Deanship of Graduate Studies

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The molecular identification of *Legionella* species from water samples in Palestine

Ashraf Rashad Nayef Zayed

M. Sc. Thesis

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Prepared By:

Ashraf Rashad Nayef Zayed

B. Sc: Medical Technology, Arab American University- Jenin

Supervisor: Dr. Dina M. Bitar

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Prepared By: Ashraf Rashad Nayef Zayed

Registration Number: 20913083

Supervisor: Dr. Dina M. Bitar

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The names and signatures of examining committee members are as follows:

1- Dr. Dina M. Bitar (Head of committee):.....

2- Dr. Omar Hamarsheh (Internal Examiner):.....

3- Dr. Kamel Adwan (External Examiner):.....

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Dedication

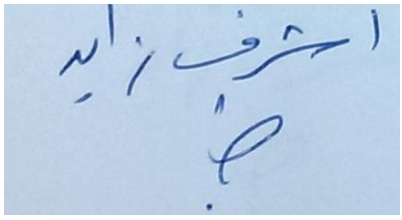
To my mother and father…
To my dear brother, Majd…
To my family…
To my friends…
To my teachers…
To all the people who supported, and encouraged me.

Ashraf Rashad Nayef Zayed

Declaration:

I certify that this thesis submitted for the degree of Master, is the result of my own research, except there otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed



Ashraf Zayed

Date: May 22, 2013

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Abstract

The isolation of *Legionella* bacteria from environmental samples poses a great challenge due to the Viable-But-Non-Culturable (VBNC) state of *Legionella*. Moreover, cultivation of this fastidious bacterium is difficult due to its slow growth and overgrowth by competing bacteria in the same sample. For that we aimed to indentify *Legionella* species (spp) from water samples using cultivation independent analysis (16S rRNA Polymerase Chain Reaction (PCR), sequencing of the 16S rRNA gene and multiple-locus variable number of tandem repeats (VNTR) assays (MLVA)).

Our sample study included 307 samples [15 (5%) water samples and 292 (95%) biofilm swabs] which were analyzed by cultivation dependent analysis (microbiological techniques) and 79 samples [15 (19%) water samples and 64 (81%) biofilm swabs] were analyzed by cultivation independent technique. The samples were collected from seven Palestinian governmental hospitals from different regions (Northern, Central and Southern) West Bank (WB), from Al-Makassed hospital in Eastern Jerusalem and from Al-Quds University main campus. Also, serological analysis was done to identify *Legionella pneumophila* serogroups (*L.pneumophila* sgs). For cultivation dependent analysis, Heterotrophic plate count (HPC) and *Legionella* count were performed. For cultivation independent analysis, DNA was extracted from the samples and analyzed for the study of bacterial population, for the presence of *Legionella* genus bacteria and for the presence of *L. pneumophila*, using 16S rRNA gene, Com, Lgsp and L1 primers respectively. The 16S rRNA gene of six *Legionella* isolates were sequenced in the Helmholtz Center for Infection Research (HZI) (Braunschweig, Germany).fourty one isolates were analysed using MLVA analysis for quality assurance, identification and classification of *Legionella* in order to identify *Legionella* strains. Furthermore, water samples were tested for physical and chemical parameters. All samples were collected, processed and analyzed according to international standard operational procedures (SOPs) ISO 11731, ISO 11731-2 and ISO 6222.

L.pneumophila was detected in all hospitals water systems and in Al-Quds University. *L.pneumophila* was isolated from 96 (31%) of 307 samples using cultivation dependent analysis and from 52 (66%) of 79 samples using cultivation independent analysis. The latter technique more than doubled the isolation rate of *Legionella* which may be due to the VBNC state of *Legionella*. *L.pneumophila* was the only *Legionella* spp that was detected in all positive samples. *L.pneumophila* sg.1 was detected in 61 (64%) of the isolates, while 35 (36%) isolates were *L.pneumophila* sg 2-14 (out of 96 isolates). Strain results showed diversity between *L.pneumophila* isolates. Indeed, classification to the strain level is important since *L.pneumophila* is an opportunistic pathogen and has a VBNC state. Strain analysis could be applied without the cultivation of the infective strains and thereby contributes to an improved surveillance of Legionnaires' disease (LD).

In this study we found that most of the critical hospital wards (pediatric, neonate, ICU, and CCU) in the WB hospitals were contaminated with *L. pneumophila*. This finding is a potential health risk to immunocompromised patients. Thus, we recommend thermal disinfection (70°C or more), regular cleaning of water systems and regular maintenance of old water systems to eliminate the risk of *Legionella* in hospitals, domestic and hotel water systems.

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List of abbreviations

ACES	N-2-acetamino-2-aminoethansulfonic acid
AFLP	Amplified fragment length polymorphism
AQU	Al-Quds University
BCYE	Buffered charcoal yeast extract
C	Celsius
CAP	Community acquired pneumonia
CFU	Colony forming unit
DALY	Disability adjusted life years
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
Dot/icm	defective organelle trafficking/ intracellular multiplication
DW	Distilled water
ELB	Enzymatic lyses buffer
ELISA	Enzyme linked immunosorbent assay
EtOH	Ethanol
EWGLI	The European working group for *Legionella* infection
FDA	Fluorescent direct antibody
FISH	Fluorescent in situ hybridization

GVPC	Glycine Vancomycine Polymyxin B Cycloheximide
HCl	Hydrochloric acid
HIA	Health impact assessment
HPC	Heterotrophic plate count
HZI	Helmholtz Center for Infection Research
IFA	Immuno fluorescence assay
ISO	International organization for standardization
KCl	Potassium chloride
KOH	Potassium hydroxide
L	liter
LD	Legionnaires' disease
LLAPs	*Legionella*- like amoebal pathogens
M	Molar
m	meter
MAb	Monoclonal antibody
mbar	millibar
mg/ml	milligram per milliliter
MIC	Minimal inhibitory concentration
min	minute
Mip	Macrophage infectivity potentiator

ml	milliliter
MLST	Multi locus sequence typing
MLVA	Multi Locus Variable number of tandem repeat Assay
µm	micrometer
MOH	Ministry of health
NaCl	Sodium chloride
ng	nanogram
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PNA	Palestinian National Authority
PWA	The Palestinian Water Authority
QMRA	Quantitative microbial risk assessment
rDNA	Ribosomal Deoxyribonucleic acid
REA	Restriction endonuclease analysis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Round per minute
rRNA	Ribosomal Ribonucleic acid
Sg	Serogroup
SOP	Standard operation procedure

Spp	Species
TEA	Tris-acetate buffer
USA	United States of America
VBNC	Viable but non culturable
WB	West Bank
WHO	World health organization

Chapter One

Introduction

1.1 Introduction

Legionella bacteria are opportunistic pathogens with widespread distribution in the water environment. The term Legionellosis includes Legionnaires' disease (LD) which is an atypical pneumonia and a nonpneumonic febrile illness called Pontiac Fever. Among the different species of the genus *Legionella*, *Legionella pneumophila* is responsible for approximately 91% of all reported community-acquired cases of Legionellosis (Adeleke et al., 2001; Benson and Fields, 1998). Many studies have demonstrated that the main sources for LD are the potable water systems in large buildings like hospitals and hotels (Fraser et al., 1977; Mavridou et al., 2008). The contamination of hospitals' water systems with *Legionella* is high risk for patients with various diseases, who may stay hospitalized for long periods of time. It is well known that LD is an important cause of hospital-acquired pneumonia (Yu and Stout, 2000).

Legionella can be found in water distribution systems, especially in main tanks, hot water plumbing when temperatures are below 50°C, showers, whirlpool spas, and cooling towers (Fields et al., 2002; Kusnetsov et al., 2003; Stout et al., 1982). Also, they are associated with biofilm that lines the inside of pipes which provides shelter and nutrients, and supports its survival and multiplication (Fields et al., 2002; Keevil, 2003; Murga et al., 2001).

Point-of-use water may be the source of the transmission of *Legionella* bacteria. Patients may be exposed to *Legionella* bacteria while showering, bathing, drinking water, and from contact to medical equipment rinsed with possibly contaminated tap water, or the hands of medical staff washed with contaminated tap water (Fiore et al., 1998; Schijven and de Roda Husman, 2005; Woo et al., 1992).

The difficulty in diagnosing LD is a common problem in many countries. This well known phenomenon may be due to the following reasons: (i) the inability to distinguish LD from other causes of pneumonia clinically, (ii) the failure to perform diagnostic tests specific for *Legionella*, (iii) the inadequate processing of specimens and lacking technical expertise for culturing and diagnosis of *Legionella*, and (iv) the shortcomings of available diagnostic tests.

Several methods are used for the identification of *Legionella*. Cultivation dependent analysis (culturing analysis) of the hospital water system for *Legionella* is the first step in the evaluation of the risk for hospital-acquired LD. This approach is recommended in the national guidelines for most of European countries, especially those encountering cases of LD (Pei-Yi et al., 2008). Also, cultivation independent analysis (molecular analysis) of the water samples is important since *Legionella* species (*Legionella* spp) occur in a Viable-But-Non-Culturable (VBNC) state. This VBNC state most likely explains why *L. pneumophila* cannot be isolated from aquatic environments that are suspected source of infection using cultivation dependent analysis (Steinert et al., 2002). Furthermore, cultivation of this fastidious bacterium is difficult due to its slow growth and overgrowth by competing bacteria in the same sample (Nederbragt et al., 2008; Steinert et al., 1997; Steinert et al., 2002).

1.2 Problem statement

Contamination of the hospital water supply with *Legionella* bacteria is a well-known risk factor for pneumonia and hospital-acquired pneumonia (Benin et al., 2002; Yu and Stout, 2000). Presence of *Legionella* bacteria in water distribution systems actually is a serious health risk to hospital staff and the patients, but the magnitude of the problem is largely

unrecognized and there are no specific guidelines for protecting patients from exposure in our hospitals (Fiore et al., 1998; Schijven and de Roda Husman, 2005; Yu and Stout, 2000).

No study was done previously in Palestine employing the molecular identification of *Legionella* spp in water samples which are considered as opportunistic pathogens that cause Legionellosis. Shareef and Mimi focused on testing hospital tap water systems as a source of Legionellosis in West Bank hospitals using bacteriological and serological methods (Shareef and Mimi, 2008). In view of the fact that *Legionella* are difficult to isolate using microbiological methods due to a viable but nonculturable (VBNC) state which leads to misdiagnosis of Legionellosis. Furthermore, cultivation of this fastidious bacterium is difficult due to its slow growth and overgrowth by competing bacteria in the same sample (Nederbragt et al., 2008; Steinert et al., 1997; Steinert et al., 2002) Therefore, we chose to identify *Legionella* by cultivation-independent methods (molecular techniques), supported with microbiological and serological techniques to give a clear picture of *Legionella* prevalence in Palestine.

1.2 Goals

The purpose of this study is to identify the level of *Legionella* contamination in West Bank hospitals water supplies using molecular, microbiological and serological techniques. Also, to examine biofilms in distribution water systems by taking swabs from interior surfaces of faucets and showerheads and analyze them for the presence of *Legionella*. Detection of *Legionella* would provide an early warning system to all collecting sites and identification of the critical sites (hot spots). Finally, to compare the detection of *Legionella* species, serogroups, and strains based on molecular and serological techniques in Palestinian water supplies to other countries.

1.4 Questions

- What is the prevalence of *Legionella* in Palestine?
- Is there a difference between *Legionella* Genus, species, and serogroups between the Middle East and Europe?

1.5 Hypothesis

Legionella are opportunistic bacteria that cause atypical pneumonia and hospital acquired pneumonia for hospitalized and immunocompromised patients. Also, *Legionella* are difficult to cultivate due to VBNC state, slow growth and overgrowth by competing bacteria in the same sample. Thus, we hypothesized to use a complete system to identify *Legionella* spp in West Bank hospital water samples using molecular techniques, microbiological and serological techniques. This complete system will reduce misdiagnosis of *Legionella* in hospital water systems.

1.6 History

In July 1976, an unidentified bacterium affected persons attending an American Legion Convention in Philadelphia, Pennsylvania, causing a common source outbreak of pneumonia. Approximately 15% of the cases were fatal. The cause of the Philadelphia outbreak was unknown for months in spite of determined laboratory examination. An epidemiologic analysis determined that the disease most likely was airborne and primarily focused at one convention hotel, which later had to be closed because of adverse publicity (Fraser et al., 1977). After six months, Joseph McDade and Charles Shepard announced that they had discovered the etiologic agent, a fastidious Gram-negative bacillus (McDade et al., 1977). Because of the historical association with the American legion convention, this disease was called Legionnaires' disease (LD) and the etiologic agent named *Legionella pneumophila*. *L. pneumophila* belongs to the family *Legionellaceae*. Soon they learned that several prior unsolved outbreaks of pneumonia had been LD, including outbreaks in the 1950s and 1960s.

In addition, members of the genus had been isolated some 25 years earlier from sporadic cases of pneumonia (Winn, 1988). Also, an unsolved outbreak of a nonpneumonic febrile illness was found to be resulted from exposure to *Legionella* bacteria; this illness was called Pontiac fever (Glick et al., 1978; Tossa et al., 2006). As with LD, past epidemics of Pontiac fever had happened as early as 1949 without solved etiology. Cases of LD have been traced to a wide variety of fabricated water sources, including cooling towers, spas, fountains, and whirlpools. (Winn, 1988).

1.7 Microbiology

Legionellaceae consists of the single genus *Legionella*. *Legionella* are Gram-negative coccobacilli that measure 0.3 µm to 0.9 µm in width and 2 to 20 µm in length. In clinical specimens and tissue, the organisms are coccobacillary; measuring 1 to 2 µm. Elongated filamentous forms may be seen after growth on some culture media. Soluble iron and L-cysteine are required for optimal growth. Also, it is essential for the initial isolation of the bacterium from both clinical and environmental sources. Iron, L-cysteine, α-ketoglutarate, and charcoal-containing yeast extract agar buffered with an organic buffer (BCYEα agar) is the ideal growth medium for clinical isolation. In addition, the pH of the agar is important and should be adjusted to pH 6.9 by adding N-2-acetamino-2-aminoethansulfonic acid (ACES) (Maiwald et al., 1998).Differently from most bacterial species, *Legionnellae* use protein for energy source rather than carbohydrates. *Legionnellae* are obligate aerobes, and grow at temperatures between 20°- 42°C and are killed at temperatures above 50 °C. The optimal conditions for *Legionella* species growth is incubation at 35°C in humidified air on BCYEα medium for 2 to 5 days. In rare cases, the isolation of unusual *Legionella* species needs incubation up to 10 days.

The number of documented species and serogroups (Sg) of the genus *Legionella* increase. There are currently 50 species (http://www.dsmz.de/bactnom/bactname.htm) comprising about 70 diverse Sgs. *L. pneumophila* comprises at least 15 different Sgs; six other species comprise two different Sgs, with the remaining species comprising only one Sg each.

Legionella species and serogroups associated with diseases are shown in (Table 1.1) (Benson and Fields, 1998; Fields et al., 2002). Some *legionella* spp. cannot grow on routine *Legionella*-specific media and have been named *Legionella*-like *amoebal* pathogens (LLAPs). These *legionellae* have been isolated and maintained by coculturing the bacteria with their protozoan hosts. One LLAP strain was isolated from the sputum of a pneumonia patient by enrichment in *amoebae* and is considered a rare human pathogen (Fig. 1.1) (Rowbotham, 1993). Additional LLAP strains may be human pathogens, but proving this is difficult because they cannot be detected by conventional techniques used for *Legionellae*.

Legionella spp. are ubiquitous. They are found in natural aquatic environments like rivers, streams, lakes and thermal pools, in moist soil and in mud. They have even been found in the sunshade of the rain forest (Koide et al., 1999; Parthuisot et al., 2010; Steele et al., 1990). Environmentally, the organisms are able to survive in moist soil for long periods and can survive temperatures of 0–68°C and a pH range of 5.0–8.5. They can survive chlorination, therefore enter water supply systems, and proliferate in thermal habitats, like air-conditioning systems, cooling towers, showerheads, hot water systems taps, whirlpool spas and respiratory ventilators. Also, *Legionellae* are found in biofilms on the surfaces of these systems (Dondero et al., 1980; Winn, 1988; Woo et al., 1992).

The majority of cases of Legionellosis can be traced to man-made aquatic environments where the water temperature is higher than ambient temperature. Coexisting microorganisms, which provide nutrients, and free-living *amoebae* in which the *Legionella* spp. can live and multiply. Two factors that can increase the risk of LD are the presence of the bacteria in an aquatic environment and warm water temperature. Also the presence of nutritional factors is important that allow the bacteria to amplify. *Legionellae* survive in aquatic and moist soil environments as intracellular parasites of free-living protozoa (Rowbotham, 1980). Thermally changed aquatic environments can shift the balance between protozoa and bacteria, resulting in rapid multiplication of *Legionellae*. However, multiple strains may colonize water-distribution systems, but only a few specific species will cause disease in patients exposed to the water.

LD is a major concern of public health professionals and individuals involved with the maintenance of water systems, such as air-conditioning systems, cooling towers, and circulating water systems. Generally, Legionellosis is considered a preventable illness because controlling or eliminating the bacterium in certain reservoirs will theoretically prevent disease. This theory of preventable illness has resulted in a number of guidelines and control strategies aimed at reducing the risk of Legionellosis in water systems. The factors that lead to outbreaks or cases of LD are not completely understood, but certain trials are considered prerequisites for infection. These include the presence of virulent bacteria in an aquatic environment, amplification of the bacterium to an unknown infectious dose, and transmission of the bacteria via aerosol to a human host that is susceptible to infection.

Approximately 90% of all Philadelphia outbreak cases of LD were caused by *L. pneumophila* Sg 1 (Benin et al., 2002; Yu et al., 2002). Despite, *L. pneumophila* Sg 1 accounts approximately 90% of American and European *Legionella* isolates, but in Australia and New Zealand, *L. pneumophila* Sg 1 accounts for only approximately 50% of cases of community-acquired legionellosis, whereas *L. longbeachae* accounts for approximately 30% of cases (Yu et al., 2002). *L. pneumophila* Sg 1 can be divided into multiple subtypes using a variety of serologic, other phenotypic and genetic methods. One particular subtype of *L. pneumophila* Sg 1 causes 67% to 90% of cases of LD. This subtype is distinguished by its reactivity with a particular monoclonal antibody, and it is termed the Pontiac, the Joly monoclonal type 2 (MAb2), or the Dresden monoclonal type 3/1 (MAb 3/1) subtype (Helbig et al., 2002). Because of the diversity within *L. pneumophila* Sg 1, clinical and environment, isolates must be matched by molecular techniques to adequately identify environmental sources of disease. These include ribotyping, amplified fragment length polymorphism (AFLP) analysis, pulsed-field gel electrophoresis (PFGE), restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP) analysis, multi locus sequence typing (MLST) and arbitrarily primed PCR (Fry et al., 1999). One of these methods, a single-endonuclease, amplified fragment length polymorphism analysis method by which the patterns are resolved by standard agarose electrophoresis, was adopted as an international standard and is now widely used by members of the European Working Group for *Legionella* Infections (EWGLI) (Fry et al., 2002; Fry et al., 2000).

Table 1.1: Table of *Legionella* species and serogroups (Benson and Fields, 1998; Fields et al., 2002).

Species	No. of Serogoups associated with diseases	Species	No. of Serogoups associated with diseases
L. pneumophila	15	*L. jordanis*	1
L. longbeachae	2	*L. dumoffii*	1
L. sainthelensi	2	*L. gormanii*	1
L. bozemanae	2	*L. lansingensis*	1
L. feeleii	2	*L. maceachernii*	1
L. hackeliae	2	*L. micdadei*	1
L. erythra	2	*L. oakridgensis*	1
L. anisa	1	*L. parisiensis*	1
L. birminghamensis	1	*L. tucsonensis*	1
L. cincinnatiensis	1	*L. wadsworthii*	1

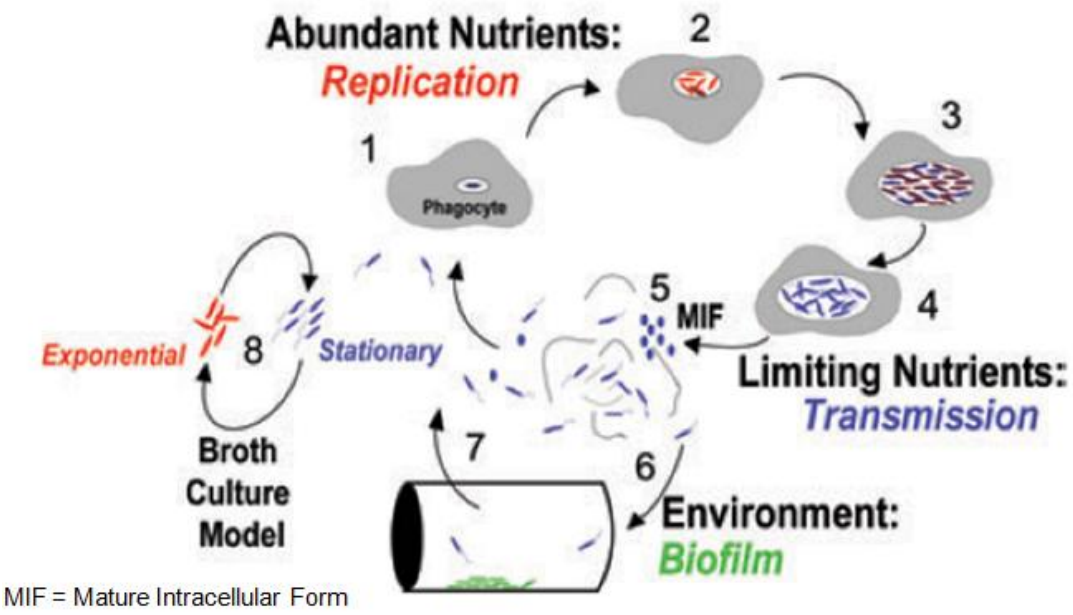


Figure 1.1: *L. pneumophila* – biphasic life cycle (Molofsky and Swanson, 2004).

1.8 Ecology

In 1979 *L.pneumophila* was isolated from water samples (Fliermans et al., 1979). Most *Legionella* spp are found in freshwater lakes, streams and municipal water distribution systems (Dennis et al., 1982; Kusnetsov et al., 2003; Stout et al., 1982).However, *L.longbeachae* has been detected in soil and potting mixes (Koide et al., 1999; Steele et al., 1990). In 1983, Rowbotham reported that *Legionellae* could multiply inside cells of *Acanthamoeba* (Bitar et al., 2004; Rowbotham, 1980). Thus, one amoebic cell could host >1000 *legionella* bacteria. At least 16 species of protozoa have been found to harbor or host *Legionella* spp (Fields et al., 2002). Also, they can persist in *amoebal* cysts, which might be important for survival and dissemination in adverse environmental conditions.

Legionellae can be found in water distribution systems, especially in hot water plumbing, when temperatures are below 50°C (Fields et al., 2002; Kusnetsov et al., 2003; Stout et al., 1982).Also, they are found growing in the biofilm that lines the inside of pipes (Fields et al., 2002; Keevil, 2003; Rogers et al., 1994).Additionally, some parts of water distribution

systems are especially prone to contain *Legionellae*, like blind loops, plumbing fixtures, showers, whirlpool spas, and cooling towers.

1.9 Environmental investigations

Legionella spp are ubiquitous in water distribution systems and in aquatic habitats (Dennis et al., 1982; Stout et al., 1982). Mostly, the infecting *Legionella* bacteria are acquired from water, especially potable water, either by microaspiration or by inhalation of aerosols (Fiore et al., 1998; Yu, 1993). Also, thermal baths have been found to be a source of infection with *L.pneumophila* (Molmeret et al., 2001) and potting mixes have been shown to be an important source for *L.longbeachae* infection. Therefore, it is important to relate patient strains to environmental isolates in epidemiological investigations.

Legionellae are cultured from water. Hot water systems and the biofilm of plumbing fixtures are the samples of choice (Kusnetsov et al., 2003; Stout et al., 1982). Currently, the method of choice for concentration of the sample by filtration of water through a black filter (pore size 0.45 μm) (Ta et al., 1995). The filter is placed on the growth medium then can be screened in a low power microscope for colonies with typical cut glass appearance. The culture of *Legionellae* from water distribution systems is performed much the same way as has been described previously for clinical samples (Edelstein, 1981; Ta et al., 1995). Acid treatment of samples and culture on selective media is of main importance.

1.10 Mode of transmission

Legionellae infect humans due to inhalation of aerosols (Fiore et al., 1998; Swanson and Hammer, 2000; Watson et al., 1994). This theory is supported by the fact that patients in case-control studies have been infected by passing outside buildings, where the source of the causative *legionella* strain has been found (Fiore et al., 1998; Watson et al., 1994). Some individuals have been at a distance of several 100 m from the source. Also, a whirlpool spa, and a shower humidifier may create an infectious aerosol (Breiman et al., 1990; Woo et al.,

1992). In the Netherlands, in the large outbreak at a flower show the proximity of individual persons to the source, a whirlpool spa, was correlated to the risk of acquiring LD (Boshuizen et al., 2001).

However, some authors suggest that aspiration is more important than inhalation in mode of transmission (Blatt et al., 1993; Pedro-Botet et al., 2002). This is likely in a hospital environment with immunocompromised patients who have poor respiratory tract reflexes. In such cases, drinking water contaminated with *Legionella* is a possible risk. In the Philadelphia outbreak, only drinking water at the hotel was the statistically significant relationship that could be associated with disease in a case control study (Fraser et al., 1977). However, Inhalation of aerosols is perhaps an important factor (Fiore et al., 1998).

The causes of transmission are cooling towers, evaporative condensers, mist machines, whirlpool spas and showers. An air conditioning system is only hazardous if a cooling tower or evaporative condenser is positioned in such a way that the generated aerosol can pass into the air intake of a building or be directly transmitted to a passerby bystander (Dondero et al., 1980; Fiore et al., 1998).

Inhalation or microaspiration of *amoebae* could be a potential risk, since one single *amoeba* might harbor more than 1000 *Legionella* cells (Rowbotham, 1980). Moreover, intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances the virulence of *L.pneumophila* (Bitar et al., 2004; Cirillo et al., 1999; Fields et al., 2002; Swanson and Hammer, 2000). Thus, it is possible that infection in humans may require the presence of both *Legionellae* and an *amoebal* host (Swanson and Hammer, 2000). This might explain why the attack rate in LD outbreaks is low, despite the presence of *Legionella* spp in the plumbing system. Breiman *et al* describe the occurrence of LD cases related to the presence of both protozoa and *L.pneumophila* (Breiman et al., 1990).

1.11 Epidemiology:

Outbreak of LD is still unclear, but certain events are considered prerequisites for infection. Like multiplication of the bacterium within protozoa, the presence of the bacterium in an

aquatic environment, and transmission of bacteria via aerosols to a human host (Balbus et al., 2004; Fields, 1996).

An essential part of public health practice in the setting of national and international policies and guidelines through to the decisions about building design and disinfection practices has become Environmental Health Impact Assessment (HIA). One vital step in HIA is the assessment of disease risk using Quantitative Microbial Risk Assessment (QMRA). QMRA can provide information on the probability of infection from exposure to pathogens in drinking and bathing water, which is not always possible with epidemiological studies. This requires not only quantitative data on infectious waterborne pathogens but also on their fate and transport in the aquatic environment. The latter may be assessed in the laboratory, pilot or field experiments under possibly relevant natural or induced climate change conditions yielding for instance pathogen inactivation rates. QMRA was successfully applied before in a pilot study predicting infection risk for waterborne pathogens in drinking and bathing water influenced by different climate change situations (Schijven and de Roda Husman, 2005). Despite this accepted QMRA model for LD, different standards and thresholds across Europe for *Legionella* detection and requested public health measures are used. For example, in the Netherlands specific safety procedures are requested if more than 1000 CFU/liter occur in the drinking water whereas in the United Kingdom this is the case if more than 100 CFU/l are detected. The USA has an even higher threshold of 10,000 CFU/l.

Yet most QMRA studies have been restricted to estimating probability of infections. Clinically, not all infections lead to relevant disease and even when disease occurs, severity can vary considerably. Most recent reports have used static estimates of the probability that infection will lead to disease and have used DALY *(*Disability-Adjusted Life Years) scores, the disease burden metric preferred by WHO, than to estimate water-related disease burden. The problem with relying on a single infection to disease ratio is that this will vary substantially from one setting to another, largely due to immunity from previous infections that may or may not be have been due to water exposure. This is likely to be a significant issue in country populations. Dynamic epidemiological risk assessment as projected by Balbus et al (Balbus et al., 2004; Hunter et al., 2009), overcomes this limitation and should be

included in this analysis. A further under-researched issue is the problem of susceptible sub-populations. People are susceptible if they are more likely to suffer from an infection or are more likely to have more severe disease. Susceptibility to waterborne disease associated with the susceptibility of people, is given e.g. for the poor, the elderly and/or immunocompromised people, and is of high relevance for the *Legionella*-based lung infections.

1.11.1 Country-specific epidemiology of Legionellosis:

According to the current epidemiological data available from the world, different *L.pneumophila* Sgs cause Legionellosis. In European, American and Australian societies, most of the cases were due to infections with *L.pneumophila* Sg1 (Coil et al., 2008; Huang et al., 2004; Palmore et al., 2009). In general, *L.peumophila* Sg1 is responsible for more than 60% approximately of cases in most European and American countries (Yu et al., 2002) but lower percentage of cases about 50% in countries such as Australia and New Zealand (Yu et al., 2002). Whereas in the Middle East there is a shortage of epidemiological data for *Legionella* Sgs. A recent survey in Israel indicates that *L.pneumophila* Sg3 might be the primary etiological agent responsible for Legionellosis (Oren et al., 2002). Also, a recent study of clinical isolates from Kuwait demonstrated the dominance (more than 80%) of *L.pneumophila* Sg3 in patients with LD (Qasem et al., 2008).

1.12 Virulence

Legionella spp have developed mechanisms for invasion and multiplication in protozoan hosts in the course of evolution. The same mechanisms of intracellular invasion that they use when multiplying in *amoebae* seem to operate in alveolar macrophages (Fields et al., 2002; Swanson and Hammer, 2000). The most important pathogenicity factor to be characterized was the macrophage infectivity potentiator protein (*mip*) which is encoded by the *mip* gene. The protein is an enzyme called peptidyl prolyl cis-trans isomerase, which is exposed to the bacteria surface, where it seems to influence intracellular establishment (Cianciotto et al.,

1989; Fields et al., 2002; Helbig et al., 2003a; Swanson and Hammer, 2000). The exact mechanism and role in pathogenicity are still unknown.

The mechanism can be summarized as, when *amoebae* or macrophages have ingested virulent *Legionella* cells, a phagosome is established; this is surrounded by endoplasmic reticulum and becomes completely isolated from the endosomal pathway (Fields et al., 2002; Swanson and Hammer, 2000). Initially, fusion with lysosomes is inhibited. It has been postulated that *L.pneumophila* converts to a replicate form in this protected environment, at which time it no longer expresses virulence traits but becomes acid and sodium chloride (NaCl) tolerant (Swanson and Hammer, 2000). Accordingly, endosomes containing the pathogen are able to fuse with lysosomes enabling the intracellular bacteria to make use of a nutrient-rich niche, which in ordinary circumstances would kill other bacteria. When the amino acid supply is depleted, the cells convert to a stationary phase form, simultaneously developing features that are needed for transmission to a new phagocyte. *Legionella* cells released from eukaryotic cells are short, thick, and highly motile. Thus, they exist in nature in two phases. In this system, a number of factors are implicated including type II and IV secretion, acquisition of iron, pore-forming toxins, and induction of apoptosis in the host cell. The intracellular establishment and trafficking of *Legionellae* are believed to be regulated by the dot/icm (defective organelle trafficking/intracellular multiplication) gene complex, which encodes the substances involved in type IV secretion (Fig. 1.2). Also, *Legionellae* produce extracellular cytotoxins. Experimental work indicates that virulence is significantly reduced when the incubation temperature of a cultured inoculum is reduced from 37°C to 24°C (Mauchline et al., 1994).

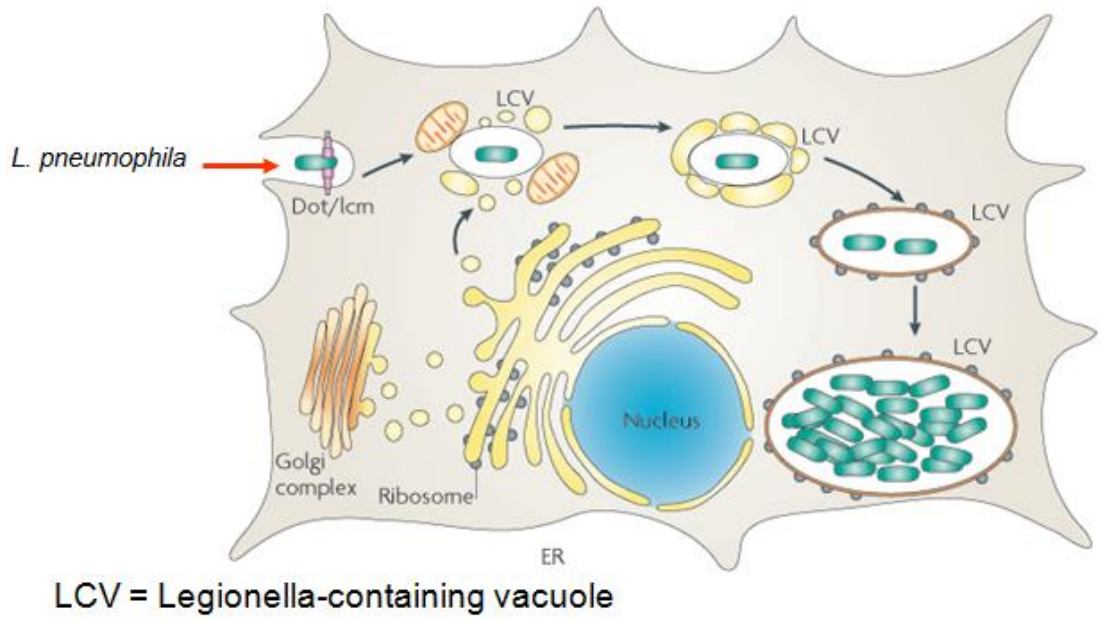


Figure 1.2: *L. pneumophila* invasion of an *amoeba*/macrophage (Isberg. and al., 2009).

1.13 Clinical features

Clinically Legionellosis provides two different diseases, first harmful disease which is LD, an atypical pneumonia with severe multisystem disease (McDade et al., 1977), and the second less harmful, a self-limited flu-like illness which is called Pontiac fever (Glick et al., 1978). In addition, many persons who are infected with *Legionellae*, will remain asymptomatic which can be confirmed by seroconversion. (Boshuizen et al., 2001). LD is transmitted from the environment by inhalation of an aerosol contaminated with *Legionellae* (Fraser, 1980). In unknown cases, microaspiration of contaminated water into the lungs could be the mode of nosocomial transmission of Legionellosis (Marrie et al., 1991). Commonly, many examples of exclusive aerosol transmission of LD exist, especially in epidemic sites where a cooling tower, water fountain, water spa or water mister are the source of disease (Fields et al., 2002).

Legionellosis can be sporadic or part of an outbreak. Legionellosis cases are reported throughout the year. Overall cases occur in the summer and autumn, because warmer weather encourages proliferation of the bacteria in water. Generally, Legionellosis occur in the

middle-aged and elderly persons, in total it occurs in people who have impaired respiratory and cardiac function, and who are heavy smokers or immunocompromised (Broome and Fraser, 1979; Marston et al., 1994).

Two to ten days is the incubation period of LD. Among patients of the Bovenkarspel outbreak, the reported incubation period was 2 - 19 days. In 16% of cases, the time before onset of illness exceeded 10 days (Boshuizen et al., 2001). A prodromal illness may occur, lasting for hours to several days, with symptoms of headache, myalgia, asthenia, and anorexia. Clinically, it is not possible to distinguish patients with LD from patients with pneumococcal pneumonia. Several studies have shown that the two diseases have nearly identical clinical and radiological picture, also the nonspecific laboratory test results cannot differentiate between the two diseases (Edelstein, 1993; Fields et al., 2002; Granados et al., 1989; Roig et al., 1991). General features of LD include fever, nonproductive cough, myalgias, rigors, dyspnea and diarrhea (Tsai et al., 1979). Neurological symptoms range begins from headache and lethargy to encephalopathy. Change in mental status is the most common neurologic abnormality (Morelli et al., 2006). Suspicion should be raised in cases of pneumonia and the presence of headache, confusion, hyponatremia, elevated creatine kinase (Pedro-Botet and Sabria, 2005). In addition, the diagnosis becomes more likely if an acute consolidating pneumonia fails to respond to several days of β-lactam antibiotic therapy, or if the pneumonia is severe enough to need intensive care unit hospitalization. Epidemiologic evidence might include use of a hot tub or recreational spa; recent pneumonia of a co-worker, relative, or fellow traveler; and recent plumbing work done at home or work. The nonspecific presentation of LD makes clinical diagnosis very difficult and mandates empiric therapy for this disease in most patients with community acquired pneumonia (CAP) of uncertain etiology. The key to diagnosis is performing appropriate microbiologic testing.

Twenty *Legionella* spp have been documented as human pathogens based on their isolation from clinical material in addition to the most common *L.pneumophila*. Pneumonia due to non-*pneumophila Legionella* species resembles, both clinically and radiographically, that due to *L. pneumophila* (Muder and Yu, 2002*)*. Similar to *L. pneumophila*, other *Legionella* species are

inhabitants of natural and man-made aqueous environments. The majority of confirmed infections involving non-pneumophila *Legionella* species has occurred in immunosuppressed patients (Muder and Yu, 2002).

Rarely, LD is a cause of pneumonia in children; most of them are immunosuppressed (Greenberg et al., 2006). All cases of LD in neonates were hospital-acquired, and most patients had potential risk factors including prematurity, bronchopulmonar dysplasia, and corticosteroid use (Greenberg et al., 2006).

Mortality rates are extremely variable with the range from less than 1% - 80% depending on the underlying health of the patient. The rapidity of specific therapy, and whether the disease is sporadic, nosocomial, or part of a large outbreak (Benin et al., 2002). Fatality rates of nosocomial disease have declined by more than 50% in the United States over the past 20 years; also, a similar but less dramatic decrease in death rates of community-acquired cases has been observed. The declines in mortality rates appear to result from better and faster disease recognition, especially through use of the urinary antigen test. In addition, widespread use of empiric therapy for pneumonia that includes drugs active against *L. pneumophila* (Benin et al., 2002).

1.14 Diagnosis

Legionella spp. diagnostic methods have improved since 1976. There is no available test able to diagnose all *Legionella* spp. with a high degree of sensitivity and specificity. Most of the data are applicable to *L. pneumophila*, since sensitivity and specificity estimates for non *pneumophila* species are not known (Formica et al., 2001; Leland and Kohler, 1991; Murdoch, 2003; Pasculle et al., 1989).

1.14.1 Culture

The best standard for diagnosis of LD is isolation of *Legionella* spp., which has a specificity of 100%. Culture diagnosis requires special media, adequate processing of specimens, and technical skill. Also, several days are required to obtain a positive result, with most *Legionella*

spp. colonies being detected within 7 days. Species other than *L. pneumophila* may grow at a slower rate and may be detectable only after 10 days of incubation (Fields et al., 2002). The standard medium used for *Legionellae* is BCYE agar supplemented with α-ketoglutarate, with or without antimicrobial agents. Commonly added antibiotics are polymyxin to control Gram-negative growth, anisomycin against yeasts, and cefamandole or vancomycin against Gram-positive bacteria. Vancomycin should be chosen if culture is aimed at species other than *L. pneumophila*, because cefamandole inhibits some *Legionella* spp. that do not produce beta-lactamases (Lee et al., 1993).

Legionella can be isolated from several sample types. The samples of choice are lower respiratory tract secretions especially sputum and bronchoscopy samples. Culture yield depends on the severity of illness, with the lowest yield from 15% to 25% in mild pneumonia and the highest yield more than 90% for severe pneumonia causing respiratory failure (Murdoch, 2003). A major limitation of sputum culture is that less than one-half of patients with LD produce sputum (Murdoch, 2003; Sopena et al., 1998; Tsai et al., 1979). Some patients with LD produce sputum that has relatively little purulence; these samples may be rejected by laboratories that discard sputum samples containing few polymorphonuclear leukocytes. However, up to 84% of *L. pneumophila*-positive samples would have been discarded by using established sputum purulence screens and they recommend acceptance of all specimens submitted for *Legionella* culture (Ingram and Plouffe, 1994). Estimated sensitivities of sputum culture range from less than 10% - 80% and vary according to different comparison standards and by individual laboratories (Fields et al., 2002; Murdoch, 2003). In practice, the better results are likely to be achieved only by laboratories with a special interest in *Legionella* infection. Because *Legionella* spp are fastidious and not easily detected by culture due to the occurrence of a Viable-But-Non-Culturable (VBNC) state known for many *Legionella* spp (Fields et al., 2002).

1.14.2 Serology

Detection of antibodies in patients was instrumental in determining the cause of the illnesses by the indirect immunofluorescence assay (IFA). Since a number of serologic test methodologies have been developed to detect antibodies to *Legionella* spp. of the various

antibody detection methods that are available, IFA and enzyme-linked immunosorbent assays (ELISA) are the most commonly used. Nowadays, ELISA assays are favored by many laboratories because they are more accurate and less subjective than IFA testing and additionally have the potential for automated performance (Boshuizen et al., 2003; Malan et al., 2003). The reported sensitivities of serological assays vary from 41% - 94% (Den Boer and Yzerman, 2004). Seroconversion may take several weeks, which is a major limitation of serological testing. Approximately 25%- 40% of patients with LD seroconvert within the first week after the onset of symptoms (Berdal et al., 1979). In most cases, a 4-fold increase in antibody titer is detected within 3-4 weeks, but in some cases, this may take more than 10 weeks (Monforte et al., 1988). Acute-phase reciprocal IFA antibody titers of $\geq$ 256 in the presence of pneumonia were once considered sufficient for a presumptive diagnosis, but this has been shown to be unreliable, especially given the high prevalence of *Legionella* antibody positivity in persons without clinical evidence of Legionellosis (Plouffe et al., 1995).

The specificity of seroconversion using *L. pneumophila* Sg 1 antigen in IFA has been reported to be approximately 99% (Wilkinson et al., 1981).In contrary, a disadvantage of serological testing is the inability to detect all *Legionella* species and Sgs accurately. Although seroconversion to *L. pneumophila* Sg 1 is generally regarded as being highly diagnostic, the sensitivity and specificity of seroconversion to other species and Sgs has not been rigorously confirmed (Murdoch, 2003).

1.14.3 Detection of *Legionella* antigen in urine

After the Philadelphia outbreak the detection of *Legionella* antigenuria has been used (Berdal et al., 1979). *Legionella* antigenuria can be detected after onset of symptoms and persists for days to weeks. In one instance, excretion of antigen was documented to occur for more than three hundred days (Kohler et al., 1981). The antigen detected is a part of the lipopolysaccharide portion of the *Legionella* cell wall and is heat stable (Kohler et al., 1981). The urinary antigen tests join logical sensitivity and high specificity with rapid results. It has revolutionized the laboratory diagnosis of LD, making it the most common laboratory test for diagnosis (Formica et al., 2001).

Two commercial kits methodologies have been widely used and available which are enzyme immunoassay (EIA) and radioimmunoassay (RIA) (Fields et al., 2002). Immunochromatographic like EIA assays have similar sensitivity and specificity (Helbig et al., 2003b). Moreover, agglutination assays have been used, but they don't have acceptable sensitivity and specificity (Leland and Kohler, 1991). The majority are most sensitive for the detection of (MAb2) monoclonal antibody type of *L. pneumophila* Sg 1 of the Pontiac fever for up to 90% of cases, less sensitive for other monoclonal antibody types of *L. pneumophila* Sg 1 approximately 60%of cases, and poorly sensitive for other *L. pneumophila* Sgs and other *Legionella* spp just for 5% of cases (Dominguez et al., 2001; Harrison et al., 1998). The average sensitivity of this test is between 70% - 80%, because the Pontiac subtype of *L. pneumophila* Sg 1 causes the majority of cases of community-acquired LD approximately 90%. An important characteristic of these tests is their high specificity approximately 99%, which is a requirement when testing a comparatively rare disease.

1.14.4 Detection of *Legionella* nucleic acid

Since *Legionella* spp are fastidious bacteria and difficult to cultivate, cultivation-independent techniques, including fluorescent antibody (FDA), fluorescent in situ hybridization (FISH) and PCR-based methods are now used to identify single species (Newton et al., 2006). High resolution identification of different serotypes is possible using Multi Locus Sequence Typing (MLST) (Luck et al., 2007) and Multi Locus Variable number of tandem repeat Assay (MLVA) (Nocker et al., 2006). It was demonstrated that high resolution genotyping of clinical material could be used to identify the environmental source of a *Legionella* infection (Luck et al., 2007).

A radiolabeled ribosomal probe specific for all strains of *Legionella* spp was the first assay designed to detect the DNA of *L. pneumophila* (Gen-Probe, San Diego, Calif.). Researchers reported varying sensitivity and specificity for this assay (Pasculle et al., 1989; Wilkinson et al., 1986). The use of the probe at one hospital resulted in 13 false-positive cases (Laussucq et al., 1988) and the assay was removed from the market immediately after this pseudo-outbreak.PCR has many advantages. PCR enables specific amplification of very small

amounts of *Legionella* DNA and can provide results within a short time. Also, it has the potential to detect infections caused by any *Legionella* spp and Sg. Real-time PCR has extra advantages to diagnosis; it minimizes manual time for the PCR and gives quantitative results. Principally, diagnostic PCR assays have targeted specific DNA regions within 16S rRNA genes (Reischl et al., 2002; Stolhaug and Bergh, 2006; Wellinghausen et al., 2001), the 23S-5S spacer region (Herpers et al., 2003), 5S rDNA (Reischl et al., 2002), or the macrophage inhibitor potentiator (*mip*) gene widely used for *L.pneumophila* (Ratcliff et al., 1998; Wellinghausen et al., 2001; Wilson et al., 2003). When testing samples from the lower respiratory tract, PCR has been shown to have sensitivity equal to or greater than culture (Cloud et al., 2000; Templeton et al., 2003). Indeed, PCR is considered the test of choice for patients who produce sputum by some authors (Murdoch, 2003). However, a number of false-positive results have been reported, with commercially available tests (Fields et al., 2002). *Legionella* DNA can be detected in several samples like urine, serum, and leukocyte samples obtained from patients with LD with sensitivities of 10%-86% (Helbig et al., 1999). The application of PCR to non-respiratory samples looks particularly attractive, because this will avoid the problem of patients who do not produce sputum. *Legionella* PCR is only available in a limited number of laboratories that use a variety of commercial assays (Fields et al., 2002; Ginevra et al., 2005).

1.15 Treatment

In the Philadelphia cases, mortality has decreased with the increased index of suspicion by physicians, early empirical treatment with antibiotics that cover *Legionella* spp. and the beginning of rapid laboratory tests. Mortality rates increased when delay in starting with appropriate therapy (Heath et al., 1996). In the period from 1980-1998 reported data from a large-scale study by the Centers for Disease Control and Prevention showing a decrease in the case-fatality rate for community-acquired *Legionella* pneumonia from 26% to 10% (Benin et al., 2002). This finding is in agreement with recent studies of patients with outbreak-related LD who received rapid diagnoses which was confirmed by urine antigen testing; these studies have reported case-fatality rates up to 5.5% (Plouffe et al., 2003; Yu et al., 2004).

The selection of empiric therapy for CAP is based on the meaning of providing optimal therapy, the epidemiological features of various microorganisms in the Netherlands, and an inference of the most likely pathogen (Aleva and Boersma, 2005). Primarily, the choice of antibiotic is based on the severity of illness according to the Dutch Working Party on Antibiotic Policy and Dutch Thoracic Society guideline (Aleva and Boersma, 2005). Rapid tests for detection of *L. pneumophila* antigen have a place in both guidelines. Mainly, empirical antibiotic therapy should target *Streptococcus pneumoniae* because of its high incidence of typical pneumonia cases. In addition, in seriously ill patients and those suspected of having LD, antibiotic therapy should target *L. pneumophila*. When a causative agent is identified, empirical therapy should be replaced with pathogen-directed therapy (Falguera et al., 2010).

Legionella spp are intracellular pathogens. Antimicrobial agents that achieve intracellular concentrations higher than the minimal inhibitory concentration (MIC) are more effective than antibiotics with poor intracellular penetration (Roig and Rello, 2003). Therefore, macrolides, quinolones, and tetracyclines are most possible antibiotics to treat Legionellosis. A number of small-uncontrolled studies of the treatment of LD exist. Prospective, adequate-size clinical trials of antimicrobial therapy for LD have not been performed. Several studies have evaluated the efficacy of macrolides (erythromycin, clarithromycin and azithromycin) versus quinolones (levofloxacin) (Sabria et al., 2005). The overall mortality was 4.5% for the macrolide group and 1.1% for the levofloxacin group but this difference was not statistically significant (Sabria et al., 2005).

The ability of a drug to inhibit intracellular *L. pneumophila* usually correlates well with its clinical effectiveness for LD. Similarly, antimicrobial therapy studies using a guinea pig model of LD correlate well with drug efficiency for the treatment of the disease in humans. *In vitro* data suggest that second and third generation of macrolides (clarithromycin and azithromycin) and many fluoroquinolone agents show the best activity against *Legionella* spp. Moreover, these antibiotics have less side effects than erythromycin (Roig and Rello, 2003).

Newer macrolides and levofloxacin are licensed by the food and drug administration for the treatment of LD and are considered preferable to erythromycin. The third generation macrolides (azithromycin) have been shown to have some additional beneficial effect. However, the lack of an intravenous formulation limits the use of newer macrolides in severely ill patients (Roig and Rello, 2003; Yu et al., 2011).

1.16 Literature review

Barbaree and others (1987) isolated *L. pneumophila* from two hospitals; the first hospital was an acute care facility in the New England Area with approximately 700 beds and 28 buildings. A total of 12 of 15 legionellosis cases were from one of six main buildings, and all isolates from patients were *L.pneumophila* serogroup 1. *L. pneumophila* were isolated from 43 of 106 samples collected (40%). *L. pneumophila* serogroup 1, 3, 5 were isolated. The second hospital was a northern Midwest pediatric hospital with approximately 300 beds. *L. pneumophila* serogroup 1 was isolated from 13 of 37 (35%) of the samples (Barbaree et al., 1987).

Borella and others (1999) reported a single case of nosocomial legionellosis was discovered in a 1000 bed hospital in Milan, Italy. The hospital's first case of hospital acquired Legionnaires' disease was a 29 year old man. He died the next day and *Legionella* was identified by immunofluorescence in lung tissue. The environmental surveillance revealed that the centralized hot water distribution system of the hospital was colonized with *L.pneumophila*. Shock heating and hyperchlorination of water was applied, which reduced the number of contaminated sites in the short term, but water was recolonised two months later. During the period of active surveillance from January 1998 to September 1999, six nosocomial cases were identified. In addition, 12 community cases were discovered (Borella et al., 1999).

Wellinghausen and others (2001) studied the contamination of hospital water systems with *Legionella* at three different hospitals belonging to the University of Ulm in Germany between October 2000 and February 2001. A total of 77 potable water samples were

collected. The rates of detection of *Legionella* were 70.1% (54 of 77) (Wellinghausen et al., 2001).

Doleans and others (2004), made a relationship between hospital water contamination with *Legionella* and hospital-acquired Legionellosis, they examined the level of *Legionella* colonization of hospital water systems in France by studying the 554 water samples predominantly collected from hospitals. The 286 positive water samples (51.6%) contained between 102 and 107 *Legionella* CFU/L, and 138 samples (48.3% of the positive samples) contained ≥103 CFU/L. Despite this frequent contamination of hospital water systems in France, hospital-acquired Legionellosis remains relatively infrequent, with about 100 cases annually (15% of all of the cases in France) (Doleans et al., 2004).

Mavridou and others (2008) studied prevalence of *Legionella* spp. in Greek hospitals. They collected water and swab samples from 13 hospitals and analyzed them for *Legionella* using cultivation independent analysis (AFLP). They detected *Legionella* in 8 out of 13 hospitals and in 22 of 130 water and swab samples. They found 72.7% of *Legionella* was *L.pneumophila* serogroup1 and 22.7% were *L.pneumophila* serogroup2-14 (Mavridou et al., 2008).

Shareef and Mimi (2008) studied hospital tap water system in West Bank hospitals. The hospitals are Jenin hospital in Jenin, Rafidia and Al-Watani hospitals in Nablus, Ramallah hospital in Ramallah, Beit Jala hospital in Bethlehem and Alia hospital in Hebron. They used cultivation dependent analysis (microbiological technique). They found *L.pneumophila* serogroup2-14 in 62% of the samples. Also, they studied the effect of thermal disinfection at 80°C as good factor to prevent from nosocomial infections (Shareef and Mimi, 2008).

Ma'ayeh and others (2008) evaluated the extent of *L.pneumophila* contamination in a dental unit water line (DUWL) at a Dental Teaching Centre in Jordan. Samples were collected from ten dental units from each teaching clinic. Sampling time was at the beginning of the working day (before the dental unit was used), after two min of flushing, and at midday.

L.pneumophila was counted between 0 and 8.35 X 10^3 CFU/ml. *L.pneumophila* was detected in 86.7% of the dental units at the beginning of the working day, 40% after two min flushing and 53.3% at midday. The highest *L.pneumophila* counts were found at the beginning of the working day which was reduced by flushing the waterlines (Ma'ayeh et al., 2008).

Kahlisch and others (2010) used single-strand conformation polymorphism (SSCP), capillary electrophoresis (CE) and real time PCR to identify *L.pneumophila*. They developed molecular techniques using multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) of *L. pneumophila* isolates for high resolution genotyping. This molecular technique will solve the problem that *L.pneumophila* is difficult to cultivate due to VBNC state. This technique facilitates detection of *L.pneumophila* (Kahlisch et al., 2010).

Lee and others (2010) studied the distribution of *Legionella* species from environmental water sources of public facilities in South Korea. They isolated 560 *Legionella* isolates from seven geographic regions (Seoul, Gyeonggi, Chungcheong, Gangwon, Gyeongsang, Jeolla and Jeju) in South Korea. 85.5% of the isolates were *L.pneumophila* with different serogroups mostly serogroup1, serogroup5, and serogroup6 with (54.7%, 11.9%, and 11.5%, respectively). The rest of the isolates (14.5%) were *non- L. pneumophila*. *Legionella anisa* and *Legionella bozemanii* predominated among *non-L. pneumophila* species (48.1% and 21.0%, respectively) (Lee et al., 2010).

Moran-Gilad and others (2012) reported clinical case of humidifier-associated pediatric Legionnaires' disease in Israel. They reported a fatal case of community-acquired LD in an infant aged less than six months. Their findings found that epidemiological and microbiological investigations suggested that a free-standing cold water humidifier using domestic tap water contaminated with *L.pneumophila* sg.1 served as a vehicle for infection. Their findings supported by sequence-based typing (SBT). Also, they reported nine pediatric cases of LD in Israel from January 2010 to July 2012. Three cases died and seven cases were nosocomial infection. Also, four cases entered ICU. *L.pneumophila* was detected using urine

Ag, PCR and culture. Two cases reported due to *L.pneumophila* sg.3 and one case due *L.pneumophila* sg.1. (Moran-Gilad et al., 2012).

1.17 Water sources in Palestine

The Palestinian Water Authority (PWA) was provided with a written informed consent prior to providing the water sources in Palestine. PWA sent a map of water sources in the West Bank (WB) (Appendix A). Jenin hospital gets its water from Al-Saadah well to Jenin Municipality water network to the Jenin hospital. Rafidia hospital gets its water from Sabastyia well, Deir Sharaf well and Ein Beit Elma' spring to Beit Elma' reservoir to Rafidia hospital. Al-Watani hospital gets its water from Al-Faraah well, Al-Bathan well, Al-Qaryoun spring and Al-Qawareen spring to Al-Jadeed reservoir to Al-Watani hospital. Ramallah hospital gets its water from Mekorot Water Company in Israel to Beitounia station to Ramallah hospital. Beit Jala hospital gets its water from Baten Al-Ghoul well to Beit Jala hospital. Al-Ahli hospital gets their water from Al-Saaer wells to Palestinian National Authority (PNA) water to Al-Ahli hospital. Alia hospital gets its water from Al-Saaer wells to Habayel Al-Reyaah reservoir to Alia hospital. Also, Al-Quds University gets its water from Al-Ezareyah reservoir. Unfortunately, we cannot get Al-Makassed hospital water sources (in occupied East Jerusalem) due to political reasons.

Chapter Two

Methodology

2.1 Inclusion criteria

Water samples and biofilm swabs from nine sites of different regions in the West Bank (WB) namely; Jenin hospital, Rafidia hospital-Nablus, Al-Watani hospital-Nablus from Northern WB, Ramallah hospital and Al-Makassed hospital- Jerusalem and Al-Quds University main campus- Abu Deis from Central WB, and Beit Jala hospital-Bethlehem, Alia hospital-Hebron and Al-Ahli hospital- Hebron from Southern WB were analyzed for the presence of *Legionella* spp in their water sources (Fig. 2.1). A written informed consent was obtained from all hospitals prior to the collection of water and biofilm samples. Also, the samples were collected with written approval from the Ministry of Health (MOH) (Appendix B). This research is part of a collaborative research project with Helmholtz Center for Infection Research (HZI) Braunschweig, Germany.

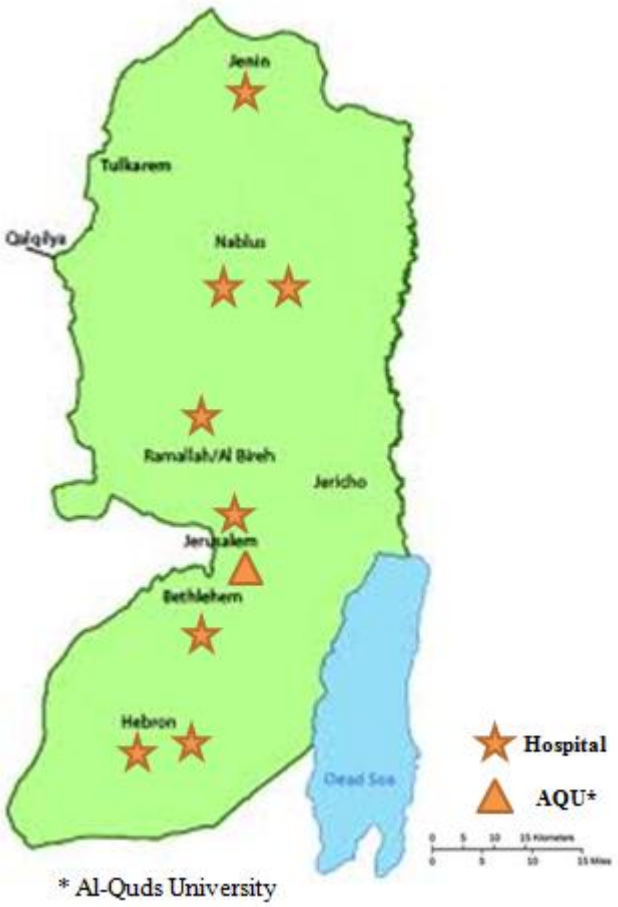


Figure 2.1: Sampling sites in the West Bank. (Jenin hospital, Rafidia and Al-Watani hospitals in Nablus district, Ramallah hospital, Al-Makassed hospital in Jerusalem district, Beit Jala hospital in Bethlehem district, Al-Ahli and Alia hospitals in Hebron district) and Al-Quds University in Abu Deis, Jerusalem.

Water samples were collected, processed and analyzed according to international standard operational procedures (SOPs). ISO 11731 for water quality; detection and enumeration of *Legionella*. ISO 11731-2 for water quality, detection and enumeration of *Legionella* part two (direct membrane filtration method for waters with low bacterial counts). ISO 6222 for water quality; enumeration of culturable microorganisms – colony count by inoculation onto nutrient agar culture medium.

2.2 Research place

The research was carried out at the Molecular Microbiology Research Lab, Al-Quds University, towers building B, Abu Dies, Jerusalem.

2.3 Scheme

Methodology scheme is summarized in (Fig. 2.2).

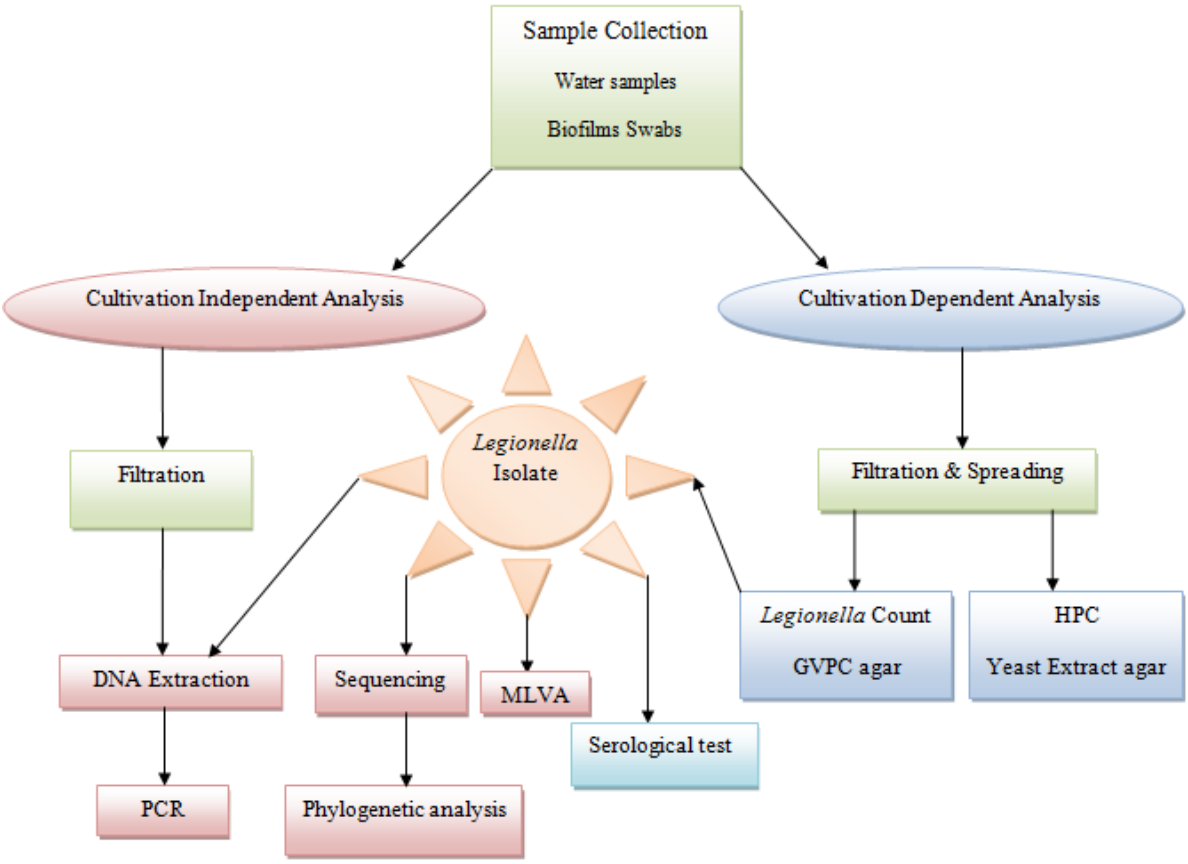


Figure 2.2: Scheme for the major analytical steps.

2.4 Sampling

2.4.1 Water samples

From November 7th through December 21st 2012, Five liters of each cold and hot water was collected for DNA extraction. One liter of each cold and hot water was collected for Heterotrophic Plate Count (HPC) and one liter of each cold and hot water was collected for *Legionella* count from eight governmental hospitals in the WB namely; Jenin, Rafidia-Nablus, Al-Watani-Nablus from Northern WB, Ramallah and Al-Makassed- Jerusalem from Central WB, and Beit Jala-Bethlehem, Alia-Hebron and Al-Ahli- Hebron from Southern WB. Samples were collected from each hospital randomly.

The samples of water were collected in accordance with the instructions for sampling, handling and preservation given in EN 25667-2 and EN ISO 5667-3.Cold and hot water samples were drawn from the main hospital tank in sterile 1L plastic bottles after a brief flow time, (2-3 min), to permit clearing the service line. Water flow was reduced to permit filling the bottles without splashing. To neutralize residual free chlorine, 0.5ml of 0.1N sodium thiosulphate was added in the sterile bottles for *Legionella* count analysis (Centers for Disease Control and Prevention CDC, 2005). Collection bottles were returned to the laboratory immediately after sampling for examination, if analysis would not begin within 24 hours, samples were kept at (5±3) °C and processed within 48 hours of collection. The test report included volume of sample, water parameters at the time of sampling, the date and time of collection of the sample, date receipt in the laboratory, and examination in the laboratory. (Appendix C).

2.4.2 Biofilm samples

From December 2011 through December 2012 a total of 292 biofilm swabs from anterior surfaces of faucets, showerheads or shower hoses were obtained for DNA extraction using sterile cotton swabs (Cotton Tipped Applicator, China) and for *Legionella* identification using transport medium (Copan, Culture swab transport system, Italy). The biofilm samples were collected from seven governmental hospitals in the WB, a Al-Makassed hospital in Eastern Jerusalem (36 swabs from Jenin, 44 swabs from Rafidia, 26 swabs from Al-Watani, 36 swabs from Ramallah, 21 swabs from

Al-Makassed, 29 swabs from Biet Jala, 24 swabs from Alia, and 33 swabs from Al-Ahli). Furthermore, 43 biofilm swabs were collected from Al-Quds University main campus. Samples were taken from each site randomly. Swabs for *Legionella* identification were processed in the laboratory by culturing on BCYE (M809, Himedia, India) and/or GVPC (M809, Himedia, India) medium immediately. The swabs for DNA extraction were kept at -20 °C for 24 hours until DNA extraction was performed.

2.4.3 Measurement of background parameters

Cold and Hot water samples were drawn in sterile bottles after a brief flow time of about three minutes. Samples were tested for temperature using electronic thermometer (ama-digit,ad 15^{th}, Germany), pH measurement and conductivity using PCE meter (PCE-PHD 1, Germany) immediately. Upon return to the laboratory water samples were tested for total iron, sensitive chlorine, nitrate, nitrite, ammonia, copper, phosphate, zinc, carbonate hardness and total hardness in water (content of calcium and magnesium salts) using quantofix sticks according to the manufacturer's instruction (Quantofix, Macherey-Nagel GmbH & co.KG, Germany).

2.5 Cultivation dependent analysis

2.5.1 Heterotrophic Plate Count (HPC) modified from ISO 6222

Yeast agar plates (Ant.Er.CP63.1, Carl Roth, Germany) were used according to manufacturer's instruction for each type of water in two sets of triplicates. 0.1ml of the water sample was spread on each agar plate using a sterile glass spreader. The plates were inverted and incubated; three plates were incubated at 36+2°C for 44+4 hours. The other three plates were incubated at 22+2°C for 68+4 hours. The plates were examined as soon as they were removed from the incubators. Any plate with confluent growth was discarded. Any colony count less than 300 was used to estimate the sample number of colony forming units per milliliter (CFU/ml) for each sample. Any count greater than 300 colonies on the plates was expressed as ≥300.

2.5.2 Enumeration and isolation of *Legionella* according ISO11731-2

2.5.2.1 Reagents and media:

2.5.2.1.1Acid buffer

3.9 ml of 0.2mol/l HCl and 25ml of sterile 0.2mol/l KCl were mixed. The pH was adjusted to 2.2 ±0.2 by adding 1mol/l solution of potassium hydroxide (KOH). Acid buffer was stored in a sterile stopped glass container in the dark at room temperature for no longer than 1 month.

2.5.2.1.2 Page´s saline

1.20 g of Sodium chloride (NaCl), 0.04 g of Magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 0.04 g of Calcium chloride ($CaCl_2 \cdot 2\ H_2O$), 1.42 g of Disodium hydrogenphosphate (Na_2HPO_4), and 1.36 g of Potassium dihydrogenphosphate (KH_2PO_4) were added to ten liters distilled water. Chemicals were allowed to dissolve, mixed well and autoclaved at (121±3) ℃ for (15±1) min.

2.5.2.1.3 GVPC medium

3g of glycine (Alfa Aesar, 10157324, UK), 0.08g of cycloheximide (01810, Fluka, Sigma-Aldrich, China), 0.002g Vancomycin (861987,Fluka, Sigma Aldrich, China),79200 I.U polymyxin B sulfate (81334,Fluka, Sigma Aldrich,China), 0.25g ferric pyrophosphate (P6526,Aldrich, Sigma Aldrich, Germany), and 0.4g L-cysteine (W326305, Aldrich, Sigma Aldrich, Germany) were added to BCYE agar medium (M809, Himedia, India) after being cooled to 50℃ according to manufacturer's instruction.

2.5.2.2 Procedure:

100ml of water sample was filtered onto membrane filter (membrane solutions, pore size 0.45µm, diameter 47mm, Whatman, England) using sterile filtration unit (Nalgene, Germany). The vacuum pump (LVP 500, South Korea) was built up to 200mbar. After filtration, 30ml of acid buffer was added on top of the membrane filter and was left for 5min. The filter was rinsed with 20ml Page´s saline. The membrane was removed from the filtration stand with sterile forceps (Dressing forceps straight 12.5cm, Narang) and placed onto the agar plate. Duplicates of BCYE and/ or GVPC (M809, Himedia, India) agar plates were used according to manufacturer's instruction. The plates were incubated inverted at (36 ± 2) °C for 10 days. Plates were checked for growth twice the third day or fourth for ten days. Final reading was done after ten days with description of colonies.

At least five colonies characteristic of *Legionella* were selected at random for each positive sample and subcultured onto BCYE and/or GVPC and blood agar (M073, Himedia, India) as negative control (L-cysteine free). Plates were incubated at 36±2°C for at least 2 days. The colonies which grew on BCYE and/or GVPC but failed to grow on blood agar medium were regarded as *Legionella*. Positive colonies were restreaked on BCYE and further identified by molecular terms. Our isolates were preserved in 40% glycerol using cryotubes (TPP, USA) at -80°C.

2.6 Cultivation independent analysis

2.6.1 Water DNA extraction

Five liters of water sample were filtered onto sandwich membrane filters composed of nucleopore-filter (Nuclepore Track-Etch Membrane, 65681 PC MB 90mm, 0.2µm, Whatman, England) and glass fiber-microfilter (GF/F) (GFF, 1825-090, 90mm, Whatman, England) using sterile filtration unit (Nalgene, Germany).The vacuum pump (KNF, N811 KN.18, Germany) was built up to 300-400mbar. The filtration time was measured till filtration was finished. Both filters from the cold and hot water samples were folded with sterile pincers

(5160173, Rostfrei, Germany) and transferred onto a sterile round aluminum foil (60220, 0.03mm, 150mm, Alu-Rundscheiben, Germany). Filters were stored at -20°C for 1 day or at -70°C for longer storage.

For the extraction of DNA from the filter sandwiches, a modified DNeasy protocol (Qiagen 69506, Germany) was used. briefly, sandwich filters were cut into small pieces and incubated with enzymatic lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X-100 (9002-93-1, Sigma- Aldrich, Germany) [pH 8.0]) containing 10 mg/ml lysozyme (62970, Fluka, Sigma-Aldrich, Switzerland) for 60 min in a 37°C water bath. After the addition of AL buffer from the kit, the samples were incubated at 78°C in a shaking water bath for 20 min. After filtration through a polyamide mesh with a 250–um pore size, absolute ethanol was added to the filtrate (ratio of filtrate to ethanol (2:1)), and the mixture was applied onto the spin column of the kit. After this step, the protocol was done according to the manufacturer's instructions. Finally, DNA was stored at -20°C until used.

2.6.2 Swab DNA extraction

For the extraction of DNA from a swab, a modified DNeasy protocol (Qiagen 69506, Germany) was used. briefly, swab was put in 2ml eppendrof tube and incubated with enzymatic lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X-100 (9002-93-1, Sigma- Aldrich, Germany) [pH 8.0]) containing 10 mg/ml lysozyme (62970, Fluka, Sigma-Aldrich, Switzerland) for 60 min in a 37°C water bath. After the addition of AL buffer from the kit, the samples were incubated at 78°C in a shaking water bath for 20 min. After filtration through a polyamide mesh with a 250–um pore size, absolute ethanol was added to the filtrate (ratio of filtrate to ethanol (2:1)), and the mixture was applied onto the spin column of the kit. After this step, the protocol was done according to the manufacturer's instructions. Finally, DNA was stored at -20°C until used.

2.6.3 *Legionella* isolates DNA extraction

One or two colonies of *Legionella* were inoculated in 100µl sterile water (W4502, Sigma Aldrich, Germany) using dry bath (DBS-001, MRC, Israel). Then, the mixture was heated at 90°C for 10 minutes (Moore et al., 2004). Finally, DNA was stored at -20°C until used.

2.7 16S rRNA PCR

2.7.1 Common primer

The PCR was used for identification of bacteria in the samples. PCR common (COM) primers were kindly provided by Prof. Manfred Hofle, HZI, Braunschweig, Germany (Eurofins, mwg operon, Germany) to amplify a PCR product of 409bp for any bacteria. The primer sequences are shown in (Table 2.1). Their location on the complete genome of *Legionella pneumophila subsp.pneumophila* ATCC 43290 is shown in (Fig. 2.3). PCR was done using PCR-ready master mix (GoTaq, Green Master Mix, Promega, USA). To each tube a mixture of 12.5µl PCR-ready Master Mix (GoTaq, Green Master Mix, Promega, USA), 7.5µl sterile water (W4502, Sigma Aldrich, Germany), 1µl (10mmol) forward primer (Com1f), 1µl (10mmol) reverse primer (Com2r), and 3µl (100µg/ml) DNA template were added. PCR amplification was done on a thermal cycler (1861096,Biorad, USA) according to the following conditions: initial denaturation at 95°C for 15 minutes followed by 35 cycles of denaturation at 95°C for 90 seconds, annealing at 55°C for 40 seconds, elongation at 72°C for 90 seconds and final elongation at 72°C for 10 minutes (Kahlisch et al., 2010). The products were analyzed using 2% agarose gel (A9539, Sigma Aldrich, Germany), and visualized by UV transilluminator (TFX-35M, Vilber lourmat, France) and documented using gel documentation system (U: Genius3, Syngene, UK). DNA of bacterial culture and *Legionella* spp. (L1-L5) is shown in (Table 2.3) were used as positive controls. Sterile water (W4502, Sigma Aldrich, Germany) was used as negative control. Moreover, twenty percent of the samples were repeated randomly. The products were evaluated according to size.

2.7.2 *Legionella* genus specific primer

The PCR was used for identification of *Legionella* genus in the samples. PCR *Legionella* genus specific (Lgsp) primers were purchased from (hy-labs, Park Tamar, 76326 Rehovot, Israel) to amplify PCR product of 426bp. The primer sequences are shown in (Table 2.1). Their location on the complete genome of *Legionella pneumophila subsp.pneumophila* ATCC 43290 is shown in (Fig. 2.3). PCR was done using PCR-ready master mix (GoTaq, Green Master Mix, Promega, USA). To each tube a mixture of 12.5µl PCR-ready Master Mix (GoTaq, Green Master Mix, Promega, USA), 7.5µl sterile water (W4502, Sigma Aldrich, Germany), 1µl (10mmol) forward primer (Lgsp17f), 1µl (10mmol) reverse primer (Lgsp28r), and 3µl (100µg/ml) DNA template were added. PCR amplification was done on thermal cycler (1861096,Biorad, USA) according to the following conditions: initial denaturation at 95°C for 15 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 66.5°C for 30 seconds, elongation at 72°C for 30 seconds and final elongation at 72°C for 10 minutes (Kahlisch et al., 2010). The products were analyzed using 2% agarose gel (A9539, Sigma Aldrich, Germany), and visualized by UV transilluminator (TFX-35M, Vilber lourmat, France) and documented using gel documentation system (U:Genius3, Syngene, UK).DNA of *Legionella* different species (L1-5) is shown in (Table 2.3) were used as positive controls.DNA of bacterial culture and sterile water was used as negative controls. Moreover, twenty percent of the samples were repeated randomly. The products were evaluated according to size.

2.7.3 *L.pneumophila* species primer

The PCR was used for identification of *Legionella pneumophila* species in the samples. PCR *L.pneumophila* species (L1) primers were purchased from (hy-labs, Park Tamar, 76326 Rehovot, Israel) to amplify PCR product of 544bp. The primer sequences are shown in (Table 2.1). Their location on the complete genome of *Legionella pneumophila subsp.pneumophila* ATCC 43290 is shown in (Fig. 2.3). PCR was done using PCR-ready master mix (GoTaq, Green Master Mix, Promega, USA). To each tube a mixture of 12.5µl PCR-ready Master Mix

(GoTaq, Green Master Mix, Promega, USA), 7.5µl sterile water (W4502, Sigma Aldrich, Germany), 1µl (10mmol) forward primer (L1f), 1µl (10mmol) reverse primer (L1r), and 3µl (100µg/ml) DNA template were added. PCR amplification was done on thermal cycler (1861096,Biorad, USA) according to the following conditions: initial denaturation at 95°C for 15 minutes followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, elongation at 72°C for 45 seconds and final elongation at 72°C for 20 minutes (Kahlisch et al., 2010). The products were analyzed using 2% agarose gel (A9539, Sigma Aldrich, Germany), and visualized by UV transilluminator (TFX-35M, Vilber lourmat, France) and documented using gel documentation system (U: Genius3, Syngene, UK).DNA of *L.pneumophila* species (L3 and L4) is shown in (Table 2.3) were used as positive controls. DNA of *Legionella non-pneumophila* species (L1, L2, and L5) is shown in (Table 2.3) was used as negative controls. Moreover, twenty percent of the samples were repeated randomly. The products were evaluated according to size.

Table 2.1: Primers used in the study.

Primer	OligoName	Sequence 5′-3′	PCR length (bp)
Bacteria Common	Com1F	5′-CAGCAGCCGCGGTAATAC-3′	409
	Com2R	5′-CCGTCAATTCCTTTGAGTTT-3′	
Legionella genus specific	Lgsp17F	5′-GGCCTACCAAGGCGACGATCG-3′	426
	Lgsp28R	5′-CACCGGAAATTCCACTACCCTCTC-3′	
Legionella pneumophila species specific	L1F	5′-CCTGGGCTTAACCTGGGAC-3′	544
	L1R	5′-CTTAGACTCCCCACCATCACAT-3′	

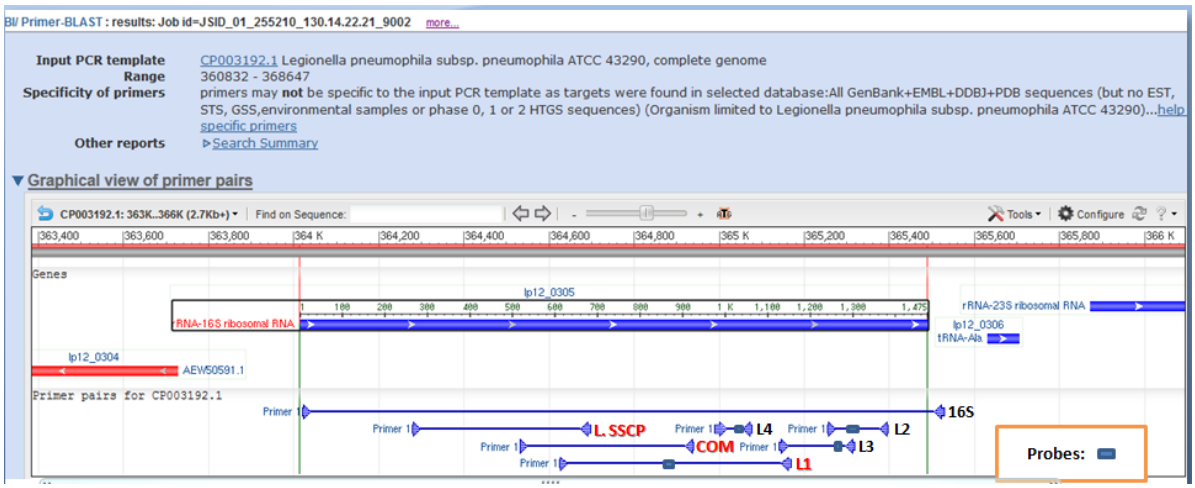


Figure 2.3: *L. pneumophila* whole genome and primers location (Dr. Manfred Hofle, HZI).

2.8 Agarose gel preparation and electrophoresis

The 2% agarose gel was prepared by dissolving 2g agarose (A9539, Sigma Aldrich, Germany) in 100 ml 1X TAE electrophoresis buffer (0.04 M Tris-Acetate, pH 8.0 and 0.001M EDTA), boiled in the microwave, and when it cooled to 50°C, 5µl of ethidium bromide (1 µg/ml) (hylabs, Israel) was added.

The PCR products were analyzed on horizontal gel electrophoresis (Multisub, Biocom, Germany). The applied voltage was 120 volts in the first 10 minutes then at 100 volts for one hour.

2.9 Sequencing of the 16S rRNA gene and phylogenetic analysis

For quality assurance, identification and classification of *Legionella*, six *Legionella* isolates were sent to HZI, Braunschweig, Germany on FTA cards (WB120205, Whatman, England). Complete sequencing of the 16S rRNA gene was kindly performed by (HZI PhD students and technicians, Braunschweig, Germany). The results were analyzed by MEGA 5 free software

and sent on phylogenetic tree using Neighbor-Joining (NJ) method. Sequences were aligned with muscle algorithm.

2.10 MLVA-8

Polymorphic tandem repeats have been successfully used for epidemiological typing studies of many bacterial species (Lindstedt, 2005). Multiple-locus variable number of tandem repeats (VNTR) assays (MLVA) are based on the analysis of short to long tandemly repeated sequences (also called microsatellites, up to 9bp, and minisatellites, more than 9bp in length). An assay is defined by a set of loci spread throughout the bacterial genome (Le Fleche et al., 2001). Previous studies of the polymorphism of tandem repeats in *L. pneumophila* suggested that VNTRs could be used for genotyping (Pourcel et al., 2003) in spite of the fact that this species is much more genetically heterogeneous than other species for which MLVA can be regarded as a reference method, such as *Mycobacterium tuberculosis* and *Yersinia pestis* (Le Fleche et al., 2001; Pourcel et al., 2004).

For quality assurance, identification and classification of *Legionella* in order to identify pathogenic strains, 41 *Legionella* isolates were sent to HZI, Braunschweig, Germany on FTA card (WB120205, Whatman, England). MLVA-8 analysis was kindly performed by (HZI PhD students and technicians, Braunschweig, Germany). MLVA-8 single PCRs were carried out by using the primer sets described by Pourcel *et al* (Pourcel et al., 2007) shown in (Table 2.2).

Table 2.2: *L.pneumophila* MLVA-8 primers (Kahlisch et al., 2010; Pourcel et al., 2007).

Primer	OligoName	Sequence 5'-3'	Repeat length (bp)	Total flanking region (bp)
Legionella pneumophila MLVA-8	Lpms1b F	5'-ACGAGCATATGACAAAGCCTTG-3'	45	205
	Lpms1b R	5'-CGGATCATCAGGTATTAATCGC-3'		
	Lpms3 F	5'-CAACCAATGAAGCAAAAGCA-3'	96	173
	Lpms3 R	5'-AGGGGTTGATGGTCTCAATG-3'		
	Lpms33 F	5'-ACCACAGCAGTTTGAACATAAT-3'	125	102
	Lpms33 R	5'-GGGAGAAGTTATAGATCTATTCG-3'		
	Lpms35 F	5'-CTGAAACAGTTGAGGATGTGA-3'	18	148
	Lpms35 R	5'-TTATCAACCTCATCATCCCTG-3'		
	Lpms13 F	5'-CAATAGCATCGGACTGAGCA-3'	24	164
	Lpms13 R	5'-TGCCTGTGTATCTGGAAAAGC-3'		
	Lpms17 F	5'-CAGCTCACCCCGTATCACTT-3'	39	200
	Lpms17 R	5'-TAACATCAATGACCGCGAAA-3'		
	Lpms19b F	5'-GAACTATCAGAAGGAGGCGAT-3'	21	89
	Lpms19b R	5'-GGAGTTTGACTCGGCTCAGG-3'		
	Lpms34 F	5'-GAAAAGGAATAAGGCGCAGCAC-3'	125	84
	Lpms34 R	5'-AAACCTCGTTGGCCCCTCGCTT-3'		

2.11 Serological test

Colonies grown on BCYE and/or GVPC were then identified by an agglutination test using (*Legionella* Latex Test, Oxoid DR0800M, England) according to manufacturer's instruction. The test allows a separate identification of *L.pneumophila* serogroup 1, serogroups 2–14 and detection of seven *Legionella* species (*L.*non- *pneumophila*), which have been implicated in human disease. Moreover, for identification and classification of *Legionella pneumophila* exactly to identify *L.pneumophila* sergroups, the sixteen *L.pneumophila* isolates form Al-Quds University were sent to HZI, Braunschweig, Germany on FTA cards (WB120205, Whatman, England). Monoclonal antibody (MAb) for identification of *L.pneumophila* exact serogroup was kindly performed by (Prof. Christian Lück team, University of Dresden, Germany).Serogroup MAb was done to identify the exact serogroup.

2.12 Controls

2.12.1 Medium control

Blood agar (M073, Himedia, India) was used as negative control since *Legionella* fails to grow on blood agar (L-cysteine free) in this study.

2.12.2 DNA control

DNA of *Legionella* as shown in (Table 2.3) kindly provided by (Prof. Manfred Hofle, HZI, Braunschweig, Germany) were used as DNA control in this study.

Table 2.3: *Legionella* reference strains.

***Legionella* Reference strains**		
Sample Name	**Code**	**DNA Conc (ng/µl)**
Legionella anisa	L1	209.8
Legionella feeleii	L2	1045.7
Legionella pneumophila str.Corby	L3	108.8
Legionella pneumophila subsp.*pneu* . Philadelphia-1	L4	200.7
Legionella jordanis	L5	50.2

2.13 Statistical analysis, figures drawing and computer software

Statistical analysis was done by Excel (Microsoft office, 2007). Figures were drawn by photo filter software program (Photo filter 6.5.2). Sequences retrieved from isolates were deposited in the GenBank. The software used to build the tree was MEGA 5.

Chapter Three

Results

3.1 Sample study

A total of 15 cold and hot water samples and 292 biofilm swabs (Table 3.1) were collected from seven governmental hospitals in the West Bank, Al-Makassed hospital in Eastern Jerusalem; (one cold water sample and 36 swabs from Jenin hospital, one cold and one hot water sample and 44 swabs from Rafidia hospital, one cold and one hot water sample and 26 swabs from Al-Watani hospital, one cold and one hot water sample and 36 swabs from Ramallah hospital, one cold and one hot water sample and 21 swabs from Al-Makassed hospital, one cold and one hot water sample and 29 swabs from Beit Jala hospital, one cold and one hot water sample and 33 swabs from Al-Ahli hospital, one cold and one hot water sample and 24 swabs from Alia hospital) and 43 swabs from Al-Quds University for cultivation dependent analysis (Table 3.1).

In addition, 15 cold and hot water samples and 64 biofilm swabs (Table 3.1) were collected for cultivation independent analysis from seven governmental hospitals in the West Bank and Al-Makassed hospital in Eastern Jerusalem; (one cold and one hot water sample and 8 swabs from each hospital except for Jenin hospital only cold water sample was collected).

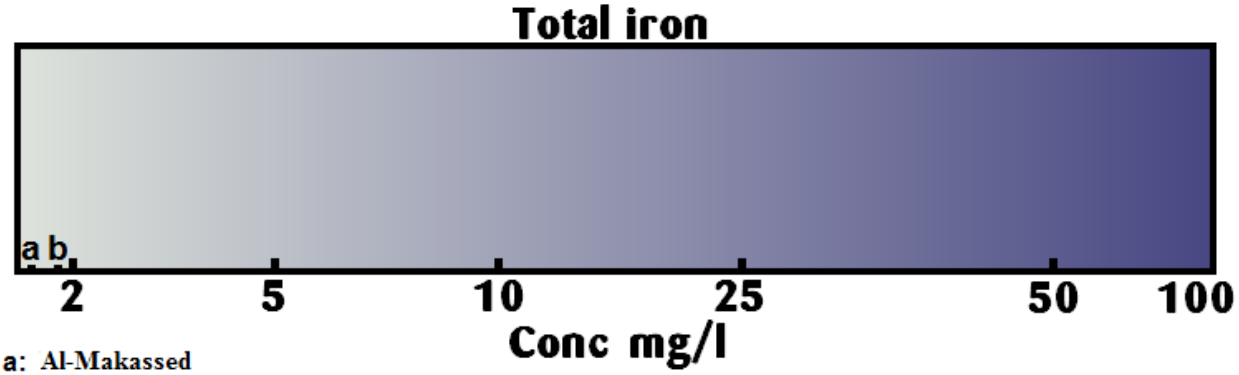
Table 3.1: Cultivation dependent analysis Vs cultivation independent analysis.

Cultivation dependent analysis Vs Cultivation independent analysis						
Sample Type	No. of collected samples		No. of positive samples		(%) of positive samples	
	CDA	CIA	CDA	CIA	CDA	CIA
Water sample	15	15	3	8	20%	53.3%
Biofilm Swab	292	64	93	44	31.8%	68.8%
Total	**307**	**79**	**96**	**52**	**31.3%**	**65.8%**

CDA: Cultivation Dependent Analysis
CIA: Cultivation Independent Analysis

3.2 Measurement of hospital water background parameters

Water samples were tested for temperature, pH and conductivity immediately upon collection. Upon return to the laboratory water samples were tested for total iron (Fig. 3.1), chlorine sensitive (Fig. 3.2), nitrate (Fig. 3.3), nitrite (Fig. 3.4), ammonia (Fig. 3.5), copper (Fig. 3.6), phosphate (Fig. 3.7), zinc (Fig. 3.8), carbonate hardness (Fig. 3.9), and total hardness.



a: Al-Makassed
b: Jenin, Rafidia, Al-Watani, Ramallah, Beit Jala, Al-Ahli and Alia
WHO: up to 2mg/l

Figure 3.1: Concentration (mg/l) of total iron (Fe^{+3}/Fe^{+2}) in hospital water samples.

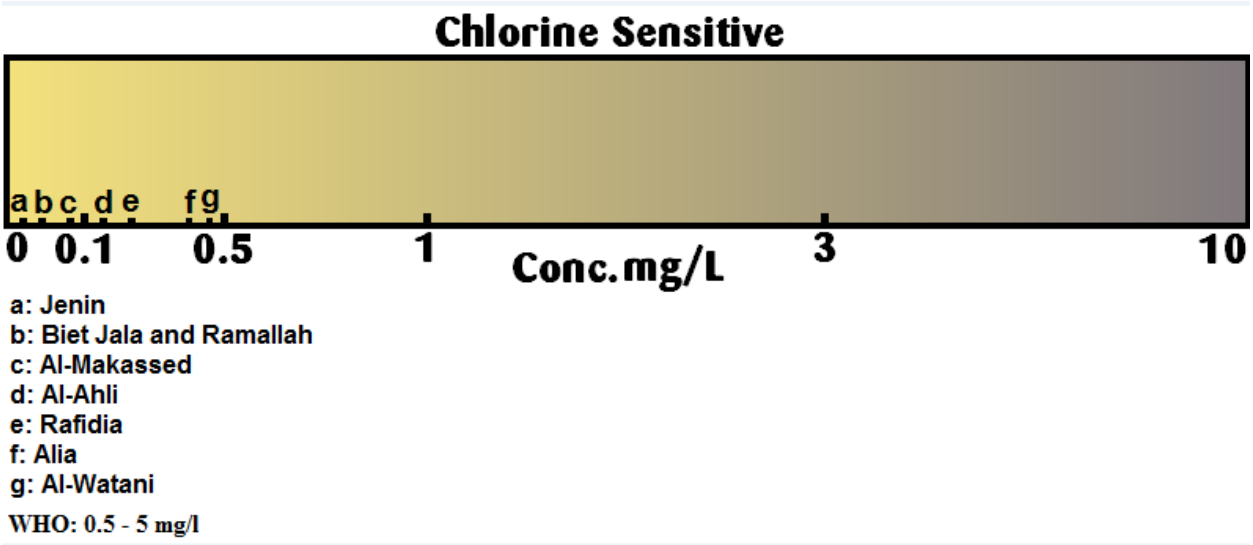


Figure 3.2: Concentration (mg/l) of chlorine sensitive (Cl_2) in hospital water samples.

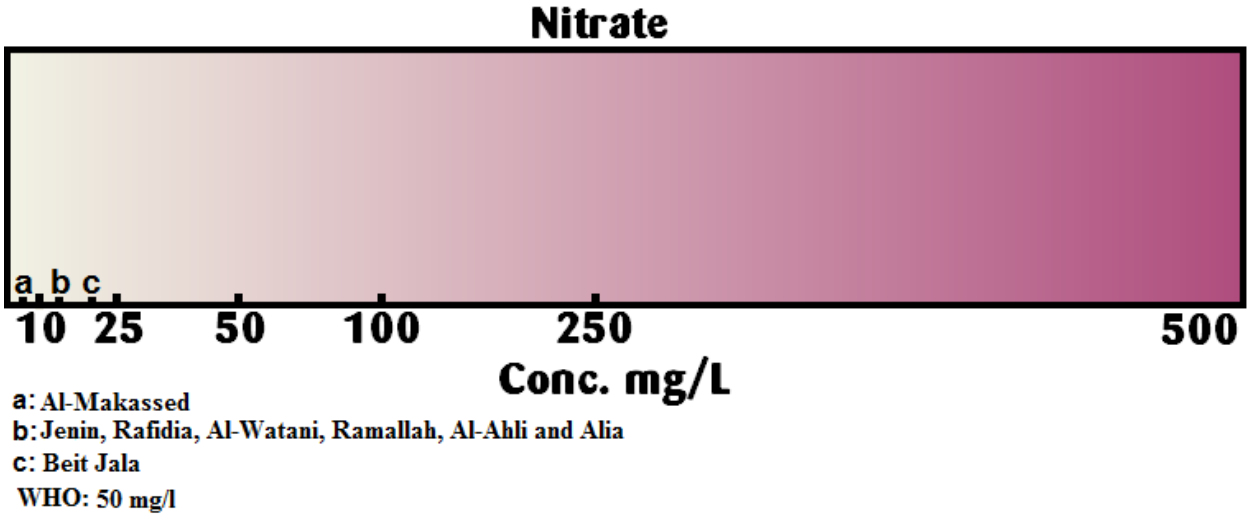


Figure 3.3: Concentration (mg/l) of Nitrate (NO_3^-) in hospital water samples.

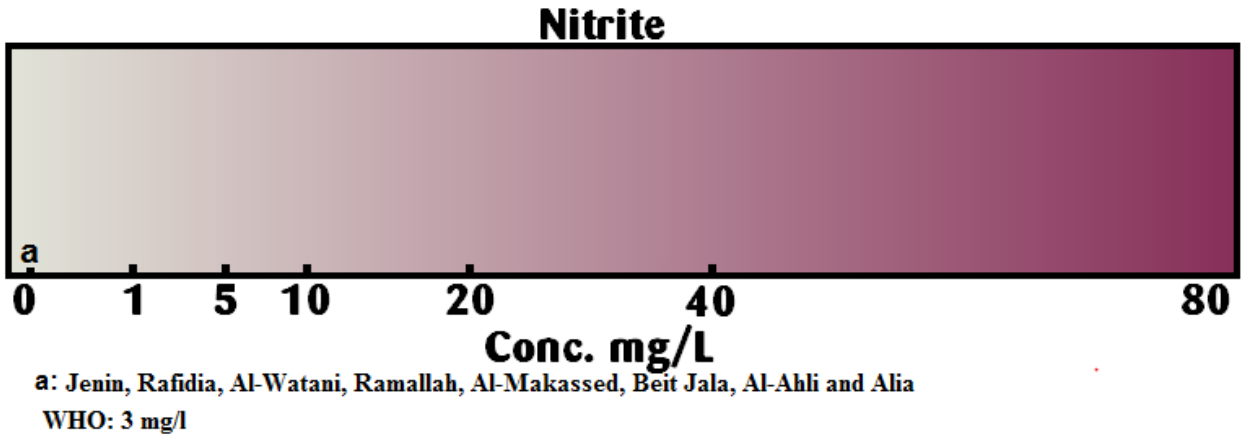


Figure 3.4: Concentration (mg/l) of Nitrite (NO_2^-) in hospital water samples.

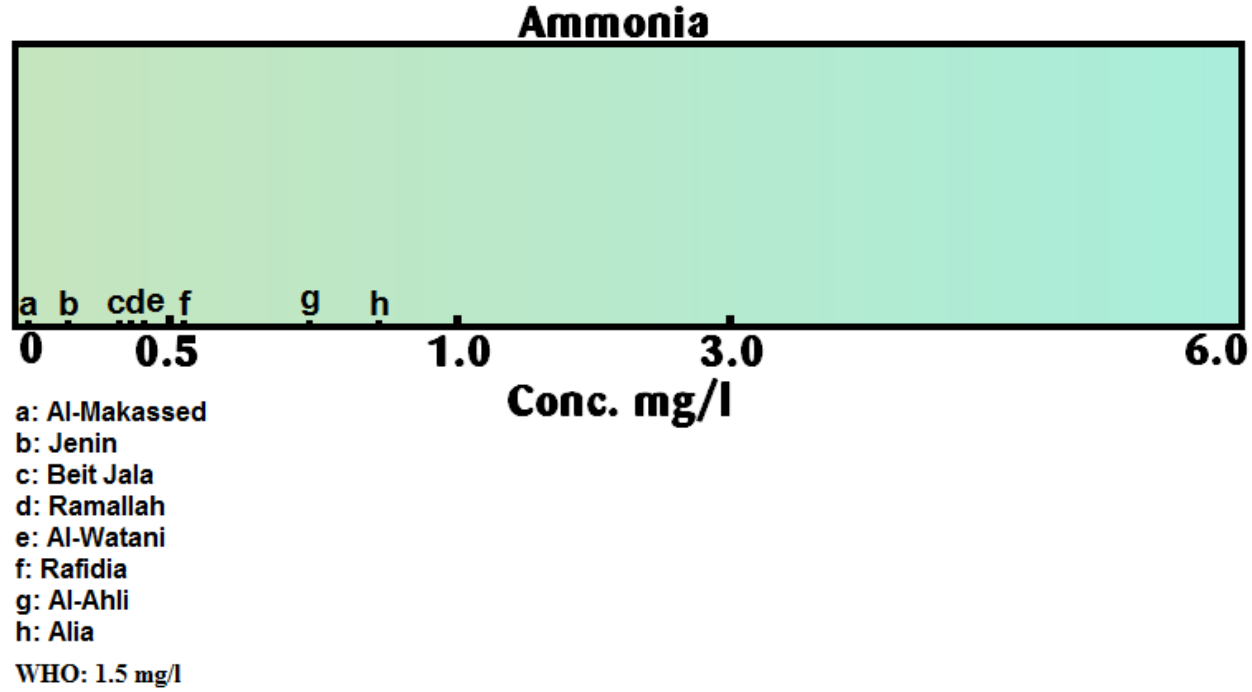
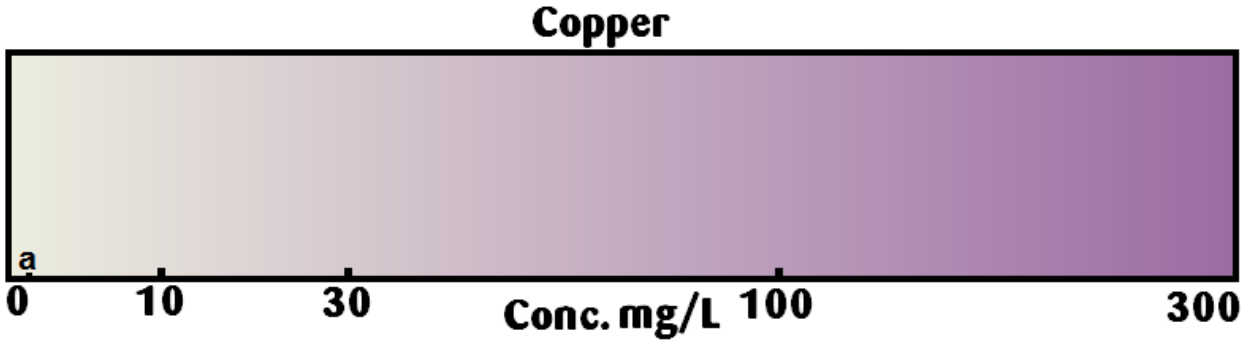
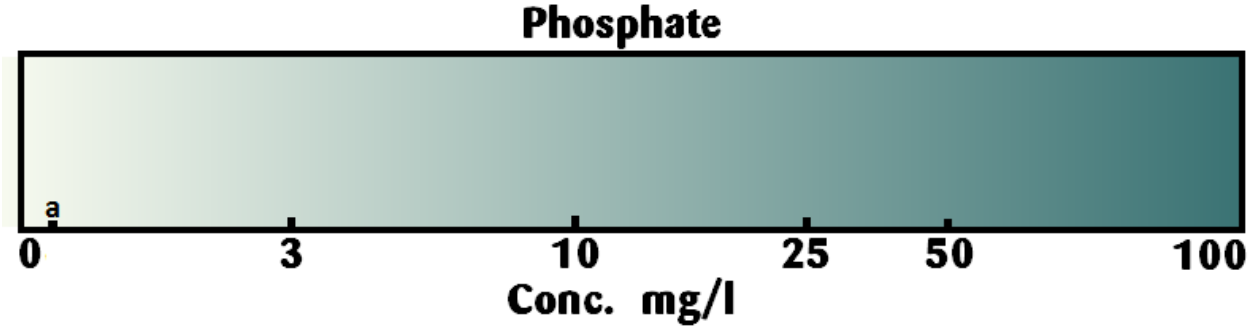


Figure 3.5: Concentration (mg/l) of ammonia (NH_3) in hospital water samples.



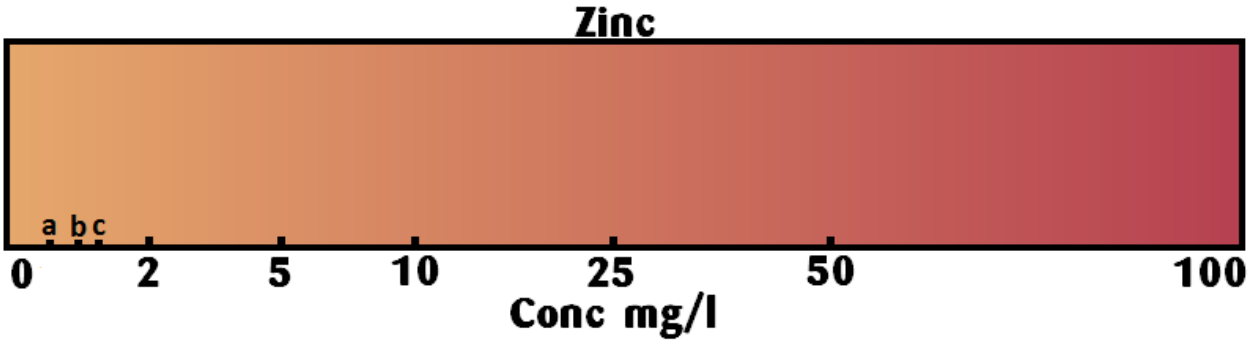
a: Jenin, Rafidia, Al-Watani, Ramallah,Al-Makassed, Beit Jala, Al-Ahli and Alia
WHO: 2 mg/l

Figure 3.6: Concentration (mg/l) of copper ($Cu^{+}/^{+2}$) in hospital water samples.



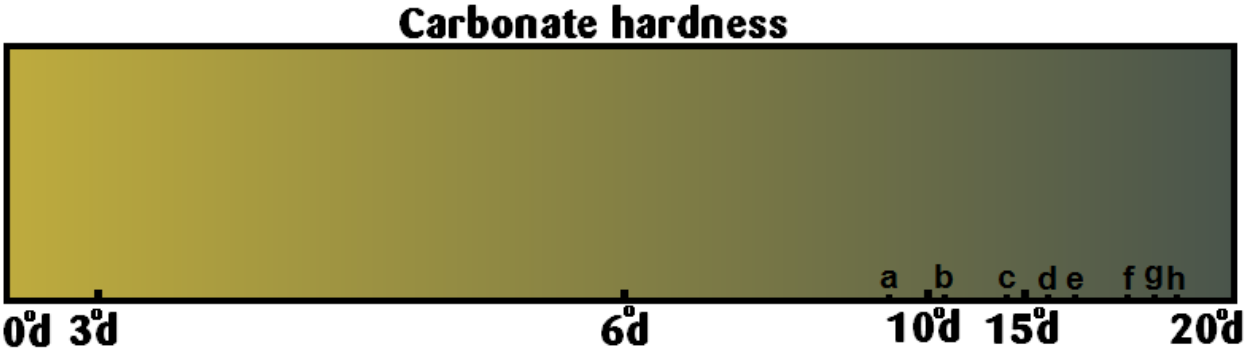
a: Jenin, Rafidia, Al-Watani, Ramallah, Al-Makassed, Beit Jala, Al-Ahli and Alia
WHO: 0 mg/l

Figure 3.7: Concentration (mg/l) of phosphate ($PO4^{3-}$) in hospital water samples.



a: Jenin, Al-Makassed and Beit Jala
b: Ramallah
c: Rafidia, Al-Watani, Al-Ahli, and Alia
WHO: 0 mg/l

Figure 3.8: Concentration (mg/l) of zinc (Zn) in hospital water samples.



a: Beit Jala
b: Al-Makassed
c: Jenin
d: Al-Ahli
e: Ramallah
f: Alia
g: Rafidia
h:Al-Watani

d = 17.8 mg/l
WHO: 200 mg/l = 11.2 d

Figure 3.9: The amount of Carbonate hardness (CO_3^{2-}) and (HCO_3^-) in hospital water samples.

For the sampling period from November 7th through December 21st 2012, no nitrite, copper, or phosphatewas detected in the water samples. The levels of iron, chlorine, ammonia and zinc detected were within acceptable levels according to WHO guidelines (World Health Organization WHO, 2008). Carbonate hardness was detected in a range from 10°d to less than 20°d, where carbonate hardness in Al-Makassed and Beit Jala hospitals were within WHO acceptable levels. However carbonate hardness in the remaining hospitals was higher than 11.2°d. Total hardness ranged from less than 15°d to less than 20°d, which is above WHO acceptable levels. The cold water temperature varied between 18.4 °C and 25.5 °C. The hot water temperature varied between 29.5°C and 70.9°C. The conductivity ranged from 610 µS to 802 µS. The pH varied from 7.6 to 8.3. Actual data on hospital water physical and chemical parameters are summarized in (Table 3.2).

Table 3.2: Hospital water physical and chemical parameters.

Parameter	Hospital background parameter results																
	Jenin	Rafidia		Al-Watani		Ramallah		Al-Makassed		Beit Jala		Al-Ahli		Alia		WHO	
	Cold	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot
Temprature °C	21.7	21	70.9	20.1	49	21.7	44.5	21.7	51.2	25.5	52.2	18.4	38.4	19.7	29.5	13±2.3	53.2±2.5
pH	7.6	7.8	7.6	8	8	7.8	7.6	7.95	8.02	7.6	7.6	8.3	8.2	7.8	8.1	6.5 - 9.5	
Conductivity µS/cm	642	711	701	761	760	818	815	774	785	610	620	798	802	620	610	400 at 20°C	
Total iron (Fe^{+3}/Fe^{+2}) mg/l	< 2	< 2		< 2		< 2		< 2		< 2		< 2		< 2		up to 2	
chlorine Sensitive (Cl_2) mg/l	< 0.1	0.1		0.5		< 0.1		0.1		< 0.1		0.1		< 0.5		0.5 - 5	
Nitrate (NO_3^-) mg/l	10	10		10		10		< 10		< 25		10		10		50	
Nitrite (NO_2^-) mg/l	0	0		0		0		0		0		0		0		3	
Ammonia (NH_3) mg/l	0	0.5		0.5		< 0.5		0		< 0.5		< 1		< 1		1.5	
Copper ($Cu^{+}/^{+2}$) mg/l	0	0		0		0		0		0		0		0		2	
Phosphate (PO_4^{3-}) mg/l	0	0		0		0		0		0		0		0		0	
Zinc (Zn) mg/l	0	< 2		< 2		< 2		0		0		< 2		< 2		0	
Carbonate hardness °d*	15	< 20		< 20		< 20		10		10		15		< 20		11.2	
Total hardness °d§	< 20	< 20		15		15		< 20		< 15		< 15		< 15		5.6	

* Carbonate ($CO3^{2-}$) and bicarbonate ($HCO3^-$)
§ Calcium and magnesium salts
°d = 17.8 mg/l

3.3 Cultivation dependent analysis

3.3.1 Heterotrophic Plate Count (HPC)

HPCs were done in triplicates, 100µl of the cold or hot hospital water was plated using spread plate technique on yeast extract agar plates. Incubation was carried out at two different temperatures according to the ISO 6222 (36°C for 48h and 22°C for 72h).The mean number of HPCs varied between 3 and 478 CFU/ml, with the exception of Rafidia hospital hot water, which showed a HPC of zero CFU/ml. For all sampling sites the HPC values were not high ranging from $>10^3$ to $5x10^5$ CFU/L. Actual data on hospital water HPCs are summarized in (Table 3.3).

3.3.2 Identification and quantification of *Legionella* spp.

A total of 15 cold and hot water samples and 292 biofilm swabs were collected from seven governmental hospitals in the West Bank and Al-Makassed hospital in Eastern Jerusalem and Al-Quds University for cultivation dependent analysis. *Legionella pneumophila* was isolated from 3 out of 15 (20%) water samples and 93 out of 292 (31%) biofilm swabs (Table 3.1).

3.3.2.1 Water samples

A total of 15 water samples were collected for identification and quantification of *Legionella* spp. On the filter used for isolation of *Legionella* spp, gray-white colonies with ground-glass opacity were observed indicating *Legionella*-like bacteria. These colonies were restreaked on GVPC to confirm the identification for *Legionella* bacteria (Fig. 3.10). *Legionella* CFUs were highest in the well-water derived water. *L.pneumophila* as confirmed by latex agglutination was isolated from 3 (20%) of 15 water samples (Jenin, Beit Jala hot water and Al-Ahli cold water). In the positive samples, the mean number of *Legionella* count varied between 35 and 260 CFU/L. Actual data on hospital water *Legionella* count are summarized in (Table 3.3).

Table 3.3: Hospitals water HPC (CFU/ml) and *Legionella* count (CFU/L).

Hospital	Type of water	Mean±SEM of HPC (CFU/ml) at		Mean±SEM of *Legionella* (CFU/l)
		36°C for 48h	22°C for 72h	
Jenin	Cold	37±12	20±12	260±28
Rafidia	Cold	10±6	7±3	0
	Hot	0	0	0
Al-Watani	Cold	3±3	10±0	0
	Hot	10±6	7±3	0
Ramallah	Cold	163±9	347±21	0
	Hot	117±9	47±13	0
Al-Makassed	Cold	170±23	140±21	0
	Hot	57±12	13±3	0
Beit Jala	Cold	487±158	187±12	0
	Hot	173±63	220±82	35±15
Al-Ahli	Cold	60±15	63±9	50±20
	Hot	37±9	47±7	0
Alia	Cold	37±17	33±15	0
	Hot	23±12	27±12	0

HPC: Heterotrophic plate count
SEM: Standard error of the mean

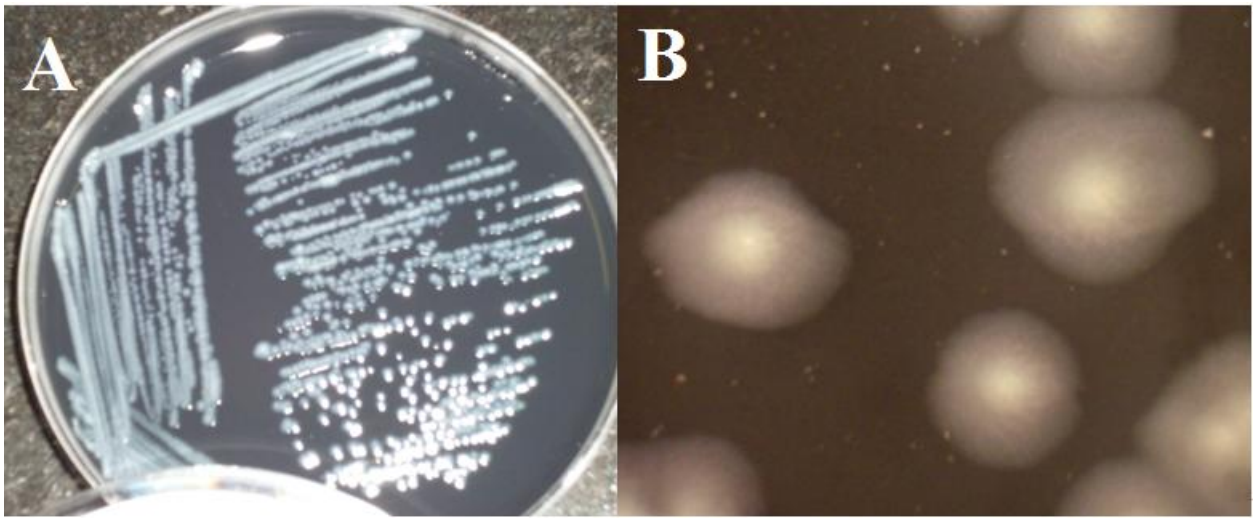


Figure 3.10: *L.pneumophila*. A: *L.pneumophila* isolate on GVPC medium.

B: *L.pneumophila* colonies under dissecting microscope.

jh 3.3.2.2 Biofilm swabs

A total of 292 biofilm swabs were collected for the identification of *Legionella* spp. *Legionella pneumophila* was isolated from 93 (31.9%) of 292 biofilm swabs (21 isolates from Jenin hospital, 17 isolate from Rafidia hospital, 3 isolates from Al-Watani hospital, 17 isolates from Ramallah hospital, 6 isolates from Al-Makassed hospital, 2 isolates from Beit Jala hospital, 1 isolate from Alia hospital, 10 isolates from Al-Ahli hospital, and 16 isolates from Al-Quds University). Actual data on cultivation dependent analysis of biofilm swabs for *L. pneumophila* are summarized in (Table 3.4).

Table 3.4: Percentage of *L.pneumophila* in biofilm samples as determined by cultivation dependent analysis.

Cultivation dependent analysis (Biofilm swabs)			
No	**Site Name**	**No. of collected swabs**	**No. (%) of positive swabs**
1	Jenin	36	21 (58.3%)
2	Rafidia	44	17 (38.6%)
3	Al-Watani	26	3 (11.5%)
4	Ramallah	36	17 (47.2%)
5	Al-Makassed	21	6 (28.6%)
6	Beit Jala	29	2 (6.9%)
7	Al-Ahli	33	10 (30.3%)
8	Alia	24	1 (4.2%)
9	Al-Quds University	43	16 (37.2%)
	Total	**292**	**93 (31.8%)**

Immunocompromised patients are the group with higher risk to have LD due to nosocomial infection. In our study it was observed that critical wards (ICU, CCU, operation, neonate and pediatric) in West Bank hospitals were contaminated with *L.pneumophila*, which is considered a real risk to their health. Data are shown in (Table 3.5).

Table 3.5: Distribution of *L.pneumophila* in the critical wards of the studied hospitals.

Hospital	No. of positive biofilm swabs in				
	ICU*	CCUδ	Neonate	Pediatric	Operation
Jenin	3	NF	3	2	3
Rafidia	1	NF	3	2	1
Al-Watani	0	NF	0	0	0
Ramallah	1	7	1	2	4
Al-Makassed	2	1	0	1	1
Beit Jala	2	NF	0	1	0
Al-Ahli	0	1	0	1	3
Alia	0	1	0	1	4

* Intensive Care Unit
δ Cardiac Care Unit

3.4 Distribution of *L.pneumophila* according to serogroups in Palestinian hospitals and Al-Quds University

The isolates were classified to serogroup 1 (sg1) or serogroup 2-14 (sg 2-14) using *Legionella* latex test. As shown in (Fig. 3.11) 61 isolates belonged to *L.pneumophila* sg1 (64%) while 35 isolates belonged to *L.pneumophila* sg 2-14 (36%).

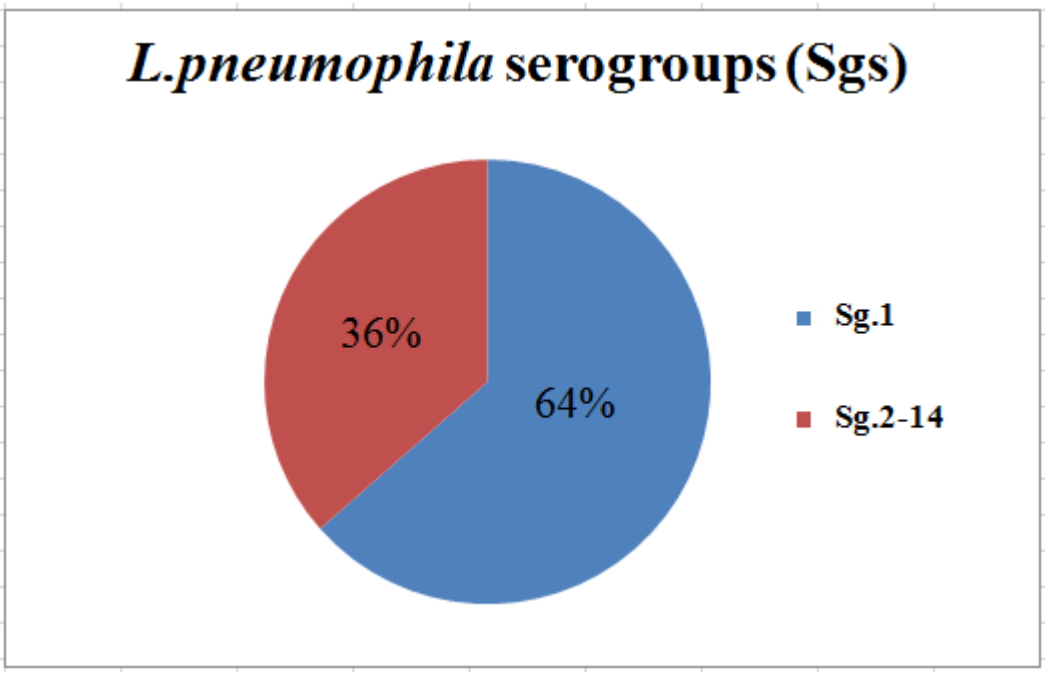


Figure 3.11: Percentages of *L.pneumophila* according to serogroups.

As shown in (Table 3.6) out of 22 isolates from Jenin hospital, 14 belonged to sg1 and 8 belonged to sg 2-14. Out of 17 isolates from Rafidia hospital, 13 belonged to sg1 and 4 belonged to sg 2-14. Out of 3 isolates from Al-Watani hospital, 2 belonged to sg1 and 1 belonged to sg 2-14. Out of 17 isolates from Ramallah hospital, 14 belonged to sg1 and 3 belonged to sg 2-14. Out of 6 isolates from Al-Makassed hospital belonged to sg1. Out of 3 isolates from Beit Jala hospital, 1 belonged to sg1 and 2 belonged to sg 2-14. Out of 11 isolates from Al-Ahli hospital, 9 belonged to sg1 and 2 belonged to sg 2-14. The isolate from Alia hospital belonged to sg 2-14. As for the 16 *L.pneumophila* isolates from Al-Quds University, 6 isolates from the Health Complex Building belonged to sg 2-14. The two isolates from the College of Science belonged to sg1. Out of 8 isolates from Arts College, 6 belonged to sg1 and 2 belonged to sg 2-14.

Table 3.6: Distribution of *L.pneumophila* according to serogroups.

***Legionella pneumophila* isolates**				
No	**Hospital Name**	**No. of isolates**	**No. (%) of sg1**	**No. (%) of sg 2-14**
1	Jenin	22	14 (63.6%)	8 (36.4%)
2	Rafidia	17	13 (76.5%)	4 (23.5%)
3	Al-Watani	3	2 (66.7%)	1 (33.3%)
4	Ramallah	17	14 (82.4%)	3 (17.6%)
5	Al-Makassed	6	0 (0%)	6 (100%)
6	Beit Jala	3	1 (33.3%)	2 (66.7%)
7	Al-Ahli	11	9 (81.8%)	2 (18.2%)
8	Alia	1	0 (0%)	1 (100%)
9	Al-Quds University	16	8 (50%)	8 (50%)
	Total	**96**	**61 (63.5%)**	**35 (36.5%)**

The 16 isolates from Al-Quds University were further classified subserogrouped using monoclonal antibody (MAb) test. As shown in (Fig. 3.12) 8 isolates belonged to *L.pneumophila* sg1 OLDA (50%), 2 isolates belonged to *L.pneumophila* sg6 (12%) and 6 isolates belonged to *L.pneumophila* sg 8 (38%). The 6 isolates from the Health Complex Building belonged to sg8. The two isolates from the College of Science belonged to sg1 OLDA. Out of 8 isolates from Arts College, 6 belonged to sg1 and 2 belonged to sg6. All isolates information is shown in (Appendix D).

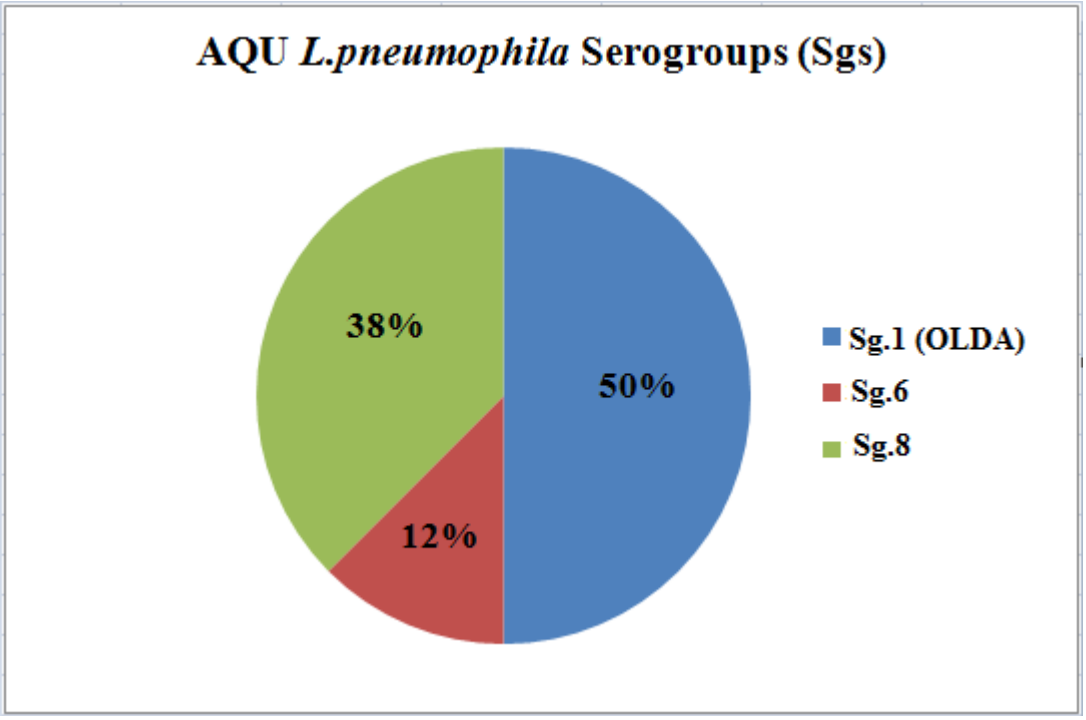


Figure 3.12: Percentage of *L.pneumophila* serogroups and subgroups in Al-Quds University.

3.5 Cultivation independent analysis

3.5.1 16S rRNA PCR

DNA extracted from the 15 cold and hot water samples and 64 biofilm swabs collected from the seven governmental hospitals in the West Bank and Al-Makassed hospital in Eastern Jerusalem were screened using 16S rRNA PCR for the presence of bacteria using the common (COM) primer, for the presence of *Legionella* using *Legionella* genus specific primer (Lgsp) and for the presence of *L.pneumophila* using L1 primer.

3.5.1.1 Screening for the presence of bacteria using Com primer

Com primer was used to identify any bacteria in the DNA extracted from the samples. Positive results were obtained in 13 (86.7%) of hospital water samples and in 56 (87.5%) of

hospital biofilm swabs. In this reaction, com primer gives PCR product 409bp (Fig. 3.13). A representative gel is shown in (Fig. 3.13). The Com primer PCR product is a 409bp. Lanes 2-8 show sandwich filter samples (water samples) where lane 3, 4, 5, 6 have a positive band equivalent to 409bp. Lanes 12-18 show biofilm swab samples, where lanes 13, 16 and 17 have a positive band.

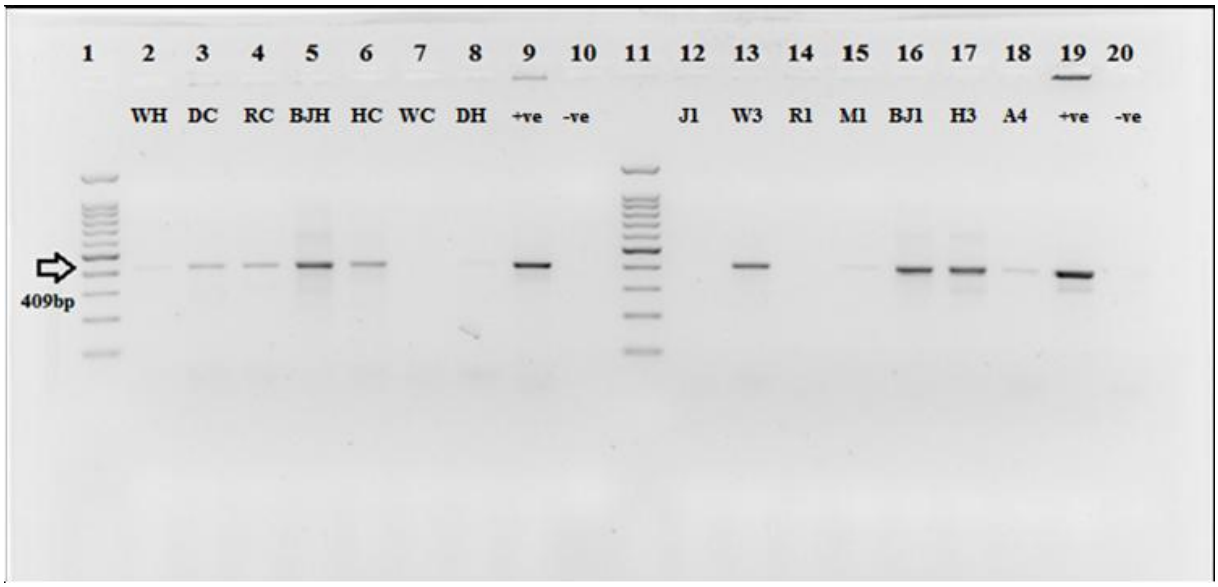


Figure 3.13: Representative 16S rRNA PCR using Com primer of DNA from hospital samples. Lane 1 represents 100bp ladder. Lane 2-8 shows sandwich filter samples (water samples), positive with 409bp and negatives results. Lane 9, positive control (*E.coli*). Lane 10, negative control (DW). Lane 11 represents 100bp ladder. Lane 12-18 show biofilm swabs samples, positive with 409bp and negatives results. Lane 19, positive control (*Legionella pneumophila* subsp.*pneu.*Philadelphia-L4-). Lane 20, negative control (DW). WH: Al-Watani hot water, DC: Rafidia cold water, RC: Ramallah cold water, BJH: Beit Jala hot water, HC: Al-Ahli cold water, WC: Al-Watani cold water, DH: Rafidia hot water, J1: Jenin operation shower hose, W3: Al-Watani pharmacy faucet, R1: Ramallah X-Ray faucet, M1: Al-Makassed labor outside faucet, BJ1: Beit Jala pediatric faucet, H3: Al-Ahli operation shower hose, and A4: Alia operation faucet 1.Complete data are shown in (Appendix E).

3.5.1.2 Screening for the presence of *Legionella* genus using Lgsp primer

The water and biofilm samples which gave a positive result with the common (com) primer were further tested using *Legionella* genus specific primer. Lgsp primer was used to identify *Legionella* genus bacteria in DNA extracted samples using 16S rRNA PCR. A total of 13 cold and hot water samples and 56 biofilm swabs collected from the seven governmental hospitals in the West Bank and Al-Makassed hospital in Eastern Jerusalem were screened. Positive results were observed in 8 (53.3%) of the hospital water samples and in 44 (68.8%) of the hospital biofilm swabs. In this reaction, Lgsp primer gives PCR aproduct 426bp (Fig. 3.14).

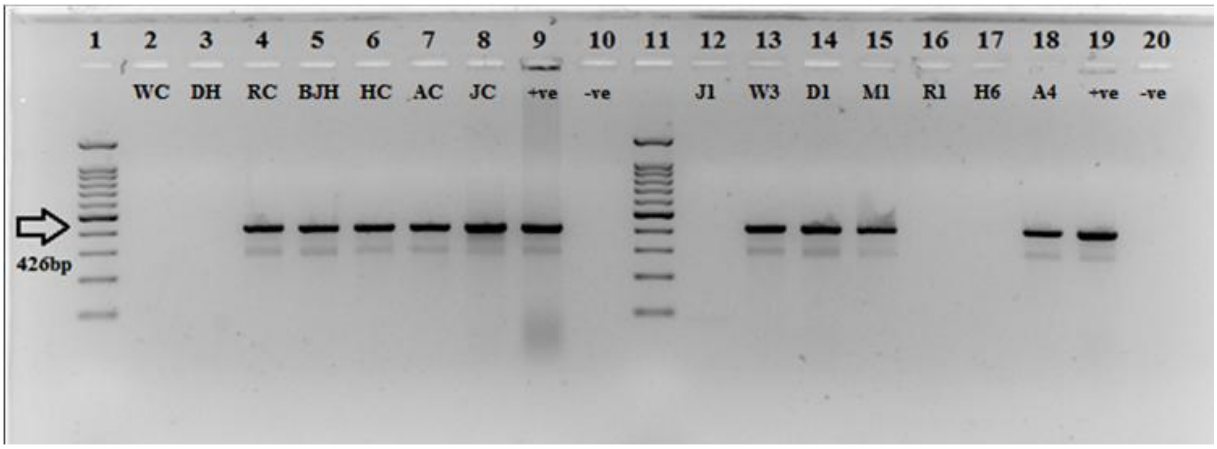


Figure 3.14: Representative 16S rRNA PCR using Lgsp primer of DNA from hospital samples. Lane 1 represents 100bp ladder. Lanes 2 to 8 represent sandwich filter samples (water samples), positive with 426bp and negative results. Lane 9, positive control (*Legionella feeleii* –L2-). Lane 10, negative control (*E.coli*). Lane 11 represents 100bp ladder. Lanes 12 to 18 represent biofilm swab samples, positive with 426bp and negative results. Lane 19, positive control (*Legionella pneumophila* subsp. *pneu.* Philadelphia-L4-). Lane 20, negative control (DW). WC: Al-Watani cold water, DH: Rafidia hot water, RC: Ramallah cold water, BJH: Beit Jala hot water, HC: Al-Ahli cold water, AC: Alia cold water, JC: Jenin cold water, J1: Jenin operation shower hose, W3: Al-watani pharmacy faucet, D1: Rafidia burn faucet, M1: Al-Makassed labor outside faucet, R1: Ramallah X-Ray faucet, H6: Al-Ahli CCU shower hose, and A4: Alia operation faucet 1.Complete data are shown in (Appendix E).

3.5.1.3 Screening for the presence of *L.pneumophila* using L1 primer

The water and biofilm samples that were positive for the com primer and the *Legionella* genus primer were screened for *L.pneumophila* using L1 primer. L1 primer was used to identify *L.pneumophila* bacteria in DNA extracted samples using 16S rRNA PCR. A total of 13 cold and hot water samples and 56 biofilm swabs collected from the seven governmental hospitals in the West Bank and Al-Makassed hospital in Eastern Jerusalem were screened. Positive results were obtained in 8 (53.3%) of the hospital water samples and in 44 (68.8%) of the hospital biofilm swabs. In this reaction, L1 primer gives PCR product 544bp (Fig. 3.15).

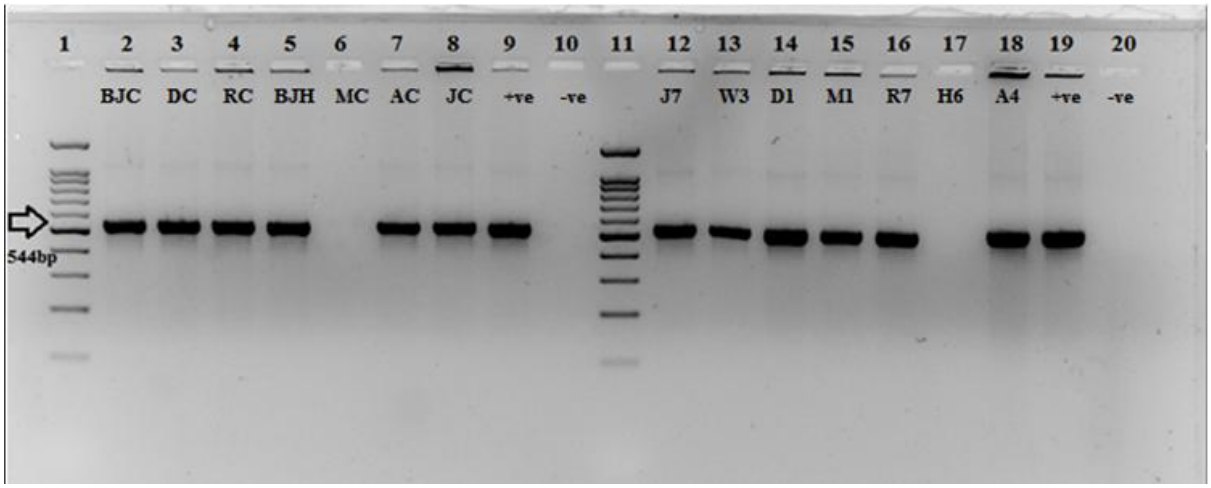


Figure 3.15: Representative 16S rRNA PCR using L1 primer of DNA from hospital samples. Lane 1 represents 100bp ladder. Lanes 2 to 8 represent sandwich filter samples (water samples), positive with 544bp and negatives results. Lane 9, positive control (*Legionella pneumophila str.Corby* –L3-). Lane 10, negative control (*Legionella anisa* –L1-). Lane 11 represents 100bp ladder. Lanes 12 to 18 represent biofilm swabs samples, positive with 544bp and negatives results. Lane 19, positive control (*Legionella pneumophila* subsp *.pneu.* Philadelphia-L4-). Lane 20, negative control (DW). BJC: Beit Jala cold water, DC: Rafidia cold water, RC: Ramallah cold water, BJH: Beit Jala hot water, MC: Al-Makassed cold water, AC: Alia cold water, JC: Jenin cold water, J7: Jenin neonate internal faucet, W3: Al-watani pharmacy faucet, D1: Rafidia burn faucet, M1: Al-Makassed labor outside faucet, R7: Ramallah CCU shower hose, H6: Al-Ahli CCU shower hose, and A4: Alia operation faucet 1. Complete data are shown in (Appendix E).

3.5.1.4 *L.pneumophila* isolates confirmation using L1 primer

We tested all 96 isolates by 16S rRNA using L1 primer and found them all to be *L.pneumophila*. In this reaction, L1 primer gives PCR product 544bp as clearly seen in (Fig. 3.16).

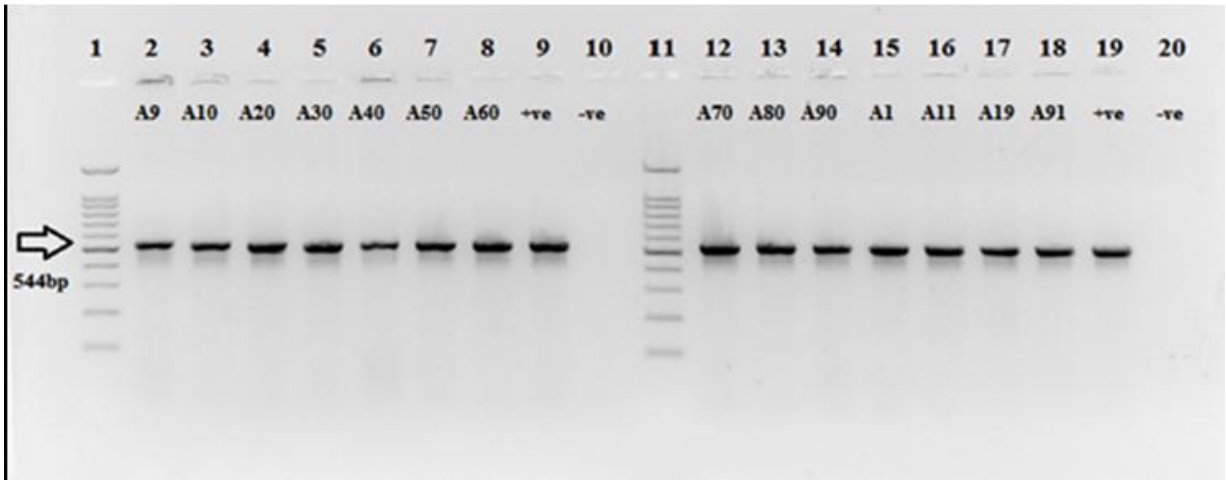


Figure 3.16: *L.pneumophila* isolates confirmation using L1 primer. Lane 1 represents 100bp ladder. Lane 2-8 shows *L.pneumophila* isolates with 544bp. Lane 9, positive control (*Legionella pneumophila str.Corby* –L3-). Lane 10, negative control (*Legionella jordanis* – L5-). Lane 11 represents 100bp ladder. Lane 12-18 show *L.pneumophila* isolates with 544bp. Lane 19, positive control (*Legionella pneumophila* subsp.*pneu.*Philadelphia-L4-). Lane 20, negative control (DW). A9: AQU College of ART 1, A10: AQU College of ART 2, A20: Beit Jala men medical ward, A30: Al-Ahli sterilization ward, A40: Jenin pediatric ward, A50: Jenin cold water, A60: Ramallah pediatric CCU ward, A70: Ramallah neonate ICU ward, A80: Rafidia labor ward1, A90: Rafidia physiotherapy ward, A1: Ramallah hospital, A11: AQU college of ART, A19: Beit Jala motor room, and A91: Al-Makassed orthopedic ward. Complete data are shown in (Appendix D).

3.5.2 Sequencing of the 16S rRNA gene and phylogenetic analysis

Phylogenetic analysis of 16S rRNA gene sequences obtained from six isolates using the Neighbor-Joining method. These isolates resulted in six *Legionella* sp. strains, which were further characterized by sequencing of the complete 16S rRNA gene (Table 3.7). A blast alignment search of the 16S rRNA sequences from the six isolates were genetically closed to four isolates could be clearly assigned as *L.pneumophila* Philadelphia namely (Sci1, LR1, LR2, and Med), one could be assigned as *L.pneumophila* Paris namely (Art2), and one could be assigned as *L.pneumophila pneumophila* ATCC 43290 namely (Art1). Phylogenetic tree is shown in (Fig. 3.17). Complete sequence is shown in (Appendix F).

Table 3.7: Characteristics of *L.pneumophila* isolates obtained from Ramallah hospital and Al-Quds University.

Isolate	source of isolaton	*L.pneumophila* PCR	16S rRNA gene sequence	Similarity
Sci1	AQU Science college	(+)	*L.pneumophila* Philadelphia	100%
LR1	Ramallah hospital	(+)	*L.pneumophila* Philadelphia	100%
LR2	Ramallah hospital	(+)	*L.pneumophila* Philadelphia	100%
Med	AQU Medicine college	(+)	*L.pneumophila* Philadelphia	100%
Art2	AQU Art college	(+)	*L.pneumophila* Paris	100%
Art1	AQU Art college	(+)	*L.pneumophila pneumophila* ATCC 43290	100%

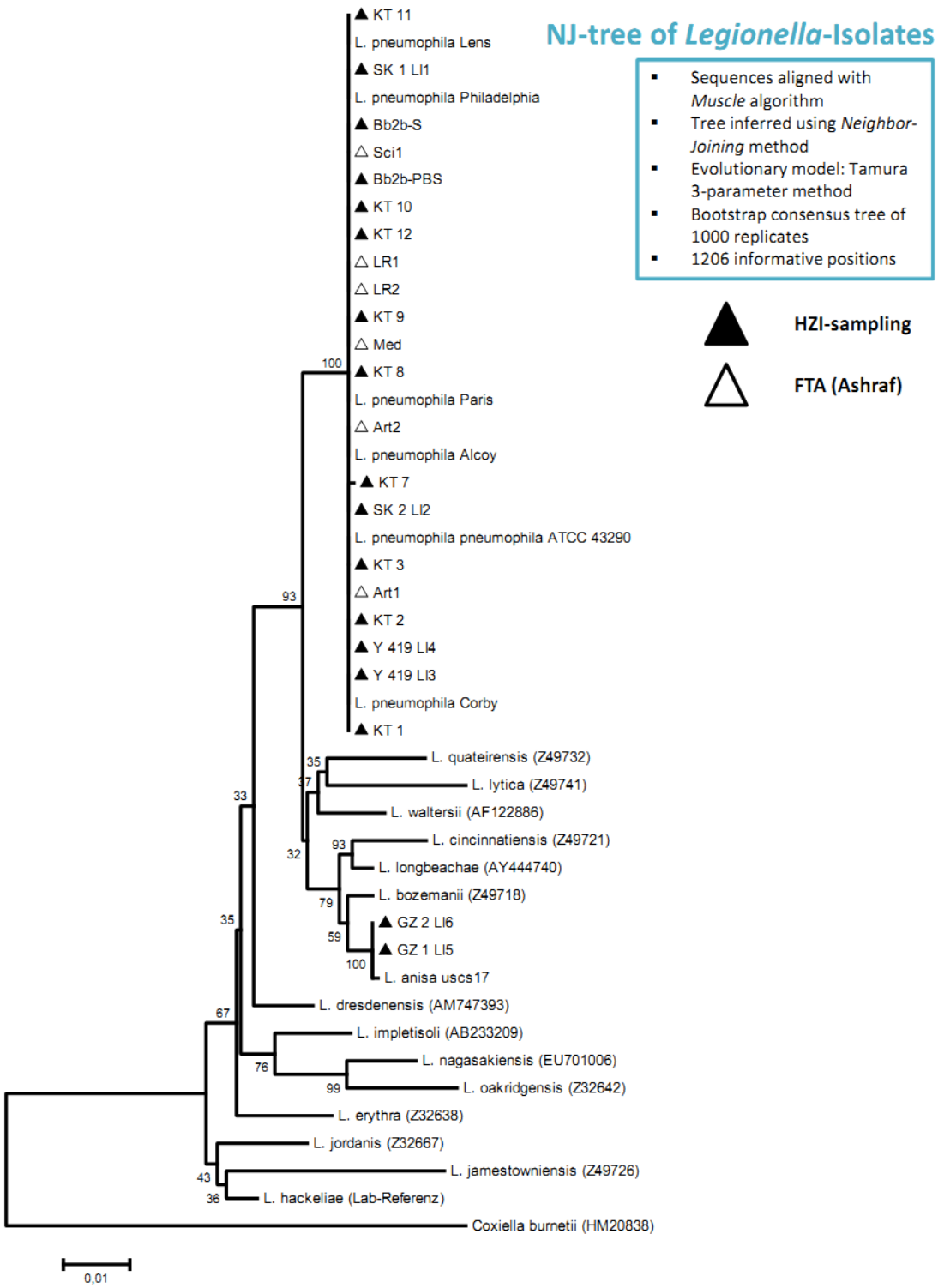


Figure 3.17: Phylogenitic tree sequence analysis of 16S rRNA gene sequences obtained for *Legionella* isolates from Ramallah hospital and Al-Quds University. (Δ Ashraf isolates) Δ Sci (College of Science), Δ LR1 Δ LR2 (Ramallah hospital), Δ Med (College of Medicine), Δ Art1 Δ Art 2 (College of Arts).

3.5.3 MLVA-8

HZI performed the clonal genotypes of 41 isolates according to the well-established MLVA-8 developed previously by Pourcel *et al* (Pourcel et al., 2003; Pourcel et al., 2007). Based on the PCR product sizes, the numbers of repeats in the alleles were calculated by subtraction of the number of flanking bases and division by the repeat unit length. Also, the observed allele sizes were compared with the sizes reported in the help file for the *Legionella pneumophila* MLVA typing website (http: //bacterial-genotyping.igmors.u-psud.fr /Legionella2006 /help .htm). This website should assist assignments when calculating allele sizes by gel-based MLVA.

As shown in (Table 3.8-a and Table 3.8-b). The 13 isolates (A1, A2, A3, A4, A5, A6, A9, A12, A20, A40, A55, A72, and A80) showed the same MLVA genotype (Gt1). A7 isolate showed MLVA genotype (Gt2). The 2 isolates from Al-Ahli hospital (A26 and A32) showed the same MLVA genotype (Gt3). The 4 isolates From Al-Quds University Health Complex (A14, A15, A17 and A18) showed the same MLVA genotype (Gt4). A64 isolate showed MLVA genotype (Gt5). The 6 isolates (A10, A37, A41, A44, A69, and A79) showed the same MLVA genotype (Gt6). A8 isolate showed nearly the same allelic profile, with only a small difference for locus 19; where no PCR product with the MLVA genotype 5. Also, A68 isolate showed nearly the same allelic profile, with only a small difference for locus 34; where no PCR product with the MLVA genotype 5. A91 isolate showed MLVA genotype (Gt7). The 4 isolates (A23, A38, A50, and A92) showed the same MLVA genotype (Gt8). The 3 isolates from Rafidia hospital (A82, A84, and A86) showed the same MLVA genotype (Gt9). A22 isolate showed nearly the same allelic profile, with only a small difference for locus 35; where no PCR product with the MLVA genotype 8. The 3 isolates (A19, A21, and A71) showed the same MLVA genotype (Gt10). MLVA analysis of the isolates showed a high degree of diversity. The diversity can be assumed to be partially a result of different water sources, because the pattern of MLVA types varied from site to site.

Table 3.8-a: MLVA-8 profile for *L.pneumophila* isolates. Genotype 1 (Gt1) was 13 isolates, 2 isolates (A1 and A2) from Ramallah hospital, 6 isolates (A3, A4, A5, A6,A9 and A12) from Al-Quds University, 1 isolate (A20) from Beit Jala hospital, 2 isolates (A40 and A55) from Jenin hospital, 1 isolate (A72) from Al-Watani hospital and 1 isolate A80 from Rafidia hospital. Gt2 was 1 isolate (A7) from Al-Quds University. Gt3 was 2 isolate (A26 and A32) from Al-Ahli hospital. Gt4 was 4 isolate (A14, A15, A17 and A18) from Al-Quds University. Gt5 was 1 isolate (A64) from Ramallah hospital.

Isolate No	Isolate designation	Site of isolation	MLVA-8 genotype at locus:								Genotype Designation
			1	3	13	17	19	33	34	35	
A1	LR1	Ramallah Hospital	7	7	10	2	4	4	2	17	Gt1
A2	LR2	Ramallah Hospital	7	7	10	2	4	4	2	17	
A3	Art1	AQU Art Faculty	7	7	10	2	4	4	2	17	
A4	Art2	AQU Art Faculty	7	7	10	2	4	4	2	17	
A5	Sci1	AQU Science Faculty	7	7	10	2	4	4	2	17	
A6	Sci2	AQU Science Faculty	7	7	10	2	4	4	2	17	
A9	Artf41a	AQU Art Faculty	7	7	10	2	4	4	2	17	
A12	Artf22	AQU Art Faculty	7	7	10	2	4	4	2	17	
A20	Jalamedmshh	Beit Jala hospital	7	7	10	2	4	4	2	17	
A40	Jenkid1	Jenin hospital	7	7	10	2	4	4	2	17	
A55	Jenchilda	Jenin hospital	7	7	10	2	4	4	2	17	
A72	Watwom	Al-Watani hospital	7	7	10	2	4	4	2	17	
A80	Rafdel1	Rafidia hospital	7	7	10	2	4	4	2	17	
A7	Med	AQU Health Complex	7	7	10	2	4	2	2	18	Gt2
A26	AhliCCU	Al-Ahli hospital	7	7	10	2	4	4	2	18	Gt3
A32	Ahlicoldw	Al-Ahli hospital	7	7	10	2	4	4	2	18	
A14	Pharmf1	AQU Health Complex	7	7	9	2	4	1	1	18	Gt4
A15	Pharmf2	AQU Health Complex	7	7	9	2	4	1	1	18	
A17	Pharmm1	AQU Health Complex	7	7	9	2	4	1	1	18	
A18	Medhandcape	AQU Health Complex	7	7	9	2	4	1	1	18	
A64	Ramopershh2	Ramallah hospital	8	7	10	2	4	4	2	17	Gt5

Table 3.8-b: MLVA-8 profile for *L.pneumophila* isolates. (Gt6) was 8 isolates, 3 isolates (A37, A41 and A44) from Jenin hospital, 2 isolates (A8 and A10) from Al-Quds University, 2 isolates (A68 and A69) from Ramallah hospital and 1 isolate (A79) from Rafidia hospital. (Gt7) was 1 isolates (A91) from Al-Makassed hospital. (Gt8) was 4 isolates, 2 isolates (A38 and A50) from Jenin hospital, 1 isolate (A23) from Al-Ahli hospital and 1 isolate (A92) from Al-Makassed hospital. (Gt9) was 4 isolates, 3 isolates (A82, A84 and A86) from Rafidia hospital and 1 isolate (A22) from Alia hospital. (Gt10) was 3 isolates, 2 isolates (A19 and A21) from Beit Jala hospital and 1 isolate (A71) from Al-Watani hospital. -1: No PCR product.

Isolate No	Isolate designation	Site of isolation	MLVA-8 genotype at locus:								Genotype Designation
			1	3	13	17	19	33	34	35	
A10	Artf41b	AQU Art Faculty	8	8	11	1	4	1	1	3	Gt6
A37	Jensur3	Jenin hospital	8	8	11	1	4	1	1	3	
A41	Jenkid2	Jenin hospital	8	8	11	1	4	1	1	3	
A44	Jenneoiso	Jenin hospital	8	8	11	1	4	1	1	3	
A69	Ramchneocli	Ramallah hospital	8	8	11	1	4	1	1	3	
A79	RafneoICUs	Rafidia hospital	8	8	11	1	4	1	1	3	
A8	Artf12	AQU Art Faculty	8	8	11	1	-1	1	1	3	
A68	Ramspemechild	Ramallah hospital	8	8	11	1	4	1	-1	3	
A91	Makortho	Al-Makassed hospital	9	8	8	2	4	2	1	18	Gt7
A23	Ahlisurshh	Al-Ahli hospital	9	8	8	2	4	2	2	18	Gt8
A38	Jensurrest	Jenin hospital	9	8	8	2	4	2	2	18	
A50	Jencold	Jenin hospital	9	8	8	2	4	2	2	18	
A92	MakICU	Al-Makassed hospital	9	8	8	2	4	2	2	18	
A82	Rafpharm	Rafidia hospital	9	8	11	2	4	3	4	17	Gt9
A84	Rafeme	Rafidia hospital	9	8	11	2	4	3	4	17	
A86	Raforthoshho	Rafidia hospital	9	8	11	2	4	3	4	17	
A22	Aliamed	Alia hospital	9	8	11	2	4	3	4	-1	
A19	Jalamotor	Beit Jala hospital	9	8	6	2	4	3	4	10	Gt10
A21	Jalahotw	Beit Jala hospital	9	8	6	2	4	3	4	10	
A71	Wateme2	Al-Watani hospital	9	8	6	2	4	3	4	10	

Chapter Four

4.1 Discussion

Presence of *Legionella* bacteria in water distribution systems is a serious health risk to hospital staff and patients (Fiore et al., 1998; Schijven and de Roda Husman, 2005; Yu and Stout, 2000), but the magnitude of the problem is largely unrecognized and there are no specific guidelines for protecting patients from exposure in West Bank hospitals. *Legionella* are difficult to isolate using microbiological methods due to a viable but nonculturable (VBNC) state. This state is leading to misdiagnosis of Legionellosis (Kahlisch et al., 2010). Furthermore, cultivation of this fastidious bacterium is difficult due to its slow growth and overgrowth by competing bacteria in the same sample (Nederbragt et al., 2008; Steinert et al., 1997; Steinert et al., 2002).

In order to address the problem, we set out to identify the levels of *Legionella* contamination in West Bank hospitals water supplies testing bulk water and biofilms using molecular, microbiological and serological techniques. This complete system will reduce misdiagnosis of *Legionella* in hospital water systems.

To reach our goal, water samples and biofilm swabs from seven governmental hospitals of different regions in the West Bank, Al-Makassed hospital in Eastern Jerusalem and from Al-Quds University main campus were analyzed for the presence of *Legionella* spp in their water sources. Cultivation dependent analysis was performed using microbiological techniques. HPC and *Legionella* identification and quantification were done using yeast extract agar and GVPC/BCYE respectively. Cultivation independent analysis was performed using 16S rRNA

PCR, MLVA-8 and complete sequencing of the 16S rRNA gene. Moreover, serological method was performed to classify *Legionella* serogroups.

Our study is in accordance with previous studies. The prevalence of *Legionella* in Palestinian water sources is evident. All the water sources tested positive for the presence of *Legionella* with 20% prevalence for the water samples by cultivation dependent analysis and the prevalence rate increased to 53.3% by cultivation independent analysis. As for the biofilms the *Legionella* prevalence is more evident, being 31.9% by cultivation dependent analysis, and increasing to 68.8% by cultivation independent analysis. Biofilms can harbor 25 times more bacteria per unit length of a pipe with 100mm diameter than in bulk water (Rogers et al., 1994). The formation of biofilm depends on nutrient availability for the bacteria and features of the supply system such as temperature, flow speed, architecture, pipe and valve materials etc (Rogers et al., 1994; Williams and Braun-Howland, 2003). Wellinghausen *et. al* (2001) studied the contamination of hospital water systems with *Legionella* at three different hospitals belonging to the University of Ulm in Germany. The rate of detection of *Legionella* was 70.1% (54 of 77) (Wellinghausen et al., 2001). Also, Doleans *et. al* (2004), made a relationship between hospital water contamination with *Legionella* and hospital-acquired Legionellosis, they examined the level of *Legionella* colonization of hospital water systems in France.(Doleans et al., 2004). Also, a Greek study studied prevalence of *Legionella* spp. in Greek hospitals. They collected water and swab samples from 13 hospitals analyzed them for *legionella* using cultivation independent analysis (AFLP). They detected *Legionella* in 8 out of 13 hospitals (Mavridou et al., 2008). In Another Italian study *Legionella* was isolated from 22.6% of the samples of hot water taken from domestic water system, (Borella et al., 2004). There are no previous studies of *Legionella* in universities. We studied *Legionella* prevalence in Al-Quds University to know the rate of *Legionella* contamination. Al-Quds University water system showed biofilms tested positive for *Legionella* with prevalence rate of 37.2%.

Cold water was sampled from hospitals beginning in November 7th, ending in December 21st 2012. The mean temperature for cold water was 21.2±2.1°C and varied, depending on the hospital, between 18.4°C in Al-Ahli hospital and 25.5°C in Beit Jala hospital (Table 3.2).

Legionella was isolated from two cold water samples from Jenin hospital and Al-Ahli hospital and one hot water sample from Beit Jala hospital. The temperature of the hot water in Beit Jala was only 52.2°C (Fields et al., 2002; Kusnetsov et al., 2003; Stout et al., 1982; Wadowsky et al., 1985). The pH of the cold water and hot water varied between 7.6 and 8.3 with a mean of 7.9±0.2. Measurements of conductivity during sampling resulted in a mean conductivity of 716.8 ± 83.2 µS/cm (Table 3.2). The number of heterotrophic counts varied strongly between 3 CFU/ml in AL-Watani hospital and 487 CFU/ml in Beit Jala hospital with incubation at 36°C for 48h and 7 CFU/ml in Rafidia hospital and 347 CFU/ml in Ramallah hospital with incubation at 22°C for 72h (Table 3.3).

Hot water temperature varied between 29.5°C in Alia hospital and 70.9°C in Rafidia hospital with a mean of 48±12.9°C. To be effective the water has to be heated to above 70°C (Kusnetsov et al., 2003; Wadowsky et al., 1985). The mean conductivity was 727.6±85.3 µS/cm. The number of heterotrophic counts varied strongly between 0 CFU/ml Rafidia hospital and 137 CFU/ml in Beit Jala hospital with incubation at 36°C for 48h and 0 CFU/ml in Rafidia hospital and 220 CFU/ml in Beit Jala hospital with incubation at 22°C for 72h (Table 3.3). Overall, heterotrophic plate countd from hot water samples were about 50% lower than from cold water. Obviously the water temperature in Rafidia hospital at 70.9°C is effective in killing the bacteria.

Heterotrophic plate counts measured in the hot water, which is heated to about 50°C (Al-Makassed and Beit Jala), was only 51.7% lower than in cold, unheated water. On the other hand, Rafidia hospital hot water, which is heated to about 70°C, completely killed the bacteria. The heating of water to temperatures of 60°C or more is widely used to reduce total bacterial numbers and inactivate the number of pathogens like *Legionella* (Kusnetsov et al., 2003; Wadowsky et al., 1985). Hence, we noticed a clear problem in West Bank hospital heating system because the hot water in these hospitals didn't reach 50°C except Al-Makassed and Beit Jala and only one hospital Rafidia used effective thermal disinfection with temperatures as high as 70°C.

Legionella can be found in hot water systems, when temperatures are around 50°C. Kusnetsov *et .al*. demonstrated that the growth of *Legionella* can be reduced from (mean $3.6x10^3$ CFU/l) to (mean 35 CFU/l) in hot water when the temperature is elevated to (60-80°C) (Kusnetsov et al., 2003).Also, Shareef and Mimi treated West Bank hospital water by heat. They heated Beit Jala hospital water to 80°C and Jenin hospital water to 70°C for 30 minutes, to demonstrate *Legionella* colonization before and after thermal disinfection. In Beit Jala hospital after thermal disinfection at 80°C, *L. pneumophila* positive samples were reduced from 100% before heat disinfection to 17% after heat disinfection. While, in Jenin hospital thermal disinfection (70°C) reduced the number of *L. pneumophila* colonization but not completely eliminated it. It was demonstrated that the high number of *L. pneumophila* in water distribution systems can be successfully reduced by heat treatment. In comparing the results after thermal disinfection between Beit Jala hospital and Jenin hospital, it was seen that, the thermal disinfection in Beit Jala hospital was more effective, since the temperature in Beit Jala hospital reached 80°C while in Jenin hospital it was reached 70°C. This was explained that the aggregation of biofilm entire water distribution system in Jenin hospital needs higher temperature and longer time period for killing bacteria(Shareef and Mimi, 2008).

During this study sampling period all measured physical and chemical bulk water parameters were within the range accepted by the World Health Organization (WHO) (World Health Organization WHO, 2008).

The main cause of poor water quality is the build-up of biofilm on the entire surface of water distribution systems and tanks, *Legionella* are often considered from the pioneers in creating the biofilm showed in water distribution systems, with time the accumulation of biofilm increases and the elimination of *Legionella* becomes difficult due to the nature of *Legionella* being tolerant to a wide variety of physical and chemical conditions, including temperature and chlorination. The two buildings in Ramallah hospital water system; (namely Al-Bahraini which is the neonatal and pediatric ward and Al-Kuwaiti which is the cardiac ward) are relatively new wards were established in 2010. However, *Legionella* was found in those two wards. We observed that there is a problem in the control of water temperature; it couldn't be raised more than 50°C, this is a suitable environment for the survival of *Legionella* within the

hospitals water system. To prevent the accumulation of biofilm, a factor known to be associated with outbreaks of Legionnaires' disease, water systems should be cleaned regularly including showerheads, shower hoses, faucets and main storage tanks.

The water reservoir of Al-Makassed and Al-Watani hospitals were analyzed for *Legionella*, and were found to be uncontaminated, whereas the swab from the distribution systems of the two hospitals grew *Legionella*. Biofilms are found in every drinking water distribution system attached to the surface where they harbor many potentially pathogenic bacteria which are not isolated from the bulk water but are in the biofilms. The biofilm community is protected against adverse environmental conditions including disinfection (Buswell et al., 1998; Rogers et al., 1994; Williams and Braun-Howland, 2003). Drinking water biofilms are considered an important reservoir for pathogens such as *L.pneumophila*, *Mycobacterium* spp, and *Helicobacter* spp (Berry et al., 2006; Donlan, 2002; Feazel et al., 2009; Parsek and Singh, 2003).

In the current study, *L.pneumophila* was the only *Legionella* species detected, according to Italian survey *L.pneumophila* is the most abundant species in potable and environmental water samples, more than 75% of positive samples were contaminated by *L.pneumophila* (Borella et al., 2004). Another study, 95.4% *L.pneumophila* was isolated from the Greek hospitals (Mavridou et al., 2008). Furthermore, a Korean study investigated the distribution of *Legionella* species from environmental water sources of public facilities in South Korea. They isolated 560 *Legionella* isolates from whole South Korea. They found 85.5% of the isolates were *L. pneumophila* mostly serogroup1 (54.7%). The rest of the isolates (14.5%) were *non-L. pneumophila*. (Lee et al., 2010). Our study showed that all of positive samples were contaminated by *L. pneumophila*.

L. pneumophila is the most pathogenic of *Legionella* species, causing up to 90% of the cases of legionellosis (Benin et al., 2002; Yu et al., 2002). Unfortunately, there are no previous data about legionellosis cases in Palestine. *L.pneumophila* serogroup1 represented (64%) of the isolates, while (36 %) of the isolates belonged to *L.pneumophila* serogroup 2-14 out of 96 isolates (Figure 3.4). According to the current epidemiological data available from the world, different *L.pneumophila* Sgs cause Legionellosis. In European, American and Australian

societies, most of the cases were due to infections with *L.pneumophila* Sg1 (Coil et al., 2008; Huang et al., 2004; Palmore et al., 2009). In general, *L.peumophila* Sg1 is responsible for more than 60% approximately of cases in most European and American countries (Yu et al., 2002) but lower percentage of cases about 50% in countries such as Australia and New Zealand (Yu et al., 2002). Mavridou *et. al.* studied prevalence of *Legionella* sp. in Greek hospitals. They found 72.7% of *Legionella* was *L.pneumophila* serogroup1 and 22.7% were *L.pneumophila* serogroup2-14 (Mavridou et al., 2008).Whereas in the Middle East a shortage of epidemiological data for *Legionella* Sgs. A recent survey in Israel indicates that *L.pneumophila* Sg3 might be the primary etiological agent responsible for legionellosis (Oren et al., 2002). Also, another study of clinical isolates from Kuwait demonstrated the dominance (more than 80%) of *L.pneumophila* Sg3 in patients with LD (Qasem et al., 2008). Shareef and Mimi (2008) studied hospital tap water system in West Bank hospitals. They found *L.pneumophila* serogroup 2-14 in 62% of samples(Shareef and Mimi, 2008). The Greek hospital serogroup results are similar to our study.

Since the minimum infectious dose for a severe infection with *L. pneumophila* is not known exactly (O'Brien and Bhopal, 1993) and depends greatly on the susceptibility of the exposed person (Roig and Rello, 2003), we quantified the detected *L. pneumophila* population in a culture dependent analysis using direct *Legionella* count on GVPC agar. We detected only low numbers (35, 50 and 260 CFU/L *L. pneumophila*) in Beit Jala, Al-Ahli and Jenin water samples respectively (Table 3.3). The *Legionella* low count may be explained due to *Legionella* VBNC state.

The results of the study show that, the hospitals water systems are contaminated with dangerous opportunistic *L.pneumophila* in ICU, CCU, Operation, Neonate and Pediatric Wards, which is a health risk especially for Immunocompromised patients (Table 3.5). Hence, hospitals should maintain high standards of water quality and should take immediate measures to prevent waterborne infections. Such measures are likely to be successful, given the large reductions in waterborne infections observed when specific guidelines are applied.

To understand infections by *L.pneumophila*, especially regarding its epidemiological aspects, an identification of strains at the subspecies level is necessary. Molecular tools based on the

analysis of bacterial DNA like MLVA, have become widely accepted in molecular typing studies of pathogenic bacteria (Harth-Chu et al., 2009). The MLVA analysis is based on polymorphic minisatellites (VNTRs) on different loci, where recombination and DNA polymerase slipping often happen. If occurring with certain frequencies, these events can result in changes of the repeat sizes between different strains at a given locus. MLVA data for *L. pneumophila* and several other pathogens, such as *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*, can be obtained from the central GPMS website (http://minisatellites.u-psud.fr/), and the amount of data is increasing steadily (Le Fleche et al., 2001). Recently, Pourcel *et al*. (Pourcel et al., 2003; Pourcel et al., 2007) developed an MLVA-8 gel-based typing profile for *L. pneumophila*. HZI performed similarly the same MLVA-8 profile to analyze 41 of 96 isolates (Table 3.8-a and Table 3.8-b). MLVA-8 analysis showed diversity in studied *L.pneumophila* strains. Moreover, HZI characterized 6 *L.pneumophila* strains that we obtained by the sampling of different points on the Al-Quds University campus and Ramallah hospital by 16S rRNA gene sequencing. Generally, all the sequences are *Legionella pneumophila subs. pneumophila.*

Interesting observations across the different hospitals sampled were the following: the molecular detection of *Legionella* was only positive in hot water when the cold water was positive. When cold and/or hot water was positive, higher fractions of the sampled biofilms were positive. High *Legionella* plate counts only occurred in water with molecular detection of *L.pneumophila*. All high *Legionella* plate counts were from well water derived source that was not submitted to storage in reservoirs.

This study illustrates the importance of protecting hospital water systems from contamination with *Legionella* bacteria and highlights the need for appropriate specific guidelines for protecting patients from exposure.

4.2 Recommendations

In conclusion, little is known and much remains to be learned about the molecular identification and quantification of *Legionella* in water. In the future we plan to compare bacterial cell counts using fluorescent microscopy versus heterotrophic plate counts. Also, we will plan to quantify *Legionella* using real time PCR. The seasonal cycle of *Legionella* in water systems is another factor to study. Furthermore we will study clinical samples.

To reduce the option that *Legionella* may increase in the water systems, we recommend systematic monitoring water samples and biofilm swabs from the water system for identification and quantification of *Legionella* species. Also, Hot water in hospitals should be maintained above 50°C for reduction of *Legionella* count as much as possible. Heat shock method should be done frequently in all hospitals, heat disinfection for hot water temperature range between (60°C-80°C) for longer than half an hour, the hot water to circulate and flush the whole water system and the outlets for a period of time. Temperature and duration of flushing depend upon the age of the water system and the thickness of accumulated biofilm. We recommend maintaining hot water free chlorine residual at 1-2 mg/l at the faucet. We also recommend frequent cleaning of showerheads, showerhose, faucets and the main storage tank. As well as employing 0.2μm filters that can be placed on faucets and showerheads to remove bacteria. Medical tools should be rinsed with sterile water, since tap water or distilled water might contain *Legionella* that can cause pneumonia. To protect patients we recommend that immunosuppressed patients be restricted from taking showers and taking ultra care for their bathroom.

There is insufficient data to understand the link between water and pneumonia. So, we recommend heightened surveillance for nosocomial infections in all West Bank hospital wards. The Ministry of Health (MOH) must put restrictions and clear protocols for water quality in West Bank hospitals. Finally, an evaluation of the prevalence of *Legionella* in domestic, hotels and Universities is recommended.

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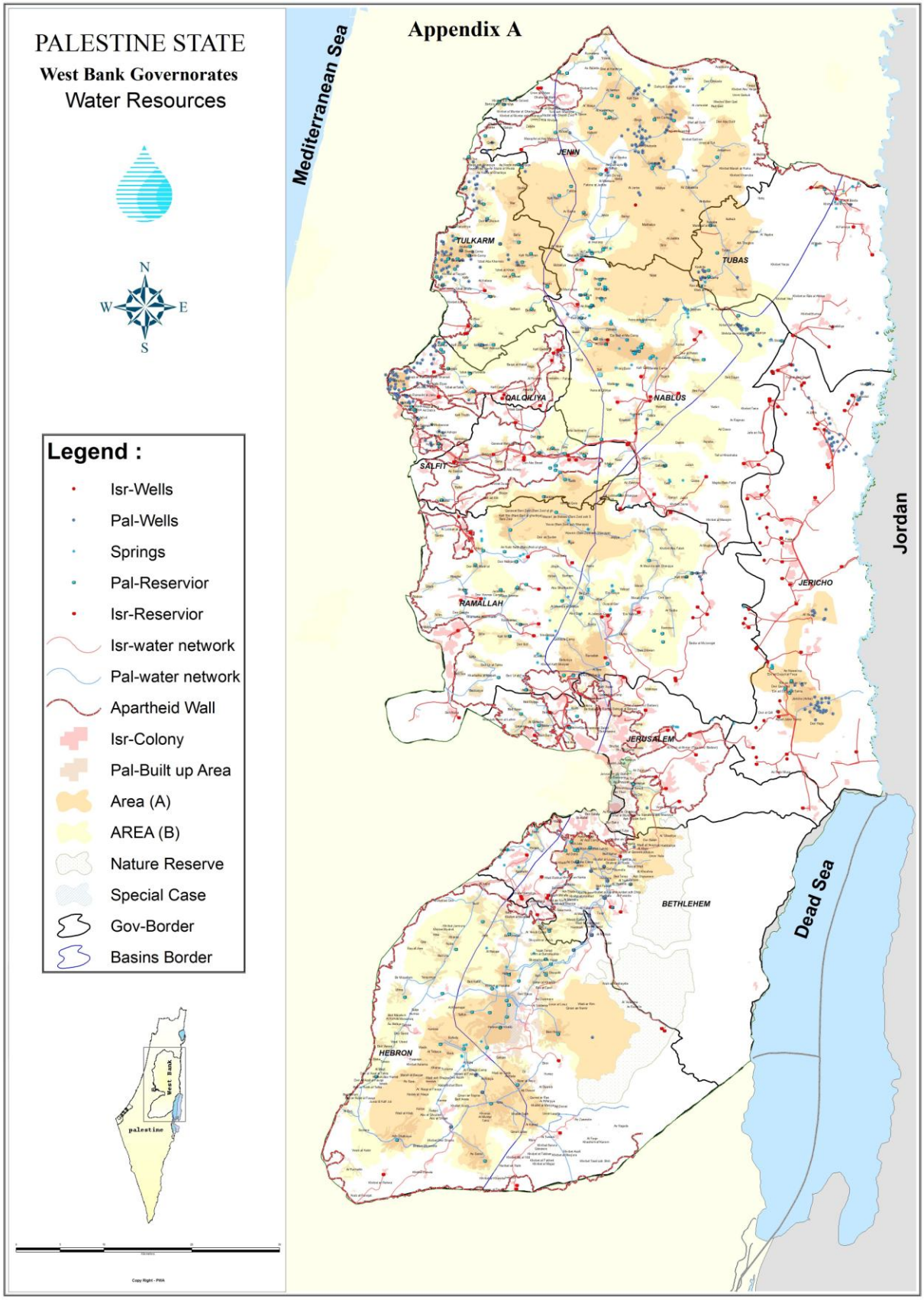
Appendices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West Bank Governorates
Water Resources
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Legend :
Isr-Wells
Pal-Wells
Springs
Pal-Reservior
Isr-Reservior
Isr-water network
Pal-water network
Apartheid Wall
Isr-Colony
Pal-Built up Area
Area (A)
AREA (B)
Nature Reserve
Special Case
Gov-Border
Basins Border
West Bank
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Appendix A
Mediterranean Sea
JENIN
TULKARM
TUBAS
QALQILIYA
NABLUS
SALFIT
RAMALLAH
JERICHO
Jordan
JERUSALEM
BETHLEHEM
HEBRON
Dead Sea

Appendix A

Al-Quds University
Faculty of Medicine
Abu-Dies, Jerusalem

جامعة القدس
كلية الطب
أبوديس – القدس

التاريخ:2013/2/9

حضرة السيدة ماجدة علاونة المحترمة
مديرة دائرة سلطة المياه

تحية طيبة و بعد،

ارجو تزويد السيد اشرف زايد بخرائط مصادر المياه لكل من:-

مستشفى جنين
مستشفى رفيديا- نابلس
مستشفى الوطني- نابلس
مجمع فلسطين الصحي – رام الله
مستشفى بيت جالا
مستشفى الأهلي- الخليل
مستشفى عالية – الخليل
جامعة القدس – الحرم الرئيس – ابوديس
مستشفى جمعية المقاصد الإسلامية الخيرية – القدس

و ذلك لحاجته لهذه المعلومات في بحثه لرسالة الماجستير
"Identification of *Legionella spp.* from hospital water systems in Palestine"

و تفضلوا بقبول فائق الإحترام

د. دينا البيطار
استاذ مشارك –الأحياء الدقيقة
دائرة الأحياء الدقيقة و المناعة
كلية الطب – جامعة القدس

P.O Box 20002
Tel 02-2799203, Fax 02-2796110

ص. ب 20002
هاتف 022799203 فاكس 022796110

Appendix B

Al-Quds University
Faculty of Medicine
Abu-Dies, Jerusalem

جامعة القدس
كلية الطب
أبوديس – القدس

التاريخ:2012/9/22

حضرة الدكتور هاني عابدين المحترم
وزير الصحة
مدير مستشفى مجمع فلسطين الطبي

الموضوع: افادة طالب

تحية طيبة و بعد،

نرجو من حضرتكم السماح للطالب اشرف زايد و هو طالب ماجستير (Clinical Lab Sciences) بأخذ عينات ماء من مستشفيات الضفة الغربية حيث انه يقوم بإعداد دراسة بعنوان:

" The molecular identification and quantification of legionella spp. From water samples"

و ذلك من المستشفيات التالية:-

1- مستشفى مجمع فلسطين الطبي
2- مستشفى جنين الحكومي
3- مستشفى الأهلي – الخليل
4- مستشفى عاليا – الخليل
5- المستشفى الوطني- نابلس
6- مستشفى بيت جالا
7- مستشفى رفيديا- نابلس

شاكرين لكم تعاونكم معنا

د. دينا البيطار
المشرفة على المشروع
كلية الطب

كلية الطب
دائرة الأحياء الدقيقة و المناعة
Faculty of Medicine

Al-Quds University
Faculty of Health Professions
Medical Laboratory Sciences Department
Jerusalem - Abu Dies

جامعة القدس
كلية المهن الصحية
دائرة العلوم الطبية المخبرية
القدس – أبوديس

التاريخ: 2012/9/21
الرقم:9/ط م م ص/594

حضرة الدكتور وائل صدقة المحترم،،
ق.أ مدير عام ادارة المستشفيات في وزارة الصحة – رام الله،،

إفادة طالب ،،

بناءا على الموافقة على كتابنا السابق للدكتور نعيم صبرة بتاريخ 2012/3/13 نرجو من حضرتكم السماح للطالب أشرف زايد وهو طالب ماجستير في (Clinical Lab Sciences) بأخذ عينات ماء من مستشفيات الضفة الغربية والقدس حيث أنه يقوم بإعداد دراسة بعنوان :

The molecular identification and quantification of Legionella *spp.* from water samples.

وذلك من المستشفيات التالية:

1) مستشفى رام الله الحكومي.
2) مستشفى جنين الحكومي.
3) مستشفى الاهلي ـ الخليل .
4) مستشفى الخليل الحكومي (عالية) – الخليل.
5) مستشفى رفيديا – نابلس.
6) المستشفى الوطني - نابلس.
7) مستشفى بيت حالا – بيت حالا.

الاستاذ المشرف: الدكتورة دينا البيطار / دائرة الأحياء الدقيقة والمناعة / كلية الطب-جامعة القدس.

شاكرين لكم تعاونكم معنا.

دائرة العلوم الطبية المخبرية
Medical Laboratory Sciences Dept.
الدكتور حاتم عيده ،،
رئيس دائرة العلوم الطبية المخبرية ،،

نسخة:الملف،،

Tel. + 02 2799753 -+ 02 2791243
Fax. 02+2791243
E- mail البريد الالكتروني
mlsdep@hpf.alquds.edu
تلفون: 02 2791243 -+ 02 2799753 +
فاكس: 02+2791243

Al-Quds University
Faculty of Health Professions
Medical Laboratory Sciences Department
Jerusalem - Abu Dies

جامعة القدس
كلية المهن الصحية
دائرة العلوم الطبية المخبرية
القدس – أبوديس

التاريخ: 2012/2/27
الرقم:9/ط م م ص/334

حضرة الدكتور بسام أبو لبدة المحترم،،
مدير مستشفى جمعة المقاصد / القدس،،

إفادة طالب

نرجو من حضرتكم السماح للطالب أشرف زايد وهو طالب ماجستير في (Clinical Lab Sciences) بأخذ عينات ماء من مستشفيات الضفة الغربية والقدس حيث أنه يقوم بإعداد دراسة بعنوان :

The molecular identification and quantification of Legionella *spp.* from water samples.

وذلك من المستشفيات التالية:

1) مستشفى رام الله الحكومي.
2) مستشفى حنين الحكومي.
3) مستشفى الاهلي _ الخليل .
4) مستشفى الخليل الحكومي (عالية) – الخليل.
5) مستشفى رفيديا – نابلس.
6) المستشفى الوطني – نابلس.
7) مستشفى بيت حالا – بيت حالا.

الاستاذ المشرف: الدكتورة دينا البيطار / دائرة الأحياء الدقيقة والمناعة / كلية الطب-جامعة القدس.

شاكرين لكم تعاونكم معنا.

د.اكرم خروبي،،
رئيس دائرة العلوم الطبية المخبرية ،،
دائرة العلوم الطبية المخبرية
Medical Laboratory Sciences Dept.

نسخة:الملف،،

Tel. + 02 2799753 -+ 02 2791243
Fax. 02+2791243
E- mail البريد الالكتروني
mlsdep@hpf.alquds.edu
تلفون: 02 2791243 -+ 02 2799753 +
فاكس: 02+2791243

Appendix C

Hospital Name		Jenin (Jenin)			
**********		Type	DNA extraction	HPC	*Legionella* count
Volume of the sample		Cold	5 Liter	1 Liter	1 Liter
		Hot	5 Liter	1 Liter	1 Liter
**********		Date		Time	
Collection sample		24-Nov-2012		8:15 AM	
Recipt in the lab		24-Nov-2012		12:50 PM	
Examination of the sample	Filtration	24-Nov-2012		1:10 PM	
	HPC	24-Nov-2012		2:00 PM	
	Swab culivation	24-Nov-2012		1:00 PM	
	Legionella count	24-Nov-2012		3:30 PM	
	DNA extraction	25-Nov-2012		9:30 AM	

Hospital Name		Rafidia (Nablus)			
**********		Type	DNA extraction	HPC	*Legionella* count
Volume of the sample		Cold	5 Liter	1 Liter	1 Liter
		Hot	5 Liter	1 Liter	1 Liter
**********		Date		Time	
Collection sample		2-Dec-2012		11:00 AM	
Recipt in the lab		2-Dec-2012		1:55 PM	
Examination of the sample	Filtration	2-Dec-2012		2:15 PM	
	HPC	2-Dec-2012		5:20 PM	
	Swab culivation	2-Dec-2012		2:15 PM	
	Legionella count	2-Dec-2012		5:30 PM	
	DNA extraction	3-Dec-2012		9:10 AM	

Hospital Name		Al-Watani (Nablus)			
**********		Type	DNA extraction	HPC	*Legionella* count
Volume of the sample		Cold	5 Liter	1 Liter	1 Liter
		Hot	5 Liter	1 Liter	1 Liter
**********		Date		Time	
Collection sample		2-Dec-2012		9:15 AM	
Recipt in the lab		2-Dec-2012		1:55 PM	
Examination of the sample	Filtration	2-Dec-2012		2:15 PM	
	HPC	2-Dec-2012		5:20 PM	
	Swab culivation	2-Dec-2012		2:15 PM	
	Legionella count	2-Dec-2012		4:50 PM	
	DNA extraction	3-Dec-2012		9:10 AM	

Hospital Name		Ramallah (Ramallah)			
**********		Type	DNA extraction	HPC	*Legionella* count
Volume of the sample		Cold	5 Liter	1 Liter	1 Liter
		Hot	5 Liter	1 Liter	1 Liter
**********		Date		Time	
Collection sample		28-Nov-2012		9:15 AM	
Recipt in the lab		28-Nov-2012		12:45 PM	
Examination of the sample	Filtration	28-Nov-2012		1:20 PM	
	HPC	28-Nov-2012		2:20 PM	
	Swab culivation	28-Nov-2012		1:00 PM	
	Legionella count	28-Nov-2012		2:00 PM	
	DNA extraction	29-Nov-2012		9:30 AM	

Hospital Name		Al-Makassed (Jerusalem)			
**********		Type	DNA extraction	HPC	*Legionella* count
Volume of the sample		Cold	5 Liter	1 Liter	1 Liter
		Hot	5 Liter	1 Liter	1 Liter
**********		Date		Time	
Collection sample		12-Dec-2012		9:30 AM	
Recipt in the lab		12-Dec-2012		12:00 PM	
Examination of the sample	Filtration	12-Dec-2012		12:20 PM	
	HPC	12-Dec-2012		1:30 PM	
	Swab culivation	12-Dec-2012		12:10 PM	
	Legionella count	12-Dec-2012		1:50 PM	
	DNA extraction	13-Dec-2012		9:30 AM	

Hospital Name		Beit Jala (Bethlehem)			
**********		Type	DNA extraction	HPC	*Legionella* count
Volume of the sample		Cold	5 Liter	1 Liter	1 Liter
		Hot	5 Liter	1 Liter	1 Liter
**********		Date		Time	
Collection sample		7-Nov-2012		9:00 AM	
Recipt in the lab		7-Nov-2012		11:45 AM	
Examination of the sample	Filtration	7-Nov-2012		12:00 PM	
	HPC	7-Nov-2012		1:00 PM	
	Swab culivation	7-Nov-2012		12:30 PM	
	Legionella count	7-Nov-2012		2:30 PM	
	DNA extraction	8-Nov-2012		9:30 AM	

Hospital Name		Al-Ahli (Hebron)			
**********		Type	DNA extraction	HPC	*Legionella* count
Volume of the sample		Cold	5 Liter	1 Liter	1 Liter
		Hot	5 Liter	1 Liter	1 Liter
**********		Date		Time	
Collection sample		11-Nov-2012		10:00 AM	
Recipt in the lab		11-Nov-2012		4:45 PM	
Examination of the sample	Filtration	12-Nov-2012		8:00 AM	
	HPC	11-Nov-2012		5:20 PM	
	Swab culivation	11-Nov-2012		5:00 PM	
	Legionella count	11-Nov-2012		5:40 PM	
	DNA extraction	12-Nov-2012		11:30 AM	

Hospital Name		Alia (Hebron)			
**********		Type	DNA extraction	HPC	*Legionella* count
Volume of the sample		Cold	5 Liter	1 Liter	1 Liter
		Hot	5 Liter	1 Liter	1 Liter
**********		Date		Time	
Collection sample		11-Nov-2012		1:00 PM	
Recipt in the lab		11-Nov-2012		4:45 PM	
Examination of the sample	Filtration	12-Nov-2012		8:00 AM	
	HPC	11-Nov-2012		5:20 PM	
	Swab culivation	11-Nov-2012		5:00 PM	
	Legionella count	11-Nov-2012		5:40 PM	
	DNA extraction	12-Nov-2012		11:30 AM	

Appendix D

	Isolation	Site of isolation	Isolate design	Sg	L1 primer	medium	sample	Frozen
A1	1-Dec-2011	Ramallah Hospital	LR1	1	(+)	BCYE	Biofilm	08-Nov-12
A2	1-Dec-2011	Ramallah Hospital	LR2	1	(+)	BCYE	Biofilm	08-Nov-12
A3	19-Jan-2012	AQU Art College	Art1	1	(+)	BCYE	Biofilm	08-Nov-12
A4	19-Jan-2012	AQU Art College	Art2	1	(+)	BCYE	Biofilm	08-Nov-12
A5	19-Jan-2012	AQU Science College	Sci1	1	(+)	BCYE	Biofilm	08-Nov-12
A6	19-Jan-2012	AQU Science College	Sci2	1	(+)	BCYE	Biofilm	08-Nov-12
A7	19-Jan-2012	AQU Medicine College	Med	(2-14)	(+)	GVPC	Biofilm	08-Nov-12
A8	14-Oct-2012	AQU Art College	Artf12	(2-14)	(+)	BCYE	Biofilm	08-Nov-12
A9	14-Oct-2012	AQU Art College	Artf41a	1	(+)	BCYE	Biofilm	08-Nov-12
A10		AQU Art College	Artf41b	(2-14)	(+)	BCYE	Biofilm	08-Nov-12
A11	14-Oct-2012	AQU Art College	Artf32	1	(+)	BCYE	Biofilm	08-Nov-12
A12	14-Oct-2012	AQU Art College	Artf22	1	(+)	BCYE	Biofilm	08-Nov-12
A13	14-Oct-2012	AQU Art College	Artm22	1	(+)	BCYE	Biofilm	08-Nov-12
A14	14-Oct-2012	AQU Pharmacy College	Pharmf1	(2-14)	(+)	BCYE	Biofilm	08-Nov-12
A15	14-Oct-2012	AQU Pharmacy College	Pharmf2	(2-14)	(+)	BCYE	Biofilm	08-Nov-12
A16	14-Oct-2012	AQU Pharmacy College	Pharmf4	(2-14)	(+)	BCYE	Biofilm	08-Nov-12
A17	14-Oct-2012	AQU Pharmacy College	Pharmm1	(2-14)	(+)	BCYE	Biofilm	08-Nov-12
A18	14-Oct-2012	AQU Medicine College	Medmhandcape	(2-14)	(+)	BCYE	Biofilm	08-Nov-12
A19	7-Nov-2012	Biet Jala hospital	Jalamotor	(2-14)	(+)	GVPC	Biofilm	18-Nov-12
A20	7-Nov-2012	Biet Jala Hospital	Jalamedmshh	1	(+)	GVPC	Biofilm	18-Nov-12
A21	7-Nov-2012	Biet Jala hospital	Jalahotw	(2-14)	(+)	GVPC	Water	18-Nov-12
A22	17-Nov-2012	Alia hospital	Aliamed	(2-14)	(+)	GVPC	Biofilm	25-Nov-12
A23	17-Nov-2012	Ahli hospital	Ahlisurshh	(2-14)	(+)	GVPC	Biofilm	25-Nov-12
A24	17-Nov-2012	Ahli hospital	Ahlisurshho	1	(+)	GVPC	Biofilm	25-Nov-12
A25	17-Nov-2012	Ahli hospital	Ahlisur	1	(+)	GVPC	Biofilm	25-Nov-12
A26	17-Nov-2012	Ahli hospital	AhliCCU	1	(+)	GVPC	Biofilm	25-Nov-12
A27	17-Nov-2012	Ahli hospital	Ahlieme	1	(+)	GVPC	Biofilm	25-Nov-12
A28	17-Nov-2012	Ahli hospital	Ahliwash	1	(+)	GVPC	Biofilm	25-Nov-12
A29	17-Nov-2012	Ahli hospital	Ahlimed	1	(+)	GVPC	Biofilm	25-Nov-12

	Isolation	Site of isolation	Isolate design	Sg	L1 primer	medium	sample	Frozen
A30	17-Nov-2012	Ahli hospital	Ahlister	1	(+)	GVPC	Biofilm	25-Nov-12
A31	17-Nov-2012	Ahli hospital	Ahliendoshho	1	(+)	GVPC	Biofilm	25-Nov-12
A32	17-Nov-2012	Ahli hospital	Ahlicoldw	1	(+)	GVPC	Water	25-Nov-12
A33	17-Nov-2012	Ahli hospital	Ahlilab	1	(+)	GVPC	Biofilm	25-Nov-12
A34	24-Nov-2012	Jenin hospital	JenICUshf	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A35	24-Nov-2012	Jenin hospital	JenICUin	1	(+)	GVPC	Biofilm	22-Dec-12
A36	24-Nov-2012	Jenin hospital	Jensur2	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A37	24-Nov-2012	Jenin hospital	Jensur3	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A38	24-Nov-2012	Jenin hospital	Jensurrest	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A39	24-Nov-2012	Jenin hospital	Jenkit	1	(+)	GVPC	Biofilm	22-Dec-12
A40	24-Nov-2012	Jenin hospital	Jenkid1	1	(+)	GVPC	Biofilm	22-Dec-12
A41	24-Nov-2012	Jenin hospital	Jenkid2	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A42	24-Nov-2012	Jenin hospital	Jenkidin	1	(+)	GVPC	Biofilm	22-Dec-12
A43	24-Nov-2012	Jenin hospital	Jenneo	1	(+)	GVPC	Biofilm	22-Dec-12
A44	24-Nov-2012	Jenin hospital	Jenneoiso	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A45	24-Nov-2012	Jenin hospital	Jenchild	1	(+)	GVPC	Biofilm	22-Dec-12
A46	24-Nov-2012	Jenin hospital	Jenadmin	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A47	24-Nov-2012	Jenin hospital	Jenlab	1	(+)	GVPC	Biofilm	22-Dec-12
A48	24-Nov-2012	Jenin hospital	Jenmen	1	(+)	GVPC	Biofilm	22-Dec-12
A49	24-Nov-2012	Jenin hospital	Jenwo	1	(+)	GVPC	Biofilm	22-Dec-12
A50	24-Nov-2012	Jenin hospital	Jencold	(2-14)	(+)	GVPC	Water	22-Dec-12
A51	24-Nov-2012	Jenin hospital	Jeneme	1	(+)	GVPC	Biofilm	22-Dec-12
A52	24-Nov-2012	Jenin hospital	Jenlabmicro	1	(+)	GVPC	Biofilm	22-Dec-12
A53	24-Nov-2012	Jenin hospital	JenICU	1	(+)	GVPC	Biofilm	22-Dec-12
A54	24-Nov-2012	Jenin hospital	Jendel	1	(+)	GVPC	Biofilm	22-Dec-12
A55	24-Nov-2012	Jenin hospital	Jenchilda	1	(+)	GVPC	Biofilm	22-Dec-12
A56	28-Nov-2012	Ramallah hospital	RamSur	1	(+)	GVPC	Biofilm	22-Dec-12
A57	28-Nov-2012	Ramallah hospital	RamCCUshh	1	(+)	GVPC	Biofilm	22-Dec-12
A58	28-Nov-2012	Ramallah hospital	RamCCU	1	(+)	GVPC	Biofilm	22-Dec-12

	Isolation	Site of isolation	Isolate design	Sg	L1 primer	medium	sample	Frozen
A59	28-Nov-2012	Ramallah hospital	RamCCUshho	1	(+)	GVPC	Biofilm	22-Dec-12
A60	28-Nov-2012	Ramallah hospital	RamChildCCU	1	(+)	GVPC	Biofilm	22-Dec-12
A61	28-Nov-2012	Ramallah hospital	RamCCUemp	1	(+)	GVPC	Biofilm	22-Dec-12
A62	28-Nov-2012	Ramallah hospital	RamoperCCU	1	(+)	GVPC	Biofilm	22-Dec-12
A63	28-Nov-2012	Ramallah hospital	RamoperCCUclear	1	(+)	GVPC	Biofilm	22-Dec-12
A64	28-Nov-2012	Ramallah hospital	Ramopershh2	1	(+)	GVPC	Biofilm	22-Dec-12
A65	28-Nov-2012	Ramallah hospital	Ramopershh1	1	(+)	GVPC	Biofilm	22-Dec-12
A66	28-Nov-2012	Ramallah hospital	Ramoper2	1	(+)	GVPC	Biofilm	22-Dec-12
A67	28-Nov-2012	Ramallah hospital	Rameme	1	(+)	GVPC	Biofilm	22-Dec-12
A68	28-Nov-2012	Ramallah hospital	Ramspemechild	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A69	28-Nov-2012	Ramallah hospital	Ramchneocli	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A70	28-Nov-2012	Ramallah hospital	RamNeoICUL	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A71	2-Dec-2012	Al-Watani hospital	Wateme2	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A72	2-Dec-2012	Al-Watani hospital	Watwom	1	(+)	GVPC	Biofilm	22-Dec-12
A73	2-Dec-2012	Al-Watani hospital	Watkid2	1	(+)	GVPC	Biofilm	22-Dec-12
A74	2-Dec-2012	Rafidia hospital	Rafmensur	1	(+)	GVPC	Biofilm	22-Dec-12
A75	2-Dec-2012	Rafidia hospital	Rafmensurshho	1	(+)	GVPC	Biofilm	22-Dec-12
A76	2-Dec-2012	Rafidia hospital	Rafwomsur	1	(+)	GVPC	Biofilm	22-Dec-12
A77	2-Dec-2012	Rafidia hospital	Rafchild	1	(+)	GVPC	Biofilm	22-Dec-12
A78	2-Dec-2012	Rafidia hospital	Rafchildshho	1	(+)	GVPC	Biofilm	22-Dec-12
A79	2-Dec-2012	Rafidia hospital	RafneoICUs	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A80	2-Dec-2012	Rafidia hospital	Rafdel1	1	(+)	GVPC	Biofilm	22-Dec-12
A81	2-Dec-2012	Rafidia hospital	Rafdel2	1	(+)	GVPC	Biofilm	22-Dec-12
A82	2-Dec-2012	Rafidia hospital	Rafpharm	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A83	2-Dec-2012	Rafidia hospital	Rafclin	1	(+)	GVPC	Biofilm	22-Dec-12
A84	2-Dec-2012	Rafidia hospital	Rafeme	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A85	2-Dec-2012	Rafidia hospital	Rafortho2	1	(+)	GVPC	Biofilm	22-Dec-12
A86	2-Dec-2012	Rafidia hospital	Raforthoshho	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A87	2-Dec-2012	Rafidia hospital	RafX	1	(+)	GVPC	Biofilm	22-Dec-12
A88	2-Dec-2012	Rafidia hospital	Rafuro	1	(+)	GVPC	Biofilm	22-Dec-12
A89	2-Dec-2012	Rafidia hospital	Rafoper2	1	(+)	GVPC	Biofilm	22-Dec-12
A90	2-Dec-2012	Rafidia hospital	Rafphysio	1	(+)	GVPC	Biofilm	22-Dec-12
A91	12-Dec-2012	Al-Makassed hospital	Makortho	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A92	12-Dec-2012	Al-Makassed hospital	MakICU	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A93	12-Dec-2012	Al-Makassed hospital	Makopheped	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A94	12-Dec-2012	Al-Makassed hospital	Makoperchroom	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A95	12-Dec-2012	Al-Makassed hospital	MakCCU	(2-14)	(+)	GVPC	Biofilm	22-Dec-12
A96	12-Dec-2012	Al-Makassed hospital	Makcath	(2-14)	(+)	GVPC	Biofilm	22-Dec-12

Appendix E

Cultivation Independent Analysis (Direct DNA Extraction)						
No	Hospital name	Site of isolation	Sample designation	Primers		
				Com	Lgsp	L1
1	Jenin	Operation shower hose	J1	(-)	(-)	(-)
2	Jenin	Neonate faucet 1	J2	(-)	(-)	(-)
3	Jenin	Neonate faucet 2	J3	(+)	(+)	(+)
4	Jenin	ICU internal faucet	J4	(-)	(-)	(-)
5	Jenin	Operation faucet	J5	(+)	(+)	(+)
6	Jenin	Orthopedic shower hose	J6	(+)	(+)	(+)
7	Jenin	Neonate internal faucet	J7	(+)	(+)	(+)
8	Jenin	ICU faucet	J8	(+)	(+)	(+)
9	Jenin	Cold water	JC	(+)	(+)	(+)
10	Al-Watani	Kidney faucet	W1	(+)	(-)	(-)
11	Al-Watani	Men faucet	W2	(+)	(-)	(-)
12	Al-Watani	Pharmacy faucet	W3	(+)	(+)	(+)
13	Al-Watani	ICU 2 faucet	W4	(+)	(-)	(-)
14	Al-Watani	Oncology faucet	W5	(+)	(-)	(-)
15	Al-Watani	Women faucet	W6	(+)	(+)	(+)
16	Al-Watani	Cardiac clinic faucet	W7	(+)	(+)	(+)
17	Al-Watani	Lab faucet 2	W8	(+)	(+)	(+)
18	Al-Watani	Cold water	WC	(-)	(-)	(-)
19	Al-Watani	Hot water	WH	(+)	(-)	(-)
20	Rafidia	Burn faucet	D1	(+)	(+)	(+)
21	Rafidia	Urology employee faucet	D2	(+)	(+)	(+)
22	Rafidia	Neonate shower hose	D3	(+)	(+)	(+)
23	Rafidia	Orthopedic shower hose	D4	(+)	(+)	(+)
24	Rafidia	Operation faucet	D5	(+)	(+)	(+)
25	Rafidia	labor operation faucet	D6	(+)	(+)	(+)
26	Rafidia	Neonate faucet 1	D7	(+)	(+)	(+)
27	Rafidia	Neonate faucet 2	D8	(+)	(-)	(-)

Cultivation Independent Analysis (Direct DNA Extraction)						
No	Hospital name	Site of isolation	Sample designation	Primers		
				Com	Lgsp	L1
28	Rafidia	Cold water	DC	(+)	(+)	(+)
29	Rafidia	Hot water	DH	(-)	(-)	(-)
30	Ramallah	X-Ray faucet	R1	(-)	(-)	(-)
31	Ramallah	Operation faucet 2	R2	(+)	(+)	(+)
32	Ramallah	CCU outside faucet 2	R3	(+)	(+)	(+)
33	Ramallah	CCU faucet 2	R4	(+)	(-)	(-)
34	Ramallah	CCU faucet 3	R5	(+)	(+)	(+)
35	Ramallah	Pediatric clinic faucet	R6	(+)	(+)	(+)
36	Ramallah	CCU shower hose	R7	(+)	(+)	(+)
37	Ramallah	Pediatric shower hose	R8	(+)	(+)	(+)
38	Ramallah	Cold water	RC	(+)	(+)	(+)
39	Ramallah	Hot water	RH	(+)	(-)	(-)
40	Al-Makassed	Labor outside faucet	M1	(+)	(+)	(+)
41	Al-Makassed	Labor faucet	M2	(+)	(+)	(+)
42	Al-Makassed	Orthopedic shower hose	M3	(+)	(-)	(-)
43	Al-Makassed	CCU outside faucet	M4	(+)	(-)	(-)
44	Al-Makassed	Catheterization faucet	M5	(+)	(-)	(-)
45	Al-Makassed	Pediatric ICU faucet	M6	(+)	(+)	(+)
46	Al-Makassed	Operation shower hose	M7	(+)	(-)	(-)
47	Al-Makassed	ICU faucet	M8	(+)	(-)	(-)
48	Al-Makassed	Cold water	MC	(+)	(-)	(-)
49	Al-Makassed	Hot water	MH	(+)	(-)	(-)
50	Beit Jala	Pediatric faucet	BJ1	(+)	(+)	(+)
51	Beit Jala	Oncology shower hose	BJ2	(+)	(-)	(-)
52	Beit Jala	Men internal shower head	BJ3	(+)	(+)	(+)
53	Beit Jala	ICU faucet 1	BJ4	(+)	(+)	(+)
54	Beit Jala	Men surgery shower head	BJ5	(+)	(+)	(+)

Cultivation Independent Analysis (Direct DNA Extraction)						
No	Hospital name	Site of isolation	Sample designation	Primers		
				Com	Lgsp	L1
55	Beit Jala	Men internal shower hose	BJ6	(+)	(+)	(+)
56	Beit Jala	Women surgery shower head	BJ7	(+)	(+)	(+)
57	Beit Jala	ICU faucet 2	BJ8	(+)	(+)	(+)
58	Beit Jala	Cold water	BJC	(+)	(+)	(+)
59	Beit Jala	Hot water	BJH	(+)	(+)	(+)
60	Al-Ahli	Washing shower hose	H1	(-)	(-)	(-)
61	Al-Ahli	ICU shower hose	H2	(-)	(-)	(-)
62	Al-Ahli	Operation shower hose	H3	(+)	(+)	(+)
63	Al-Ahli	Labor faucet	H4	(+)	(+)	(+)
64	Al-Ahli	Pediatric shower hose	H5	(+)	(+)	(+)
65	Al-Ahli	CCU shower hose	H6	(-)	(-)	(-)
66	Al-Ahli	Operation faucet	H7	(+)	(+)	(+)
67	Al-Ahli	CCU shower head	H8	(-)	(-)	(-)
68	Al-Ahli	Cold water	HC	(+)	(+)	(+)
69	Al-Ahli	Hot water	HH	(+)	(-)	(-)
70	Alia	ENT shower hose	A1	(+)	(+)	(+)
71	Alia	Emergency faucet	A2	(+)	(+)	(+)
72	Alia	CCU shower hose	A3	(+)	(+)	(+)
73	Alia	Operation faucet 1	A4	(+)	(+)	(+)
74	Alia	Pediatric shower head	A5	(+)	(+)	(+)
75	Alia	Operation faucet 2	A6	(+)	(+)	(+)
76	Alia	Operation shower hose	A7	(+)	(+)	(+)
77	Alia	Operation faucet 3	A8	(+)	(+)	(+)
78	Alia	Cold water	AC	(+)	(+)	(+)
79	Alia	Hot water	AH	(+)	(+)	(+)

Appendix F

>LR1
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الكشف الجزيئي (Molecular Identification) عن بكتيريا اللجيونيلا (*Legionella*) من عينات المياه في فلسطين

اعداد: أشرف رشاد زايد

اشراف: د. دينا بيطار

ملخص:

ان عزل بكتيريا اللجيونيلا (*Legionella*) من العينات البيئية يشكل تحديا كبيرا بسبب حاله خاصه بهذه البكتيريا تدعى (VBNC) اي انها قابلة للحياه لكن غير قابله للزراعه. زد على ذلك ان اللجيونيلا من البكتيريا التي تصعب زراعتها (fastidious) فهي بطيئه النمو و تواجه منافسه شديده من انواع البكتيريا المائية الاخرى التي تنمو بسرعه. لذلك كان هدف الدراسة الرئيسي هو الكشف عن وجود بكتيريا اللجيونيلا في المياه باستخدام طرق الكشف الجزيئية (Molecular identification).

اشتملت عينة الدراسة على جمع عينات ماء وعينات مسح الغلاف الحيوي (Biofilm swabs) من سبعة مستشفيات حكومية في شمال ووسط وجنوب الضفه الغربيه ومن مستشفى المقاصد في مدينه القدس و من جامعه القدس في ابوديس. تم فحص هذه العينات باستخدام طريقتين مختلفتين هما:- اولا: طرق الكشف التقليدية (cultivation dependent analysis) باستخدام (تقنيات زراعة الاحياء الدقيقة (microbiological techniques)). ثانيا: طرق الكشف الجزيئية (cultivation independent analysis) (باستخدام التفاعل السلسلي (PCR) للحمض النووي وتسلسل الحمض النووي (sequencing) ومتعدد الاعداد المتغير من مكان يكرر جنبا الي جنب (MLVA). اضافة الى الفحوصات المصلية (serological tests) لمعرفة السلالات المصلية (serogroups) للجيونيلا الرئوية (*L.pneumophila*).حيث تم جمع 307 عينة و فحصها باستخدام طرق الكشف التقليدية و 79 عينة وفحصها باستخدام طرق الكشف الجزيئية. كذلك تم

فحص الخصائص الفيزيائية و الكيميائية للمياه. و لاضفاء الطابع التقني على الدراسة، لقد تم جمع و معالجة و تحليل العينات وفقا للاجراءات القياسية الدولية رقم ISO11731 ، ISO11731-2 و ISO6222 .

أظهرت نتائج الدراسة انه تم عزل بكتيريا اللجيونيلا باستخدام طرق الكشف التقليديه بنسبه 31% بينما تم الكشف عن 66% من بكتيريا اللجيونيلا باستخدام طرق الكشف الجزيئية. هذا يثبت مدى أهمية استخدام طرق الكشف الجزيئيه للكشف عن اللجيونيلا. كذلك اظهرت نتائج الدراسة أن اللجيونيلا الرئوية هي النوع الوحيد من انواع اللجيونيلا التي تم الكشف عنها في هذه الدراسة.حيث تم الكشف على ما نسبته 64% من اللجيونيلا الرئوية تعود الى السلالة المصلية رقم 1 (sg.1) و 36% تعود الى السلالات المصلية 14-2 (sg 2-14). كذلك اظهرت نتائج الدراسه تنوعا في سلالات اللجيونيلا الرئوية. في الواقع ان التصنيف بالاعتماد على السلالات مهم جدا بسبب ان اللجيونيلا الرئوية هي بكتيريا ممرضة وتحمل صفه (VBNC) ويمكن تطبيق تحليل سلالات دون زراعة السلالات المعدية، وبالتالي يسهم في تحسين مراقبة مرض الالتهاب الرئوي اللجيونيلي (Legionnaires' disease).

في هذه الدراسة، كانت الاقسام الحساسة (وحده العناية المكثفه، وحده العنايه القلبية، قسم الاطفال، قسم حديثي الولادة والخدج و العمليات) في مستشفيات الضفة الغربية ملوثة باللجيونيلا الرئويه. هذا يشكل خطرا حقيقيا على حياه هؤلاء المرضى الذين يعانون نقصا في المناعه. لذا توصي الدراسة باستخدام الحرارة العالية في تعقيم المياه (اكثر من 70 درجة مئويه)، تنظيف مصادر المياه و الصيانة الدورية لشبكات المياه القديمة للتخلص من اللجيونيلا الرئويه.