* was above the threshold proposed by the manufacturer (*A. baumannii*) (Matthias *et al*., 2015). Another study in Basraha, Iraq one third of community- acquired pneumonia cases are caused by atypical pneumonia agents. These agents are *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila*. The laboratory diagnosis of these organisms is difficult and time-consuming by conventional microbiological techniques. Polymerase chain reaction (PCR) is one of the important tools which can resolve this problem. Among 45 bronchoalveolar lavage specimens taken from patients presented clinically with community-acquired pneumonia. PCR results revealed that 10(22.2%) cases gave positive for *M. pneumoniae*, 8 (17.7%) cases gave positive for *C. pneumoniae* and 3 (6.6%) cases gave positive results for *L. pneumonphila*. The PCR method is a rapid, sensitive and specific technique that has been applied to the detection of many infectious pathogens (Mohammed, 2015).

### 2.2.1.3. Causative agents

#### 2.2.1.3.1. *Mycoplasma pneumoniae*

*Mycoplasma pneumoniae* **(***M. pneumoniae*), a common agent of respiratory tract infections that is transmitted from person to person through aerosolization. The infection occurs in all age groups, but older children and young adults are affected at a higher frequency than other age groups. Clinical manifestations range from

mild cases of tracheobronchitis to severe atypical pneumonia and can be followed by a broad spectrum of extra pulmonary complications. *M. pneumoniae* infection is characterized by incidence peaks every years (Von *et al*., 2009, Polkowska *et al*., 2012, Uldum *et al*., 2012, Blystad *et al*., 2012). The organism is responsible for up to 25% of all cases of CAP (Atkinson *et al*., 2008). Reports from Europe and Asia have shown a notable increase in the frequency of infections caused by *M. pneumoniae* during 2011–2012 (Chalker *et al*., 2012, Linde *et al*., 2012, Pereyre *et al*., 2013). For clarification of the epidemiology of *M. pneumoniae* infection and identification of the relevant periods of incidence peaks, molecular typing of the prevalent strains can be an efficient tool. The strains can be divided into subtypes and variants according to sequence differences in the gene coding for the immunodominant main adhesion P1type. It has been hypothesized that the specific antibody level in the host population can influence further infections and lead to a change of the dominating P1 type (Dumke *et al*., 2010). Knowledge of the strain's genotype identity currently has no therapeutic consequences. Because mycoplasmas, which do not have cell walls, are not susceptible to β-lactam antimicrobial drugs, macrolides are generally accepted as first choice agents for treatment, especially in children. However, mutations in the 23S rRNA locus of *M. pneumoniae* have been shown to result in complete macrolide resistance rates range from >90% in China (Sun *et al*., 2013) to <10% in Europe, requiring periodic monitoring of strains to identify possible new resistance or resistant strains (Dumke *et al*., 2010).

The organism is known primarily as mucosal pathogens that reside extracellular lyon epithelial surfaces. However during the past few years, the potential for several mycoplasmal species to fuse with and enter host cells that are not normally phagocytic has been demonstrated (Rottem, 2002). Such an occurrence should not be unexpected for microorganisms lacking a rigid cell wall that are typically

closely associated with host cell surfaces. Rottem, (2002) has summarized current knowledge concerning the features that enable *M. penetrans* and *M. fermentans* to invade host cells, some of which may be relevant to enhance understanding of similar events that may occur with *M. pneumoniae*. Dallo and Baseman, (2000) described the ability of *M. pneumoniae* to survive, synthesize DNA, and undergo cell replication in artificial cell culture systems over a 6-month period. An intracellular existence that sequesters *M. pneumoniae* could facilitate the establishment of latent or chronic states circumvent mycoplasmacidal immune mechanisms, 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 *Mycoplasma* under clinical conditions (Rottem, 2002). Fusion of the mycoplasmal cell membrane with that of the host may also result in release of various hydrolytic enzymes produced by the *Mycoplasma* as well as insertion of mycoplasmal membrane components into the host cell membrane, a process that could potentially alter receptor recognition sites and affect cytokine induction and expression (Rottem., 2002).

*M. pneumoniae* is primarily an extracellular pathogen requiring close association with host cells to survive as its highly reduced genome renders it incapable of *de novo* synthesis of amino acids, nucleotides, and other essential molecules. Also, this organism is unique among bacteria in their growth requirement for host cholesterol. Adherence to the host respiratory epithelium is believed to be the initiating event that facilities local cell injury, tissue disruption, and cytotoxic effects (Waite and Talkington, 2004). Several protein components of the adhesion complex have been identified including the P1 protein. The two *M. pneumoniae* subtypes, type 1 and type 2, were established based on P1 sequence polymorphisms. Hydrogen peroxide and superoxide radicals are known virulence factors of *M. pneumoniae*. The Community-acquired Respiratory Distress

Syndrome (CARDS) toxin, an ADP-ribosylating and vacuolating toxin of *M. pneumoniae*, is capable of inducing pulmonary inflammation and airway hyper reactivity (Kannan *et al*., 2005; Kannan and Baseman, 2006; Dandekar *et al*., 2008; Hardy *et al*., 2009; Medina *et al*., 2012; Medina *et al*., 2014). Inappropriate host immune responses also contribute to the pathogenesis of *M. pneumoniae* infection. The molecular mimicry by *M. pneumoniae* adhesion proteins and glycolipid of various host cell components may trigger autoimmune disorders that involve multiple organ systems (Waites and Talkington, 2004: Sharma *et al*., 2011). *M. pneumoniae* may also be a facultative intracellular pathogen; viable bacteria have been shown to move into the interior of human cells *in vitro* (Dallo and Baseman, 2000). This aspect of the organism's life cycle and the ability to form biofilms on epithelial tissue likely contribute to the establishment of chronic infection (Simmons *et al*., 2013). Whole genome sequencing has greatly facilitated understanding of *M. pneumoniae*. The genome of the type 1 strain M129 (ATCC 29342) was sequenced by using a laborious approach involving the construction of an ordered cosmid library. The sequence was reported in 1996 and reannotated in 2000 as having 816,394 bp, 730 genes, and an average GC content of 40 % (Dandekar *et al*., 2008). The genome sequences of the type 2 strains FH and 309 were completed using next generation sequencing methods (Roche 454 sequencers) (Krishnakumar *et al*., 2010; Kenri *et al*., 2012). Another strain, M29 was recently submitted (accession number GCA_00733995.1) and has not yet been annotated. A preliminary comparison of the first three genomes indicated that they are very similar, except for variation in a 6-kb insertion region coding lipoproteins (Kenri *et al*., 2012).

It is well known that *M. pneumoniae* is one of the most prevalent causes for respiratory tract infections worldwide (Waites and Talkington, 2004). As a human pathogen, it was described for the first time in 1944, after it had been isolated from

the sputum of a patient with atypical pneumonia. Transmission usually occurs through aerosols from person to person. Once inside the human host, the bacteria colonize the mucosa of the lower respiratory tract leading to atypical pneumonia. Although the respiratory tract is the typical habitat, *M. pneumoniae* has also been isolated from several extra pulmonary infection sites like the synovial, the cerebrospinal and the pericardial fluid. Importantly, extra pulmonary manifestations are present in up to 25% of all infected persons (Waites and Talkington, 2004). While, in general, *M. pneumoniae* infections are rather mild, they can cause worse disease patterns in children and immunocompromised patients leading to complications like meningitis, myocarditis or rheumatoid arthritis (Ramirez *et al*., 2005). Due to their natural lack of a cell wall, *Mycoplasma* infections cannot be with treated with common β-lactam antibiotics like penicillin which target the cell wall synthesis machinery. Instead, tetracycline and macrolide-antibiotics are used (Blanchard and Bébéar, 2011).

*M. pneumoniae* is a human pathogenic bacterium, which belongs to the group of Firmicutes (Ciccarelli *et al*., 2006). The term "Mollicutes" can be translated as "soft skin", describing the absence of a cell wall in these bacteria, due to the lack of genes for peptidoglycan synthesis. Therefore, *Mycoplasma* is not surrounded by rigid boundaries which give them defined forms like cocci or rods, but they exhibit pleomorphic cell shapes. A typical cell of *M. pneumoniae* is filamentous or flask-shaped, with a knobby tip and is about 1-2 μm long and 0.1-0.2 μm wide. The tip has important functions as attachment organelle, in gliding and in cell division (Miyata, 2008).

The Mollicutes are the smallest bacteria that are capable of independent life. They are characterized by extremely reduced genomes as result of a long time degenerative evolution, probably due to their parasitic life style with constantly high nutrient availability and stable conditions in their habitat. Among the

*Mycoplasma* spp., the genome size varies between 0.58 Mb in *M. genitalium* and 1.36 Mb in *M. penetrans*. The complete genome sequence of *M. pneumoniae* has been available since 1996. It has a size of 0.86 Mb and contains 688 open reading frames (Dandekar *et al*., 2000). The minimal gene set is not only reflected in the lack of peptidoglycan synthesis. Also, the bacterium lacks the genes for most anabolic pathways. Genes coding for amino acid- and vitamin biosynthesis are completely absent and the organism is not able to perform respiration. Concerning the catabolism, glycolysis is the only central catabolic pathway which is complete. The tricarboxylic acid (TCA) cycle is entirely missing and the pentose phosphate shunt is incomplete. Since it lacks its oxidative part, the predominant role of the pentose phosphate shunt is supplying the cell with phosphoribosyl pyrophosphate (PRPP) for nucleotide biosynthesis. Glucose is the carbon-source that is taken up most efficiently and allows the best growth. *M. pneumoniae* can additionally use glycerophosphocholine (GPC), fructose, mannose, glycerol and probably also glycerol-3-phosphate as carbon sources, with all of them entering the glycolysis (Halbedel *et al*., 2004).

In *M. pneumoniae*, substrate-level phosphorylation in the glycolysis and the pyruvate metabolism is the only way to produce ATP. The degradation of one molecule glucose via the glycolysis yields two molecules pyruvate and two molecules ATP. Pyruvate is converted to either lactate or, after several steps, acetate and ATP. Both lactate and acetate are secreted from the cell and lead to an acidification of the surrounding medium. Due to its constant conditions in the host tissue, the bacterium has no need to adapt to drastic changes which would require an elaborate regulatory network. Therefore, comparably few genes for regulatory proteins are found. Whereas in other bacteria, like *Pseudomonas aeruginosa* or *Streptomyces coelicolor*, transcription factors account for about 10% of the genome, *M. pneumoniae* possesses only an hand full of potential regulators, which

make up less than 0.5% of the genome (Stülke *et al*., 2009). However, this does not mean that *M. pneumoniae* constantly expresses its genes and does not react to extracellular clues. Transcriptome analyses show that *M. pneumoniae* gene expression is indeed altered, e.g. in the presence of different carbon sources, during oxidative stress, heat stress, iron-depletion or temperature imbalance. Also, the regulatory roles of small RNAs and antisense-RNAs were described (Güell *et al*., 2009). In addition, there have been several evidences for regulation on a post-translational level, like phosphorylation and acetylating (Halbedel *et al*., 2004; Schmidl *et al*., 2010). In fact, the amount of acetylated targets in *M. pneumoniae* is three times as high as in *E. coli* (Van Noort *et al*., 2012).

Bronchiolitis is an acute lower respiratory tract infection that primarily involves terminal and respiratory bronchioles. The disease may extend to the adjacent alveolar ducts and alveolar space (Zentz, 2011**)**. *Mycoplasma* is the smallest free-living, self-replicating microorganism. It is highly transmissible and is a frequent cause of respiratory tract infection in children. The symptoms of *M. pneumoniae* upper respiratory tract infection are usually mild; one-fifth of infected individuals being asymptomatic, but in some cases, *M. pneumoniae* causes severe conditions such as organizing pneumonia (Defilippi, 2008; Natori, 2010; Hadi *et al*., 2011; Hoffmann, 2012). Acute MP infection may also exacerbate asthma or cause asthmatic symptoms. Because *M. pneumoniae* is difficult to isolate in culture and infections are most often confirmed by polymerase chain reaction (PCR) gene amplification or serology. In children with bronchiolitis, detection rates of 75.8% have been reported and 2.7% for *M. pneumoniae* 12. In recent years, the incidence of *M. pneumoniae* -caused bronchiolitis has been rising. A study of 211 cases of bronchiolitis reported an *M. pneumoniae* -positive rate of 7.1%; and as with RSV, it was frequently detected in moderate or severe cases (Watanabe *et al*., 2014). In another report14, *M. pneumoniae* was identified in 34.3% of children with

bronchiolitis who were between 6 months and 2 years of age, and who comprised 52.2% of the study subjects. The evidence shows that *M. pneumoniae* has become an important cause of bronchiolitis in children patients (Cosentini, 2008).

Complications of mycoplasmal pneumonia included lobar consolidation, abscess, pleural effusion (15-20%), empyema (rare), bronchiolitis obliterans and necrotizing pneumonitis.

Although most cases of pneumonia cause by *M. pneumoniae* are mild and self-limited, fulminant disease can occur and result in acute respiratory distress syndrome, respiratory failure extra pulmonary complications may occur as a result of *M. pneumoniae* infection, although the incidence is less than 10% when compared to respiratory problems. In many of the suspected extra pulmonary problems, it is unclear if the disease entity is caused by the organism itself, or by an immune response triggered by the *M. pneumoniae* infection.

Cardiac involvement in *M. pneumoniae* infection manifests as conduction abnormalities, either rhythm disturbances or heart blocks, seen on the Electrocardiography (ECG). Chest pain from pericarditis or myocarditis can be a clinical symptom, and these entities have been linked to anti-cardiolipin antibodies **(**Nagashima *et al*., 2010). Congestive heart failure is another extra pulmonary complication of *M*. *pneumoniae* infection. Myocardial damage has been reported in children with *M*. *pneumoniae* pneumonia (Li *et al*., 2013).

Central nervous system involvement is rare in most *M pneumoniae* infections, but hospitalized children are at particular risk of developing encephalitis, aseptic meningitis, transverse myelitis, peripheral neuropathy, or cerebellar ataxia. These complications can be seen in adults, although less frequently (Smith et al., 2000; Daxboeck et al., 2006). Some of the Central Nervous System (CNS), sequelae may be permanent. How *M*. *pneumoniae* causes neurologic damage is unclear, but may be linked to an immunologic reaction to antigens produced by the infection.

Hemolytic anemia may develop if the IgM antibodies to *M pneumoniae* antigens cross react to antigens on human erythrocytes, causing destruction. Hemolysis in sickle cell patients with an M. pneumoniae infection is concerning, but rarely fatal (Smith and Eviatar, 2000). *M. pneumoniae* infection has been associated with erythema multiform, macular exanthems, vesicular exanthems, urticaria, erythema nodosum, and Stevens-Johnson syndrome. *M. pneumoniae* has been associated with arthralgia and myalgias, although arthritis is rare. Rhabdomyolysis has been linked with *M. pneumoniae* infections, with very high Creatine phosphokinase (CPK) and myoglobin levels reported (Khan and Sayed, 2012). Gastrointestinal symptoms are nonspecific, include hepatitis and pancreatitis, and are thought to be related to circulating antibodies to the *M. pneumoniae* organisms.

The ophthalmologic manifestation of *M. pneumoniae* infection is most commonly conjunctivitis, but cranial neuropathies, optic papillitis, and anterior uveitis can occur (Liu and Janigian, 2013). Glomerulonephritis is a rare complication of *M pneumoniae* infection, and is likely caused by immune complex deposits in the glomerulus. Mycoplasmal species that infect animals and humans are particularly well known for their ability to induce chronic disease states in which clearance of the organism is extremely difficult. Intracellular localization, immunomodulatory effects and surface-antigen variations may all contribute to this process. Infection by *M. pneumoniae* is suspected to play a role in some chronic human diseases, including adult rheumatoid arthritis, juvenile idiopathic arthritis, Crohn's disease and asthma (Sutherland *et al*., 2004; Ramirez *et al*., 2005). A role for *M. pneumoniae* in the pathogenesis of asthma was suggested more than 30 years ago, and the support for this theory is now strong, implicating this organism both in pathogenesis as well as in exacerbation of acute attacks (Sutherland *et al*., 2004). Evidence implicating *M. pneumoniae* in asthma, summarized by Waites and Talkington (Waite and Talkington, 2004) includes the following:

A) The organism can be isolated in higher prevalence from asthmatics than from healthy persons.
B) Administration of macrolide antibiotics can result in improvement in pulmonary function in asthma patients who are known to have mycoplasmal infection.
C) Follow-up studies in children have demonstrated prolonged airway dysfunction consistent with a persistent infection.

D) *M. pneumoniae* induces a number of inflammatory mediators such as IgE, substance P and neurokinin 1, and IL-5, implicated in the pathogenesis of asthma that may play a role in exacerbations, which often include wheezing.

Models of chronic respiratory infection in mice have demonstrated that the organisms can produce pneumonia, and stimulate cytokine production, airway hyper-responsiveness resembling chronic asthma and a Th2-dominant airway inflammatory process that potentiates organism survival in the lungs. The possibility of mycoplasmal association in the pathogenesis of asthma has stimulated experimental work using rodent models of chronic respiratory infection. Prior allergic sensitization of mice to hen egg ovalbumin has been shown to be associated with down regulation of TLR-2 expression and decreased clearance of *M. pneumoniae* in mouse lungs **(**Chaplin *et al*., 2007). A Th17-dominant inflammatory response appears to be important in organism clearance, however, mycoplasma infection of mouse lungs can be prolonged when alveolar macrophages are depleted and IL-23-mediated IL-17F production is neutralized **(**Martin *et al*., 2001 **;** Lai *et al*., 2007). Another effect of allergic pulmonary inflammation in this model is to decrease the humeral immune response to the pathogen. Interestingly, in a study of 55 adult, chronic stable asthmatics, Martin *et al.* found a 43% prevalence of PCR positivity for *M. pneumoniae* in bronchoalveolar lavage fluid and bronchial biopsies, but none of the subjects had

elevated levels of antibody (Martin *et al*., 2001). In a 5-year prospective study of 82 allergic children, significantly fewer were found to be positive for IgG antibody compared with controls, although there was no difference in the detection of IgM or genomic DNA (Atkinson *et al*., 2005). Thus, humans with allergic sensitization of the lungs may be at greater risk of developing chronic airway colonization by *M. pneumoniae*, with resultant augmentation of airway hyper-reactivity, even in the absence of a measurable humeral immune response.

*M. pneumoniae* accounts for as many as 10-30 per cent of all cases of commonly acquired pneumonia (CAP) in general population and for 25- 71 per cent in closed populations (Waits and Talkingt, 2004), such as students and military recruits living in dormitories. *M. pneumoniae* has been frequently observed in patients suffering with respiratory illness and is also reported to be associated with acute exacerbation of bronchial asthma and chronic obstructive pulmonary disease (COPD) (Varshney *et al*., 2009), acute respiratory distress syndrome (ARDS) (Chaudhry *et al*., 2010), polyarthritis (Chaudhry *et al*., 2003), stroke (Ngeh and Goodbourn, 2005), Guillain-Barre syndrome (Gorthi *et al*., 2006) and coronary artery diseases (CAD) (Goyal *et al*., 2007).

Mammalian cells parasitized by *M. pneumoniae* can exhibit a number of cytopathic effects and impaired metabolism as a result of the local damage that occurs through mechanisms described above. Cells of the respiratory epithelium typically lose their cilia, appear vacuolated, and show a reduction in oxygen consumption, glucose utilization, amino acid uptake and macromolecular synthesis, ultimately resulting in exfoliation. These sub cellular events in the airways of the lung are manifested clinically by the persistent, hacking cough typical of *M. pneumoniae* bronchitis and pneumonia (Waites *et al*., 2007). Damage to cells of the upper respiratory tract and the ensuing local inflammatory response is reflected by pharyngitis, hoarseness, coryza, earache, conjunctivitis and cervical adenopathy.

Mycoplasmal species that infect humans are primarily mucosal pathogens that cause clinical disease by their deleterious effects on the host-cell epithelium, but this concept has been changing recently as evidence accumulates that some species can invade eukaryotic cells. Data obtained from the complete sequencing of the *M. pneumoniae* genome revealed the elimination of a number of genes essential for independent existence, consistent with the life cycle of an intracellular organism. Although intracellular growth and replication has been described for *M. pneumoniae in vitro*, this process has not been proven to occur during natural infections. However, some of the clinical characteristics of *M. pneumoniae* infections are consistent with what may be expected from an intracellular pathogen, including the establishment of latent or chronic infections, limited efficacy of some antimicrobials, necessity for prolonged treatment to eradicate infection in some instances and circumvention of the host immune response (Waites and Talkington, 2004).

Biological properties of *M. pneumoniae* its true role as a human pathogen have been hindered significantly over the years by its very slow replication rate (~6 h), fastidious demands for successful laboratory cultivation and the relatively low sensitivity and specificity of the earliest complement fixation serological tests, which were much better suited for less antigenically complex viral pathogens. Until recent years, as more sophisticated laboratory techniques have become available, dependence on non standardized serological tests performed in reference laboratories requiring measurement of antibodies in acute and convalescent sera meant that laboratory confirmation of mycoplasmal infection was seldom sought. Physicians could not easily distinguish mycoplasmal respiratory infection from clinically similar illnesses caused by several other bacteria including *Chlamydophila pneumoniae* and various respiratory viruses, and therefore did not appreciate how often it occurred in their patient populations (Ken et *al*., 2008). A

frequent, but incorrect assumption was that mycoplasmal respiratory infection was uncommon, rarely significant from a clinical standpoint and limited to select age groups. Primary-care physicians seldom considered a mycoplasmal etiology when patients presented with a more severe respiratory infection, or extra pulmonary manifestations, or when an elderly person, very young child or infant was involved. Moreover, the benefit of antimicrobial therapy was not always appreciated, allowing untreated persons to continue to spread the infection within their families, schools and communities. Spread among susceptible populations is also facilitated by the fact that many infectious persons are asymptomatic or very mildly ill and may not take precautions to limit exposure to others (Ken et *al*., 2008).

Knowledge gained over the past several years has proven that *M. pneumoniae* is a significant respiratory pathogen in persons of all ages, sometimes causing severe respiratory disease, and it may induce clinically significant manifestations in extra pulmonary sites by direct invasion and/or immunologic effects. Although most cases can be managed on an outpatient basis, *M. pneumoniae* is estimated to cause more than 100,000 adults hospitalizations each year in the USA (Waites and Talkington, 2004). Cytadherence and subsequent close association of the organism on the respiratory tract mucosa lead to a variety of effects that induce local inflammation and stimulate the host immune system to produce additional manifestations. The ability to detect acute *M. pneumoniae* infection has improved substantially owing to the development and commercialization of improved serological immunoassays, some of which are now point-of-care tests, and the introduction of molecular-based nucleic acid-amplification assays available in some clinical reference laboratories. Despite these significant advances, much remains to be learned about how this organism invades the body, interacts with the host immune system and produces disease. The biological properties of *M.*

*pneumoniae* and typical clinical manifestations of infection were comprehensively reviewed in 2004 (Waites and Talkington, 2004).

In the treatment of mycoplasmal pneumonia, antimicrobials against *M pneumoniae* are bacteriostatic, not bactericidal. Tetracycline and erythromycin compounds are very effective. The second-generation tetracycline's (doxycycline) and macrolides are the drugs of choice. Macrolide resistance has been reported in several areas of the world, but most experts agree that macrolides are the antibiotics of choice for treating *M. pneumoniae* infections in adults and children (Uh *et al*., 2013; Kawai *et al*., 2013; Cardinale *et al*., 2013; Biondi *et al*., 2014).

### 2.2.1.3.2. *Chlamydia pneumoniae*

*Chlamydia pneumoniae* (*C. pneumoniae*) was a leading cause of community-acquired atypical pneumonia; *C. pneumoniae* caused a spectrum of chronic diseases ranging from atherosclerosis to adult-onset asthma (Grayston, 2000). It is broad association with diseases in multiple organ- systems underscores the pathogen's capacity to infect a variety of host cell types. For example, whereas *C. trachomatis* is limited to productive infection within epithelial cells and fibroblasts, *C. pneumoniae* is capable of infecting and multi- plying within epithelial, endothelial and smooth muscle cells in addition to fibroblasts and macrophages. The organism is a strictly human pathogen that is transmitted by respiratory droplets. It therefore initially accesses the body through the respiratory system. Upper respiratory infection leads to subclinical disease or pharyngitis, sinusitis or bronchitis. Transmission into the lower respiratory tract is followed by productive infection of alveolar macrophages and subsequent development of community-acquired atypical pneumonia. It is thought that the inflammatory response that ensues leads to micro vascular damage, which enables the pathogen to escape into the vasculature. Monocytes or lymphocytes may serve as the vehicle of transport

for the obligate intracellular bacterium. The pathogen thus may seed multiple sites within the vasculature, including the coronary arteries, abdominal aortic aneurysms or carotid bifurcations to induce or contribute to atherosclerotic heart and vessel disease (Campbell *et al*., 2000).*C. pneumoniae* is an obligatory intracellular pathogen that causes upper and lower respiratory tract infections. Most adults have been exposure to this pathogen at some time during their life, and the seroprevalence increases with age due to recurring infections or reactivation of chronic infections. Like other Chlamydial species, also *C. pneumoniae* has a tendency to cause persistent infections (Hogan *et al*., 2004).

The bacterium was first isolated in 1965 in Taiwan from the eye of a child In 1999, a new taxonomy was proposed for the order Chlamydiales based on 16S and 23S rRNA comparisons; it included two new families, Parachlamydiaceae and Simkaniaceae to contain the recently identified *Chlamydia*-like species, and divided the family Chlamydiaceae into two genera *Chlamydia* and *Chlamydophila*. The genus Chlamydia contained, *C. trachomatis*, *C. muridarum*, and *C. suis* whereas *C. pneumoniae*, *C. pecorum*, *C. psittaci*, and the three new species (*C. abortus*, *C. caviae*, and *C. felis*) derived from *C. psittaci*, formed the new genus *Chlamydophila* (Everett *et al*. 1999). This new taxonomy has not generally been accepted among chlamydiologists; especially the division into two genera, based on minor differences in the 16S and 23S rRNA sequences, was criticized (Schachter *et al*. 2001). Also > 95% identity as a division criterion was considered inappropriate for intracellular organisms that are not fast-growing and do not commonly exchange DNA. Now, when the genomic sequences of several chlamydial species and strains are available, it has become apparent that there is an almost 80% conservation of genes and gene order between *C. abortus*, *C. trachomatis*, and *C. pneumoniae*, whereas in free-living bacteria it is common that almost no discernable conservation is seen in gene order beyond the operand

between strains of the same species (Stephens, 2008). The new taxonomy has also brought confusion to the field and two different names for *C. pneumoniae* can be found in publications. However *C. pneumoniae* is more widely used than *Chlamydophila pneumoniae*, and it has been proposed that "a one-genus, multiple-species" system should again be adopted (Stephens, 2008).

*C. pneumoniae* is an obligatory intracellular pathogen that has a two-phase developmental cycle typical to chlamydial species. Small (a diameter of approximately 0.3 μm) and metabolically inert elementary bodies (EBs) are the infectious form. The typical chlamydial EB is round and has a very little periplasmic space, The EBs may, however, sometimes be pear-like and contain a large periplasmic space. EBs attaches to the cell surface and then enters the cell through receptor-mediated endocytosis in clathrin-coated pits, pinocytosis in non-clathrin-coated pits, or by phagocytosis (Wyrick, 2000). The receptors used for entry may be different for different chlamydial species. It has been suggested that, to enter and infect endothelial cells, *C. trachomatis* may use a manning receptor and *C. pneumoniae* mannose a 6-phosphate/insulin-like growth factor 2 receptor (Puolakkainen *et al*., 2005). Inside the host cell, EBs inhibits fusion of phagosome with lysosome and instead associates with exocytic vesicles. For *C. trachomatis*, homotypic endosomal fusion results in one vacuole with several EBs, whereas *C. pneumoniae* and *C. psittaci* infections result in several inclusions per host cell (Wyrick, 2000). In the early phases of infection, *Chlamydia* secures their intracellular growth by inhibiting apoptosis (Peters *et al*., 2007). EBs then transform into larger (diameter ~1.0 μm) reticulate bodies (RBs), which are the replicative form. The RBs then begin to divide by binary fission and an inclusion 2–12 μm in diameter, consisting of up to thousands of bacteria, is formed. The shape of *C. pneumoniae* inclusions in HeLa cells is oval and dense. Growing *Chlamydia* acquire amino acids, nucleotides, and lipids from the pools of the host

cell. Membrane lipids like glycerophospholipids, sphingolipids, and cholesterol are acquired by selectively rerouting Golgi-derived exocytic vesicles and multivesicular bodies. It was recently shown that *C. trachomatis* also transports cytoplasmic lipid droplets into inclusions, possibly by a mechanism dependent on a chlamydial secreted protein Lda3 and an inclusion membrane protein IncA (Cocchiaro *et al*., 2008). In the late phase of the developmental cycle, different forms of *Chlamydia* can be found in the inclusion: dividing RBs, intermediate forms. At the end of the reproductive cycle, EBs is again formed and they escape from the cell by host cell lyses or exocytosis. *Chlamydia* may also enter into a persistent phase where the RBs or aberrant bodies (AB) are viable but abnormal looking, non-dividing, and their metabolic activity is low. This persistence may be triggered by cytokines like IF-γ, by antibiotic treatment, or by depletion of certain nutrients (Hogan *et al*., 2004).

In a primary *C. pneumoniae* infection, IgM antibody response develops approximately 2–3 weeks after infection, and after 2–6 months it usually cannot be detected anymore. IgG and IgA responses develop more slowly and may reach high titres only 6–8 weeks after infection and may then remain elevated for long periods. Especially low titres may persist for several years. However, due to the shorter half-life of IgA antibodies, IgA positivity disappears faster than IgG positivity. IgG and IgA responses are faster in re infections and can often be detected within 1–2 weeks after infection, whereas *C. pneumoniae* is found worldwide, and seroepidemiological studies show that over 50% of adults have been an exposed to this pathogen at some time during their life. In addition, seroprevalence increases with age due to e.g. recurring infections or reactivation of chronic infections (Dowell *et al*., 2001). In industrialized countries, the most prominent increase in seroprevalence is seen during the years 5 to 20, and in the elderly population seroprevalence is up to around 75%. In a study with children in

Finland, it has been shown by an enzyme immunoassay (EIA) method that primary *C. pneumoniae* infections occur at early ages (0.6–1.1 years), but that the IgG antibodies caused by these early infections usually decline rapidly; whereas an IgA response was seldom detected (Paldanius *et al*., 2005). Seroprevalence increased clearly at school age; IgG responses were more persistent and IgA antibodies were produced more often. No difference in IgG and IgA prevalence was seen between boys and girls (Paldanius *et al*., 2005).

*C. pneumoniae* is thought to cause ~10% of acute pneumonia cases that are often mild with prolonged onset, and ~5% of acute bronchitis and sinusitis cases. Pharyngitis is frequently associated with *C. pneumoniae* infection and is often relatively severe. In addition, common colds are frequent in *C. pneumoniae* infections or the infections may also be asymptomatic. Epidemics have been reported in all seasons of the year, and their course is usually long and the spread of infection from case to case is relatively inefficient. Re infections are thought to be common especially in elderly people, and among them fewer can be severe and last for long periods. Generally, re infections can, however, be either milder or more severe than primary infections. Co infections, especially with *Streptococcus pneumoniae* and *M. pneumoniae,* may occur (Monno *et al*., 2002).

In the case of *Chlamydia* the term "persistence" refers to a situation where a long-term association between them and their host cell is established; this situation is characterized by *Chlamydia* remaining viable but culture-negative. At the organism level, persistent infection means the immune system of the host does not eliminate the pathogen but it remains inside the host and may over time continue to cause damage. In the case of *Chlamydia,* this persistence may last from several months to years, and often without any obvious illness as an outcome. *In vitro* persistent infection, characterized by an altered developmental cycle and development of aberrant forms, can be triggered by IFN-γ, nutrient depletion, and

antibiotics (Hogan *et al*., 2004). Treatment of host cells with medium levels of IFN-γ during infection results in large RBs with abnormal metabolism (Pantoja *et al*., 2001). IFN-γ induces indoleamine 2, 3-dioxy hlamydial growth by nitric oxide (NO) induction, and intracellular iron depletion. The effect of IFN-γ treatment is reversible upon removal of IFN-γ and as a result internal reorganization of the enlarged forms and emergence of morphologically normal RBs and EBs is seen. Depletion of amino acids or glucose in a culture medium has been described to cause persistent forms of *C. trachomatis* in McCoy cells. Interestingly, amino acid concentrations seen in blood were found to induce aberrant forms. However the minimum requirement of each amino acid by *Chlamydia*, rather than the total amino acid concentration within cells, may be the limiting factor in this case (Harper *et al*., 2000). Iron participates in important cell functions like electron transport and DNA synthesis, and it is likely that *Chlamydia* transport and use iron from their host cells. Depletion of iron by treatment with deferoxamine mesylate (DAM), which removes free cellular iron, has been reported to cause persistent forms of *C. pneumoniae* in a cell culture, and this inhibition has been shown to be reversible by addition of iron-saturated transferrin. This suggests that iron levels may affect the outcome of chlamydial infections also in *vivo* (Al-Younes *et al*., 2001) Treatment of infected cells with antibiotics may result in persistent forms of *Chlamydia*, with the type and amount of antibiotics, and the time of the developmental cycle during treatment determining the primary molecular target. Mainly, treatment that targets bacterial protein or RNA synthesis results in inhibition of differentiation either from EBs to RBs or from RBs to EBs and treatment that targets DNA or peptidoglycan synthesis prevents RB-to-EB differentiation. (Hogan *et al*., 2004), Sub inhibitory concentrations of antibiotics used for treatment of *C. pneumoniae* infection have also been shown to induce persistence in HeLa cells (Gieffers *et al*., 2004). It is well known that chlamydial

species have a tendency to cause persistent infections in vivo. In the case of *C. trachomatis*, persistent infections have been associated with trachoma and tubal factor sub fertility (Den *et al*., 2006, Wright *et al*., 2008). In humans, persistent pulmonary *C. pneumoniae* infections and cases where extended antibiotic treatment has only caused temporary improvement have been described (Miyashita *et al*., 2002). In addition, persistent *C. pneumoniae* infection has been associated with several different diseases. These diseases themselves are also often chronic in nature, and include e.g. cardiovascular diseases like coronary heart disease, acute myocardial infarction, stroke, transient ischemic attack, and abdominal aortic aneurysm; neurological diseases like multiple sclerosis and Alzheimer's disease; respiratory tract diseases like asthma and chronic obstructive pulmonary disease; and other disease like lung cancer, non-Hodgkin lymphoma, and reactive arthritis (Saikku, 2002).

Prevalence of *C. pneumoniae* in Community Acquired pneumonia is 6 to 20% *C. pneumoniae* causes diseases in humans, including bronchitis, and sinusitis. It is also associated with atherosclerosis, coronary heart disease, and hyperlipidemia (Zheng *et al*., 2015). There are multiple reports validating the presence of *C. pneumoniae* in respiratory secretion fluid, nasal, tracheal and lung tissues of the patients with inflammatory lung disease (Teig *et al*., 2005; Brandén and Gnarpe, 2007; Loens *et al*., 2008). Moreover, *C. pneumoniae* can efficiently propagate in blood cells, in particular in mononuclear cells and lymphocytes (Anil *et al*., 2009). The presence of *C. pneumoniae* in the blood cells predetermines the possibility of pathogen dissemination from respiratory system to different organs and tissues. Besides respiratory organs *C. pneumoniae* can be detected in specimens from atherosclerotic plagues (Iriz *et al*., 2007). Cerebrospinal fluid (Schmeck *et al*., 2008). Which have been associated with diseases like coronary heart disease, stroke, abdominal aortic aneurysm, Alzheimer's disease, asthma, and reactive

arthritis (Saikku, 2002). *In vitro*, persistent infection can be triggered by interferon antibiotics, and depletion of nutrients (Hogan *et al*., 2004).

Cardiovascular disease (CVD) is a major health problem in developed countries with over 17 million deaths per year and the main pathological process underlying this disease is the atherosclerosis. Atherosclerosis typically begins with endothelial injury followed by low-density lipoprotein (LDL) oxidation and accumulation within vascular cells, triggering the pro-inflammatory cascade [interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α] and the subsequent proliferation of smooth muscle cells. Through this complex process, a sequence of events, including foam cell formation followed by fibrous cap and thrombus formation in the advanced plaque occurs leading to cardiovascular diseases, such as coronary heart diseases (angina, myocardial infarction), stroke, and peripheral vascular diseases (Laboz and Mostaza, 2007). Current opinion is that the most implicated infectious agent in the pathogenesis of CVDs is *C. pneumoniae*, known as the etiologic agent of respiratory tract infections (Rosenfeld and Campbell, 2001; Sessa *et al*., 2014). *C. pneumoniae* is an intracellular obligate pathogen with a unique developmental cycle, characterized by two alternating functionally and morphologically distinct forms: The elementary body, the metabolically inert and infectious form, and the reticulate body, the intracellular replicative form. In the last years, the attention has been drawn to a third non-replicating and non-infectious form, called persistent form, described as involved in the pathogenesis of chronic inflammatory diseases such as atherosclerosis. Indeed, chlamydial persistent form may endure for a long time inside host cells, since it is able to evade the host immune response leading to a chronic inflammatory state in the vascular wall (Hogan *et al*., 2004; Schoborg, 2011; Di *et al*., 2012).

To treat acute respiratory *C. pneumoniae* infections, antibiotics like macrolides (erythromycin, roxithromycin, clarithromycin, and azithromycin), fluoroquinolone

(the newer ones like Levofloxacin, moxifloxacin, gemifloxacin, and garenoxacin being more efficient), or tetracycline's can be used, and intensive, prolonged therapy is recommended (Blasi, 2004). However, it has been shown in a continuous cell culture model that 30-day treatment with azithromycin, clarithromycin, or Levofloxacin with concentrations similar to those achieved in epithelial lining fluid reduced but did not eliminate *C. pneumoniae* (Blasi, 2004). In a study that focused on eradication of replicating *C. pneumoniae* from coronary endothelial cells and smooth muscle cells, all the antibiotics tested were found to be effective, with rifampin showing the best results. Macrolide roxithromycin and quinolone moxifloxacin were the most efficient drugs (Gieffers *et al*., 2001). However, rifampin and azithromycin were not able to eradicate *C. pneumoniae* from cultured human Monocytes, but instead a persistent infection was developed (Gieffers *et al*., 2001).

Vaccination against C. *pneumoniae* infections could help prevents not only respiratory diseases caused by the pathogen but, if efficient against persistent infection, possibly also cardiovascular diseases. However, no such vaccine is available to date. The design of vaccines against *C. pneumoniae* infection is complicated by exacerbated immunopathology, possibly due to immune response against some chlamydial components. Thus, vaccine research has mainly focused on subunit vaccines and DNA vaccines against e.g. MOMP, Omp2, and Hsp60 (Finco *et al*., 2005). Some new vaccine candidates have been proposed in recent studies. In a genome-wide screening of multiple vaccine candidates, 53 recombinant proteins that induced mouse *C. pneumoniae*-binding antibodies were identified, and in further studies six of these were reported to induce *in vitro* neutralizing antibodies and four (Pmp2, Pmp10, OmpH-like, and enolase) also inhibited dissemination of *C. pneumoniae* in a hamster model (Finco *et al*., 2005). Vaccination with LcrE (a putative lid of the type III secretion system) fusion

protein was recently found to induce CD4+ and CD8+ T cell activation, type 1 cytokine response, and neutralizing antibodies, and to be effective in eliminating C. pneumoniae infection in mice (Thorpe *et al*., 2007).

### 2.3.1.2.3 *Legionella pneumophila*

(*L. pneumophila*) Legionellaceae family contains only one genus *Legionella* and over 52 species and 64 serogroup, which is one of the most important causes of respiratory disease in humans. The bacteria are abundant in man-made aquatic environments and water resources. The most important species of this genus is *L. pneumophila*, which has 15 serogroup. This bacterium is operating more than 90% of the legionary, which is an acute respiratory disease. Serogroup 1 and 6 are the causes of two thirds of cases of *Legionella* infection (Yu *et al*., 2002). Mortality rate in elderly and immunocompromised patients with *L. pneumophila* may be more than 30% (Bonetta *et al*., 2010). Various reports suggest that 1-5% of CAP as well as over 30% of hospital-acquired pneumonia infections is caused by *Legionella* (Berger *et al*., 2006). Children younger than two years or elderly adults and patients with immunodeficiency are at highest risk of infection. The infection is not contagious and only a breath of aerosols contaminated with this bacterium may cause infection and clean air is an important parameter in the prevention of respiratory infection (Shadrach *et al*., 2005). Timely diagnosis and treatment of infection disease is effective in reducing the mortality rate. Epidemiological findings indicate that this bacterium is transmitted through aerosols released of infected water sources and involves the respiratory system. Hospital environment as a growth area and people at risk of aerosol transmission are potential pre-disposing factors for growth and spread of these bacteria. *Legionella* are widely dispersed in natural and man- made water sources (Reischl *et al*., 2002). Legionnaire's outbreak depends on the contamination of water sources and sensitivity of individuals (Bonetta *et al*., 2010).

In 1976, an explosive outbreak of pneumonia of unknown etiology occurred among American Legion members attending a convention in Philadelphia. A total of 221 cases were documented, the death rate in this outbreak was 16% (Fraser *et al*., 1977). It was six month later that a Gram negative bacterium, presumed to be the etiological agent was isolated and characterized. Because of the historical association with the American Legion convention, the pneumonia is called Legionnaires' disease (LD), and the etiologic agents belong to the family Legionellaceae, with *Legionella pneumophila* being the species responsible for this outbreak. The availability of various detection methods for the disease showed that several prior unsolved outbreaks of pneumonia had been Legionnaires' disease. *Legionella* has been retrospectively identified as the cause of outbreaks of Legionnaires' disease since 1947 (McDade *et al*., 1977). Since then, Legionnaires' disease is identified as a cause of pneumonia all over the world.

The Legionellaceae are small obligatory aerobic Gram-negative bacilli with fastidious growth requirements. Proteins rather than carbohydrates are used as an energy source, the bacteria grows at temperatures ranging from 20° to 42°C. The *Legionella* bacteria are in the taxonomic order Legionellales, which includes the families Coxiellaceae and Legionellaceae. The Legionellaceae and *C. burnetti*, the cause of Q-fever, have similar intracellular lifestyles, and may have common genes associated with the infection processes in their hosts. Three different genera have been proposed for the Legionellaceae: *Legionella*, *Fluoribacter*, and *Tatlockia*; however, the later two generic names have never been widely used or accepted, and the single genus *Legionella* is almost universally used to describe all species. At least 58 different *Legionella* species have been described. In 25 of these species some strains have been reported to infect humans (Muder *et al*., 2010). *L. pneumophila* contains at least 15 different serogroup; ten other species can be subtypes into at least two serogroup, with the remaining species containing only

one serogroup each (Bartram *et al*., 2007; Helbig *et al*., 2007). Serogroup 1 caused the 1976 Philadelphia outbreak and is the cause of 70 % to 90 % of all cases of Legionnaires' disease for which there has been a bacterial isolate. Like *L. pneumophila*, other *Legionella* species are widely distributed in aquatic habitats and soil. Recovery of these species is generally less frequent and technically more demanding than is recovery of *L.* pneu*m*ophila. Twenty-five *Legionella* species have been documented to cause human infection based on isolation from clinical material (Muder *et al*., 2010). Isolates of the other species are limited to water and soil, although several have been implicated in human infection based on seroconversion in the absence of isolation.

The natural habitat for *Legionella* appears to be aquatic bodies including rivers, streams, and thermally-polluted waters (Brooks *et al*., 2004). *Legionella* bacteria have been detected in all segments of water distribution – from the source water (rivers and ground water) to the tap. Natural aquatic bodies contain only small numbers of Legionella. The presence of *Legionella* in a water distribution system is not necessarily an indication that the system is poorly maintained, as this bacterium may be a normal constituent of the microbial population of water distribution systems. It has been estimated that *Legionella* are found in approximately 50% of large building water systems and 10-30% of home water systems in the U.S. (Stout and Yu, 2011) and detection methods are becoming increasingly sensitive. Depending on the study and methods, a range of 12-70% of hospital water systems are estimated to be colonized with *Legionella* (Stout and Yu, 2011). Recent publications have demonstrated the presence of 'nonculturable' cells of *L. pneumophila* and methods for their resuscitation to ensure that colony counts are not underestimated (Ducret *et al*., 2014).

In study to use more sensitive molecular techniques *Legionella* genetic material was detected in 50% of cold water samples (Donohue *et al*., 2014). Cooling towers

and, to a lesser degree, evaporative condensers were implicated in the earlier outbreaks prior to recognition of potable water as a reservoir (Bentham, 2000; Nguyen *et al*., 2006). The emphasis of cooling towers in the dissemination of *Legionella* has been challenged. Reports of cooling towers as reservoirs for legionellosis have dwindled in comparison to those linked to building water distribution systems. *Legionella* are not completely eliminated from drinking water by standard water treatment practices. For example, *Legionella* are comparatively more resistant to chlorine than *Escherichia coli* (Kim, 2002; Hosein, 2005; Zhang *et al*., 2007; Garcia and Pelaz, 2008). *Legionella* growth and proliferation occur in engineered habitats, especially water distribution systems, which provide favorable water temperatures (25°-42°C), surfaces for biofilms formation, and nutrients (Murga *et al*. 2001; Donlon and Costerton 2002). One important factor appears to be water temperature. Buildings with recirculating hot water distribution systems colonized with *L. pneumophila* were significantly more likely to have lower hot water heater temperatures (< 60° C) than systems that were not colonized (Darelid, 2002). The microorganism is readily found in biofilms and detritus at the bottom of hot water tanks. Bacteria, protozoa, and amoebae also colonize water pipe surfaces, some of which have been shown to promote *Legionella* replication (Buse *et al*., 2014). *Legionella* and other microorganisms attach to surfaces and form biofilms on pipes throughout the water distribution system. Cold-water sources such as ice from ice machines and water from fountains with stable, biofilms colonized surfaces have also been implicated as a source of infection (O'Loughlin *et al*., 2007). The prevalence of community-acquired and healthcare-associated legionellosis are both increasing. One-quarter (25%) of *Legionella* spp. infections are healthcare-associated (Neil and Berkelmann, 2008). As it has been shown that *L. pneumophila* is present in drinking water distribution systems and household water (Borella *et al*., 2005; Donohue et al. 2014), persons at risk for Legionnaires'

disease should take precautions. Risk factors for Legionnaires' disease include: reduced immune competence, smoking, alcoholism, and older age (CDC, 2011). Case reports are highest in summer (CDC, 2011). After transmission from the environment to humans by inhalation of an infectious aerosol or by aspiration, *Legionella* spp. can cause pneumonia with severe multisystem disease (Legionnaires' disease). It is an atypical pneumonia, nonproductive cough and with no clinical differences as compared to other pneumonias. Apart from pneumonia, *Legionella* is associated with a self-limited influenza-like respiratory infection (Pontiac Fever). Some *L. pneumophila* strains are more virulent than others, although the precise factors causing virulence remain unclear. *Legionella* produces a number of exotoxins, including a hemolysin, cytotoxin, deoxyribonuclease, ribonuclease, and various proteases. Legionellaceae also produce a weak lipopolysaccharide endotoxin capable of activating the complement pathway. Some individuals may be more susceptible to Legionnaires' disease if conditions that hinder mucociliary clearance, e.g., smoking or underlying disease are present. Surgery and organ transplantation are major risk factors for acquisition nosocomial *Legionella* infection. Chronic obstructive pulmonary disease and immunnosuppression have also consistently been implicated as risk factors for acquisition of Legionnaires' disease. The key to diagnosis is performing appropriate microbiological testing (Fields *et al*., 2002).

Legionnaires' disease lacks characteristic symptoms or signs, there is no typical syndrome, and not everyone exposed to the organism will develop symptoms of the disease (Boshuizen *et al*., 2001; Fields *et al*., 2002). A large epidemiological study of a major outbreak of Legionnaires' disease associated with a flower show in the Netherlands found that 16% of cases had incubation times longer than 10 days, with the average being 7 days (Jonker *et al*., 2004). Legionnaires' disease generally starts with fever, headache, fatigue, muscle aches and cough, initially

characterized by anorexia, malaise and lethargy (Lettinga *et al*., 2002). Legionnaires' disease patients may develop a mild and unproductive cough. Before full-blown pneumonia is present, chest pain, diarrhea, confusion, shaking chills and shortness of breath may be seen. Fewer than one half of the patients with Legionnaires' disease produce purulent sputum, and about one third develops blood-streaked sputum or cough up blood (haemoptysis). Chest pain, either pleuritic (i.e. involving infection of the lung lining) or non-pleuritic, is prominent in about 30% of patients, and may be mistaken for blood clots in the lungs when associated with haemoptysis. Gastro- intestinal symptoms are prominent, with up to half of patients having watery diarrhea, and 10–30% suffering nausea, vomiting and abdominal pains (Muder and Yu, 2002; Darby and Buising, 2008). If untreated, Legionnaires' disease usually worsens during the first week and can be fatal. The most frequent complications are respiratory failure, shock and acute renal and multi-organ failure. According to the source of infection, Legionnaires' disease is commonly classified as community-acquired nosocomial, or travel associated. *Legionella* is an important pathogen in health-care acquired (nosocomial) pneumonia, particularly in immunocompromised patients. *Legionella* spp. can also cause community-acquired pneumonia (CAP), the more severe cases of pneumonia required hospitalization or admission into intensive care. A confirmed case of Legionnaires' disease is a patient presenting clinical and radiological signs of pneumonia with at least one of the following laboratory criteria:

**1.** Isolation of *Legionella* spp. from respiratory secretions or any normally sterile site.

**2.** Detection of *L. pneumophila* antigen in urine.

**3.** *L. pneumophila* serogroup 1 specific antibody response.

A probable case of Legionnaires' disease is defined as a patient presenting clinical and/or radiological signs of pneumonia with at least one of the following laboratory criteria:

**1.** Detection of *L. pneumophila* antigen in respiratory secretions or lung tissue e.g. by direct fluorescent antibody staining,

**2.** Detection of *Legionella* spp. nucleic acid in a clinical specimen,

**3.** *L. pneumophila* non-serogroup 1 or other *Legionella* spp. specific antibody response,

**4.** A single high titer in specific antibody response for *L. pneumophila* serogroup 1, other serogroup or other *Legionella* spp.

After experiencing Legionnaires' disease, almost half of patients suffer from disorders related to the nervous system, such as confusion, delirium, depression, disorientation and hallucinations (Lettinga *et al*., 2002). These disorders may also occur in the first week of the disease. Other neurological deficits that can arise after a severe infection include residual cerebellar dysfunction. Minor problems may include persistent pulmonary scars and restrictive pulmonary disease in some patients who experience severe respiratory failure, retrograde amnesia, and neurological symptoms (Morgan *et al*., 2004). Appropriate treatment usually results in full recovery; however, long-term pathological conditions resulting from the disease may occur (Lettinga *et al*., 2002). The mortality rate among patients with Legionnaires' disease caused by *L. pneumophila* varies from 0% to 26 %, depending on the clinical setting, severity of disease, patient population and timely antimicrobial treatment (Lettinga *et al*., 2002). Early recognition, through correct diagnostic methods, enables appropriate antimicrobial treatment which is potentially life-saving (Gacouin *et al*., 2002). *Legionella* spp. is facultative

intracellular bacteria that can survive and multiply in human macrophages. The intracellular location of this pathogen is thought to be relevant for the efficacy of the antimicrobial agent in the treatment of the disease. Antimicrobials that achieve intracellular concentrations higher than the minimal inhibitory concentration (MIC) are regarded as more effective than antibiotics with poor intracellular penetration. The antimicrobial agents most commonly used for treatment are fluoroquinolone, macrolides and rifampicin (Barlett *et* al., 2000; Yu *et al*., 2004).

Pontiac fever, or non-pneumonic Legionellosis, is a milder form of infection associated with *Legionella* spp. It is an acute, self-limiting, influenza-like illness without pneumonia. Pontiac fever occurs after exposure to aerosols of water colonized with *Legionella* spp. Unlike Legionnaires' disease, Pontiac fever has a high attack rate, often in the range 50% to 80%, affecting 95% or more of exposed individuals. The influenza-like syndromes are fever, chills, headache, and muscle pain, with or without nausea. This type of Legionellosis is a mild, self-limited illness of short duration, the incubation period is 1-3 days, and does not require antimicrobial treatment (Darby and Buising, 2008).

The accidental human infection is a dead end for *L. pneumophila* replication. Human hosts act neither as a reservoir nor a viable vector for transmission of *Legionella*, rendering any infection futile. The lack of LD transmission among humans remains with no satisfactory explanation to date.

The distribution of LD cases by age and sex are homogenous among countries, being children the less affected age-class. Most cases correspond to men older than 50 years (74-91%). The mortality rate is normally between 8-12% and the case-fatality rate in Europe is 10% (range 0-27% in countries reporting at least 30 cases) and 8% in the USA (Dominguez and Alvarez, 2009; Joseph and Ricketts, 2010). Nosocomial cases have a higher case-fatality rate, in the 15-34% range. Comparisons of the incidence of legionellosis cases in different countries are

highly dependent on the number of laboratory confirmations but also on the notification rates. LD is considered as underreported because clinicians prescribe broad-spectrum antibiotics that cover for Legionella spp. and rarely ask for laboratory confirmation. Besides, although being tested, positive results are not always notified to health authorities (De *et al*., 2012). Most of the cases (69%) were acquired in a community, 20% were travel-associated and 8% linked to healthcare facilities. *L. pneumophila* was found as the causal agent in 98% of the cases confirmed by culture, 85% of them corresponding to serogroup 1. These notified cases included 99 clusters, the largest one involving 42 cases in a hotel in Spain (Vanaclocha *et al*., 2012). The incidence of LD has increased in the USA in the period 2000-2009, with the north-eastern states reporting most of the cases123. Australia and New Zealand have an additional peak of LD cases due to *L. longbeachae* potentially linked to potting soil and gardening activities (Currie *et al*., 2014).

Numerous outbreaks of Legionnaire's disease have occurred since the discovery of the bacterium, which in the grand scheme of known infectious agents was relatively recent. This late emergence can be attributed to increased urbanization and development and use of anthropogenic water systems, as witnessed by outbreaks from water towers and presence of the bacteria in household water systems and air conditioners (Nguyen *et al*., 2006). Of particular hazard are ventilation and cooling systems in hospitals, which put already immunocompromised patients at high risk of infection by *L. pneumophila*, nearly all cases arise from artificial water sources, as the bacteria are generally incapable of replicating independently of a host in natural water sources(Sabria and Yu, 2002).

In its freshwater environment, the bulk of *L. pneumophila* colonizes pre-existing biofilms (Murga *et al*., 2001), where the bacteria cycle between replicative and

infections phases. This biphasic lifestyle is reflected by major changes in gene expression (Brüggemann *et al*., 2006). The natural host for *L. pneumophila* is the free-living and soil dwelling *Acanthamoeba castellanies*. It is viability is not limited to *A. castellanies*, but extends to a variety of amoebae or protozoa including *Hartmannella vermiformis*, Dictyostelium discoideum (Hagele *et al*., 2000; Solomon *et al*., 2000), *Tetrahymena pyriformis* and *Naegleria fowleri* among others. Multiple mammalian cell lines can also support replication. In spite of *L. pneumophila* broad host range for intracellular replication, extracellular growth in the laboratory is only supported with special media. It was proposed that *L. pneumophila* co-evolved with its natural host resulting in a selection of virulence factors which can also support infection in other organisms (Greub and Raoult, 2004; Brüggemann *et al*., 2006). Namely, human alveolar macrophages bear significant resemblance to *A. castellanies*, and as such are the target of *L. pneumophila* infection in humans. The organism has a very plastic survival mechanism, employing several strategies to evade host defenses and exploit host factors to create a replication-permissive niche in a vacuole derived from the host's plasma membrane (PM) (Hubber and Roy, 2010).

The most important etiological agent of Legionnaires' disease (LD) is *L. pneumophila* serogroup 1, accounting for more than 90% of human infections in North America and Europe (Roig & Rello, 2003; Doleans *et al*., 2004). The mortality rate among patients with *L. pneumophila* infections continues be high up to 26% (Lettinga *et al*., 2002). Therapeutic failure and unsuccessful treatment of *L. pneumophila* infection has also been documented, but delay in medical attention or untimely or inappropriate antimicrobial treatment has usually been attributed as the main cause (Pedro and Yu, 2006).

In study carried out to evaluate the performance of different diagnostic tests for Legionnaires' disease in a clinical setting where *L. pneumophila* PCR had been

introduced. Electronic medical records at the Cleveland Clinic USA were searched for *Legionella* urinary antigen (UAG), culture, and PCR tests ordered from March 2010 through December 2013. For cases where two or more test methods were performed and at least one was positive, the medical record was reviewed for relevant clinical and epidemiologic factors. Excluding repeat testing on a given patient, 19,912 tests were ordered (12,569 UAG, 3,747 cultures, and 3,596 PCR) with 378 positive results. The positivity rate for each method was 0.4% for culture, 0.8% for PCR, and 2.7% for UAG. For 37 patients, at least two test methods were performed with at least one positive result: 10 (27%) cases were positive by all three methods, 16 (43%) were positive by two methods and 11 (30%) were positive by one method only. For the 32 patients with medical records available, clinical presentation was consistent with proven or probable Legionella infection in 84% of the cases. For those cases, the sensitivities of culture, PCR, and UAG were 50%, 92%, and 96%, respectively. The specificities were 100% for culture and 99.9% for PCR and UAG (Derrick *et al*., 2015).

### 2.2.1.3.4. Diagnosis

## A) Serological methods

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 cornerstone of *M. 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 percent- age 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 that have been used in the past

have 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.

During seventies a number of serologic assays were developed and evaluated to detect antibodies to *Legionella* spp (Boshuizen *et al*., 2003). Of the various antibody detection methods that are available, indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assays (EIA) are the most used ones. For serodiagnosis of the disease it is recommended that an acute-phase serum sample be collected early and a follow-up serum specimen be collected within 2-6 weeks. A fourfold rise in antibody titer between an acute-phase serum specimen and a convalescent-phase specimen is considered suggestive of infection with *Legionella* spp (Boshuizen *et al*., 2003). In most cases unfortunately, the development of a diagnostic fourfold rise in antibody titer can be slow and may occur in no more than 75% to 80% of patients who ultimately are shown to have Legionnaires' disease (Boshuizen *et al*., 2001). Antibodies in patients with culture-confirmed Legionnaires' disease are not detectable within 3 weeks and immunosuppressed patients may not produce detectable antibodies (McWhinney *et al*., 2000; Yu and

Stout, 2008). Single elevated titers may be suggestive of *Legionella* infection; a high titer does not necessarily indicate recent infection because high titers can still be detected 48 month after disease onset. In the normal population elevated anti-*Legionella* titers have been found to vary from 1 to 36% (Fields *et al*., 2002; Edelstein, 2002; Den and IJzerman, 2004). Also high antibody titer can persist in healthy persons with no current clinical evidence of Legionnaires' disease. It is also known that using antigens for antibody testing has the potential for cross-reacting with serum from patients with other kinds of infections. A disadvantage of serological testing is the inability to detect accurately all *Legionella* species and serogroup. Seroconversion (fourfold increase of antibody titer) may take several weeks and therefore not useful as a diagnostic tool. The use of serologic methods remains important for epidemiological studies of outbreaks, prevalence studies etc (Elverdal *et al*., 2011).

The urine immunochromatographic (ICT) was developed for detection of *L. pneumophila* serogroup l, which is the cause of most *Legionella* infections (80%) causing CAP.4 In the outbreak of legionnaires' disease (LD) in 1999 in the Netherlands the urine ICT showed a sensitivity of 72% and after concentration of the urine samples a not statistically significant increase in sensitivity to 81% (Yzerman *et al*., 2002), demonstrated an association between sensitivity and clinical severity. A high sensitivity was seen in patients with severe community acquired pneumonia, while the urinary antigen test was less reliable in milder cases of LD. That the urine ICT had a specificity of 99% since *L. pneumophila* is an infrequent cause of CAP in our region, the urine ICT does not have to be performed on all patients admitted with CAP. Instead, the urine ICT is indicated when a *L. pneumophila* infection is considered in the differential diagnosis or as a routine diagnostic investigation in patients referred to ICU, considering the strong association between *Legionella* infection and the need for admission to an ICU.'

These test results will remain positive for weeks even in the presence of adequate antibiotic therapy (Lettinga *et al*., 2002).

Direct fluorescent antigen (DFA) is a diagnostic test used for detection of *L. pneumophila* antigen directly in respiratory specimens and tissue samples. This technique has the advantage of providing a result within 2-3h which allows preliminary information useful in guiding treatment. Method with high specificity estimated at 94% although can be less specific with inexperienced laboratory personnel, due the possibility of cross-reactions with other bacteria, including *Pseudomonas* spp. Values of sensitivity have a range of 25 to 70% once is technical demanding and depending on sputum sample. For this all reasons DFA is only considered a probable diagnosis of *Legionella* infections according to European Working Group for *Legionella* Infections (EWGLI) (Fields, 2002; Murdoch, 2003; Pedro, 2011).

The microimmunofluorescence (MIF) test measures *C. pneumoniae*-specific antibodies quantitatively and is the serologic assay of choice to detect antibodies to *C. pneumoniae* (Wang, 2000; Dowell *et al*., 2001). However, the MIF test is technically demanding and requires expertise in fluorescence microscopy. Findings can vary with reagents, antigens, incubation time and temperature, the microscope, and experience of the technician. Studies have linked infection with *C. pneumoniae* (as defined by titers of immunoglobulin G [IgG] or IgA antibodies with an increased risk of several chronic diseases. However, results have been inconsistent (Danesh *et al*., 2000). It is important to understand if the weak associations observed in some studies are due to measurement error in detection of IgA or IgG antibody levels or to true weak or null effects. Because no "gold standard" for antibody measurement exists. Thus, a key measure of error in IgA and IgG antibody levels is the reliability of repeated testing of specimens on the same subjects at different laboratories (Danesh *et al*., 2002).

## B) Molecular Detection

Polymerase chain reaction (PCR) is technique that amplifies a single or a few copies of apiece of DNA across several orders of magnitude generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences comp- lementary the target region along with a DNA polymerase (after which the method is named) is key components to enable selective and repeated amplification. As PCR progresses¸ the DNA generated is itself used as template for replication¸ setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulation (Murdock, 2004). PCR is an appealing tool for the diagnosis of *M. pneumoniae*. However, false positive results, due to primer specificity or carry over contamination from previous PCR reactions, can occur (Murdoch, 2003), while the presence of inhibitory substances in the clinical sample could produce false negative results. Most studies have used lower respiratory tract samples, such as sputum. A major limitation of lower respiratory tract samples is that they are difficult to collect, requiring invasive techniques (Murdoch, 2003). In contrast, (Murdoch, 2003) suggested that nasopharyngeal secretions, sputum, endotracheal aspiration, pleural fluid and lung tissue were acceptable specimens for PCR, but the highest sensitivity and specificity was obtained with sputum. Different target sequences, within specific genes, have been used for PCR detection. Examination of the 16S rRNA sequence has revealed the existence of highly conserved regions, which display sequence variability at the genus and species levels, thus allowing the selection of genus and species specific primers. The P1 adhesin gene is an appropriate target for PCR, because of its repetitive nature within the genome. Although similar P1 adhesin genes have been

found in other *Mycoplasma* species, some of the highly conserved regions are unique to *M. pneumoniae* enabling PCR primers directed at these regions to be species specific .Various PCR assays have been used for the detection of *M. pneumoniae*, such as real time PCR (Daxboek *et al*., 2003), capillary PCR (Honda *et al*., 2000), multiplex PCR (Loens *et al*., 2003), loop-mediated isothermal amplification (LAMP) (Saito *et al*., 2005), nucleic acid sequence-based amplification and the enzyme- linked gel assay (Loens *et al*., 2003). PCR has been successfully used to detect *Legionella* DNA in clinical and environmental samples and are promising for a rapid diagnosis of legionellosis. There are several techniques available using rRNA (ribosomal RNA): rRNA 5S, 16S rRNA gene MIP (macrophage infectivity potentiator) among others, which makes a non standardized method due to different degrees of sensitivity and specificity. Therefore, any result should be interpreted as a probable diagnosis. The technique of real time PCR (RT-PCR) combined with hybridization probe enables the specific amplification of *Legionella* DNA, providing results in a short time and confirming the reduction cases of cross- contamination (Murdock, 2004).

## C) Sequence alignments Tools

Sequence alignments provide a powerful way to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be inferred from well designed queries and alignments. BLAST (Basic Local Alignment Search Tool), provides a method for rapid searching of nucleotide and protein databases. Since the BLAST algorithm detects local as well as global alignments, regions of similarity embedded in otherwise unrelated proteins can be detected. Both types of similarity may provide important clues to the function of uncharacterized proteins (Schwede *et al*., 2003).

## D) Sequence-based typing

Sequence based typing (SBT) is a variant of multilocus sequence typing that employs variations from multiple chromosomal locations, or genes. Currently, the European SBT panel includes six *L. pneumophila* genes: flaA, pilE, asd, MIP, mompS, proA. Thus, an SBT type comprises a string of the individual allele numbers of each of these genes separated by commas. The major advantages of SBT are stability of the marker, good discriminatory power if appropriate loci are selected, and flexibility, since additional gene loci can be investigated if necessary. Data are readily exchanged among laboratories either as sequence data or as designated alleles. Furthermore, SBT reduces the need to transport live bacteria, since nucleotide sequence determination from PCR products can be achieved from killed-cell suspensions, purified DNA, or clinical material. While SBT is particularly suited to long-term and global epidemiology, as it identifies a variation which is accumulating slowly within a population, the data can also be used to investigate single cases or outbreaks (Gaia *et al*., 2005). Currently, the allocation of the allele formula can be done using the EWGLI website.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1. Study Design

### 3.1.1. Study type

This is a descriptive cross-sectional study.

### 3.1.2. Study area

Greater Khartoum is located between latitudes 15º26 and 15º45 N and longitudes 32º25 and 32º40 E, at an altitude of 405.6 m above sea level. Khartoum State lies at the junction of the two rivers, the White and the Blue Niles in the North Eastern part of central Sudan. A total area is about 20,736 km2. The Khartoum State consists of three locality with a total population 5274321 peoples were distributed as fellows; Omdurman Locality (2215330), Al-Khartoum Locality (1582027) and Bahri Locality (1247745). The people residence in Khartoum is from different ethnic groups. From the total population of Khartoum State about 61% of the population is aged 15–64 years indicating that, the more productive groups are migrating to Khartoum. Omdurman is the most populous part of the city, housing 43.5 per cent of total population (Central Statistical Organization: http://www.cbs.gov.sd/en/files.php?id=7#&panel1-2). (Khartoum state police:

http://www.ksp.gov.sd/en/index.php?option=com_content&view=article&id=1&Itemid=2).

The specimens for this study were collected from three major Localities in Khartoum State including Al-Khartoum Locality, Bahri Locality and Omdurman Locality. Serology and molecular parts were carried out in the Research

Laboratory, Sudan University of Science and Technology (SUST). Gene sequencing was done by Macrogen, Inc. Korea.

### 3.1.3. Target population

Patients suspected to have atypical community-acquired pneumonia and presented to the chest units of Omdurman Teaching Hospital, Abo Anja Teaching Hospital, Bahry Teaching Hospital and Al-Shaab Teaching Hospital were enrolled. The age groups of the target population were divided into three categories as follows; young adult; 13-30 years old, middle age; 31-60 years old and elderly more than 60 years old (Salama *et al.*, 2012).

### 3.1.4. Study duration

This study was carried during the period from March 2015 to January 2016.

## 3.2. Inclusion criteria

Patients presenting with symptoms of pneumonia regardless age or sex were included in this study.

## 3.3. Exclusion criteria

Patients with symptoms of pneumonia and under treatment with antibiotics or refuse to sign consent were excluded from this study.

## 3.4. Sample size

The sample size was determined according to the following formula (Wood and Mark, 1999),

$N=Z^2\times ð^2/Se^2$

Where:

N= sample size

$Z^2$= tabulated level of confidence limited medical research 95%=$1.96^2$=3.84

$ð^2$= the expected population value. In this study it was equal to 50%, because there is no previous study.

$Se^2$= standard error chance in medical research, which can be accepted in the result, =5%

N= $[(1.96) \times (50/100)]^2 / (0.05)^2$ =380.

## 3.5. Data collection

Full information of each patient was collected using a predesigned questionnaire (Appendix I) including age, sex, hospital, residence and date of collection.

## 3.6. Ethical considerations

The proposal of the study was approved by the College Ethical Committee, College of Medical Laboratory Science, SUST. The information regarding risk factors (if any) was explained to all patients under the study. Maintaining confidentiality of information obtained from patients. Consent to collect the specimens and seciodemographic data were obtained from patients and hospitals administration participated in this study (Appendix II).

## 3.7. Target pathogenic bacteria

All fastidious atypical bacterial pneumonia such as *M. pneumoniae, C. pneumoniae and L. pneumophila* were considered target pathogenic bacteria.

## 3.8. Sampling technique

Expectorated sputum specimens were collected in sterile wide mouth containers with screw caps. Five ml of venous blood were also collected using a disposable sterile plastic syringe and transferred to sterile plane tubes. All specimens were transported to the laboratory as soon possible. Serum was separated from blood cells by centrifugation for 10 minutes at 5000 round per minute (rpm) at room temperature. The sera were preserved at -20°C till use.

## 3.9. Detection of atypical pathogens

### 3.9.1. *M. pneumoniae*

Anti- *M. pneumoniae* ELISA test (Euroimmun kit-Germany) was done according to manufacturer's instructions to detect *M. pneumoniae* in patients as follows.

1. All reagents were brought to room temperature (+18°C to +25°C) for at least 30 minutes before use.
2. 100 µl of the calibrator, positive and negative controls or diluted patient serum were transferred into the individual micro plate wells according to the pipetting protocol.
3. The plate was incubated for 30 minutes at room temperature, then washed 3 times using 300 µl of working strength wash buffer for each wash; the wash buffer in each well was left 30 to 60 seconds per washing cycle, and then discarded.
4. 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgM) was added to each well of the micro plate than incubated for 30 minutes at room temperature.
5. The wells were washed as described above.
6. 100 µl substrate of Chromogen/substrate solution was added into each of the micro plate wells, then incubated for 15 minutes at room temperature (protected from direct sunlight).
7. 100 µl of stop solution was added into each well in the same order and at the same speed as the Chromogen/substrate solution was introduced to stop the reaction.
8. Photometric measurement of the color intensity was carried out at a wave-length of 450 nm and a reference wavelength between 620 nm and 650 nm within 30 minutes of added the stop solution.

9. Prior to measure the micro plate was shacked slightly to ensure a homogeneous distribution of the solution.
10. results were interpreted as follows;

Ratio <0.8: negative

Ratio 0.8 to <1.1: borderline

Ratio 1.1: positive

### 3.9.2. *L. pneumophila*

This organism was detected using Anti-*Legionella pneumophila* immunofluorescence test IIFT (IgG, IgAGM), (Euroimmun kit-Germany), following the manufacturer's instructions as follows;

1. Reagents were brought to room temperature for at least 30 minutes before used.
2. To prepare PBS-Tween one pack of "salt for PBS" was dissolved in 1 liter of distilled water and mixed with 2 ml of Tween 20 for 20 min until homogeneous.
3. The serum was diluted 1:100 in PBS-Tween and mixed thoroughly for 4 seconds.
4. 30 µl of diluted serum was added to each reaction field of the reagent tray.
5. Incubation started by fitted the BIOCHIP slide in to the corresponding recesses of the reagent tray, of the each sample was contact with BIOCHIP put individual sample not came into contact with each other.
6. The tray was incubated for 30 minutes at room temperature (+2°C to +25°C).
7. BIOCHIP slides was rinsed and flushed with PBS-Tween using a beaker and was immersed them immediately.

8. The cuvette containing PBS-Tween was shacked for at least 5 minutes with a rotary shaker.
9. 30 µl of fluorescein-labelled anti-human globulin was applied to each field of a clean reagent tray; all droplets were added before continuing incubation by using a stepper pipette.
10. BIOCHIP Slide was removed from the PBS-Tween within five seconds, blot the back and the long edges was cleaned with a paper towel and immediately the put into the recesses of the reagent tray.
11. The BIOCHIP Slide was rinsed then incubated for 30 min at room temperature with a flush of PBS-Tween using a beaker and put in a cuvette containing PBS-Tween for at least 5 min.
12. 10 drops of Evans Blue (150 µl) was added to 150 ml phosphate buffer as a counterstaining, then glycerol/PBS was placed onto a cover glass.
13. The BIOCHIP Slide was removed from the PBS-Tween using a polystyrene embedding template.
14. The back and all four edges of the BIOCHIP as well as the surface around were dried with a paper towel.
15. The BIOCHIP Slide was put facing downwards, onto the prepared cover glass which was properly fitted into the recesses of the slide.
16. Finally the BIOCHIP Slide was read under the fluorescence microscope, and the result was recorded as positive and negative.

### 3.9.3. *C. pneumoniae*

Anti- *C. pneumoniae* immunofluorescence test IIFT (IgM) (Euroimmun kit-Germany) was used to detect *C. pneumoniae*. The procedure was done according to the manufactures instructions.

1. Reagents were brought to room temperature for at least 30 minutes before used.
2. To prepare PBS-Tween one pack of “salt for PBS” was dissolved in 1 liter of distilled water and mixed with 2 ml of Tween 20 for 20 min until homogeneous.
3. The serum was diluted 1:100 in PBS-Tween and mixed thoroughly for 4 seconds.
4. 30 µl of diluted serum was added to each reaction field of the reagent tray.
5. Incubation started by fitting the BIOCHIP slide in to the corresponding recesses of the reagent tray, of the each sample was contacted with BIOCHIP put individual sample not came into contact with each other.
6. The try was incubated for 30 minutes at room temperature (+2°C to +25°C).
7. BIOCHIP slides was rinsed and flushed with PBS-Tween using a beaker and was immersed immediately.
8. The cuvette containing PBS-Tween was shacked for at least 5 minutes with a rotary shaker.
9. 30 µl of fluorescein-labelled anti-human globulin was applied to each field of a clean reagent tray; all droplets were added before continuing incubation by using stopper pipette.
10. BIOCHIP Slide was removed from the PBS-Tween within five seconds, blot the back was blotted and the long edges were cleaned with a paper towel and immediately the put into the recesses of the reagent tray.
11. The BIOCHIP Slide was rinsed then incubated for 30 min at room temperature with a flush of PBS-Tween using a beaker and put in a cuvette containing PBS-Tween for at least 5 min.
12. 10 drops of Evans Blue (150 µl) where added to 150 ml phosphate buffer as a counterstaining, then glycerol/PBS was placed onto a cover glass.

13. The BIOCHIP Slide was removed from the PBS-Tween using a polystyrene embedding template.
14. The back and all four edges of the BIOCHIP as well as the surface around were dried with a paper towel.
15. The BIOCHIP Slide was put facing downwards, onto the prepared cover glass which was properly fitted into the recesses of the slide.
16. Finally the BIOCHIP Slide was read under the fluorescence microscope, and the result was recorded as positive and negative.

## 3.10. Molecular techniques

### 3.10.1. DNA extraction

The DNA extraction was done by bacterial DNA Preparation Kit, (Jena Bioscience, Germany), following the manufacturer's instructions. The procedure was carried out as follows.

1. 1 ml of sputum was transferred into a 1.5 ml micro tube.
2. The sputum was centrifuged at 15,000 rpm for 1 min then the supernatant was discarded.
3. 300 µl of Cell Lyses Solution was added to resuspend the pellet.
4. 1.5 µl of RNase a Solution was added and mixed well.
5. Incubation was done at 37 °C for 15-30 min.
6. 100 µl of Protein Precipitation Solution was added and vortexed done vigorously for 20-30 sec, then the mixture was centrifuged at 15,000 rpm for 5 min.
7. The supernatant was transferred to a clean 1.5 ml micro tube containing 300 µl Isopropanol >99 %, mixed by inverting gently for 1 min, then mixed and centrifuged at 15,000 rpm for 1 min. At the end of this step, the DNA was a visible as a small white pellet.

8. The supernatant was discarded and the tube was drained carefully on clean absorbent paper, then 500 µl washing buffer was added and the tube was inverted several times to wash the DNA pellet, centrifuged at 15,000 rpm for 1 min and the ethanol was discarded, and air dried at room temperature for 10-15 min.
9. A moment of 50-100 µl of DNA hydration solution was added to the dried DNA pellet and then hydrated by incubating at 65 °C for 60 min.
10. The obtained DNA was stored at -20°C until used.

### 3.10.1.1. Electrophoresis of the extracted DNA in agarose gel

The extracted DNA was checked by electrophoresis on agarose gel and stained with Ethidium bromide, fluorescence was excited by ultra-violet radiation when it complexes with nucleic acids. The gel was prepared by mixing 1.5 gm agarose, 100 ml 1X TBE buffer and 4 µl of Ethidium bromide (10 mg/ml), and then 2-3µl of the extracted DNA was mixed with 2-3 µl of loading dye. 4µl of DNA was loaded on the gel. Gel was run in 1X TBE running buffer and electrophoresis was carried out at 100 to 145 volts for 10-20 min. Then the gel was viewed under UV light and photographed.

## 3.10.2. Polymerase chain reaction (PCR)

### 3.10.2.1. Primers

The following primers were used for detection of *M. pneumonia*, *C. pneumonia* and *L. pneumophila*.

**Table 1. Primers of *M. pneumoniae, C. pneumoniae* and *L. pneumophila.***

| Primer specify | Primer | Primer pair Séquence (5' ---3') | Size | Reference |
|---|---|---|---|---|
| *M. pneumonia* | F<br>R | 5-AAGGACCTGCAAGGGTTCGT-3<br>5-CTCTAGCCATTACCTGCTAA -3 | 277bp | (Zibo *et al.*,2015) |
| *C. pneumonia* | F<br>R | 5-TGACAACTGTAGAAATACAGC-3<br>5- CGCCTCTCTCCTATAAAT--3 | 463bp | (Jafer *et al.*,2013) |
| *L. pneumophila* | F<br>R | 5-GCTACAGACAAGGATAAGTTG-3<br>5-GTTTTGTATGACTTTAATTCA -3 | 649bp | (Jafer *et al.*,2013) |

## 3.10.2.2. Standard PCR reaction

The experiment consists of the experimental DNA, a positive control and a negative control. 2μl DNA was transferred to PCR tube and the following solutions were added in a total volume of 20 μl:

10X Taq buffer (final concentration 1X).

2.5 mM 4dNTP stock (final concentration 200 μmol).

Ten pmol/μl primer F.

Ten pmol/μl primer R.

Hundred ng of genomic DNA template.

$MgCl_2$ (final concentration 1.5μm).

$H_2O$ (up to the total volume of 20μl).

2.5u Taq Polymerase.

### 3.10.3. Preparation of controls

### 3.10.3.1. Preparation of *M. pneumoniae* DNA control

The DNA control of *M. pneumoniae* (Vircell, Spain) was centrifuged for 1 minute at 1000 rpm; 100 μl of control to reconstitute the solution was added and mixed well until completely reconstituted. The final concentration was 10000-20000 copies/ μl, shacked with vortex for 30 seconds to dissolve and homogenize completely. DNA control was diluted 1: 10, by adding 10 μl of DNA control to 90μl of reconstitution solution.

### 3.10.3.2. Preparation of *C. pneumoniae* DNA control

The DNA control of *C. pneumoniae* (Vircell, Spain) was centrifuged for 1 minute at 1000 rpm; 100 μl of control to reconstitute the solution was added and mixed well until completely reconstituted. The final concentration was 10000-20000 copies/ μl, shacked with vortex for 30 seconds to dissolve and homogenize completely. DNA control was diluted 1: 10, by adding 10 μl of DNA control to 90μl of reconstitution solution.

### 3.10.3.3. Preparation of *L. pneumophila* DNA control

The DNA control of *L. pneumophila* (Vircell, Spain) was centrifuged for 1 minute at 1000 rpm; 100 μl of control to reconstitute the solution was added and mixed well until completely reconstituted. The final concentration was 10000-20000 copies/ μl, shacked with vortex for 30 seconds to dissolve and homogenize completely. DNA control was diluted 1: 10, by adding 10 μl of DNA control to 90μl of reconstitution solution.

### 3.10.4. PCR Programming:

### 3.10.4.1. Protocol used for amplification of 16s rRNA genes for *C. pneumoniae*

The amplification was done using CONVERGYS® Ltd peltier thermal cycle (Germany). DNA amplifies of 16S rDNA gene was done using Maxime PCR PreMix kit (iNtRON, Korea).

The PCR program consisted of an initial denaturation step at 94°C for 5-min, followed by 30 cycles DNA denaturation at 95°C for 30 sec, primer annealing at 54°C for 30 sec, elongation at 72°C for 30 sec and the final extension was done at 72°C for 5 min (Jafer *et al*., 2013).

### 3.10.4.2. Protocol used for amplification of *MIP genes* for *L. pneumophila*

The amplification was done using CONVERGYS® Ltd peltier thermal cycle (Germany). DNA amplifies of *MIP* gene was done using Maxime PCR PreMix kit (iNtRON, Korea).

The PCR program consisted of an initial denaturation step at 94°C for 5-min, followed by 30 cycles DNA denaturation at 95°C for 30 sec, primer annealing at 55°C for 1min, elongation at 72°C for 1 min and the final extension was done at 72°C for 5 min (Jafer *et al*.,2013).

### 3.10.4.3. Protocol used for amplification of 16SrRNA *M. pneumoniae*

The amplification was done using CONVERGYS® Ltd peltier thermal cycle (Germany). DNA amplifies of 16S rDNA gene was done using Maxime PCR PreMix kit (iNtRON, Korea).

The PCR program consisted of an initial denaturation step at 94°C for 5-min, followed by 30 cycles DNA denaturation at 95°C for 30 sec, primer annealing at 55°C for 45 sec, elongation at 72°C for 45 sec and the final extension was done at

72°C for 5 min (Zibo *et al.*,2015).

### 3.10.5. Gel electrophoresis

The gel electrophoresis was analyzed in 1.5% agarose gel in TBE buffer. The gels were run at 75V for 30 minutes in 1x TAE containing 0.05 mg/L Ethidium bromide. The PCR products were visualized with UV light. The bands were matched with 100-1000 ladder (Macrogen, Korea).

## 3.11. Sequence similarities and phylogenetic analysis

The PCR products obtained were sent to Macrogen (Korea) for standard sequence DNA analysis. The same primers as above and an automated sequencer were used for this purpose. The sequence was compared for similarity level with the references of *M. pneumoniae, C. pneumoniae and L. pneumoniae* in genomic database banks, using the NCBI Blast available at the ncbi-nlm-nih.gov Web site. The obtained results were viewed using BioEdit v7.0.9 software (www.mobioncsu.edu/bioedit/bioedit.html). The software ClustalW2 (http://www.ebi.ac.uk/Tools/Msa/ClustalW2) was used to calculate the identities, similarities and differences among queries sequenced.

To construct the phylogenetic tree of the queries sequenced the results were submitted to the NCBI data bank.

## 3.12. Data analysis

The collected data were recorded and then analyzed using statistical package of social science (SPSS, version 11) program and chi-square test. *P*. values of $< 0.05$ were considered statistically significant.

# CHAPTER FOUR

# RESULTS

In this study a total of 400 patients with pneumonia were enrolled. Among them 242(60.5%) were males and 158(39.5%) were females (Fig 1). The highest frequency (54.5%) age group was 31-60 years followed by age group 13-30 years (30.5%) while the lowest frequency was age group 61-91years (15%) (Fig 2). Specimens number for patients attended to major hospitals were distributed (Fig 3).

Serological tests revealed 125(30.3%) positive, while 275(69.7%) were negative. These were 50(12.5%) positive for *L. pneumophila*, while 350(87.5%) were negative followed by 43(10.8%) positive for *C. pneumoniae*, while 357(89.2%) were negative followed by 32(8.0%) positive IgM of *M.* p*neumoniae*, while 368(92%) were negative (Table 2),

DNA extraction was done for all sputa analysis of the extracted genomic DNA in 1.0% agarose gel with 1X TBE buffer to check the purity of DNA (Figure 4).

PCR technique was done for all specimens, specific primers for atypical bacterial pneumonia showed 175(43.75%) positive, while 225(56.25%), were negative. These were 69(17.3%) positive for 16SrRNA gene of *C. pneumoniae*, while 331(82.7%) were negative followed by 57(14.3%) positive for *MIP* gene of *L. pneumophila*, while 343(85.7%) were negative followed by 49(12.3%) positive for 16Sr RNA gene of *M. pneumoniae*, while 351(87.7%) were negative (Table 3). The relationship between Hospitals and *M. pneumoniae, C. pneumoniae* and *L pneumophila* were insignificant were the (*P*-value= 0.192), (*P*-value= 0.211), (*P*-value= 0.301) respectively (Tables 4, 5 and 6).

The relationship between genders and *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila* were insignificant were the (*P*- value= 0.346), (*P*- value= 0.119) and (*P*- value= 0.365) respectively (Tables 7).

The relationship between age groups and *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila* were significant were the (*P*- value= 0.012), (*P*- value= 0.015) and (*P*- value= 0.002) respectively (Tables 8, 9 and 10).

The probability values between *M. pneumoniae* PCR, *C. pneumoniae* PCR and *L. pneumophila* PCR with Serology were significant were the (*P*-value= 0.000) (Table 11).

The curve of age distribution is normal, the Standard Deviation= 16.81, mean= 42.1, n= 400 its samples size (Figure 12).

Sequencing test done for PCR products, the results showed identity range and query cover range by Blast analysis of *L. pneumophila* (01, 02 and 03) the identity range between 99% to 100% were the query cover range between 86% to 100%, *M. pneumoniae* (01, 02 and 03) the identity range between 97% to 100% were the query cover range between 74% to 100% and *C. pneumoniae* identity range between 92% to 100% were the query cover range between 74% to 100% (Figers 13, 16,19,22, 25, 28, 31, 34 and 37).

Multiple Sequence alignment done for *L. pneumophila* (01, 02 and 03), *M. pneumoniae* (01, 02 and 03) and *C. pneumoniae* (01, 02 and 03) separately and to gather (Figers 14, 17, 20, 23, 26, 29, 32, 35, 38, 40, 42 and 44). Phylogenetic tree done for *L. pneumophila* (01, 02 and 03), *M. pneumoniae* (01, 02 and 03) and *C. pneumoniae* (01, 02 and 03) separately and to gather (Figers 15, 18, 21, 24, 27, 30, 33, 36, 39, 41, 43 and 45) (Appendix V).

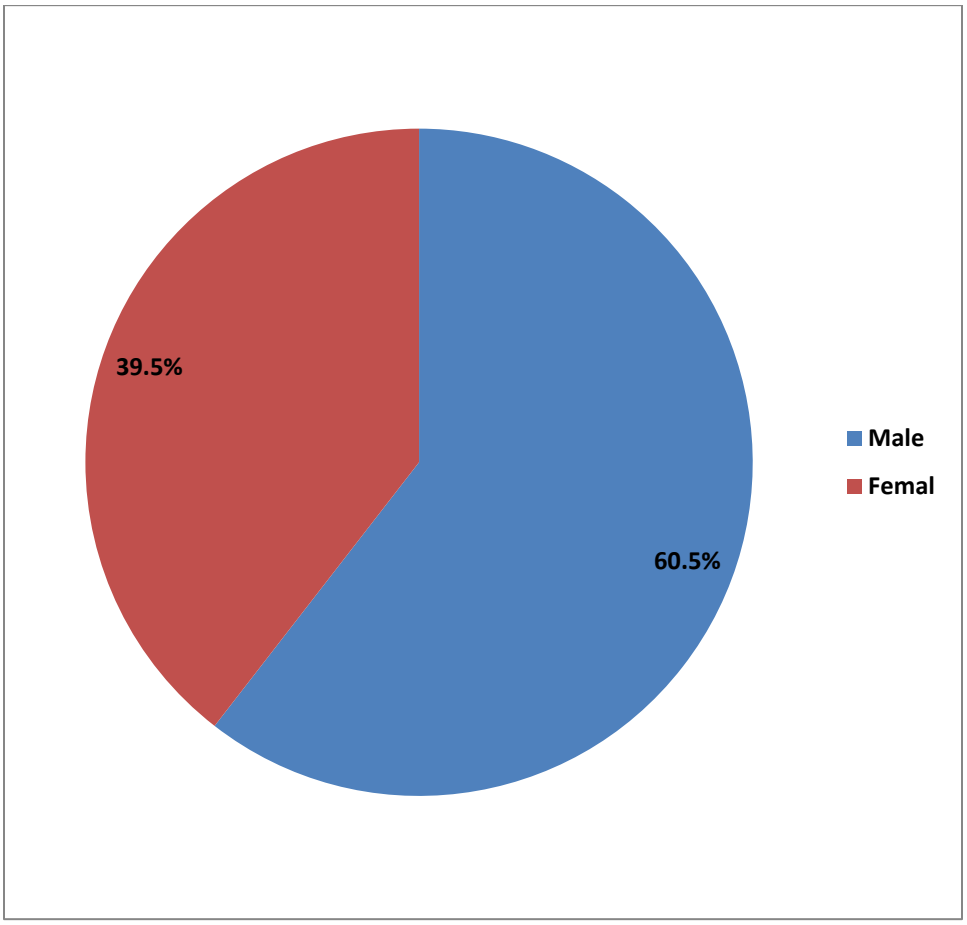


**Fig 1. Distribution of specimens according to sex**

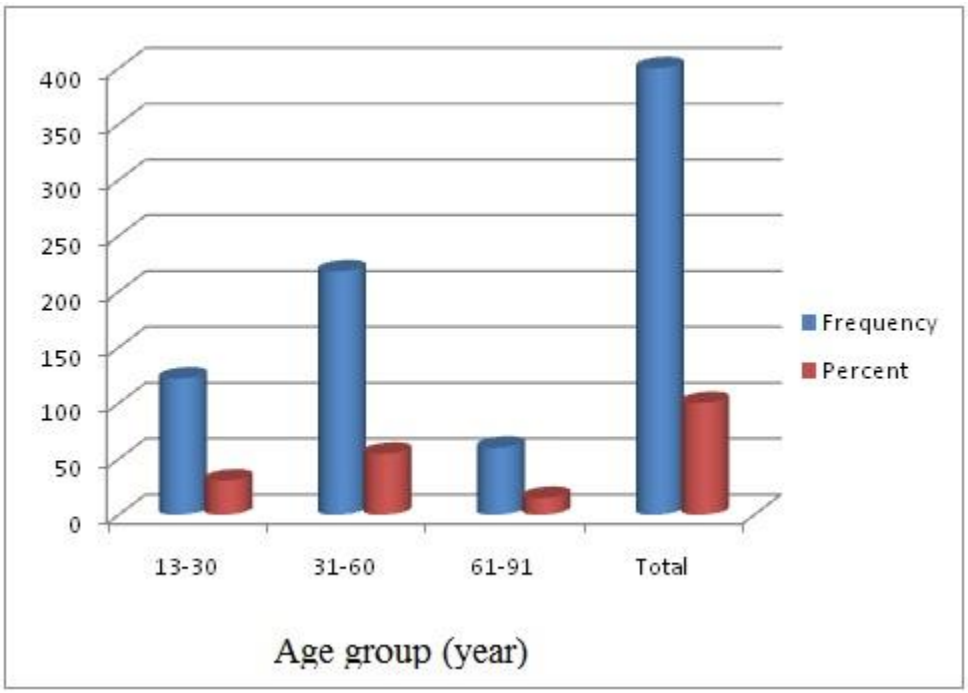


**Fig 2. Distribution of patients according to age group**

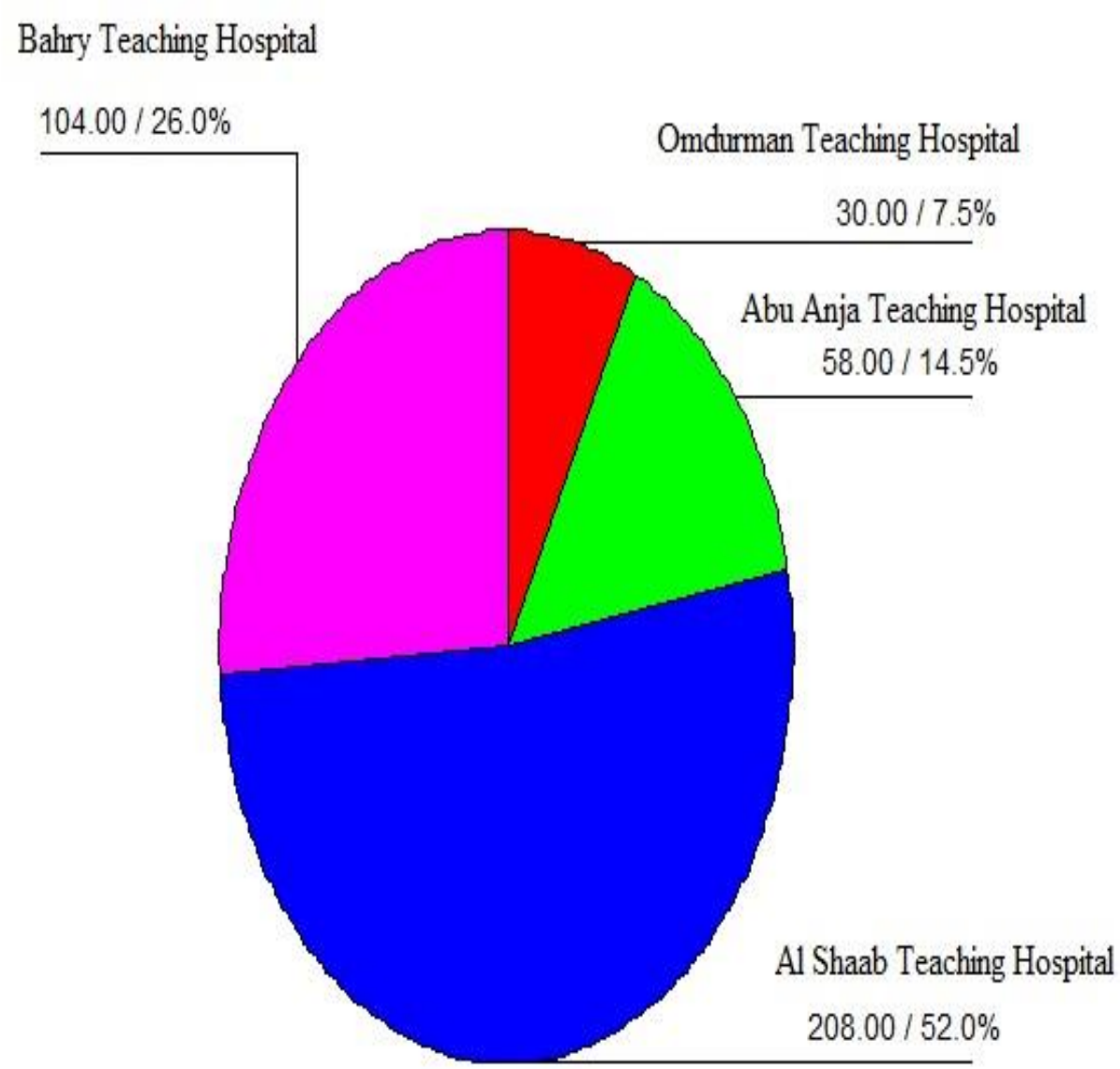


**Fig 3. Distribution specimens according to the Hospitals**

**Table 2. Sero detection of atypical bacterial pneumoniae among enrolled patients (n=400)**

| Result | Ssrodetection of | | |
|---|---|---|---|
| | ***C. pneumoniae*** **No (%)** | ***L. pneumophila*** **No (%)** | ***M. pneumoniae*** **No (%)** |
| Positive | 43(10.8) | 50(12.5) | 32(8.0) |
| Negative | 357(89.2) | 350(87.5) | 368(92.0) |

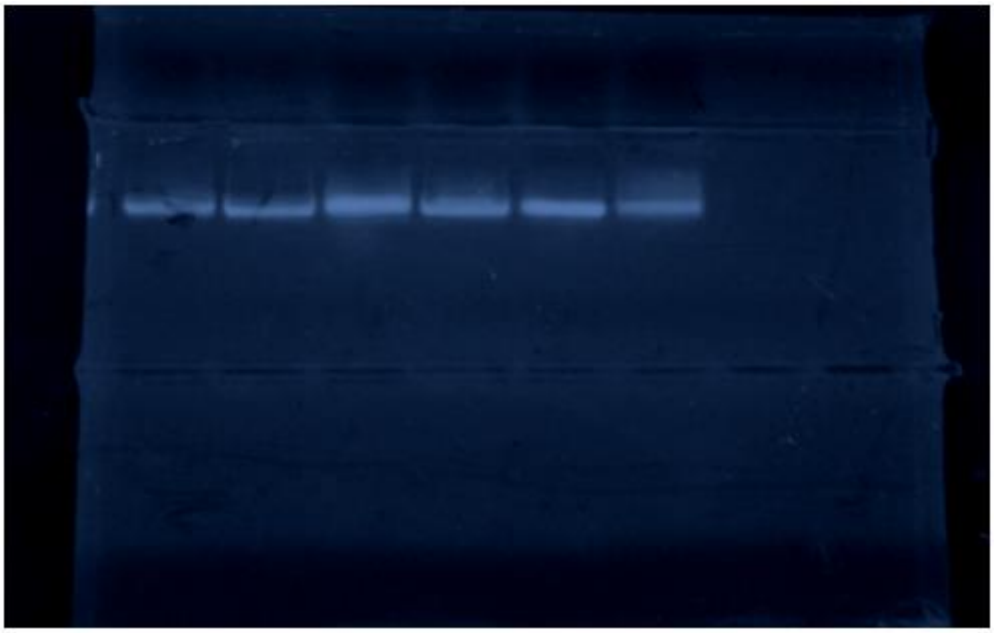

**Fig 4. Agarose gel of genomic DNA**

**Table 3. Molecular detection of atypical bacterial pneumoniae among enrolled patients (n=400)**

| Result | Molecular detection of | | |
|---|---|---|---|
| | ***C. pneumoniae*** **No (%)** | ***L. pneumophila*** **No (%)** | ***M. pneumoniae*** **No (%)** |
| Positive | 69(17.3) | 57(14.3) | 49(12.3) |
| Negative | 331(82.7) | 343(85.7) | 351(87.7) |

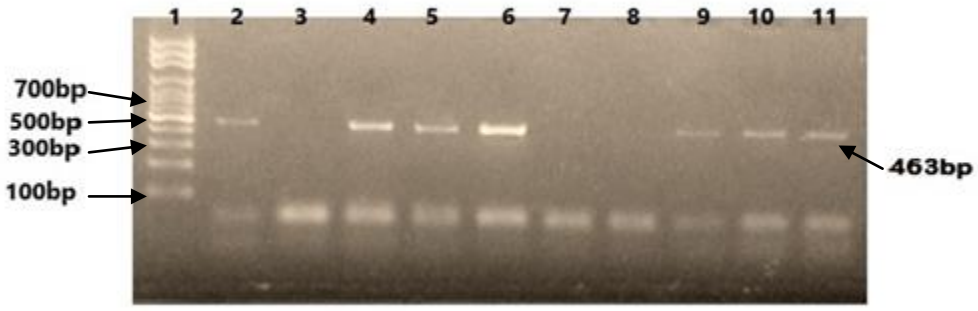


**Fig 5. Agrose gel of PCR products of 16SrRNA gene of *C. pneumoniae***

1.5 % agarose gel electrophoresis of *C. pneumoniae* by PCR, lane (1) Mw 100 – 1000 bp fragments – lane (2) controls Positive. The pictorial showed all (6) isolates (4, 5, 6, 9, 10, 11), with a band typical in size (463bp) which are positive for 16SrRNA gene, (3) negative control while (7, 8) isolates were negative.

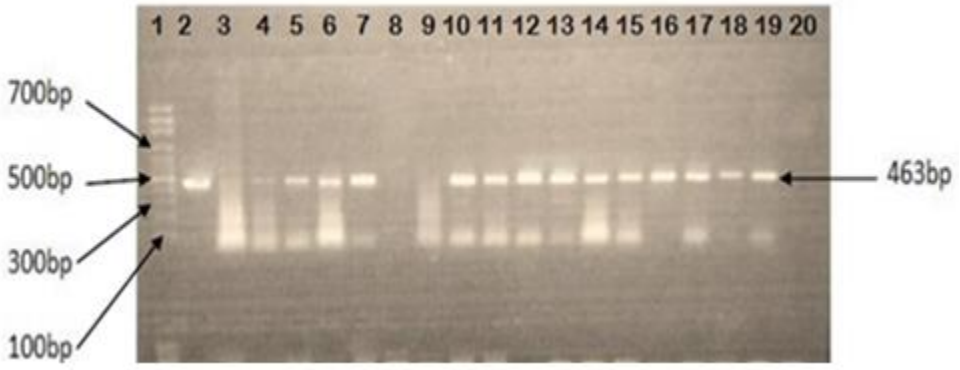


**Fig 6. Agrose gel of PCR products of 16SrRNA gene of *C. pneumoniae***

1.5 % agarose gel electrophoresis of *C. pneumoniae* by PCR. Lane (1) Mw 100 – 1000 bp fragments – lane (2) controls Positive. The pictorial showed all (14) isolates (4, 5, 6, 7, 10- 19), with a band typical in size (463bp) which are positive for 16SrRNA gene, (3) negative control, (8, 9, 20) isolates are negative.

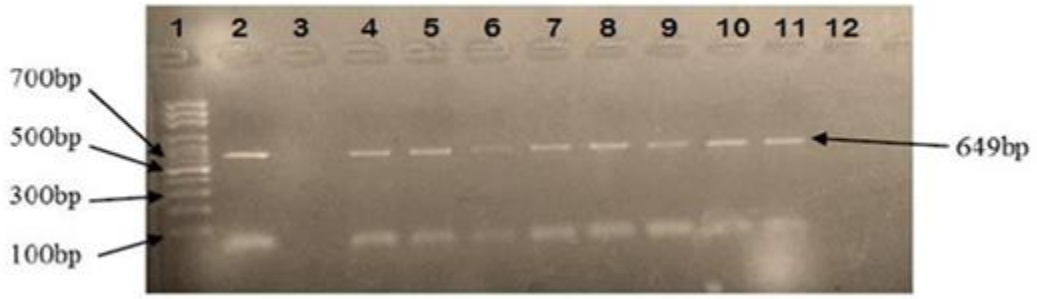


**Fig 7. Agrose gel of PCR products of *L. pneumophila***

1.5 % agarose gel electrophoresis of *L. pneumophila* by PCR. Lane (1) Mw 100 – 1000 bp fragments – lane (2) controls Positive. The pictorial showed all (8) isolates (4, 5, 6, 7, 8, 9, 10, 11), with a band typical in size (649bp) which are positive for *MIP* gene, (3) negative control while (12) isolates were negative.

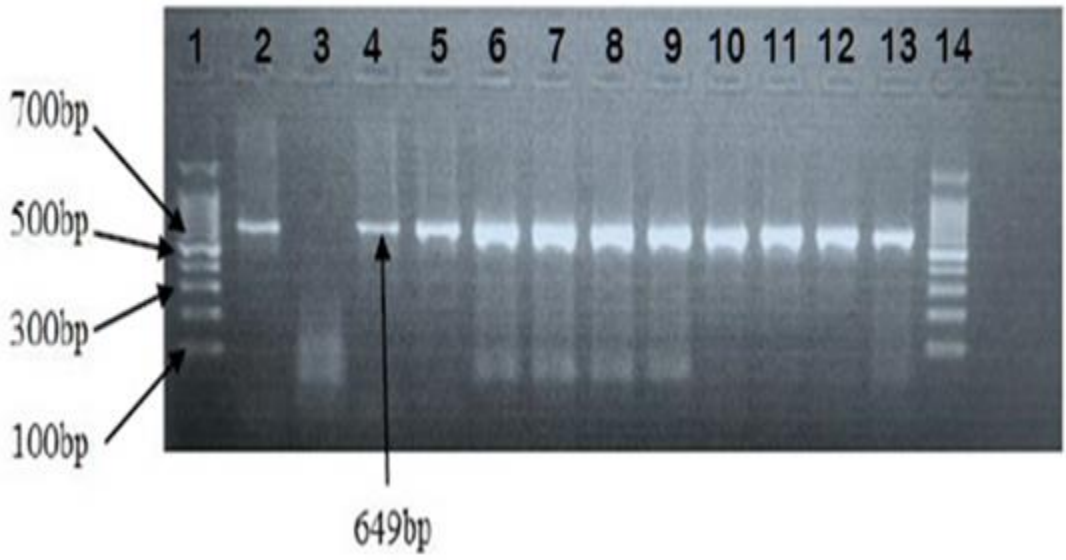


**Fig 8. Agrose gel of PCR products of *L. pneumophila***

1.5 % agarose gel electrophoresis of *L. pneumophila* by PCR. Lane (1) and (14) Mw 100 – 1000 bp fragments – lane (2) controls Positive. The pictorial showed all (10) isolates (4, 5, 6, 7, 8, 9, 10, 11, 12, 13), with a band typical in size (649bp) which are positive for *MIP* gene, (3) negative control.

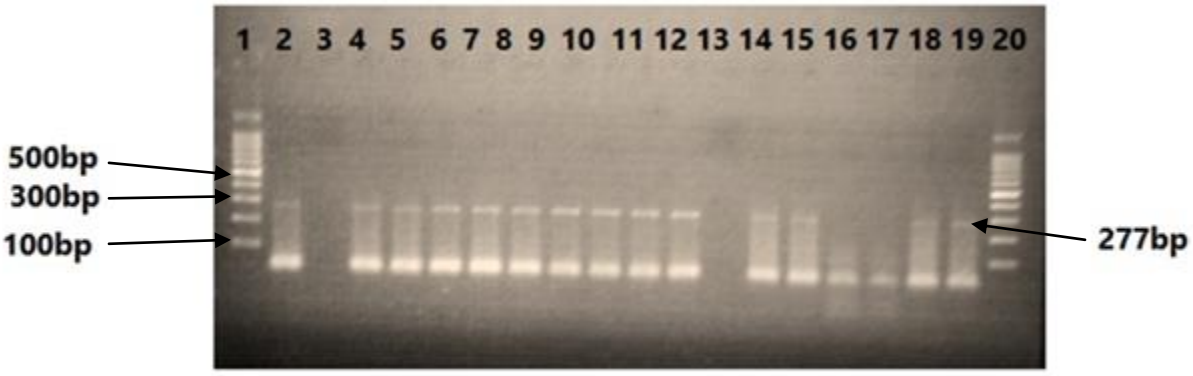


**Fig 9. Agrose gel of PCR products of 16Sr RNA gene of *M. pneumoniae***

1.5 % agarose gel electrophoresis of *M. pneumonia*e by PCR. Lane (1) Mw 100 – 1000 bp fragments – lane (2) controls Positive, land three negative controls. The a pictorial showed all 13 isolates (4-12,14,15,18,19), with a band typical in size ( 277bp ) which are positive for 16SrRNA gene, (13,16,17 ) isolates were negative 16SrRNA gene and land (20) M. Mw 100 – 1000 bp fragments.

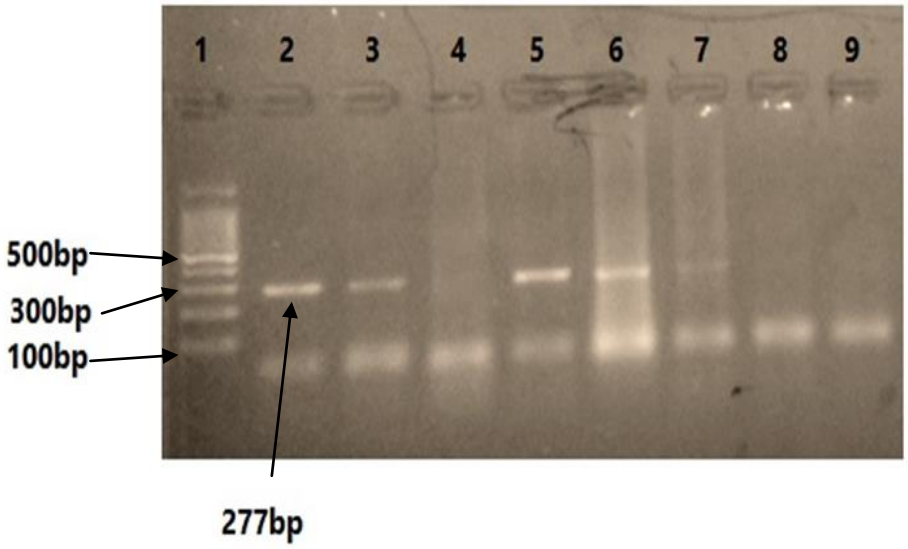


**Fig 10. Agrose gel of PCR products of 16Sr RNA gene of *M. pneumoniae***

1.5 % agarose gel electrophoresis of *M. pneumonia*e by PCR. Lane (1) Mw 100 – 1000 bp fragments – lane (2) controls Positive. The a pictorial showed all 4 isolates (3,5,6,7), with a band typical in size ( 277bp ) which are positive for 16SrRNA gene, (4 ) negative control and (8, 9) were negative.

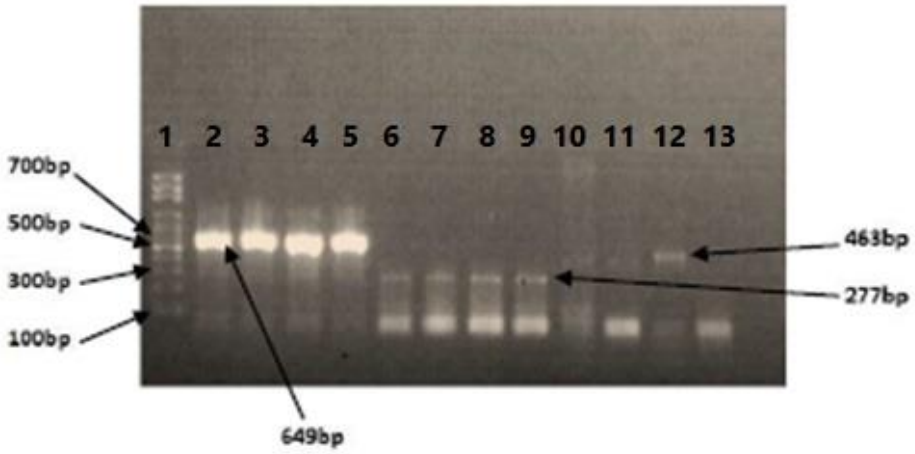


**Fig 11. *L. pneumophila, M. pneumoniae* and *C. pneumoniae***

1.5 % agarose gel electrophoresis of *M. pneumoniae* (277bp), *L. pneumophila* (649) and *C. pneumoniae* (463) by PCR. Land (1) Mw 100 – 1000 bp fragments – lane 2-5, two control positive,3-5 positive samples for *L. pneumophila*. Lane 6-9, 6 control positive 7-9 positive samples for *M. pneumoniae*. Lane 10-12, 10, 11 positive samples, 12 positive controls for *C. pneumoniae* and 13 negative controls.

**Table 4. Relationship between hospitals and *C. pneumoniae***

| Hospital | *C. pneumoniae* | | Total |
|---|---|---|---|
| | **Positive** | **Negative** | |
| Omdurman Teaching Hospital | 8(2.0%) | 22(5.5%) | 30(7.5%) |
| Abu anja Teaching Hospital | 11(2.8%) | 47(11.8%) | 58(14.5%) |
| Al shaab Teaching Hospital | 38(9.5%) | 170(42.4%) | 208(52.0%) |
| Bahry Teaching Hospital | 12(3.0%) | 92(23.0%) | 104(26.0%) |
| Total | 69(17.3%) | 331(82.7%) | 400(100.0%) |

*P*= 0.211

**Table 5. Relationship between hospitals and *L. pneumophila***

| Hospital | *L. pneumophila* | | Total |
|---|---|---|---|
| | **Positive** | **Negative** | |
| Omdurman Teaching Hospital | 7(1.8%) | 23(5.8%) | 30(7.5%) |
| Abu anja Teaching Hospital | 5(1.3%) | 53(13.3%) | 58(14.5%) |
| Al shaab Teaching Hospital | 31(7.8%) | 177(44.2%) | 208(52.0%) |
| Bahry Teaching Hospital | 14(3.5%) | 90(22.5%) | 104(26.0%) |
| Total | 57(14.3%) | 343(85.7%) | 400(100.0%) |

*P*= 0.301

**Table 6. Relationship between hospitals and *M. pneumoniae***

| Hospital | *M. pneumoniae* | | Total |
|---|---|---|---|
| | **Positive** | **Negative** | |
| Omdurman Teaching Hospital | 3(0.8%) | 27(6.8%) | 30(7.5%) |
| Abu anja Teaching Hospital | 6(1.5%) | 52(13.0%) | 58(14.5%) |
| Al shaab Teaching Hospital | 21(5.3%) | 187(46.7%) | 208(52.0%) |
| Bahry Teaching Hospital | 19(4.8%) | 85(21.3%) | 104(26.0%) |
| Total | 49(12.3%) | 351(87.7%) | 400(100.0%) |

*P*= 0.192

**Table 7. Correlation between atypical bacterial pneumonia and gender**

| Agent | Gender | | ***P* –Value< 0.05** |
|---|---|---|---|
| | **M (%)** | **F (%)** | |
| *C. pneumoniae* | 48(12.0) | 21(5.3) | 0.119 |
| *L. Pneumophila* | 38(9.5) | 19(4.8) | 0.365 |
| *M. pneumoniae* | 27(6.8) | 22(5.5) | 0.346 |
| Total | 113(28.3) | 62(15.6) | |

**Table 8. Relationship between age group and *M. pneumoniae***

| Age group (year) | *M. pneumoniae* | | Total |
|---|---|---|---|
| | Positive | Negative | |
| 1-30 | 10(2.5%) | 112(28.0%) | 122(30.5%) |
| 31-60 | 25(6.3%) | 193(48.3%) | 218(54.5%) |
| 61-91 | 14(3.5%) | 46(11.5%) | 60(15.0%) |
| Total | 49(12.3%) | 351(87.7%) | 400(100.0%) |

*P*= 0.002

**Table 9. Relationship between age group and *C. pneumoniae***

| Age group (year) | *C. pneumoniae* | | Total |
|---|---|---|---|
| | Positive | Negative | |
| 1-30 | 31(7.8%) | 91(22.8%) | 122(30.5%) |
| 31-60 | 31(7.8%) | 187(46.8%) | 218(54.5%) |
| 61-91 | 7(1.8%) | 53(13.3%) | 60(15.0%) |
| Total | 69(17.3%) | 331(82.7%) | 400(100.0%) |

*P*= 0.003

**Table 10. Relationship between age group and *L. pneumophila***

| Age group (year) | *L. pneumophila* | | Total |
|---|---|---|---|
| | Positive | Negative | |
| 1-30 | 12(3.0%) | 110(27.5%) | 122(30.5%) |
| 31-60 | 28(7.0%) | 190(47.5%) | 218(54.5%) |
| 61-91 | 17(4.3%) | 43(10.8%) | 60(15.0%) |
| Total | 57(14.3%) | 343(85.7%) | 400(100.0%) |

*P*= 0.002

**Table 11. Comparison between Serological and molecular techniques in detection of atypical pneumonia**

| Agent | Serology | PCR |
|---|---|---|
| | No. (%) | No. (%) |
| *C. pneumoniae* | 43(10.75%) | 69(17.25%) |
| *L. Pneumophila* | 50(12.5%) | 57(14.3%) |
| *M. pneumoniae* | 32(8.0%) | 49(12.3%) |

*P*= 0.000

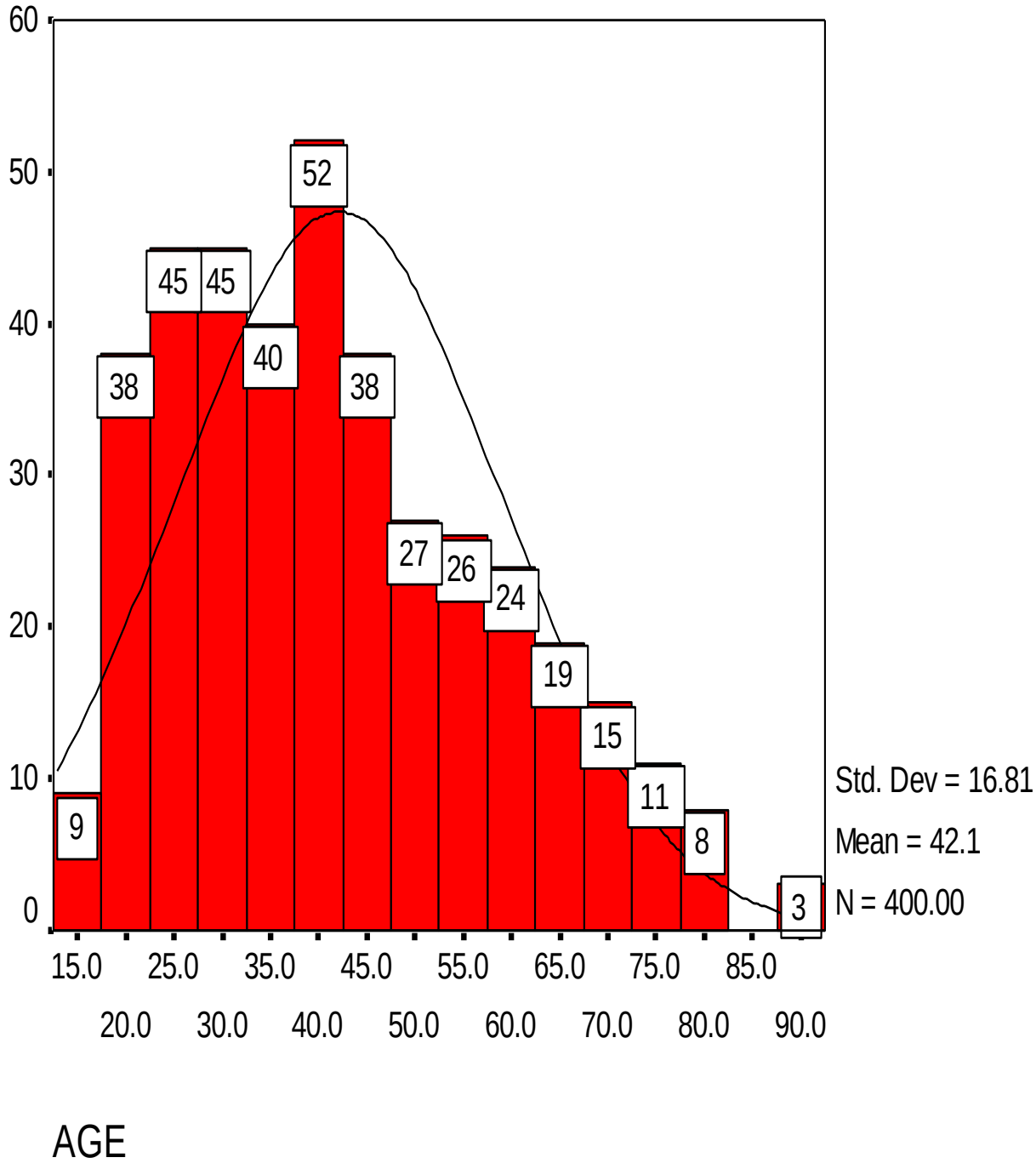


**Fig 12. Normal age distribution**

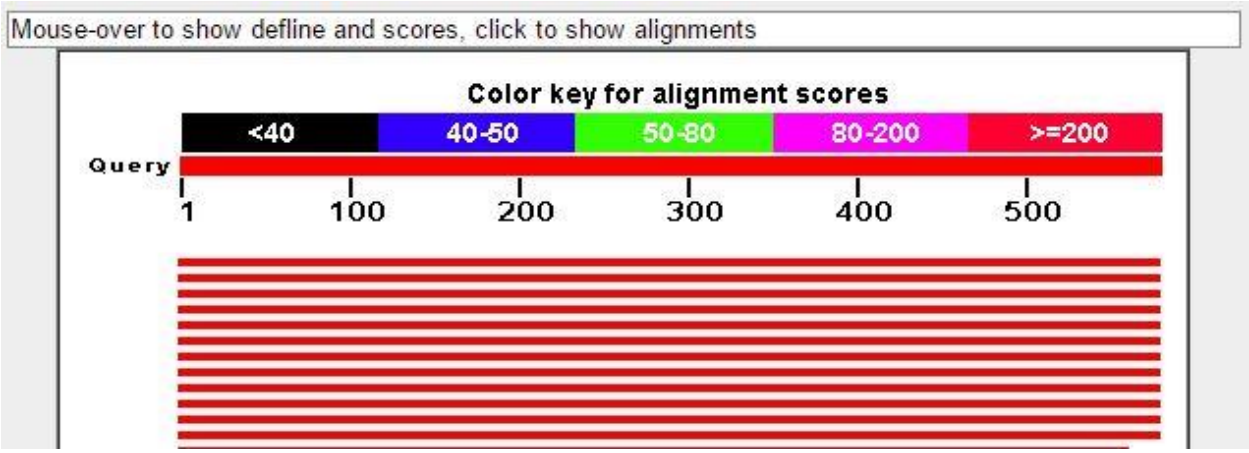


**Fig 13.** BLAST analysis of *L. pneumophila* (LEG-01) 649bp showed (100%) identity to nucleotide sequence of *L. pneumophila* subsp-pneumophila LPE509 with ex. No (CP003885.1) with query cover (100%).


**Fig 14. Multiple sequence alignment of *L. pneumophila* (LEG-01)**

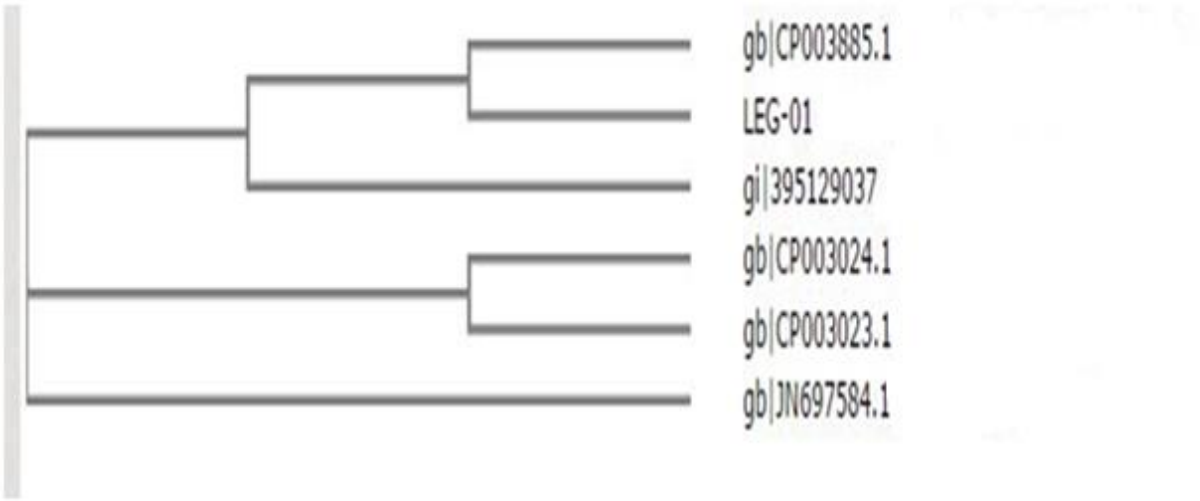


**Fig 15. Phylogenetic tree result of *L. pneumophila* (LEG-01)**

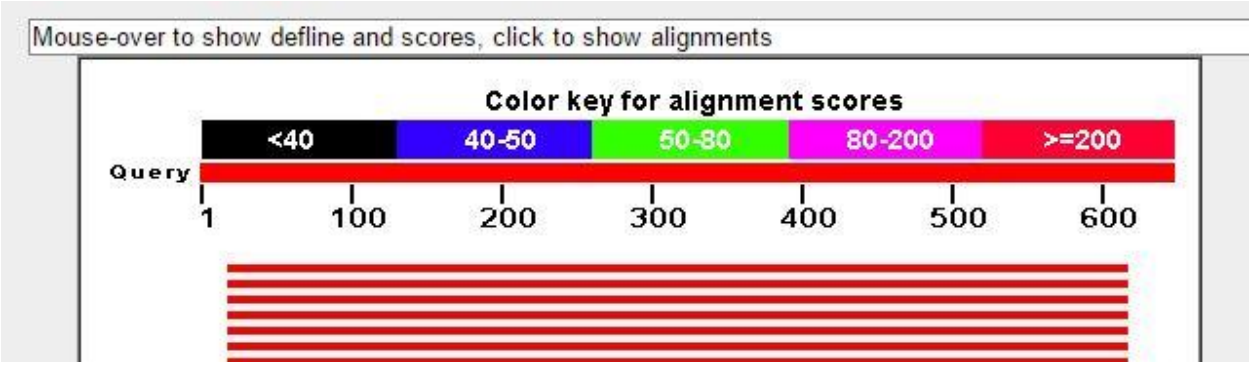


**Fig 16.** BLAST analysis of *L. pneumophila* (LEG-02) 649bp showed (100%) identity to nucleotide sequence of *L. pneumophila* subsp-pneumophilaHL0604101035 with ex. No (Kj160936.1) with query cover (86%).

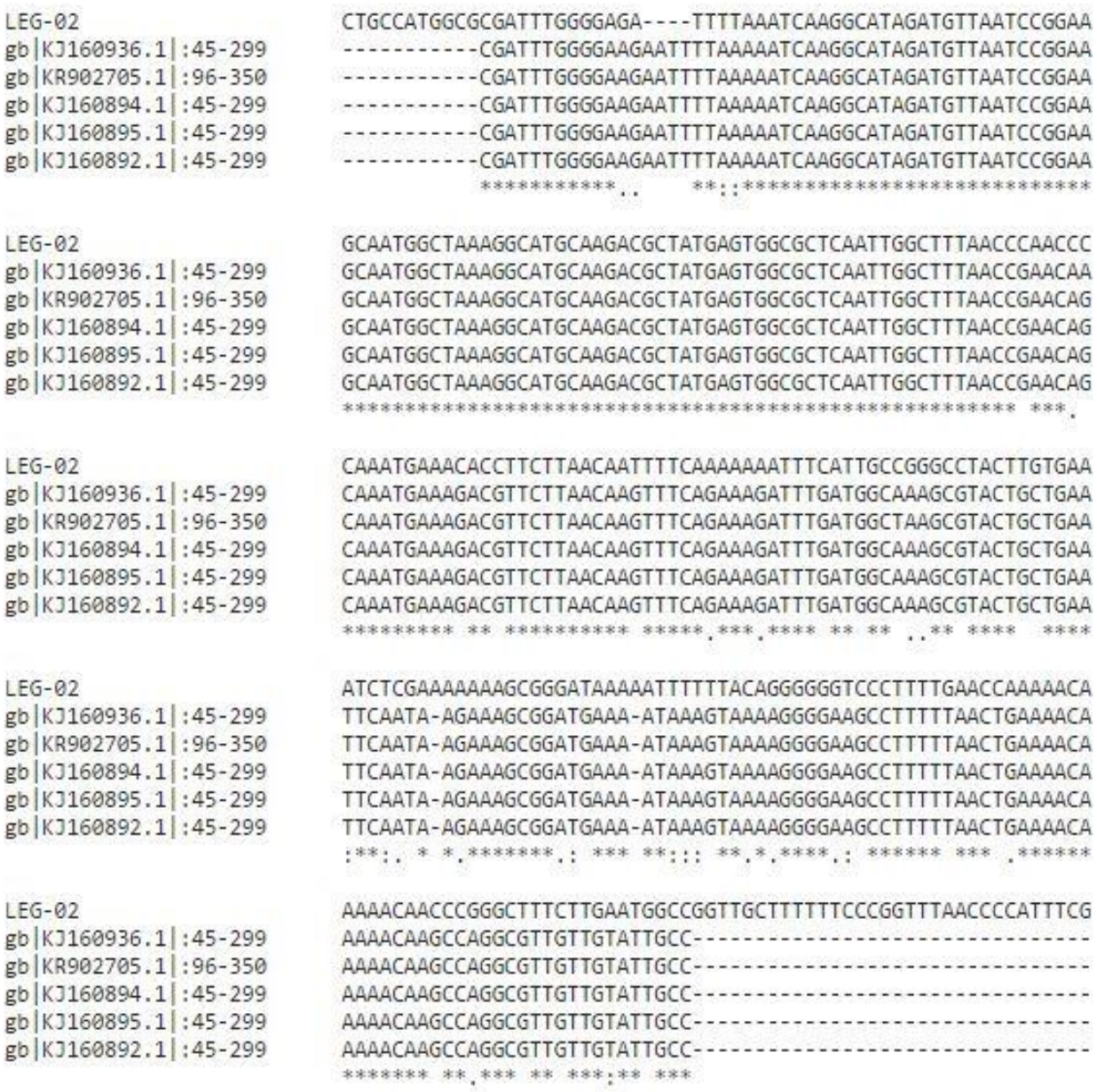


**Fig 17. Multiple sequence alignment of *L. pneumophila* (LEG-02)**

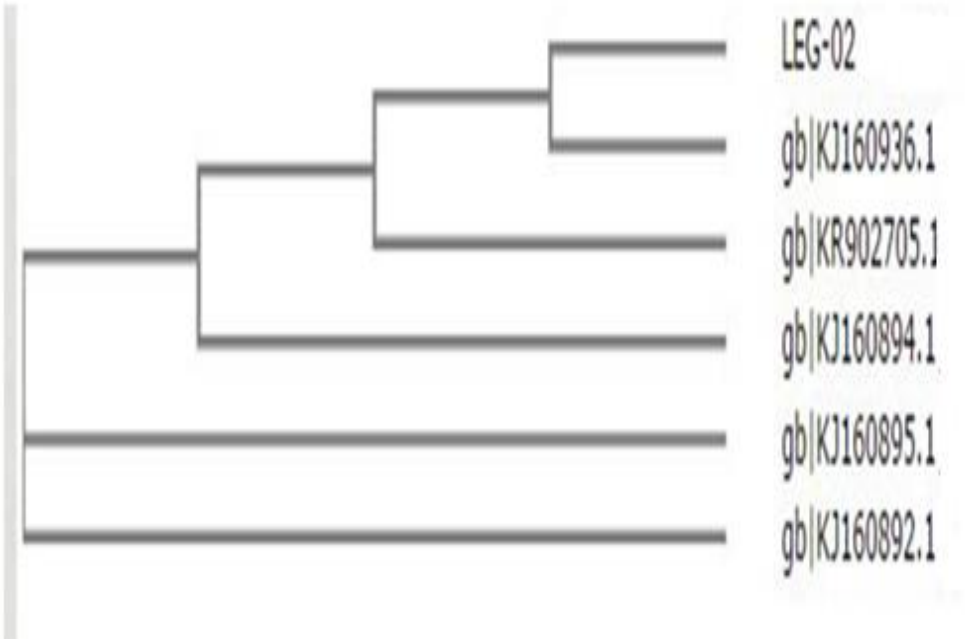


**Fig 18. Phylogenetic tree result of *L. pneumophila* (LEG-02)**

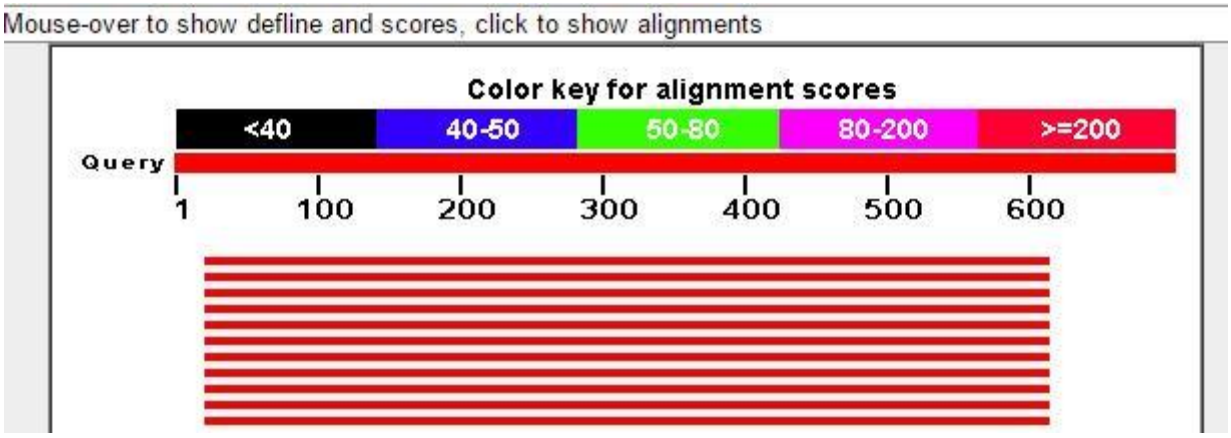


**Fig 19.** BLAST analysis of *L. pneumophila* (LEG-03) 649bp showed (99%) identity to nucleotide sequence of *L. pneumophila* LPE 509 with ex. No (HQ645035.1) with query cover (97%).

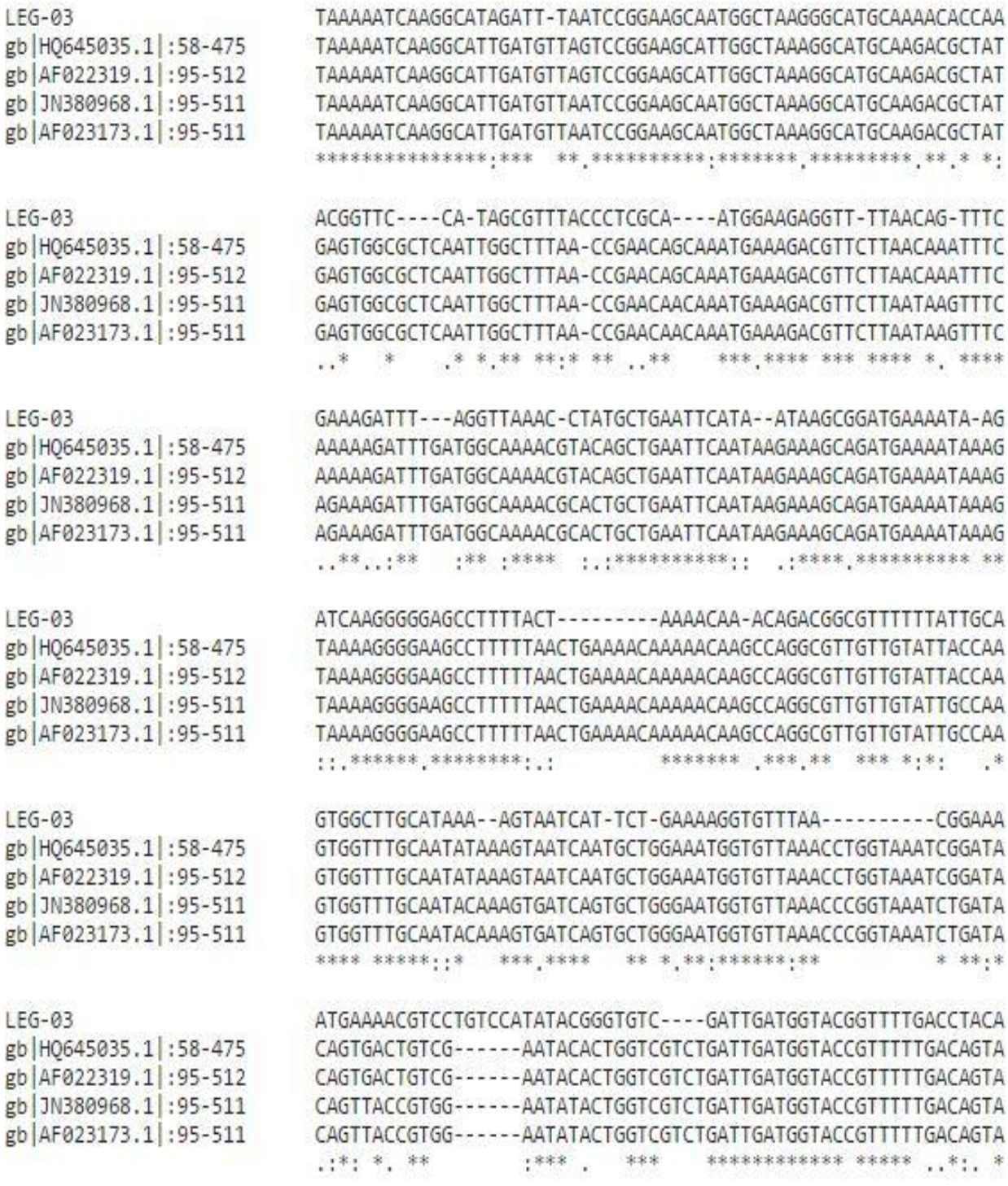


**Fig 20. Multiple sequence alignment of *L. pneumophila* (LEG-03)**

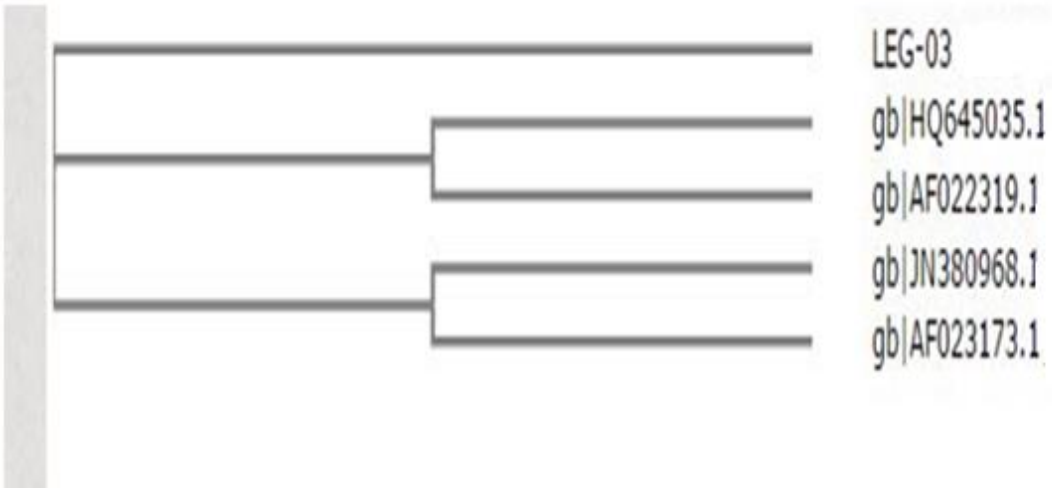


**Fig 21. Phylogenetic tree result of *L. pneumophila* (LEG-03)**

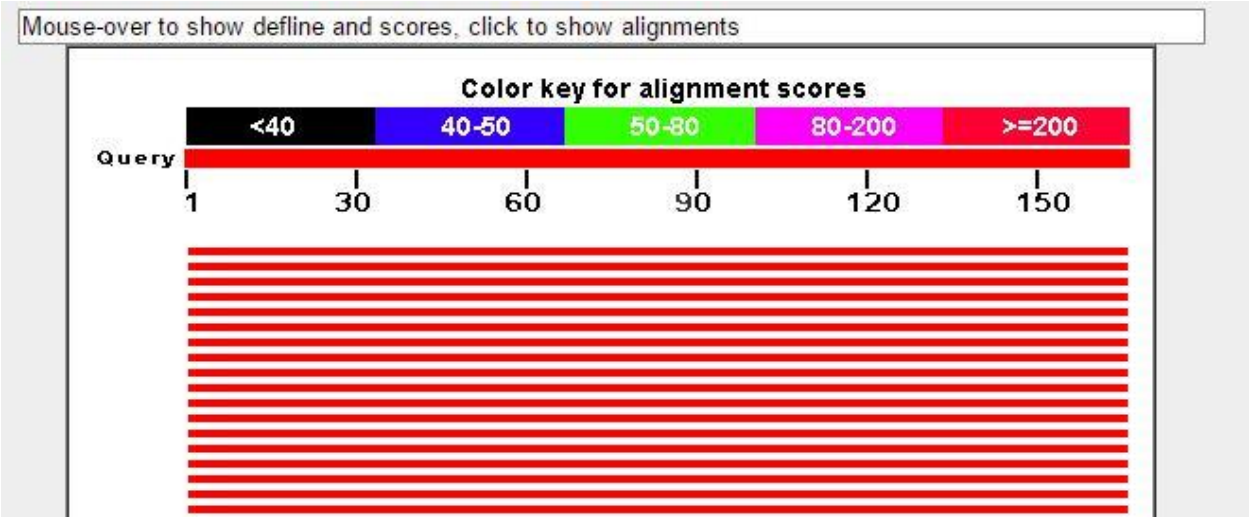


**Fig 22.** BLAST analysis of (*M. pneumoniae* (MYC-01) 277bp showed (100%) identity to nucleotide sequence of *M. pneumoniae* strain s355 ex. No (CP010551.1) with query cover (99%).

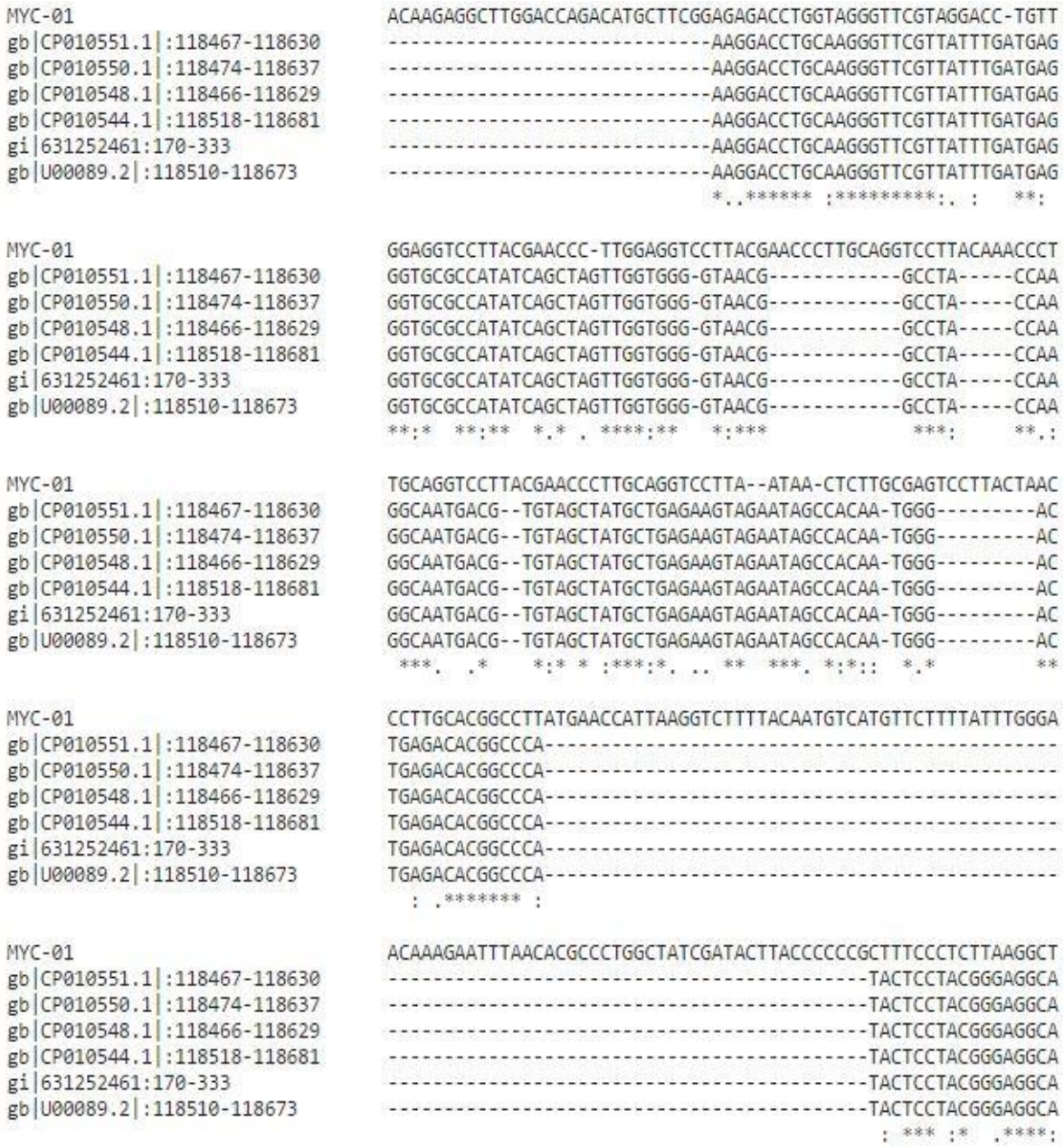


**Fig 23. Multiple sequence alignment of *M. pneumoniae* (MYC-01)**

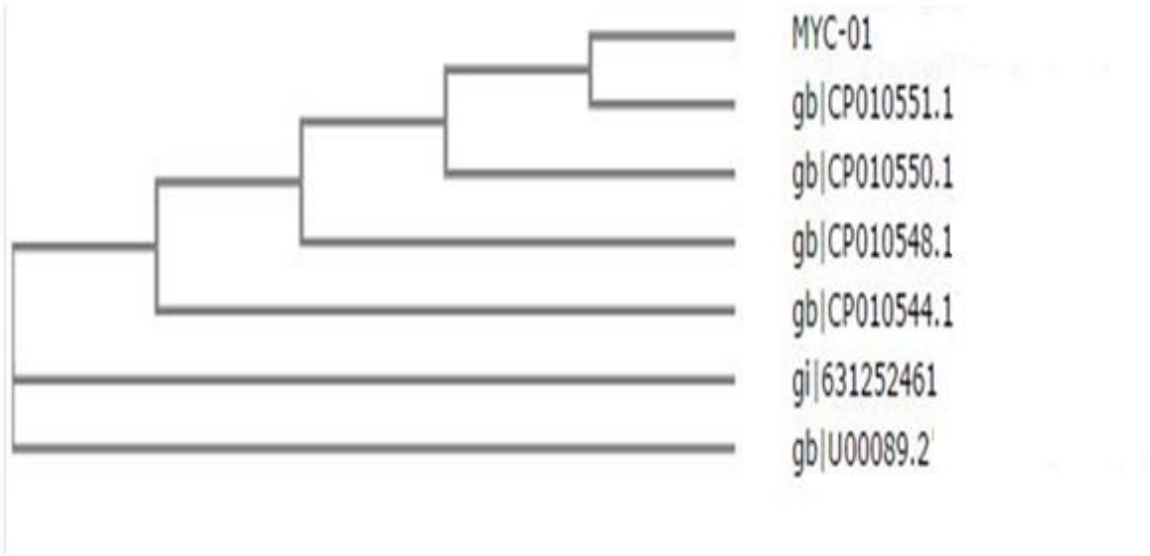


**Fig 24. Phylogenetic tree result of *M. pneumoniae* (MYC-01)**

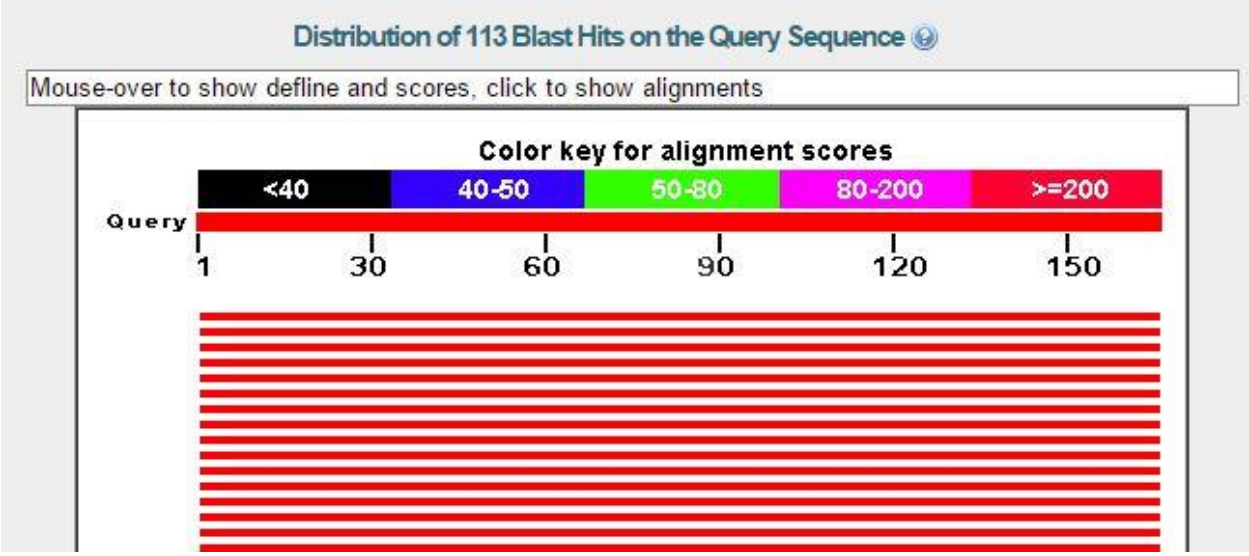


**Fig 25.** BLAST analysis of *M. pneumoniae* (MYC-02) 277bp showed (99%) identity to nucleotide sequence of *M. pneumoniae* strain s355 ex. No (CP013829.1) with query cover (86).


**Fig 26. Multiple sequence alignment of *M. pneumoniae* (MYC-02)**

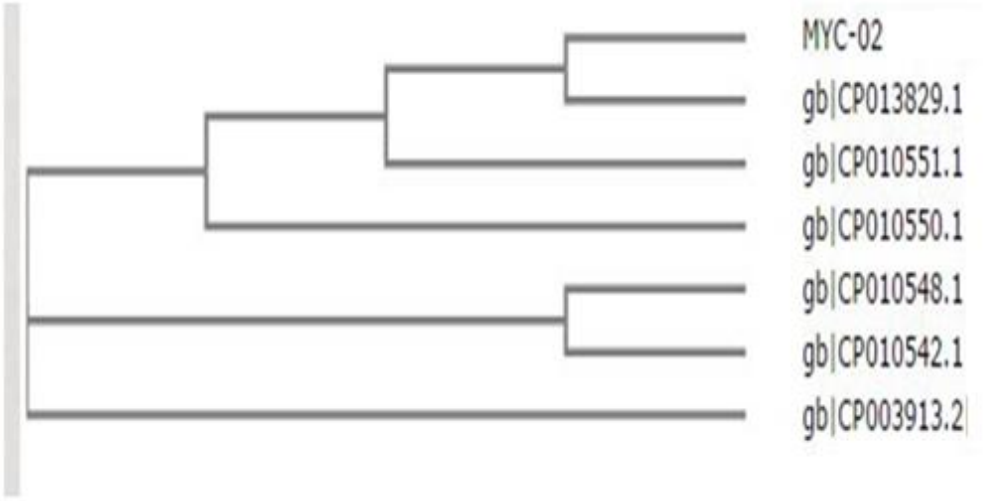


**Fig 27. Phylogenetic tree result of *M. pneumoniae* (MYC-02)**

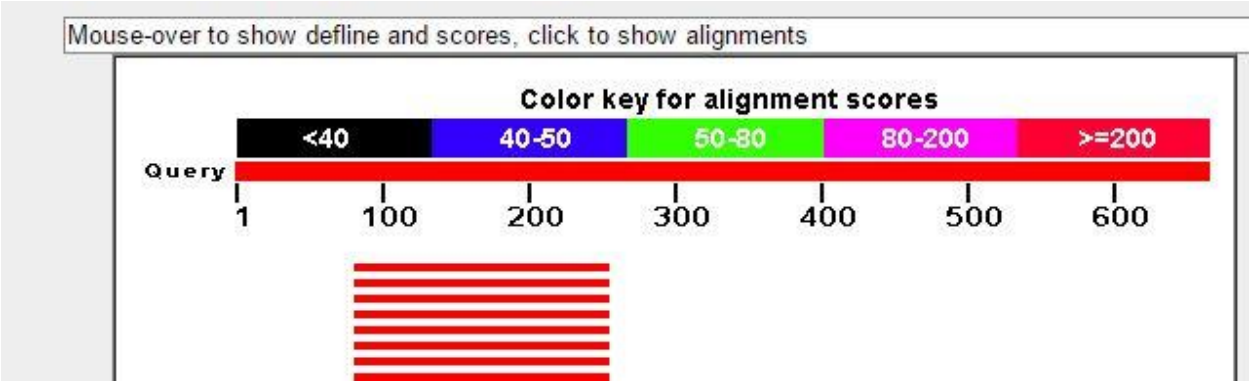


**Fig 28.** BLAST analysis of *M. pneumoniae* (MYC-03) 277bp showed (97%) identity to nucleotide sequence of *M. pneumoniae* strain s355 ex. No (CP01055.1) with query cover (74).


**Fig 29. Multiple sequence alignment of *M. pneumoniae* (MYC-03)**

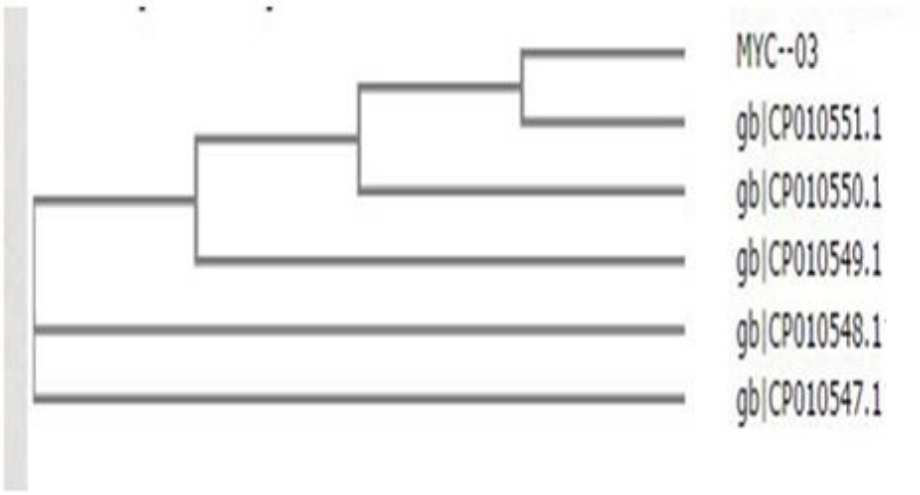


**Fig 30. Phylogenetic tree result of *M. pneumoniae* (MYC-03)**

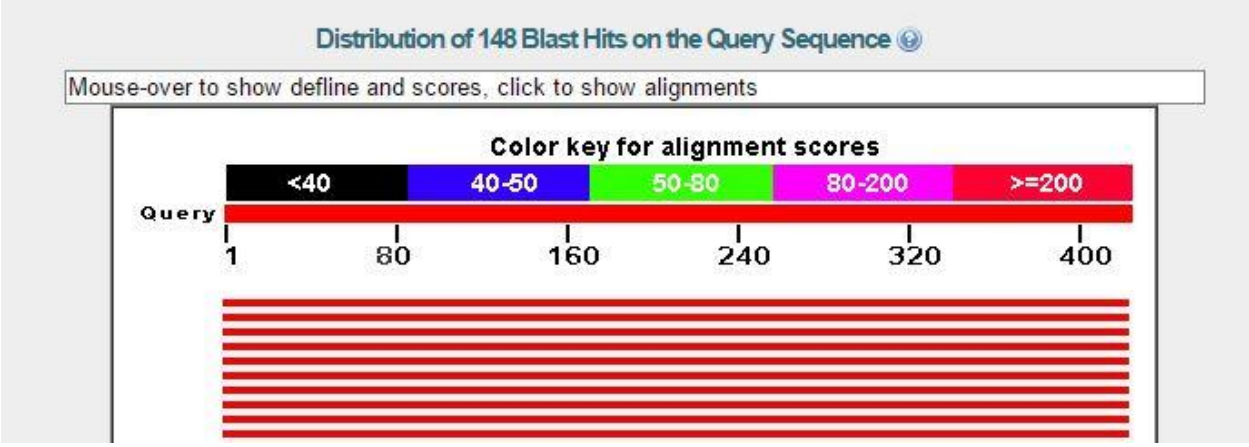


**Fig 31.** BLAST analysis of *C. pneumoniae* (CHL-01) 463bp showed (100%) identity to nucleotide sequence of *C. pneumoniae* strain ex. No (LN80676372.1) with query cover (99).

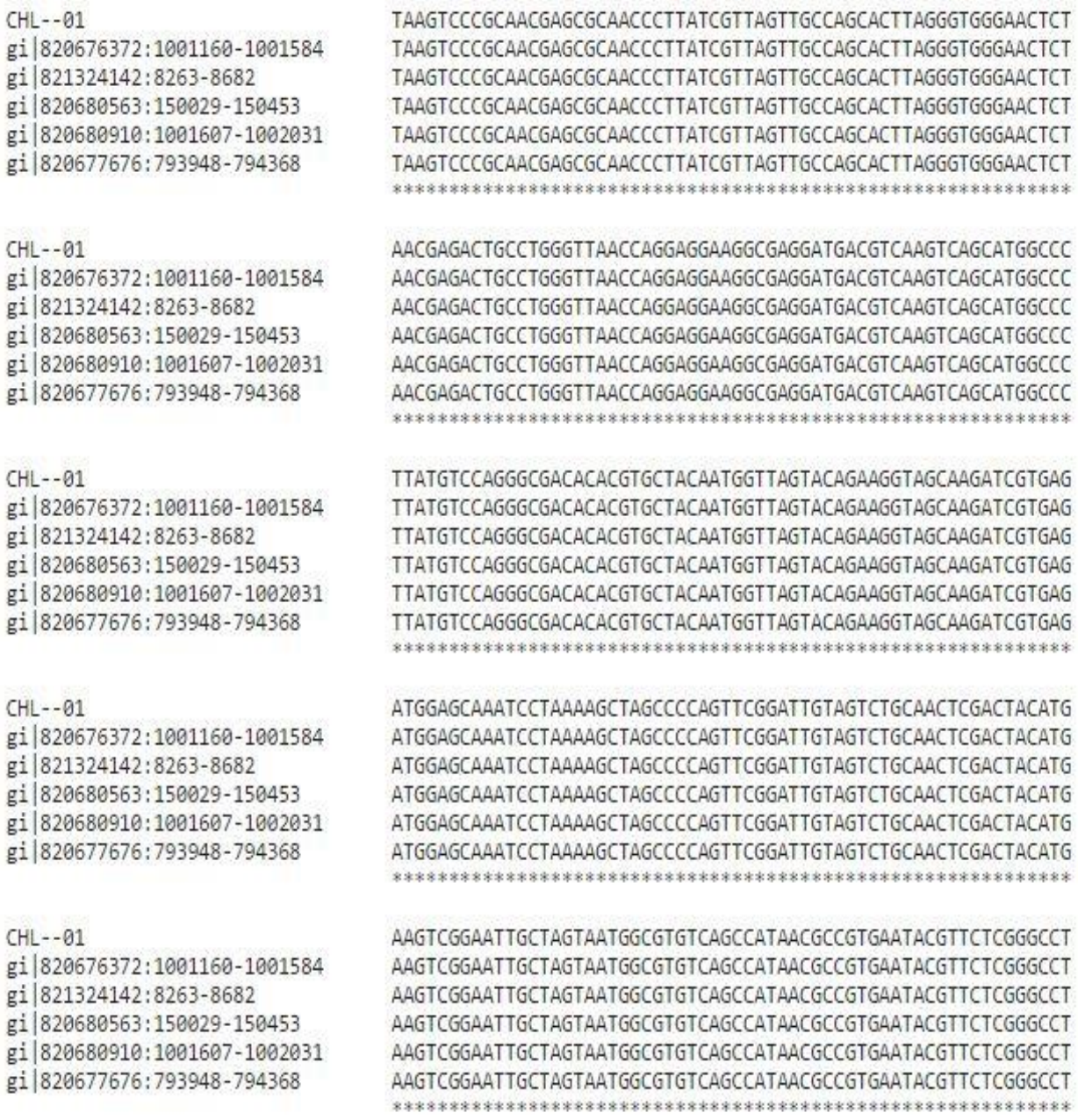


**Fig 32. Multiple sequence alignment of *C. pneumoniae* (CHL- 01)**

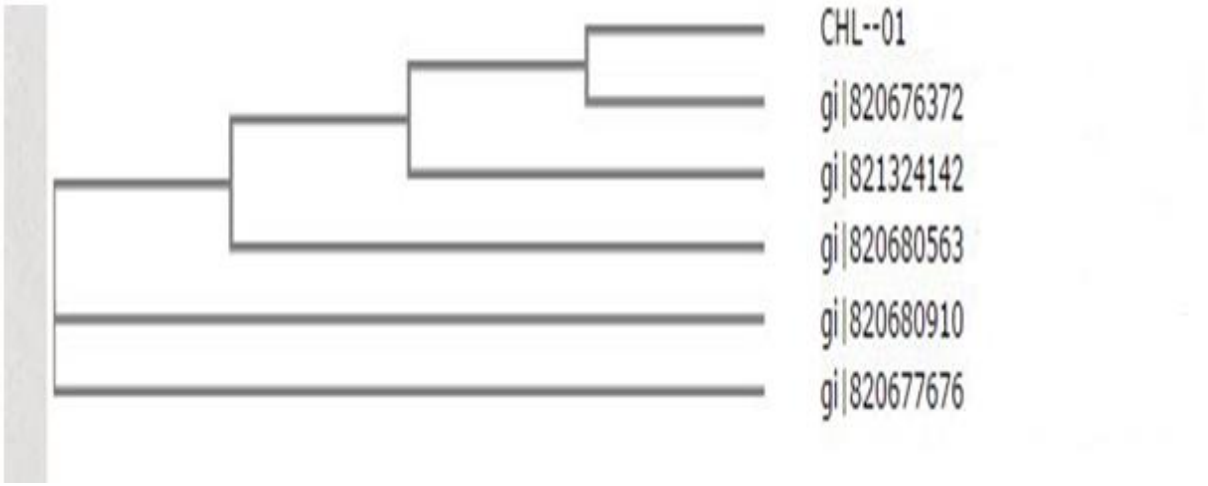


**Fig 33. Phylogenetic tree result of *C. pneumoniae* (CHL- 01)**

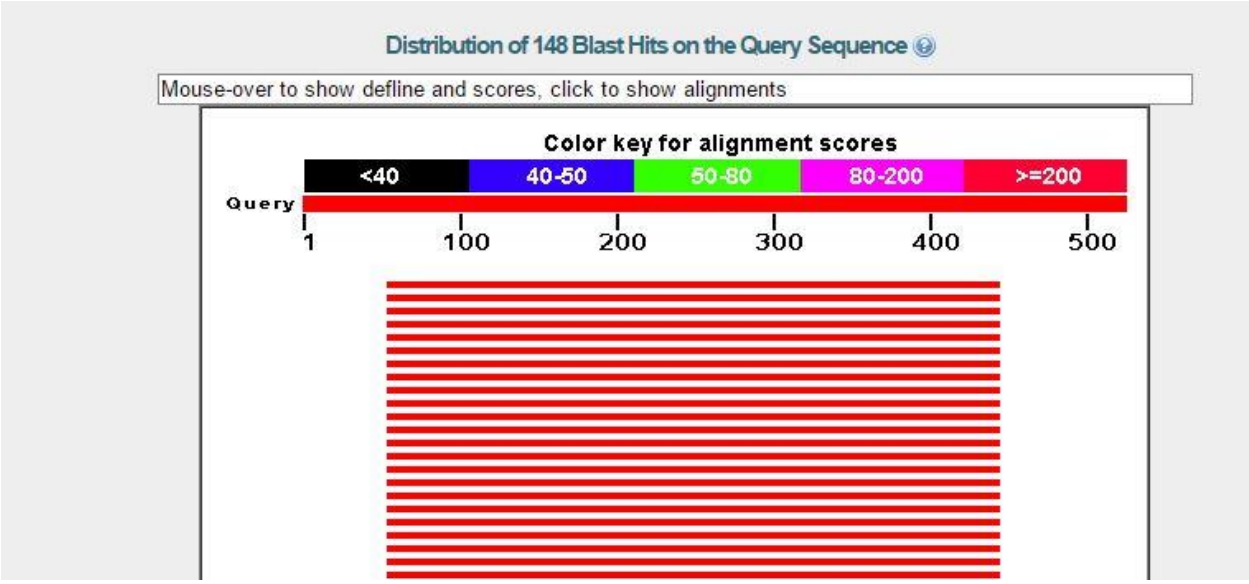


**Fig 34.** BLAST analysis of *C. pneumoniae* (CHL- 02) 463bp showed (92%) identity to nucleotide sequence of *C. pneumoniae* strain ex. No (gi 820677676) with query cover (74).

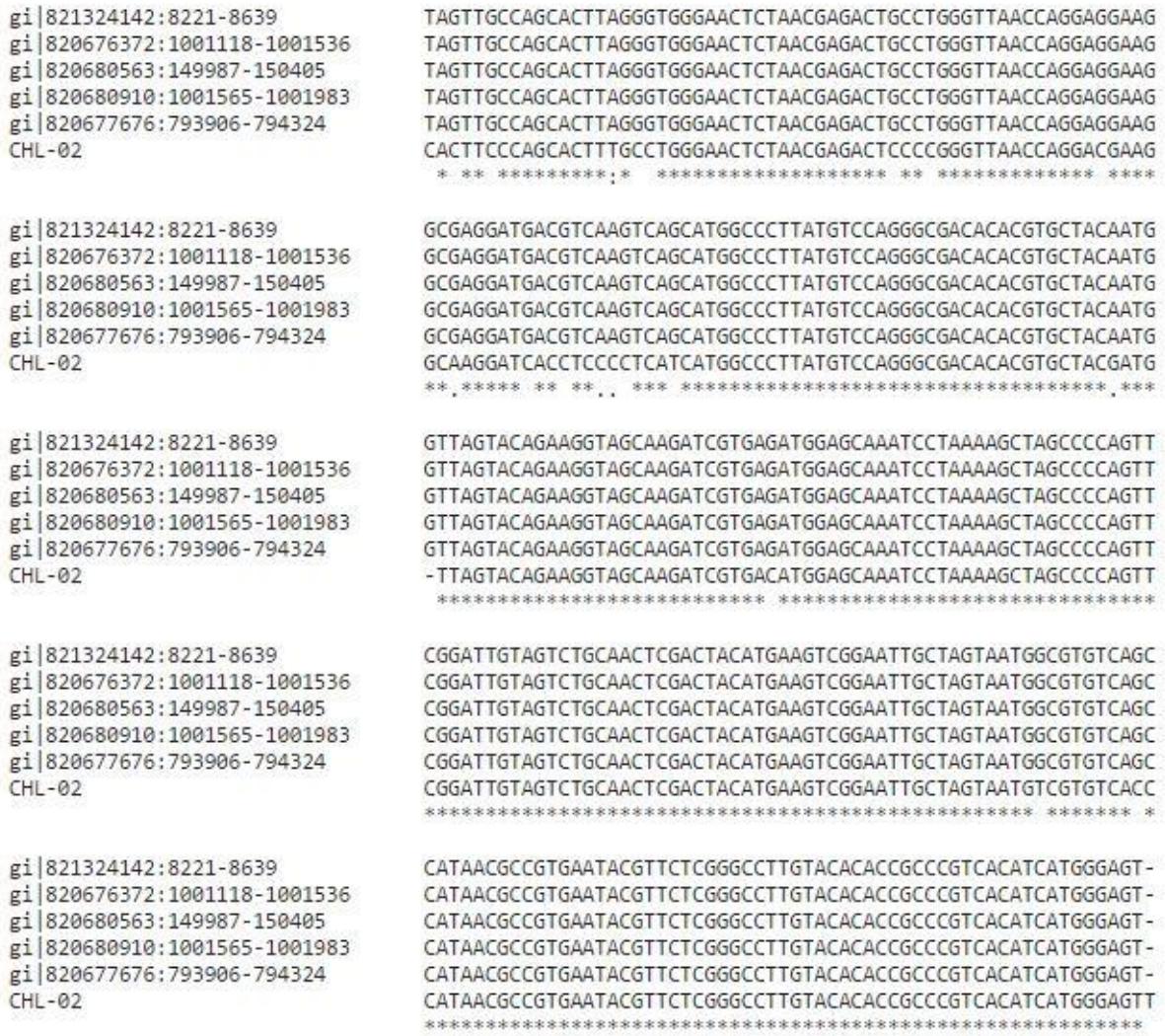


**Fig 35. Multiple sequence alignment of *C. pneumoniae* (CHL- 02)**

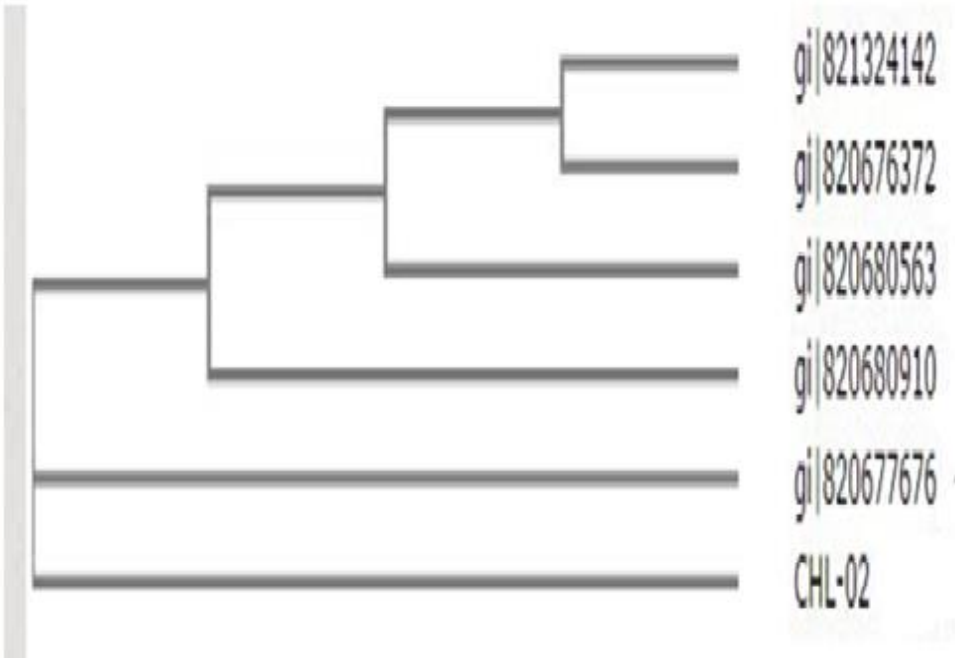


**Fig 36. Phylogenetic tree result of *C. pneumoniae* (CHL-02)**

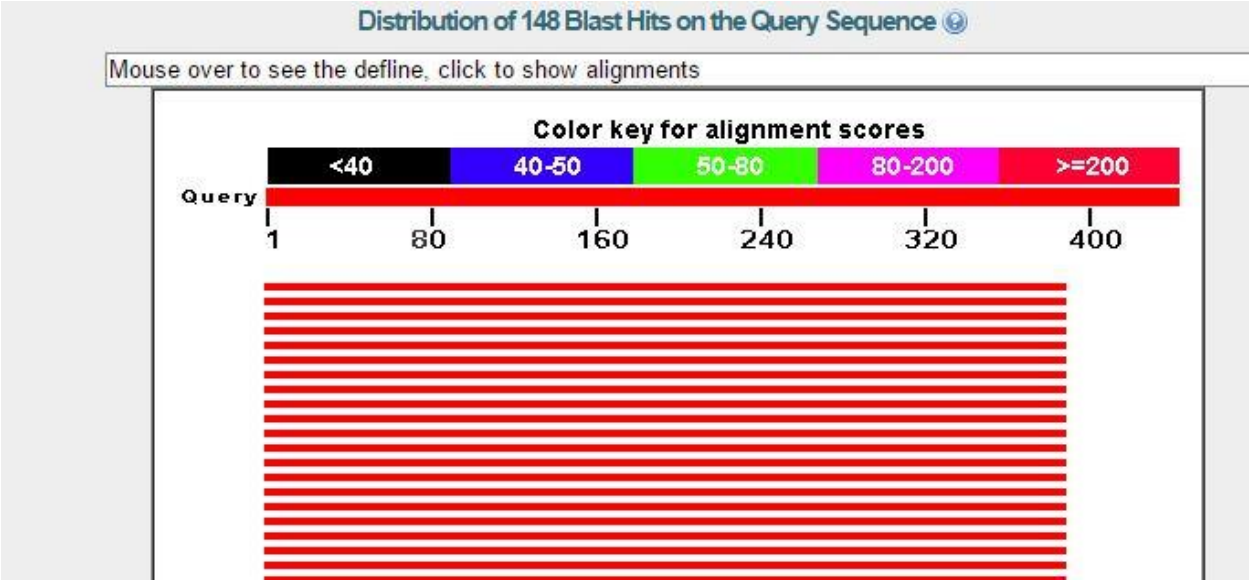


**Fig 37.** BLAST analysis of *C. pneumoniae* (CHL-03) 463bp showed (94%) identity to nucleotide sequence of *C. pneumoniae* strain s355 ex. No (LN81324142) with query cover (87%).


**Fig 38. Multiple sequence alignment of *C. pneumoniae* (CHL-03)**

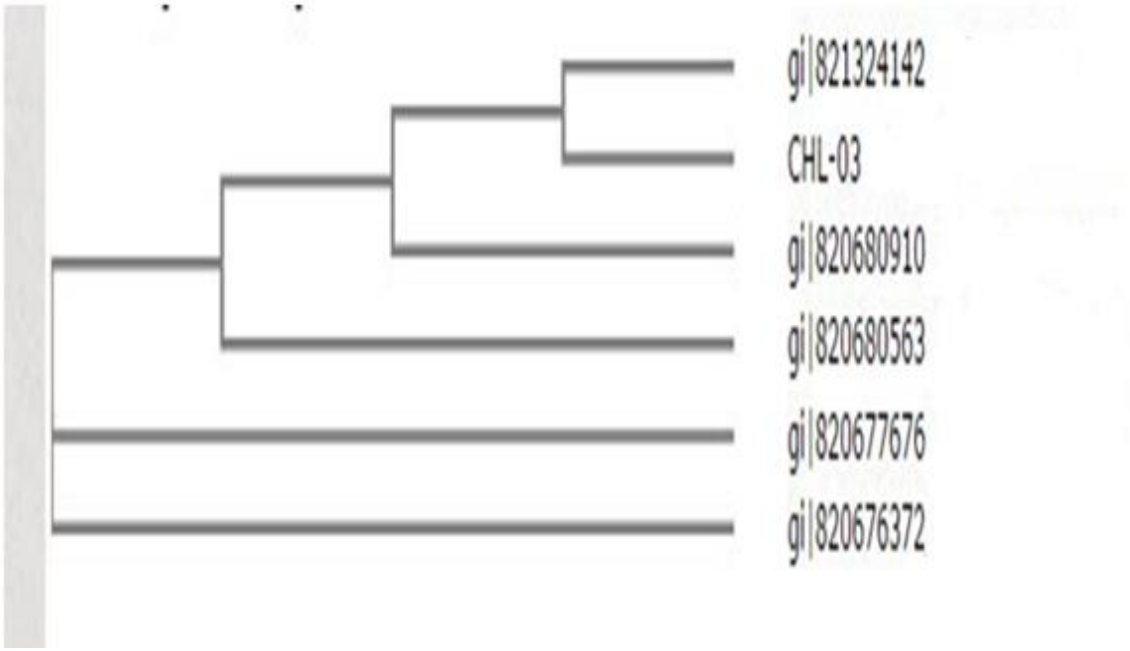


**Fig 39. Phylogenetic tree result of *C. pneumoniae* (CHL-03)**

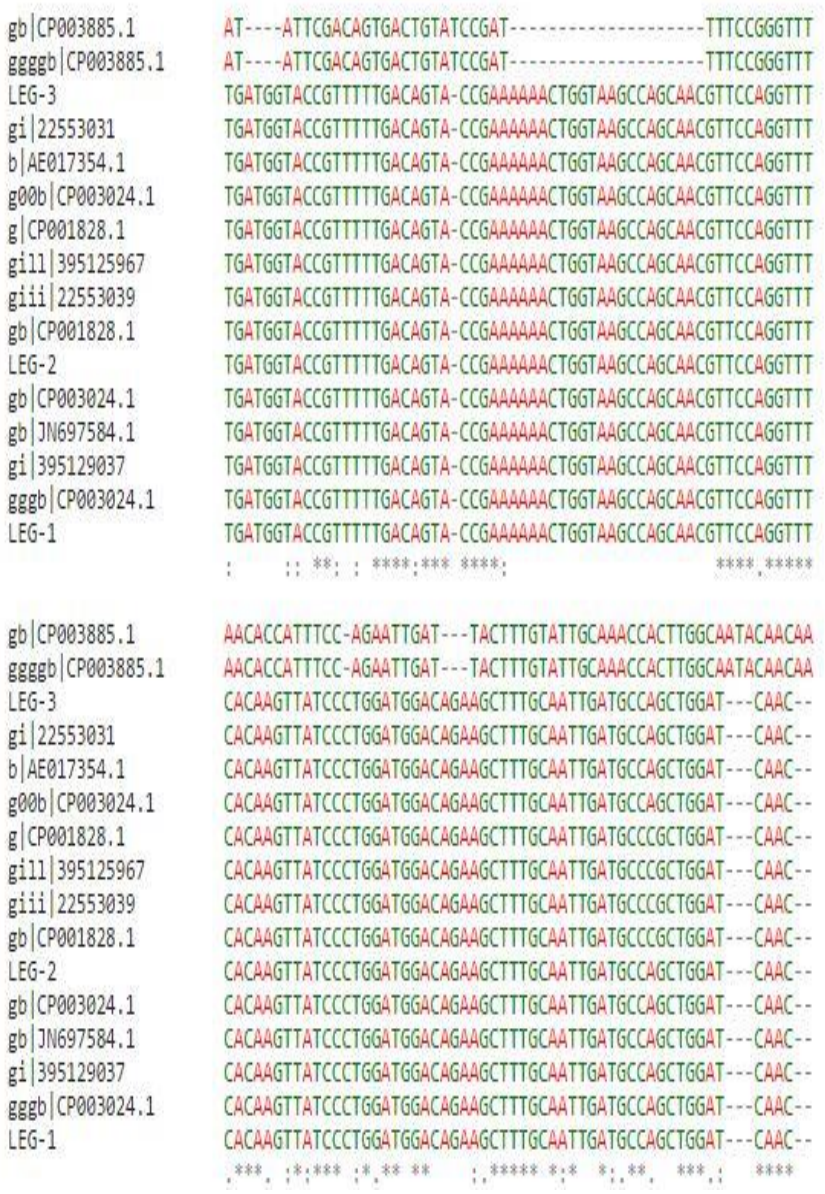


**Fig 40. Multiple sequence alignment of *L. Pneumophila* (LEG-1, 2 and 3)**

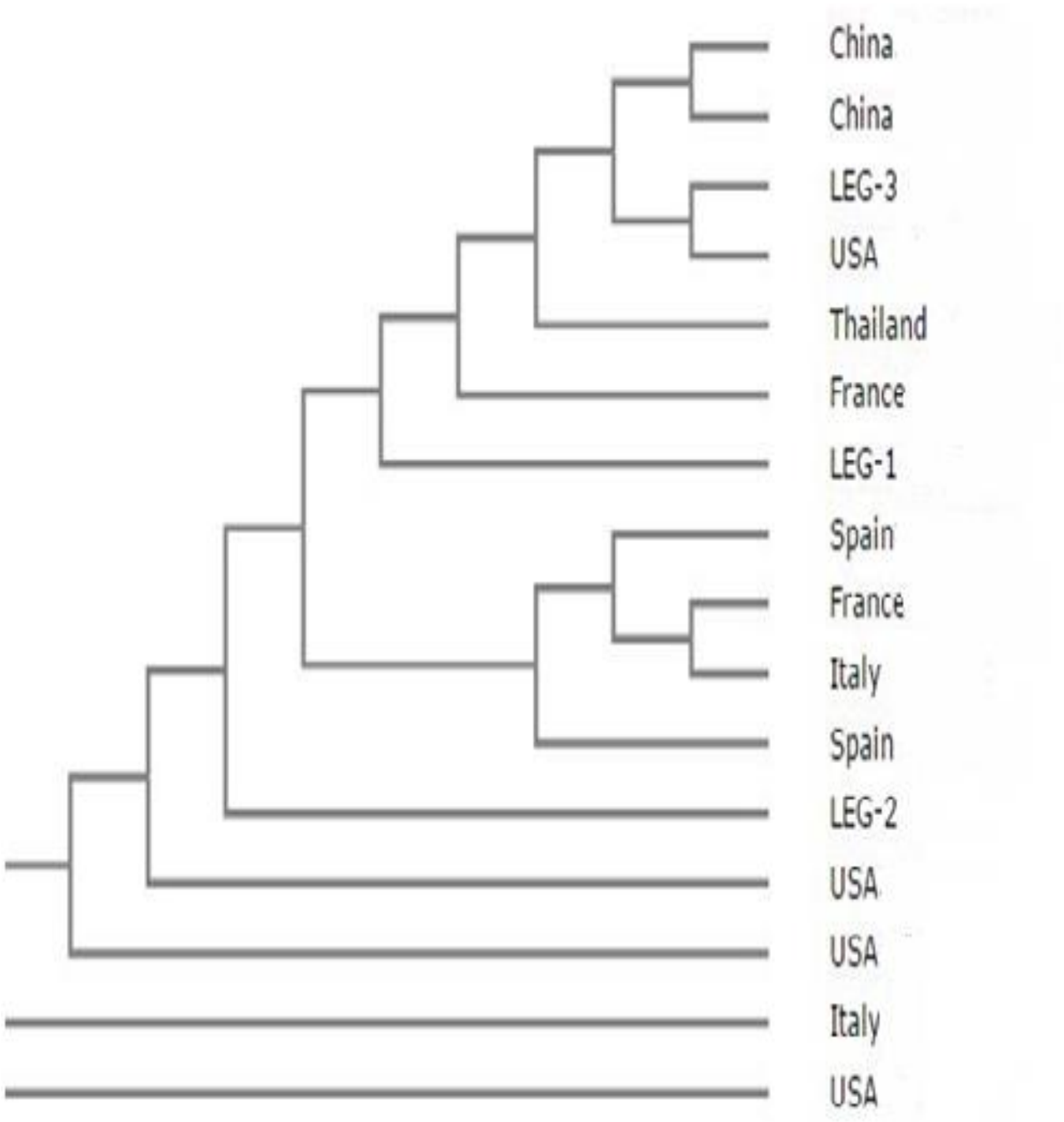


**Fig 41. Phylogenetic tree result of *L. pneumophila* (LEG-1, 2 and 3)**

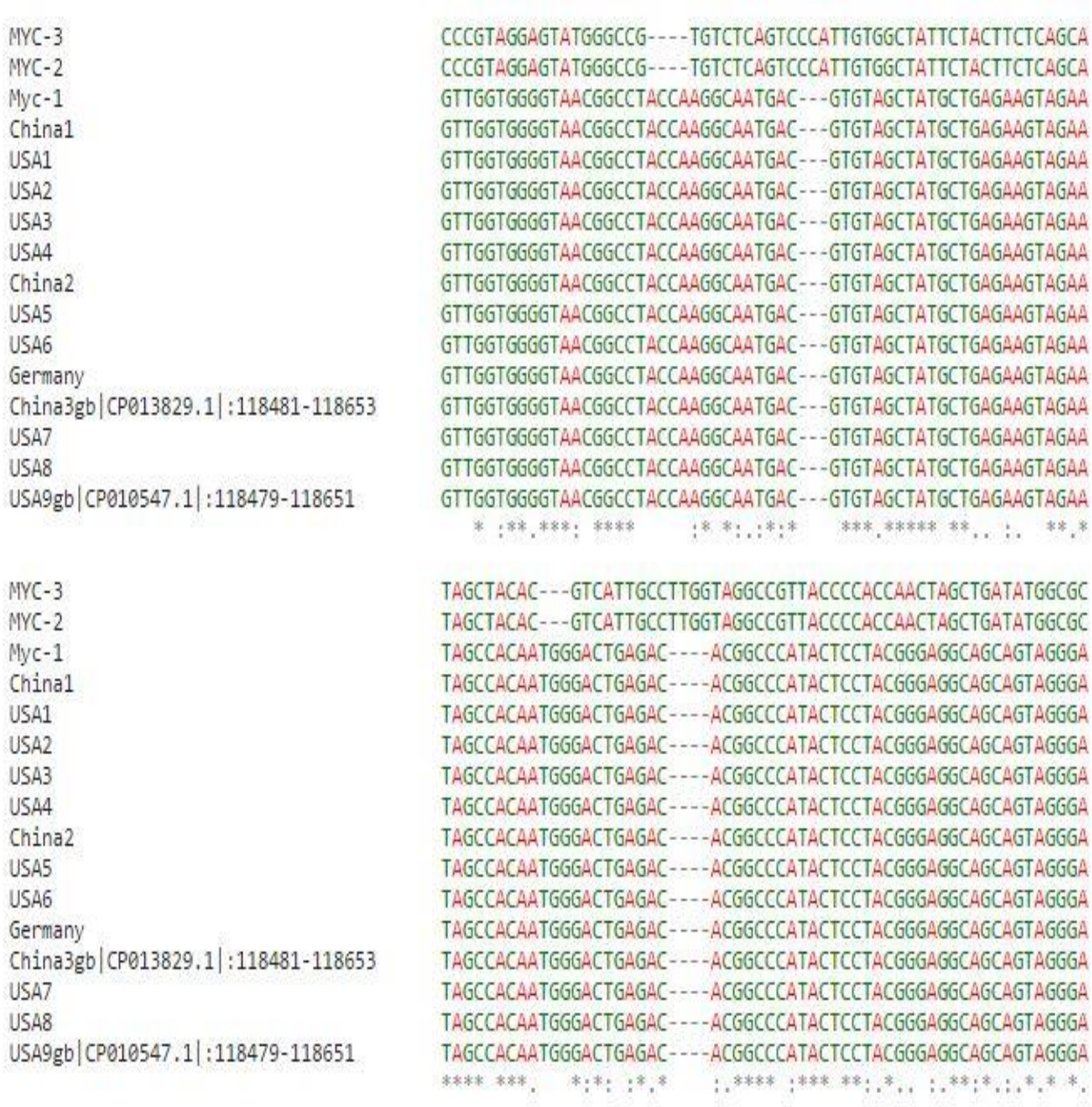


**Fig 42. Multiple sequence alignment of *M. pneumoniae* (MYC-1, 2 and 3)**

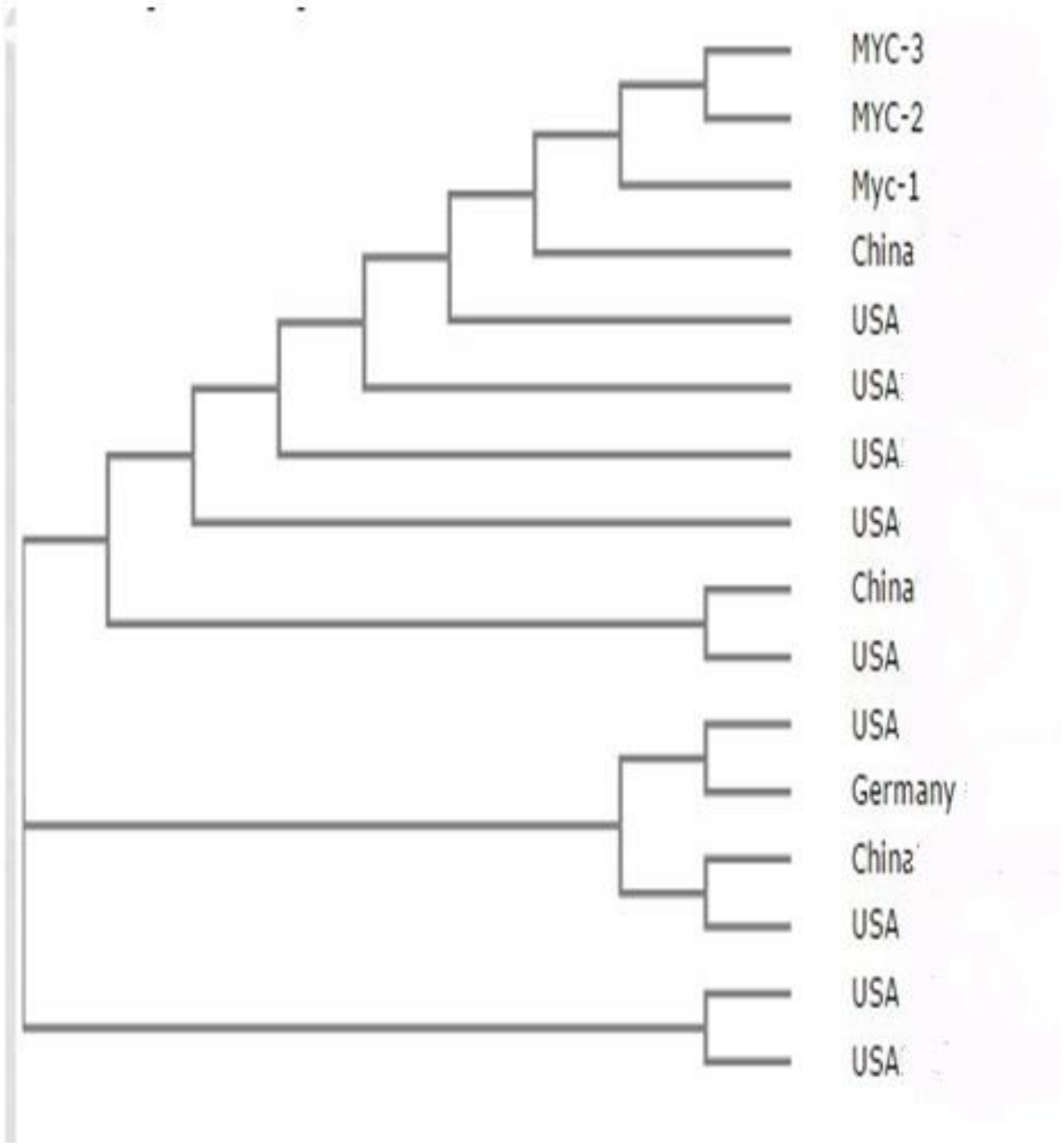


**Fig 43. Phylogenetic tree result of *M. pneumoniae* (MYC-1, 2 and 3)**

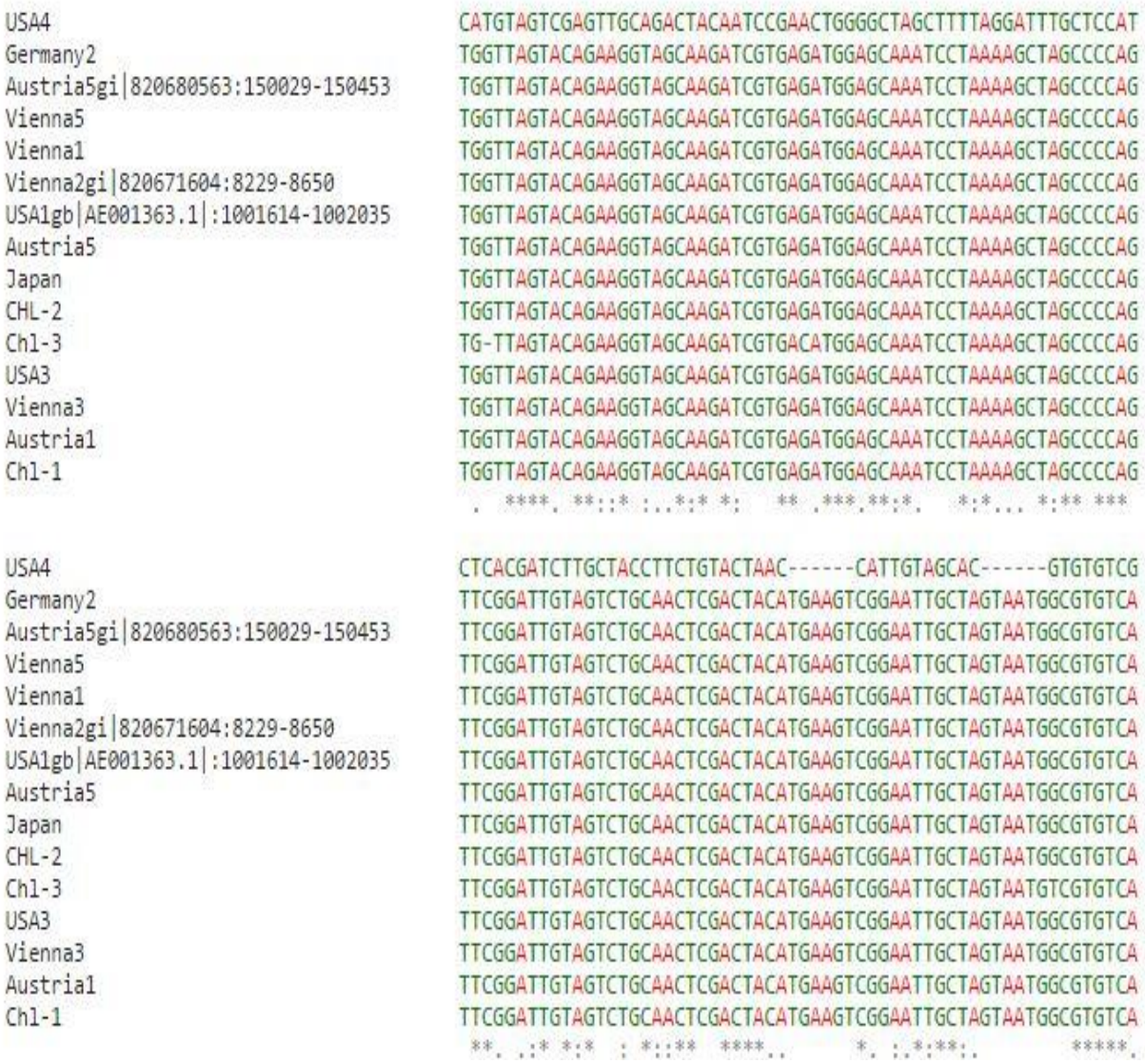


**Fig 44. Multiple sequence alignment of *C. pneumoniae* (CHL-1, 2 and 3)**

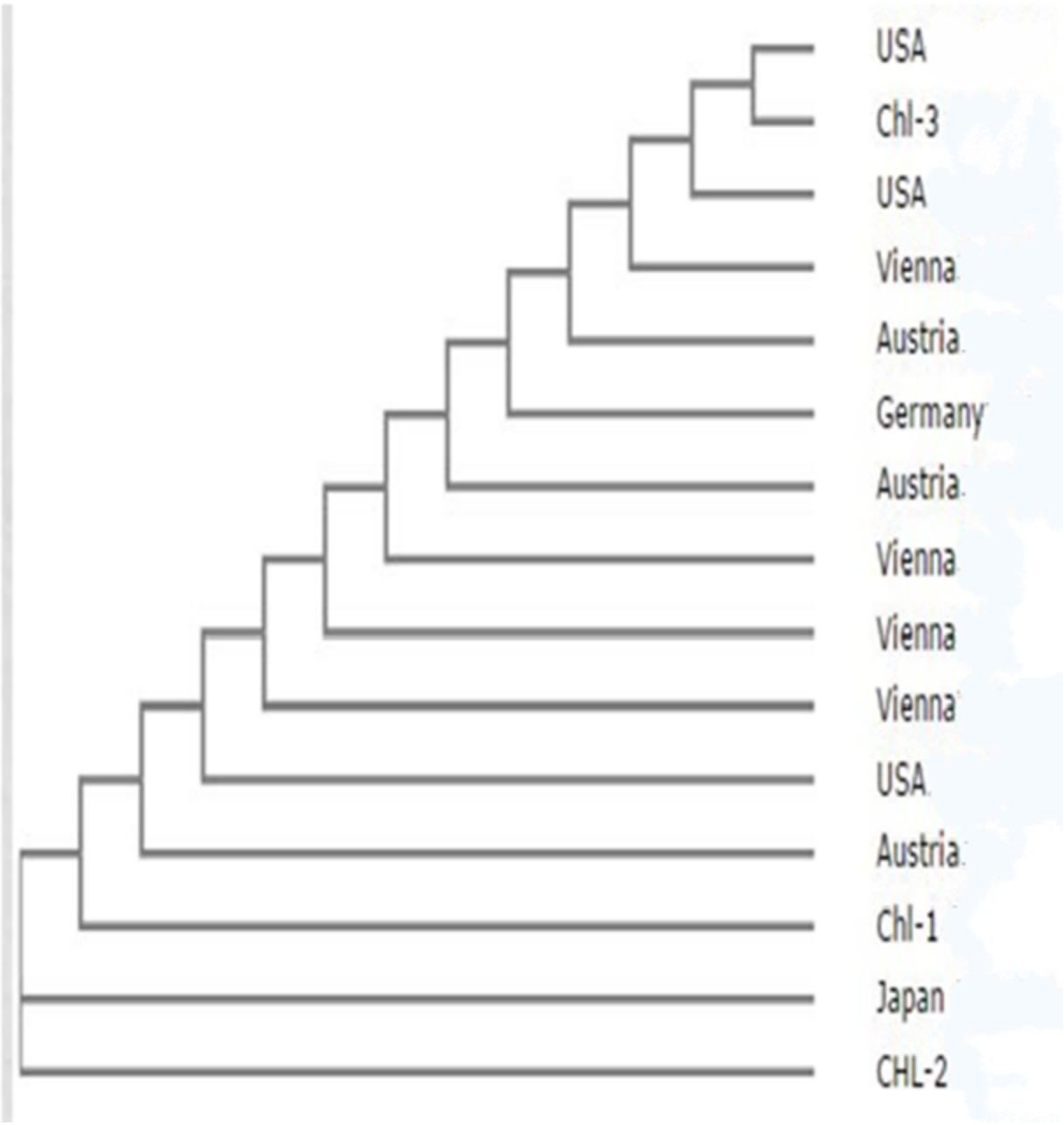


**Fig 45. Phylogenetic tree result of *C. pneumoniae* (Chl-1, 2 and 3)**

# CHAPTER FIVE

## 5.1. Discussion

Community-acquired pneumonia (CAP) is a worldwide cause of morbidity and mortality (Alvarez and Torres, 2004; Arnold *et al*., 2007). In spite of the advances in diagnostic methods, the etiology of CAP often is uncertain and therapy is empirical (Mandell *et al*., 2007). The low sensitivity of blood and sputum cultures and the occurrence of a heterogeneous bacterial flora in the upper respiratory tract confuse the interpretation of the tests (Forbes *et al*., 2007). Identification by molecular techniques might accelerate appropriate treatment of both ventilators associated pneumonia (VAP) and CAP (Wang *et al*., 2012), which is of special importance for patients treated in intensive care units (ICU). The etiologic diagnosis of infections with atypical pathogens such as *C. pneumoniae*, *M. pneumoniae* and *L. pneumophila* still remains difficult. This is mainly due to difficulties in culturing and to the delayed results associated with conventional methods (Mustafa *et al*., 2011).

This result revealed that 242(60.5%) were males and 158(39.5%) were females this showed insignificant relationship between gender and atypical organisms the p-value between gender and *M. pneumoniae* is (0,346), *C. pneumoniae* (p=0.119) and *L. pneumoniae* (p=0.365) (Table 7). The prevalence percentage of *M. pneumoniae* in males is 27 (6.8%) and 22 (5.5%) in females. But there was no statistical significant (p-value 0,346) association found between gender and *M. pneumoniae* positivity. Chaudhry *et al*., (2013) in their study found 27 of 92 (69%) males and 16 of 42 (38%) females to be infected with *M. pneumoniae*. Kashyap *et al*., (2008) reported 12 out of 46 males (26.09%) and 4 out of 29 females (13.79%) to have *M. pneumoniae* infection. No statistically significant association between sex and *M. pneumoniae* infections were found in the above studies, which

concords the current findings. Surinder and his collogue from India showed there were 127 (63.5%) males and 73 (36.5%) females in the 200 cases investigated. The presence of *C. pneumoniae* antibody was higher in 10 (7.87%) males than in 2 (2.74%) females, though this difference was statistically non-significant (P value 0.22) (Surinder *et al*., 2011).

Another part of our investigation highlights the age and sex-dependent distribution of *L. pneumophila*. The investigation even insignificant result showed that age and sex of patients are predisposing factors for prevalence of *L. pneumophila* in males 39(9.5%) where in females 19(4.8%) this is in agreement with Fatemeh and her colleagues from Iran (Fatemeh *et al*., 2015), who showed men usually have more contact with the external contaminated environment. They work outdoors, while women usually stay at home and are not in close contact with contaminated environments. Therefore, it is clear that the prevalence of *L. pneumophila* in men (14.81%) was higher than that in women (8.69%). Nagalingam from Trinidad and Tobago showed Hospitals, gender and ethnicity did not significantly ($p > 0.05$; $\chi 2$) affect the seroprevalence of *L. pneumophila* (Nagalingam *et al*., 2005).

According to the results revealed there is insignificant relationship between hospitals and atypical microorganisms (p=0.192) *M. pneumoniae*, (p=0.211) *C. pneumoniae* and (p=0.301) *L. pneumophila*. This may depends on the specialized hospital and specimen's numbers example Al shaab hospital specialized in chest and cardiology, patients attended to this hospital more than other and from different area of Khartoum Stat.

The results of the present study showed significant relationship between age group and *M. pneumoniae* (P- value= 0.012) (Table 1). The high prevalence was found in age group 31-60 (6.3%) than another age group 61-91(3.5%), and age group 13-30 (2.5%). This slightly agree with Hassan from KSA who showed 71 cases showed that 45 (63. 3%) cases were between30-70 years of age, as our group of patients

were living in an air conditioned closed atmosphere throughout the year. This is a very strong predisposing factor for a susceptible person to got CAP especially *L. pneumophila* (Hassan *et al*., 2006). Our study is in agreement with Salama from Egypt who carried out study with a wide age-range and classified patients into 3 groups (below 30, between 30 and 60 and above 60 years). She found that *C. pneumoniae* infection is more prevalent in the middle age group between 30–60 years (66.7%) (Salama *et al*., 2012). Similar result was obtained by Nader from Iran who showed eleven samples had positive results using real time PCR analysis of 16s rRNA gene fragments specific for *L. pneumophila*. Of the total positive cases, six were males, one female and four infants. The seven adults aged 40-65 years (Nader *et al*., 2015).

The etiologic diagnosis of infections with atypical pathogens such as *C. pneumoniae*, *M. pneumoniae* and *L. pneumophila* still remains difficult. This is mainly due to difficulties in culturing and to the delayed results associated with conventional methods (serology and culture). Technology has developed a PCR-based assay for the detection of these pathogens (Nadia *et al*., 2010). Molecular diagnostic techniques are promising tools for the rapid etiologic diagnosis of many infections including CAP, saving both time and cost. Thus, simultaneous detection of multiple CAP pathogens is possible and desirable for rapid diagnosis of pneumonia (Templeton *et al*., 2005; Johansson *et al*., 2010). In our study the most frequently detected pathogens by PCR and serology were *C. pneumoniae* 69(17.3%), 43(10.8%), *M. pneumoniae* 49(12.3%), 32(8.0%) and *L. pneumophila* 57(14.3 %), 50(12.5%). In this study there is no over lapping results and none of the patients got two atypical pathogens at the same time. These results are similar to Vivian and his coworkers who enrolled 256 specimens and showed *M. pneumoniae* and *C. pneumoniae* were detected in 32 (9%) and 28 (7.9%) cases, respectively, but only in 13 cases for each agent were they the sole pathogen. For *M*

*pneumonia*, PCR and serology yielded almost similar results (69.7% and 72.7%, respectively; 42.4% of cases were positive by both tests). *C. pneumoniae* was detected by PCR (19/28) and serology (15/28), with 6 of 28cases (21.4%) detected by both tests. *L. p*eumophila was detected in 13/256 cases (5.07%), 10/256 by antigen detection (Vivian *et al*., 2013). Results disagrees with Grace from Hong Kong were showed among 134 (28.6%) patients had atypical pneumoniae infections, including *M. pneumoniae* (n = 78), *C. pneumoniae* (n = 55), *L. pneumophila* (n = 1) and *C. burnetii* (n = 2). Two patients had dual *mycoplasma*/ *chlamydophila* infections (Grace *et al*., 2009). Our results agree with studies gave variable positivity rates for *M. pneumoniae*, ranging from 1% to 27 % (Deory *et al*., 2000). Disagrees with Luna, Argentina *M. pneumoniae*, present in 19 (13%), *C. pneumoniae*, present in 12 cases (8%) (Luna *et al*., 2000). Positive cases for these atypical pathogens more than likely depend on the patient population, socioeconomic factors, age and possibility of exposure. The high level of *C. pneumoniae* in our study more than *M.pneumoniae* this result agrees with Naoyuki in Japan who reported *C. Pneumoniae* (7.2%), *M.pneumoniae* (4.8%) (Naoyuki *et al*., 2004), and Seung in Korea who reported *C. Pneumoniae* (12.3%), *M.pneumoniae* (8.6%) (Seung *et al*., 2002). The variable results showing *C. pneumoniae* more than *M. pneumoniae* because of the large number of specimens from Alshaab hospital (cardiac and chest infection hospital), most of outpatients may have complication chest and cardiac infections because chlamydial persistent form may endure for a long time inside host cells, since it is able to evade the host immune response leading to a chronic inflammatory state in the vascular wall (Schoborg, 2011; Di *et al*., 2012).

The seroprevalence of *L. pneumophila* was found to be (12.5%) which is to disagrees with the percentage obtained by Sabah and his co-workers (27.4%) (Sabah, *et al* 2010) and Rabih from Sudan (22.7%) (Rabih *et al*., 2014). Our results

similar to Lower seroprevalence of L. *pneumophila* among community acquired pneumonia were reported by Chaudhry (15%) (Chaudhry *et al*., 2000). This may be the different in the specimen's number and the population area.

The *M. pneumoniae* PCR in our study showing 49(12.25%). This result is in agreement with Roger from Germany (12.3%) (Roger *et al*., 2015). The PCR is more accurate than serology. Similar result reported by Jiuxin from China that IgM testing (7.4%, 28.6% and 1.45) compare with PCR (40.7%, 50% and 3.63) on three months (Jiuxin *et al*., 2013), Xiao found The positive percentage of *M. pneumoniae*-DNA was higher than that of *M. pneumoniae* -IgM (Xiao *et al*., 2013), Kate approved that 12 (11.3%) were positive by all the molecular methods whereas serology with acute sample and convalescent samples detected 6 (5.6%) and 9 (8.5%), Bineeta from India showing PCR positive results diagnosed *M. pneumoniae* infection in 18 (24%) than Serological evidence of *M. pneumoniae* infection was observed in 16(21.3%) (Bineeta *et al*., 2008). The reason for the different between PCR and serology because Serological methods lack adequate sensitivity in the acute phase of the disease, an perfect diagnosis with convalescent phase samples is often made many days after the beginning of disease (Thacker *et al*., 2000), Sensitivity and specificity values are between 55 and 100%, depending on the serological method used and the patient population tested. PCR has been shown to offer the latent of increased sensitivity and rapidity compared to other diagnostic tests (Ferwerda *et al*., 2001).

The seroprevalence of IIFA showed 43(10.75%) and PCR technique showed 69(17.25%) of 16SrRNA gene. The serology sensitivity 62.3% and specifity 92.7% compare with PCR that mean the PCR is more sensitive and more specific than serology this results are in agreement with several studies showed the PCR percentage is more than serology , Mohammad from Iran found positive PCR detected in 19.6% (10/51) of cases and positive IgM detected in 9.8%

(5/51)(Mohammad *et al*., 2014), Cheuk results showing the PCR more sensitive than MIF serology test (Cheuk *et al*., 2005), Nele study showed CAP was caused by *C. pneumoniae* in 5/546 cases (0.9%). Antibody testing by microimmunofluorescence was done in 376 of 546 patients. All patients were negative for IgM antibodies the conclusion PCR is more sensitive technique (Nele *et al*., 2006), Hem from India reported 29.67% (27/91) patients were positive for *C. pneumoniae* using nested PCR compared with presence of *C. pneumoniae* specific IgA, IgA IgG and IgG antibodies 11(12%) were IgA positive, 13(14.2%) were IgA IgG positive and only1 (1.1%) was IgG positive (Hem *et al*., 2007), Ali reported serological acute infection for *C. pneumoniae* was not detected among patients with positive PCR results (Ali *et al*., 2009). Our results disagree with Zheng that the sensitivity, specificity, and concordance rate of the mAb-based IIF and ELISA tests more specific were compared with those of polymerase chain reaction (PCR) ( Zheng *et al*., 2015). That because he used a novel monoclonal antibodies (mAbs) against a recombinant protein equivalent to the immunodominant region of chlamydial protease-like activity factor (CPAF) from *C. pneumoniae*. With the added sophistication and modernization of amplification processes like multiplex PCR and real-time PCR, technology has enabled testing to be more proficient and accurate. As with all molecular biology-based amplification methods, contamination and false positive results are always a risk. Good molecular biology practices in the laboratory and experience reduce this to a very low level (Khanna *et al*., 2005).

Sequencing test done for nine positive PCR products; three selected randomly for each bacterium of *M. pneumoniae, C. pneumoniae* and *L. pneumophila* were selected to more confirmations. The results showed identity range between 92% to 100% and the query cover range between 74% to 100% when compare with the

database. This result gave amore identification with PCR test and serology test to detection of fastidious bacteria.

## Conclusion

-The prevalence of CAP among pneumonic patients is high.

-The prevalence of atypical bacterial pneumonia i.e. *M. pneumoniae*, *L. pneumophila* and *C. pneumoniae* are High.

-*C. pneumoniae* is dominant of atypical bacterial pneumonia.

-ELISA and IIFA techniques are efficient in detection of atypical bacterial pneumoniae antibodies in infected patients.

-The sensitivity and specifity of the IIFA technique is high.

-The seroprevalence of *L. pneumoniae* is high but in agreement with other studies carried out in Sudan.

-IFA IgG, IgA, and IgM can differentiate between the past and persistent infections.

-ELISA technique is faster than IIFA test because no need the professional technician (Electron microscope slides reader) to do the test.

-The prevalence of atypical bacterial pneumonia showed insignificant with Hospitals and gender but showed significant with age groups.

- PCR Technique showed more accurate than serological test.

-Serology and PCR are suitable for detection of fastidious bacteria i.e. *M. pneumoniae*, *L. pneumophila* and *C. pneumoniae*.

-Sequencing is confirmation techniques for identification of atypical bacterial pneumonia.

## Recommendations

**1-** The IIFA test IgM, IgA, IgG is suitable test for detection of fastidious bacteria i.e. *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila.*

**2-** Serological test is the primary and fast test to give the physician a guideline for treatment with another confirmation test like PCR and culture.

**3-** Clinicians should select reliable PCR technology (while available) plus serology as diagnostic screening for the patients suspected, combination of serology and PCR is recommended to provide rapid, reliable, and accurate diagnosis of fastidious bacteria like *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila*.

**4-** In general, the diagnosis based on the analysis of two serum samples in two to three weeks apart is currently recommended.

**5-** *C. pneumoniae* is very important because associated with cardio vascular disease. However, more studies in larger groups of strains are necessary to confirm these finding.

**6-** PCR is a promising test that allows detection of atypical pneumoniae DNA in all phases of infection, including early periods when the serum may be negative for antibody and a new technique like RT-PCR, Nested PCR (when available) is a highly recommended.

**7-** Application of PCR based *MIP* gene of the *L. pneumophila* and serological IIFA IgG, IgA, IgM are the suitable for diagnosis.

**8-** Further in-depth studies including large sample size and other locations are recommended

## References


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# Appendixes

## Appendix I

**Sudan University of Science and Technology**

**Community-acquired atypical bacterial pneumonia among Sudanese: A serological and molecular study**

**Questionnaire**

**Hospital name: …….**

**-Patient No: ……..**

**-Name ……**

**-Age: ……..**

**-Gender:**

**-Male ……..**

**-Female ……..**

**Researcher signature: ……..**

**Donor signature: ……..**

**Date: …../…../……**

# Appendix II

## Informed consent

**إعلام موافقة**

**هذه دعوة منى: الباحث /علي فاضل كاظم طالب دكتوراه - مختبرات طبية – بجامعة السودان للعلوم والتكنولوجيا لمشاركتكم في برنامج بحث هدفه الكشف عن الألتهاب البكتيري الرئوي الغير نمطي في مجتمع السودان بدراسة مصلية وجزيئية.**

**إذا رغبتم في إنجاح هذا البرنامج فإني وفريق البحث سنقوم:-**

**أخذ عينة بلغم للكشف عن البكتريا المسببة لهذا المرض.**

**أخذ عينة من الدم لقياس مستوي الأجسام المضادة لهذه البكتريا..**

**بملء إستمارة بمعلومات تخصكم لها علاقة بموضوع البحث.**

**أي معلومة تخصكم في الإستمارة سوف تكون سرية.**

**مشاركتكم في البرنامج تسعدنا وتساعد في إنجاح هدف البحث.**

**لكم كامل الحرية في إختيار عدم المشاركة, المشاركة أو الإنسحاب من برنامج البحث في أي وقت تشاءون.**

**يمكنكم الحصول علي إجابة لأي سؤال عن برنامج البحث.**

**التاريخ .....**

**توقيع المتبرع .....**

**توقيع الباحث .....**

# Appendix II

## Preparation of reagents

## A) Preparation of 10 X TBE buffer

Amount of 108 g Tris base were weighed and added to 55gm of boric acid and 40ml of 0.5 EDTA then dissolved into 1 liter deionized water PH 8.0

## B) Preparation of 1X TBE buffer .

Ten ml of 10 X TBE buffer was added to 90 ml deionized water and heated until completely dissolved.

## C) Preparation of Ethidium bromide

Five milligrams of Ethidium bromide powder were dissolved into 500 µl deionized water, and kept into brown bottle.

## D) Preparation of loading dye

Three ml of glycerol were added to 7ml of D.W and 2.5 g of bromophenol blue was dissolved into 100 ml D.W .The mixture was as a loading dye.

## E) Preparation of agarose gel

Amount of 1.5% of agarose powder was dissolved by boiling in 100 ml 1X TBE buffer (AppliChem). Then was cooled to 55°C in water bath. Then, 5 µl of (10mg\ml). Ethidium bromides were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed

## F) Visualization of PCR products

The gel casting tray was put into the electrophoresis, tank flooded with 1x TBE buffer just to cover the gel surface, 5 µl of PCR products from each samples was mixed with 0.5 µl of loading dye and then electrophoreses 5 µl of DNA ladder (marker) was mixed with 0.5 µl of loading dye and were added to the well in each run. The gel electrophoresis apparatus was connected to power supply (Primer, 125v, 500 mA, UK). The electrophoresis was carried at 75v for 30 minutes after electrophoresis period, the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized by u.v transilluminater (Uvitec – UK).

## G) Trisbase Boric acid EDTA (TBE) Buffer For 500 ml

89 mM Trisbase 54 gm
89 mM Boric acid 27.5 gm
2 mM EDTA 3.72 g

# Appendix III

| No. | Age | Gender | Hospital | *L. pneumophila* | | *C. pneumoniae* | | *M.pneumoniae* | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | pcr | serology | pcr | serology | pcr | serology |
| 1 | 32 | F | Omdurman | + | + | - | - | - | - |
| 2 | 25 | M | = | - | - | - | - | + | - |
| 3 | 19 | M | Abu anja | - | - | - | - | - | - |
| 4 | 40 | M | = | - | - | - | - | - | - |
| 5 | 42 | M | = | + | - | - | - | - | - |
| 6 | 27 | F | = | - | - | - | - | - | - |
| 7 | 23 | M | = | - | - | - | - | + | + |
| 8 | 39 | F | = | - | - | - | - | - | - |
| 9 | 30 | F | = | - | - | - | - | - | - |
| 10 | 37 | F | = | - | - | - | - | - | - |
| 11 | 30 | F | = | - | - | - | - | - | - |
| 12 | 42 | M | = | - | - | - | - | - | - |
| 13 | 30 | F | = | - | - | - | - | - | - |
| 14 | 32 | M | = | - | - | - | - | - | - |
| 15 | 26 | M | = | - | - | - | - | - | - |
| 16 | 23 | M | = | - | - | + | + | - | - |
| 17 | 72 | M | = | + | + | - | - | - | - |
| 18 | 35 | M | = | - | - | - | - | - | - |
| 19 | 27 | F | = | - | - | - | - | + | + |
| 20 | 38 | F | = | - | - | - | - | - | - |

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|---|---|---|---|---|---|---|---|---|---|
| **21** | **24** | **F** | **=** | - | - | - | - | + | - |
| **22** | **50** | **F** | **=** | - | - | - | - | - | - |
| **23** | **32** | **F** | **=** | - | - | - | - | - | - |
| **24** | **56** | **M** | **Omdurman** | - | - | - | - | - | - |
| **25** | **33** | **M** | **=** | - | - | - | - | - | - |
| **26** | **56** | **M** | **=** | - | - | - | - | - | - |
| **27** | **47** | **M** | **=** | - | - | - | - | - | - |
| **28** | **39** | **M** | **=** | - | - | - | - | - | - |
| **29** | **43** | **F** | **Abu anja** | - | - | - | - | + | + |
| **30** | **25** | **M** | **=** | - | - | - | - | - | - |
| **31** | **41** | **M** | **=** | - | - | - | - | - | - |
| **32** | **65** | **F** | **=** | + | + | - | - | - | - |
| **33** | **22** | **F** | **=** | - | - | - | - | - | - |
| **34** | **20** | **M** | **=** | - | - | - | - | - | - |
| **35** | **20** | **M** | **=** | - | - | - | - | - | - |
| **36** | **27** | **M** | **=** | - | - | - | - | - | - |
| **37** | **23** | **M** | **=** | - | - | - | - | + | - |
| **38** | **35** | **M** | **=** | - | - | - | - | - | - |
| **39** | **55** | **M** | **=** | - | - | - | - | - | - |
| **40** | **50** | **M** | **=** | - | - | + | + | - | - |
| **41** | **35** | **M** | **=** | - | - | + | - | - | - |
| **42** | **31** | **M** | **=** | - | - | - | - | - | - |
| **43** | **17** | **F** | **=** | - | - | - | - | - | - |
| **44** | **13** | **M** | **=** | + | + | - | - | - | - |
| **45** | **42** | **M** | **Alshaab** | - | - | + | - | - | - |

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| **46** | **40** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **47** | **70** | **F** | **=** | **+** | **-** | **-** | **-** | **-** | **-** |
| **48** | **40** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **49** | **80** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **50** | **37** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **51** | **76** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **52** | **81** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **53** | **40** | **F** | **=** | **+** | **-** | **-** | **-** | **-** | **-** |
| **54** | **50** | **M** | **=** | **+** | **-** | **-** | **-** | **-** | **-** |
| **55** | **38** | **M** | **Omdurman** | **-** | **-** | **+** | **-** | **-** | **-** |
| **56** | **73** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **57** | **32** | **F** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **58** | **59** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **59** | **38** | **F** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **60** | **17** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **61** | **16** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **62** | **44** | **F** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **63** | **47** | **F** | **Al shaab** | **+** | **+** | **-** | **-** | **-** | **-** |
| **64** | **30** | **F** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **65** | **35** | **M** | **=** | **+** | **-** | **-** | **-** | **-** | **-** |
| **66** | **35** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **67** | **20** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **68** | **58** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **69** | **70** | **M** | | **+** | **+** | **-** | **-** | **-** | **-** |
| **70** | **45** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |

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| **71** | **50** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **72** | **18** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **73** | **20** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **74** | **26** | **F** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **75** | **88** | **F** | **=** | **-** | **-** | **-** | **+** | **+** | **+** |
| **76** | **59** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **77** | **47** | **F** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **78** | **31** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **79** | **57** | **F** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **80** | **72** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **81** | **50** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **82** | **25** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **83** | **32** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **84** | **62** | **F** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **85** | **40** | **F** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **86** | **15** | **F** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **87** | **28** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **88** | **70** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **89** | **20** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **90** | **25** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **91** | **40** | **F** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **92** | **36** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **93** | **50** | **F** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **94** | **40** | **F** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **95** | **16** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **96** | **50** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |

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| **97** | **24** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **98** | **24** | **M** | **Abu anja** | **-** | **-** | **+** | **-** | **-** | **-** |
| **99** | **37** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **100** | **65** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **101** | **25** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **102** | **30** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **103** | **23** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **104** | **35** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **105** | **23** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **106** | **32** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **107** | **18** | **F** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **108** | **26** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **109** | **25** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **110** | **36** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **111** | **57** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **112** | **60** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **113** | **21** | **F** | **Omdurman** | **-** | **-** | **+** | **+** | **-** | **-** |
| **114** | **16** | **M** | **Alshaab** | **-** | **-** | **-** | **-** | **-** | **-** |
| **115** | **38** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **116** | **55** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **117** | **35** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **118** | **53** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **119** | **54** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **120** | **38** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **121** | **26** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |

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| **122** | **55** | **F** | **Omdurman** | **-** | **-** | **-** | **-** | **+** | **-** |
| **123** | **18** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **124** | **18** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **125** | **30** | **F** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **126** | **52** | **F** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **127** | **35** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **128** | **55** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **129** | **51** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **130** | **57** | **M** | **Alshaab** | **-** | **-** | **-** | **-** | **-** | **-** |
| **131** | **78** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **132** | **41** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **133** | **24** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **134** | **37** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **135** | **35** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **136** | **50** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **137** | **27** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **138** | **65** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **139** | **19** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **140** | **39** | **F** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **141** | **60** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **142** | **37** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **143** | **35** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **144** | **65** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **145** | **60** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **146** | **65** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |

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| **147** | **35** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **148** | **38** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **149** | **65** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **150** | **75** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **151** | **65** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **152** | **38** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **153** | **31** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **154** | **28** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **155** | **32** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **156** | **21** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **157** | **45** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **158** | **50** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **159** | **32** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **160** | **39** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **161** | **65** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **162** | **41** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **163** | **20** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **164** | **50** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **165** | **62** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **166** | **23** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **167** | **18** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **168** | **18** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **169** | **62** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **170** | **45** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **171** | **25** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **172** | **18** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |

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| **173** | **60** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **174** | **50** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **175** | **26** | **F** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **176** | **26** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **177** | **27** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **178** | **17** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **179** | **70** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **180** | **27** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **181** | **27** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **182** | **60** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **183** | **66** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **184** | **20** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **185** | **60** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **186** | **55** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **187** | **53** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **188** | **18** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **189** | **82** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **190** | **38** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **191** | **36** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **192** | **57** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **193** | **65** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **194** | **58** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **195** | **70** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **196** | **37** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **197** | **43** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **198** | **53** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |

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| **200** | **60** | **F** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **201** | **36** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **202** | **16** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **203** | **50** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **204** | **40** | **F** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **205** | **38** | **F** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **206** | **42** | **F** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **207** | **70** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **208** | **60** | **F** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **209** | **35** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **210** | **26** | **F** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **211** | **28** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **212** | **20** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **213** | **60** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **214** | **26** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **215** | **46** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **216** | **43** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **217** | **24** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **218** | **30** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **219** | **70** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **220** | **45** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **221** | **65** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **222** | **45** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **223** | **19** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **224** | **32** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |

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| **226** | **60** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **227** | **28** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **228** | **42** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **229** | **26** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **230** | **57** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **231** | **40** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **232** | **59** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **233** | **71** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **234** | **45** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **235** | **65** | **F** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **236** | **48** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **237** | **40** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **238** | **50** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **239** | **55** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **240** | **60** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **241** | **29** | **M** | **Omdurman** | **-** | **-** | **+** | **-** | **-** | **-** |
| **242** | **22** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **243** | **24** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **244** | **42** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
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| **246** | **53** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **247** | **42** | **F** | **Abu anja** | **-** | **-** | **-** | **-** | **-** | **-** |
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| **251** | **32** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **252** | **30** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **253** | **25** | **F** | **Alshaab** | **-** | **-** | **-** | **-** | **-** | **-** |
| **254** | **27** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **255** | **29** | **F** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **256** | **50** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **257** | **40** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **258** | **50** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **259** | **35** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **260** | **21** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **261** | **40** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **262** | **45** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **263** | **47** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **264** | **38** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **265** | **26** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **266** | **50** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **267** | **40** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **268** | **56** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **269** | **74** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **270** | **38** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **271** | **66** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **272** | **45** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **273** | **37** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **274** | **46** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **275** | **18** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |

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| **277** | **29** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **278** | **46** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **279** | **65** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **280** | **32** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **281** | **39** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **282** | **45** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **283** | **25** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **284** | **19** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **285** | **91** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **286** | **70** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **287** | **21** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **288** | **79** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **289** | **60** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **290** | **29** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **291** | **42** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **292** | **38** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **293** | **24** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **294** | **21** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **295** | **36** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **296** | **25** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **297** | **41** | **M** | **Bahry** | **-** | **-** | **-** | **-** | **-** | **-** |
| **298** | **71** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
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| **300** | **30** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **301** | **29** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |

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| **302** | **35** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **303** | **79** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **304** | **65** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **305** | **35** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **306** | **22** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **307** | **43** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **308** | **44** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **309** | **27** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **310** | **51** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **311** | **38** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **312** | **70** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **313** | **45** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **314** | **36** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **315** | **36** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **316** | **54** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **317** | **75** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **318** | **44** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **319** | **43** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **320** | **59** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **321** | **33** | **F** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **322** | **51** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **323** | **49** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **324** | **20** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **325** | **33** | **F** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **326** | **61** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **327** | **28** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |

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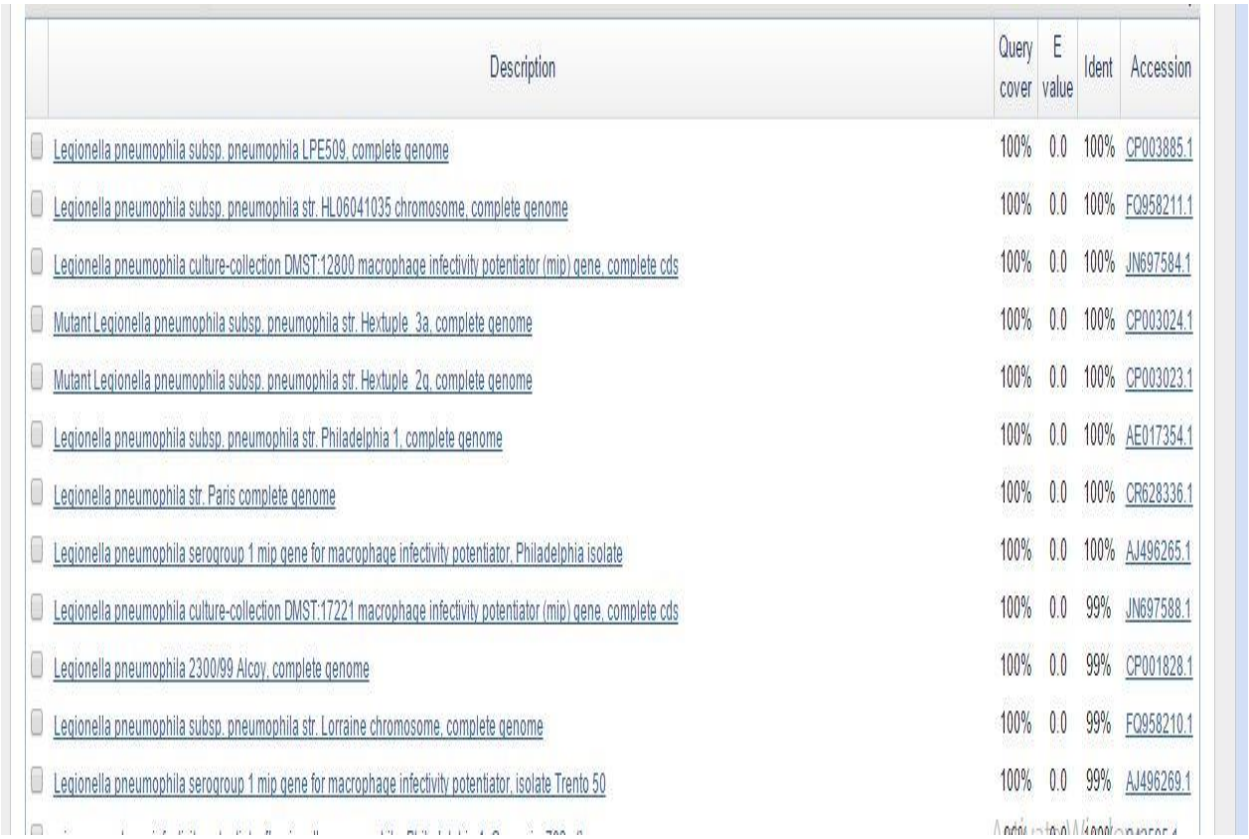
| **328** | **55** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **329** | **47** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **330** | **57** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **331** | **49** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **332** | **24** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **333** | **52** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **334** | **56** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **335** | **45** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **336** | **19** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **337** | **90** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **338** | **60** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **339** | **50** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **340** | **41** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **341** | **29** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **342** | **33** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **343** | **65** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **344** | **40** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **345** | **71** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **346** | **20** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **347** | **29** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **348** | **29** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **349** | **30** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **350** | **37** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **351** | **79** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **352** | **65** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **353** | **36** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **354** | **22** | **F** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **355** | **46** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **356** | **45** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **357** | **42** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **358** | **39** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **359** | **27** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **360** | **50** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **361** | **40** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **362** | **55** | **F** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **363** | **73** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **364** | **37** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **365** | **31** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **366** | **71** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **-** |
| **367** | **20** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **368** | **29** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **369** | **29** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **370** | **30** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **371** | **79** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **372** | **65** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **373** | **36** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **374** | **22** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **375** | **46** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **376** | **45** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **377** | **42** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **378** | **39** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **379** | **27** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **380** | **50** | **F** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **381** | **40** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **382** | **55** | **F** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **383** | **73** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **384** | **36** | **F** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **385** | **47** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **386** | **47** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **687** | **19** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **388** | **40** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **389** | **65** | **M** | **=** | **+** | **+** | **-** | **-** | **-** | **-** |
| **390** | **33** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **391** | **75** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **392** | **30** | **M** | **=** | **-** | **-** | **+** | **-** | **-** | **-** |
| **393** | **46** | **M** | **=** | **-** | **-** | **+** | **+** | **-** | **-** |
| **394** | **36** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **395** | **47** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **396** | **36** | **F** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **397** | **47** | **F** | **=** | **+** | **-** | **-** | **-** | **-** | **-** |
| **398** | **19** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |
| **399** | **75** | **M** | **=** | **-** | **-** | **-** | **-** | **+** | **+** |
| **400** | **30** | **M** | **=** | **-** | **-** | **-** | **-** | **-** | **-** |

# Appendix V

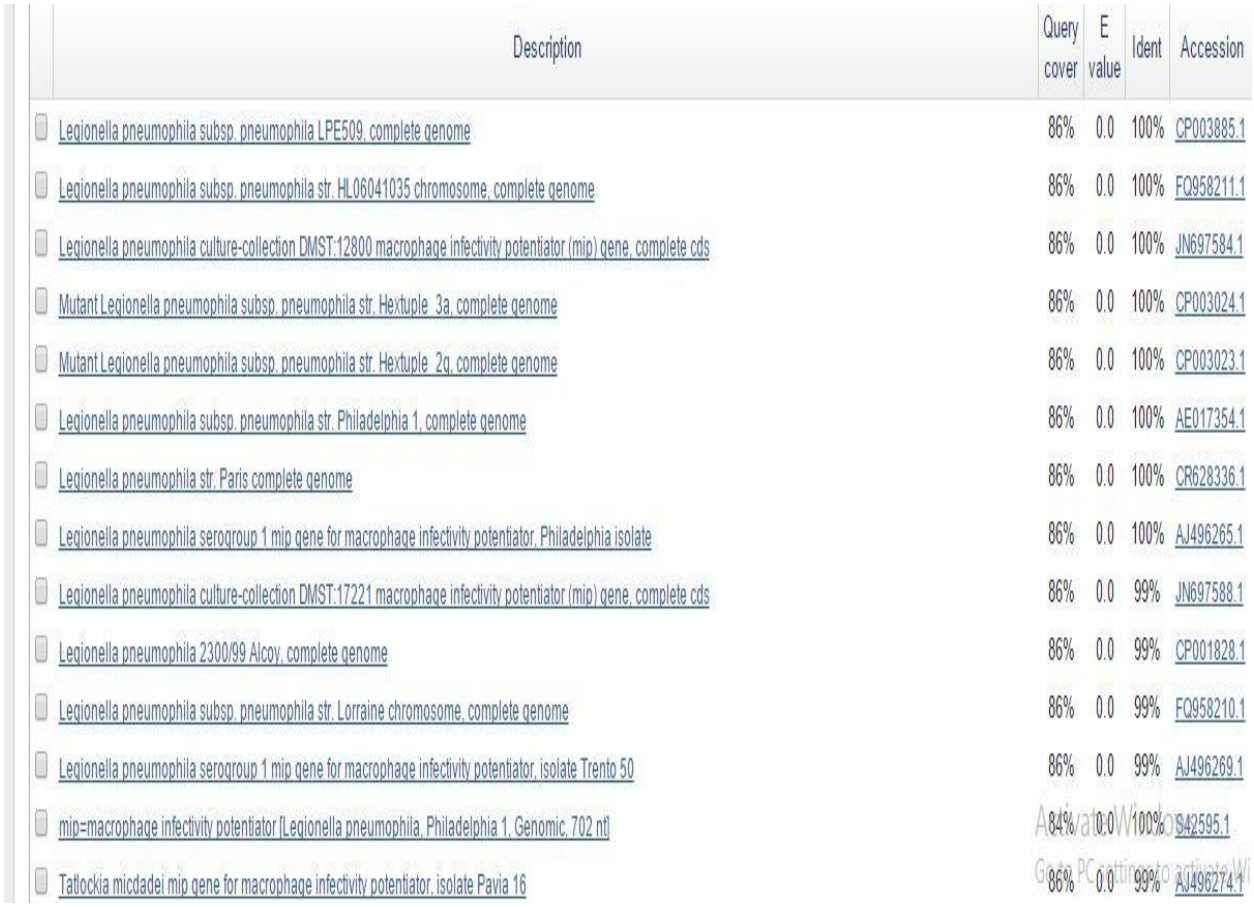
## Descriptions and Neighbor joining tree



| Description | Query cover | E value | Ident | Accession |
|---|---|---|---|---|
| Legionella pneumophila subsp. pneumophila LPE509, complete genome | 100% | 0.0 | 100% | CP003885.1 |
| Legionella pneumophila subsp. pneumophila str. HL06041035 chromosome, complete genome | 100% | 0.0 | 100% | FQ958211.1 |
| Legionella pneumophila culture-collection DMST:12800 macrophage infectivity potentiator (mip) gene, complete cds | 100% | 0.0 | 100% | JN697584.1 |
| Mutant Legionella pneumophila subsp. pneumophila str. Hextuple 3a, complete genome | 100% | 0.0 | 100% | CP003024.1 |
| Mutant Legionella pneumophila subsp. pneumophila str. Hextuple 2g, complete genome | 100% | 0.0 | 100% | CP003023.1 |
| Legionella pneumophila subsp. pneumophila str. Philadelphia 1, complete genome | 100% | 0.0 | 100% | AE017354.1 |
| Legionella pneumophila str. Paris complete genome | 100% | 0.0 | 100% | CR628336.1 |
| Legionella pneumophila serogroup 1 mip gene for macrophage infectivity potentiator, Philadelphia isolate | 100% | 0.0 | 100% | AJ496265.1 |
| Legionella pneumophila culture-collection DMST:17221 macrophage infectivity potentiator (mip) gene, complete cds | 100% | 0.0 | 99% | JN697588.1 |
| Legionella pneumophila 2300/99 Alcoy, complete genome | 100% | 0.0 | 99% | CP001828.1 |
| Legionella pneumophila subsp. pneumophila str. Lorraine chromosome, complete genome | 100% | 0.0 | 99% | FQ958210.1 |
| Legionella pneumophila serogroup 1 mip gene for macrophage infectivity potentiator, isolate Trento 50 | 100% | 0.0 | 99% | AJ496269.1 |

## Descriptions of *L. pneumophila* (LEG-01)

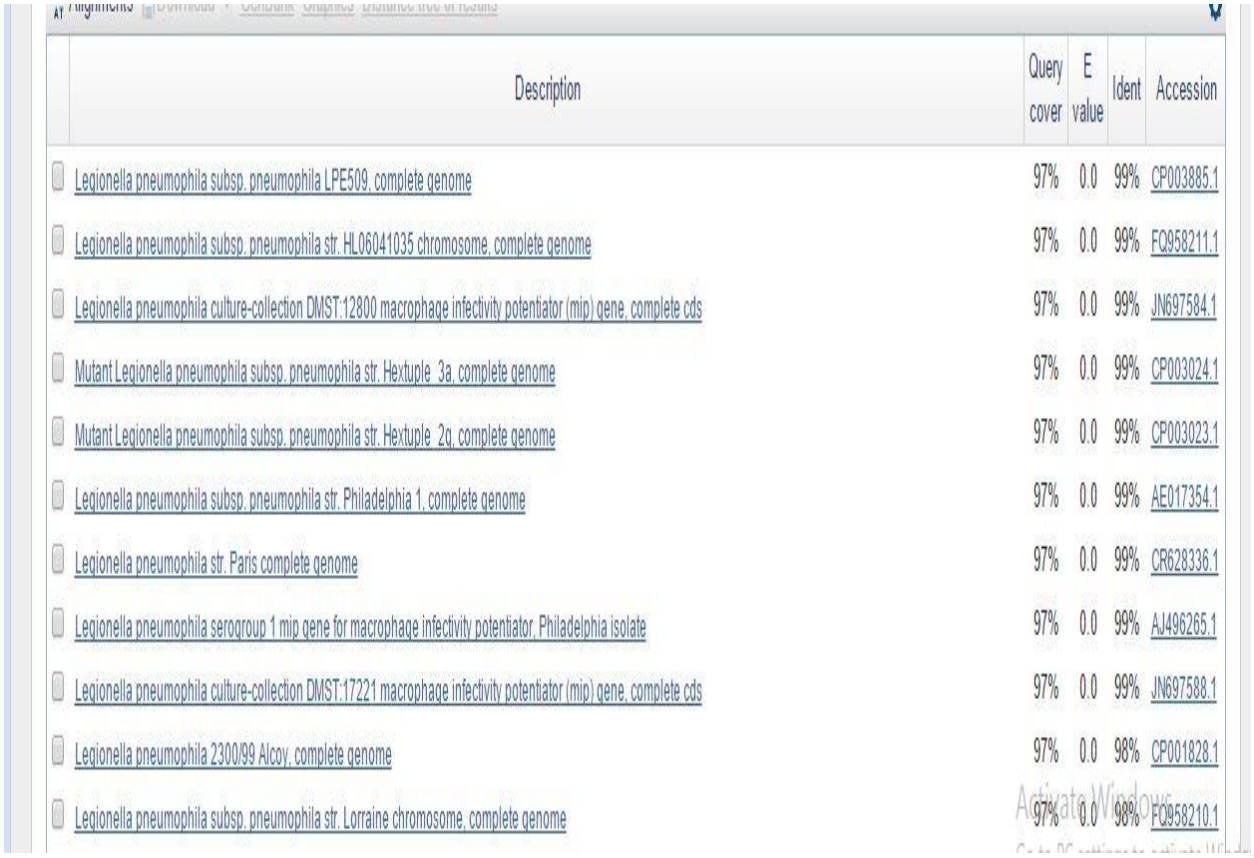

**Neighbor joining tree of *L. pneumophila* (LEG-01)**



| Description | Query cover | E value | Ident | Accession |
|---|---|---|---|---|
| Legionella pneumophila subsp. pneumophila LPE509, complete genome | 86% | 0.0 | 100% | CP003885.1 |
| Legionella pneumophila subsp. pneumophila str. HL06041035 chromosome, complete genome | 86% | 0.0 | 100% | FQ958211.1 |
| Legionella pneumophila culture-collection DMST:12800 macrophage infectivity potentiator (mip) gene, complete cds | 86% | 0.0 | 100% | JN697584.1 |
| Mutant Legionella pneumophila subsp. pneumophila str. Hextuple_3a, complete genome | 86% | 0.0 | 100% | CP003024.1 |
| Mutant Legionella pneumophila subsp. pneumophila str. Hextuple_2g, complete genome | 86% | 0.0 | 100% | CP003023.1 |
| Legionella pneumophila subsp. pneumophila str. Philadelphia 1, complete genome | 86% | 0.0 | 100% | AE017354.1 |
| Legionella pneumophila str. Paris complete genome | 86% | 0.0 | 100% | CR628336.1 |
| Legionella pneumophila serogroup 1 mip gene for macrophage infectivity potentiator, Philadelphia isolate | 86% | 0.0 | 100% | AJ496265.1 |
| Legionella pneumophila culture-collection DMST:17221 macrophage infectivity potentiator (mip) gene, complete cds | 86% | 0.0 | 99% | JN697588.1 |
| Legionella pneumophila 2300/99 Alcoy, complete genome | 86% | 0.0 | 99% | CP001828.1 |
| Legionella pneumophila subsp. pneumophila str. Lorraine chromosome, complete genome | 86% | 0.0 | 99% | FQ958210.1 |
| Legionella pneumophila serogroup 1 mip gene for macrophage infectivity potentiator, isolate Trento 50 | 86% | 0.0 | 99% | AJ496269.1 |
| mip=macrophage infectivity potentiator [Legionella pneumophila, Philadelphia 1, Genomic, 702 nt] | 84% | 0.0 | 100% | S42595.1 |
| Tatlockia micdadei mip gene for macrophage infectivity potentiator, isolate Pavia 16 | 86% | 0.0 | 99% | AJ496274.1 |

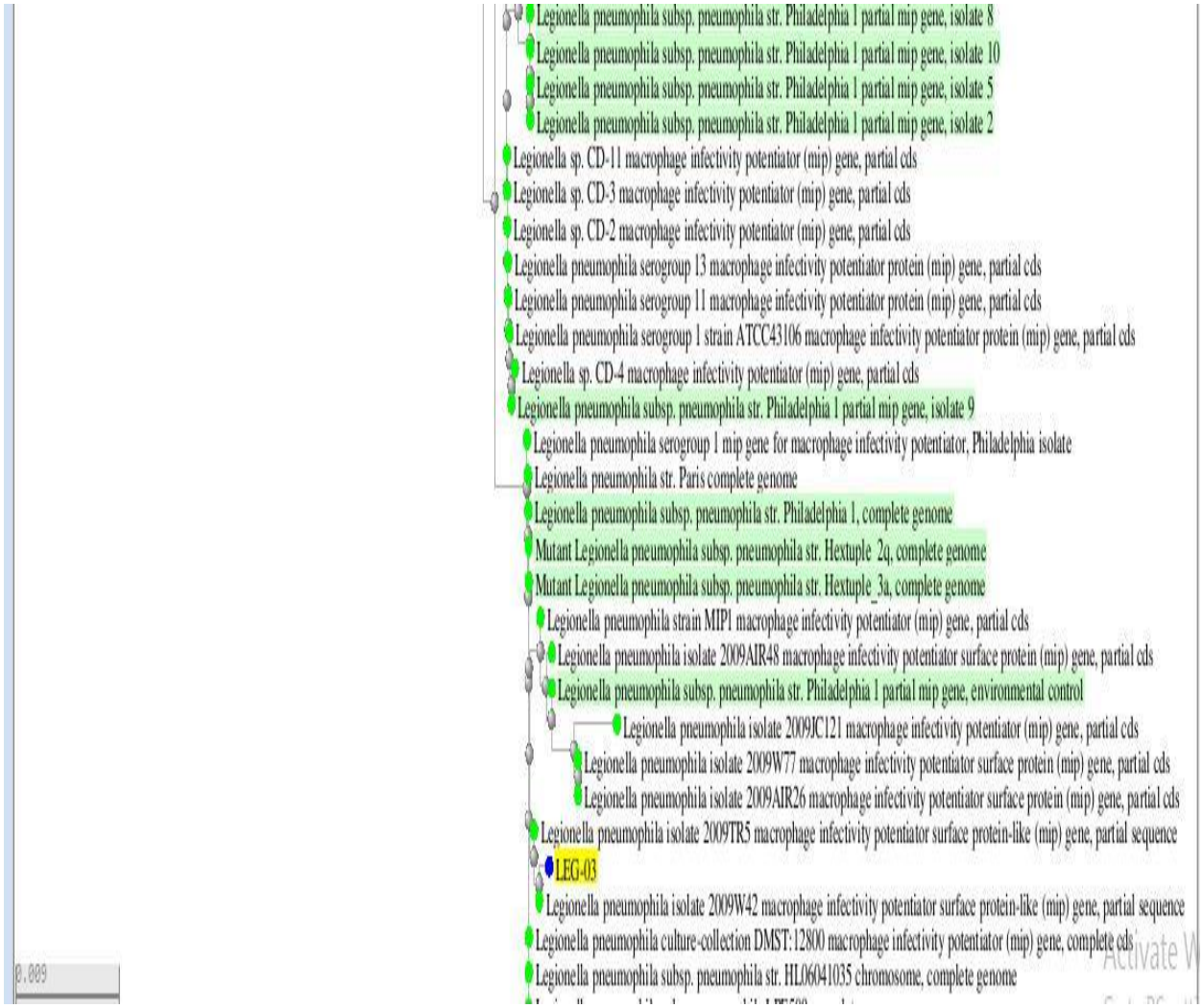
## Descriptions of *L. pneumophila* (LEG-02)


**Neighbor joining tree of *L. pneumophila* (LEG-02)**



| Description | Query cover | E value | Ident | Accession |
|---|---|---|---|---|
| Legionella pneumophila subsp. pneumophila LPE509, complete genome | 97% | 0.0 | 99% | CP003885.1 |
| Legionella pneumophila subsp. pneumophila str. HL06041035 chromosome, complete genome | 97% | 0.0 | 99% | FQ958211.1 |
| Legionella pneumophila culture-collection DMST:12800 macrophage infectivity potentiator (mip) gene, complete cds | 97% | 0.0 | 99% | JN697584.1 |
| Mutant Legionella pneumophila subsp. pneumophila str. Hextuple_3a, complete genome | 97% | 0.0 | 99% | CP003024.1 |
| Mutant Legionella pneumophila subsp. pneumophila str. Hextuple_2g, complete genome | 97% | 0.0 | 99% | CP003023.1 |
| Legionella pneumophila subsp. pneumophila str. Philadelphia 1, complete genome | 97% | 0.0 | 99% | AE017354.1 |
| Legionella pneumophila str. Paris complete genome | 97% | 0.0 | 99% | CR628336.1 |
| Legionella pneumophila serogroup 1 mip gene for macrophage infectivity potentiator, Philadelphia isolate | 97% | 0.0 | 99% | AJ496265.1 |
| Legionella pneumophila culture-collection DMST:17221 macrophage infectivity potentiator (mip) gene, complete cds | 97% | 0.0 | 99% | JN697588.1 |
| Legionella pneumophila 2300/99 Alcoy, complete genome | 97% | 0.0 | 98% | CP001828.1 |
| Legionella pneumophila subsp. pneumophila str. Lorraine chromosome, complete genome | 97% | 0.0 | 98% | FQ958210.1 |

**Descriptions of *L. pneumophila* (LEG-03)**

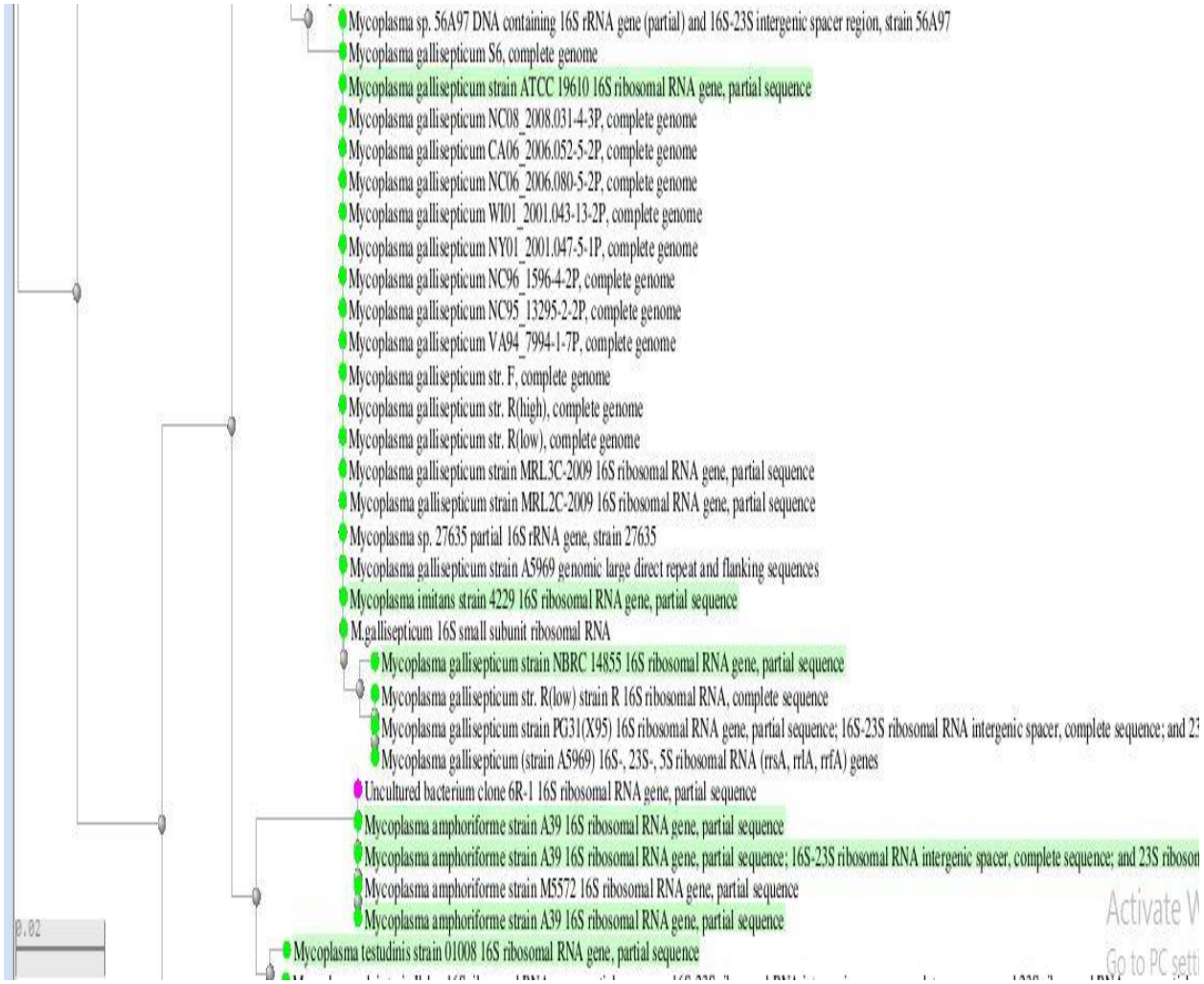



**Neighbor joining tree of *L. pneumophila* (LEG-03)**

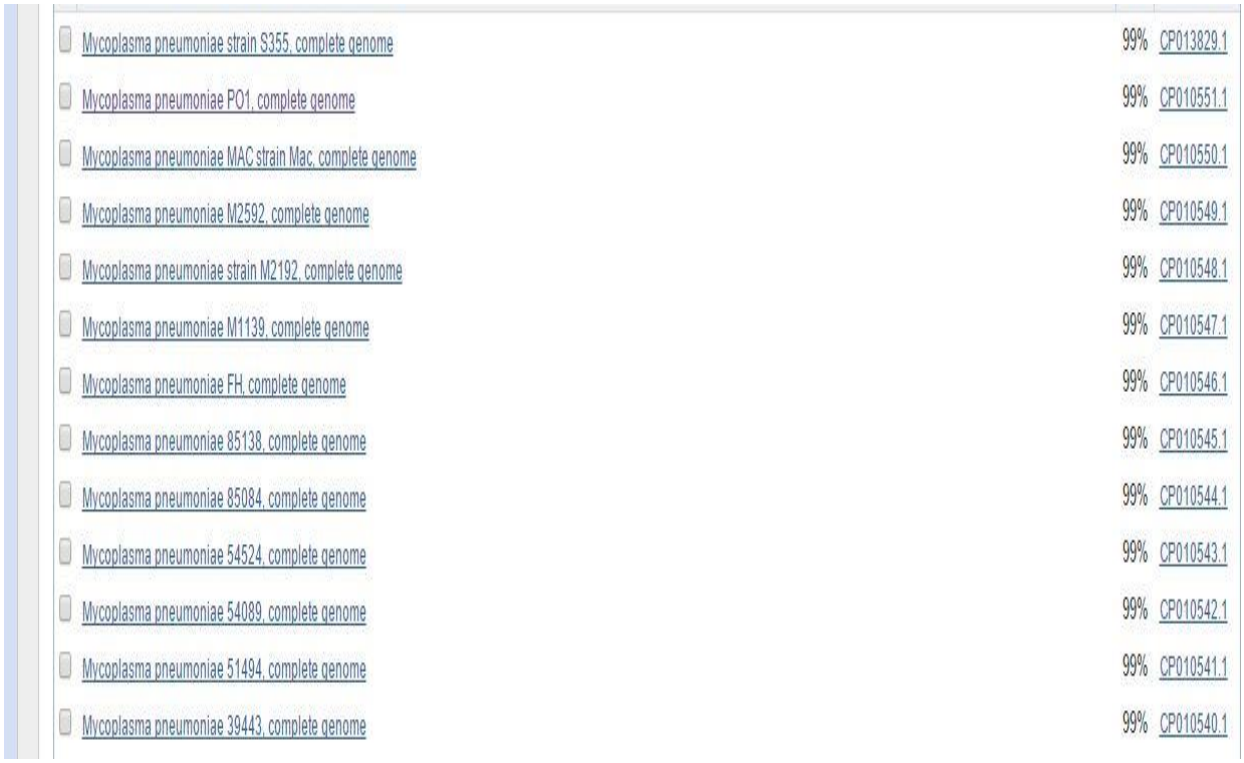
| Description | Query cover | Ident | Accession |
|---|---|---|---|
| Mycoplasma pneumoniae PO1, complete genome | 99% | 100% | CP010551.1 |
| Mycoplasma pneumoniae MAC strain Mac, complete genome | 99% | 100% | CP010550.1 |
| Mycoplasma pneumoniae M2592, complete genome | 99% | 100% | CP010549.1 |
| Mycoplasma pneumoniae strain M2192, complete genome | 99% | 100% | CP010548.1 |
| Mycoplasma pneumoniae M1139, complete genome | 99% | 100% | CP010547.1 |
| Mycoplasma pneumoniae FH, complete genome | 99% | 100% | CP010546.1 |
| Mycoplasma pneumoniae 85138, complete genome | 99% | 100% | CP010545.1 |
| Mycoplasma pneumoniae 85084, complete genome | 99% | 100% | CP010544.1 |
| Mycoplasma pneumoniae 54524, complete genome | 99% | 100% | CP010543.1 |
| Mycoplasma pneumoniae 54089, complete genome | 99% | 100% | CP010542.1 |
| Mycoplasma pneumoniae 51494, complete genome | 99% | 100% | CP010541.1 |
| Mycoplasma pneumoniae 39443, complete genome | 99% | 100% | CP010540.1 |
| Mycoplasma pneumoniae 19294, complete genome | 99% | 100% | CP010539.1 |
| Mycoplasma pneumoniae PI 1428, complete genome | 99% | 100% | CP010538.1 |



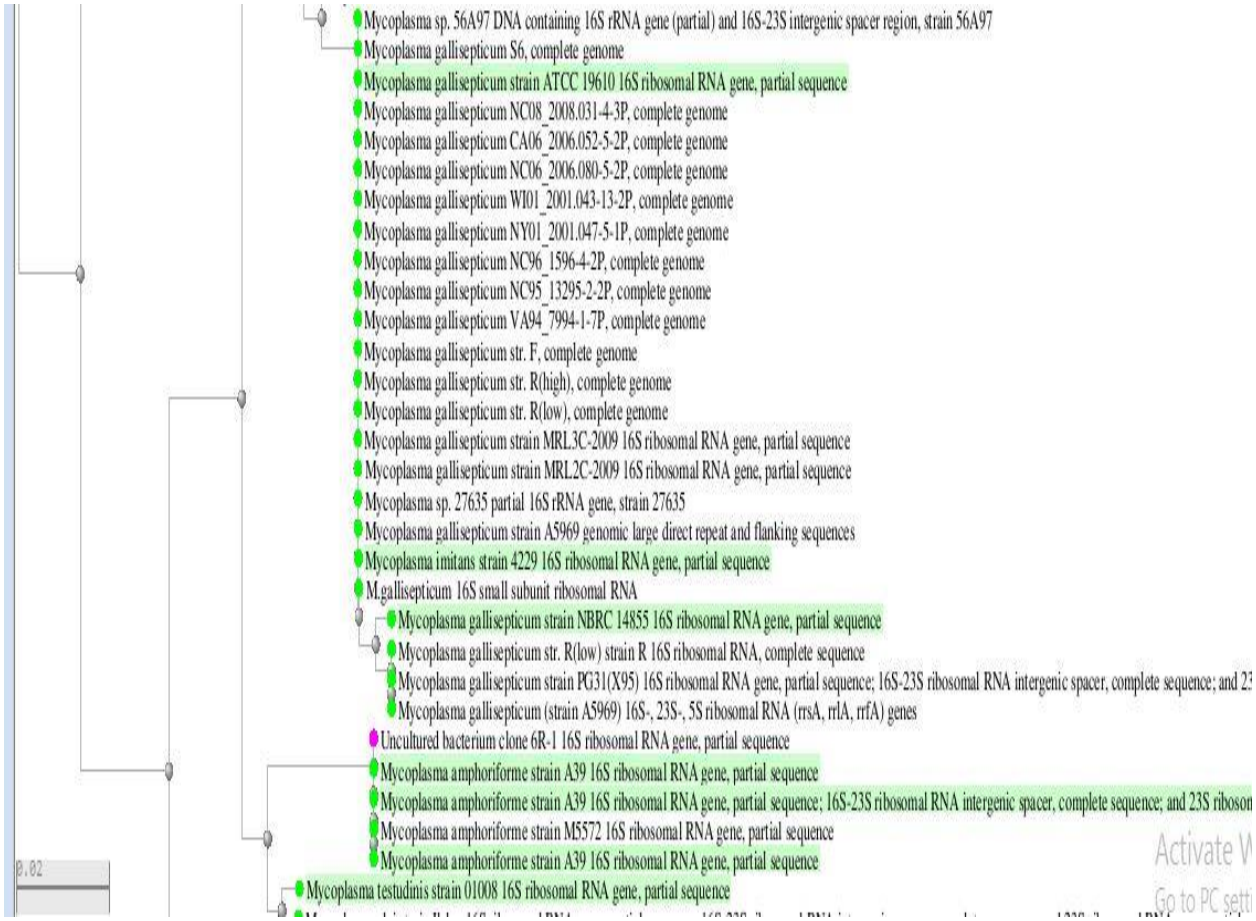
**Descriptions of *M. pneumoniae* (MYC-01)**




**Neighbor joining tree of *M. pneumoniae* (MYC-01)**




**Descriptions of *M. pneumoniae* (MYC-02)**

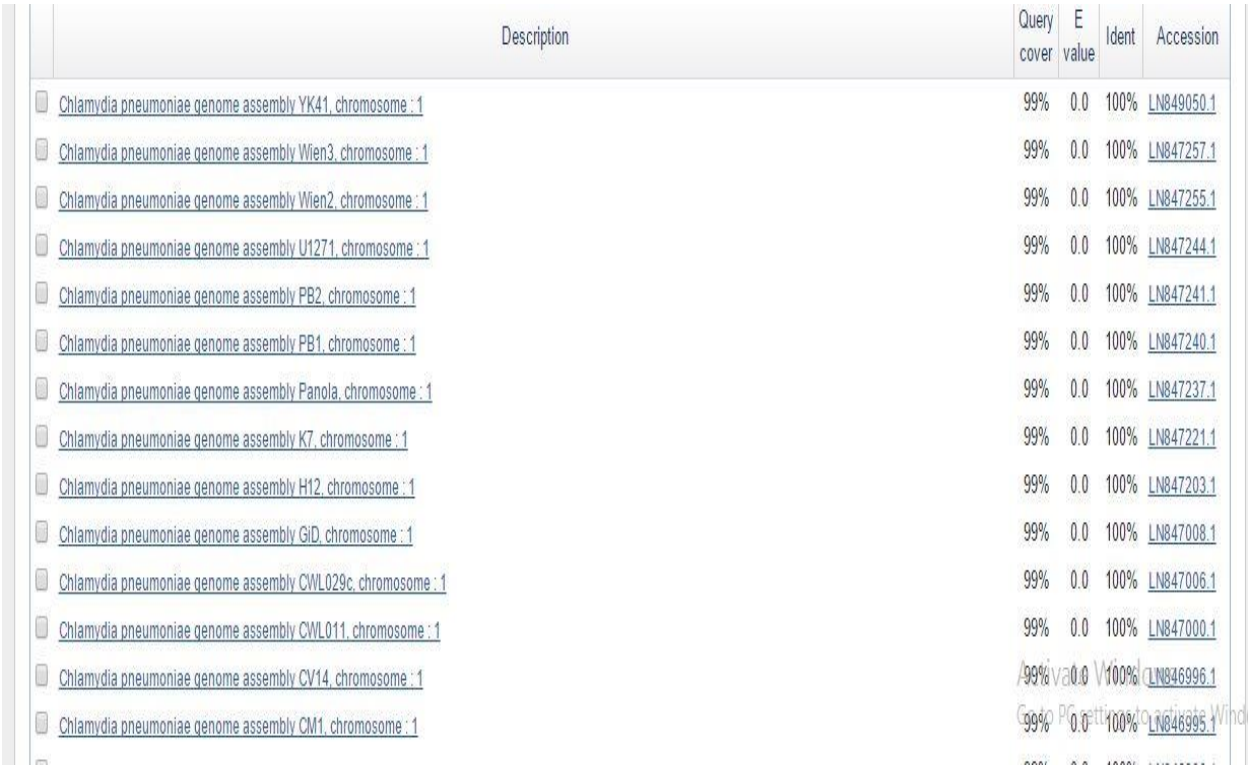



**Neighbor joining tree of *M. pneumoniae* (MYC-02)**

| Description | Ident | Accession |
|---|---|---|
| Mycoplasma pneumoniae strain S355, complete genome | 97% | CP013829.1 |
| Mycoplasma pneumoniae PO1, complete genome | 97% | CP010551.1 |
| Mycoplasma pneumoniae MAC strain Mac, complete genome | 97% | CP010550.1 |
| Mycoplasma pneumoniae M2592, complete genome | 97% | CP010549.1 |
| Mycoplasma pneumoniae strain M2192, complete genome | 97% | CP010548.1 |
| Mycoplasma pneumoniae M1139, complete genome | 97% | CP010547.1 |
| Mycoplasma pneumoniae FH, complete genome | 97% | CP010546.1 |
| Mycoplasma pneumoniae 85138, complete genome | 97% | CP010545.1 |
| Mycoplasma pneumoniae 85084, complete genome | 97% | CP010544.1 |
| Mycoplasma pneumoniae 54524, complete genome | 97% | CP010543.1 |
| Mycoplasma pneumoniae 54089, complete genome | 97% | CP010542.1 |
| Mycoplasma pneumoniae 51494, complete genome | 97% | CP010541.1 |

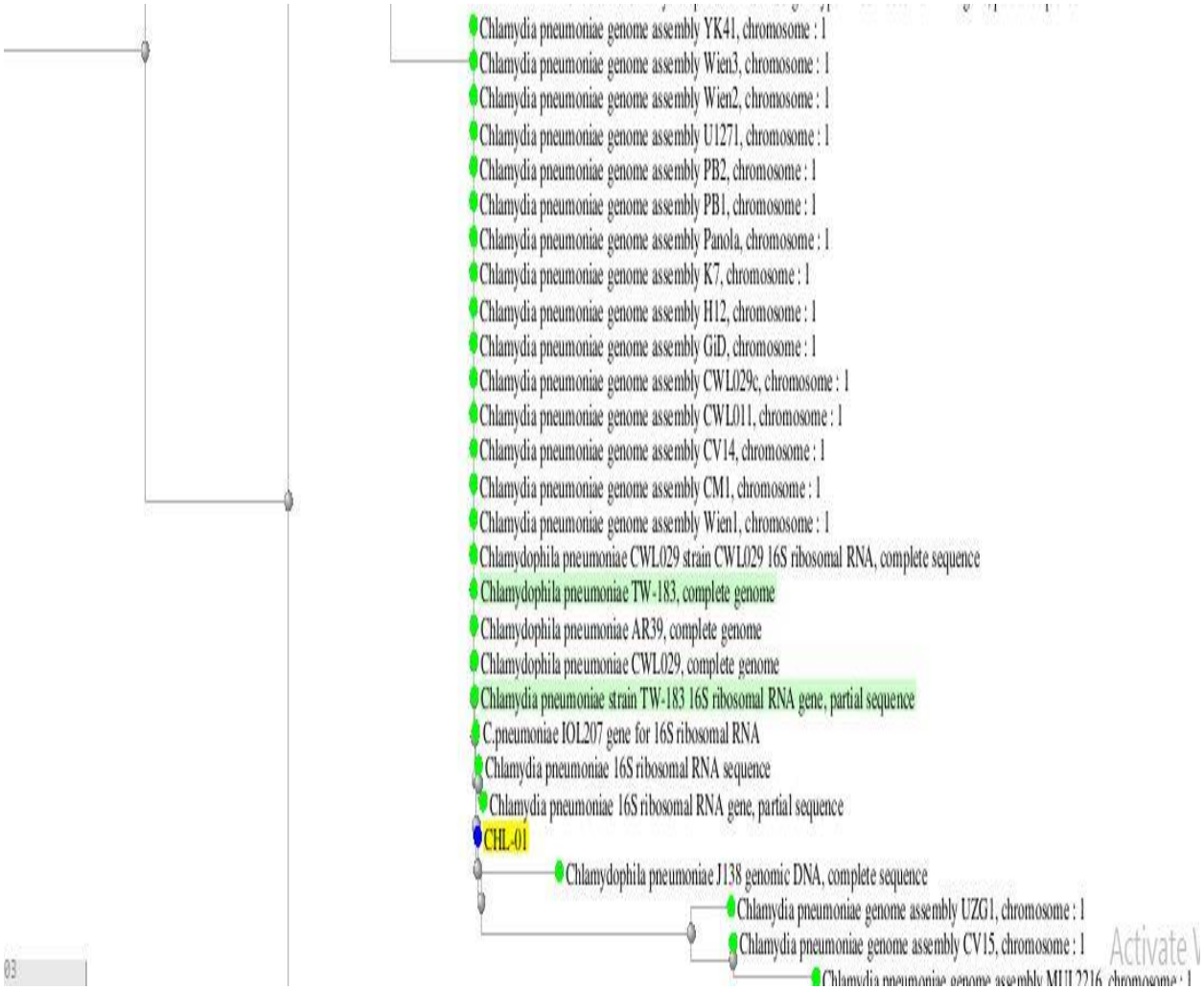
**Descriptions of *M. pneumoniae* (MYC-03)**


## Neighbor joining tree of *M. pneumoniae* (MYC-03)

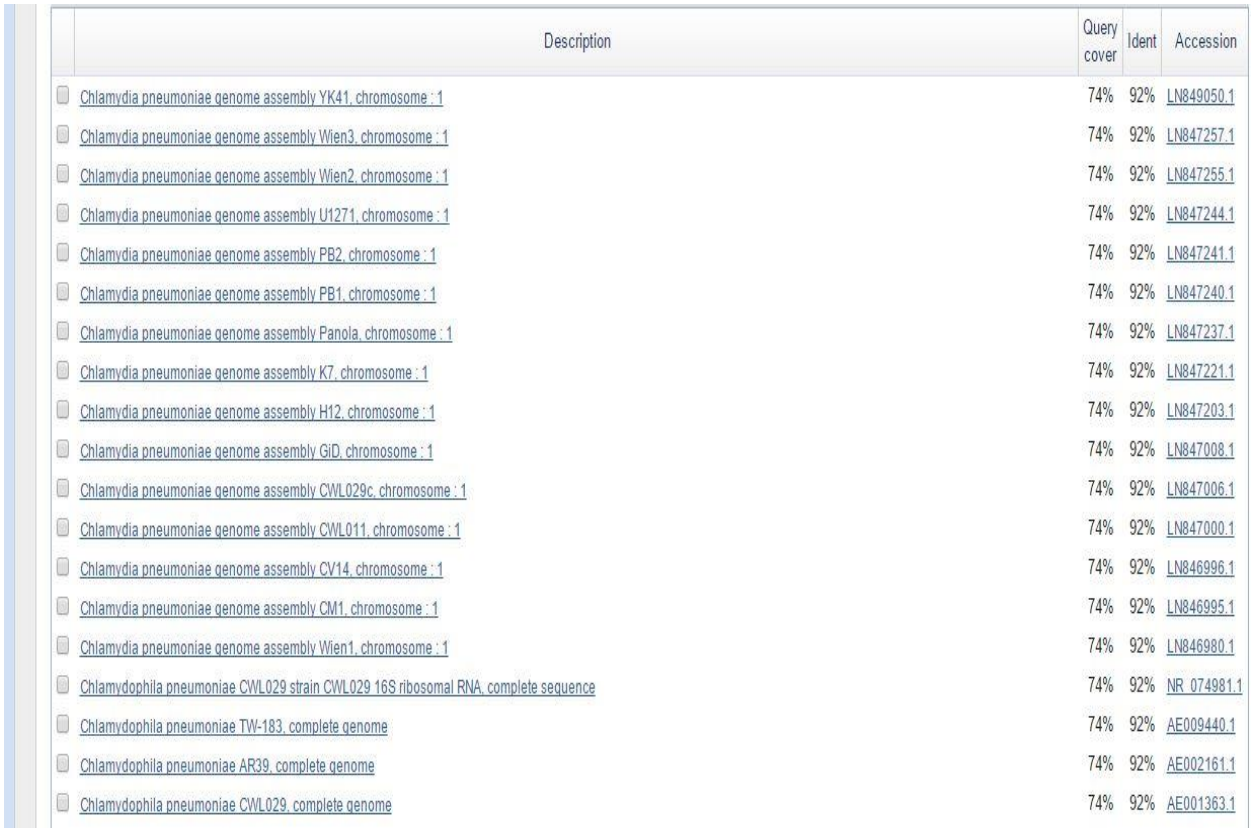


| Description | Query cover | E value | Ident | Accession |
|---|---|---|---|---|
| Chlamydia pneumoniae genome assembly YK41, chromosome : 1 | 99% | 0.0 | 100% | LN849050.1 |
| Chlamydia pneumoniae genome assembly Wien3, chromosome : 1 | 99% | 0.0 | 100% | LN847257.1 |
| Chlamydia pneumoniae genome assembly Wien2, chromosome : 1 | 99% | 0.0 | 100% | LN847255.1 |
| Chlamydia pneumoniae genome assembly U1271, chromosome : 1 | 99% | 0.0 | 100% | LN847244.1 |
| Chlamydia pneumoniae genome assembly PB2, chromosome : 1 | 99% | 0.0 | 100% | LN847241.1 |
| Chlamydia pneumoniae genome assembly PB1, chromosome : 1 | 99% | 0.0 | 100% | LN847240.1 |
| Chlamydia pneumoniae genome assembly Panola, chromosome : 1 | 99% | 0.0 | 100% | LN847237.1 |
| Chlamydia pneumoniae genome assembly K7, chromosome : 1 | 99% | 0.0 | 100% | LN847221.1 |
| Chlamydia pneumoniae genome assembly H12, chromosome : 1 | 99% | 0.0 | 100% | LN847203.1 |
| Chlamydia pneumoniae genome assembly GiD, chromosome : 1 | 99% | 0.0 | 100% | LN847008.1 |
| Chlamydia pneumoniae genome assembly CWL029c, chromosome : 1 | 99% | 0.0 | 100% | LN847006.1 |
| Chlamydia pneumoniae genome assembly CWL011, chromosome : 1 | 99% | 0.0 | 100% | LN847000.1 |
| Chlamydia pneumoniae genome assembly CV14, chromosome : 1 | 99% | 0.0 | 100% | LN846996.1 |
| Chlamydia pneumoniae genome assembly CM1, chromosome : 1 | 99% | 0.0 | 100% | LN846995.1 |

## Descriptions of *C. pneumoniae* (CHL-01)

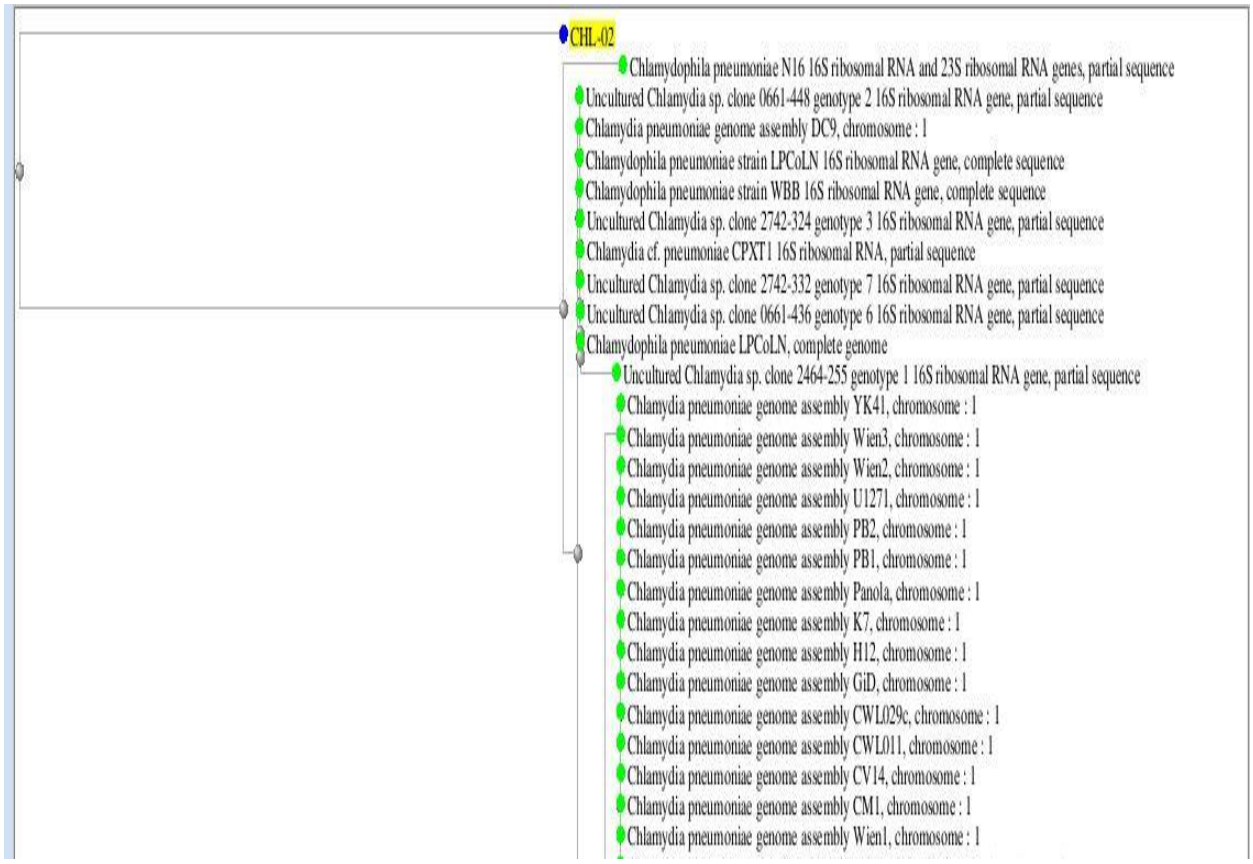



**Neighbor joining tree of *C. pneumoniae* (CHL-01)**



| Description | Query cover | Ident | Accession |
|---|---|---|---|
| Chlamydia pneumoniae genome assembly YK41, chromosome : 1 | 74% | 92% | LN849050.1 |
| Chlamydia pneumoniae genome assembly Wien3, chromosome : 1 | 74% | 92% | LN847257.1 |
| Chlamydia pneumoniae genome assembly Wien2, chromosome : 1 | 74% | 92% | LN847255.1 |
| Chlamydia pneumoniae genome assembly U1271, chromosome : 1 | 74% | 92% | LN847244.1 |
| Chlamydia pneumoniae genome assembly PB2, chromosome : 1 | 74% | 92% | LN847241.1 |
| Chlamydia pneumoniae genome assembly PB1, chromosome : 1 | 74% | 92% | LN847240.1 |
| Chlamydia pneumoniae genome assembly Panola, chromosome : 1 | 74% | 92% | LN847237.1 |
| Chlamydia pneumoniae genome assembly K7, chromosome : 1 | 74% | 92% | LN847221.1 |
| Chlamydia pneumoniae genome assembly H12, chromosome : 1 | 74% | 92% | LN847203.1 |
| Chlamydia pneumoniae genome assembly GiD, chromosome : 1 | 74% | 92% | LN847008.1 |
| Chlamydia pneumoniae genome assembly CWL029c, chromosome : 1 | 74% | 92% | LN847006.1 |
| Chlamydia pneumoniae genome assembly CWL011, chromosome : 1 | 74% | 92% | LN847000.1 |
| Chlamydia pneumoniae genome assembly CV14, chromosome : 1 | 74% | 92% | LN846996.1 |
| Chlamydia pneumoniae genome assembly CM1, chromosome : 1 | 74% | 92% | LN846995.1 |
| Chlamydia pneumoniae genome assembly Wien1, chromosome : 1 | 74% | 92% | LN846980.1 |
| Chlamydophila pneumoniae CWL029 strain CWL029 16S ribosomal RNA, complete sequence | 74% | 92% | NR_074981.1 |
| Chlamydophila pneumoniae TW-183, complete genome | 74% | 92% | AE009440.1 |
| Chlamydophila pneumoniae AR39, complete genome | 74% | 92% | AE002161.1 |
| Chlamydophila pneumoniae CWL029, complete genome | 74% | 92% | AE001363.1 |

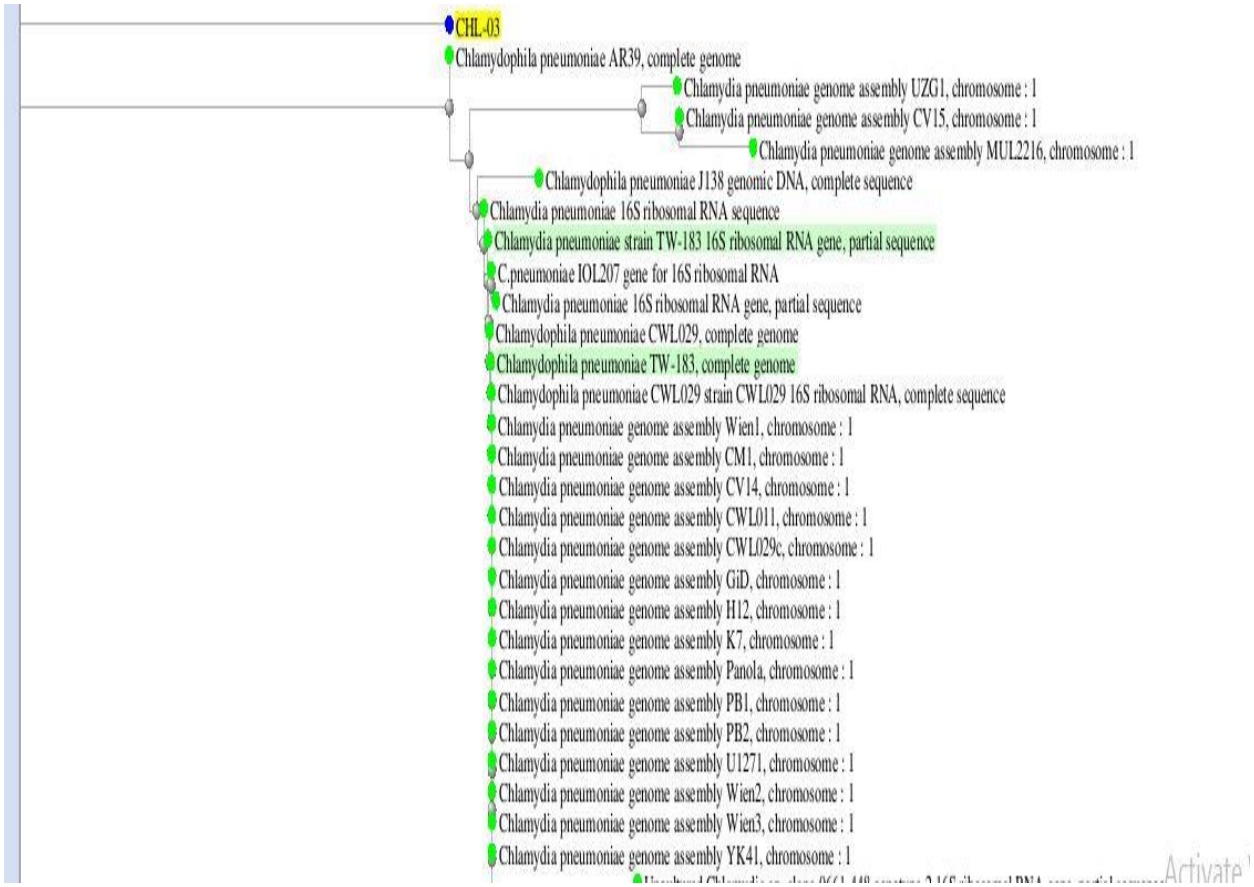
## Descriptions of *C. pneumoniae* (CHL-02)




**Neighbor joining tree of *C. pneumoniae* (CHL-02)**

| Description | Query cover | Ident | Accession |
|---|---|---|---|
| Chlamydia pneumoniae genome assembly YK41, chromosome : 1 | 87% | 94% | LN849050.1 |
| Chlamydia pneumoniae genome assembly Wien3, chromosome : 1 | 87% | 94% | LN847257.1 |
| Chlamydia pneumoniae genome assembly Wien2, chromosome : 1 | 87% | 94% | LN847255.1 |
| Chlamydia pneumoniae genome assembly U1271, chromosome : 1 | 87% | 94% | LN847244.1 |
| Chlamydia pneumoniae genome assembly PB2, chromosome : 1 | 87% | 94% | LN847241.1 |
| Chlamydia pneumoniae genome assembly PB1, chromosome : 1 | 87% | 94% | LN847240.1 |
| Chlamydia pneumoniae genome assembly Panola, chromosome : 1 | 87% | 94% | LN847237.1 |
| Chlamydia pneumoniae genome assembly K7, chromosome : 1 | 87% | 94% | LN847221.1 |
| Chlamydia pneumoniae genome assembly H12, chromosome : 1 | 87% | 94% | LN847203.1 |
| Chlamydia pneumoniae genome assembly GiD, chromosome : 1 | 87% | 94% | LN847008.1 |

**Descriptions of *C. pneumoniae* (CHL-03)**




**Neighbor joining tree of *C. pneumoniae* (CHL-03)**