

CHAPTER ONE

INTRODUCTION

1.1. Introduction

More than 30 years have passed since *Legionella pneumophila* the causative agent of Legionnaires' disease was identified as a new human pathogen. First recognized due to epidemic of pneumonia that followed the 1976 legionnaires' convention in Philadelphia, USA, *Legionella* is still a disease of medical and public interest, *Legionella* commonly found in aquatic habitats, where its ability to survive and multiply within different protozoa equips the bacterium to be transmissible and pathogenic to human (Michele and Klaus, 2008).

The history of legionnaires' disease began at least 33 years before the 1976 Philadelphia epidemic, when *Legionella micdadei* was isolated from human blood multiple isolation of several different *Legionella* Spp. Were made prior to 1976. It was known by 1968 that tetracycline therapy prevented deaths in *L. pneumophila* infected chicken embryos. The 1976 epidemic provided the scientific focus and resources necessary to determine that *Legionella pneumophila* caused epidemic pneumonia and to show that epidemics of legionnaires' disease had occurred worldwide many years before 1976 (Michele and Klaus, 2008).

Pontiac fever disease of unknown etiology is a self limiting and short duration febrile illness that has been associated with exposure to *L. pneumophila*. Because of non specific clinical findings that overlap with other diseases accurate diagnosis of Pontiac fever in non outbreak is impossible. Legionnaires' disease can be diagnosed specifically by specialized laboratory tests but not by clinical finding alone. Antimicrobial

therapy of legionnaires' disease requires the use of drugs that are active against intracellular *Legionella* spp such as tetracycline, macrolides, azalides and antibacterial quinolones. Legionnaires' disease is a type of pneumonia caused by *Legionella* spp. which is environmental Gram negative bacteria (Michele and Klaus, 2008).

The majority of legionnaires' diseases are caused by *L. pneumophila* and in particular *L. pneumophila* serogroup 1. In the 1976 epidemic of Legionnaires disease occurs in person attending an American legion convention in Philadelphia, Pennsylvania, Congress was first recognized occurrence of legionnaires' disease it led to naming of disease and isolation and characterization of *L. pneumophila*. Prompt and specific antibiotic treatment of legionnaires' disease can reduce the fatality rate of the disease which 15-80% of patients die. Depending on underlying disease, host immunity and duration and severity of illness before treatment. Unfortunately there are no clinical features that allow clinicians to specifically diagnose the disease with sufficient accuracy which places great emphasis on the need for empiric antibiotic therapy for the disease (Michele and Klaus, 2008).

1.2. Rationale

Legionella pneumophila (*L. Pneumophila*) has been increasingly recognized as an emerging pathogen responsible for atypical pneumonia and community acquired pneumonia incidence worldwide. There is an estimated incidence of 25,000 to 35,000 cases per year. With mortality rate 5-30%. If untreated mortality can reach 80%. In the US there is an incidence of 8,000 to 18,000 cases reported per year. Approximately only 5-10% of the total *L. pneumophila* cases are actually reported (Schaechter *et al*; 1999). Despite the outbreak in many countries, previous studies carried out in Sudan to determine the prevalence of *L. pneumophila* as a cause of community acquired pneumonia could not be found.

1.3. OBJECTIVES

1.3.1. General objective

To study the association of *L.pneumophila* with chest infection/pneumonia among patient's attending selected hospitals in Khartoum State.

1.3.2. Specific objectives

- 1- To detect *L.pneumophila* nucleic acid by Polymerase Chain Reaction (PCR) in the serum of patients with chest infection/pneumonia.
- 2- To detect *L. pneumophila* antibodies by Enzyme linked immunosorbent assay (ELISA) in the serum of patient with pneumonia.
- 3 - To identify the risk factors predisposing to infection by *L.pneumophila*.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Historical Background

The *Legionellaceae* were not documented until 1976, when a detrimental outbreak of pneumonia occurred in Philadelphia at an American Legion Convention. Thirty four of the 221 people who became ill after exposure died within the first few weeks after the convention. The culprit, *L. pneumophila*, was isolated first by inoculation of postmortem lung tissue into guinea pigs and then by subculture into a rich artificial medium. Then by indirect immunofluorescent antibody assay, it was found that over 90% of those that felt ill had at least four times the concentration of antibody in their blood (fourfold rise in titer) against this organism. The same method was used to screen previously saved sera from earlier outbreaks of unexplained respiratory disease and they discovered that a number of them were associated with seroconversion to *L. pneumophila*, including a “rickettsia-like” organism (fig 2.1 and 2.2). The organism was isolated by guinea pig inoculation from the blood of a feverish patient(Lederberg, 2000).



Fig (2.1): Image of *Legionella pneumophila*

By:Brenner DJ, Steigerwalt AG, McDade JE (1979).

2.2. General characteristic

The *Legionellaceae* are fastidious Gram-negative bacteria that reside in aquatic environments all over the globe. In their natural environment, the *Legionellaceae* are intracellular parasites of free-living protozoa. These organisms may also inhabit man-made water distribution systems. The family *Legionellaceae* consists of a single genus, *Legionella*. More specifically, this genus includes the species *Legionella pneumophila*, is non-encapsulated, aerobic bacilli (Lederberg, 2000).

L.pneumophila belongs to the family *Legionellaceae*.Currently 50 species and subspecies and 71 serological types of *Legionella* have been isolated from either human specimens, environmental sources or both(Kingetal; 1988).

Within the species *L. pneumophila*, human infection is caused primarily (but not exclusively) by a limited number of serogroups (serogroups 1, 4, and 6).

L. pneumophila is the most frequent cause of human legionellosis, better known as Legionnaire's disease in the *Legionellaceae* family. It is also a relatively common cause of community-acquired and nosocomial pneumonia in adults (Lederberg, 2000).*L. pneumophila* serogroup 1 alone is responsible for 70-90% of cases (Rathore, 2006).

2.2.1. Genomic structure

The *Legionella* genome structure continues to be searched. Three different genomes of *L. pneumophila* were completed over a span of three years. First, *Legionella pneumophila* spp. *pneumophila* strain. *philadelphia 1* (3,397,754 bp, 3002 genes) was completed in October 2001. Plasmid pLPP of *Legionella pneumophila* str. *Paris* was completed in October 2004; in addition to the full genome (3,503,610 bp, 3136 genes). Plasmid pLPL of *Legionella pneumophila* str. *Lens* was also completed in October 2004, in

addition to the full genome (3,345,687 bp, 3001 genes) (National center Biotechnology information site).

2.2.2. Cell structure and metabolism

L. pneumophila is a Gram-negative, non-encapsulated, aerobic bacillus with a single, polar flagellum. The organism is approximately 2µm in length and 0.3-0.9µm in width (fig 2.1, fig 2.2). But in nutrient-deficient media, it may become long and filamentous. It is surrounded by a Gram-negative cell wall and pili are sometimes identified. The cell envelope is composed of branched-chain fatty acids and distinctive ubiquinones, whose structural differences have been used to classify different *Legionella* species. The outer-membrane is comprised of a lipopolysaccharide (LPS), which is “fully sequenced and found to have several novel features which have pathophysiologic consequences” and is noticeably less endotoxic than enterobacterial LPS since it has weak interactions with the CD14 receptor on monocytes. The interactions are probably inhibited by the long-chain fatty acids of *L. pneumophila* lipid A, which are two times the length of enterobacterial lipid A. A single, major protein also makes up the outer membrane and functions as a porin and as a target for human complement fixation. *L. pneumophila* serogroup 1LPS also has a repeating O antigen. It is a homopolymer of an uncommon sugar, called legionaminic acid. LPS is the immunodominant antigen of the *Legionellaceae*, and the O antigen is the determinant of serogroup specificity within the genus (Lederberg, 2000).

L. pneumophila is non-sporulating and unable to hydrolyse gelatin or produce urease, they are also non-fermentative. *L. pneumophila* is neither pigmented nor does it autofluoresce. It is oxidase- and catalase-positive, and produces beta-lactamase (Rayn and Ray, 2004).

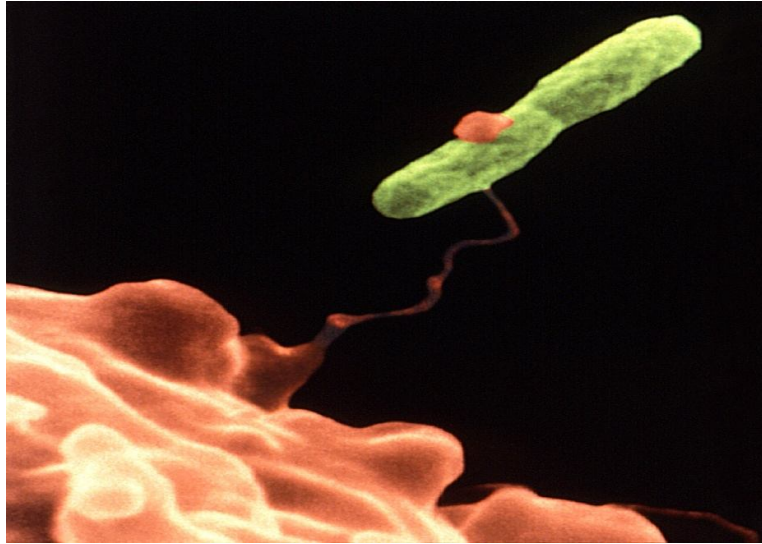


Fig (2.2): This electron micrograph depicts an amoeba, *Hartmannella vermiformis* (orange), as it entraps a *Legionella pneumophila* bacterium (green) with an extended pseudopodium. (Image courtesy of the Centers for Disease Control and Prevention and Dr. Barry S Fields).

2.3. Pathology

L. pneumophila can only be acquired from an environmental source; therefore, infection never occurs between humans or humans and animals. Another interesting thing is, unlike other pathogens that cause bacterial pneumonia, *L. pneumophila* do not inhabit the upper respiratory tract. Once inhaled, they are small enough to avoid the defenses of the upper airway. In the lung, pulmonary alveolar macrophages (macrophages of the air sacs in the lung) and sometimes type II alveolar epithelial cells take up *L. pneumophila* where it begins to grow intracellularly. It seems intracellular infection is necessary for producing infection because mutants of *L. pneumophila* are unable to cause disease. This is true also because *L. pneumophila* is not sensitive to antimicrobials (e.g. penicillin, cephalosporin, aminoglycosides) that are excluded by the plasma membrane, and therefore, it is treated only with antibiotics that can enter the host cells (e.g., macrolides, quinolones, tetracyclines) (Lederberg, 2000).

Then the bacteria undergo a series of events that begins with phagocytosis. Most, but not all strains of *L. pneumophila* are taken up by “coiling phagocytosis”—when the macrophage coils around the bacteria to take it up, which is mediated by the CR1 and CR3 receptors (complement receptors of the macrophage) with or without fixation of complement serum factors like C3. Also during uptake the plasma membrane engulfing the bacteria is altered; some membrane proteins, like MHC I and II (both molecules that aid in immune response), are specifically excluded whereas others, like CR3 and 5'-nucleotidase are preserved. Two hours later, the phagosome (the vacuole formed around the pathogen) is found near mitochondria, smooth vesicles, or the nuclear membrane. Normally, phagosomes will fuse with lysosomes and pathogenic microorganisms are killed, but in this case acidification of the phagosome does not take place, and its membrane does not get late endosomal markers, like rab7 and LAMP-1. This results in the failure of phagosolysosomal fusion which allows bacteria to avoid intracellular killing. Mutants of *L. pneumophila* on the other hand cannot avoid it and are transported to the lysosome within 30 minutes of uptake. Fully virulent organisms on the other hand will take up an endosome that has become enclosed by the rough Endoplasmic Reticulum(ER) (a response to cellular amino acid starvation i.e. autophagy) 4 hours after uptake. It is by this endosome enclosure that bacterial multiplication proceeds. While the bacteria are multiplying inside their host cells, they also “begin to express flagella, motility, cellular toxicity, and sensitivity to physiologic concentrations of sodium chloride. The genes that mediate intracellular trafficking and necrosis (accidental cell death) are the same genes that introduce the cytotoxic phenotype of *L. pneumophila*. Introduction of cytotoxicity in response to cellular amino acid starvation in the host may be a key mechanism that signals the bacteria to exit host cells and infect new cells (Lederberg, 2000).

A particular feature of *Legionella* is its dual host system allowing the intracellular growth in protozoa (amoebae), and during infection in human pulmonary alveolar macrophages. Like macrophages, amoebae ingest *L. pneumophila* by phagocytosis, which can be mediated by amoebic-specific receptors. After ingestion, the bacteria evade phagosolysosomal fusion and localize to a membrane-vesicle surrounded by endoplasmic reticulum where it grows and acquires motility before release from the cell. This growth process of *L. pneumophila* to infect mammalian phagocytes strongly supports the theory that it evolved from protozoa. Since *L. pneumophila* are not transmitted between mammalian hosts or return to the natural environment by infected individuals, this hypothesis explains why this pathogen is adapted to intracellular life in the human lung without any apparent selection for pathogenic traits in the microenvironment” (Lederberg, 2000).

The Mip gene, a 24 kDa bacterial envelope protein, was the first virulence determinant of *L. pneumophila*. The consequence of a Mip mutation is a 1.5 to 3 log reduction in infectivity of explanted alveolar macrophages, alveolar epithelial cells, and amoebae (Lederberg, 2000).

Iron is essential for all pathogenic bacteria. Without it, the growth of intracellular *L. pneumophila* becomes restricted. It was found that gamma-interferon restricts the growth of *L. pneumophila* by downregulating the expression transferring receptors on macrophages and the cellular concentration of ferritin. Naturally, *L. pneumophila* taken from iron-deficient cultures grown are defective in cellular infection. *L. pneumophila* has a homolog, which regulate the expression of an aerobactin synthetase homolog. Even though siderophores have yet to be identified in *L. pneumophila*, a mutation of this iron acquisition gene will cut intracellular infection by 80-fold. Studies on intracellular survival of *L. pneumophila* have shown two chromosomal regions to encode functions that are essential for establishing intracellular infection. The genes have been designated icm

(intracellular multiplication) by one group and dot (defect in organelle trafficking) by the other. Mutation in almost any of these genes results in either or complete loss of cellular infectivity. In all cases, the loss of infectivity is associated with a failure to evade phagolysosomal fusion, as well as a loss of the immediate contact cytotoxicity of *L. pneumophila* at high rates of infections. Mutations in several of the dot-icm genes—not just those with sequence homology to known conjugation genes have been shown to terminate plasmid transfer. These same mutations also result in loss of this toxicity coupled with the insertion of a pore into host cell membranes. The proteins encoded by these loci probably either combine to form the pore or participate in its transfer or both (Lederberg, 2000).

Legionellosis is the infection caused by *L. pneumophila* and can cause either: Legionnaires' disease, which is characterized by fever, myalgia, cough, pneumonia, or Pontiac fever, a milder illness without pneumonia. The symptoms of Legionnaire's disease range from a mild cough and low fever to rapidly progressive pneumonia, coma, and death. Early symptoms include slight fever, headache, aching joints and muscles, lack of energy or tiredness, and loss of appetite. Later symptoms include high fever, cough, and difficulty breathing/shortness of breath, chills, chest pain, common gastrointestinal symptoms including vomiting, diarrhea, nausea, and abdominal pain. The symptoms of Pontiac fever include flu-like symptoms such as fever, headache, tiredness, loss of appetite, muscle and joint pain, chills, nausea, and a dry cough. Patients usually reach full recovery within two to five days without medical attention and no deaths have been reported (Center Disease Control and Prevention Site).

2.3.1. Clinical feature of Pontiac fever and Legionnaires' disease:

Pontiac fever is a self-limited short duration febrile illness of unknown aetiology. It's unclear whether the illness results from non-*Legionella* spp.

Bacteria endotoxin inhalation from inhalation of live or dead *Legionella* and non *Legionella* microbe and their toxins (Edelstein, 2007).

It's unknown how to specifically diagnose single cases of Pontiac fever, and case reports of sporadic cases should be viewed with skepticism. The reason for this skepticism is that *Legionella* spp are commonly found in environmental water without causing disease and because up to 25-40% of healthy people may have detectable *Legionella* spp antibodies especially against non *Legionella pneumophila* (Edelstein 2006 b).

The incubation period after exposure to a bacteria contaminated aerosol has a wide range (4-120 hours). The sites of Pontiac fever outbreaks have included workplaces, hotels, recreational spaces and restaurants, but disease can occur almost anywhere there is possibility of encountering a bacterial aerosol. Attack rates are very high, with more than 80-90% of such exposed people becoming ill. Fever, myalgia, headache and fatigue are the dominant symptoms. Cough, dyspnoea, anorexia, arthralgia and abdominal pain occur less frequently. There is little information about physical examination findings in the first day of the illness, 2-5 days after onset may show fever and tachypnoea but pneumonia does not occur (Castor *et al.*, 2005).

Fatigue and non focal neurological complaints have been reported to persist for up to several months in the minority of affected patients. Most people with Pontiac fever recover within 2-4 days although some are sick for up to a week illness severe enough to result in hospitalization is exceptional, so much so that a requirement for this should bring into question the diagnosis the possibility of other disease including legionnaires' disease. (Michele and Klaus, 2008b).

2.4. Legionnaires' disease:

Legionnaires' disease is pneumonia caused by *Legionella* spp than may or may not be associated with extrapulmonary infection. There are very rare

case reports of *legionella* spp extrapulmonary infection in the absence of pneumonia (Michele and Klaus, 2008b).

2.4.1. Clinical findings:

Patients with legionnaires' disease have pneumonia and in addition may have clinical findings suggestive of a systemic disease (Edelstein and Cianciotto, 2005).

Symptoms and signs of the disease are often quite variable. The majority of patients have fever, which is usually one of the earliest signs of illness. Accompanying the fever may be anorexia, myalgia, rigors and headache. Chest pain, shortness of breath and cough may or may not be prominent findings.

The cough may or may not be productive and when it is productive the sputum can be bloody, purulent, or scant and mucoid. In some cases the absence of purulent sputum production, chest pain and cough may fool the clinician into discarding pneumonia as a possibility. When chest pain and haemoptysis are prominent the patients may be suspected of having pulmonary infarction. Abdominal pain, diarrhea, nausea and vomiting may occur as well symptoms that have led to consideration of intra abdominal infections and inflammatory conditions.

As the untreated disease progresses, the major findings include fever or hypothermia, dense consolidation of the lung, and often respiratory failure. The majority (70%) of non immunocompromised patients without significant underlying disease recover without specific therapy after being quite ill for 5-7 days. Death is usually the consequence of respiratory failure, often combined with sepsis syndrome.

Hyponatraemia, leucopenia or leucocytosis, thrombocytosis or thrombocytopenia, elevated serum creatinine kinase and elevated liver associated tests are all non specific laboratory findings that can be commonly

observe. In the presence of severe respiratory failure thrombocytopenia and intravascular coagulation is common chemical evidence of pancreatitis is an occasional finding as is evidence of myocarditis. Chest radiography always reveals alveolar filling infiltrates often with consolidation (Kirby *et al.*, 1979, Ten *et al.*, 2000).

Purely lung infiltrates are very uncommon. cavitation of prior are as consolidation occurs in up to 10% of immunocompromised patients pleural effusions are seen in about 40% of patients often in patients with other causes such as heart failure and renal failure. Patients treated with specific antibiotic therapy usually improve promptly; confusion and sepsis clearing most rapidly up to a week may be required for patients to become completely afebrile, although the fever starts to decrease within 12 hours of therapy. Immunocompromised patients or patients with very advanced pneumonia may have either no response or a very slow response to specific therapy with prolonged fever and respiratory failure. Fatality rates are the highest in patients treated late in disease with the fatality rate approaching 70%. Not all of the clinical manifestations of legionnaires' disease may be due to infection with *legionella* spp as up to 10% of patients with this disease have co infection with other microbes, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus* and many other organisms (Meyer *et al.*, 1980; Ruutu *et al.*, 1987; Marrie *et al.*, 1992; Edelstein and Cianciotto, 2005).

Legionella spp infection in the absence of legionnaire's disease is very uncommon (McClelland *et al.*, 2004). Most such cases involve direct inoculation of injured tissues with water containing *legionella* spp (Lowry *et al.*, 1991).

2.4.2. Distinguishing Legionnaires ' disease from other causes of pneumonia:

Rapid diagnosis of Legionnaires ' disease allows the use of therapy specific for the disease and prompt notification of public health authorities that may curtail epidemic. However the presenting signs and symptoms of legionnaires' disease are indistinguishable from those found in people with other common causes of community acquired pneumonia, such as that due to *Streptococcus pneumoniae*. Although *Legionella* specific laboratory testing is required to accurately diagnose the disease these test may be expensive, intensive, and unavailable or require many days to perform. The similarity of the clinical finding of legionnaires' disease to these of other pneumonia and the deficits of specific diagnostic testing, led to interest in defining specific clinical findings that could be used to diagnose the disease quickly without resort to specific laboratory testing (Michele and Klaus, 2008b).

It was originally thought that legionnaires' disease was a distinct and easily distinguishable clinical syndrome, characterized by rigors absence of productive cough high fever with low pulse rate, headache, myalgia, anorexia, nausea and diarrhea, in addition it was thought that several non specific abnormalities were characteristic of the disease (Tsai *et al.*, 1979; Kirby *et al.*, 1980; Lattimer and Omsbee, 1981). However a seminal study by Yu and colleagues in 1982 showed that it was impossible to distinguish the presenting clinical findings of legionnaires' disease from other common cause of community acquired pneumonia.

2.5. Laboratory Diagnosis:

Specialized laboratory tests are necessary to establish the diagnosis. These tests must be specifically requested from the clinical-microbiology laboratory because they are not routinely performed.

2.5.1. Culture of *Legionella* Spp

Culture is still the gold standard among the diagnostic methods for *Legionella* infections. The medium necessary for the cultivation of *Legionella* is buffered charcoal yeast extract (BCYE) agar supplemented with antibiotics. Some *Legionella* strain might be susceptible to the antibiotic in selective media. Therefore antibiotic free agar should be used as an additional culture medium for material with no or low content of normal flora. Since *Legionella* are environmental, aquatic organisms which do not colonize humans and cannot be isolated from healthy person, the specificity of culture is estimated to be 100% (Stout *et al.*, 2003). False positive results may occur if clinical sample are contaminated with water containing *legionellae*, although reports of such cases are very rare (lightfoot *et al.*, 1991). The sensitivity of culture for the diagnosis of Legionnaires ' disease has been estimated to be in the range of 11-65% (Hayden *et al.*, 2001; DenBoreet *al.*, 2004; Lindsay *et al.*, 2004). Bronchoalveolar lavage fluid, bronchial aspirates, lung biopsies, post mortem tissue specimens and sputum are suitable for culture where as pleural fluid is less suitable (Edelstein, 2000; Field *et al.*, 2002, BoneBore *et al.*, 2004). *Legionella* colonies usually form within 3-5 days. Suspected colonies are subcultured on BCYE agar or Columbia blood agar. One of the limiting factors for cultivation of *Legionella* Spp seems to be the experience of the laboratory staff (Edelstin, 2000). *Legionella* colonies have unusual morphology that might not be recognized (Stout *et al.*, 2003).

2.5.2. Identification of *legionella* species:

The most important technique for the identification of *Legionella* in the clinical laboratory is serological characterization of isolated strains. A fluorescein conjugated monoclonal antibody (MAb) which recognizes an outer membrane protein of *L.pneumophila* is commercially available. This species-specific MAb detects all serogroups and can be used for rapid

identification of *L.pneumophila* from clinical samples or environmental specimens (Helbig *et al.*, 2007).

The serogroup specificity is based on chemical composition of lipopolysaccharide, and the division into serogroup is based on reactivity with polyclonal antisera and monoclonal antibodies. A few serogroups mainly Sg1 of *L.pneumophila* can be divided into MAb subtype which is used in epidemiological investigation (Joly *et al.*, 1986; Helbig *et al.*, 2002).

Polyclonal antisera either fluorescein conjugated or coupled to latex beads, are commercially available for many but not all *Legionella* species. Recently an Immunochromatographic assay that utilizes monoclonal antibodies was developed and evaluated. This method is suitable to confirm the majority of *Legionella* species isolated from clinical and environmental samples (Helbig *et al.*, 2006). Fluorescently labelled oligonucleotide probes can also be used to verify that given isolate belongs to the genus *Legionella* (Buchbinder *et al.*, 2004).

2.5.3. Detection of legionella antigen in urine:

An antigen excreted with urine has been characterized as heat stable resistant to enzymatic cleavage and about 10KDa molecular weight (Helbig *et al.*, 2007).

All assays of the detection of *L.pneumophila* urinary antigens show sufficient recognition of the antigens which are not homologous to the serogroup monoclonal subgroup used as immunogen for preparation of the antisera.

Several ELISA are commercially available the specificities of all assays which were mostly evaluated by testing urine specimens from patients with urinary tract infection or pneumonias caused by other pathogens have been reported to be > 99.9% . Recently it has been reported by several investigators that the sensitivity of the urinary antigen correlates with the severity of

illness. Concentrated urine samples increase the sensitivity without decrease the specificity. Besides these rapid immunochromatographic assay (Binax Now) has been on the market for several years to detect antigenuria within a very short time and no laboratory equipment is required. The (Binax Now) is slightly less sensitive than the ELISA. The advantages of urinary antigen detection are striking. Specimens are easy to obtain and can be investigated repeatedly. Antigenuria is detectable very early, the test is very rapid and it has very high specificity.

In most cases the antigenuria ends after 10 to 14 days. Despite the initiation of appropriate treatment antigenuria may persist for some weeks to months, but this persistence of antigenuria does not reflect a failure of treatment. It is significantly associated with immunosuppressive therapy. Despite the easiness and advantages of urinary antigen the negative result never excludes *Legionella* infection (Micheles and Klaus 2008c).

2.5.4. Detection of *legionella* by direct fluorescent antibody testing:

Direct fluorescent antibody (DFA) testing of respiratory specimens is a rapid method for the detection of *Legionella* antigen in respiratory sample. A monoclonal, fluorescein conjugated antibody against an outer membrane protein of *L.pneumophila* is commercially available. This reagent is highly specific but gives occasional false positive results with *Staphylococcus aureus* due to non specific binding to protein A. The sensitivity of DFA testing range from 27 to 70% (Hayden *et al.*, 2001; Lindsay *et al.*, 2004), however the sensitivity depends on the type of specimen used, the technical equipment and the experience of the laboratory staff. The protein antigen detected by this assay is not degraded after fixation with formalin hence the test allows the detection of the aetiological agent in formalin, fixed lung tissue which is not possible with other methods available (Micheles and Klaus 2008c).

2.5.5. Detection of *legionella* by fluorescence in situ hybridization (FISH):

Fluorescently labelled oligonucleotide probes can also be used to detect *legionellae* in clinical samples. And requires an experienced laboratory staff. (Haydeu *et al.*, 2001; Buchbinder *et al.*, 2004).

2.5.6. Detection of *legionella* nucleic acids in clinical samples:

Detection of nucleic acid has been more frequently used to identify *Legionella* in clinical samples in the last years. Depending on the primers used the polymerase chain reaction (PCR) assay detect either *L.pneumophila* or several or all of the known species of the genus *legionella* (DenBoer *et al.*, 2004).

Since *Legionella* is not considered to be part of the normal flora, the presence or absence of *Legionella* DNA in specimens is the main clinical criterion rather than the quantity of the pathogen (Micheles and Klaus 2008c).

2.5.6.1. Detection of nucleic acid in respiratory samples

The first application of PCR for respiratory specimens was reported in 1992 by Jaulhac *et al* 1992 in bronchoalveolar lavage (BAL) fluid specimens. The high sensitivity for the detection of *Legionella* DNA in respiratory samples demonstrated by several studies suggests that PCR may exceed culture in its ability to detect *legionella* (Cloud *et al.*, 2000., Hyden *et al.*, 2001; Rantakokko and Jalava , 2001; Reischl *et al.*, 2003; DenBore *et al.*, 2004; Koide *et al.*, 2004). External quality assurance (EQA) system has been operating in Germany since 2004 the result of (EQA) scheme revealed certain problems in term sensitivity and specificity that occur in routine laboratories.

2.5.6.2. Detection of *legionella* nucleic acid in urine

The excretion of DNA fragments in the urine has been described for several bacterial pathogens suggesting the suitability of urine PCR for the detection of *Legionella* DNA. The reported sensitivity varies from 17 to 72%. (Maiwald *et al.*, 1995).

2.5.6.3. Detection of *legionella* nucleic acid in serum samples

The detection of *Legionella* in serum sample has been described (Lindsay *et al.*, 1994; Murdoch *et al.*, 1996; Matsiota-Bernard *et al.*, 2000; Diederer *et al.*, 2007; Luck *et al.*, 2006). *Legionella* DNA is detectable in all acute and convalescent phase sera from patients with confirmed legionellosis but not in the sera from over 100 patients without evidence of legionellosis, this kind of clinical samples is inexpensive and simple to perform. The sensitivity of the DNA detection in sera varies from 29 to 82% no DNA was detected in serum samples from patients with pneumonia due to other organisms (Micheles and Klaus 2008c).

2.5.7. Antibody detection in human sera:

The indirect fluorescent antibody (IFA) test has been evaluated and standardized. ELISA tests are available from different suppliers but are of lower specificities. The sensitivity of serology is generally limited by the time required to develop a detectable antibody response during the course of the infection. The sensitivity of serology from 70 to 80. The *Legionella* serology performed with polyvalent conjugates detecting IgG, IgM and IgA antibodies, since the immune response in the course of *Legionella* infections varies with respect to different immunoglobulin classes (Micheles and Klaus 2008c).

2.6. Therapy

Delay in instituting appropriate therapy for *Legionella* pneumonia significantly increases mortality (Heath *et al* 1996). Therefore, empirical antilegionella therapy should be included in the treatment of severe community-acquired pneumonia (Neiderman *et al.*, 1993). Erythromycin has historically been the drug of choice, but the newer macrolides, especially azithromycin, have superior in vitro activity and greater intracellular and lung-tissue penetration. The gastrointestinal intolerance, the requirement for the administration of large volumes of fluid, and ototoxicity related to the 4-g dose of erythromycin (Swanson *et al* 1992). Have made this drug less attractive. Azithromycin, clarithromycin, josamycin, and roxithromycin have been efficacious in anecdotal reports. With the intravenous formulation of azithromycin now available, it may displace erythro-mycin as the drug of choice. Quinolones also have greater in vitro activity and better intracellular penetration than the macrolides (Baker and Brown, 1995, Stout *etal.*, 1994, Edelstin. 1995).

Numerous anecdotal successes with the quinolones, especially ciprofloxacin, have been reported. Given the pharmacologic interaction of the macrolides and rifampin with the immunosuppressive medications used after transplantation, we recommend ciprofloxacin or levofloxacin for transplant recipients with Legionnaires ' disease. Rifampin is highly active in vitro and in vivo against *Legionella*. And is recommended as part of combination therapy (with a macrolide or a quinolone) for patients who are severely ill. Tetracycline proved efficacious in the original American Legion outbreak, and successes with minocycline and doxycycline have also been documented. Imipenem, trimethoprim–sulfamethoxazole, ofloxacin, and clindamycin have proved efficacious in isolated reports (Edelstin, 1995).

Parenteral therapy should be given until there is an objective clinical response; most patients become afebrile within three days. Then, oral therapy

can be substituted. The total duration of therapy is 10 to 14 days. But a 21-day course has been recommended for immunosuppressed patients or those with extensive evidence of disease on chest radiographs. Five to 10 days of azithromycin therapy is sufficient. A newer macrolide may be the antibiotic of choice for immunocompetent patients with community-acquired pneumonia, since such an agent would cover both the typical bacterial pathogens (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus*) and atypical pathogens (*Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and *L. pneumophila*). If an undiagnosed pneumonia is severe enough to warrant admission to the intensive care unit, empirical coverage for legionella is warranted (Janet and Victor 1997).

2.7. Prevention

One approach to preventing Legionnaires' disease is to identify the environmental source and then eradicate the organism. Allegheny County, Pennsylvania, has formulated guidelines for routine culturing of the water supply for *Legionella* in all hospitals in the county. The guidelines recommend an annual environmental survey of all hospitals, including those with no known cases of legionellosis. All hospitals were included because hospital-acquired legionellosis can easily be overlooked unless specialized laboratory tests are readily available. A minimum of 10 distal sites (faucets and showerheads) and all hot-water tanks are cultured. If the organism is found, then physicians should have a high index of suspicion for *Legionella* in hospital-acquired pneumonias, and specialized laboratory tests should also be made available for patients with nosocomial pneumonia. Disinfection should be considered on the basis of the number of positive culture sites and prior experience with hospital-acquired cases (Janet and Victor 1997).

Over the past 13 years, numerous methods of disinfection have been tried with variable success. Three methods are now being used, but no method is ideal. Superheating the water to 70 to 80°C, with flushing of the distal sites; installing

copper–silver ionization units, and hyperchlorinating the water (chlorine concentration, 2 to 6 ppm). The advantage of the first approach is that it can be instituted quickly to halt an outbreak. The long-term efficacy of both superheating and hyperchlorination has been problematic. Copper–silver units have proved cost effective for hospitals whose plumbing systems have been damaged by years of hyperchlorination (Janet and Victor1997).

In summary, legionnaires' disease has been insightfully characterized as a disease that is overtreated and underdiagnosed(Bartlett, 1993).

With the introduction of rapid diagnostic tests into hospital laboratories, especially the urinary antigen assay and PCR, this trend may be reversed (Janet and Victor1997).

2.8. Transmission

Infection normally occurs after inhaling an aerosol (fine airborne particles) containing *Legionella* bacteria. Such particles could originate from any infected water source. When mechanical action breaks the surface of the water, small water droplets are formed, which evaporate very quickly. If these droplets contain bacteria, the bacteria cells remain suspended in the air, invisible to the naked eye and small enough to be inhaled into the lungs (UK health protection Agency). This often occurs in poorly ventilated areas such as prisons where a condensating air conditioner can spread it throughout the entire room, infecting anyone not immune to the strain of bacteria. Potential sources of such contaminated water include cooling towers (some 40% to 60% of one's tested) used in industrial cooling water systems as well as in large central air conditioning systems, evaporative coolers, nebulizers,

humidifiers, whirlpool spas, hot water systems, showers, windshield washers, architectural fountains, room-air humidifiers, ice making machines, misting equipment, and similar disseminators that draw upon a public water supply (Cooling technology institute).

The disease may also be transmitted from contaminated aerosols generated in hot tubs if the disinfection and maintenance program is not done rigorously (Cilivianch and Celebrity, 2001). Freshwater ponds, creeks, and ornamental fountains are potential sources of *Legionella* (Winn, 1996). The disease is particularly associated with hotels, fountains, cruise ships and hospitals with complex potable water systems and cooling systems. The development of bacterial infections may cause Legionnaires' disease. Respiratory care devices such as humidifiers and nebulizers used with contaminated tap water may contain *Legionella*. Using sterile water is very important, especially when using respiratory care devices (Woo *et al.*, 1992).

2.9. Action levels

The European Working Group for *Legionella* Infections (EWGLI) was established in 1986 within the European Union framework to share knowledge and experience about potential sources of *Legionella* and their control. This group has published guidelines about the actions to be taken to limit the number of colony forming units (i.e., the "aerobic count") of microorganisms per ml at 30 °C (minimum 48 hours incubation)(www.hpa.org.uk/web).

Table (2.1): Action levels of *L.pneumophila*

<i>Legionella</i> bacteria,CFU/litre	Action required (35 samples per facility are required, including 20 water and 10 swabs)
1000 or less	System under control
More than 1000 up to 10,000	Review program operation by re-sampling. If similar count found again, a review of the control Measure and risk assessment should be carried out.
More than 10,000	Implement corrective action.The system immediately be re-sample. The risk assessment and control review to identify remedial action.

Almost all natural water sources contain *Legionella* and their presence should not be taken as an indication of a problem. The tabled figures are for total aerobic plate count, cfu/ml at 30 °C (minimum 48 hours incubation) with colony count determined by the pour plate method according to ISO 6222(21) or spread plate method on yeast extract agar. *Legionella* isolation can be conducted using the method developed by the US Center for Disease Control using buffered charcoal yeast extract agar with antibiotics. Many other governmental agencies, cooling tower manufacturers, and industrial trade organizations have developed design and maintenance guidelines for preventing or controlling the growth of *Legionella* in cooling towers. However, in the US, there are no regulations requiring testing or maintaining any specified levels in these facilities (Ewgli.org).

2.10. Breeding ground

The bacteria grow best in warm water, like the kind found in hot tubs, cooling towers, hot water tanks, large plumbing systems, or parts of the air-

conditioning systems of large buildings. Indoor ornamental fountains have been confirmed as a cause of Legionnaires' disease outbreaks, in which submerged lighting as a heat source was attributed to the outbreak in all documented cases. Controlling the growth of *Legionella* in ornamental fountains is touched on in many of the listed guidelines. However, specific guidelines for solar water heating systems fountains have also been published (*Legionella* Risk management).

Adding an antibacterial agent to the automobiles' windshield system's reservoir is also recommended (Woo *et al.*, 1992). *Legionellae* have been discovered in up to 40% of freshwater environments and have been in up to 80% of freshwater sites by PCR hybridization assay (Field *et al.*, 2002).

2.11. Regulations and ordinances

The guidance issued by the UK government's Health and Safety Executive (HSE) now recommends that microbiological monitoring for wet cooling systems, using a dip slide, should be performed weekly. The guidance now also recommends that routine testing for *Legionella* bacteria in wet cooling systems be carried out at least quarterly, and more frequently when a system is being commissioned, or if the bacteria have been identified on a previous occasion (UK health and safety executive microbiology monitoring)

Further non-statutory UK guidance from the Water Regulations Advisory Scheme now exists for pre-heating of water in applications such as solar water heating systems. The City of Garland, Texas requires yearly testing for *Legionella* bacteria at cooling towers at apartment buildings (Dallasnews.com).

Malta requires twice yearly testing for *Legionella* bacteria at cooling towers and water fountains. Malta prohibits the installation of new cooling towers

and evaporative condensers at health care facilities and schools (www.doi.gov/EN/legalnotices).

The Texas Department of State Health Services has provided guidelines for hospitals to detect and prevent the spread of nosocomial infection due to *Legionella* (Texas Legionellosis).

2.12. Limiting growth

Legionella bacteria themselves can be inactivated by UV light. However, *Legionella* bacteria that grow and reproduce in amoebae or that are sheltered in corrosion particles cannot be killed by UV light alone.

Legionella will grow in water at temperatures from 20 to 50 °C (68 to 122 °F). However, the bacteria reproduce at the greatest rate in stagnant water at temperatures of 35 to 46 °C (95 to 115 °F).

Copper-Silver ionization is an effective industrial control and prevention process to eradicate *Legionella* in potable water distribution systems and cooling towers found in health facilities, hotels, nursing homes and most large buildings. In 2003, ionization became the first such hospital disinfection process to have fulfilled a proposed four-step modality evaluation; by then it had been adopted by over 100 hospitals (Stout and Yu, 2003). A 2011 study by Lin and others found Copper-Silver ionization to be the only *Legionella* control technology which has been validated through a 4 step scientific approach.

2.13. Prognosis

The fatality rate of Legionnaires' disease has ranged from 5% to 30% during various outbreaks and approaches 50% for nosocomial infections, especially when treatment with antibiotics is delayed. According to the journal *Infection*

Control and Hospital Epidemiology, hospital-acquired *Legionella* pneumonia has a fatality rate of 28%, and the principal source of infection in such cases is the drinking-water distribution system (Stout *et al.*, 2007).

2.14. Epidemiological subtyping of isolated *Legionella* strains

So far, a positive culture is the only method that allows the comparison of patient and environmental *Legionella* strains necessary to confirm or exclude a given environmental reservoir as the source of the infection.

Epidemiological investigations of legionellosis are often complicated by the ubiquity of *legionellae* in nature. Because the incubation period varies from 2 to 10 days, the length of stay in a hotel, private accommodation, public building or hospital before onset of clinical signs does not establish with certainty where the infection has been acquired. Recognized sources of Legionnaires' disease confirmed in epidemiological investigations are: warm and cold water supplies (shower, taps) in private homes, hospitals, hotels, public buildings, cruise ships, cooling towers with 'water based cooling', whirlpools, thermal springs, moistener/respirators, decorative fountains, humidifiers for food display cabinets, car washers. Often different species, serogroups, and monoclonal subtypes of *Legionella* are isolated from a given environmental source (Lück *et al.*, 1998; Visca *et al.*, 1999; Beyrer *et al.*, 2006). In contrast to this, simultaneous infection with multiple *Legionella* strains seems to be a rare event (Horbach *et al.*, 1988; Lück *et al.*, 1998; Buchbinder *et al.*, 2004).

In some cases, no corresponding environmental isolate could be found, though all suspected water sources were investigated (Jonas *et al.*, 2000). In such cases, it might be that the patient acquired the infectious strain from other environmental sources that were not investigated, e.g. outside the hotel or hospital, during overnight stays in the nearby private accommodation, etc. On the other hand, the causative strain might not have been isolated and

subsequently typed because the source of infection was decontaminated or the bacterial population in the environment had changed and/or the causative strain had been overlooked.

All the above mentioned problems lead to discussions as to the number of colonies which should be typed after primary isolation and to the preferable typing method(s). The present, we serotype at least six colonies from environmental samples. Further, a combination of antigenic and genomic typing systems applied as a step-by-step procedure is recommended for the identification of *Legionella* strains that cause the infection (Michel and Claus, 2008)

2.15. Monoclonal antibody typing

Subtyping of *Legionella pneumophila* strains by using monoclonal antibodies (MAb) was the first technique used in epidemiological studies and is now a well established method (Joly *et al.*, 1986). As major advantages, it is technically simple and quick to perform. The reactivity patterns are stable, although changes have been observed due to point mutations and deletions in genes involved in the synthesis of lipopolysaccharide (LPS) (Zou *et al.*, 1999; Lück *et al.*, 2001; Bernander *et al.*, 2003). In general, genetic variation is of minor relevance, since such events occur at a relative low frequency under natural conditions.

The major disadvantages of MAb typing are the time-consuming and expensive establishment, maintenance and quality assurance of the hybridoma cell lines producing MAbs. Furthermore, the antigenic diversity, or number of subtypes, is limited. Thus, the index of discrimination, an important characteristic which reflects the ability of a typing system to recognize different strains as different, is in the range of 0.8. Nevertheless, MAb subtyping is applicable to *L. pneumophila* sg 1, which can be divided into at least 12 subtypes (Joly *et al.*, 1986; Helbig *et al.*, 2002). When the MAb type of the clinical isolate does not match that of environmental

strain(s), these reservoirs can with high probability is excluded as the source of the infection. At times, the use of subgroup-specific and cross-reacting antibodies has allowed the subtyping of strains belonging to serogroups 2-15 (Helbig *et al.*, 2007).

All MAb subtypes are named according to a reference strain that shows this particular reactivity pattern. This kind of data can easily be exchanged between laboratories. In a multicentre evaluation of typing methods for the epidemiological typing of *L. pneumophila* serogroup 1, the epidemiological concordance was very close to 1 (Fry *et al.*, 1999).

2.16. Macrorestriction analysis (MRA) by pulsed-field gel electrophoresis

Subtyping of *Legionella pneumophila* strains by MRA is an excellent tool for subtyping *Legionella* species and was considered to be the gold standard for more than 15 years (Lück *et al.*, 1998; Fry *et al.*, 1999; Jonas *et al.*, 2000). Macrorestriction patterns can be analysed both visually and by computer-aided methods. The index of discrimination is greater than 0.95, the value which is required for a good subtyping system. However, the intra- and inter-laboratory standardization and exchange of data are difficult (Fry *et al.*, 1999). Nowadays, sequences based typing (SBT) has replaced MRA.

2.17. Amplified fragment length polymorphism (AFLP) typing

This technique uses a combination of DNA restriction by endonuclease and amplification by the polymerase chain reaction. It was the first typing system standardized within the European Working Group on *Legionella* Infection (EWGLI) (Fry *et al.*, 1999, Jonas *et al.*, 2000).

2.18. 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.

2.19. Antibiotic therapy *in vitro* susceptibility of *Legionella*

In vitro susceptibility testing can be performed on artificial media to screen for active agents. It must be considered that components of the media (e.g. charcoal) might influence the *in vitro* susceptibility data. Macrolides, quinolones, ketolide, quinupristin/dalfopristin, doxycyclin, imipenem, rifampicin and tigecyclin are *Legionella* factors that are active *in vitro* (Stout *et al.*, 1998; Nielsen *et al.*, 2000a; Hammerschlag *et al.*, 2001; Edelstein *et al.*, 2003; Stout *et al.*, 2005).

Resistance to all clinically relevant substances can be induced in laboratory experiments (Dowling *et al.*, 1985; Nielsen *et al.*, 2000b; Jonas *et al.*, 2003). However, so far, there is no evidence that resistance occurs in clinical situations. Case reports describing clinical failure and/or prolonged clinical illness were never related to the development of resistance to antimicrobials (Rudin *et al.*, 1984; Kurz *et al.*, 1988; Tan *et al.*, 2001; Glaser *et al.*, 2005).

2.20. Intracellular activity of antimicrobial agents against *Legionella*

As *legionellae* are intracellular pathogens, *L. pneumophila* has been cultivated *in vitro* in a number of macrophage-like cell lines. In this way, the intracellular activity of antimicrobials can be assessed (Stout *et al.*, 1998; Jonas *et al.*, 2003; Stout *et al.*, 2005). Generally, these results show a good correlation with animal experiments. In earlier studies it was shown that although bacterial growth is inhibited by erythromycin and rifampicin, it recurs when the drugs are removed from the cells. This is in contrast to newer fluoroquinolones and macrolides, which kill intracellular *Legionella* bacteria and do not permit bacterial regrowth. Azithromycin is the most active macrolide, and it has a much higher activity than erythromycin against intracellular *L. pneumophila* (Edelstein *et al.*, 1991, Fitzgeorge *et al.*, 1993; Jonas *et al.*, 2003).

2.21. Clinical experience in the treatment of legionellosis

The clinical experience of azithromycin in the treatment of Legionnaires' disease is known to be safe and efficacious (Edelstein, 1995; Plouffe *et al.*, 2003). The vast majority of patients who receive monotherapy with intravenous azithromycin for 2-7 days, followed by oral azithromycin, are cured.

The efficacy of levofloxacin was reported in a study analysing six clinical trials encompassing a total of 1997 patients with community-acquired pneumonia. More than 90% of mild-to-moderate and severe cases of *Legionella* infection were cured; no deaths were reported (Yu *et al.*, 2004). Recently, three observational studies comparing levofloxacin vs. macrolides (not Azithromycin) in the treatment of Legionnaires' diseases have been published (Blázquez-Garrido *et al.*, 2005; Mykietiuk *et al.*, 2005; Sabria *et al.*, 2005). There were no significant differences in clinical outcomes among the groups of patients with mild or moderate pneumonia; but, in patients with severe pneumonia, levofloxacin was slightly more effective. The

combination of rifampicin and levofloxacin provides no additional benefit but does increase the rate of side effects.

In summary, as compared with older macrolides in the treatment of Legionnaires' disease, levofloxacin appears to be associated with better clinical outcomes, including a faster resolution of pneumonia symptoms, a more rapid achievement of clinical stability, and a shorter hospital stay. Thus, monotherapy with levofloxacin might be regarded as first-line antimicrobial for treatment of Legionnaires' disease. However, it must be underlined that the direct comparison of azithromycin and levofloxacin in the treatment of *Legionellapneumophila* has not been performed. Thus, it can be recommended to use fluoroquinolones or azithromycin, rather than older macrolides, for the treatment of Legionnaires' disease (Pedro-Botet *et al.*, 2006).

Delay in the initiation of appropriate antibiotic therapy for *Legionella pneumophila* significantly increases mortality (Heath *et al.*, 1996; Gacouin *et al.*, 2002; Lettinga *et al.*, 2002). It is therefore recommended that anti-*Legionella* agents be included early in the empiric therapy of severe community-acquired pneumonia. However, there are different viewpoints regarding the first line antimicrobial therapy that is generally recommended for treating pneumonia (File *et al.*, 2004).

Blanquer *et al.*, (1991) carried study in the Valencia region of Spain to determine the cause of community acquired pneumonia (CAP). The study included patients with pneumonia (281) cases. 208 cases were found to have bacterial, 60 viral, and 13 mixed infections. The common organisms were *Streptococcus pneumoniae* (14.5%), *Legionella spp*(14%), Influenza virus (8%), *Mycoplasma pneumoniae* (4%). There was a higher incidence of *Legionella spp* than in other studies. Other study was performed by Jacob *et al.*, (1999) to investigate an increase in reports of Legionnaires' disease by multiple hospital in SanAntonio,Texas, and to study risk factor for

nosocomial transmission of legionnaires disease and determinants for *Legionella* colonization of hospital hot- water system. For 3 years twelve cases of nosocomial Legionnaires disease were identified. The rise in cases occurred shortly after physician started requesting *Legionella* urinary antigen tests. *Legionella* was isolated from the water system of 11 out of 12 hospitals. It was concluded that use of monochloramine by municipalities for residual drinking water disinfection may help prevent Legionnaires disease.

In 1999 Mauricio, *et al* studied the etiology of community acquired pneumonia (CAP) over all 395 consecutive patients with CAP during 15 month's period. Examination of sputum, blood culture and serology showed that the most frequent pathogens, were *Strept pneumoniae* (29%), *H. influenzae* (11%), Influenza virus A&B (10%), *Legionella spp* (8%), *Chlamydia pneumoniae* (7%), Gram negative enteric bacilli (6%), *Pseudomonas aeruginosa* (5%). In 1998, Lim *et al* (2001) in Britain studied the etiology of CAP in adults admitted to hospital over 12 months period. 309 patients with CAP had their sample from blood sample, sputum and urine collected for microbiological testing by standard culture technique and new serological and urine antigen detection method. The most frequent pathogen were *Strept pneumoniae* (48%), Influenza A virus (19%), *Chlamydia pneumoniae* (13%), *Haemophilus influenzae* (7%), *Mycoplasma pneumoniae* (3%), *Legionella pneumophila* (3%), other *Chlamydia spp* (2%), *Moraxella catarrhalis* (2%), *Coxiella burnetii* (0.7%), and others (3%). Miquel *et al* (2004) studied for 5 years, twenty hospitals in Catalonia, Spain. The result of this study showed the increased of nosocomial Legionnaires disease due to using urinary antigen test in their laboratories.

The outbreak of legionnaires disease in Ontario, Canada from September to October 2005 resulted in the death of 23 resident and the illness of 112 other people in response molecular methods were developed to detect *Legionella pneumophila* in clinical and environmental samples and these molecular

typing methods confirmed the outbreak source as a contaminated air condition cooling tower(Mathew *et al.*, 2007).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Study design

The present study is a cross sectional study.

3.2. Study Area

This Study was carried out at selected hospitals (Omdurman teaching Hospital, Jaafar Ibn Auf Hospital and Elswedy Hospital) in addition to (Elhikmma Center, Wadnobawi Center, and special clinics) in Khartoum State.

3.3. Study duration

This study was conducted during the period from September 2012-September 2013.

3.4. Study population

Patients attending the study area during the period of study diagnosed as pneumonia/ chest infection, with symptoms such as (fever, cough, chest pain, gastrointestinal symptoms). Patients in this study included males and females with different age.

3.5. Sample technique

Three hundred (n= 300) patients attending the above mentioned health facilities during study period were randomly selected to participate in this study.

3.6. Data Collection

After having a verbal consent of each patient or his relative to participate in the study, personal and clinical data were collected using questionnaires (Appendix II). The required information included sex, age, residences and clinical information.

3.7. Specimen collection

Five mls of venous blood were collected under aseptic condition and drawn into plain container. Blood was allowed to clot and after clot retraction centrifuged and the serum separated into another container to be stored at -20 and tested later.

3.8. Laboratory examination

All sera were then tested for *L.pneumophila* IgG antibodies using Enzyme linked Immunosorbent Assay(ELISA). And detection of *L.pneumophila* nucleic acid in serum by Polymerase Chain Reaction (PCR).

3.8.1. Enzyme linked Immunosorbent Assay

Anti-Legionella pneumophila ELISA (IgG) –EUROIMMUN-German.

The ELISA kit provides a semiquantitative or quantitative in vitro assay for human antibodies of the IgG class against *Legionella pneumophila* in serum or plasma. The test kit contains microtitre strips each with 8 break-off reagent wells coated with *Legionella pneumophila* antigens. In the first reaction step, diluted patient samples are incubated with the wells. In the case of positive samples, specific IgG antibodies will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (enzyme conjugate) catalyzing a colour reaction.

3.8.1.1. Sample dilution:

- The serum samples were diluted 1:101 with sample buffer. 10 µl serum in 1.0 ml sample buffer and was mixed well by vortexing.

3.8.1.2. Methodology of the test

- 100µl of the calibrators, positive, negative controls and diluted patient samples were transferred into the individual microplate wells according to the pipetting protocol. Incubated for 30 minutes at room temperature. The wells were emptied and subsequently washed 3 times using 300 µl of working strength wash buffer per each wash. Wash buffer were left in each well for 30 to 60 seconds per washing cycle, and then the wells were emptied. After washing throughly all the liquid were disposed from the microplate by tapping it on absorbent paper with the opening facing downwards to remove all residual wash buffers. 100µl of enzyme conjugate (peroxidase-labelled anti-human IgG) were pipetted into each microplate wells. Incubated for 30 min, at room temperature. The wells were emptied and washed as described above. 100µl of chromogen/substrate solution was pipetted into each of the micro plate's wells. Incubated for 15 minutes at room temperature (protected from direct sunlight). 100µl of stop solution (0.5 M sulphuric acid) were pipette into each of microplate wells. The colour intensity was photometrically measured of wavelength of 450nm and a reference wavelength between 620-650 nm within 30 minutes of the stop solution was added. By (TECAN- Euroimmune, AG-D-23560 Lubeck-Seekamp 31).

3.8.1.3. Validation of test:

The test was considered valid and hence accepted when:

{The negative control ratio 0-0.7}.

{The positive control ratio 1.4-3.8}.

{The Optical Density(OD) of calibrator 2 > 0.140}.

3.8.1.4. Calculation of Results:

Semiquantitative: results were evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator 2. The ratio was calculated according to the following formula:

$$\frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator 2}} = \text{Ratio}$$

3.8.1.5. Interpretation of the result:

Euroimmun recommended interpreting results as follows

Ratio < 0.8 = negative.

Ratio > 0.8 to <1.1 = Borderline.

Ratio > 1.1 = positive.

*The sample showed result near to (0.8) were considered as negative.

*The sample showed result near to (1.1) were considered as positive.

3.8.2. Detection of *legionella pneumophila* nucleic acid in serum samples

3.8.2.1. Extraction of *Legionella pneumophila* DNA from serum sample

The DNA extracted from serum by using G-DEXIIb Genomic Extraction Kit. 300µl of cell lysis solution were added to 300µl serum and mixed thoroughly using the pipette to lysed the cells. Sample was chilled to room temperature, then 100µl Protein precipitation buffer (PPT buffer) were added to cell lysate, and vortexed vigorously at high speed for 20 seconds. In order to remove the protein contamination. In some case the sample was put in ice for 5 min then centrifuged at 13,000-16,000xg for 3-5 minutes. The precipitated proteins form a tight white pellet. 300µl of supernatant containing the DNA (leaving behind the precipitated protein pellet) were transferred into 1.5 ml test tube. 300µl of 100% Isopropanol were added and sample was mixed by inverting

it up and down gently several times. Then centrifuged at 13.000-16.000xg for 1 minutes. The DNA would be visible as a small white pellet. Supernatant was poured off and the tube drained briefly on clean absorbent paper. 1 ml of 70% Ethanol was added and inverted the tube several times to wash the DNA pellet. And centrifuged at 13000-16000xg for 1 minute. Ethanol was poured off carefully otherwise, pellet maybe lost. So, Ethanol was poured slowly and pellet was watched for. The tube was inverted and drained on the clean absorbent paper and allowed to air dry for 10-15 minutes. Then the supernatant was discarded. Care must be taken not to discard the DNA pellet. Over drying was avoided because too much dried DNA might not dissolve very well in DNA Hydration buffer. 150µl of DNA Rehydration buffer were added, and DNA incubated at 4°C for overnight. For long term storage collected DNA was stored at -20°C or -80°C. DNA purity was measured O.D 260:280 ratio.

3.8.2.2. Primer

The primer used in this study were purchased from MACROGen.

The primer used in this study to detect *L.pneumophila* were (Lmp-1, Lmp-2) described by Lindsay *et al*(1994), Jaulhac *et al* (1992) and Massoud *et al* (2004). Were chosen to detect MIP gene, which codes for a virulence protein seemed to be a promising target for the diagnosis of legionellosis. Two 20-base oligonucleotides.(Lpm-1, Lpm-2) bracketing a 600-bp DNA fragment was synthesized as primers. Lpm-1 (5'-GGTGACTGCGGCTGTTATGG-3') was located at nucleotides 853 to 872 from the coding strand; Lpm-2 (5'-GGCCAATAGGTCCGCCAACG-3') was located at nucleotides 1465 to 1484 complementary to the coding strand.

3.8.2.2.1. Preparation of Primers

The primers sequences were first checked for quality assurance, spin for few seconds, definite volume of sterile water was added according to

manufacture instruction in bio safety hood used sterile filter tips, labelled with date of preparation. The primers solution were mixed well and kept in refrigerator at 4°C for overnight. Then stored in -20°C.

3.8.2.2.2. Primers dilution

The primers aliquot were prepared by adding 10 µL of primers stock (100 pmol/ µL) to 0.5 ml sterile eppendorf tube containing 90 µL distilled water to obtain final primers concentration of 10 pmol/ µL then labelled and stored at -20°C where it was later used in PCR reaction.

3.8.2.3. Control of *Legionella Pneumophila*

Control of *Legionella pneumophila* from Statens Serum Institute-Denmark. The concentration of control 10^{-2} (100,000,00 copy). The Control was diluted to 10^{-4} . By 0.1 ml of stock control added to 0.99 ml of DW (stock 1), then 1 ml from stock 1 added to 99 DW to final concentration 10^{-4} .

3.8.2.4. Preparation of reaction mixture of mip gene

PCR mixes were prepared by using Maxime PCR premix Kit (i-taq) from Intron biotechnology (Korea). The product was mixed every components for i-Taq DNA polymerase, dNTP mixture, reaction buffer in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every component for PCR, so we can do PCR just by adding a template DNA, primer set and D.W. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. The components for 20µl reaction (i-Taq polymerase (5U/µl) =2.5U, dNTPs= 2.5mM for each, reaction buffer 10X=1X, Gel loading buffer=1X).

PCR mixes were prepared in total volume containing 20 µl (Template DNA 3-5µl, 1.5µl for each primer, 12-14µl and 12-14 µl distilled water

3.8.2.5. PCR amplification

The amplification was done using CONVERGYS® peltier thermal cycle (Germany). The machine was adjusted based on the as following protocol.

Table (3.1): PCR protocol for *L. pneumophila*

Amplification step	Temp and duration
Initial denaturation	95°C for 5 min
Denaturation	95°C for 1 min
Annealing	62°C for 2min
Extension	72°C for 2 min
Final extension	72°C for 10 min
Cycle number	40 Cycle

3.8.2.6. Preparation of Agarose Gel

Agarose gel was prepared in a concentration of 1.5% as follows; 1.5gm of agarose powder (Ambion-USA) was dissolved by boiling in 100 ml 1X trisbase boric acid EDTA(TBE buffer)(AppendixI). Then was cooled to 55°C in water bath, then, 5 µl of (10mg/ml) Ethidium bromide were added, mixed 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.

3.8.2.7. Visualization of PCR Product

The gel casting tray was put into the electrophoresis tank, flooded with 1XTBE buffer as just to cover the gel surface. 5 µl of the reaction mixture (PCR product) from each sample were put in the well and then the gel was electrophoresed at 80Vfor 30min (Serva Blue Power 500, Germany). The gel was visualized by UV transilluminator (SYNGENE, UK).

3.8.2.8. Interpretation of PCR Results

A blotting chart was drawn to compare the size of each ampilicon against the DNA marker (100 bp). Positive results of *Legionella pneumophila* produced a band of 600 bp for mip gene.

3.8.3. Detection of *Legionella pneumophila* mip gene by Real Time PCR

100 samples randomly selected from 300 sample enrolled in study were tested by using Real Time PCR.

3.8.3.1. Protocol for Real time PCR

By using Real MOD™ Green Real-time PCR master mix Kit (2X).

Into Real time PCR tube for 25 µl. 10 µl of 2X Real MOD™ Green Real-time PCR master mix solution(T044-690203-44) INTRON-Biotechnology were added,1.5 µl for each primer mention above, 0.5ul from High Resolution Melt dye (HRM dye) R146-690201-44) INTRON-Biotechnology , 7 µl template DNA,4.5 µl distilled water.

The upper reaction mixtures were mixed thoroughly and aliquot appropriate volume into the Real-time PCR tube. Then The Real time PCR instrument from Q Agen- Roter gene Q were programmed as mention above (table 3.1), the Real time PCR tube were placed in the Real time PCR instrument and cycling program was started. After the reaction was completed , the amplification curve was verified.

3.8.3.2. Interpretation of results.

Results obtained in real-time PCR were expressed as threshold cycle (Ct) values, corresponding to the cycle at which PCR entered the exponential phase. If no increase in fluorescent signal was observed after 40 cycles, the sample was assumed to be negative.

3.9. Data analysis

Data was analyzed using manual and Statistical Package for the Social Sciences (SPSS) version-11.5. A descriptive analysis was used to describe the population enrolled in this study. The differences between the proportions were tested using the Chi-square(X2) test ($P \leq 0.05$).and manual to detect Relative Risk(RR).

Data presentation:Data were presented in form of tables, Chart and graphs.

3.10. Ethical consideration

- 1- This study was approved by the National Ethical committee, Ministry of Health, Khartoum State, Sudan (Appendex III).
- 2- Orally consent was obtained from every patient before being enrolled in the study.
- 3- Maintaining confidentiality of information obtained from participants investigated.

CHAPTER FOUR

4. RESULTS

4.1. Demographic data

A total of 300 patients were included in this study. All ages range from less than 15 years to more than 60 years were recruited to participate in this study table (4.1). The majority 55% were males and 45% females fig (4.1). The majorities were from Omdurman (47.3%) fig (4.2). All patients enrolled in this study have pneumonia with different symptoms fig (4.3). The majority of patients do not use air conditioning fig (4.4).

Table (4.1): Distribution of patients according to the age group

Age group/ years	Frequency	Percentage %
0-15	101	33.7
16-30	57	19
31-45	57	19
46-60	33	11
> 60	52	17.3
Total	300	100

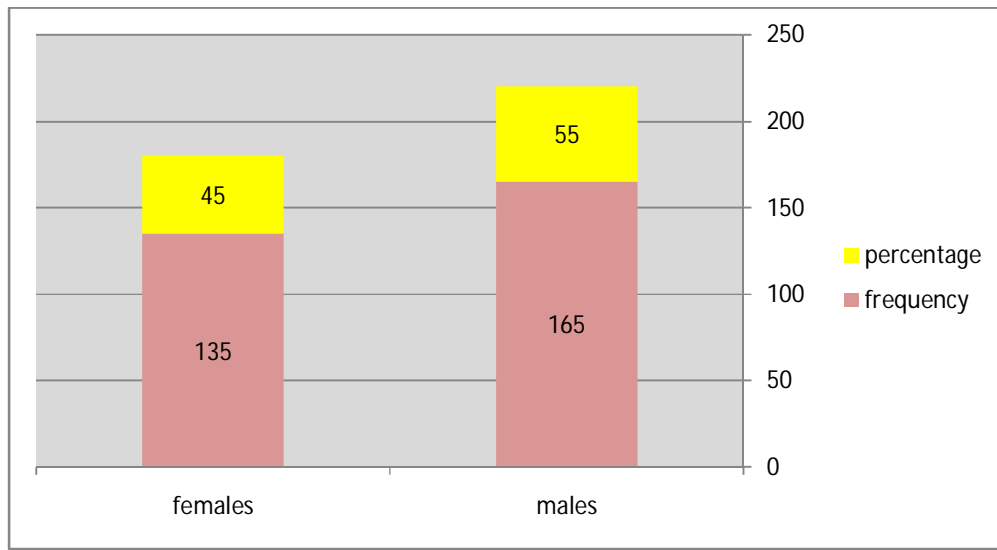


Fig (4.1): Distribution of the studied population according to gender

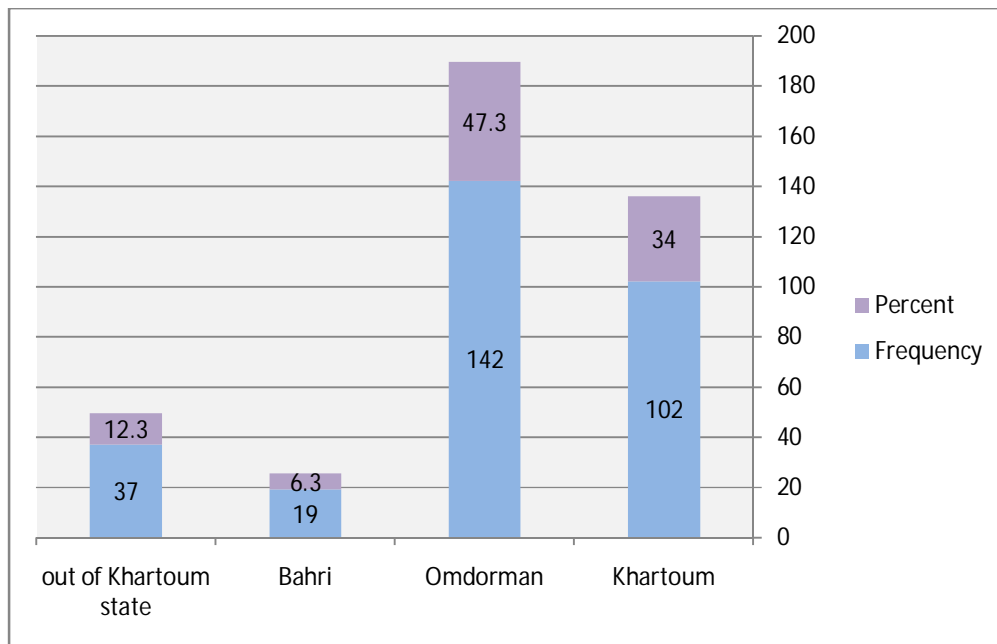


Fig (4.2): Distribution of studied population according to residences (n=300).

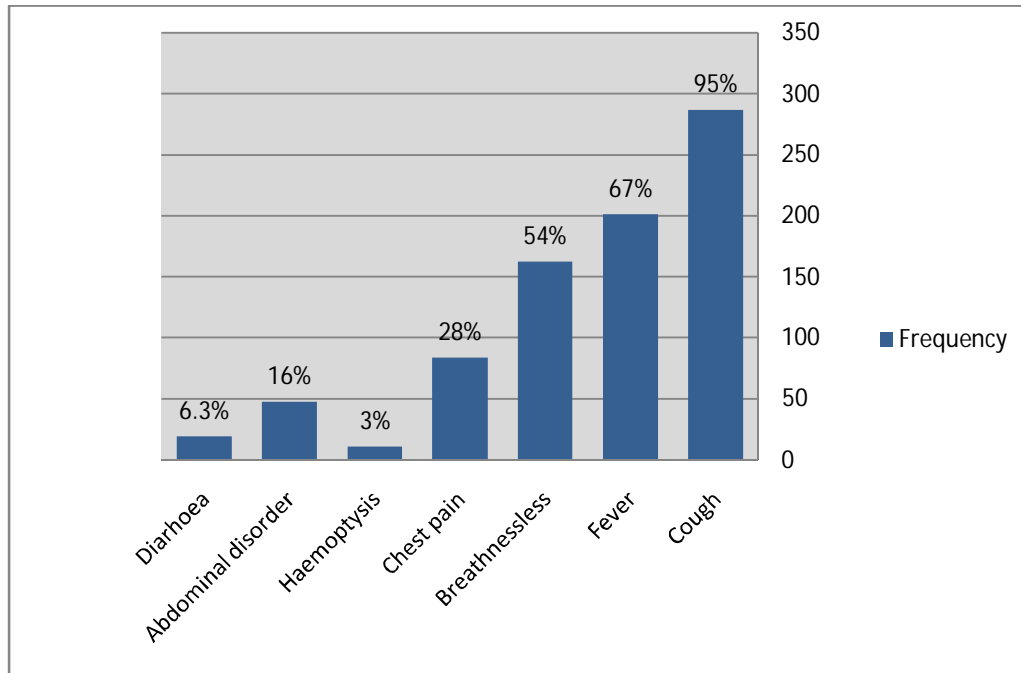


Fig (4.3): Distribution of studied population according to symptoms (n=300).

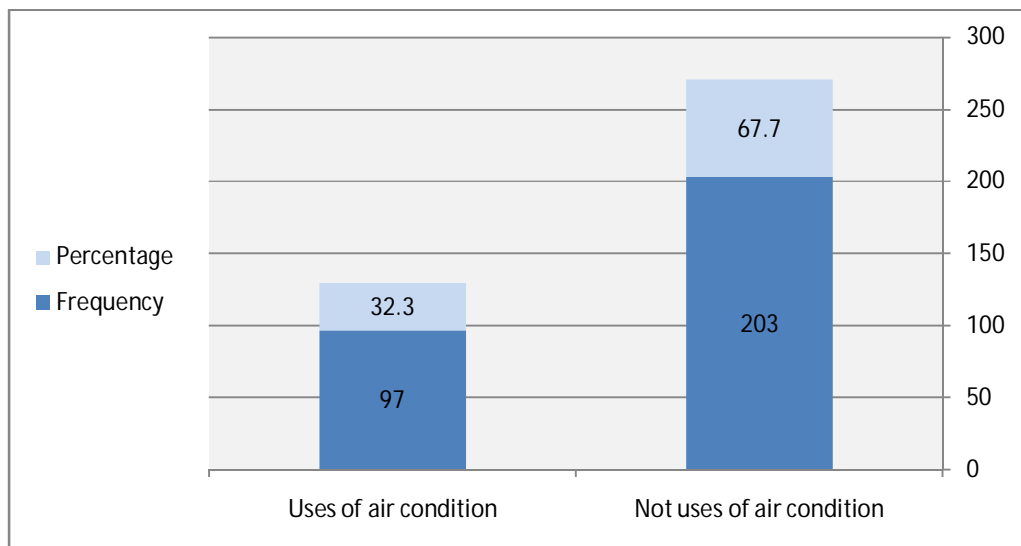


Fig (4.4): Distribution of studied population according to uses of air condition(n=300).

4.2. Results of ELISA

The overall seroprevalence of *Legionella pneumophila* IgG antibodies was found to be 22.7 % (68 out of 300). The highest prevalence of *Legionella pneumophila* IgG 36.8% was found in the age group 31-45 years (table (4.2)). The seroprevalence of *L.pneumophila* was almost equal in both males and females (23% and 22.2%) respectively Fig (4.3). Patients residing in Omdurman were found to have the highest rate of *L.pneumophila* IgG table(4.3). Seropositivity with *L.pneumophila* was high in patients running nose and hemoptysis (Relative Risk (RR) 2.0 and 1.6 respectively) table (4.4).

Table (4.2): Distribution of *L.pneumophila* IgG antibodies according to age group

Age group / years	Total examined	<i>L.pneumophila</i> IgG +ve	
		Frequency	Percentage %
≤15	101	13	12.9
16-30	57	13	22.8
31-45	57	21	36.8
46-60	33	10	30.3
≥60	52	11	21.2
Total	300	68	22.7

(Key: $X^2=0.010 < P < 0.05$)

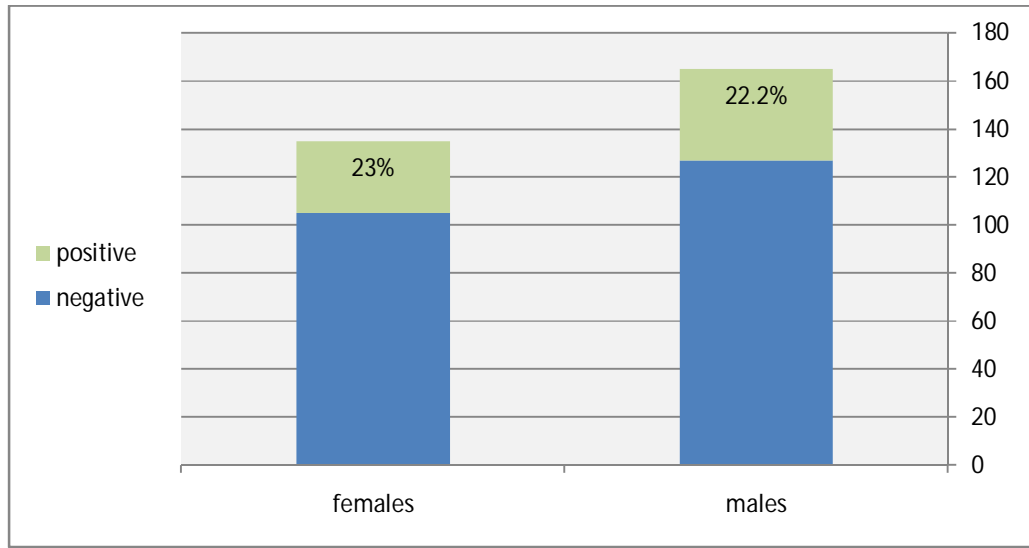


Fig (4.5): distribution of *L. pneumophila* IgG antibodies according to gender (n=300). $X^2=0.868 > P > 0.05$

Table (4.3): distribution of *L.pneumophila* IgG antibodies according to residence

Residence	Total	+ve <i>L.P</i> IgG	
		Frequency	Percentage %
Khartoum	102	15	14.7
Omdurman	142	40	28.1
Bahri	19	5	26.3
Out of Khartoum	37	8	21.6
Total	300	68	100

($X^2=0.098 > P > 0.05$)

Table(4.4):The rate of *L.pneumophila* IgG positive case s in relation to the clinical symptoms

Sign symptoms	Positive for the symptoms			Negative for the symptoms			RR
	Total	+vef or <i>L.p</i> IgG	Rate of of infection 1	Total	+ve for <i>L.p</i> IgG	Rate of of infection 2	
Cough	287	64	0.222	13	4	0.308	0.72
Breathnessless	162	42	0.259	138	26	0.18.8	1.37
Chest pain	84	21	0.25	216	47	0.217	1.2
Fever	201	42	0.209	99	26	0.262	0.8
Hemoptysis	11	4	0.364	289	64	0.221	1.6
Abdominal disorder	48	14	0.292	252	54	0.212	1.4
Diarrhoea	19	5	0.263	281	63	0.224	1.2
Headache	26	5	0.192	274	63	0.23	0.8
Sweating	8	2	0.25	292	66	0.226	1.1
Running nose	16	7	0.438	284	61	0.215	2.0
Fatigue	11	3	0.273	289	65	0.225	1.2

($X^2 > P > 0.05$)

RR: Relative Risk = rate of infection 1/ rate of infection 2

If the RR > 1 there are association between symptoms and disease.

If the RR < 1 there are no association between symptoms and disease.

4.3. Results of convential PCR:

All 300 samples of DNA extracted from serum of patient's sufferings from pneumonia/ chest infection were negative for *L.pneumophila* fig (4.4), (4.5), (4.6).

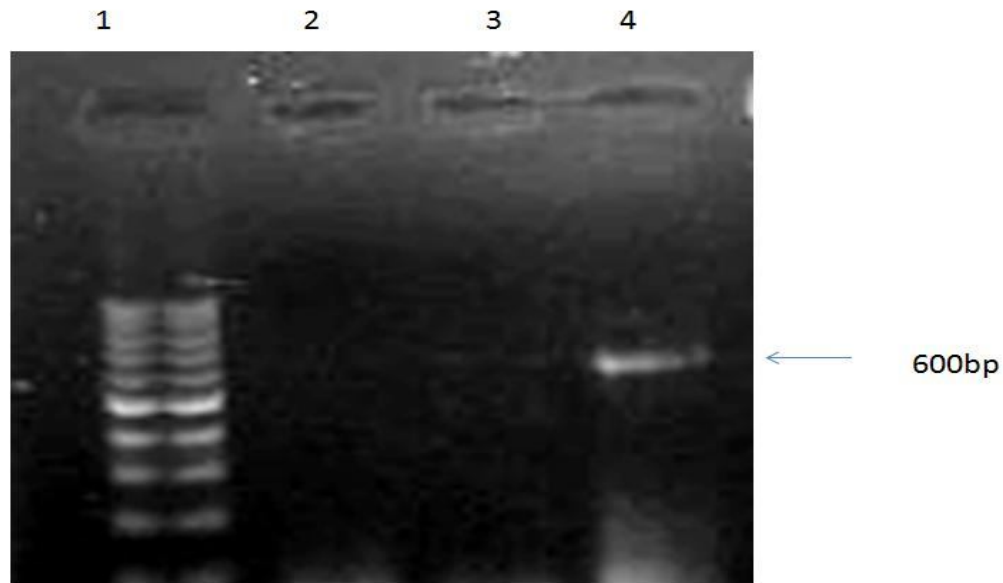
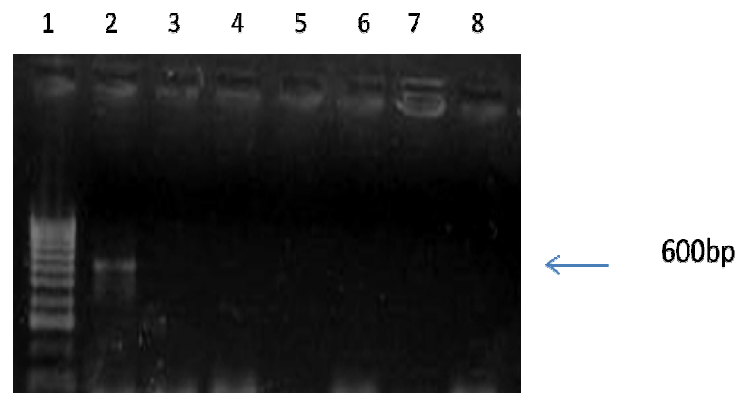


Fig (4.6):result of gel electrophoresis of PCR product obtained from patient sample. Lane 1 DNA Marker 100 bp , lane 2 negative control, lane 3 patient sample, lane 4 positive control for *L.pneumophila*.



Fig(4.7): result of electrophoresis of PCR product.Lane 1 DNA marker, Lane 2 psitive control mip gene for *legionella pneumopila*, Lane 3,4,5,6,7,8 negative sample from patients.

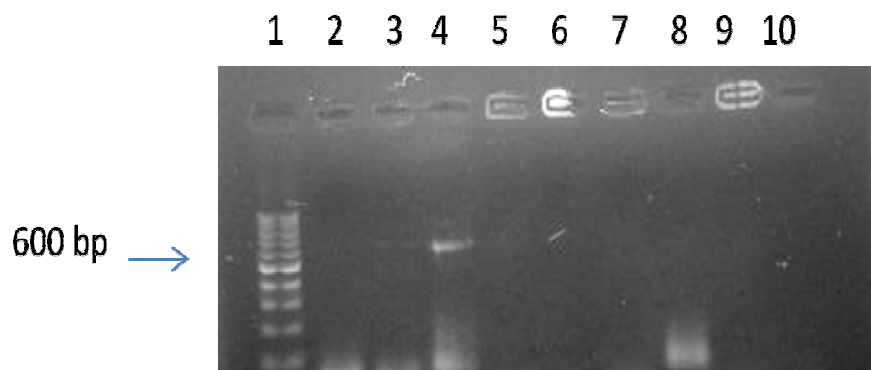


Fig (4.8): result of PCR product. Lane 1 DNA Marker, lane 2,3,negative sample, lane 4 positive control of *L.pneumophila* 600pb, lane 5,6,7,8,9 negative samples, lane 10 negative control.

4.4. Results of Real Time PCR

10specimenout of the 100 specimens (selected randomly from the total number of sample size, were found to be positive by real time PCR. (Fig4.9).

Based on the results of Real time PCR, the highest rate of *L.pneumophila* infection was within the age group 46-60 years,table (4.5). There was no significant difference between the incidence of *L.pneumophila* between males and females(10.5%, 9.3%) respectively fig (4.10).The highest

incidence of *L.pneumophila* in the selected 100 specimens(14.8%) was among patient residing in Omdurman table (4.6).

The present infection of *Legionella pneumophila* with patients presented with haemoptysis 25% , then headache 20%, diarrhea 20%, breathlessness 14.2% with high relative risk with Breathlessness(3.1), haemoptysis (2.6), diarrhoea and headache (2.1) .table (4.7).

EndPoint Graph

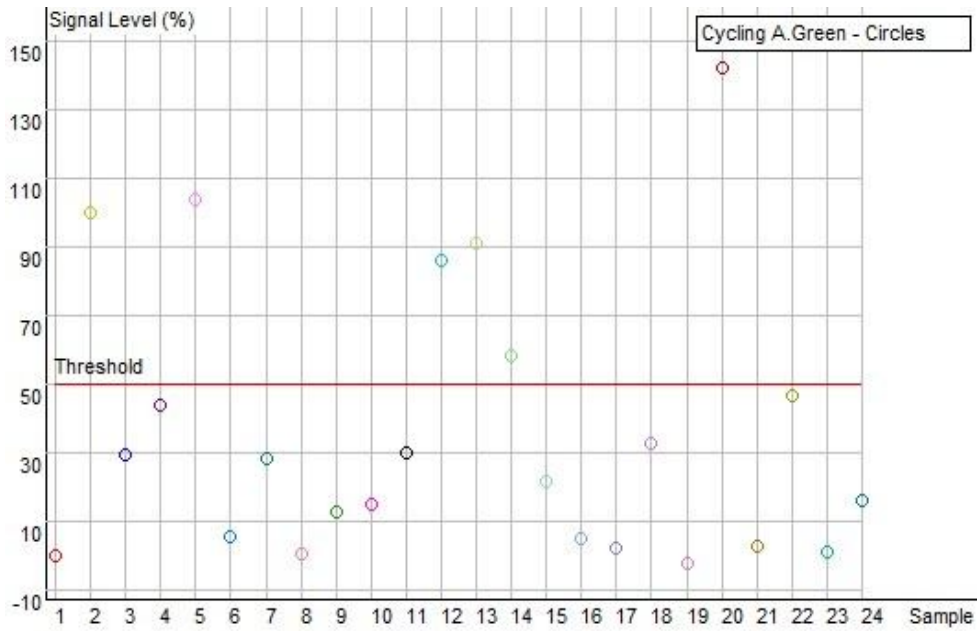


Fig (4.9): analysis of A Green by Endpoint

Key: point above threshold line is positive sample for *L.pneumophila* DNA and point under threshold line is negative for *L.pneumophila* DNA.

Table (4.5): Distribution of *L.pneumophila* according to age group

Age group / years	Total examined	<i>L.pneumophila</i> DNA+ve	
		Frequency	Percentage %
≤15	34	3	8.8
16-30	16	0	0
31-45	18	2	11.1
46-60	11	2	18.1
≥60	21	3	14.3
Total	100	10	10

$X^2=0.541 > P>0.05$

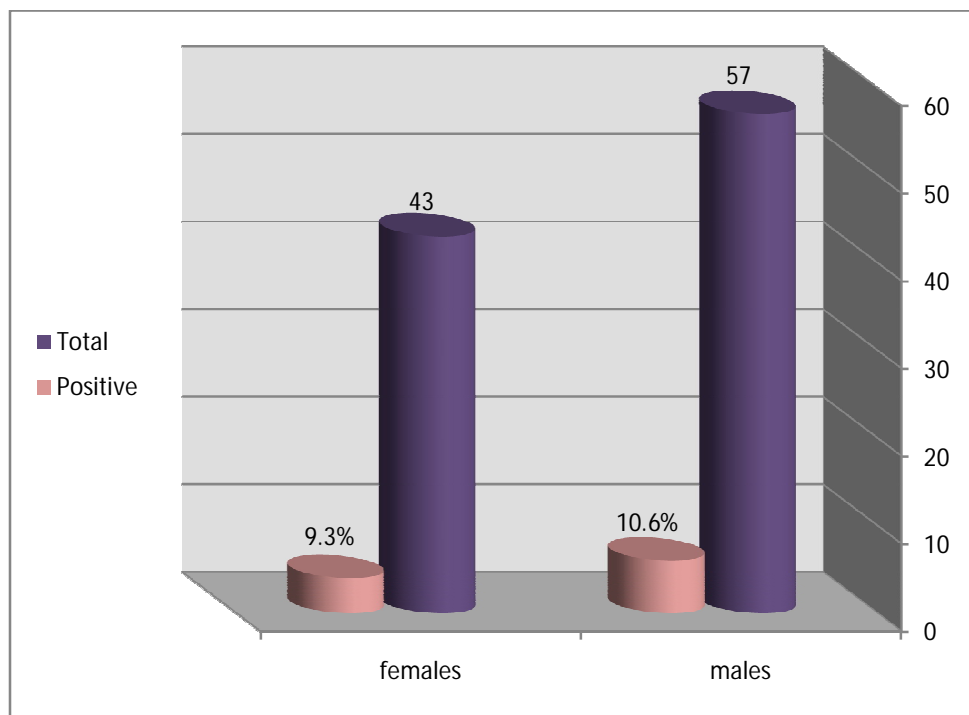


Fig (4.10): Results of *L.pneumophila* PCR in males and females.

$X^2=0.840 > P>0.05$

Table (4.6): Distribution of *L.pneumophila* according to Residence

Residence	Total	+ve <i>L. pneumophila</i>	
		Frequency	Percentage %
Khartoum	27	2	7.4
Omdurman	54	8	14.8
Bahri	3	0	0
Out of Khartoum	16	0	0
Total	100	10	10

$X^2=0.295>P>0.05$

Table(4.7):The rate of *L.pneumophila* positive cases in relation to the clinical symptoms & signs

Signs or symptoms	Positive for the symptoms			Negative for the symptoms			RR
	Total	+ve for <i>L.p</i>	Rate of infection 1	Total	+ve for <i>L.p</i>	Rate of infection 2	
Cough	94	10	0.106	6	0	0	0
Breathlessness	56	8	0.142	44	2	0.045	3.1
Chest pain	28	1	0.035	72	9	0.125	0.28
Fever	66	6	0.09	34	4	0.117	0.76
Haemoptysis	4	1	0.25	96	9	0.094	2.6
Abdominal disorder	16	1	0.062	84	9	0.107	0.57
Diarrhoea	5	1	0.20	95	9	0.095	2.1
Headache	5	1	0.20	95	9	0.095	2.1
Sweating	3	0	0	97	10	0.1	0
Running nose	5	0	0	95	10	0.1	0
Fatigue	11	0	0	97	10	0.1	0

($X^2 > P > 0.05$)

RR: Relative Risk = rate of infection 1/ rate of infection 2

If the RR > 1 there are association between symptoms and disease.

If the RR < 1 there are no association between symptoms and disease.

CHAPTER FIVE

5. 1. DISCUSSION

In this study, the seroprevalence of *L.pneumophila* IgG antibodies was found to be 22.7% (68out300) which is to some extent in agreement with the percentage obtained by Sabah *et al*(2010) (27.4%) ,and Bahl *et al*1997(21%). But Lower seroprevelances of *L.pneumophila* among community acquired pneumonia were reported by Chaudhy 2000 (15%), and Agrawalet *al*1991 (9%).

Although Agrawalet *al* reported avery high rate of *L. pneumophila* in the environmental specimens (76%) compared to 6.8% as reported by Mohamed and Hamedelnil 2012 from Sudan. But the rate of community acquired pneumonia caused by *L.pneumophila* obtained in the current study was far higher than what was reported by Agarawalet *al*1991. This could be because of the small sample size in Agarwal study compared to our study (45 versus300) respectively.

In this study *L.pneumophila* (DNA) was detected in 10 % of the selected 100specimens.This is in agreement with the results of 2 studies done in Spain the reported incidence of 8% and 14 %(Mauricioet *al* 1999 and Blanquer, 1991).Murdoch *et al* (1996) reported a similar rate of infection with *L.pneumophila* in USA. A similar result was repeated by another study done in USA (Muderet *al*, 1983).

The incidence of *L.pneumophilawas* by studies done in Germany were lower than our study (Lindsay *et al* 1994, Ruff *et al* 1989).

In 2001 in UK Limet *al*(3%) of patients with community acquired pneumonia to have *L.pneumophila* and this result is far lower than our study.

In this study all patients with Legionnaires' disease diagnosed by pneumonia and this agree with Edelstin and Cianciotto 2005. And this study showed

that it was impossible to distinguish the legionnaires' disease from other causes of community acquired pneumonia. And this agree with Yu *et al* (1982), and agree with DenBore and Yzerman 2004 *L.pneumophila* can be difficult to diagnose because the sign and symptoms are non specific and don't distinguish *Legionella* infection from other causes of pneumonia. In spite of In this study the predominant symptoms are haemoptysis, breathlessness, headache, and abdominal disorders. Kirby *et al* 1979 and Ten *et al* 2000 their result showed that the majority of cases have fever.

In this study males and females can be affected by the disease and there was no difference between them but Frank 2009 found that men were affected more frequently than women.

Legionella pneumophila affect all age groups especially mild age and older and this study show high percent of *Legionella pneumophila* in age group (46-60 years) (18.1%) and this agree with Burke and Micheel (2014) their study showed risk of *L. Pneumophil* in older age with the mean age 52.7 increasing until 79 years. and also agrees with Center for Disease Control and Prevention (2013) their report showed people in 50 years or older at high risk of legionnaires' disease. Mauricio and tem work in 1999 their study detect patients less than 60 years were at risk and this also agree with our present study. Lim and *et al* 2001 detected Legionnaires' disease in young patient which disagree with our study.

In this study *L.pneumophila* was detected as one of important atypical pneumonia.

5.2. Conclusion

Among patient attending major hospitals in Khartoum State with pneumonia/ chest infection the seroprevalence of *L.pneumophila* IgG antibodies was found to be 22.7%.

L.pneumophila IgG antibodies were found in all age groups and the highest prevalence found in the age group 31-45 year.

L.pneumophila DNA was detected in 10% of patients with pneumonia / chest infection. *L.pneumophila* was found in all age group and high percentage in age group 46-60 years. All patients had fever, cough, the most of them had breathlessness, and high percentage of haemoptysis, headache, diarrhoea in patients with legionellosis.

This study revealed that *legionella* may be an important water pathogen and important causes of community acquired pneumonia and atypical pneumonia in Sudan as in many countries. There are no specific symptoms and signs to distinguish Legionnaires' disease from the other pneumonia.

6.2. Recommendations

- Further in depth studies including large sample size and other locations are recommended.
- Uses of Real time PCR for detection of *L.pneumophila* DNA rather than conventional PCR in serum sample.
- Introduction of some tests for diagnosis *L.pneumophila* in hospital laboratory such as urinary antigen test and antibodies detection (ELISA, IgM, IgG).

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Appendix I

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 gm

Appendix II

Questionnaire

Association of *Legionella Pneumophili* with chest infection among patients attending selected hospitals in Khartoum state

No:.....

Residence.....

Hospital:.....

Name.....

Age.....

Gender Male` male

Symptoms: cough Fever ssnesschestpain emoptysis
diarrhea others

Uses of air condition: yes No

If yes:Types of condition: water fe

Sources of water used: Tap water yers hers

Types of specimens: Serum

ELISA result: Positive Negative

PCR result: Positive Negative