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Cultural and Molecular Evidence of *Legionella pneumophila* in Dental Unit Waterlines in the West Bank, Palestine

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Dedication

To my mother and father…

To my dear brother, Mohammed…

To my sister, Maram…

To my family…

To my friends…

To my teachers…

To all the people who supported, and encouraged me.

Mutasem Zuheir Hilmi Burghal

Al-Quds University
Deanship of Graduate Studies
Biochemistry and Molecular biology

Thesis Approval

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Jerusalem – Palestine

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Declaration:

I certify that this thesis submitted for the degree of Master, is the result of my own research, except where 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

Mutasem Burghal

Date: 19-1-2020

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Cultural and Molecular Evidence of *Legionella pneumophila* in Dental Unit Waterlines in the West Bank, Palestine

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Abstract

Legionella spp. is a Gram-negative, rod-shaped, a strictly aerobic and nutritionally fastidious bacterium. *Legionella pneumophila* is ubiquitous in aquatic environments and water distribution systems, including dental unit waterlines (DUWLs). Legionellosis is the disease caused by *Legionella* bacteria including Legionnaires' disease (LD),a fatal type of pneumonia, and the less acute form Pontiac fever, a flu-like illness. Among the 59 species and 70 serogroups of *Legionella spp*., *L. pneumophila* is the major cause of sporadic and outbreak legionellosis (91.5%), and serogroup 1 is the predominant serotype (84.2%).

Many studies have demonstrated bacterial contamination of dental unit waterlines (DUWLs). When *Legionella* enters the DUWL from the main water reservoir, biofilms are formed on the inner surface of the waterlines. Biofilm provides suitable conditions for colonization and growth of *Legionella* within plumbing systems. Infection with *Legionella* occurs as a result of inhalation of aerosolized *Legionella* or aspiration of *Legionella* contaminated water by susceptible patients, health workers and dentists. The contamination of DUWLs with *Legionella* poses a serious health hazard for patients with chronic diseases and an impaired immune system.

Previous work in the Microbiology Research Laboratory performed a three-year (2012-2015) environmental surveillance of *Legionella* in the hospitals' water systems of eight hospitals across the West Bank.The study used culture and polymerase chain reaction (PCR) for the detection of *Legionella.* Their results showed low prevalence for *Legionella spp.* of 8.3% for water samples by culture, however this percentage increased to 50% by PCR. As for biofilms, The *Legionella* in biofilms was higher, being 16.8% by culture vs. 61% by PCR.

In this study we undertook to determine the prevalence of *Legionella* in water and biofilm samples from Tap and DUWLs collected from the dental clinics in the faculty of dentistry

at Al-Quds University (AQU) in Abu Deis Jerusalem and Arab American University in Jenin (AAUP), and dental clinics located in three major Palestinian cities; Nablus, Tulkarem, and Hebron in the West Bank.

The study samples included 185 samples, 89 (48%) water samples and 96 (52%) biofilm swabs, which were analyzed by cultivation dependent analysis (microbiological techniques) and by the cultivation-independent technique, namely PCR. For cultivation dependent analysis, the *Legionella* count was performed as well as serotyping of the isolates into serogroup 1 or serogroup 2-14. For cultivation-independent analysis, DNA was extracted from the samples and analyzed for the study of; the bacterial population, the presence of *Legionella* genus bacteria and for the presence of *L. pneumophila*, using 16S rRNA gene, Com, Lgsp, and L1 primers respectively. Partial sequencing of the 16S rRNA gene for seven *Legionella* isolates was done for further analysis for quality assurance and identification. Furthermore, water samples (Tap and DUWL) 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.

L. pneumophila was isolated from 28 (15%) of 185 samples using cultivation dependent analysis and was detected in 142 (77%) of 185 samples using cultivation-independent analysis (PCR). PCR was 5x more sensitive than the culture technique, due to the Viable-But-Non-Culturable (VBNC) state of *L. pneumophila*. *L. pneumophila* was the only *Legionella spp.* that was detected in positive samples. *L. pneumophila* sg.1 was detected in 23/28 (82%) of the isolates, while 5/28 (18%) isolates were *L. pneumophila* sg. 2-14. All seven *Legionella* isolates' DNA sequenced for the 16SrRNA gene identified with *L. pneumophila >95.7%*. To ensure the quality of the water samples, their physical and chemical characteristics were measured; all were within acceptable ranges compared to WHO guidelines, except for carbonate hardness which was above WHO levels in 12 clinics and total hardness were above the WHO acceptable range in all clinics.

These results show that DUWLs of the examined dental clinics are contaminated with *L. pneumophila*. This finding reveals a serious potential health risk for infection of immunocompromised patients, health workers and dentists post-exposure.

The Ministry of Health (MOH) and the Palestinian Water Authority should put limitations and guidelines for water quality and microbiological monitoring, should advise washing of DUWLs with disinfectants such as chlorhexidine gluconate (CHX) or pure water and using

softener filters as well as routine periodic checking of DUWLs for bacterial contamination to ensure the health safety of patients and dentists.

List of tables

List of figures

List of Appendices

List of abbreviations

AE	Elution buffer
AP-PCR	Arbitrarily primed PCR
ACES	N-2-acetamino-2-aminoethansulfonic acid
AFLP	Amplified fragment length polymorphism
AQU	Al-Quds University
AAUP	Arab American University
ATS	American Thoracic Society
AW1	Wash buffers 1
AW2	Wash buffers 2
BTS	British Thoracic Society
BCYE	Buffered charcoal yeast extract
C	Celsius
CAP	Community-acquired pneumonia
CO_2	Carbon dioxide
CFU	Colony-forming unit
CHX	Chlorhexidine gluconate
DALY	Disability-adjusted life years
DNA	Deoxyribonucleic acid
Dot/icm	Defective organelle trafficking/ intracellular multiplication
DUWL	Dental unit waterlines
DCU	Dental chair unit
DW	Distilled water

ELB Enzymatic lysis buffer

ELISA Enzyme-linked immunosorbent assay

EtOH Ethanol

EWGLI The European working group for *Legionella* infection

GU Genomic units

FDA Fluorescent direct antibody

FISH Fluorescent in situ hybridization

GVPC Glycine Vancomycin Polymyxin B Cycloheximide

HCl Hydrochloric acid

HIA Health impact assessment

HPC Heterotrophic plate count

HZI Helmholtz Center for Infection Research

ICU Intensive Care Unit

IDSA Infectious Diseases Society of America

IFA Immunofluorescence assay

ISO International organization for standardization

KCl Potassium chloride

KOH Potassium hydroxide

L Liter

LD Legionnaires' disease

LLAPs *Legionella*- like amoebal pathogens

M Molar

mM millimolar

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
µl	Microleter
µm	Micrometer
µS	Micro Siemens
MOH	Ministry of health
NaCl	Sodium chloride
NAATs	Nucleic Acid Amplification Tests
Ng	Nanogram
ppm	Parts per million
PCR	Polymerase chain reaction
PVC	Polyvinyl Chloride
EPS	Extracellular polymeric substances
PFGE	Pulsed-field gel electrophoresis
PWA	The Palestinian Water Authority

PPIase Peptidylprolyl cis/trans isomerase

QMRA Quantitative microbial risk assessment

rep-PCR Repetitive element PCR

rDNA Ribosomal Deoxyribonucleic acid

RFLP Restriction fragment length polymorphism

RNA Ribonucleic acid

Rpm Round per minute

rRNA Ribosomal Ribonucleic acid

SATs Slide agglutination tests

Sg Serogroup

SOP Standard operation procedure

Spp Species

TEA Tris-acetate buffer

TVCs The total viable counts

USFAD US Food and Drug Administration

USA United States of America

VBNC Viable but non-culturable

WB West Bank

WHO World health organization

Table of Contents

Chapter One

Introduction

1.1 Introduction

Legionella is a ubiquitous, aquatic, opportunistic Gram-negative bacteria found in many ecosystems, including surface water, groundwater and water systems made by humans. *Legionella* is responsible for a severe disease called legionellosis. *Legionella* causes nosocomial and community-acquired pneumonia (Moosavian and Dashti, 2011). Legionellosis includes two forms; Legionnaires' disease (LD) which is an atypical severe type of pneumonia and the less severe form, flu-like febrile illness called Pontiac fever. (Principe et al., 2017; Prussin et al., 2017). Legionellosis occurs in immunocompromised individuals following inhalation of aerosolized droplets of *Legionella*. LD is not a communicable disease and is not transferred from human to human (Mizrahi et al., 2017).

The genus *Legionella* includes currently 59 species of which about half have been associated with be pathogenic to humans, and the majority are considered as virulent (Mercante and Winchell, 2015). *L. Pneumophila* is responsible for almost 90% of human disease. *L. Pneumophila* is classified into 15 serogroups (sgs) of which sg 1 is the most prevalent and is responsible for up to 90% of *L. pneumophila* infections.

Dental Unit Waterline (DUWL) is part of the environment, which allows *Legionella* and other aquatic bacteria to grow. The problem of bacterial water contamination and biofilm formation in DUWLs has been examined since the moment the first dental chair units (DCUs) were built. In 1963 Bake was the first researcher who reported bacterial contamination in DUWL (Blake, 1963). This was followed by many studies describing both chemical (sodium hypochlorite, chlorhexidine gluconate, hydrogen peroxide) and mechanical (rinsing, filtration) control of microorganisms or biofilm in DUWL. (Liaqat and Sabri, 2010; Sedlata Juraskova et al., 2017; Walker and Marsh, 2004).

Effective control of infections is one of the basic principles of good clinical practice. Pathogenic opportunists including *Pseudomonas aeruginosa, Enterococci spp., Streptococci spp., Legionella spp.* were found contaminating DUWLs and ultrasonic

scalers (Singh et al., 2013). While having dental treatment, patients and dentists are subjected together by unit turbin, including ultrasonic devices, to direct contact with *legionella* -contaminated water as drizzle and contaminated water aerosol emitted during the operation. (Kumar et al., 2010).

L. pneumophila, the causative agent of LD was discovered in 1976 following an outbreak of pneumonia cases from persons attending the American Legion Convention in Philadelphia, Pennsylvania, United States of America (USA). (Zhan and Chao-Hui Hu, 2015). Ninety percent of LD cases are caused by infection with *L. pneumophila* (Tabatabaei et al., 2016), and humans may be infected with *Legionella*, after inhalation of contaminated droplet aerosols or by microaspiration of contaminated water. (Borges et al., 2012; Yong et al., 2010).

The main sources of LD are the drinkable water systems in hotels and hospitals (Mavridou et al., 2008). According to the CDC, the hospitalization rate caused by legionellosis accounts for 8,000 to 18,000 in the USA each year. Common habitats for this bacterium are hospitals that have susceptible patients. The first infection outbreak was recorded in 1957. The prevalence rate of legionellosis outbreaks in hospitalized patients was reported to be between 0 - 47% in the USA. (Khaledi et al., 2018).

Hospital water systems or Dental clinics, when contaminated with *Legionella*, pose a high risk for patients with various diseases who may stay in the hospital for a long time and are immunocompromised. It is well known that LD is a major cause of hospital-acquired pneumonia. (Yu and Stout, 2000).

Acanthamoeba is a particularly suitable host for many bacteria in natural and man-made water systems. including *L. pneumophila*. In the amoebae, the *Legionella* are preserved from changes in pH, temperature, and disinfection. Moreover, passage through amoebae appears to promote their pathogenicity and to refresh viable but nonculturable (VBNC) *Legionella* (Scheikl et al., 2016). In addition to that, *Legionella's* ability to multiply in ecological protozoa has allowed the *Legionella* to replicate alveolar macrophages in humans (Yong et al., 2010).

Legionella spp. are pervasive in the ecosystem, soil, and water (Yong et al., 2010), and extremely inhabit human-made aquatic environments such as hot water plumbing when

water temperature is below 50^0C, whirlpool spas, decorative fountains, and cooling towers (Kusnetsov et al., 2003; Lapierre et al., 2017; Lévesque et al., 2016; Simon. et al., 2016). The outbreaks of legionellosis were associated with *L. pneumophila* in household supplies of hot water, showerheads, cooling water and other water services in large buildings such as hotels and hospitals (Blanky et al., 2015).

Furthermore *Legionella spp.* is correlated with a biofilm that lines the inside of pipes and on the surface of DUWL, which provides refuge and nutrients and supports their existence and multiplication (Fields et al., 2002; Keevil, 2003). Water-using tools can also be the source of transmission of *Legionella* bacteria. Patients may be exposed to these bacteria during bathing, showering and from contact with medical devices washed with contaminated tap water, or the hands of medical staff rinsed with contaminated tap water (Fiore et al., 1998; Schijven and de Roda Husman, 2005; Woo et al., 1992).

Major risk factors for developing legionellosis pneumonia mostly happens in patients with suppression of the cellular immune system, chronic heart or lung diseases, renal failure, and old age, such as people treated with cortisol, cigarette smokers, alcohol abusers and use of well water (Mizrahi et al., 2017; Szymańska, 2004).

Diagnosis of LD in many countries is very difficult, this is a well-known issue because of the following reasons; the inability to distinguish between LD from other pneumonia infections on clinical grounds, the diagnostic laboratory does not do routine testing for *Legionella*, the unsuitable handling of samples and lack of technical expertise for culturing and diagnosis of *Legionella*, and the deficiency of available diagnostic tests.

In the past, several techniques were used to identify *Legionella*. Cultivation is the primary method for diagnosing infection of bacteria (Cultivation dependent analysis). However this culture method when used to test *Legionella spp*. it can give false-negative data or bacterial counts are underestimated (Ditommaso et al., 2016). Culture analysis of the hospital water system for *Legionella spp.* is the first step in the risk assessment of hospital-acquired LD. This approach is recommended in the national guidelines for most European industrial countries, particularly those who encounter cases of LD (Yu et al., 2008). In addition to the health recommendations for dental surgeries, Italian guidelines for the prevention of LD relatively recently in 2015, recommended tracking their DUWLs at least annually in order to ensure that the DUWL is free from *Legionellae* (Ditommaso et al., 2016).

In addition, cultivation-independent analysis (molecular analysis) for water specimens is necessary since *Legionella spp.* occur in Viable-But-Non-Culturable (VBNC) status. VBNC state most likely explains why *L. pneumophila* sometimes cannot be isolated from the aquatic environment that is suspected to be the source of infection by cultivation analysis only. (Steinert et al., 2002a). Moreover, cultivation of this bacteria is very hard because it requires at least 2-5 days to grow, thus can be easily overgrown by other bacteria in the same sample, as well as it's fastidious growth requirements. (Ditommaso et al., 2016; Nederbragt et al., 2008).

1.2 Problem statement

Contamination of Dental unit waterline (DUWL) is considered a source of water-borne opportunistic microorganisms (Ajami et al., 2012). Typically, *Legionella* may contaminate the DUWL from the main water reservoir where they can multiply in the biofilm (Pankhurst et al., 2003). Furthermore, as most DUWL handling methods have restrictions, biofilms are complicated to remove (Porteous et al., 2011). Low- and high-speed handpieces, ultrasonic instruments and air-water syringes make air-water aerosols, which may be a source of *Legionella* infection. Both the dental staff and the patient are exposed to the infected aerosols by inhaling them, so the presence of *Legionella* bacteria in the water distribution poses a health risk to dental staff and patients. The extent of the problem is big and undefined and there are no specific guidelines to protect the patients from exposure in dental clinics (Fiore et al., 1998; Schijven and de Roda Husman, 2005; Szymańska, 2004; Yu and Stout, 2000).

The first study which dealt with the identification of *Legionella* in water systems in Palestine was done in 2008, whereby the researchers examined the West Bank hospital water for aquatic organisms including *Legionella* using only bacteriological and serological methods (Shareef and Mimi, 2008). Because *Legionella* is difficult to isolate by using microbiological culture methods due to the viable but nonculturable (VBNC) state, moreover, the cultivation of these bacteria is difficult because of the slow growth and competitive growth of other bacteria in the same sample (Nederbragt et al., 2008; Steinert et al., 2002a) our laboratory did a three years proactive environmental surveillance (2012-2015) of *L. pneumophila* in the water distribution systems of eight hospitals in the West

Bank, using both cultivation dependent (cultural analysis) and cultivation-independent methods (molecular methods). *L. pneumophila* was detected in all hospitals' water systems. Most of the isolates were identified as serogroup 1 (62%). (Ashraf Zayed thesis, 2013).

Since there are no previous studies regarding the prevalence of *Legionella* in water and biofilm samples from DUWL in dental clinics in Palestine, we set out to investigate this problem and to identify *Legionella* by microbiological culture, serological testing and confirmed by molecular methods (PCR) and sequencing.

1.3 Aim of the Study

The aim of this study is to evaluate the microbiological quality of Dental Unit Waterline (DUWL) and to determine the prevalence of *Legionella spp.* from DUWL, Tap water, and biofilm samples collected from two colleges of dentistry, the clinics in Al-Quds University, Arab American University in Jenin and dental clinics across the West Bank using both standard culture methods and molecular method (PCR).

1.4 Objectives:

To reach this goal the following objectives were approached:

1. Bacteriological isolation of *L. pneumophila*, from water (Tap water and DUWL) and biofilm (Tap and DUWL) samples, using the standard cultural method.

2. Molecular identification of *L. pneumophila*, from water (Tap water and DUWL) and biofilm (Tap and DUWL) samples using 16S rRNA PCR.

3. Assessment of infection routes for waterborne *Legionella* infections in dental clinics.

4. Recommendation of management guidelines for freshwater systems in DUWL to prevent *Legionella* infections.

1.5 Questions

- How sensitive is PCR compared to standard culture in the identification of *L. pneumophila* in DUWL?
- Is there a difference between *Legionella spp*., and serogroups recovered from water systems between Palestine and neighboring countries?
- Are DUWLs being a potential risk factor for legionellosis?

1.6 Hypothesis

The health safety of dental patients and dentists requires adequate microbiological water quality in dental units. *Legionella* species are pathogenic microorganisms that can be transmitted via aerosols to the patients in the dental clinic and may be a cause of atypical pneumonia. Knowing that *Legionella* is hard to cultivate due to VBNC state, slow growth and competitive growth of other bacteria in the same sample, thus we hypothesized to use a complete system to identify *Legionella spp.* in DUWL in the West Bank, Palestine, using microbiological, serological and molecular techniques. This complete system will decrease the misdiagnosis of *Legionella* in DUWL.

1.7 History

It has been more than forty years since the isolation of the bacterium *L. pneumophila* for the first time. A severe pneumonia epidemic occurred in the summer of 1976 among the people attending an American Legion Convention in Philadelphia, Pennsylvania, USA. A total of 221 individuals were infected with severe pneumonia, approximately 15% of the cases died from pneumonia. The reason for the Philadelphia outbreak was unknown for months in spite of extensive laboratory examinations (Brenner, 1987; Mcdade et al., 1977). An epidemiologic analysis detected that the disease most likely was airborne (Fraser et al., 1977). Dr. Joseph McDade later, in January 1977 with Charles Shepard of the Center for Disease Control (CDC), isolated the bacterium from the air conditioning system where the convention took place. A fastidious Gram-negative bacillus namely *L. pneumophila* was the etiological agent of the disease and became the first-named member of the family,

Legionellaceae. Receiving the name *Legionella* to honor legionnaires affected in the USA and the Greek word pneumophila, meaning "lung-loving". Because of the historical association with the American Legion Convention, this disease was called Legionnaires' disease (LD) (Fang et al., 1989; Mcdade et al., 1977).

Next, they learned that several previous unresolved severe pneumonia outbreaks were LD, including outbreaks in the 1950s and the 1960s, the first was in Washington, DC "District of Columbia" in 1965, 14 out of 81 people died. (Mcdade et al., 1977; Thacker et al., 1978), and the second was an outbreak of non-pneumonia that happened in 1968 in Pontiac, MI, where no deaths were reported in 144 cases (Glick et al., 1978; Mcdade et al., 1977). Also, this unsolved outbreak of a non-pneumonic febrile illness was found to have resulted also caused by exposure to *Legionella* bacteria; this disease has been called Pontiac fever (Burillo et al., 2017; Tossa et al., 2006).

Over the years several new members of the genus *Legionella* have been discovered (Casati et al., 2009; Currie et al., 2014; Pravinkumar et al., 2010; Travis et al., 2012). As with LD, previous Pontiac fever epidemics occurred in 1949 without addressing the causative agent. LD specific cases were connected to a wide range of man-made sources of water, including cooling towers, spas, fountains and whirlpools. (Winn, 1988).

After these outbreaks, the monitoring systems and controls were evaluated and managed any future outbreaks in big industrial countries like the USA, Japan, Europe, Australia, and New Zealand (Phin et al., 2014). However, there is no surveillance for *Legionella* in most Arab countries including Palestine.

1.8 Microbiology and Morphology

The *Legionellaceae* are obligatory aerobic and nutritionally fastidious Gram-negative coccobacilli. They are unencapsulated and non-spore forming. Most species are motile utilizing one to three polar or lateral flagella (Winn, 1988). There may be a lack of motility during growth under artificial circumstances. Bacterial length varies depending on growth conditions, stage of growth and whether the bacterium is grown in eukaryotic cells or extracellular environment. When grown on solid media, the *Legionella* bacteria are usually at least a rod length of 5-40 μm, with a cell width of 0.3 - 0.9 μm. All *Legionella* are

weakly positive for catalase. *Legionella* are saccharolytic, oxidase reaction is usually weakly positive, but maybe negative, they liquefy gelatine without reducing nitrate or hydrolyzing urea. The test for hydrolysis of sodium hippurate is positive for *L. pneumophila* and negative for most other species of *Legionella* (Benson R.F. and Fields B.S., 1998). Although the biochemical tests and the ability of the rods to produce fluorescence under ultraviolet light help distinguish the species. Presently, DNA analysis and antigenic analysis of different proteins and peptides are the best techniques to classify *Legionella spp.* These organisms are non-fermentative. Amino acids are used as an energy source rather than carbohydrates (Brenner, 1987). On Buffered Charcoal Yeast Extract (BCYE) agar, they can grow (Arora et al., 2012) and Mueller- Hinton medium supplied with 1% hemoglobin and 1% Isovitalex. L-cysteine, α-ketoglutarate and charcoal-containing yeast extract agar buffered with an organic buffer (BCYEα agar) are required for growth and iron are required for initial isolation from the environment or clinical samples. It is important to adjust the pH of the agar to pH 6.9 by adding N-2-acetamido-2-aminoethanesulfonic acid (ACES) (Maiwald et al., 1998). The pH and optimum temperature for *Legionella* in vitro growing 6.8 - 7.0 and 25 - 42°C respectively and are killed at temperatures above 50°C. Below 20°C, the bacteria become dormant but stay viable for months (DH Estates, 2006). At 66^0C *Legionella* die within two minutes and are immediately destroyed at a temperature of more than 70^0 C. (Dimitriadi and Velonakis, 2014). *Legionella* has been isolated from ecological sources of pH ranging from 2.7 to 8.3 (Sheehan et al., 2005).

Between 35 and 37 ° C, optimum growth occurs in vitro in humidified air on BCYEα medium for 2-5 days. In rare cases, it takes up to 10 days to isolate unusual *Legionella* species.

The cell wall of *Legionella* is different from most Gram-negative bacteria, it contains long-chain hydroxyl fatty acid that is unique for the family. The problem of cell staining is affected by ubiquinones with a side chain of 9-14 isoprene units in a fatty acid chain in the cell wall (Garrity et al., 2005). Also, the polysaccharide epitopes of the lipopolysaccharides in the cell wall are specific for *Legionella* and can be used for serological grouping (Helbig et al., 1997).

The number of the *Legionella* genus documented species and serogroups (sg) is increasing. Fifty-nine *Legionella* species comprising 70 distinct serogroups have been confirmed to date (Fields et al., 2002). Twenty *Legionella spp.* have been recorded to cause human pneumonia, although more than 90% of all infections are caused by *L. pneumophila*. *Legionella* species and serogroups related to human disease are shown in (Table 1.1) (Arora et al., 2012; Muder and Yu, 2002).

Table 1.1: *Legionella* species and serogroups associated with human disease

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

Some *Legionella spp.* cannot be cultivated on routine *Legionella*-specific media and have been termed *Legionella*-like *amoebal* pathogens (LLAPs). LLAP strain has been isolated from the sputum of a patient with pneumonia.by enrichment in amoebae (Fig. 1.1) (Rowbotham, 1993).

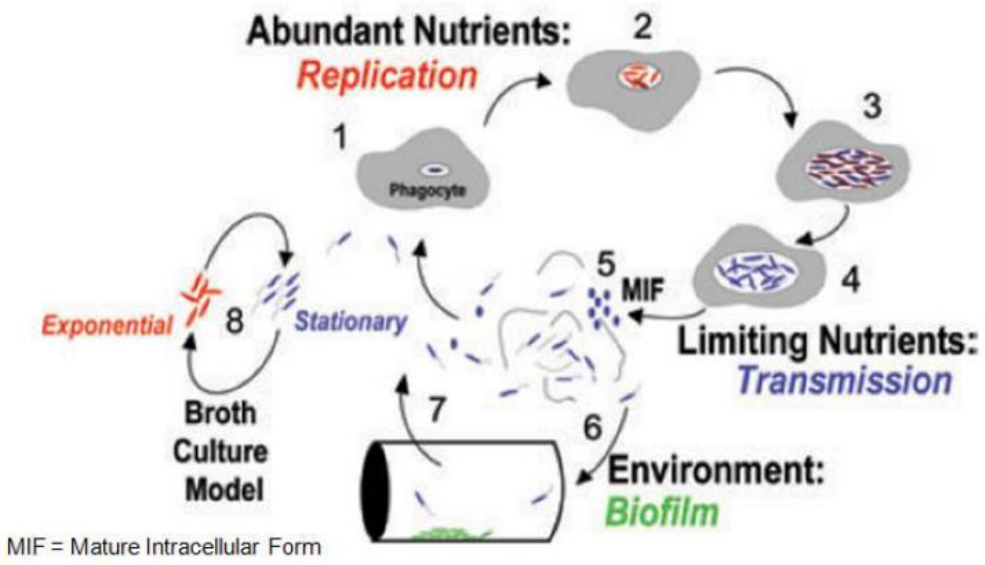


Figure 1.1: *L. pneumophila* – biphasic life cycle (Molofsky and Swanson, 2004)

Multiplication of *legionella* in aquatic environments depends on its symbiotic association with other microorganisms. Experiments have shown that *Legionella* shows long-term survival in sterile tap water but does not multiply, while *Legionella* in non-sterile water has been shown living and multiplying (Surman et al., 1994). In fact, the viability of *Legionella* is retained when paired with algae in cultivation, while the viability of *Legionella* decreases once the algae are absent. (Winn, 1988). Actually, *Legionella* is known to infect a total of 16 amebae species including *Acanthamoeba*, *Naegleria* and *Hartmanella spp*. and two species of ciliated protozoa *Tetrahymena pyriformis, Tetrahymena vorax* (Rowbotham, 1986; Wadowsky et al., 1985). *Legionella* also can proliferate intracellularly within protozoal hosts (Vandenesch et al., 1990). *Legionella* strains that proliferate inside protozoa have been shown to be more virulent, possibly because of increased bacterial numbers (Kramer and Ford, 1994). In these symbiotic hosts, the ability to multiply provides *Legionella* with protection against otherwise harmful environmental conditions. *Legionella* can, therefore, thrive in environments with higher temperatures, be more resistant to water treatment with biocides, chlorine, and other disinfectants, and flourish under harsh conditions when encapsulated in cysts. Their enhanced resistance to water treatment has major inclusion for both disease transmittance and water treatment methods. *Legionella* grows on the surface of biofilms together with other aquatic bacteria and produces extracellular polymeric substances (EPS) (Kramer, M. H., & Ford, 1994; Liaqat and Sabri, 2010). Biofilms provide nutrients for the *Legionella* to grow and also provide protection against adverse environmental conditions (including water disinfection).

Legionella spp. arevubiquitously found in the environment, they are found in rivers, lakes, streams, and hot pools, in moist soil and the mud. They have even been found in the showers of the rain forest (Koide et al., 1999; Parthuisot et al., 2010; Steele et al., 1990). *Legionella* can live in different water conditions, temperatures ranging from 0-63 ° C, a pH range of 5.0-8.5, and a concentration of dissolved oxygen in the water of 0.2-15 parts-per-million ppm. (Nguyen et al., 1991). They can live in chlorinated water, therefore enter water supply systems, and grow in hot habitats, like air-conditioning systems, cooling towers, showerheads, hot water faucet system, whirlpool spas, respiratory ventilators, and Dental unit. Also, *Legionella* can be found on the surfaces of these structures in biofilms

(Declerck, 2010; Walker and Marsh, 2004; Winn, 1988; Woo et al., 1992). Figure 1.2 shows the temperature-dependent growth function *of L. pneumophila* bacteria in water and in biofilm.

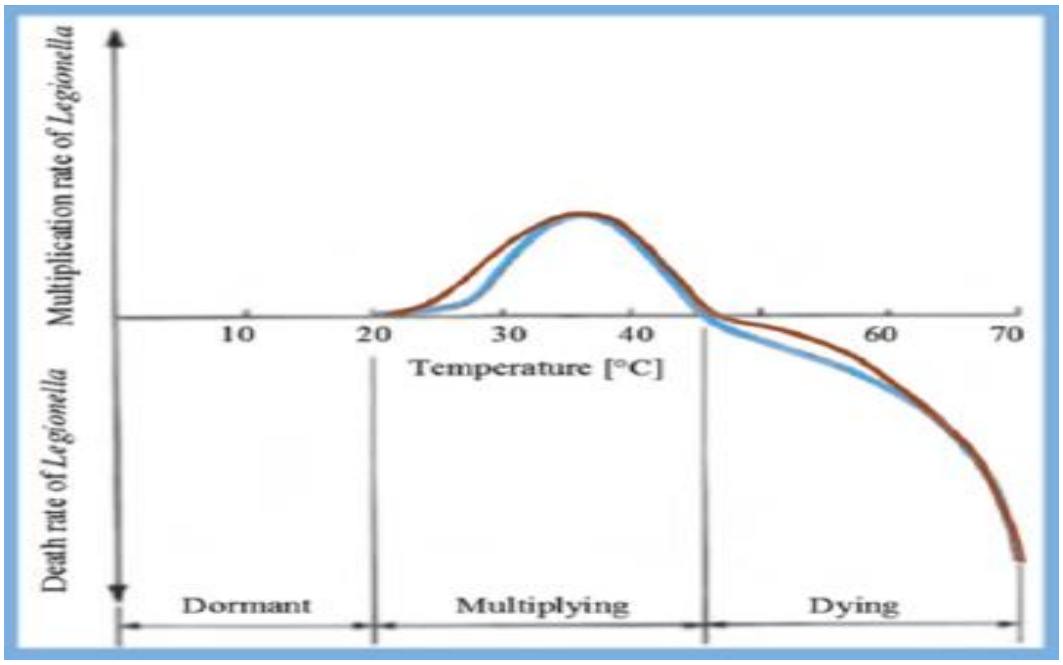


Figure1.2: "Growth function of *Legionella pneumophila* in water (blue) and in biofilm (brown)" (Van Kenhove et al., 2017).

Cases of legionellosis can be traced to man-made aquatic systems, where the water temperature exceeds the ambient temperature, where symbiotic microorganisms provide nutrients, and the *Legionella spp*. can grow. The presence of *Legionella* in the water environment and the temperature of thermal water are two factors that may increase the risk of LD. *Legionella* live in aquatic and moist soil environments parasitizing free-living amoebae (Rowbotham, 1980). The thermally changed aquatic character will change the balance between bacteria and protozoa, resulting in *Legionella* rapid growth. Different strains that colonize water distribution systems, but, when exposed to water, only a small specific species can cause the disease to patients. LD is a significant concern among public health workers and people interested in water system management, such as air conditioning systems, water circulation systems, and cooling towers. Generally, legionellosis is considered a preventable disease because controlling or removing the bacterium in certain tanks will theoretically prevent the disease. This theory of preventable disease has resulted in some guidelines and control strategies aimed at decreasing the dangers of legionellosis in water systems. The factors leading to outbreaks or LD cases are not fully known, but

some infection prerequisites include; the presence of pathogenic bacteria in the aquatic environment, the proliferation of the bacterium to an unknown infectious level, and the transmission of the bacteria by aerosol to an infectious human host. *L. pneumophila* sg 1 can be subdivided into multiple subtypes by serology or by phenotypic or genetic methods. Other species known to cause pneumonia in humans include *L. micdadei*, *L. bozemanii*, *L. dumoffii*, and *L. longbeachae* (Arora et al., 2012; Bartram et al., 2007). The focus of the study on *L. pneumophila* is more than other *Legionella spp.* because, *L. pneumophila* sg 1 causes approximately 90% of all Philadelphia outbreak of LD (Benin et al., 2002; Yu et al., 2002). In America and Europe, *L. pneumophila* sg 1 accounts approximately to 90% of *Legionella* isolates, But in New Zealand and Australia, *L. Longbeachae* accounts for about 30% of cases and *L. pneumophila* sg 1 accounts for around 50% of the cases of legionellosis acquired by the population (Yu et al., 2002).

It is always important to match clinical and environmental isolates to recognize the environmental origin of the disease, based on molecular techniques including; ribotyping, amplified fragment length polymorphism (AFLP) analysis, pulsed-field gel electrophoresis (PFGE), restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP) analysis, multilocus sequence typing (MLST) and arbitrarily primed PCR (Fry et al., 1991). One method, a single-endonuclease, amplified fragment length polymorphism analysis is currently widely used by members of the European Working Group for *Legionella* Infections (EWGLI) (Fry et al., 2002).

1.9 Ecology

L. pneumophila was isolated from water samples in 1979 (Fliermans et al., 1979). *Legionella spp.* are ubiquitous in freshwater habitats, including rivers, lakes, streams, ponds, hot springs, muds and groundwater, and are a normal part of microbial ecosystems. (Ortiz-Roque and Hazen, 1987; Qin et al., 2013). While, *L*. The first person to report the proliferation of *Legionella spp.* in close association with free living amoeba genera like *Acanthamoeba*, *Tetrahymena* and *Naegleria* was Rowbotham (Bitar et al., 2004; Declerck, 2010; Sheehan et al., 2005). Thus, one amoeboid cell could host more than 1000 *Legionella*. Sixteen species of protozoa have been found to refuge or harbor *Legionella spp*. (Fields et al., 2002). This is very important in life cycles of *Legionella* to motivate

virulent bacterial characteristics, and assist in the distribution and defend against harmful or bactericidal environmental conditions, such as excess heat and chlorine (Berk et al., 1998; Bigot et al., 2013; Neumeister et al., 2000). *Legionella spp*. can survive in different water temperatures in hot and cold water at (25°C to 37°C) but may grow and proliferate at temperatures above and below this range and may even grow at limited temperatures of 20°C and 55°C (Arvand et al., 2011). Moreover, they are found growing in the biofilm that lines the inside of faucets (Fields et al., 2002) also, some parts of water distribution systems are especially prone to contain *Legionella*, like blind loops, plumbing fixtures, showers, whirlpool spas, and cooling towers (Rogers et al., 1994).

1.10 Environmental investigation

Legionella is omnipresent in natural and manmade aqueous environments (Fields, 1996). In nature, *Legionella* seems to be at least facultative, but may even be an obligatory intracellular parasite of free-living amoeba and protozoa (Moosavian et al., 2019). *L. pneumophila* has been isolated and recovered from both natural and human-made habitats, from lakes, muds, and streams to air-conditioning cooling towers, fountains, and spa baths (Bitar et al., 2004; Lettinga et al., 2002; Simon. et al., 2016). But *L. longbeachae* are the only exception and differ from the environmental niche of *L. pneumophila* which inhabit primarily soil, mud and infections are often associated with exposure to soil (Steele et al., 1990). Therefore, it is important to link the patient strains to ecological isolates in epidemiological investigations. This association between *Legionella* and amoeba can develop the resistance to biocides, antibiotics, acid, osmotic and temperature stress (Cirillo et al., 1999, 1994). Furthermore, amoeba associated *L. pneumophila* bacteria can be found in biocide-resistant vesicles that can act as an airborne factor for bacterial transmission (Berk et al., 1998).

In human-made water systems, such as in buildings, hospitals, and dental clinics water systems, *Legionella* along with other aquatic microorganisms produce a microbial community at the intersurface between liquid and surface, which is called biofilm. The replication of *L. pneumophila* within the biofilm depends on the presence of an amoeba host (Henne et al., 2012; Murga et al., 2001). The bacteria inside the biofilms are relatively

resistant to standard water disinfection procedures (Liaqat and Sabri, 2010; Salam et al., 2017).

Legionella are isolated from water, thermal water systems and the biofilm of plumbing fixtures (Bédard et al., 2015; Kusnetsov et al., 2003; Stout et al., 2003). Currently, the concentration of the sample by filtration of water through the filter is the best method (pore size 0.45μm) (Ta et al., 1995). The filter is placed on the growth plate medium, and then it can be examined under a low power microscope for colonies with typical cut-glass appearance.

LD is mostly related to human-made aquatic environments that contain water at hot temperatures. Instead, several disease outbreaks are related to cooling towers and evaporative condensers, which can produce water aerosols contaminated with *L. pneumophila* that are inhaled (Newton et al., 2010). The pathogenesis of LD is at large due to *L. pneumophila* ability to infect and grow in the lungs' macrophages (Rowbotham, 1986). Therefore, the development of virulence traits in *L. pneumophila* has resulted largely from the requirement of the organism to multiply in an intracellular host (Newton et al., 2010).

1.11 Mode of transmission

Many modes of transmission of *Legionella* to humans have been reported. In reality, the evidence is overwhelming that the vast majority of LD patients contracted the disease by inhalation of *Legionella* bacteria that float in the air. *L. pneumophila* transmission is believed to occur mainly through inhalation of contaminated aerosols or through inhaling aerosol or dust from bacteria-laden water droplets or by coughing (Khazaei et al., 2015; Swanson and Hammer, 2000; Szymańska, 2004). This can occur primarily in patients with swallowing disorders or with nasogastric feeding (Johnson et al., 1985) and not from other infected persons; to date, only one case of human-to-human transmission has been documented (Lapierre et al., 2017). Aerosols floating through the air are considered as the vehicles responsible for transporting the bacteria. Particles 5 μm less in diameter can reach the alveoli of the lung.

The phenomenon of inhalation seems not to be limited to patients that develop legionellosis. A seroprevalence study in the Netherlands has shown that with aging the presence and quantity of measurable IgG antibodies (Ab's) against *Legionella* was elevated, while IgM was reduced. Moreover, Some authors suggest aspiration more serious than inhalation in the mode of transmission (Blatt et al., 1993; Pedro-Botet et al., 2002). LD is a respiratory disease caused by inhalation of *Legionella*-containing aerosols formed by showers, faucets, air conditioning cooling towers, whirlpool spas, fountains, dental devise like triple syringe, turbine handpiece and other devices produce aerosols (Bennett et al., 2014; García-Fulgueiras et al., 2003; George et al., 2016a) (Fig 1.3).

The causes of transmission are cooling towers, condensers for evaporation, vapor machines, hot pool, and showers. An air conditioning system is only dangerous if a cooling tower or evaporative condenser is set in such a way that the produced aerosol can be transmitted into the air intake of a building or be directly in contact with a passerby bystander (Dondero et al., 1980; Fiore et al., 1998). Amebae microaspiration could pose a potential risk as one single amoeba could contain more than 1000 *Legionella* cells. (Rowbotham, 1980). When exposure to, or contact with aerosols containing free-living amoebae infections may occur, amoebae can act a “Trojan horses” for *Legionella* (Ditommaso et al., 2016). Moreover, intracellular multiplication in *Acanthamoeba castellanii* affects macrophages entry mechanisms and the promotion of virulence of *L. pneumophila*. Therefore, infection in humans can require both *Legionella* and an amoeba host to be present. (Bitar et al., 2004; Swanson and Hammer, 2000). At the same time, the problem is especially important in hospitals, where medical devices can also cause infection (Endoscopes, devices for artificial respiration and oxygen therapy, dental devices, etc.) (Van Heijnsbergen et al., 2015).

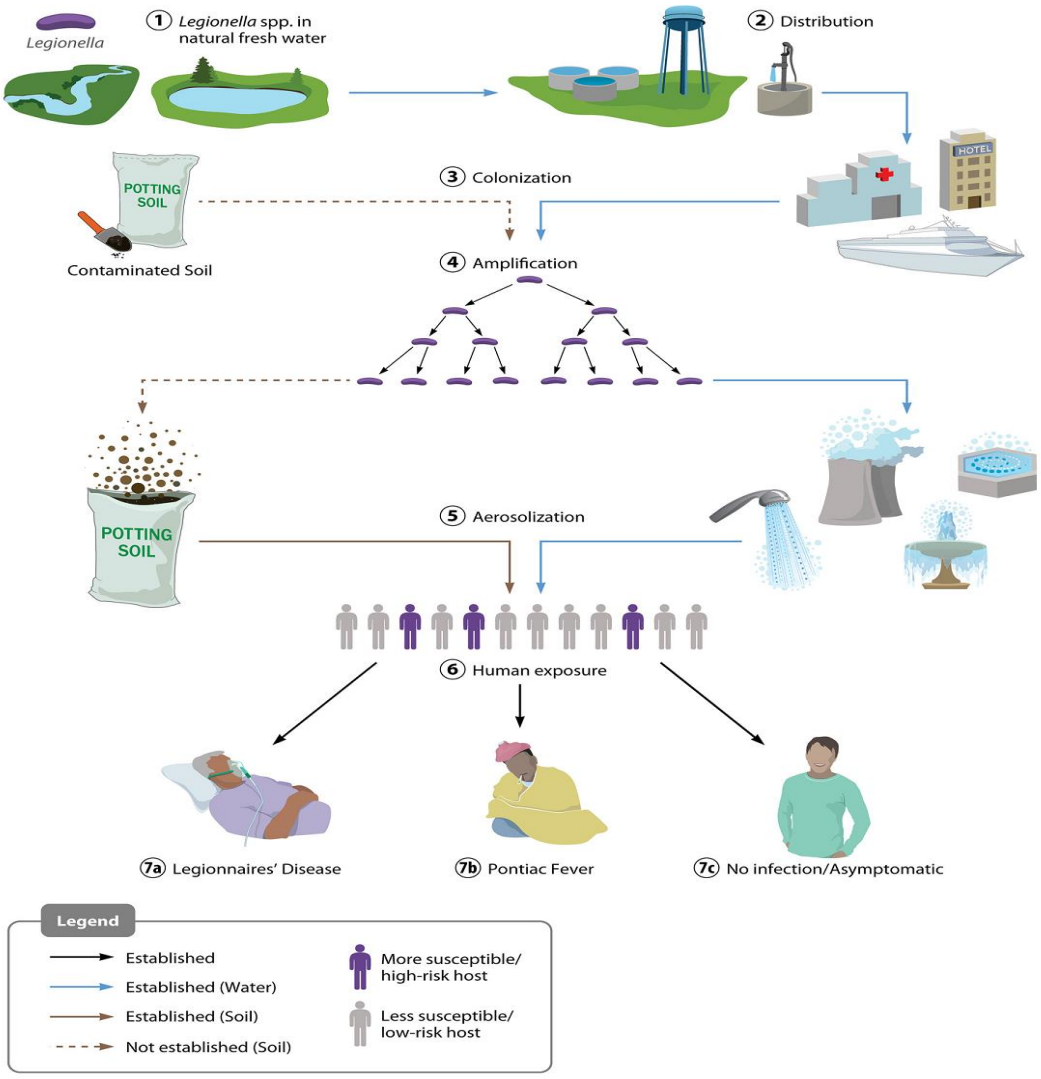


Figure 1.3: "Modes of *Legionella* dissemination from natural waters to development of LD and/or Pontiac fever. *Legionella* from freshwater sources (1) is distributed at low concentrations from points of water purification (2) to colonize downstream local plumbing networks and cooling systems (among other sites) (3) and amplifies under permissive environmental conditions (4). Subsequent aerosolization (5) exposes a human population, which may include individuals with increased susceptibility (6), leading to a potential disease spectrum. More susceptible individuals (due to age or underlying medical conditions) are at a higher risk of LD than those less susceptible, and both groups are at risk for Pontiac fever. The route of LD caused by contaminated soil is less well understood but also appears to involve aerosol exposure". Adapted from (Mercante and Winchell, 2015).

1.11.1 Recognized and potential sources of *Legionella* infection

All sources or possible sources of *Legionella* bacteria are the following:

- Water systems with a cooling tower
- Water systems with an evaporative condenser
- Hot and cold water tanks
- Swimming pools
- Natural hot springs and their systems of allocation
- Respiratory therapy equipment
- Moisturizers
- Dental unit waterlines
- Decorative Fountains/sprinklers
- Water-cooled machine tools
- Vehicle washes
- Potting manure/soil in warmer environments
- Factories containing water systems with temperature above 20^0C or have an electrical part that can pass heat and cause localized heating and that can emit a spray or aerosol.

1.12 Epidemiology

An outbreak is when two or more cases of legionellosis are diagnosed from the same geographical area or have been exposed to the same potential source of infection in the previous six months (National guidelines for the control of Legionellosis in Ireland, 2009). The outbreak of LD is unknown; nevertheless, such events are considered preconditions for infection. Like intracellular multiplication of the bacteria in protozoa, bacteria involvement in the aquatic environment, and transmission of bacteria by aerosols to the human host (John Balbus et al., 2004). Studies have demonstrated that LD accounts for 0.5% to 10% of community-acquired pneumonia requiring hospitalization in adults (Bartram et al., 2007). *L. pneumophila* was second to *S. pneumoniae* as the most frequently known etiological agent (Vergis et al., 2000). Many *Legionella spp.* and serogroups can be found in hospital water systems. In spite of the fact that *L. pneumophila* sg 1 accounts for

the majority of cases of LD, other serogroups have also been correlated with infection in healthcare settings (Goetz et al., 1998; Perola et al., 2002). This has significant clinical drawbacks because the urinary antigen test is the most commonly used method for LD diagnosis, which is specific for *L. pneumophila* sg 1 only. LD rarely occurs in children (Greenberg et al., 2006). In Cyprus, a major outbreak was reported in a private hospital's neonatal unit, 11 cases were reported and 3 deaths (USFCD,2008).

An essential part of public health practice in governing national and international architecture and disinfection policies and guidelines called 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 risk of contamination with pathogens in drinking water and bathing water, which is not always possible for epidemiological studies. This requires not only quantitative data on infectious waterborne pathogens but also on their fate and transport in the aquatic nature. 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. In a pilot study forecasting the risk of infection with waterborne microbes in drinking water and bathing water affected by different climate change conditions, QMRA was successfully applied (Schijven and de Roda Husman, 2005). Despite this accepted QMRA form 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 potable water while 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 limited to estimating the 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 phenomenon 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 not 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 (J. Balbus et al., 2004; Hunter et al., 2009). A further under-researched issue is the problem of susceptible subpopulations. 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.

1.12.1 Country-specific epidemiology of legionellosis:

North and South America, Asia, Australia, New Zealand, Europe, and Africa have reported cases of LD (Bartram et al., 2007). According to the world's current epidemiological data, specific *L. pneumophila* sgs are responsible for legionellosis. In American, European, 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). National surveillance programs currently being conducted in the United States, 24 European countries, Australia, and New Zealand. The true detection of legionellosis is difficult to know because the identification of cases needs adequate surveillance. Research suggests that LD is under-reported to national surveillance systems (Bartram et al., 2007; Marston et al., 1994). The recognition of the disease depends on the physician's awareness and the resources available to diagnose the disease.

In the USA, the number of cases per million population increased from 3.5 in 1984 to 6.3 in 1994 and then decreased to 4.7 in 1996. (Bartram et al., 2007; Marston et al., 1994). The Centre for Disease Control and Prevention (CDC) has been estimated that between 10,000 and 20,000 LD cases occur annually in the USA. (Arora et al., 2012) In England and Wales between 1980 and 2002, 4402 cases of the LD were identified (Arora et al., 2012; Fraser et al., 1977).

Outbreaks of the disease occur periodically throughout the year, most of the epidemics of the disease occur in late summer and autumn, presumably due to the proliferation of *Legionella* in water reservoirs during the hot months. Table 1.2 Number of reported cases of LD and age-standardized rates per 100,000 population (Beauté, 2017)

Table 1.2: Number of reported cases of LD per 100,000 populations in different European countries from 2011 to 2015

Country	2011	2012	2013	2014	2015
Austria	**96**	**104**	**100**	**133**	**160**
Belgium	**79**	**84**	**155**	**200**	**196**
Czech Republic	**57**	**56**	**67**	**100**	**120**
Denmark	**123**	**127**	**113**	**158**	**185**
France	**1170**	**1298**	**1262**	**1348**	**1389**
Germany	**635**	**628**	**810**	**832**	**865**
Greece	**18**	**29**	**38**	**27**	**29**
Hungary	**37**	**33**	**29**	**32**	**58**
Italy	**1021**	**1346**	**1363**	**1510**	**1556**
The Netherlands	**311**	**304**	**308**	**348**	**419**
Norway	**33**	**25**	**40**	**51**	**60**
Poland	**18**	**8**	**11**	**12**	**23**
Portugal	**89**	**140**	**94**	**588**	**145**
Slovenia	**44**	**81**	**77**	**59**	**106**
Spain	**706**	**972**	**815**	**925**	**1024**
Sweden	**127**	**102**	**122**	**136**	**142**
United Kingdom	**251**	**401**	**331**	**370**	**412**

L. peumophila sg1 is responsible for more than 60% approximately of cases in most European countries and America (Yu et al., 2002) but a smaller percentage of cases in countries like Australia and New Zealand are around 50%. (Yu et al., 2002). A recent survey study in Israel demonstrated 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 indicated dominance (more than 80%) of *L. pneumophila* sg3 in patients with LD (Qasem et al., 2008).

1.13 Virulence

The main feature of *Legionella* pathogenesis is its ability to replicate within macrophages.. However the ability of *Legionella* to infect both protozoa and macrophages, including the attachment of bacterial cells to host cells, intracellular replication, and cell-to-cell spread, all indicate pathogenesis. (Zhan and Hui Hu, 2015). 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 bacterial surface, where it seems to influence intracellular establishment (Fields et al., 2002; Helbig et al., 2003; Swanson and Hammer, 2000). The exact role and mechanism in pathogenicity are still unknown.

The mechanism can be illustrated as, when amoebae or macrophages have engulfed virulent *Legionella,* a phagosome is formed; this is surrounded by the endoplasmic reticulum and is totally isolated from the endosomal track (Fields et al., 2002; Swanson and Hammer, 2000). Firstly, lysosome fusion is impaired. It has been supposed that *L. pneumophila* converts to a replicative form in this protected environment, it no longer expresses virulence features but becomes acid and sodium chloride (NaCl) resistant (Motaharinia et al., 2010). Also, endosomes containing the microbe can integrate with lysosomes allowing the intracellular bacteria to use a nutrient-rich niche that would kill other bacteria under normal circumstances. When the stock of amino acids is depleted, the cells transform into a stationary phase state and simultaneously acquire the characteristics required for transfer to a new phagocyte. *Legionella* is small, dense and highly mobile released from eukaryotic cells. Thus, they are present in the environment in two phases. In this system, there are many factors involved, including type II and IV secretion systems, iron accumulation, pore-forming toxins, and apoptosis activation in the host cell. *Legionella* intracellular establishment and trafficking are thought to be regulated by the *dot / icm* (defective organelle trafficking/intracellular multiplication) gene complex, which encodes the substances involved in type IV secretion system (McAdam et al., 2014)

(figure 1.3). Additionally, *Legionella* produces cytotoxins that are extracellular. Experimental data indicate that when the incubation temperature of a cultured inoculum reduces from 37 ° C to 24 ° C, virulence decreases significantly. (Mauchline et al., 1994).

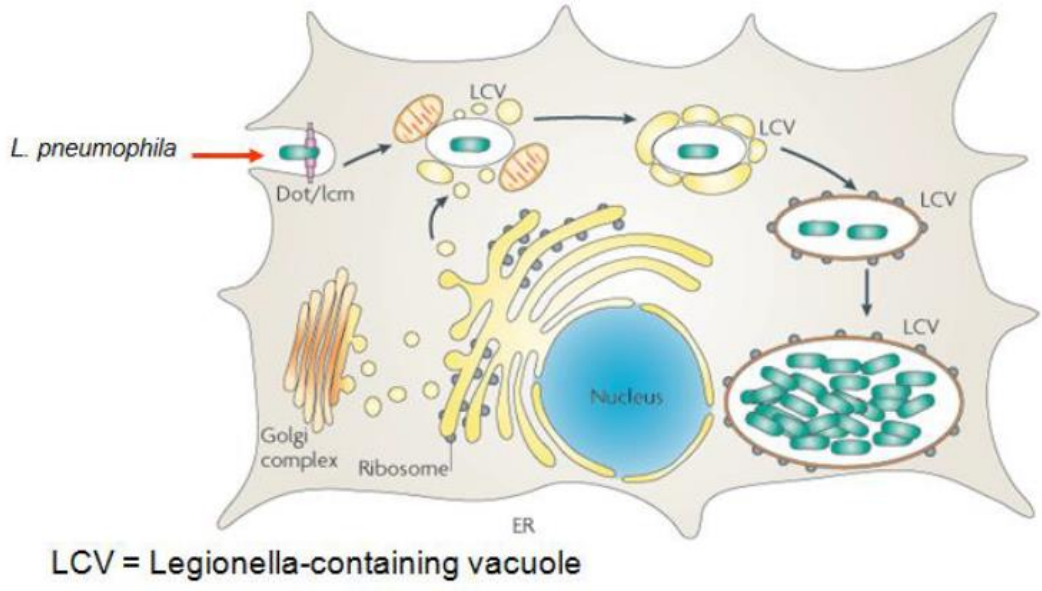


Figure 1.4*: L. pneumophila* invasion of an amoeba/macrophage (Isberg., and al., 2009).

1.14 Clinical features

Legionellosis presents clinically in two forms, Legionnaires' disease (LD) and Pontiac fever which is a mild flu-like illness (Tabatabaei et al., 2016). Many persons may be infected with *Legionella*, but remain asymptomatic which can be confirmed by seroconversion (Boshuizen et al., 2001). A person is infected with LD by inhaling aquatic aerosols, such as drinking water, cooling towers, showerheads, spa, whirlpools, and other man-made devices that generate aerosols. (Mizrahi et al., 2017).

In unknown cases, the microaspiration of contaminated aquatic aerosols into the lungs could be the mode of nosocomial and community-acquired pneumonia (CAP) transmission of legionellosis (Marrie et al., 1991). Microaspiration of contaminated water supposed to be the mode of transmission (Kao et al., 2017). There are often many examples of LD transmission by contaminated aerosols, especially in the epidemic region. where a cooling tower system, water fountain, or water spa exists. (Lapierre et al., 2017). Legionellosis can be a sporadic epidemic or part of it. Legionellosis cases are documented over the year.

Most cases occur in the summer and fall due to hot weather encouraging the bacteria's proliferation in water.

Generally, legionellosis occurs in middle-aged and elderly persons, in total it occurs in people who have chronic heart and lung disease, and who are heavy smokers or immunocompromised (Mizrahi et al., 2017). LD lacks clinical symptoms or signs, there is no typical syndrome and not every person exposed to the organism will develop symptoms of the disease (Bartram et al., 2007).

The incubation period of LD from two to ten, rarely up to 20 days was noted in one outbreak (Den Boer and Yzerman, 2004). Pontiac fever from 3 -5 hours (most commonly 24 -48 hours) and is more common in younger people (Tossa et al., 2006). Most patients with LD have a fever, non-productive cough, headache, myalgia, dyspnea anorexia, and lethargy. Clinical syndromes may include diarrhea, nausea, chest pain, vomiting, liver and kidney dysfunction, thrombocytopenia, hyponatremia, and neurological disorders (Zarogoulidis et al., 2011). Neurological signs range from neurological abnormality is headache and lethargy to encephalopathy. (Morelli et al., 2006), fever may also include organ-specific signs and symptoms, such as diarrhea or confusion, or both multisystem disease fever, including kidney failure pneumonia rhabdomyolysis with extrapulmonary features and severe fulminant disease (Chidiac et al., 2012).
Rarely, LD is a cause of pneumonia in children most of them are immunosuppressed (Greenberg et al., 2006).

A mortality rate is standard in most people ranging from 8–12% but may increase in people who are at higher risk including elderly, have preexisting medical conditions, cigarette smokers, are nosocomial cases or are delayed in their diagnosis and treatment and in individuals with suppressed immune system has been reported even to 80% (Khazaei et al., 2015; Mojtaba. et al., 2017). The average case-fatality rate in Europe is 10% (range 0–27% in countries reporting a total of 30 cases) and 8% in the USA. For nosocomial cases, the case-fatality rate is higher and ranges from 15% to 34% (Beauté, 2017).

1.15 Diagnosis

The effort of finding a predictive result that identifies *L. pneumonia* in individual patients has been unsuccessful. *Legionella spp.* diagnostic tests have improved since 1976. There are no available methods able to diagnose all *Legionella spp*. with high sensitivity and specificity. Symptoms for LD and *Legionella* pneumonia are often similar. This overlap makes it difficult to establish a checklist of features to identify *Legionella* infected individual patients. However, LD's clinical symptoms are typically more severe than most "atypical" pneumonia cases. In LD, diarrhea and hyponatremia are more severe than in other types of pneumonia. (Arora et al., 2012).

Most of the data is for *L. pneumophila* since nonpneumophila species sensitivity and specificity estimates are not known(Kalogeropoulou and Vrioni, 2008; Kenagy et al., 2017). A case of LD will be unknown unless special laboratory tests are done. Unluckily, these tests are not routinely available in many countries. Many hospitals in the USA have only recognized cases of LD following extensive examination of pneumonia patients. Similarly, several additional cases were reported in hospitals where only one to three cases of LD were identified over several months after the intensification of surveillance. Community-acquired pneumonia studies have also shown that increased surveillance leads to better diagnoses (Fields et al., 2002).

Identification of *L. pneumophila* can be done by various methods such as isolation of the bacteria on culture media, identification of bacterial via serology, urinary antigen test, detection of bacteria in the body fluids or tissues using an immunofluorescent microscope like direct immunofluorescent assay (DFA) and the detection of bacterial DNA by the Polymerase Chain reaction (PCR) (Aksono and Hermadi, 2017). These molecular techniques were evaluated to increase the specificity and sensitivity in the clinical diagnosis of LD and early detection and monitoring (Figure 1.5).

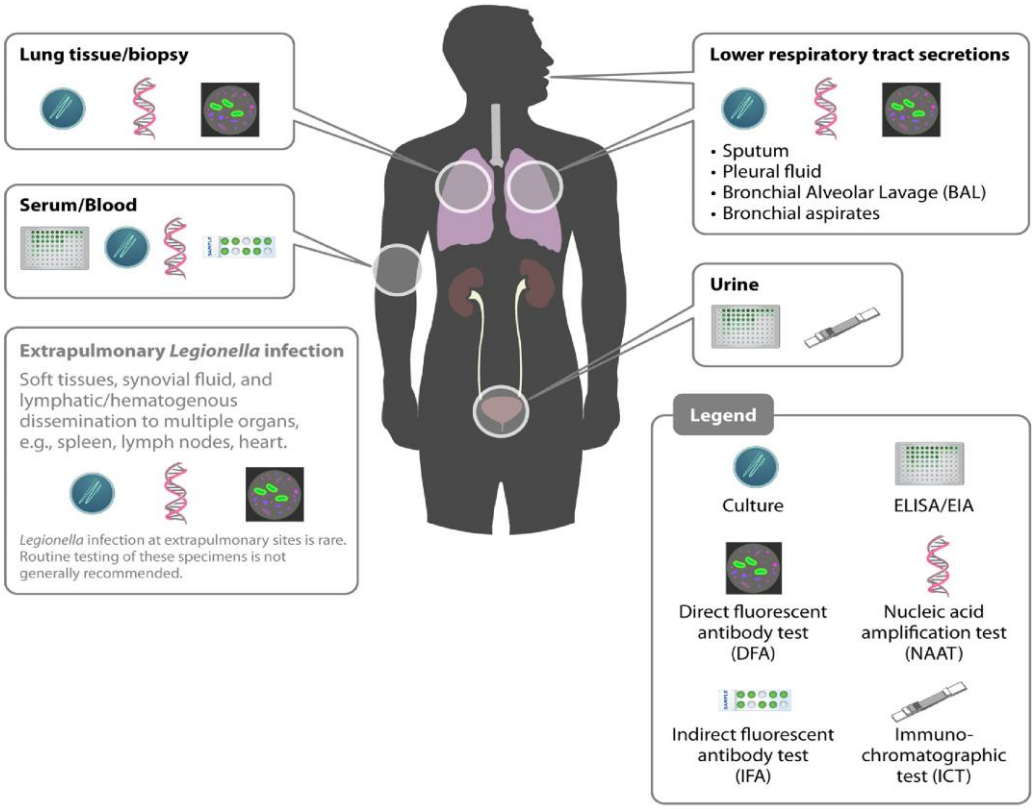


Figure 1.5: "specimen types and diagnostic tests: In this figure, we can see many of the samples and diagnostic tests for the detection of *Legionella pneumophila*. Some assays apply to multiple samples types, such as culture and nucleic acid amplification. In general, the success of detecting *Legionella* is dependent on the severity of the disease, the safety of the sample, the technical skill of the laboratory, and particular test features" (Adapted from (Mercante and Winchell, 2015).

Identification of *Legionella spp*. and serogroups are not adequate for epidemiological investigation of LD. It is important to use subtyping methods, which differentiate between the strains at the molecular level.

1.15.1 Culture

Before an in vitro medium was established that could isolate *Legionella* (Feeley et al., 1979) *Legionella* was grown in guinea pigs or chicken eggs (Morris et al., 1979).

The best standard method for diagnosis of LD is the isolation of *Legionella spp.* from the environmental or clinical samples. The "gold standard" is culture on Buffered Charcoal Yeast Extract (BCYE) agar added to L-cysteine (Tabatabaei et al., 2016; Tronel and

Hartemann, 2009) as well as a culture on GVPC (Glycine-Vancomycin-Polymyxin-Cycloheximide) selective media (George et al., 2016a) which has a specificity of 100%. Culture diagnosis requires selective media, adequate processing of samples, and technical proficiency. It also takes a lot of days to get a positive result, with most *Legionella spp*. Culture plates are incubated for up to 14 days at 36 ± 1 ° C and tested every 2 to 3 days, colonies were detected within 7 days. Even the presence of one or several colonies is necessary to confirm the diagnosis. (Bartram et al., 2007). Depending on their growth on the BCYE agar, *Legionella* should be distinguished from *Francisella tularensis*, *Bordetella pertussis*, and certain thermophilic spore-forming bacilli (Arora et al., 2012).

Certain *Legionella* species can grow at a slower rate and can only be detected after 10 days of incubation. (Fields et al., 2002). When patients take appropriate antibiotics, the appearance of colonies may be delayed, as well as when the samples are infected with oth other microorganisms (Lück PC, Helbig JH, 2002). Before antibiotic treatment is begun, the specimens should be taken for cultivation. Respiratory samples that are especially difficult to get, for example, lung tissue, pleural fluid or bronchoalveolar lavage (BAL) should be cultured immediately if routinely obtained (Stout et al., 2003). BCYE agar supplemented with α-ketoglutarate, glycine, with or without antimicrobial agents is the main medium used for *Legionella*. Polymyxin is commonly added antibiotics to control Gram-negative growth, anisomycin against yeast, 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, 2.5% CO2 atmosphere (Lee et al., 1993).

Legionella can be isolated from different specimen types. The samples of choice are lower respiratory tract secretions especially sputum and bronchial alveolar lavage (BAL) samples, pleural fluid. The yield of culture depends on the severity of the disease, with the lowest yield from 15% to 25% in slight pneumonia and the highest yield for severe pneumonia with respiratory failure of more than 90% (Murdoch, 2003). *Legionella* was successfully isolated from samples of the lower respiratory tract including BAL, transtracheal aspiration, endotracheal suction specimens, pleural fluid, lung biopsy, and expectorated sputum. In the early phase of the disease, legionellosis is often accompanied by little phlegm, while the few organisms outside the lungs and the oral flora's inhibitory effect decrease the sensitivity of the cultivation method (Bartram et al., 2007).

A major limitation of sputum culture is that sputum is produced by less than half of LD patients. Many patients with LD produce sputum with relatively few purulences; laboratories discarding sputum samples containing few pus cells may not accept these samples. Sputum culture has an estimated range of sensitivities of 10% to 80% and differs by various comparative criteria and by individual laboratories (Murdoch, 2003). In practice, the best results are likely to be achieved only by laboratories with a special interest in *Legionella* infection. Furthermore, *Legionella spp* are fastidious and not easily detected by culture because of the occurrence of a Viable-But-Non-Culturable (VBNC) state known for many *Legionella spp* (Ditommaso et al., 2016; Fields et al., 2002). Some species of the *Legionella* are unable to grow on standard media of *Legionella* culture and have been termed *Legionella*-like amoebal pathogens (LLAP) because they multiply in certain host species of amoeba (Fard et al., 2012). By cocultivating the bacteria with their protozoan hosts, these species were isolated and preserved in culture. (Bartram et al., 2007).

1.15.2 Serological and antibody-based assays

For the detection of *L. pneumophila*, the serology tests for IgG and IgM antibodies against *Legionella* in blood serum was important in the original Philadelphia outbreak investigation (Beauté et al., 2013), and one of the major LD diagnostic methods used in the early 1980s. The number of serological tests used in the clinical laboratory has reduced significantly with the increase of standardized culture media, techniques and more definitive analyses such as the rapid urine antigen test and molecular methods (Benin et al., 2002).

Detection of urine antigen of *Legionella spp.* by enzyme immunoassay (EIA) is an adequate detection method with a specificity of 100% and a sensitivity of 70-100% (Qasem et al., 2008). The indirect immunofluorescence assay (IFA) is important in the identification of patients' antibodies and was instrumental in identifying the cause of the disease. From the different antibody detection tests that are available, IFA and enzyme-linked immunosorbent assays (ELISA) are the most commonly used. Most laboratories are now preferring ELISA assays because they are more precise and less subjective than IFA testing and also have the possibility of automated results (Malan et al., 2003). Using enzyme immunoassays (EIAs) to detect *L. pneumophila* antigen in urine allows early

diagnosis of LD in the infection. Sensitivities of serological assays varied from 41% - 94% (Den Boer and Yzerman, 2004). The antigen can be found in most patients from 1 to 3 days after symptoms start and can last for a few weeks or months (Birtles et al., 1990). Other tests such as direct fluorescent antibody test (DFA) assays, slide agglutination tests (SATs), and Monoclonal Antibody (MAb). The screens are based on antibodies, but the patient's serum is not tested directly. For the SATs and MAb test, the isolation of pure culture is required. DFA assays can be done on cultures, tissues of the patient or fluid secretions. However, their use for *Legionella* respiratory antigen detection in the clinical laboratory was decreased to minimal, from a rate of 1% in 1996 to 1/10 of 1% in 2010 (Beauté et al., 2013).

1.15.3 Urinary antigen test

Following the Philadelphia outbreak, the *Legionella* antigen was found in the urine (Berdal et al., 1979). *Legionella* antigen can be found in the urine after symptoms have occurred and for days to weeks, it lasts. In one case, excretion of antigen was reported to persist for more than three hundred days (Kohler et al., 1981). The identified antigen is part of the *Legionella* cell wall's lipopolysaccharide component and is heat stable (Kohler et al., 1981). Urinary antigen test (UAT) is significantly outpaced other laboratory methods for diagnosis, representing 82%- 92% of the diagnostic tool used to confirm LD in Europe and the USA. And has a high degree of sensitivity and specificity, showing a successful identification of > 90% of cases in less than 15 minutes (Control, C. f. D., and Prevention report, 2011).

The publicity and ubiquity of the UAT are attributed to its quick, relatively inexpensive, uncomplicated procedure, and easier collection of the specimen. *Legionella* specific antigenuria can be detected in the majority of *L. pneumophila* infections shortly after clinical symptoms, infections appear (2 to 3 days) and can be excreted even during antibiotic treatment and after disease resolution for several days to 10 months (Jarraud et al., 2013; Qin et al., 2016)

Two commercial kits methodologies have been widely used and available; the enzyme immunoassay (EIA) *L. pneumophila* sg 1 antigen detection kits are available., and the

radioimmunoassay (RIA) method for detecting *Legionella* antigens in urine was also used (Fields et al., 2002). The most significant trouble with the UAT is that it is highly specific only to *L. pneumophila* sg 1, therefore a positive test indicates LD, but a negative test cannot be neglected.

A quick immunochromatographic test to detect *L. Pneumophila* sg 1 antigen is also available in the urine. This test detects antigeneuria. laboratory equipment is not required and in a very short time performed (Helbig et al., 2001). The concentration of urine increases the sensitivity of both the EIA and immunochromatographic assays, without decreasing their specificity. Furthermore, agglutination assays can be used, but they don't have acceptable sensitivity and specificity (Leland, D.S., and Kohler, 1991).

1.15.4 Nucleic acid-based molecular diagnostics

The serology methods, when compared with molecular methods, are less sensitive, specifically in the early stage of the disease during which antibody level is low (Ayala et al., 2014). Furthermore, molecular techniques have more sensitivity and specificity compared to serology methods (Khazaei et al., 2015).

The challenge of growing *Legionella* isolates from environmental and clinical samples to the evolution of rapid molecular techniques for the detection of *Legionella* DNA (Yong et al., 2010). Culture has some troubles, the sensitivity variable and highly dependent on the laboratory technical proficiency, molecular techniques (cultivation-independent techniques) that are 100 percent sensitive and quick were sought. In the mid-1980s Nucleic acid-based research for *Legionella* detection, diagnosis and typing started (Mercante and Winchell, 2015).

Polymerase Chain Reaction (PCR) was first used as a tool for detecting *Legionella* in 1989, at Stanford University when researchers had combined PCR with Southern blot to detect *Legionella* DNA in water (Starnbach et al., 1989). The development of PCR-based strategies continued into the 1990s for epidemiological studies with ecological samples, also evaluated and validated this strong new method in a variety of forms, including water from cooling towers, rivers, and hot water bath as well as sputum, BAL fluid, serum, and urine (Miyamoto et al., 1997). In the early 2000s, Real-time PCR was used for the quantitative and monitoring of *L. pneumophila*.

High sensitivity and specificity are the advantages of Nucleic Acid Amplification Tests (NAATs), and quick turnaround time. NAATs like traditional PCR and real-time PCR (single and multiplex). *Legionella* detection protocols were developed and characterization. Real-time PCR has more advantages to diagnosis; it reduces the manual time for the PCR and gives quantitative results (Peci et al., 2016).

The majority of NAAT-based assays are highly specific (near 100%), and the rising consensus is that the sensitivity of PCR is equal to or more than that of culture-based detection using samples from the lower respiratory tract or environmental water samples (Jespersen et al., 2009). Detection of mild LD cases can be eminent or detection post-exposure. PCR has been shown to have sensitivity equal to or greater than culture when examining specimens from the lower respiratory tract. (Cloud et al., 2000). Can detect *Legionella* DNA in a different specimen like urine, serum, and leukocyte samples obtained from patients with LD with sensitivities ranging between 10% to 86% (Helbig et al., 1999; Lu et al., 2016).

The most common targets include a preserved portion of the rRNA genes for the 5S and 16S subunits, the 16S-23S spacer and/or the protein macrophage inhibitor (*mip*), found primarily in the *Legionella* genus and strongly preserved in all *L. pneumophila* isolates (Divan Khosroshahi et al., 2015). The current "gold standard" in molecular diagnosis is based on the detection of the *mip* gene-specific for *L. pneumophila* and 16S rRNA for the identification of the *Legionella* genus. The *mip* gene is the first gene linked to *L. pneumophila* capacity. multiplying in eukaryotic cells and encodes a surface located peptidylprolyl cis/trans isomerase (PPIase) (Yong et al., 2010). Also, the *ssrA* gene is a target (for all *Legionella spp*), and *wzm* for *L. pneumophila* sg 1(for Lp1) genes (Mercante and Winchell, 2015).

Besides detection and diagnosis, NAATs are generally used for *legionella* typing, mainly in conjunction with traditional MAb or serology. Additionally, cultivation-independent techniques, including fluorescent antibody (FDA), fluorescent in situ hybridization (FISH) (Newton et al., 2010). 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).

1.15.5 Molecular Typing

In epidemiological studies the detection and identification of *Legionella* bacteria are not adequate, so scientists developed methods with more distinct discrimination for further subtyping.

Many subtyping techniques can be used on *L. pneumophila* bacteria; pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), ribotyping, arbitrarily primed PCR (AP-PCR), repetitive element PCR (rep-PCR), amplified fragment length polymorphism (AFLP) analysis, and phylogenetic comparison of various *Legionella spp.* and strain-specific genes, including *ftsZ* and *sidA*, among others (Gilmour et al., 2007; Ratcliff, 2013).

Subtyping methods of *L. pneumophila* strains are considered the most special methods for typing and subtyping however they needed isolates (Fry et al., 2002). PCR and RT-PCR performed directly from clinical specimens were evaluated for the diagnosis of LD and showed high sensitivity and specificity (Mercante and Winchell, 2015).

Epidemiological techniques have been developed over the past few years to be used directly by PCR-based typing methods such as with clinical samples; Multiple locus variable tandem-repeat (MLVA) assays are based on the division and scale of repetitive sequences from short to long tandem. Sequence-Based Typing (SBT) is an effective method based on seven gene loci sequencing and is recognized as the new EWGLI (European Working Group for *Legionella* Infections) gold standard tool for *L. pneumophila* typing (Pancer, 2013).

1.16 Treatment

The cases of Philadelphia outbreak mortality decreased with a doctor's increased suspicion index, early antibiotic treatment covering *Legionella spp.* and the beginning of quick laboratory tests. Mortality rates increased when there is a delay in starting with adequate treatment (Heath et al., 1996).

Legionella spp. is a common cause of community-acquired pneumonia (CAP) requiring hospitalization, causing 2%–16% of all cases (Plouffe et al., 2003). Documented data from a large-scale study conducted by the Center for Disease Control and Prevention showed a

decrease in the case-fatality rate for community-acquired *L. pneumophila* in the period 1980-1998, between 26% and 10% (Benin et al., 2002).

This finding is in harmony with recent studies of patients with outbreak-related LD, who received early diagnoses which were confirmed by urinary antigen test; these studies have reported case-fatality rates up to 5.5% (Plouffe et al., 2003). The selection of clinical therapy for CAP is based on the sense of the best treatment, the epidemiological features of various microorganisms in the Netherlands, and an inference of the most likely pathogen (Aleva et al., 2005). The choice of antimicrobial treatment for LD should be driven by disease frequency, immunocompromised degree, and the quality and possible toxicity of medicines (Diederen, 2008).

The first empirical curative approach an antimicrobial agent effective against *Legionella* spp should be included in severe cases of CAP. (Roig and Rello, 2003). Quick tests for the detection of *L. pneumophila* antigen have a position in both guidelines. Mainly, due to its high occurrence of typical cases of pneumonia, theoretical antibiotic therapy will target *Streptococcus pneumoniae*. Also, antibiotic therapy will target *L. pneumophila* in seriously ill patients and those suspected of LD. Empirical therapy should be replaced with pathogen-directed therapy when a causative agent is identified (Falguera et al., 2010). The presence of antibiotics in nature may promote the development of microbial resistance mechanisms. For *Legionella spp*, this is particularly important. Because it can colonize natural water systems where they can be exposed to antibiotics from medical, veterinary or other micro-organisms (De Giglio et al., 2015)

Legionella spp. are intracellular pathogens. Antimicrobial agents that exceed concentrations intracellular above the minimal inhibitory concentration (MIC) are more active than antibiotics with poor intracellular penetration (Roig and Rello, 2003). Therefore, the safest antibiotics to treat legionellosis are macrolides, quinolones, and tetracyclines. Several few-uncontrolled studies on the treatment of LD exist. There has been no retrospective, adequate-size clinical studies of antimicrobial therapy for LD.

Three observational studies suggest that levofloxacin therapy may be superior to macrolide therapy, these should be interpreted with caution (Mykietiuk et al., 2005; Sopena et al., 1998).

Several research measured macrolide activity (erythromycin, clarithromycin, and azithromycin) versus quinolone activity (levofloxacin)(Pedro-Botet, M.L., and Sabria, 2005). US Food and Drug Administration approves levofloxacin and newer macrolides for the treatment of LD and is considered preferable to erythromycin. Usually, two to three weeks of therapy are suggested. The British Thoracic Society (BTS) guidelines advise clarithromycin ± rifampicin as the preferred treatment for LD with a fluoroquinolone (Mcfariene, 2004). The more recent Infectious Diseases Society of America (IDSA)/ American Thoracic Society (ATS) consensus guidelines on the management of community-acquired pneumonia (CAP) in adults, recommended fluoroquinolone or azithromycin as the preferred Doxycycline antimicrobials as an alternative for the treatment of LD. (Mandell et al., 2003).

1.17 DCU and DUWL

A single dental chair unit can be used in many patients every day, and microbial contamination of specific components can be a significant potential source of cross-infection. (Kotaka, 2012). The dental chair unit (DCU) is the most important equipment available for dental practice and is defined as a medical device in compliance with the Medical Devices Directive of the European Union. Over the past 40 years, the DCU function has developed from simple physical support to sophisticated designs and installations that include several complexes, interconnected equipment systems that provide all the facilities (e.g. water, air supply, electricity, and suction) and dental tools required for a wide range of dental treatment procedures. Dental instruments connected to DCUs (e.g. ultrasonic scalers, air scalers, high-speed dental turbine handpieces, and standard dental handpieces) are cooled by DCU-supplied water, which also provides three-way air/water syringes for irrigating and cooling dental surfaces. Furthermore, water is also supplied to the DCU cup filler outlet used by patients for oral cleaning, as well as to the bowl-rinse outlet that rinses the spittoon DCU. -DCU is equipped with a network of interconnected narrow bore tubes (DUWLs) with flexible plastics. (Kumar et al., 2010) That supplies water to all the instruments supplied by the DCU (Boyle et al., 2011).

DUWL is comprised of ~6 m of narrow bore elastic polyurethane or PVC (Polyvinyl Chloride) plastic tubing (2 mm diameter) with several brass couplings and other plastic

couplings that are not flexible (4 mm diameter). Narrow bore tubing has a very wide area-to-volume ratio (6:1) that facilitates the formation of biofilms. (Kumar et al., 2010). In the opened water system, the source of water is municipal water supply and water is supplied from a tank belonging to the unit in a closed water system. In some large dental clinics supplied with many DCUs, water also comes from a large holding tank supplied with water, whereas in smaller dental clinics, DCUs may have individual systems. (Oleiwi, 2017).

Aquatic bacteria in the environment interact with biofilm surfaces, a technique built to help survival and maximize the nutrients available (Henne et al., 2012). Due to water retention in microbore tubing and intermittent patterns, biofilm formation occurs on the inner surfaces of waterlines. Biofilm will grow to achieve a top microcolonies group embedded in a six-day preservative amorphous extracellular matrix (Salam et al., 2017; Szymańska and Sitkowska, 2013). Biofilms resist penetration by a wide range of chemical agents including disinfectants, chlorine detergents and biocides, antibiotics and other antimicrobials (Coleman et al., 2013).

That leads to serious concern regarding the potential occupational danger in the dental office, taking into account the rising numbers of dental patients. It may be that some of them are immunocompromised, elderly, smokers, alcoholics, organ transplant and blood transfusion recipients, AIDS and cancer patients, diabetics, people with autoimmune disease and people with chronic organic disorders (Oleiwi, 2017).
Several microorganisms are found in the waterlines of dental units, the most important of which being; *P. aeruginosa*, *Streptococcus spp.*, *Enterococci spp.,* and *L. pneumophila* (Ghalyani et al., 2015; Oleiwi, 2017). Freshwater amoebae containing *Legionella* have been detected in dental unit water samples (Ditommaso et al.,2016). Furthermore, oral microbial normal flora of patients can also enter into the waterlines via suctioning of saliva by the head of the handpiece known as backward contamination (Ghalyani et al., 2015). Several studies have shown that DUWL biofilms contain a diverse population of microorganisms and at least 40 bacterial genera have been described at the molecular level. (Porteous et al., 2011).

Legionella typically enter the waterlines of the dental unit (DUWL) from the water supply, where they can proliferate in the biofilm. (Pankhurst, 2003). Dentists have a higher prevalence of *L. pneumophila* infections compared to other individuals. Patients and dentists are usually exposed to aerosols produced by water spray and handpiece linked to the dental unit. Contaminated water can also come in the waterlines of the dental scalers and expose other patients as well as the dentists. Thus, it is of extreme importance to estimate possible microbial contamination of this water (Ghalyani et al., 2015; Szymańska, 2004). Patients and dentists are both exposed to direct contact with bacteria-contaminated water in the form of splatter and contaminated water aerosol sprayed through dental treatment during work by unit handpieces, including rotating and ultrasonic instruments (Szymańska and Sitkowska, 2013). The aerosol droplets produced by dental handpieces are of sufficient size and stability to enter the lung alveoli. (Pankhurst, 2003). The safety of dental patients and dentists, therefore, requires an adequate microbiological quality of water used in dental units.

1.18 Literature review

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

Borella et al reported reported a single case of nosocomial legionellosis found in Milan, Italy, 1000-bed hospitals, a 29-year-old man was the first case of the hospital-acquired LD, the next day he died and *Legionella* was identified in the lung tissue by immunofluorescence. Environmental testing showed that the hospital's centralized network of hot water delivery was colonized with *L. Pneumophile*. Shock heating and water hyper chlorination were applied, which in the short term reduced the number of contaminated sites, but two months later the water was recolonized. Six nosocomial cases were identified

during the period of active surveillance between January 1998 and September 1999. However, 12 population cases have been identified (Borella et al., 1999).

Atlas and colleagues in 1995 reported that, a Californian dentist may have died from LD due to exposure to water in the dental device. (Atlas et al., 1995a).

Pankhurst et al researched the occurrence of *Legionella* in general dental practices in London and rural Northern Ireland. And whether the organism exists in the Dental Unit Waterline (DUWL) at a sufficiently high frequency and magnitude to pose a threat to the health of dentists. Dental surgeries randomly selected two hundred and sixty-six (166 London, 100 Northern Ireland). In order to measure the prevalence of *Legionella*, standardized 250 ml water samples were taken from the DUWL and 1-liter samples from the cold water tap. For the *Legionella* Ab level, the dentists donate a blood sample. *Legionella* prevalence was very small (0.37%), *Legionella* were not isolated from Dental unit waterline (DUWL) or surgery basin taps in Northern Ireland. *Legionella spp* were isolated from the DUWL and surgical basin of one practice in London and from the cold water source of three additional practices. The prevalence of *L. pneumophila* antibodies was less than that seen in a comparable group of London blood donors (Pankhurst et al., 2003).

Doleans et al established established a relationship between water contamination in hospitals and *Legionella* and hospital-acquired legionellosis, the level of colonization of *Legionella* water systems in France was investigated by 554 water samples. Most of them were collected from hospitals. Between 102 and 107 *Legionella* CFU / L, 286 positive water samples (51.6%), and 138 samples (48.3% of the positive samples) contained ≥103 CFU/L. Despite this frequent contamination of hospital water systems in France, legionellosis acquired in hospitals remains relatively rare, Approximately 100 cases a year. (15 % of all French cases) (Doleans et al., 2004).

Ma'ayeh et al assessed the extent of *L. pneumophila* contamination in a dental unit waterline (DUWL) at a Dental Teaching Centre in Jordan, Samples from 10 dental units in each teaching clinic from each teaching clinic, namely conservative dentistry, periodontology, and prosthodontics. Samples were collected from the air/water syringe,

high-speed handpiece and water cup filler. The sampling time was at the start of the working day (before use of the dental unit), after 2 minutes of flushing, and at noon. *L. pneumophila* number range from 0 to 8.35 to 10^3 (CFU / ml). At the beginning of the working day, 86.7% of the dental units *L.pneumophila* was detected 40% after 2 min flushing and 53.3% at midday. At the beginning of the working day. *L. pneumophilia* counts were found to have been decreased by flushing the waterlines (Ma'ayeh et al., 2008).

Mavridou et al studied the prevalence of *Legionella spp*. in Greek hospitals. They collected and analyzed water and biofilms samples from 13 hospitals. *Legionella* using cultivation-independent analysis (AFLP). In 8 out of 13 hospitals, they found *Legionella* and in 22 of 130 water and swab samples. They found 72.7% of *Legionella* was *L. pneumophila* sg and 22.7% were *L. pneumophila* sg 2-14 (Mavridou et al., 2008).

Shareef and Mimi studied the hospital faucet 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* sg 2-14 in 62% of the samples. Also, they studied the effect of thermal disinfection at 80°C as a good factor to prevent nosocomial infections (Shareef and Mimi, 2008).

Göksay et al studied the level of microbial and mycological contamination of dental unit waterline (DUWLs.). DUWLs provide an appropriate environment for microbial biofilm and multiplication primarily due to the high tube surface and the fluid dynamics characteristic of narrow, smooth-walled waterlines. Samples of water were collected from DUWLs from 20 private dental offices. Just 2 (3.4%) of the 59 dental unit water samples met the DUWL water quality requirement (< 200 CFU / ml). By the American Dental Association (ADA). 14 (24 %) of the 59 water samples tested were positive for Pseudomonas sp. And 18 (30.5%) had a positive effect on fungi, fourteen bacterial strains and seven fungi, 57.1% of bacterial strains, were isolated: many bacterial species are known as *Pseudomonas fluorescens, Pasteurella haemolytica, Photobacterium damsela, Ochrobacter anthropi, Moraxella sp., Aspergillus flavus, Penicillium expansum.*

Legionella spp. was not detected in any water sample (Göksay et al., 2008a; Kadaifciler et al., 2013).

Ajmi and colleagues in 2009 wanted to determine if the dental unit waterline (DUWL) device was contaminated with *L. Pneumophila* sg1 occurred at the Mashhad Faculty of Dentistry. Total Water samples of 52 dental units from all Mashhad Faculty of Dentistry clinical departments were selected. Water samples from water/air spray outlets, high-speed dental handpieces and water cup fillers were obtained. Testing of samples using the ELISA method. At the beginning of the workday, a total of 36.1% of dental units were contaminated by *L. pneumophila* sg 1.(Ajami et al., 2012).

Al Matawah et al investigated the incidence of *L. pneumophilia* in water systems of residential facilities in Kuwait, between November 2007 and November 2011. A total of 204 water samples obtained from the bathroom tap and showerheads (n = 82), kitchen taps (n = 51) and water tanks (n = 71), From various residential locations in Kuwait, both have been tested for *L. Pneumophila* by standard method of culture and by polymerase chain reaction in real-time (RT-PCR Of the 204 samples, 89 (43.6%) for *Legionella spp*, were positive 48 (23.5%) samples were detected by the standard cultural method, and through RT-PCR, 85 (41.7%) were detected. Counts ranged between 10 to 2250 CFU/L for culture-positive *Legionella* samples. Serological typing of the 48 isolates of *Legionella*, 6 (12.5 %) belonged to *L. pneumophila* sg 1, 37 (77.1%) isolates from L. Serogroup 3 and 1 isolate (2.1%) each belonged to serogroups 4, 7, and 10. In 2015, Al Matawah also performed a similar study of Kuwait's air conditioning cooling tower systems (Al-Matawah et al., 2015, 2012).

Moran-Gilad et al reported a clinical case of humidifier-associated pediatric LD in Israel. In an infant under the age of six months, they reported a fatal case of community-acquired LD. Their findings showed that epidemiological and microbiological research suggests a free-standing cold water humidifier using domestic faucet contaminated water with *L. pneumophila* sg1 was used as an infectious device. Their findings confirmed 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 a nosocomial infection. Also, four cases entered ICU. *L. pneumophila* was detected using urine antigen test, PCR, and

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

Al-Sulami et al investigated the *L. pneumophila* incident in Iraq. in various sources of drinking water in the Basra Governorate as well as isolate resistance to several antibiotics. In 2008–2009, total water samples 222 were collected: 49 samples from water purification plants, 127 Faucet water samples; and 46 reverse osmosis water supply samples from reservoirs and plants. The results supported the presence of *L. pneumophila* in sources of crude water, drinking water supplies and drinking water tankers in general. 77.1 % were serogroup 1 and 22.9 % were serogroup 2–14 out of 258 isolates. (Al-Sulami et al., 2013).

Ashraf Zayed and colleagues in 2013 aimed to use high-resolution molecular typing and serotyping methods for a better understanding of the geographic distribution and environmental reservoirs of this pathogen in the West Bank. Three-year proactive environmental surveillance of *L. pneumophila* in the water distribution system of eight hospitals was carried out. A collection of 226 environmental *L. pneumophila* isolates were characterized by serotypes and monoclonal antibodies (Sg-Mab). A subset of 180 isolates was analyzed using Multilocus Variable-number tandem repeat (MLVA). MLVA-8 (12) (Eight and twelve-locus-comprising) scheme was applied to the isolates using an optimized multiplex PCR followed by electrophoresis. *L.pnumophila* sg1 represented 61% of the isolates (Ashraf, 2013).

Szymańska and Sitkowska set out the determination of qualitative and quantitative water contamination with aerobic and possible anaerobic bacteria in the DUWL. Water samples obtained from 107 reservoirs of dental units housed in public health center dental surgeries. In order to identify microorganisms, they used traditional microbiological methods. The study showed that water contamination in the DUWL is normal with aerobic and facultative anaerobic bacteria. The mean concentration of mesophilic bacteria in the dental unit waterline exceeded 1.1×105 CFU/ml. The predominant species were family Gram-negative bacteria *Burkholderiaceae*, *Pseudomonadaceae*, *Ralstoniaceae*, and *Sphingomonadaceae*. Many bacteria were *Ralstonia pickettii*, constituting 49.33 % of all the identified aerobic and facultative anaerobic bacteria. The most numerous of the Gram-positive rods were genus bacteria *Brevibacterium* (5.83 %), in comparison, the highest

percentage shares (13.25 %) of all Gram-positive microorganisms were found to be *Actinomyces spp*. Their research has not studied *Legionella spp* existence. (Szymańska and Sitkowska, 2013).

Khaledi et al investigated the prevalence of *Legionella spp.* in water resources of Iran by a systematic review and meta-analysis of Iran's water resources, according to papers linked to the prevalence of *Legionella* in Iran's water resources by use of the collection of scientific information in both English and Persian. Each cohort and cross-sectional study that reported the contamination of water with *Legionella* was included in the study. In Iran's water resources, the prevalence of *Legionella spp* was 27.3%, in hospital water, 28.8%, in dental settings water, 23.6% other water resources. The most common *Legionella* species were *L. pneumophila* with a prevalence of 60.5%, and the prevalence of all other species was 52.5%. The highest prevalence was reported in Isfahan with 55.7% *L. pneumophila* (Khaledi et al., 2018).

Ditommaso et al designed to assess prevalence *L. pneumophila* in the dental waterline (DUWL) and tap water by using a molecular, propidium monoazide (PMA) qPCR, and standard culture methods. Also determined in the samples were the total viable counts (TVCs) of aerobic heterotrophic bacteria. Eighty-six water samples from 26 private dental clinics have been collected. The PMA-qPCR process identifies 100% of *Legionella spp* in the samples, whereas the culture method detects *Legionella* in just 7% of the samples. No published evidence of clusters or outbreaks of legionellosis linked to dental care, just one case of 82 elderly Italian women with LD following appointment to dental care, and a report of a fatal case of *Legionella* infection based pneumonia in a dentist in the USA. (Ditommaso et al., 2016).

Ghalyani et al assessed water contamination in dental clinics in Isfahan city. Water samples were collected from 50 private offices; Each scaler collected 10 mL of dental unit water and used a sample of city tap water as a control. They used a 3-step polymerase chain reaction (PCR) for the detection of *L. pneumophila*. The extracted DNA has been analyzed using spectrophotometry for the presence of the *mip* gene sequence. The control samples for any bacteria were negative. Thirty-two samples of the understudy bacteria were also negative, but 18 DCU positively screened for *L. pneumophila* The results show

that harmful bacteria can be contained in the biofilm dental network. (Ghalyani et al., 2015).

1.19 Water sources in Palestine

The Palestinian Water Authority (PWA) has provided us with maps of water sources of concern to this study, in the West Bank (WB), Palestine. (Appendix A). Al-Quds University (AQU) Faculty of Dentistry gets its water from Al-Ezareyah reservoir. Arab American University (AAUP), Faculty of Dentistry in Jenin gets its water from Qabatiya well which then goes to Al Zababdeh Municipality water network and then to the University. Nablus City, Rafidia area water is provided from Sabastyia well, Deir Sharaf well and Ein Beit Elma' spring which then goes to Beit Elma' reservoir then to Rafidia area. Center of Nablus city water is provided from Al-Qaryoun spring which then goes to Al-Jadeed reservoir then to the city center. Askar camp water is provided from Wadi Al Far'a well, Al-Bathan well, and Askar spring then to Al Masaken reservoir then to Askar camp. Balata camp gets its water from the Rujeeb well and Balata spring which then goes to the Nablus Municipality water network to Balata camp.

Tulkarem City, Nur Shams camp water is provided from Ra'fat Al Qubbaj well to Al Mahajar reservoir to Nur Shams camp. Tulkarem camp gets its water from the Dennabeh well, to the Tulkarem Municipality water network to Tulkarem. Center of Tulkarem city, water is provided from Shufa well, Kafr Zibad well and Shweikeh well to the Tulkarem Municipality water network to Tulkarem City Center.

Hebron City Center (Ras Aljora) water is provided from Sa'ir well to the Hebron Municipality water network to Hebron City Center.

Chapter Two

Methodology

2.1 Inclusion criteria

Water samples and biofilm swabs were collected from; Al-Quds University (AQU) Faculty of Dentistry clinics, in Abu Deis Jerusalem, from Arab American University in Jenin (AAUP), Faculty of Dentistry clinics and from dentists' clinics in different regions in the West Bank (WB); twelve in Nablus, five in Tulkarem and two in Hebron. Water and biofilm samples were analyzed for the presence of any bacteria, *Legionella spp*. and *L. pneumophila* in their water sources (Fig. 2.1). The samples were collected after written approval from the Deanships of the two Faculties of Dentistry, AQU, AAUP and written informed consent from all the dentists' clinics (Appendix B).

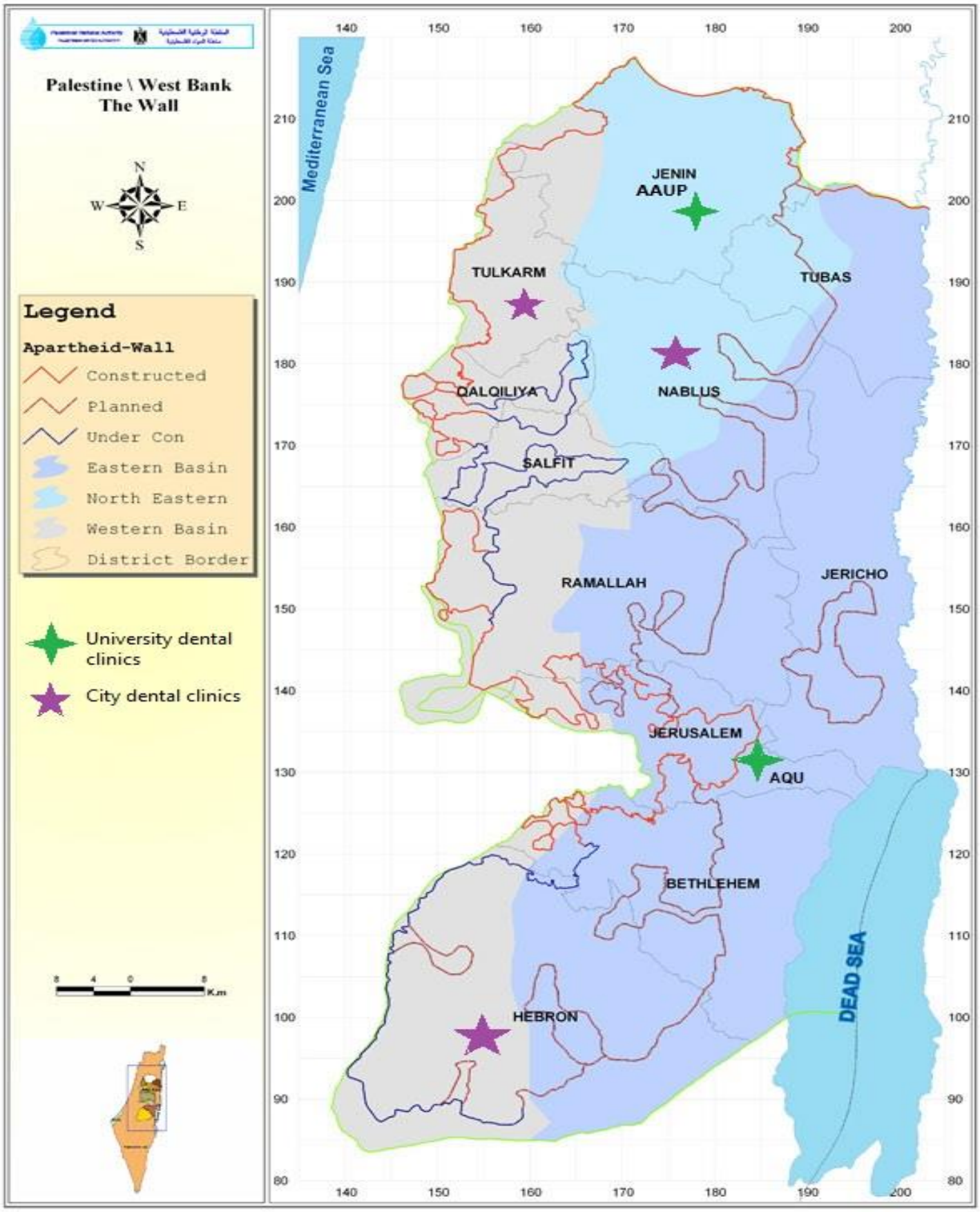


Figure 2.1: Sampling sites in the West Bank; Al-Quds University (AQU), Faculty of Dentistry in Abu Deis Jerusalem, Arab American University in Jenin (AAUP), Faculty of Dentistry, Dentists clinics in Nablus, Tulkarem, and Hebron. (Map adapted from Palestinian water authority PWA 2019).

Water samples were collected, processed and analyzed according to international standard operational procedures (SOPs). For water quality; detection and enumeration of *Legionella* ISO 11731 were used. For water quality, detection, and enumeration of *Legionella* part two (direct membrane filtration method for waters with low bacterial counts) ISO 11731-2 was used.

2.2 Research place

The research was carried out at the Microbiology Research Laboratory, AQU towers building B, Abu Dies, Jerusalem.

2.3 Work planner

Methodology work planner is summarized in (Fig. 2.2).

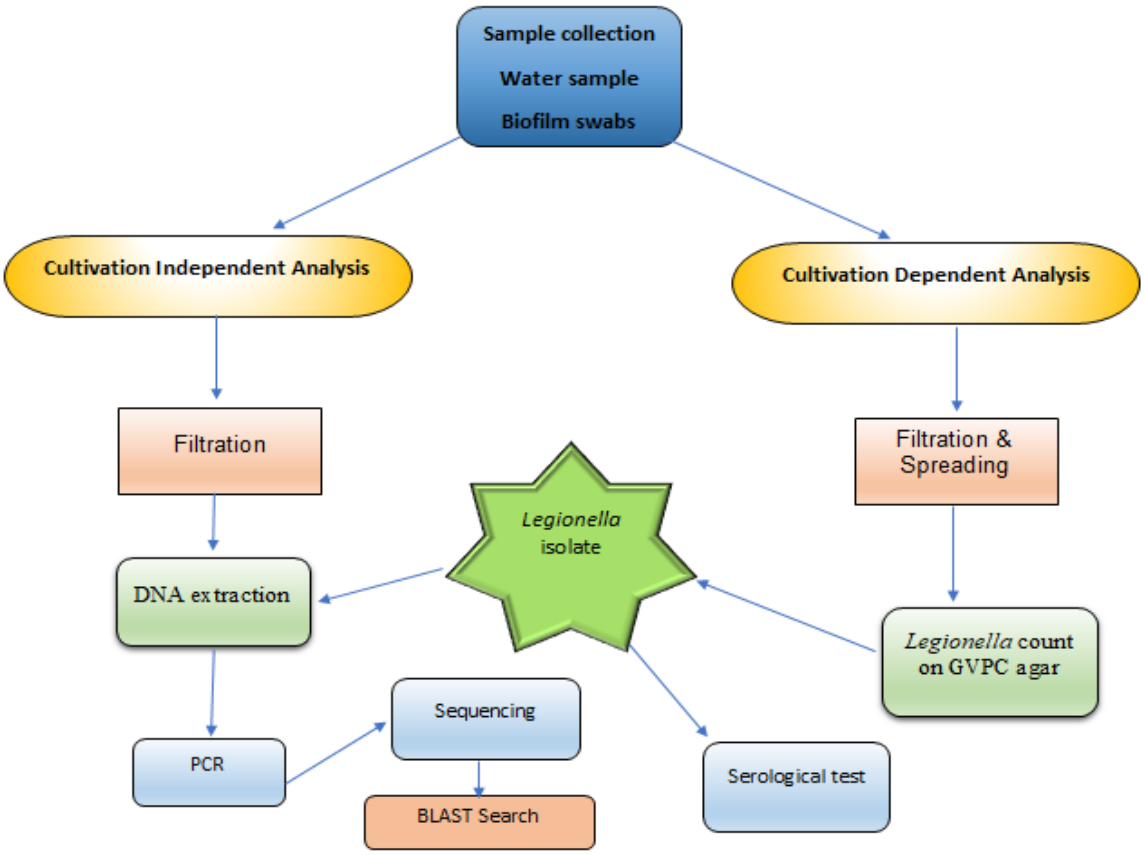


Figure 2.2: Work planner for the major analytical steps

2.4 Sampling

2.4.1 Water samples

From February through October 2018, a total of 89 water samples were collected. One liter of each Dental Unit Waterline (DUWL) and Tap water was collected for DNA extraction. Also, one liter of each DUWL and Tap water was collected for *Legionella* count from two Faculties of Dentistry clinics and from different cities in the WB namely; AQU Faculty of Dentistry clinics, AAUP, Faculty of Dentistry clinics and from dentists' clinics in different regions in the West Bank (WB); twelve in Nablus, five in Tulkarem and two in Hebron.

Tap water samples were collected in sterile 1L polyethylene bottles, after a brief flow time (2-3min), 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 0.1 M sodium thiosulphate ($Na_2S_2O_3.5H_2O$) (0.1 gm /100 ml) was added in the sterile bottles for *Legionella* count analysis (Centers for Disease Control and Prevention CDC, 2005).

From the DUWL, sampling was done by collecting and mixing about 200 ml of water (for a total of 1 liter) from each of the following:

1. Air-water syringe
2. Micro-engine
3. Turbine
4. Ablator
5. Cup filler

The water samples were kept at refrigeration temperature at 4 to 8ºC until analysis, culture and DNA extraction (Fabio et al.,2007). Samples were delivered to the Microbiology Research laboratory within one day (Diederen et al., 2007).

Temperature, pH, and conductivity were measured on-site for every water sample (EWGLI 2005 manual).

The test report included the 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 on the same day. (Appendix C).

2.4.2 Biofilm samples

From February 2018 through October 2018 a total of 96 biofilm swabs from anterior surfaces of faucets of DUWL and clinic taps 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; AQU Faculty of Dentistry clinics, from AAUP, Faculty of Dentistry clinics and from dentists' clinics in different regions in the West Bank (WB); twelve in Nablus, five in Tulkarem and two in Hebron. From AQU 22, from AAUP 16, from Nablus 24, from Tulkarem 16, and from Hebron 4 biofilm swabs. Samples were taken from each site randomly. Biofilm swabs for *Legionella* identification were processed in the laboratory by culturing on BCYE (CM0655, OXOID, UK) and/ GVPC (Glycine-Vancomycin-Polymyxin-Cycloheximide) (CM0655, OXOID, UK) medium immediately. The swabs for DNA extraction were kept at -80°C until DNA extraction was performed.

2.4.3 Measurement of physical and chemical background parameters

Tap and DUWL water samples were tested for temperature using an electronic thermometer (ama-digit, ad 15th, Germany), pH measurement and conductivity using PCE meter (PCE-PHD 1, Germany) on site. Upon arrival, to the Microbiology Research 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 Enumeration and isolation of *Legionella* was performed according to ISO11731-2.

2.5.1.1 Reagents and media:

2.5.1.1.1Acid buffer

Fresh acid buffer was prepared and stored in a sterile closed glass container in the dark at room temperature for not more than 1 month. Acid buffer was prepared by mixing 3.9 ml of 0.2mol/l HCl and 25ml of sterile 0.2mol/l KCl. The pH was adjusted to 2.2 ± 0.2 by adding a 1mol/l solution of potassium hydroxide (KOH).

2.5.1.1.2 Page´s saline

Page's saline was prepared by adding 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 dihydrogen phosphate (KH_2PO_4) to ten liters distilled water. Chemicals were allowed to dissolve completely, mixed well and then autoclaved at (121±3) °C for (15±1) min.

2.5.1.1.3 GVPC medium

It is a modified BCYE agar. GVPC (Glycine-Vancomycin-Polymyxin-Cycloheximide) medium was prepared by adding 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) to BCYE agar medium (CM0655, OXOID, UK) after being cooled to 50°C according to manufacturer's instruction.

2.5.1.2 Procedure:

2.5.1.2.1 Acid treatment

In triplicate, each water specimen (100 ml) was filtered onto membrane filter (pore size 0.22µm, diameter 47mm, MILLIPORE, Ireland) using a sterile filtration unit (Nalgene, Germany). The vacuum pump (LVP 500, South Korea) pressure was approximately

200mbar. For acid treatment, 30ml of acid buffer was added on top of the membrane filter and was left for 5min. The filter was then rinsed with 20ml Page´s saline. Page's saline is recommended for bacterial concentration including *Legionella* organisms by membrane filtration for *Legionella* detection and enumeration The membrane was then aseptically placed onto the agar plate. Triplicates of BCYE and/ or GVPC (CM0655, OXOID, UK) agar plates were used with a chemical enrichment supplement recommended for enhancing *Legionella* species growth. The 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. The final reading was done after ten days with a description of colonies.

At least four colonies are circular, glistening, entire, and measured up to 4 mm of *Legionella* were selected at random for each positive sample and subcultured onto BCYE and/or GVPC and blood agar (M089, Himedia, India) as a 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*. *Legionella* colonies were restreaked on BCYE and further identified by molecular analysis. All isolates were preserved at -80°C in 30% glycerol using cryotubes (TPP, USA).

2.6 Serological test

L. pneumophila colonies grown on BCYE and/or GVPC were then identified by an agglutination test using (*Legionella* Latex Test, Oxoid DR0800M, England) DR0801 *L. pneumophila* sg1, DR0802 *L. pneumophila* sgs 2-14, DR0803 *Legionella spp*, DR0804 positive control suspension, DR0805 negative control, DR0806 control latex, DR0807 suspension buffer , by the following method:

1. Latex reagent is allowed to warm to room temperature
2. One drop of each reagent is placed per well.
3. One drop of diluent buffer is added to the suspension to each of the 4 tests without mixing the reagents
4. Using the bacteriological loop one or two colonies are suspended in each test
5. The reagents and suspension are mixed together and spread with the loop

6. The mixture is gently shaken in a circular motion and read after 1 minute.

The test allows a separate identification of *L. pneumophila* sg 1, sgs 2–14 and *Legionella spp*.

2.7 Cultivation independent analysis

2.7.1 Water DNA extraction

One liter of each water sample was 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) with vacuum pump (KNF, N811 KN.18, Germany) pressure up to 300-400mbar. The filtration time was measured until filtration was finished. Filters from the DUWL and Tap 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 -80°C for longer storage.

For DNA extraction from the filter sandwiches, a modified DNeasy protocol (Qiagen 69506, Germany) was used. Sandwich filters were cut into pieces and incubated with 1.5 ml ELB 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. 37.5µl protein kinase K was added, vortexed and incubated at 56 °C for 30 min with 500 rpm shaking. 1350 µl AL buffer was added from the kit, the samples were incubated at 78°C for 20 min (with shaking at 500 rpm). After filtration through a polyamide mesh with a 250µm pore size, 800 µl ethanol (96-100%) was added and mixed by vortexing, and the mixture was applied onto the spin column of the kit. It was centrifuged at max speed for 2 min, the liquid was discarded and the filter was placed in new tubes. Then 500 µl of AW2 buffer was added and centrifuged at max speed for 2 minutes, the liquid was discarded, then the spin column was placed in a new labeled Eppendorf (2 ml collection tube) after waiting for 1 min, 25 µl of AE buffer was added then centrifuged at 6500x g, another 25 µl of AE buffer was added and re-centrifuge at 6500x g. The extracted DNA was stored at -80°C.

2.7.2 Biofilm swab DNA extraction

For DNA extraction from the biofilm swab, a modified DNeasy protocol (Qiagen 69506, Germany) was used. The biofilm swab was placed in 2ml Eppendorf tube and 200 µl ELB 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) was added. Then 20 µl protein kinase K was added, vortexed and incubated at 37°C for 20 min with shaking. 350 µl AL buffer from the kit was added, then the samples were incubated at 78°C (with shaking at 500 rpm) for 5 min. The Eppendorf tube is inverted upside down the swab, loading all the liquid into a new label Eppendorf and the tubes with the swabs were centrifuge at max speed for 2 min until the swabs are dry, the liquid was transferred into new labeled tubes. 200 µl ethanol (96-100%) was added and mixed by vortexing, the mixture was applied onto the spin column of the kit. Followed by Centrifugation at max speed for 2 min, the liquid was discarded. Then 500 µl of AW1 buffer was added and centrifuged at max speed for 2 min, the liquid was discarded. Then 500 µl of AW2 buffer was added and centrifuged at max speed for 2 minutes, the liquid was discarded, then the spin column was placed in a new labeled Eppendorf (2 ml collection tube) and after waiting for 1 min, 30 µl of AE buffer was added then centrifuged at 6500x g, and another 30 µl of AE buffer was added and re-centrifuged at 6500x g. The extracted DNA was stored at -80°C.

2.7.3 *Legionella* isolates DNA extraction

Nuclease free sterile water (E476, Amresco, Isreal) (100µl) was used to suspend two to three colonies of *Legionella* isolates, using a dry bath (DBS-001, MRC, Israel), the mixture was heated at 90°C for 10 minutes (Moore et al., 2004). Finally, DNA was stored at -80°C until used.

2.7.4 Bacterial isolates DNA extraction

E.coli, Pseudomonas spp, Staphylococcus spp, Streptococcus spp colonies (2 colonies) were inoculated in 100µl sterile pure Nuclease free water (E476, Amresco, Isreal). Using a dry bath (DBS-001, MRC, Israel), the mixture was heated at 90°C for 10 minutes (Moore et al., 2004). Finally, DNA was stored at -80°C until used.

2.8 16S rRNA PCR

2.8.1 Common primer

The first PCR using the extracted DNA was performed for the identification of bacteria in the samples. PCR common (COM) primers were purchased by (hy-labs, Park Tamar, 30853 Rehovot, Israel) to amplify a PCR product of 409bp for the identification of 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 a 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 Nuclease free water (E476, Amersco, Isreal), 1µl (10mM) forward primer (Com1F), 1µl (10mM) reverse primer (Com2R), and 3µl (100µg/ml) DNA template were added. PCR amplification was done using a thermal cycler (1861096, Biorad, USA): 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 such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus spp.* was used as positive controls. Nuclease free water (E476, Amresco, Isreal) was used as a negative. The products were evaluated according to size.

2.8.2 *Legionella* genus-specific primer

The second PCR was used for the identification of *Legionella* genus in the samples. PCR *Legionella* genus-specific (Lgsp) primers were purchased from (hy-labs, Park Tamar, 30853 Rehovot, Israel) to amplify the PCR product of 426bp. The primer sequences are shown in (Table 2.1). Their location on the complete genome of *L. pneumophila* subsp. pneumophila ATCC 43290 is shown in (Fig. 2.3). PCR was done using a 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 Nuclease free water (E476, Amersco, Isreal), 1µl (10mM) forward primer (Lgsp17F), 1µl

(10mM) reverse primer (Lgsp28R), and 3µl (100µg/ml) DNA template were added. PCR amplification was done on thermal cycler (1861096, Biorad, USA): 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 spp*, (*Legionella feeleii* code L2, DNA concentration 1045.7ng/µl) kindly provided by Prof. Manfred Hofle, HZI, Braunschweig, Germany, were used as positive controls. Nuclease free water (E476, Amersco, Isreal) was used as a negative control. The products were evaluated according to size.

2.8.3 *L. pneumophila* species primer

The third PCR was used for the identification of *Legionella pneumophila* species in the samples. PCR *L. pneumophila* species (L1) primers were purchased from (hy-labs, Park Tamar, 30853 Rehovot, Israel) to amplify the PCR product of 544bp. The primer sequences are shown in (Table 2.1). Their location on the complete genome of *L. pneumophila* subsp. pneumophila ATCC 43290 is shown in (Fig. 2.3). PCR was done using a 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 Nuclease free water (E476, Amersco, Isreal), 1µl (10mM) forward primer (L1F), 1µl (10mM) reverse primer (L1R), and 3µl (100µg/ml) DNA template were added. PCR amplification was done on thermal cycler (1861096, Biorad, USA): 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* was used as a positive control. Nuclease free water (E476, Amersco, Isreal) was used as a negative control. The products were evaluated according to size.

Table 2.1: Primers used in this 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′	

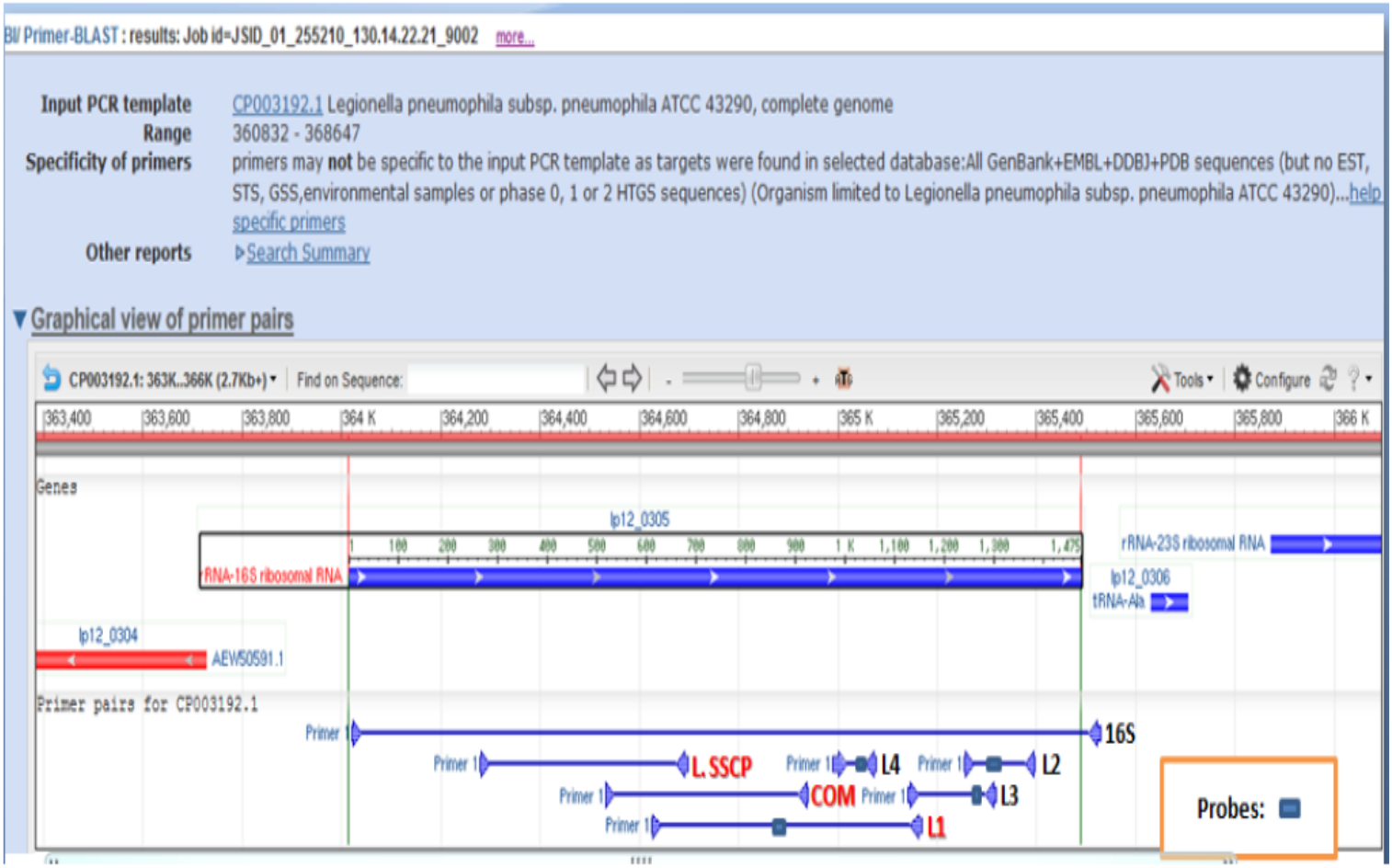


Figure 2.3: *L. pneumophila* whole-genome and primers location (adapted from Ashraf Zayed thesis, 2013).

2.9 Agarose gel preparation and electrophoresis

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

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

2.10 Sequencing of the 16S rRNA gene and BLAST search

Seven *Legionella* isolates were sent to Alisteshari Hospital, Ramallah for partial sequencing of the 16S rRNA gene for quality assurance and identification. In Alisteshari Hospital, sequencing for a PCR product is performed through Gel Electrophoresis, EXO-SAP, Big-Dye Sequencing, Xterminator cleaning, and sequencing on the ABI3500 genetic analyzer. Basic Local Alignment Search Tool (BLAST) https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome was used to compare for similarity to sequences in GeneBank.

2.11 Controls

2.11.1 Medium control

Blood agar (M089, Himedia, India) was used as a negative control since *Legionella* fails to grow on blood agar (L-cysteine free).

2.11.2 *Legionella* DNA control

DNA of *L. pneumophila* was used as a positive control, and *Legionella spp*, (*Legionella feeleii* code L2, DNA concentration 1045.7ng/µl) was kindly provided by Prof. Manfred Hofle, HZI, Braunschweig, Germany, was used as DNA control in this study.

2.12 Statistical analysis, figures drawing and computer software

Excel (Microsoft Office, 2019) was used for statistical analysis. The photo filter software program (Photo filter 6.5.3) was used for figure drawing. BLAST was used to analyze the results of sequencing. Sequences retrieved from isolates were deposited in the GenBank.

Chapter Three

Results

3.1 Sample study

A total of 89 Tap and DUWL water samples and 96 Tap and DUWL biofilm swabs were collected from AQU, Faculty of Dentistry clinics and AAUP, Faculty of Dentistry clinics and from dental clinics in 3 different cities in the WB namely; twelve clinics from Nablus, seven from Tulkarem and two from Hebron (Table 3.1).

Table 3.1: Water samples (Tap and DUWL) and biofilm swabs (Tap and DUWL) from sampling areas.

Sample area	No of collected samples (Dental clinics)	Tap water	DUWL	Tap biofilm	DUWL biofilm
Nablus City	12	11	11	12	12
Tulkarem City	7	9	8	7	7
Hebron City	2	2	2	2	2
AQU Faculty of Dentistry	14	9	13	8	14
AAUP Faculty of Dentistry	16	8	16	16	16
Total	51	39	50	45	51

3.2 Measurement of Tap water and DUWL physical and chemical parameters

The pH, temperature, and conductivity of the water samples were tested at the site of collection by PCE meter. Upon arrival to the Microbiology Research 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 by using quantolfix sticks.

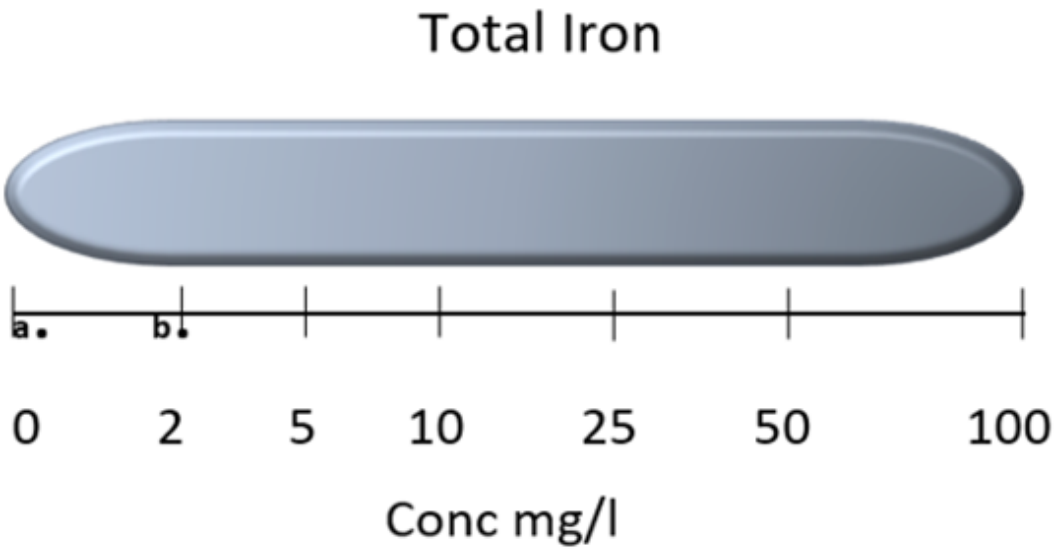


Figure 3.1: Concentration (mg/l) of total iron (Fe^{+3}/Fe^{+2}) in DUWL and tap water samples

a: Z.A: Zaid Atabeh, U.Z: Usama Zaloom, A.SH: Alaa Alshekh, QU: Al-Quds University Faculty of Dentistry (QUF1: Dental surgery first floor, QUF2: Periodontics second floor, QUF4: Pediatric fourth floor, G10: Clinics in Arab American University Faculty of Dentistry.

b: F.D: Fadi Daghlas, S.H: Saed Habash, A.H: Adib Halob, M. AB: Moath Abubaker, M.S: Monia Sabbah, N.H.C: Nurshams Heath Center, T.H.C: Tulkarem Heath Center, G12, G2: Clinics in Arab American University

WHO: up to 2 mg/l

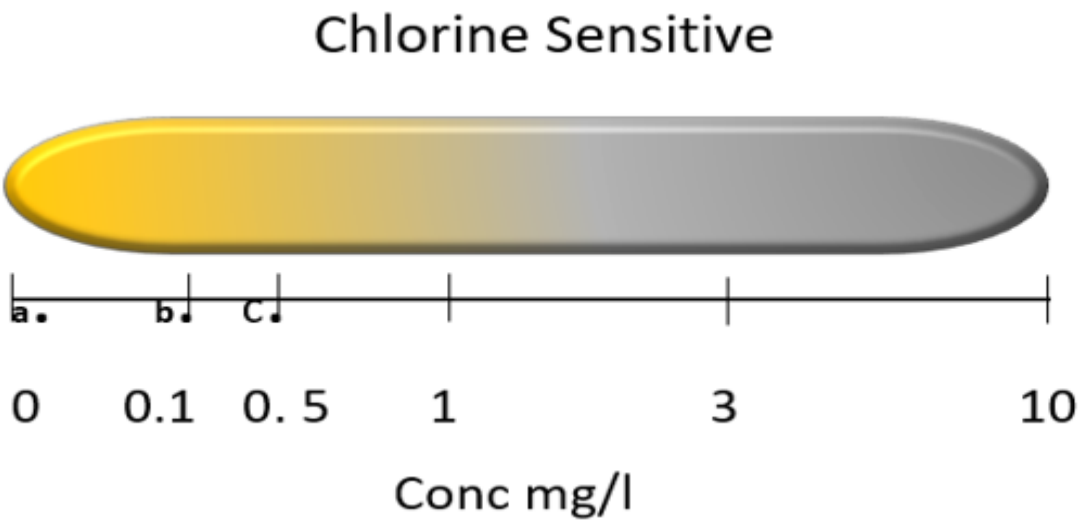


Figure 3.2: Concentration (mg/l) of chlorine sensitive (Cl_2) in DUWL and Tap water samples.

a: T.N: Tayseer Nana, M.T: Mohammed Tammam, U.Z: Usama Zaloom, QUF1, QUF2, QUF4: Clinics in AQU, G14, G13, G12, G11, G10, G9: Clinics in AAUP.

b: Z.A: Zaid Atabeh F.D: Fadi Daghlas, S.H: Saed Habash, A.H: Adib Halob, G2: Clinics in AAUP, N.H.C: Nurshams Heath Center, T.H.C: Tulkarem Heath Center.

c: M. AB: Moath Abubaker, M.S: Monia Sabbah, A.SH: Alaa Alshekh.

WHO: 0.5 - 5 mg/l

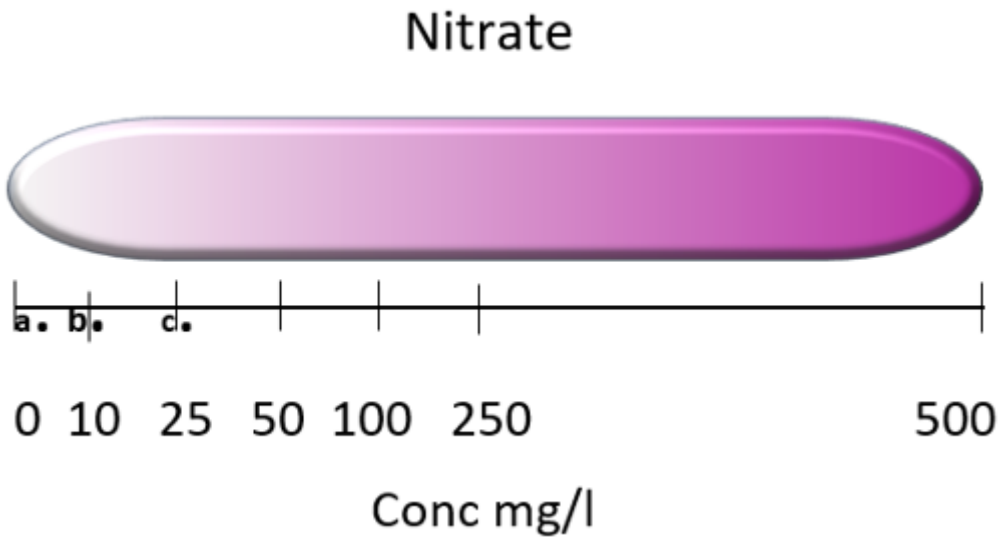


Figure 3.3: Concentration (mg/l) of Nitrate (NO_3^-) in DUWL and tap water samples.

a: QUF1, QUF2, QUF3: Neurosurgery third floor, QUF4: Clinics in AQU, G14, G13, G12, G11, G10, G1, G2: Clinics in AAUP, T.H.C: Tulkarem Heath Center, N.H.C: Nurshams Heath Center,

b: Z.A: Zaid Atabeh, F.D: Fadi Daghlas, U.Z: Usama Zaloom, A.SH: Alaa Alshekh.

c: A.H: Adib Halob, M. AB: Moath Abubaker, M.S: Monia Sabbah, M.H: Mohammed Hafi.

WHO: up to 50 mg/l

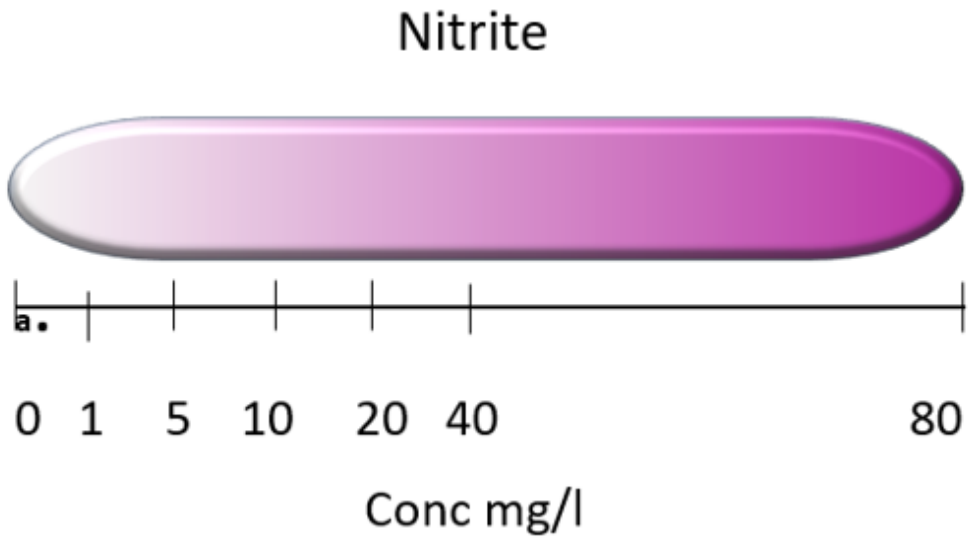


Figure 3.4: Concentration (mg/l) of Nitrite (NO_2^-) in DUWL and tap water samples.

a: QUF1, QUF2, QUF3, QUF4: Clinics in AQU, G14, G13, G12, G11: Clinics in AAUP, T.H.C: Tulkarem Heath Center, Z.A: Zaid Atabeh, F.D: Fadi Daghlas, U.Z: Usama Zaloom, A.SH: Alaa Alshekh, A.H: Adib Halob, M. AB: Moath Abubaker, M.S: Monia Sabbah,
M.H: Mohammed Hafi.

WHO: up to 3 mg/l

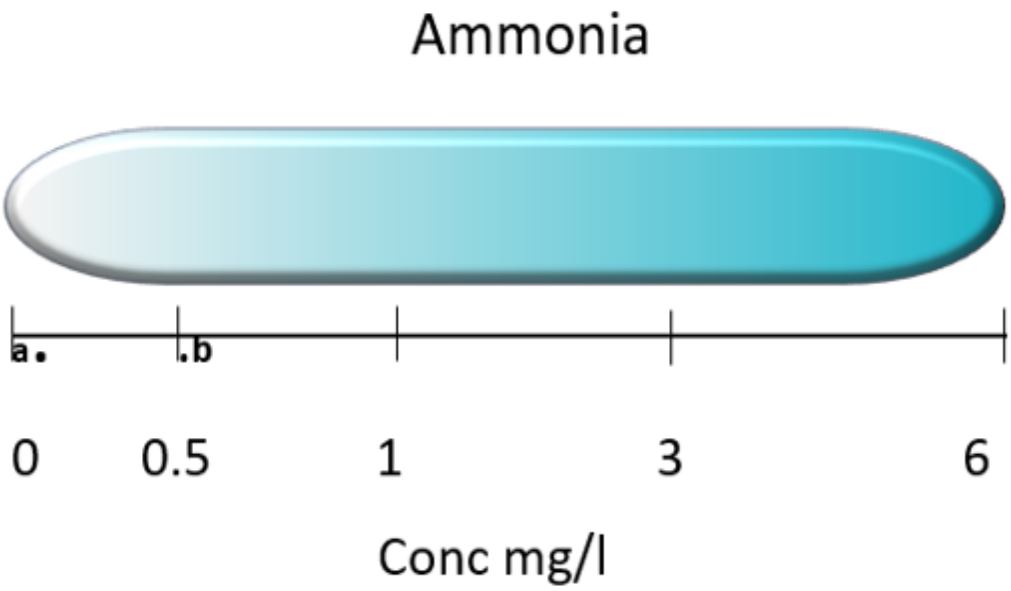


Figure 3.5: Concentration (mg/l) of ammonia (NH_3) in DUWL and tap water samples.

a: QUF1, QUF2, QUF4: Clinics in AQU, G14, G13, G12, G11, G1, G2: Clinics in AAUP, T.H.C: Tulkarem Heath Center, Z.A: Zaid Atabeh, U.Z: Usama Zaloom, A.SH: Alaa Alshekh, Fadi Daghlas, S.H: Saed Habash, A.H: Adib Halob, M. AB: Moath Abubaker, M.S: Monia Sabbah

b: QUF3: Clinics in AQU, G9: Clinics in AAUP, M.T: Mohammed Tammam.

WHO: 1.5 mg/l

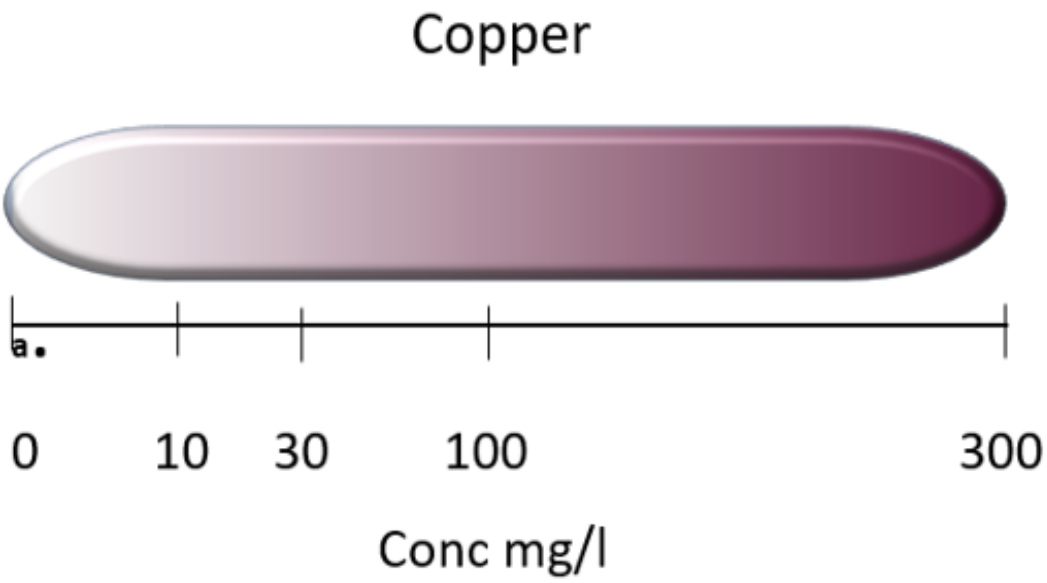


Figure 3.6: Concentration (mg/l) of copper ($Cu^{+/+2}$) in DUWL and tap water samples.

a: QUF1, QUF2, QUF3, QUF4: Clinics in AQU, G14, G13, G12, G11, G10: Clinics in AAUP, T.H.C: Tulkarem Heath Center, Z.A: Zaid Atabeh, F.D: Fadi Daghlas, T.N: Tayseer Nana, U.Z: Usama Zaloom, A.SH: Alaa Alshekh, A.H: Adib Halob, M. AB: Moath Abubaker, M.S: Monia Sabbah.

WHO: 2 mg/l

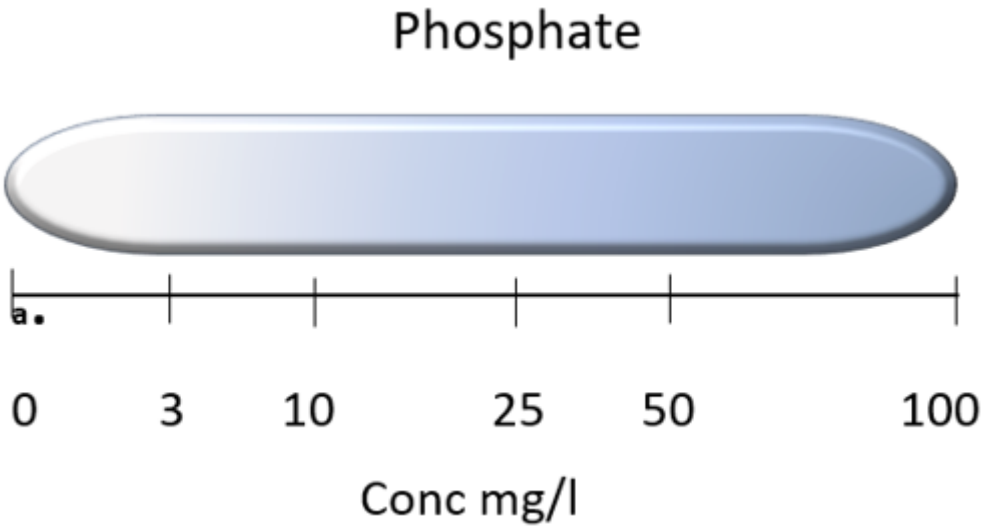


Figure 3.7: Concentration (mg/l) of phosphate (PO_4^{3-}) in DUWL and tap water samples.

a: QUF1, QUF2, QUF3, QUF4: Clinics in AQU, G14, G13, G12, G11, G10: Clinics in AAUP, N.H.C: Nurshams Heath Center, Z.A: Zaid Atabeh, S.H: Saed Habsh, T.N: Tayseer Nana, U.Z: Usama Zaloom, AB.SH: Abdlkarem Abusherekh A.SH: Alaa Alshekh, A.H: Adib Halob, M. AB: Moath Abubaker, M.S: Monia Sabbah.

WHO: 0 mg/l

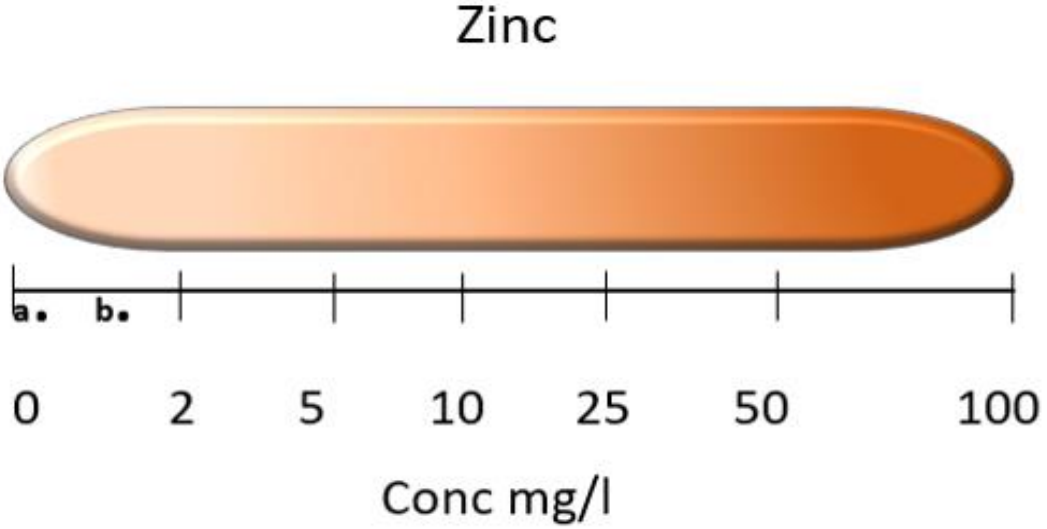


Figure 3.8: Concentration (mg/l) of zinc (Zn) in DUWL and tap water samples.

a: QUF1, QUF2, QUF3, QUF4: Clinics in AQU, G14, G13, G12, G11, G10: Clinics in AAUP, N.H.C: Nurshams Heath Center, T.H.C: Tulkarem Heath Center.
b: Z.A: Zaid Atabeh, S.H: Saed Habsh, T.N: Tayseer Nana, U.Z: Usama Zaloom, AB.SH: Abdlkarem Abusherekh, A.W: Arafat Alwazani.

WHO: 0 mg/l

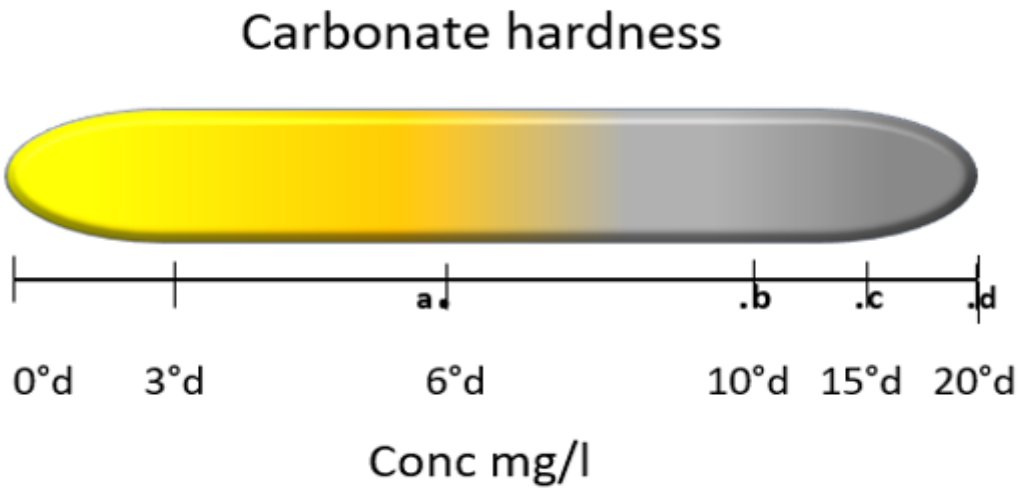


Figure 3.9: The amount of Carbonate hardness (CO_3^{2-}) and (HCO_3^-) in DUWL and tap water samples.

a: QUF1, QUF3: Clinics in AQU, G13: Clinics in AAUP, T. N: Tayseer Nana, A. Sar: Amera Sartawi, AB.SH: Abdlkarem Abusherekh,
b: QUF2: Clinics in AQU, G12, G10, G1, G2: Clinics in AAUP, M.H: Mohammed Tammam, M.SH: Mosab Shorishi, F.D: Fadi Daghlas, A.W: Arafat Alwazani,
U.Z: Usama Zaloom.
c: QUF4: Clinics in AQU, G14: Clinics in AAUP, A.H: Adib Halob, M. AB: Moath Abubaker, A.SH: Alaa Alshekh, M.S: Monia Sabbah, M.H: Mohammed Hafi
d: G9, G11: Clinics in AAUP, N.H.C: Nurshams Heath Center, T.H.C: Tulkarem Heath Center, Z.A: Zaid Atabeh, S.H: Saed Habsh.

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

Upon testing of the chemical properties of a random sample from the total water samples, no nitrite, copper, or phosphate was detected in the water samples. The levels of iron, chlorine, ammonia, and zinc detected were within the acceptable range according to WHO guidelines (World Health Organization WHO, 2011). Carbonate hardness was detected ranging from 5°d to 20°d, whereas carbonate hardness in 12 dental clinics (Z.A, S.H, G11, T.H.C, N.H.C, A.Halo, A.SH, M.AB, M.Sab, M.Haf, QUF4, G14) was above WHO levels. However, carbonate hardness in the remaining dental clinics was less than 11.2°d. Total hardness ranged from 15°d to 20°d, which is above the WHO acceptable range.

The temperature of Tap and DUWL varied between 11.7 °C and 25 °C. The conductivity ranged from 59 microsiemens (µS) to 1078 µS. The pH varied from 7.9 to 9.2. Actual data regarding the dental clinics' (Tap and DUWL) water physical and chemical parameters are summarized in (Table 3.2). All the data on the dental clinics' (Tap and DUWL) water physical and chemical parameters are shown in (Appendix F).

Table 3.2: Tap and DUWL water physical and chemical parameters.

Parameter	Dental clinic (Tap + DUWL) Water physical and chemical parameters.										
	Nablus		Tulkarem		Hebron		AQU		AAUJ		WHO
	Z. A		A. Halo		U. Z						
	Tap	DUWL	Tap	DUWL	Tap	DUWL	Tap	DUWL	Tap	DUWL	
Temperature °C	21±0.5	22±0.5	24±0.6	24±0.6	18±1	20±1	17±08	18±0.8	10±0.3	11±0.3	13±2.3
pH	7.9	8.8	8.8	8.7	8.6	8.7	9.1	9.2	8.5	8.6	6.5-9.5
Conductivity µS/cm	683	650	819	984	534	544	508	546	1078	998	400 at 20 °C
Total iron (Fe^{3+}/ Fe^{2+}) mg/l	0	0	2	2	0	0	0	0	0	0	Up to 2
Chlorine sensitive (Cl_2) mg/l	0.1	0	0.5	0.5	0	0	0	0	0	0	0.5-5
Nitrate (NO_2^-) mg/l	10	10	25	25	10	10	0	0	0	0	50
Nitrite (NO_3^-) mg/l	0	0	0	0	0	0	0	0	0	0	3
Ammonia (NH_3) mg/l	0	0	0	0	0	0	0	0	0	0	1.5
Copper ($Cu^{+/2+}$) mg/l	0	0	0	0	0	0	0	0	0	0	2
Phosphate (PO_4^{3-}) mg/l	0	0	0	0	0	0	0	0	0	0	0
Zinc (Zn) mg/l	<2	<2	0	0	<2	<2	0	0	0	0	0
Carbonate hardness *d°	20 d	20 d	15 d	20 d	10 d	10 d	5 d	5 d	15d	15 d	11.2
Total hardness ¥d°	10 d	20 d	20 d	20 d	20 d	20 d	15d	15 d	20 d	20 d	5.6

*Carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-)
¥Calcium and magnesium salts
°d =17.8 mg/L

3.3 Cultivation dependent analysis

3.3.1 Identification and quantification of *L. pneumophila* from Tap and DUWL

A total of 89 Tap and DUWL water samples and 96 biofilm swabs were collected from two Faculties of Dentistry clinics and private clinics in 3 different cities in the West Bank (WB) namely; AQU Faculty of Dentistry, AAUP Faculty of Dentistry in Jenin and from dental clinics in three different regions in the WB; twelve from Nablus, five from Tulkarem and two from Hebron for cultivation dependent analysis. *L. pneumophila* was isolated from 7 out of 39 (18%) Tap water samples and from 6 out of 50 (12%) DUWL. Also, 9 out of 45 (20%) Tap biofilm swabs yielded positive culture for *L. pneumophila* and 6 out of 51 (12%) DUWL biofilm swabs (Table 3.8). Data are shown in (Appendix D).

3.3.1.1 Water samples *Legionella* count

A total of 89 water samples (Tap and DUWL) were collected for the identification and quantification of *L. pneumophila*. Thirteen samples were positive by the culture method (CDA). On the filter used for isolation of *Legionella spp*, gray-white colonies with ground-glass appearance were noted. The number of colonies on triplicate plates was counted to determine the colony-forming units (CFU)/L. These colonies were sub-cultured on GVPC to confirm the identification as *Legionella* bacteria (Fig. 3.10) and for further testing of the *Legionella* isolates in order to determine the *L. pneumophila* serogroup by latex agglutination.

The mean number of *Legionella* count varied between 27 and 177 CFU/L. The actual data of the dental clinic DUWL *Legionella* count are summarized in (Table 3.3).

Table 3.3: Dental Clinic water (Tap and DUWL) *Legionella* count (CFU/L).

Dental Clinic	Type of Water sample	Mean ± SEM of *Legionella* CFU/L
QUF1 Surgical dentistry	**Tap Water**	**43 ± 3**
QUF2 Periodontology	**Tap Water**	**37 ± 7**
QUF3 Neurosurgery	**Tap Water**	**60 ± 6**
	DUWL	**57 ± 12**
QUF4 Pediatric dentistry	**DUWL**	**50 ± 12**
G9	**Tap Water**	**57 ± 12**
	DUWL	**50 ± 6**
G10	**DUWL**	**55 ± 6**
T.H.C	**Tap Water**	**177 ± 12**
	DUWL	**40 ± 6**
	DUWL	**43 ± 3**
A. Haw	**Tap Water**	**27 ± 9**
N.H.C	**Tap Water**	**53 ± 9**

SEM: Standard error of the mean from triplicate plates.

Thirteen water (Tap and DUWL) samples out of 89 total water samples yielded a positive culture of *L. pneumophila* as shown in Table 3.4. Percentages of positive water samples (Tap and DUWL) for *L. pneumophila* by culture in this study, shown in (Table 3.5). Actual data are shown in (Appendix D).

Table 3.4: Percentage of *L. pneumophila* isolates from water (Tap and DUWL) as determined by culture

Sample area	Tap water	% of positive Tap water	DUWL	% of positive DUWL	Total No. of Water samples	% of positive water
Nablus	**11**	**1 (9%)**	**11**	**0%**	**22**	**1 (5%)**
Tulkarem	**9**	**2 (22%)**	**8**	**2 (25%)**	**17**	**4 (24%)**
Hebron	**2**	**0%**	**2**	**0%**	**4**	**0**
AQU	**9**	**3 (33%)**	**13**	**2 (15%)**	**22**	**5 (23%)**
AAUP	**8**	**1 (13%)**	**16**	**2 (13%)**	**24**	**3 (25%)**
Total	**39**	**7 (18%)**	**50**	**6 (12%)**	**89**	**13 (15 %)**

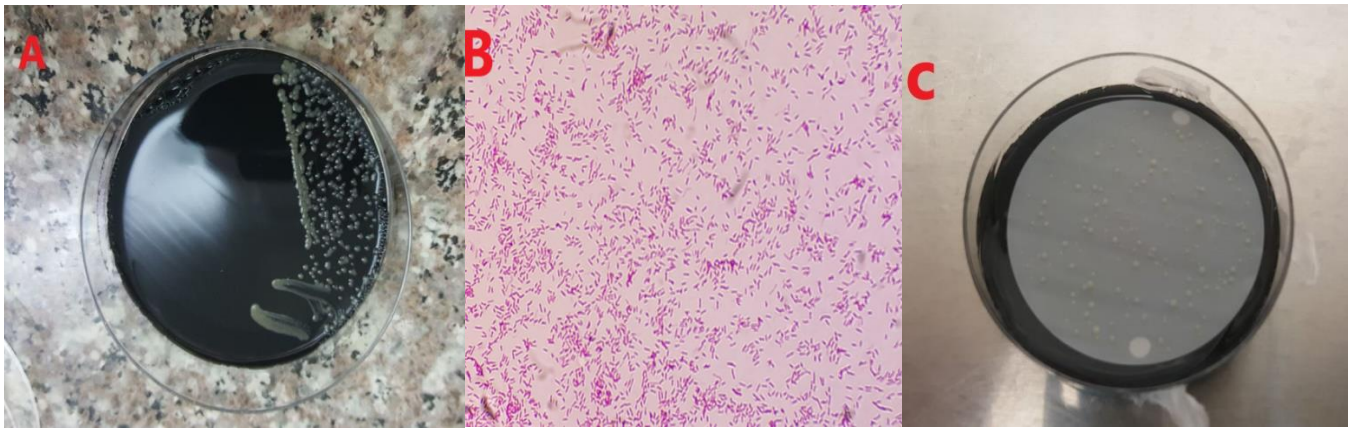


Figure 3.10: *L. pneumophila*. A*: L. pneumophila* isolate culture on GVPC medium

B: Gram stain of *L. pneumophila* isolates (1000x magnification)

C: *L. pneumophila* isolates on Nucleopore filter on GVPC medium

3.3.1.2 Biofilm Swabs

A total of 96 biofilm swabs were collected for the identification of *L. pneumophila*. Results of cultivation dependent analysis (CDA) of biofilm swabs for *L. pneumophila* are summarized in (Table 3.5). Fifteen out of 96 total biofilms (Tap and DUWL) swabs

yielded a positive culture of *L. pneumophila* as shown in Table 3.5. Actual data are shown in (Appendix D).

Table 3.5: *L. pneumophila* isolated from biofilm swabs (Tap and DUWL) as determined by culture

Cultivation Dependent Analysis of Biofilm Swabs (Tap and DUWL)						
Sample area	**No. of collected Tap biofilm**	**No. (%) of positive Tap biofilm**	**No. of collected DUWL biofilm**	**No. (%) of positive DUWL biofilm**	**Total No. of collected biofilm**	**Total No. (%) of positive biofilm**
Nablus	**12**	**3 (25%)**	**12**	**1 (8%)**	**24**	**4 (17%)**
Tulkarem	**7**	**2 (29%)**	**7**	**1 (14%)**	**14**	**3 (21%)**
Hebron	**2**	**0**	**2**	**0**	**4**	**0**
AQU	**8**	**1 (13%)**	**14**	**1 (7%)**	**22**	**2 (9%)**
AAUP	**16**	**3 (19%)**	**16**	**3 (19%)**	**32**	**6 (19%)**
Total	**45**	**9 (20%)**	**51**	**6 (12%)**	**96**	**15 (16 %)**

3.4 Distribution of *L. pneumophila* according to serogroups in dental clinics

As shown in (Fig. 3.11) the majority of cultured *L. pneumophila* isolates belonged to serogroup 1; 23 isolates belonged to sg1 (82 %) while 5 isolates belonged to *L. pneumophila* sg 2-14 (18 %).

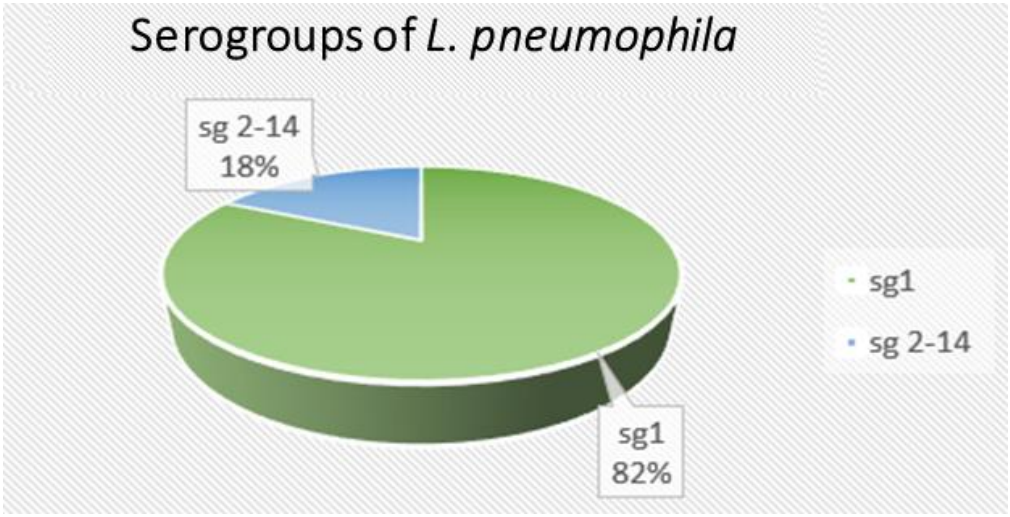


Figure 3.11: Percentages of *L. pneumophila* according to serogroups

Table 3.6: Distribution of *L. pneumophila* isolates according to serological groups.

***L. pneumophila* isolates**			
Site Name	**No. of Isolates**	**No. % of Sg1**	**No. % of Sg 2-14**
Nablus	**5**	**4 (80%)**	**1 (20%)**
Tulkarem	**7**	**6 (86%)**	**1 (14%)**
Hebron	**0**	**0**	**0**
AQU	**7**	**6 (86%)**	**1 (14%)**
AAUP	**9**	**7 (78%)**	**2 (22%)**
Total	**28**	**23 (82 %)**	**5 (18%)**

As shown in (Table 3.6) out of 5 isolates from Nablus, 4 belonged to sg1 and 1 belonged to sg 2-14. Out of 7 isolates from Tulkarem, 6 belonged to sg1 and 1 belonged to sg 2-14. No isolate was obtained from Hebron by culture-dependent analysis. Out of 7 isolates from AQU Faculty of dentistry clinics, 6 belonged to sg1and 1 belonged to sg 2-14. Out of 9 isolates from AUPF dental clinics, 7 belonged to sg1 and 2 belonged to sg 2-14.

3.5 Cultivation independent analysis

3.5.1 16S rRNA PCR

For cultivation-independent analysis DNA was extracted from 89 water samples (Tap and DUWL) and 96 biofilm swabs (Tap and DUWL). The extracted DNA from each sample was screened using 16S rRNA PCR for the presence of bacteria by 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 any bacteria using Com primer

The DNA extracted from water (Tap and DUWL) samples, 87/89 (98%) and from 96/96 (100%) biofilm swabs (Tap and DUWL) gave a PCR product of 409bp using the Com primer (Fig. 3.12) and (Fig. 3.13) indicating the presence of bacteria.

A representative gel is shown in (Fig. 3.12) The Com primer PCR product is a 409bp. Lanes 2-8 show water samples (Tap and DUWL) where lanes 2, 3, 4, 5, 6,7.8 have a

positive band equivalent to 409bp. Lanes 12-18 show water samples, where all lanes have a positive band, indicating the presence of bacteria

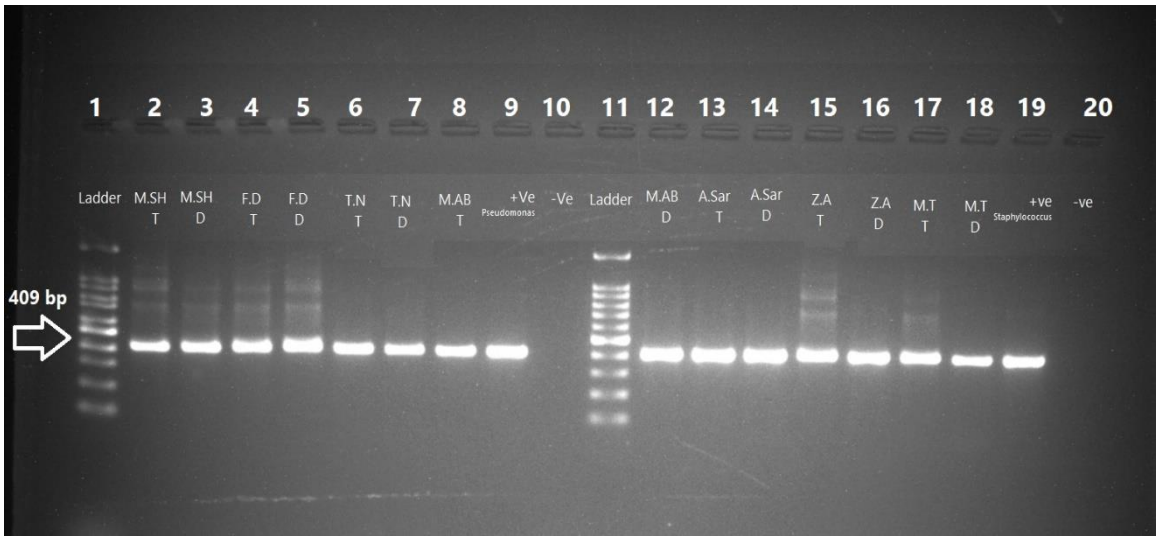
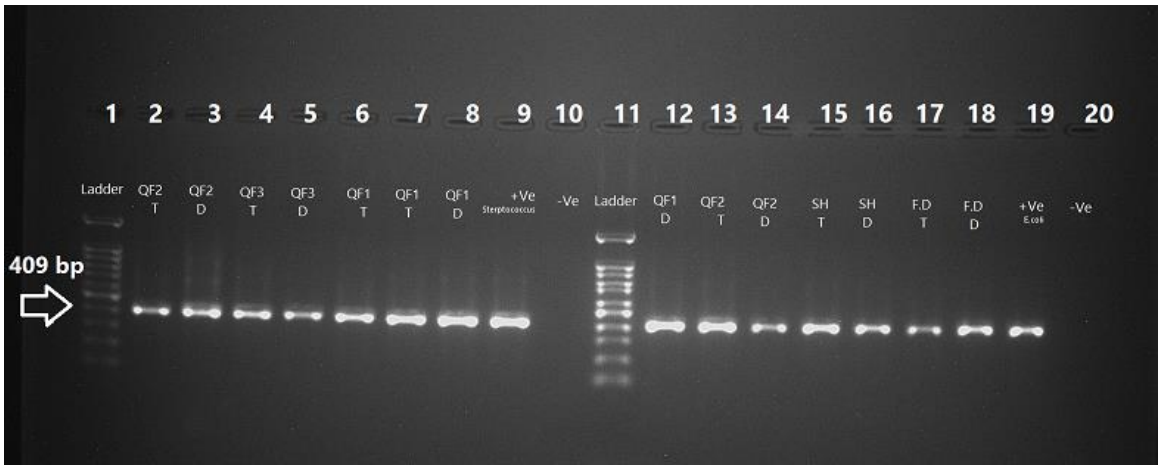


Figure 3.12: Representative 16S rRNA PCR using the Com primer of DNA from the Dental clinics. (T: Tap, D: DUWL). Lane 1 represents a 100bp ladder. Lanes 2-8 show water samples (Tap and DUWL) positive with 409bp. Lane 9, positive control (*P. aeruginosa*). Lane 10, negative control (nuclease-free water). Lane 11 represents a 100bp ladder. Lanes 12-18 water samples (Tap and DUWL), positive with 409bp. Lane 19, positive control (*Staphylococcus*). Lane 20, negative control (nuclease-free water). M.SH/T: Mosaab Jorishi Tap water, M.SH/D: Mosab Jorishi DUWL, F.D/T: Fadi Daghlas Tap water, F.D/D: Fadi Daghlas DUWL, T.N/T: Tayser Nana tap water, T.N/D: Tayser Nana DUWL, M.AB/T: Moaath Abu baker Tap water, M.AB/D: Moaath Abu baker DUWL, A.Sar/T: Ameera Sartawi Tap water, A.Sar/D: Ameera Sartawi DUWL, Z.A/T: Zaid Atabeh tap water, Z.A/D: Zaid Atabeh DUWL, M.T/T: Mohammed Tammam Tap water, and M.T/D: Mohammed Tammam DUWL. Complete data are shown in (Appendix E).



(Figure 3.13): Representative 16S rRNA PCR using the Com primer DNA from the Dental clinics and from AQU Faculty of Dentistry clinics, (T: Tap, D: DUWL). Lane 1 represents a 100bp ladder. Lanes 2-8 show biofilm (Tap and DUWL) positive with 409bp. Lane 9, positive control (*Streptococcus*). Lane 10, negative control (nuclease-free water). Lane 11 represents a 100bp ladder. Lane 12-18 biofilm (Tap and DUWL), positive with 409bp. Lane 19, positive control (*E. coli*). Lane 20, negative control (nuclease-free water). QF2/T: AQU Faculty of Dentistry Department of Periodontics second floor (Tap biofilm), QF2/D: (DUWL biofilm), QF3/T: AQU Faculty of Dentistry Department of neurosurgery third floor (Tap biofilm), QF3/D: (DUWL biofilm), QF1/T: AQU Faculty of Dentistry Department of Dental surgery first floor (Tap biofilm), QF1/D: (DUWL biofilm), S.H/Saeed Habash Tap biofilm, S.H/D: Saeed Habash (DUWL biofilm), F.D/T: Fadi Daghlas (Tap biofilm), F.D/D: Fadi Daghlas (DUWL biofilm). Complete data are shown in (Appendix E).

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

The extracted DNA of a total of 87 water samples (Tap and DUWL) and 96 biofilm swabs which gave a positive result by the common (Com) primer, were screened for the presence of *Legionella spp*. by using Lgsp primer 16S rRNA PCR

Positive results were observed in 62/87 (71%) of Dental clinics water (Tap and DUWL) and in 85/96 (89%) of dental clinics biofilm swabs (Tap and DUWL). The Lgsp primer gives PCR a product 426bp (Fig. 3.14) and (Fig. 3.15). The biofilm swabs gave a higher yield of positive samples for *Legionella spp.* than the water samples, 89% versus 71%.

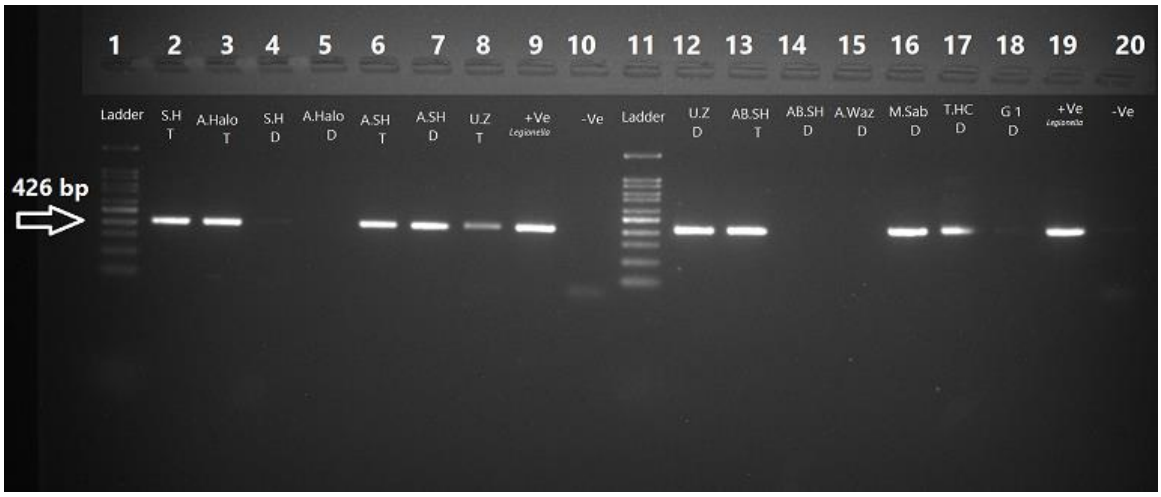


Figure 3.14: Representative 16S rRNA PCR using Lgsp primer of DNA from the Dental clinics (T: Tap, D: DUWL). Lane 1 represents the 100bp ladder. Lanes 2-8 show water samples (Tap and DUWL), positive with 426 bp and negative results. Lane 9 positive control (*Legionella feeleii*). Lane 10, negative control (nuclease-free water). Lane 11 represents a 100bp ladder. Lanes 12 to 18 represent water samples (Tap and DUWL samples), positive with 426bp and negative results. Lane 19, positive control (*Legionella pneumophila*). Lane 20, negative control (nuclease-free water).S.H/T: Saeed Habash Tap water, A.Halo/T: Adib Halob Tap water, S.H/D: Saeed Habash DUWL, A.Halo/D: Adib Halob DUWL, A.SH/T: Alaa Sheikh Tap water, A.SH/D: Alaa Sheikh DUWL, U.Z/T: Usama Zallom Tap water, U.Z/Usama Zallom DUWL, AB.SH/T: Abd AlKareem Abu Sharekh Tap water, AB.SH/D: Abd AlKareem Abu Sharekh DUWL, A.W/D: Arafat Alwazani DUWL, M.Sab/D: Monia Sabbah DUWL, T.H.C/D: Tulkarem UNRWA Health Center DUWL, G1/D: G Clinic in AAUP DUWL. Complete data are shown in (Appendix E).

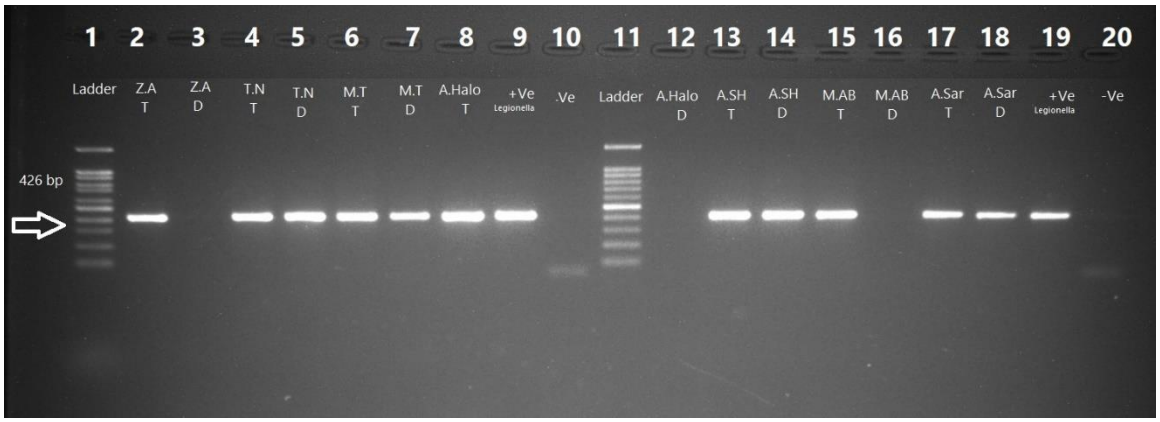


Figure 3.15: Representative 16S rRNA PCR using Lgsp primer of DNA from the Dental clinics (T: Tap, D: DUWL). Lane 1 represents a 100bp ladder. Lanes 2-8 show biofilm swabs (Tap and DUWL), positive with 426 bp and negative results. Lane 9, positive control (*Legionella feeleii*). Lane 10, negative control (nuclease-free water). Lane 11 represents a 100bp ladder. Lanes 12 to 18 represent biofilm swabs (Tap and DUWL samples), positive with 426bp and negative results. Lane 19, positive control (*L. pneumophila*). Lane 20, negative control (nuclease-free water). Z.A/T: Zaid Atabeh Tap biofilm, Z.A/D: Zaid Atabeh(DUWL biofilm), T.N/T: Tayseer Nana (Tap biofilm), T.N/D: Tayseer Nana (DUWL biofilm), M.T/T: Mohammed Tammam (Tap biofilm), M.T/D: Mohammed Tammam (DUWL biofilm), A.Halo/T: Adib Halob (Tap biofilm), A.Halo/D: Adib Halob (DUWL biofilm), A.SH/T: Alaa Sheikh (Tap biofilm), A.SH/D: Alaa Sheikh (DUWL biofilm), M.AB/T: Moath Abu baker (Tap biofilm), M.AB/Moath Abu baker (DUWL biofilm), A.Sar/T: Ameera Sartawi (Tap biofilm) and A.Sar/D: Ameera Sartawi (DUWL biofilm).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 which were positive using the Com primer and the *Legionella* genus primer were tested for *L. pneumophila* using L1 primer. A total of 60 water samples (Tap and DUWL) and 85 biofilms (Tap +DUWL) were screened. Positive results were obtained in 59/62 (95%) of Dental clinics water (Tap and DUWL) and in 83/85 (98%) of dental clinics biofilm swabs (Tap and DUWL). In this reaction, L1 primer gives PCR product 544bp (Fig. 3.16) and (Fig. 3.17). The biofilm swabs gave a higher yield of positive samples for *L. pneumophila* than the water samples, 83/96 (86%) versus 59/89 (66%).

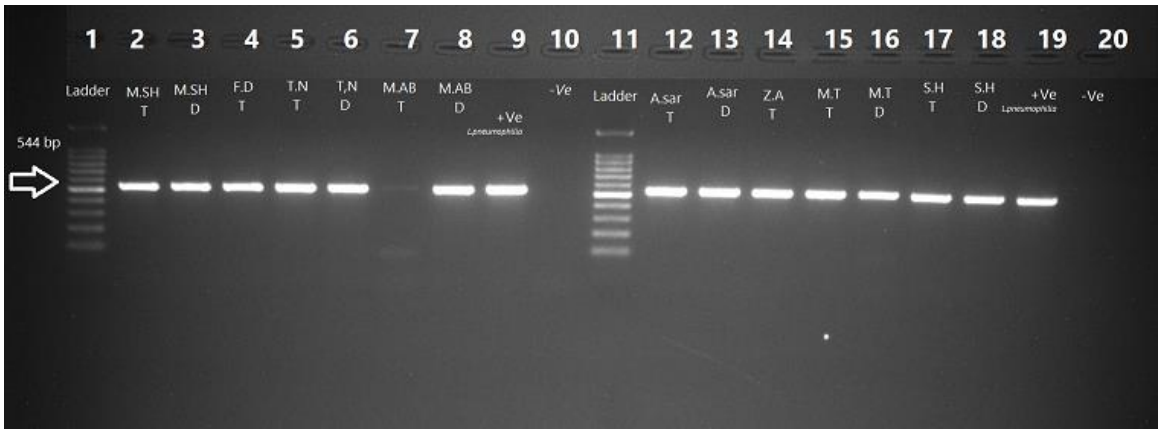


Figure 3.16: Representative 16S rRNA PCR using L1 primer of DNA from the Dental clinics. (T: Tap, D: DUWL). Lane 1 represents the 100bp ladder. Lanes 2-8 show water samples (Tap and DUWL), positive with 544 bp and negative results. Lane 9, (*L. pneumophila*). Lane 10, negative control (nuclease-free water). Lane 11 represents a 100bp ladder. Lanes 12 to 18 represent water samples (Tap and DUWL), positive with 544 bp. Lane 19, positive control (*L. pneumophila*). Lane 20, negative control (nuclease-free water). M.SH/T: Mosab Jorishi Tap water, M.SH/D: Mosab Jorishi DUWL, F.D/T: Fadi Daghlas Tap water, T.N/T: Tayseer Nana tap water, T.N/D: Tayseer Nana DUWL, M.AB/T: Moath Abu Baker Tap water, M.AB/D: Moath Abu Baker DUWL, A.Sar/T: Ameera Sartawi Tap water, A.Sar/D: Ameera Sartawi DUWL, Z.A/T: Zaid Atabeh Tap water, M.T/T: Mohammed Tammam Tap water, and M.T/D: Mohammed Tammam DUWL, S.H/T: Saeed Habash Tap water and S.H/D: Saeed Habash DUWL. Complete data are shown in (Appendix E)

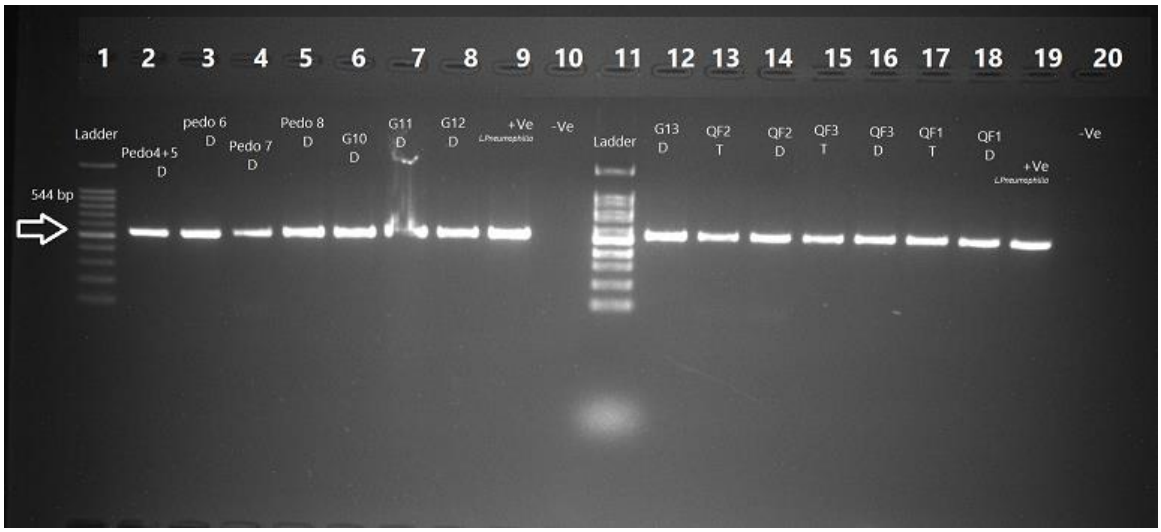


Figure 3.17: Representative 16S rRNA PCR using L1 primer of DNA from AQU Faculty of dentistry and AAUP in Jenin, Lane 1 represents the 100bp ladder. Lanes 2-8 shows biofilm swabs (Tap and DUWL samples), positive with 544 bp. Lane 9, positive control (*L. pneumophila*). Lane 10, negative control (DW free nuclease). Lane 11 represents a 100bp ladder. Lanes 12 to 18 represent biofilm swabs (Tap and DUWL samples), positive with 454 bp. Lane 19, positive control (*Legionella pneumophila*). Lane 20, negative control (nuclease-free water). Pedo 4+5/D: Dental Clinic in AQU College of Dentistry Pediatric fourth floor (DUWL biofilm), Pedo 6/D: Dental Clinic AQU (DUWL biofilm), Pedo 7/D: Dental Clinic AQU (DUWL biofilm), Pedo 8/D: Dental Clinic in AQU (DUWL biofilm), G10/D, G11/D, G12/D, G13/D: G Dental Clinic in AAUP (DUWL biofilm), QF2/T: clinic in AQU (Tap biofilm), QF2/D: (DUWL biofilm), QF3/T: clinic in AQU (Tap biofilm), QF3/D: clinic in AQU (DUWL biofilm), QF1/T: clinic in AQU (Tap biofilm), QF1/D: (DUWL biofilm).Complete data are shown in (Appendix E)

3.5.1.4 *L. pneumophila* isolates confirmation using L1 primer

L. pneumophila isolates obtained by culture were confirmed by 16S rRNA PCR using L1 primer. In this reaction, L1 primer gives PCR product 544bp as clearly seen in (Fig. 3.18).

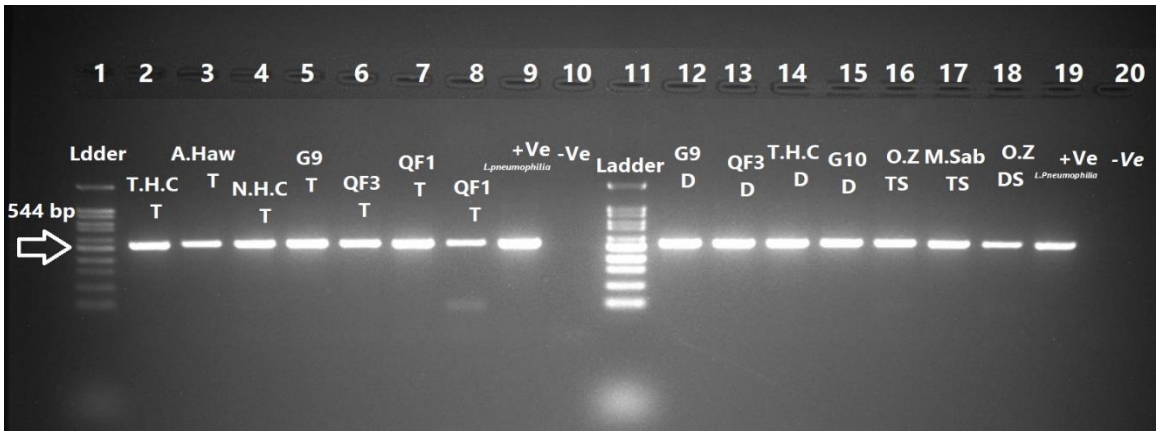


Figure 3.18: *L. pneumophila* isolates confirmation using an L1 primer. (T: Tap, D: DUWL). Lane 1 represents a 100bp ladder. Lane 2-8 shows *L. pneumophila* isolates with 544bp. Lane 9, positive control (*L. pneumophila*). Lane 10, negative control (nuclease-free water). Lane 11 represents a 100bp ladder. Lanes 12-18 show *L. pneumophila* isolates with 544bp. Lane 19, positive control (*L. pneumophila*). Lane 20, negative control (nuclease-free water). T.H.C/Tulkarem UNRWA Health Center Tap water, A.Haw/T: Ammar Halaweh tap water, N.H.C: Nurshams UNRWA Health Center Tap water, G9/T: G Dental Clinic in AAUP in Jenin Tap water, QF3/T: clinic in AQU Tap water, QF1/T: clinic in AQU Tap water, G9/D: G: Dental Clinic in AAUP (DUWL water), QF3/D: DUWL, T.H.C/D: Tulkarem UNRWA Health Center DUWL, G10/D G Dental Clinic in AAUP DUWL, O.Z/TS: Omar Zagha Tap biofilm swab, M.Sab/TS: Monia Sabbah Tap biofilm swab, O.Z/DS: Omar Zagha DUWL biofilm swab. Complete data are shown in (Appendix E).

3.5.2 Sequencing of the 16S rRNA gene and BLAST search

Seven *L. pneumophila* isolates, three from water and four from biofilm isolates were further characterized by sequencing of the partial 16S rRNA gene. Sequencing results are shown in (Table 3.7). Complete sequences are shown in (Appendix G). All seven isolates were confirmed as *L. peumophila* and identified to > 95.7% with *L. peumophila* Philadelphia *or L. peumophila* JCM 7571 *or L. peumophila* Los Angelos.

Table 3.7: Characteristics of *L. pneumophila* isolates obtained from isolation areas.

Isolate	Sample type	Source of isolation	*L. pneumophila* PCR	16S rRNA gene	% Query Cover	% Identity	Accession No
T.H.C	**DUWL**	**Tulkarem health center**	**(+)**	***L. pneumophila fraseri* strain Los Angelos**	**97%**	**96.36%**	**NR_104921.1**
QUF1	**Tap water**	**Dental clinic in AQU surgery department**	**(+)**	***L. pneumophila* fraseri strain Los Angelos**	**67%**	**95.7%**	**NR_104921.1**
N.H.C	**Tap water**	**Nurshams health center**	**(+)**	***L. pneumophila* JCM 7571**	**91%**	**96.87%**	**NR_113235.1**
M.S	**Tap biofilm**	**Monia Sabah/ Tulkarem**	**(+)**	***L. pneumophila* JCM 7571**	**79%**	**96.41%**	**NR_113235.1**
Y. Z	**Tap biofilm**	**Yahiya Zanoun /Nablus**	**(+)**	***L. pneumophila* Philadelphia**	**96%**	**99.61%**	**NR_074231.1**
O. Z	**DUWL biofilm**	**Omar Zagha / Nablus**	**(+)**	***L. pneumophila* Philadelphia**	**96%**	**100%**	**NR_074231.1**
G10	**Tap biofilm**	**Dental clinic in AAUP**	**(+)**	***L. pneumophila* Philadelphia**	**95%**	**99.8%**	**NR_074231.1**

3.6 Cultivation Dependent Analysis Vs Cultivation Independent Analysis

All the collected water (89) and biofilm samples (96) were analyzed for the presence of *L. pneumophila* in their water sources by microbiological culture, for cultivation dependent analysis (CDA) (Table 3.2). *L. pneumophila* was isolated from 7 out of 39 (18%) Tap water samples, and from 6 out of 50 (12%) DUWL samples.

L. pneumophila was isolated from 9 out of 45 (20%) faucet biofilm swabs and from 6 out of 51 (12%) DUWL biofilm. The yield from the biofilm was higher than from the water samples. The isolation rate was higher for *L. pneumophila* from the tap water samples than the DUWL whether the sample was a water sample or a biofilm swab.

In addition, the 89 Tap and DUWL water samples and 96 biofilm swabs (Table 3.8) were analyzed by cultivation-independent analysis (CIA) namely by PCR for the presence of *L. pneumophila.* The number of positive samples by (CIA) PCR was, 4.5x higher for Tap water (7/32), DUWL (6/27) and Tap biofilm swab (9/41) and up to 7x higher for DUWL biofilm swabs (28/142) (Table 3.8). Data are shown in Appendices D and E.

Table 3.8: 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
Tap Water	39	39	7	32	18%	82%
DUWL	50	50	6	27	12%	54%
Tap biofilm	45	45	9	41	20%	91%
DUWL biofilm	51	51	6	42	12%	82%
Total	185	185	28	142	15%	77%

CDA: Cultivation Dependent Analysis

CIA: Cultivation Independent Analysis.

Chapter Four

Discussion

4.1 Discussion

DUWL water is an ideal environment for the presence of biofilm and microbial contamination of due to the nature of the tubing of the DUWL. This phenomenon has been well documented as well as the difficulties in biofilm cleaning and the prevention of regrowth (Szymańska and Sitkowska, 2013). With regard to exposure of the dental patient, the health worker and the dentist in a dental clinic, the nature of the use of the DUWL in dentistry helps in the production of aerosols and splatter, generated by working handpieces, two important means of transmission of *Legionella* bacteria. The dental patient is also exposed to contamination from DUWL from backward contamination which may occur when oral normal flora of patients enters the waterlines via suctioning of saliva by the head of the handpiece (Ghalyani et al., 2015).

Dentists and patients can be exposed to opportunistic or pathogenic microorganisms including *Legionella* bacteria by inhaling droplets and aerosols produced by dental instruments connected to DUWLs (James et al., 2015; Sedlata Juraskova et al., 2017), but the extent of the problem is generally unrecognized and there are no specific guidelines for protecting patients and dentists from exposure to aerosols contaminated with *Legionella*. Biofilm is a complex heterogeneous microbial cluster that forms on any non-sterile moist surface. Being an aquatic organism *Legionella* is found growing in the biofilm that lines the inside of pipes and water lines (James et al., 2015; Liaqat and Sabri, 2010; Singh et al., 2013). *Legionella pneumophila* is considered an important pioneer colonizer in aquatic environments especially DUWL (Aprea et al., 2010).

Isolation of *Legionella* is difficult by microbiological methods due to a VBNC state, because of this state misdiagnosis of legionellosis may occur (Scheikl et al., 2016). Furthermore, the cultivation of this fastidious bacterium is difficult due to its slow growth and overgrowth by other bacteria (Nederbragt et al., 2008; Steinert et al., 2002b).

This is the first study which identifies *Legionella* by cultural and molecular methods in DUWL in the WB, Palestine, we set out to detect the levels of *Legionella* contamination in

DUWL water supplies, testing water and biofilms using molecular, microbiological and serological techniques.

To achieve this aim, water and biofilm samples from Tap and DUWL were collected from AQU clinics, AAUP clinics, and dental clinics in three different regions in the WB. All samples were analyzed for the presence of *Legionella spp.* in their water sources. Cultivation dependent analysis was performed using microbiological techniques, *Legionella* identification and quantification was done using GVPC or BCYE. Latex agglutination was performed on the isolates to identify *Legionella* serogroups. Cultivation independent analysis was performed using 16S rRNA PCR, and partial sequencing of the 16S rRNA gene.

All sites except Hebron tested positive for *Legionella*. The Tap water tested, positive for *Legionella* with 18% prevalence by cultivation dependent analysis (CDA) and the prevalence rate increased to 82% by cultivation-independent analysis (CIA). Whereas the prevalence in DUWL was 12% by CDA and this prevalence increased to 54% by CIA (Table 3.2). As expected for the biofilm samples, the *Legionella* prevalence was higher, being 20% by CDA for Tap biofilm and it increased to 91% by CIA. Similarly, the prevalence rate of *Legionella* positive samples for DUWL biofilms was 12% by CDA and it increased to 82% by CIA (Table 3.8). Using CDA, the number of *L. pneumophila* isolates from biofilm (15 isolates) was higher than isolates from water samples (13 isolates).

Biofilms provide *Legionella* with nutrients for growth and protection from adverse ecological conditions like water disinfection. (Ma'ayeh et al., 2008) studied the contamination of DUWL water systems with *Legionella* in the University of Science and Technology in Jordan, the rate of detection of *Legionella* from DUWL was 86.7% at the beginning of the day, 40% after 2 min of flushing, 53.5% at midday (Ma'ayeh et al., 2008). Another study (Ajami in 2009) reported the rate of detection of *Legionella* sg1 from DUWL was 36.1% (9/52): 17.3% at the beginning of the day, 5.7% after 2 min of flushing, 5.7% at midday (Ajami et al., 2012). Also, (Ghalyani et al., 2015), assessed DUWL contamination in private dental clinics in Isfahan city, the prevalence of *Legionella* 15% in DUWL in Isfahan City Olewi did a study did a study to detect the microbial contamination in DUWL of several clinics in Baghdad city; the average bacterial growth was 40.4% (20 samples of 45) (Oleiwi, 2017). Globally, various studies have revealed that DUWLs have

high levels of microbial contamination; in 1995 Challacombe and Fernandes studied 194 DUWL to detect the presence of *Legionella*, they found very low concentration in 49/194 (25%) and 145/194 (75%) were negatives (Challacombe and Fernandes, 1995). A study in the USA examined 28 dental clinics in six U.S. states that tested positive for the presence of *L. pneumophila* and other *Legionella spp.* by PCR (Atlas et al., 1995a). Williams and colleagues studied 47 DUWL biofilms and found 62% of them having *Legionella* and 19% concentration exceeding 100 CFU / ml. (Szymańska, 2004; Williams et al., 1993). A study in Istanbul revealed the prevalence of many types of bacteria and mycological microorganisms such as *Pseudomonas fluorescens, Pasteurella haemolytica, Photobacterium damsela, Ochrobacter anthropi, Moraxella sp., Aspergillus flavus, Penicillium expansum,* however, *Legionella spp*. were not detected in DUWL water samples (Göksay et al., 2008b; Kadaifciler et al., 2013). In 2015 another study in the Czech Republic, in the faculty of Medicine and Dentistry from a total of 50 samples cultured, 18 samples (36.0 %) tested positive for *Legionella spp*. James and colleagues in India assessed the microbial quality of water in dental unit waterlines DUWL in a dental school at Mangalore (James et al., 2015). A recent study in Torino/Italy by Ditommaso et al in 2016 determined the prevalence of *Legionella* in DUWLs and tap water samples by using PMA-qPCR propidium monoazide (PMA) quantitative PCR (qPCR) and standard culture methods, the results showed the level of *Legionella spp.* very low. Detection of contaminated water by CDA does not reflect the true scale of the problem, so they needed to do heterotrophic plate count on yeast extract agar, based on the assumption that *Legionella* is part of the components of biofilms, and *Legionella* are fastidious bacteria, affected by overgrowth of other bacteria. (Ditommaso et al., 2016).

All the samples in our study were collected at the beginning of the day to detect the highest level of *Legionella* by CDA. *Legionella* was isolated from six DUWL samples; 2 from AQU, 2 from AAUP, and 2 isolate from clinics in Tulkarem City. (Table 3.4), and seven isolates from Tap water, as well as 15 from biofilm swabs (Table 3.5). The *Legionella* count from the water samples (Table 3.3) varied between 27 CFU/L and 177 CFU/L. The American Dental Association (ADA) in 1996, set a limit for DUWL to contain less than 200 CFU/ml. The Center for Disease Control and Prevention (CDC) in 2003 recommended ≤500 CFU/ml for non-surgical dental procedures (Singh et al., 2013). Theoretically, the potential health hazard of *Legionella* to humans is associated with cell concentration above

10^4 to 10^5 CFU/L of water (Al-Matawah et al., 2012). Persistence of *L. pneumophila* in aquatic systems is a health hazard and this is reflected in the medical research focus on this fastidious bacteria (Tabatabaei et al., 2016). In Palestine, there is a need for surveillance for *Legionella* contamination of water systems as well as legislation regarding the limits allowed especially in hospital water systems and in dental clinics.

In this study, the predominant serogroup of the *L. pneumophila* isolates (total 28 isolates) was serogroup 1 representing 82% of the isolates and serogroup 2-14 only 18% of the isolates. This is in accordance with many other studies. (Yu et al., 2002) reported that *L. pneumophila* constituted 91.5% of 508 isolates, and the most prevalent serogroup was sg1 (84.2 %) followed by sg 2-13 (7.3%) and the rest of the isolates belonged to other species. Aurell et al studied studied 691 isolates of *Legionella* by pulsed-field gel electrophoresis (PFGE) and found (98.6%) *L. pneumophila*, sg1 (88.7%) and (11.7 %) sg 2-14. (Aurell et al., 2003).

During this study, water samples that were collected and cultured during the winter and spring seasons showed no growth for *Legionella*. The seasonal distribution of *Legionella* in water systems is well known, Rodriguez et.al 2015 reported higher detection of *Legionella* in the Summer and Spring than Autumn and Winter. Water temperatures between 20 C and 45 C favor *Legionella* growth. The heating of water at >60°C is widely used to decrease total bacterial numbers and inactivate the number of pathogens like *Legionella* (Kusnetsov et al., 2003).

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). Shareef and Mimi also treated the West Bank hospital water by using heating treatment (Shareef and Mimi, 2008). Some researchers suggest using disinfectant instead of hot water for rinsing DUWL. Cycloheximide CHX 0.12 %, hydrogen peroxide, chlorine dioxide, and sodium phosphate has been used instead of distilled water or tap water (Boyle et al., 2011; Leoni et al., 2005; Singh et al., 2013), he recommended two minutes flushing regimen to reduce bacterial contamination. According to CDC guidelines, the morning flushing with 0.12% chlorhexidine gluconate (CHX) or Hydrogen peroxide 3%, for 2 min and for 20-30 s between patients should be followed during dental work. Sedlata Juraskova et al studied 50 water samples from tap and DUWL

and results were 13/50 (36%) positive for *Legionella*. Ten positive DUWL samples were selected and disinfected using a solution (1% stabimed) solution for 30 min. Upon further culture, nine of the ten samples no longer showed any presence of *Legionella* (Sedlata Juraskova et al., 2017).

The mean temperature for Tap and DUWL was 19.5 ± 0.8°C and varied, depending on the dental clinic, between 10°C and 25°C. The pH of the Tap water varied between 9.1 and 7.9 with a mean of 8.7±0.1. Measurements of conductivity during sampling of Tap water resulted in a mean conductivity of 611.6 ± 48.2 µS/cm. The pH of the DUWL varied between 9.2 and 5.1 with a mean of 8.7±0.1. Measurements of conductivity during sampling DUWL resulted in a mean conductivity of 682.5 ± 46.4 µS/cm (Table 3.2). All data are shown in Appendix E.

During this study-sampling period, almost all measured physical and chemical bulk water parameters were within the acceptable ranges according to WHO guidelines (World Health Organization WHO, 2011). The Carbonate hardness (Carbonate and bicarbonates) and the total hardness (Calcium and Magnesium salts) were above the acceptable level in the tested sites. Hardness in water is caused by dissolved polyvalent metallic ions from sedimentary socks, seepage, and runoff from soils. Calcium and Magnesium the two principal ions can be found in many sedimentary rocks, the most common being limestone and chalk (WHO 2011). In the West Bank and according to the report of the Palestinian Water Authority, the sources of water for the tested sites are mainly wells (groundwater) or springs which explains the levels of hardness. In addition, the hardness of water contributes to scales build up in water lines, which helps in the build-up of biofilms.

The most common cause of DUWL contamination is the build-up of biofilms on the surfaces of tubing within the dental-unit, entire surfaces of water distribution systems and tanks by microorganisms. The structure of the dental units supports biofilm formation and bacterial contamination of DUWLs (Liaqat and Sabri, 2010; Salam et al., 2017). Most of the DUWL biofilm studies tested dental units using the municipal water supply source water. Several studies tested the dental unit as distilled water (DW) with source water and found that DW alone did not prevent the formation of biofilms without a regular, intermittent DUWL cleaning system at the same time. But no former study reported the formation of biofilm in ultrapure water, this type of water has dissolved solids in parts per

billion (ppb) (Porteous et al., 2011). *Legionella* is often regarded as one of the first colonizers in the creation of the biofilm seen in water systems, with time the accumulation of biofilm increases and the elimination of *Legionella* becomes complicated due to the nature of *Legionella* being tolerant to a wide range of physical and chemical factors, including chlorination and temperature.

In this study, few dentists used DW for supplying DUWL, such as Zaid Atabeh clinic in Nablus and Usama Zallom clinic in Hebron, *Legionella* was not found in (Usama Zallom) clinic in Hebron by cultivation method however *Legionella* was identified by PCR in Hebron (Fig 3.14) Lane 12. The water samples of some dental clinics in this study were found to be uncontaminated, whereas the biofilm swab from the same DUWL systems of these clinics grew *Legionella*. Biofilms can be found in any waterline distribution system attached to the surface where many potentially pathogenic bacteria are present, which are not isolated from the bulk water but are in the biofilms. The biofilm population is shielded from adverse environmental conditions, including disinfection (Lauritano et al., 2017; Liaqat and Sabri, 2010; Salam et al., 2017).

In the current study, *L. pneumophila* was the only *Legionella* species recovered, according to a recent Italian survey (Ditommaso et al., 2016) *L. pneumophila* is the most abundant species in potable and environmental water samples.

The rate of isolation of *Legionella spp.,* varies from 0 % to 100% when DUWL are tested and the counts of these bacteria in DUWL samples ranges from 0 to 10^6 CFU/ml. (Ajami et al., 2012; Al-Hiyasat et al., 2007; Atlas et al., 1995b; Challacombe and Fernandes, 1995; Ma'ayeh et al., 2008; Marston et al., 1994; Walker and Marsh, 2004). There is wide variability in the counts of *Legionella* due to several factors such as the amount of available soluble organic compounds, presence of heavy metals, temperature, and level of free chlorine in municipal water distribution systems. Compairing culture, PCR, or immunofluorescence staining, the gold standard method for determining the quantity of these bacteria in the water may be qPCR, due to their ability to detect low levels of target nucleic acids, including those of viable *Legionella*, VBNC *Legionella*, and *Legionella* within amoebae (Ditommaso et al., 2016). In Ditommaso study, they used the culture method to detect *Legionella* in the DUWL samples; they observed a low rate of contamination 4/56 (6.6%) in DUWL and 2/24 (7.7%) from tap water. In our study, the

positive samples observed were 6/50 (12%) from analyzing 50 DUWL and 7/39 (18%) from 39 tap water samples by culture method. In contrast, when Ditommaso used PMA-qPCR to detect *Legionella*, he found that 100% of the samples were positive for *Legionella* (Ditommaso et al., 2016). In our study *Legionella* was identified by conventional PCR in 60/87 (69%) of dental clinics water (Tap and DUWL) and in 85/96 (89%) of dental clinics biofilm swabs (Tap and DUWL). The biofilms showed a higher prevalence of *Legionella* both by culture and PCR than the water samples.

Unfortunately, there are no previous data about legionellosis cases in Palestine. With regards to dental patients, a recent case report in 2012, talked about a healthy 82-year-old Italian woman who contracted LD after a dental appointment and a report of a fatal case of legionellosis based pneumonia in a dentist in the United States (Atlas et al., 1995a; Ditommaso et al., 2016). Reinthaler, Fotos and colleagues showed that dentists do develop raised antibody titer to *L. pneumophila* after varying periods of clinical work, which showed that exposure to the bacteria is sufficient to initiate an immunological response (Challacombe and Fernandes, 1995).

L. pneumophila is the most pathogenic of *Legionella spp*, causing up to 90% of the cases of legionellosis (Ajami et al., 2012; Carvalho et al., 2007; George et al., 2016b; Kahlisch et al., 2010; Tabatabaei et al., 2016).

L. pneumophila sg 1 represented 23/28 (82%) from the total isolates, while 5/28 (18%) of the isolates belonged to *L. pneumophila* sg 2-14 (Figure 3.11). According to the current epidemiological data available from the world, different *L. pneumophila* sgs cause legionellosis. In general, *L. peumophila* sg1 is responsible for approximately 75% of cases in most American and European countries and 20-30% are caused by other serogroups, and the remaining 5% caused by *Legionella* nonpneumophila species (Bartram et al., 2007). Mavridou et. al. studied the prevalence of *Legionella spp*. in Greek hospitals, they found 72.7% of *Legionella* was *L. pneumophila* sg1 and 22.7% were *L. pneumophila* sg 2-14 (Mavridou et al., 2008). Furthermore, a Korean study investigated the distribution of *Legionella spp* from environmental water sources of public facilities in South Korea. They isolated 560 *Legionella* isolates from all South Korea. They found 85.5% of the isolates were *L. pneumophila* sg (54.7%), the rest of the isolates (14.5%) were non- *L. pneumophila* (Lee et al., 2010). Whereas in the Middle East there is a shortage of

epidemiological data for *Legionella* sgs. A recent study in Israel indicated that *L. pneumophila* sg3 might be the primary causative agent responsible for legionellosis (Oren et al., 2002). Blanky et al.,2015 in Israel revealed 23 water samples were *Legionella* positive. *L. pneumophila* sg 1(87%), serogroup 3(21%) and serogroups (2, 4–14) (18%) (Blanky et al., 2015). Similarly, another study in Kuwait on clinical isolates demonstrated dominance (more than 80%) of *L. pneumophila* sg3 in patients with LD (Qasem et al., 2008). The second Study in Kuwait by Al Matawah revealed the 46 *L. pneumophila* isolates, the majority of the isolates belonged to serogroup 3 (80%), followed by serogroup 1 (13%), serogroup 7 (2%), serogroup 10 (2%), and serogroup 4(2%) (Al-Matawah et al., 2012). The third Study in Basra / Iraq by Al Sulami revealed 188 *L. pneumophila* isolates from 258 isolates, the majority of the isolates belonged to serogroup 1 (77.1%) and 59 isolates belonged to serogroup 2 -15(22.9%) (Al-Sulami et al., 2013).

Shareef and Mimi studied the hospital's tap water system in the West Bank hospitals. They found *L. pneumophila* sg 2-14 in 62% of samples (Shareef and Mimi, 2008). Ashraf Zayed et al (2013) studied the hospital's tap water system in the West Bank hospitals and Almakased Hospital in Jerusalem. They found *L. pneumophila* sg 1 (64%) of samples and sg 2-14 in (36%) (Ashraf, thesis 2013).

In another clinical study about LD prevalence in Palestine (Jaber et al., 2018) cultured 195 respiratory specimens collected from suspected pneumonia patients, only one specimen yielded a positive culture *L. pneumophila* sg 1. The very low yield by culture is explained by the heavy use of antibiotics by patients in Palestine prior to hospitalization. However, 16S rRNA PCR analysis of the 195 respiratory samples for *L. pneumophila* revealed 23% positive and 77% negative samples.

Mizrahi et al studied 133 clinical sputum samples from Israel which were positive for *Legionella* by PCR 9/133 (6.8 %) and only one sample out of the nine was also positive by culture and belonged to *L. pneumophila* sg 1 (Mizrahi et al., 2017).

The results of our study show that Dental unit waterline (DUWL) is contaminated with *L. pneumophila* in; AQU dental clinics, AAUP dental clinics, and dental clinics in three different regions in Nablus, Tulkarem, and Hebron (Table 3.5) and(Table 3.6). Hence, the Ministry of Health (MOH) and the Palestinian Water Authority should maintain high standards for water quality and should take immediate measures to prevent waterborne

infections and set guidelines for the prevention of LD and other infections caused by waterborne opportunistic pathogens. Such measures are likely to be successful, given the large reductions in waterborne infections observed when specific guidelines are applied (Boyle et al., 2011).

Interesting observations across the different dental units sampled were the following: the molecular detection of *Legionella* was positive in DUWL or Tap water when the culture was negative. When DUWL or Tap water was positive for *Legionella*, higher fractions of the sampled biofilms were positive.

This study illustrates the importance of protecting patients, dental health workers and dentists from contamination with *L. pneumophila* bacteria by inhalation of aerosols, which may cause LD and highlights the need for appropriate specific guidelines for protecting patients from exposure.

4.2 Recommendations

1. The microbiological monitoring of a water system should not target individual organisms, such as *Legionella*, *E. coli*, *Pseudomonas*, and *Acinetobacter* bacteria, but it should treat the bioburden that encompasses them.
2. The washing and cleaning of dental units should be assessed by counting the concentrations of aerobic heterotrophic bacteria because contamination by these organisms is a clear indicator of the potential presence of *Legionella*.
3. Softener filter, if used, must be tested regularly to check any problems that may appear, it is an environment suitable for growing pathogens.
4. Disinfection procedures of the DUWL system should be considered and applied to remove biofilm-harboring bacteria including *L. pneumophila.*
5. Medical tools should be rinsed with sterile water since tap water or distilled water may contain *Legionella* that can be transmitted to susceptible hosts and may cause pneumonia.
6. DUWL must be routinely disinfected with a disinfectant or a solution that eliminates biofilm from the DCU such as chlorhexidine gluconate, Hydrogen peroxide, chlorine dioxide, and sodium phosphate mouth rinse is recommended.

7. Dental health workers and dentists should be educated regarding water quality, biofilm formation, water treatment, and maintenance protocols.
8. A variety of commercial DUWLs cleaning products and systems are available. Dentists should contact their specific DCU model manufacturer for advice on waterline disinfection products and procedures.
9. Information about legionellosis is inadequate in Palestinian medical teams, therefore, we recommend to do workshops educating medical personnel about legionellosis, and about routes of its transmission, diagnosis, and treatment.
10. Immunosuppressed patients must wear a mask during dental treatment.
11. Determining the concentrations of *Legionella* in DUWL outputs by the cultivation method only is insufficient for determining the exact size of the problem.
12. The molecular identification and quantification of *Legionella* in water sources are preferably recommended over the culture method.
13. The Ministry of Health (MOH) and the Palestinian Water Authority (PWA) should put limitations, guidelines and clear protocols for water quality in the West Bank hospitals, dental clinics, universities, hotels, spas, resorts, springs, wells, and all aquatic environments.

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 PMA qPCR. The seasonal cycle of *Legionella* in water systems in Palestine is another factor to study. Furthermore, we will measure the antibody level for *Legionella* in serum obtained from dentists, health workers, and patients (Pankhurst et al.,2003).

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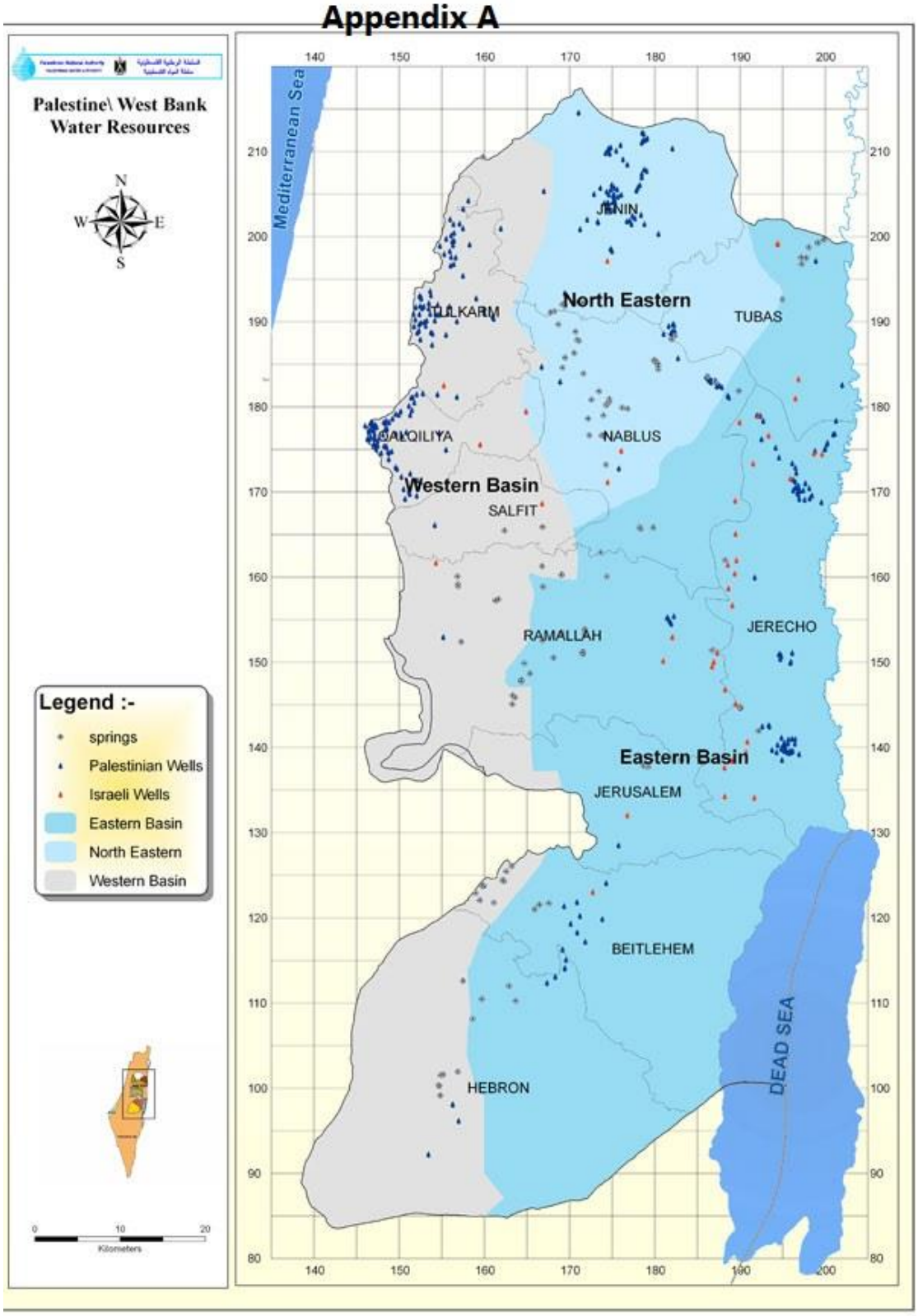
Appendix A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Appendix A

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

جامعة القدس
كلية الطب
أبوديس – القدس

التاريخ:2019/11/24

حضرة المهندس مازن غنيم المحترم
رئيس سلطة المياه الفلسطينية

تحية طيبة وبعد،

الرجاء تزويد السيد معتصم يرغال بخوائط مصادر المياه لكل من:-

- نابلس – منطقة الدوار، بلاطة ، عسكر و رفيديا.
- طولكم – منطقة مخيم نور شمس، الحارة الغربية و الدوار.
- جامعة القدس – ابوديس.
- الجامعة العربية الأمريكية – الزبابدة.
- الخليل – منطقة راس الجورة.

و ذلك لجاجته لهذه المعلومات في بحثه لرسالة الماجستير :" Cultural and molecular evidence of *Legionella pneumophila* in dental unit water lines (DUWL) in the West Bank Palestine".

الرجاء التواصل اذا امكن مع السيد معتصم يرغال على البريد الإلكتروني mutasem_burghal@hotmail.com

و تفضلوا بقبول فائق الإحترام

د. دينا البيطار
رئيس دائرة الأحياء الدقيقة و المناعة
كلية الطب
E-mail: dbitar@staff.alquds.edu

كلية الطب Faculty of Medicine

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Appendix B

Al-Quds University
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Abu-Dies, Jerusalem

جامعة القدس
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التاريخ:2017/12/10

حضرة الدكتور محمد ابو يونس المحترم
عميد كلية طب الأسنان

تحية طيبة وبعد،

ارجو من حضرتكم الموافقة لطالب الماجستير معتصم برغل(21510102) جمع عينات ماء من وحدات الأسنان في العيادات و ذلك ليتمكن من اجراء بحث الماجستير **" Cultural and molecular evidence of *Legionella pneumophila* in Dental unit waterlines (DUWL) in the West Bank, Palestine"** ابتداء من شهر يناير 2018 و لغاية شهر يوليو 2018.

و دمتم ذخرا للتقدم العلمي

مع فائق الإحترام

د. دينا البيطار
رئيس قسم الأحياء الدقيقة و المناعة

كلية الطب
Faculty of Medicine

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جامعة القدس
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التاريخ:2018/3/10

حضرة الدكتور رشدي كلاب المحترم
عميد كلية طب الأسنان في الجامعة الأمريكية في جنين

تحية طيبة وبعد،

ارجو من حضرتكم الموافقة لطالب الماجستير في جامعة القدس معتصم برغل (21510102) جمع عينات من وحدات الأسنان في العيادات في جامعتكم الموقرة و ذلك ليتمكن من اجراء بحث الماجستير :

" Cultural and molecular evidence of ***Legionella pneumophila*** in Dental Unit Waterlines (DUWL) in the West Bank, Palestine".

ابتداء من شهر اذار 2018 و لغاية شهر يوليو 2018.

و دمتم ذخرا للتقدم العلمي

مع فائق الإحترام

د. دينا البيطار
رئيسة دائرة الإحياء الدقيقة و المناعة

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التاريخ:2017/12/10

حضرة الدكتور محمد الخليلي المحترم
مدير الصحة في الضفة الغربية
UNRWA

بواسطة الدكتور عدنان قرمش المحترم
مدير الصحة في منطقة نابلس
UNRWA

تحية طيبة وبعد،

ارجو من حضرتكم الموافقة لطالب الماجستير معتصم برغل(21510102) جمع عينات ماء من وحدات الأسنان في العيادات و ذلك ليتمكن من اجراء بحث الماجستير **" Cultural and molecular evidence of *Legionella pneumophila* in Dental unit waterlines (DUWL) in the West Bank, Palestine"**
ابتداء من شهر يناير 2018 و لغاية شهر يوليو 2018.

و دمتم ذخرا للتقدم العلمي

مع فائق الإحترام

د. دينا البيطار
رئيس قسم الأحياء الدقيقة و المناعة

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التاريخ:2017/12/10

حضرة الطبيب د. عبد الكريم محمد ابو سنينة المحترم

تحية طيبة وبعد،

ارجو من حضرتكم الموافقة لطالب الماجستير معتصم برغل (21510102) جمع عينات ماء من وحدة الأسنان في عيادتكم و ذلك ليتمكن من اجراء بحث الماجستير:

" Cultural and molecular evidence of *Legionella pneumophila* in Dental unit waterlines (DUWL) in the West Bank, Palestine"

و دمتم ذخرا للتقدم العلمي

مع فائق الإحترام

د. دينا البيطار
رئيس قسم الأحياء الدقيقة و المناعة

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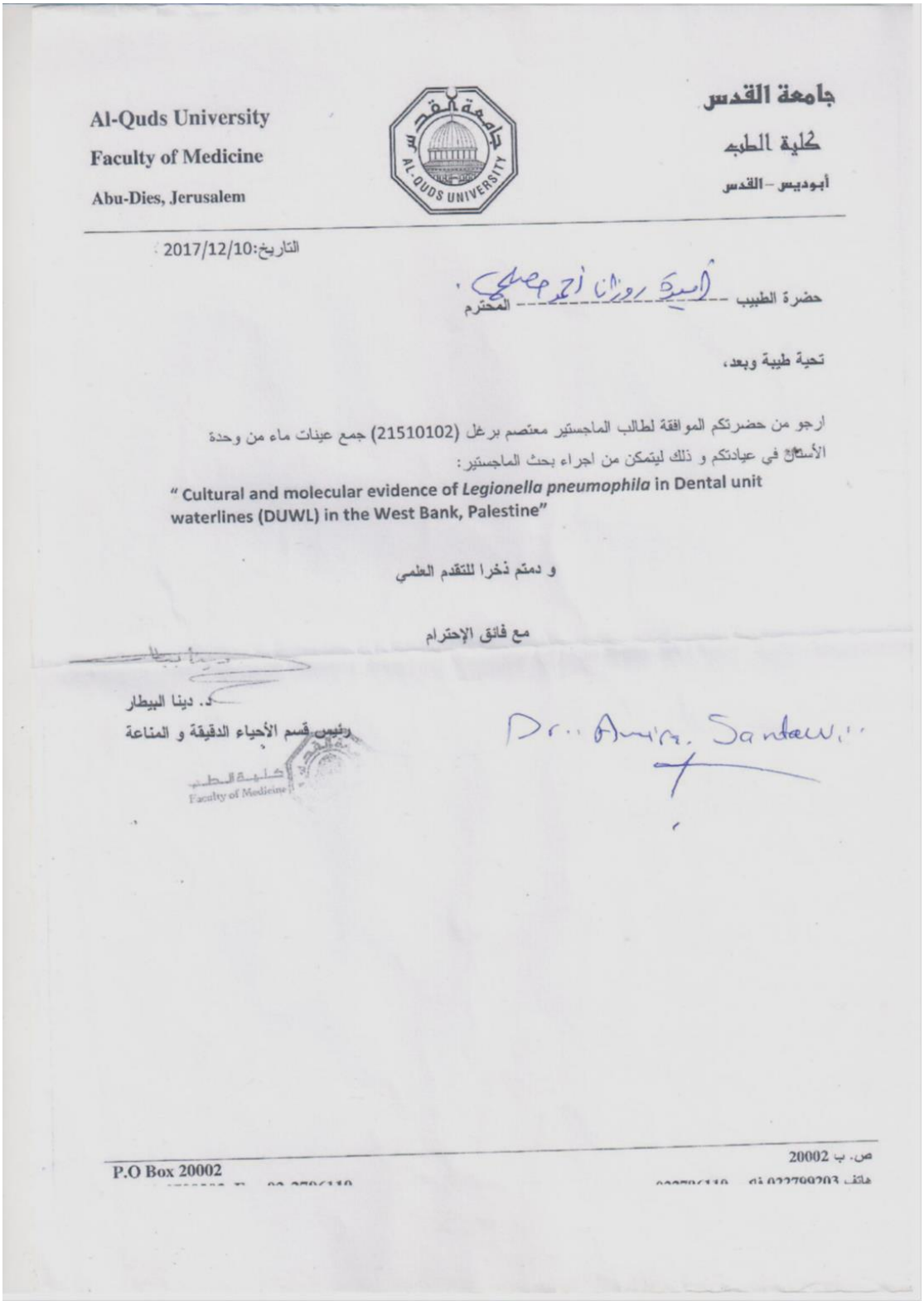
Appendix B



جامعة القدس
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Al-Quds University
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التاريخ:2017/12/10

حضرة الطبيب المحترم أسيل روان أحمد صبحي

تحية طيبة وبعد،

ارجو من حضرتكم الموافقة لطالب الماجستير معتصم برغل (21510102) جمع عينات ماء من وحدة الأسنان في عيادتكم و ذلك ليتمكن من اجراء بحث الماجستير:

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مع فائق الإحترام

د. دينا البيطار
رئيس قسم الأحياء الدقيقة و المناعة

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Dr. Amin. Sanduw...

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جامعة القدس
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التاريخ:2017/12/10

حضرة الطبيب مصعب الجوريشي -- المحترم

تحية طيبة وبعد،

ارجو من حضرتكم الموافقة لطالب الماجستير معتصم برغل (21510102) جمع عينات ماء من وحدة الأسنان في عيادتكم و ذلك ليتمكن من اجراء بحث الماجستير:

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و دمتم ذخرا للتقدم العلمي

مع فائق الإحترام

د. دينا البيطار
رئيس قسم الأحياء الدقيقة و المناعة

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حضرة الطبيب ---محمد نايل النجار--- المحترم

تحية طيبة وبعد،

ارجو من حضرتكم الموافقة لطالب الماجستير معتصم برغل (21510102) جمع عينات ماء من وحدة الأسنان في عيادتكم و ذلك ليتمكن من اجراء بحث الماجستير:

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مع فائق الإحترام

د. دينا البيطار
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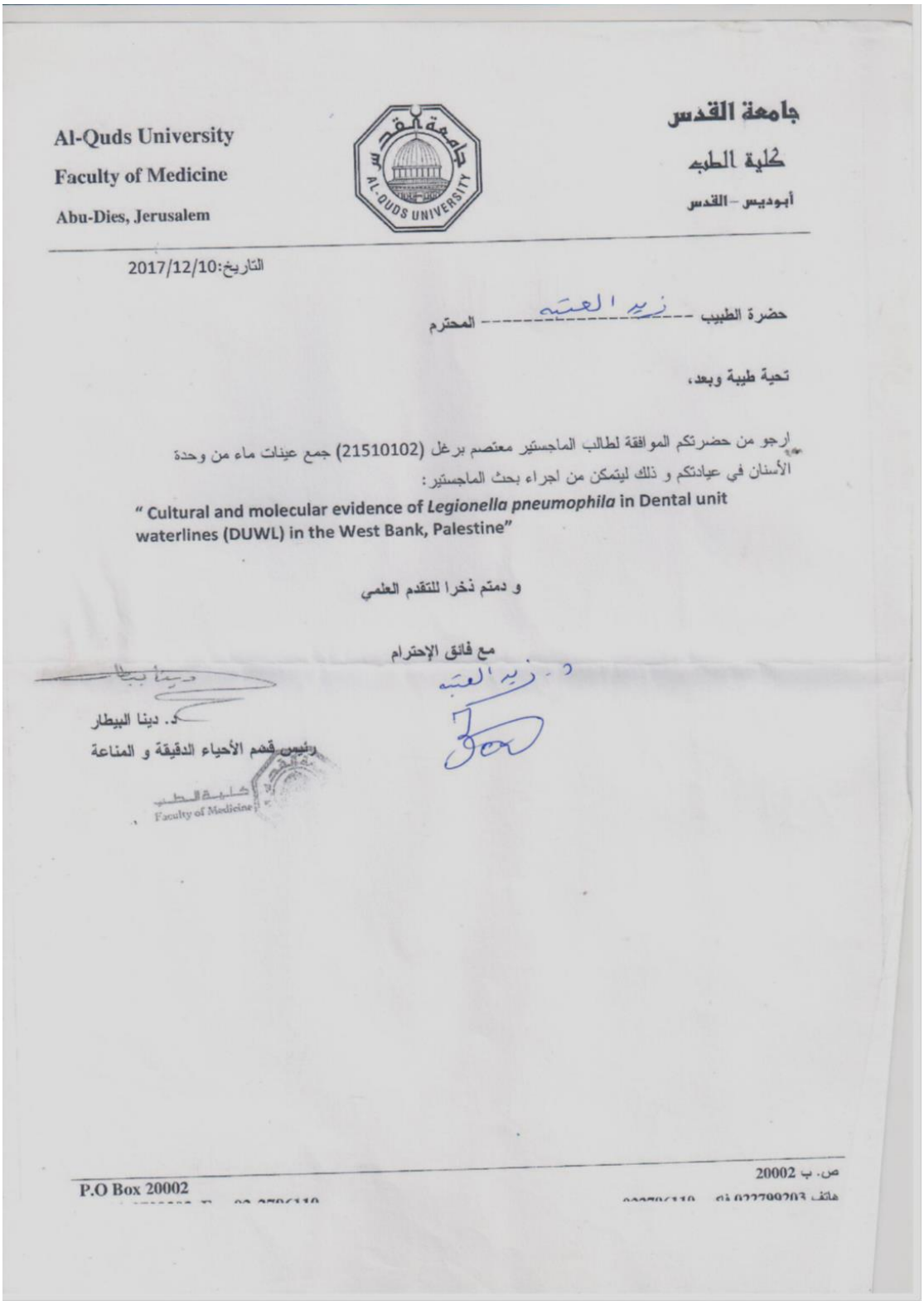

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حضرة الطبيب ---زيد العتبه--- المحترم

تحية طيبة وبعد،

ارجو من حضرتكم الموافقة لطالب الماجستير معتصم برغل (21510102) جمع عينات ماء من وحدة الأسنان في عيادتكم و ذلك ليتمكن من اجراء بحث الماجستير:

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مع فائق الإحترام

د. دينا البيطار
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د. زيد العتبه

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حضرة الطبيب خالد دناس ------ المحترم

تحية طيبة وبعد،

ارجو من حضرتكم الموافقة لطالب الماجستير معتصم برغل (21510102) جمع عينات ماء من وحدة الأسنان في عيادتكم و ذلك ليتمكن من اجراء بحث الماجستير:

" Cultural and molecular evidence of *Legionella pneumophila* in Dental unit waterlines (DUWL) in the West Bank, Palestine"

و دمتم ذخرا للتقدم العلمي

مع فائق الإحترام

د. دينا البيطار
رئيس قسم الأحياء الدقيقة و المناعة

كلية الطب
Faculty of Medicine

P.O Box 20002
ص. ب 20002

Appendix B

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

جامعة القدس
كلية الطب
أبوديس – القدس

التاريخ:2017/12/10

حضرة الطبيب ---------- المحترم

تحية طيبة وبعد،

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Faculty of Medicine
Abu-Dies, Jerusalem

جامعة القدس
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أبوديس – القدس

التاريخ:2017/12/10

حضرة الطبيب ---------- المحترم

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و دمتم ذخرا للتقدم العلمي

مع فائق الإحترام

د. دينا البيطار
رئيس قسم الأحياء الدقيقة و المناعة

كلية الطب
Faculty of Medicine

P.O Box 20002

ب 20002

Appendix C

Tap water samples collection							
No	**Name**	**Area**	**Sample design**	**Volume for *Legionella* count**	**Volume for DNA extraction**	**Date of collection**	**Time**
1	Zaid Atabeh	Nablus	Z. A	1 L 1 L	1 L 1 L	2.02.2018	10:30 AM
2	Tayser Alnana	Nablus	T. N	1 L	1 L	4.02.208	11:00 AM
3	Mohammed Altammam	Nablus	M. T	1 L	1 L	4.02.2018	11:30 AM
4	Fadi Daghlas	Nablus	F. D	1 L	1 L	2.02.2018	10:00 AM
5	Saed Habash	Nablus	S. H	1 L	1 L	4.02.2108	8:30 AM
6	Mosab jorishi	Nablus	M. SH	1 L	1 L	8.02.2018	10:00 AM
7	Amera Mesleh Sartawi	Nablus	A. Sar	1 L	1 L	8.02.2018	10:30 AM
8	Ammar Halaweh	Nablus	A. Haw	1 L	1 L	27.08.2018	9:00 AM
9	Arafat Alwazani	Nablus	A. W	1 L	1 L	2.09.2018	9:15 AM
10	Alaa Yasen	Nablus	A. Y	1 L	1 L	2.09.2018	9:30 AM
11	Omar Alzaghah	Nablus	O. Z	1 L	1 L	2.09.2018	9:00 AM
12	Adib Halob	Tulkarem	A. H	1 L	1 L	12.02.2018	9:15 AM
13	Ala Alsheikh	Tulkarem	A. SH	1 L	1 L	12.02.2018	9:30am
14	Moath Abu Baker	Tulkarem	M. AB	1 L	1 L	12.02.2018	9:45 AM
15	Tulkarem	Tulkarem	T. H .C	1 L	1 L	17.05.2018	8:00 AM
16	Nurshams Health center	Tulkarem	N. H. C	1 L	1 L	18.05.2018	8:15 AM
17	Monia Sabbah	Tulkarem	M. Sab	1 L	1 L	22.09.2018	8;30 am
18	Mohammed Hafi	Tulkarem	M. haf	1 L	1 L	22.09.2018	9:00 AM
19	Tulkarem Health center	Tulkarem	T. H .C	1 L	1 L	17.08.2018	8:30 AM
20	Nurshams Health center	Tulkarem	N. H. C	1 L	1 L	9.10.2018	8:00 AM
21	Abd alkarem Abu sharkh	Hebron	AB. SH	1 L	1 L	10.02.2018	9:00 AM
22	Usama Zallom	Hebron	U. Z	1 L	1 L	10.02.2018	9:30 AM
23	AQU Surgery	AQU Abu Deis	QUF1	1 L	1 L	18.02.2018	9:15 AM
24	AQU Surgery	AQU Abu Deis	QUF1	1 L	1 L	30.9.2018	8:30 AM

25	AQU periodontology	AQU Abu Deis	QUF2	1 L	1 L	19.02.2018	9:15 AM
26	AQU periodontology	AQU Abu Deis	QUF2	1 L	1 L	19.02.2018	9:30 AM
27	AQU periodontology	AQU Abu Deis	QUF2	1 L	1 L	30.9.2018	8:40 AM
28	AQU periodontology	AQU Abu Deis	QUF2	1 L	1 L	30.9.2018	9:00 AM
29	AQU Neurosurgery	AQU Abu Deis	QUF3	1 L	1 L	24.02.2018	8:30 AM
30	AQU Neurosurgery	AQU Abu Deis	QUF3	1 L	1 L	30.9.2018	8:30 AM
31	AQU Neurosurgery	AQU Abu Deis	QUF3	1 L	1 L	30.9.2018	9:00 AM
32	AAUP G Clinics	Jenin	G 1	1 L	1 L	28.04.2018	8:00 AM
33	AAUP G Clinics	Jenin	G 2	1 L	1 L	28.04.2018	8:15 AM
34	AAUP G Clinics	Jenin	G 9	1 L	1 L	28.04.2018	8:30 AM
35	AAUP G Clinics	Jenin	G 10	1 L	1 L	28.04.2018	8:45 AM
36	AAUP G Clinics	Jenin	G 11	1 L	1 L	29.04.2018	8:30 AM
37	AAUP G Clinics	Jenin	G 12	1 L	1 L	29.04.2018	8:45 AM
38	AAUP G Clinics	Jenin	G 13	1 L	1 L	29.04.2018	9:00 AM
39	AAUP G Clinics	Jenin	G 14	1 L	1 L	29.04.2018	9:15 AM
Total				39	39		

Appendix C

DUWL samples collection							
No	Name	Area	Sample design	*Legionella* count	DNA extraction	Date of collection	Time
1	Zaid Atabeh	Nablus	Z. A	1 L	1 L	2.02.2018	10:30 AM
2	Tayser Alnana	Nablus	T. N	1 L	1 L	4.02.208	11:00 AM
3	Mohammed Altammam	Nablus	M. T	1 L	1 L	4.02.2018	11:30 AM

4	Fadi Daghlas	Nablus	F. D	1 L	1 L	2.02.2018	10:00 AM
5	Saed Habash	Nablus	S. H	1 L	1 L	4.02.2108	8:30 AM
6	Mosab jorishi	Nablus	M. SH	1 L	1 L	8.02.2018	10:00 AM
7	Amera Mesleh Sartawi	Nablus	A. Sar	1 L	1 L	8.02.2018	10:30 AM
8	Ammar Halaweh	Nablus	A. Haw	1 L	1 L	27.08.2018	9:00 AM
9	Arafat Alwazani	Nablus	A. W	1 L	1 L	2.09.2018	9:15 AM
10	Alaa Yasen	Nablus	A. Y	1 L	1 L	2.09.2018	9:30 AM
11	Omar Alzaghah	Nablus	O. Z	1 L	1 L	2.09.2018	9:00 AM
12	Adib Halob	Tulkarem	A. H	1 L	1 L	12.02.2018	9:15 AM
13	Ala Alsheikh	Tulkarem	A. SH	1 L	1 L	12.02.2018	9:30am
14	Moath Abu Baker	Tulkarem	M. AB	1 L	1 L	12.02.2018	9:45 AM
15	Tulkarem	Tulkarem	T. H .C	1 L	1 L	17.05.2018	8:00 AM
16	Nurshams Health center	Tulkarem	N. H. C	1 L	1 L	9.10.2018	8:00 AM
17	Monia Sabbah	Tulkarem	M. Sab	1 L	1 L	22.09.2018	8;30 am
18	Mohammed Hafi	Tulkarem	M. haf	1 L	1 L	22.09.2018	9:00 AM
19	Tulkarem Health center	Tulkarem	T. H .C	1 L	1 L	17.08.2018	8:30 AM
20	Abd alkarem Abu sharkh	Hebron	AB. SH	1 L	1 L	10.02.2018	9:00 AM
21	Usama Zallom	Hebron	U. Z	1 L	1 L	10.02.2018	9:30 AM
22	AQU Surgery	AQU Abu Deis	QUF1	1 L	1 L	18.02.2018	9:15 AM
23	AQU Surgery	AQU Abu Deis	QUF1	1 L	1 L	30.9.2018	8:30 AM
24	AQU periodontology	AQU Abu Deis	QUF2	1 L	1 L	19.02.2018	9:15 AM
25	AQU periodontology	AQU Abu Deis	QUF2	1 L	1 L	19.02.2018	9:30 AM
26	AQU periodontology	AQU Abu Deis	QUF2	1 L	1 L	30.9.2018	8:40 AM

27	AQU periodontology	AQU Abu Deis	QUF2	1 L	1 L	30.9.2018	9:00 AM
28	AQU Neurosurgery	AQU Abu Deis	QUF3	1 L	1 L	24.02.2018	8:30 AM
29	AQU Neurosurgery	AQU Abu Deis	QUF3	1 L	1 L	30.9.2018	8:30 AM
30	AQU Neurosurgery	AQU Abu Deis	QUF3	1 L	1 L	30.9.2018	9:00 AM
31	AAUP G Clinics	Jenin	G 1	1 L	1 L	28.04.2018	8:00 AM
32	AAUP G Clinics	Jenin	G 2	1 L	1 L	28.04.2018	8:15 AM
33	AAUP G Clinics	Jenin	G 9	1 L	1 L	28.04.2018	8:30 AM
34	AAUP G Clinics	Jenin	G 10	1 L	1 L	28.04.2018	8:45 AM
35	AAUP G Clinics	Jenin	G 11	1 L	1 L	29.04.2018	8:30 AM
36	AAUP G Clinics	Jenin	G 12	1 L	1 L	29.04.2018	8:45 AM
37	AAUP G Clinics	Jenin	G 13	1 L	1 L	29.04.2018	9:00 AM
38	AAUP G Clinics	Jenin	G 14	1 L	1 L	29.04.2018	9:15 AM
39	AQU Surgery	AQU Abu Deis	QUF1	1 L 1 L	1 L 1 L	30.9.2018	8:30 AM
40	AQU Neurosurgery	AQU Abu Deis	QUF3	1 L 1 L	1 L 1 L	30.9.2018	8:40 AM
41	AQU periodontology	AQU Abu Deis	QUF2	1 L 1 L	1 L 1 L	30.9.2018	9:00 AM
42	AQU Pediatric	AQU Abu Deis	QUF42	1 L 1 L	1 L 1 L	30.9.2018	9:15 AM
43	AAUP G Clinics	Jenin	G 1	1 L	1 L	15.07.2018	8:30 AM
44	AAUP G Clinics	Jenin	G 2	1 L	1 L	15.07.2018	8:40 AM
45	AAUP G Clinics	Jenin	G 9	1 L	1 L	15.07.2018	8:50 AM
46	AAUP G Clinics	Jenin	G 10	1 L	1 L	15.07.2018	9:00 AM

47	AAUP G Clinics	Jenin	G 11	1 L	1 L	16.07.2018	8:30 AM
48	AAUP G Clinics	Jenin	G 12	1 L	1 L	16.07.2018	8:45 AM
49	AAUP G Clinics	Jenin	G 13	1 L	1 L	16.07.2018	9:00 AM
50	AAUP G Clinics	Jenin	G 14	1 L	1 L	16.07.2018	9:15 AM
Total				50	50		

Appendix C

Name	Area	Sample Design	Cultre tab biofilm	DUWL cultre biofilm	Tab biofilm DNA extraction	DUWL DNA extraction	Date	Time
Zaid Atabeh	Nablus	Z. A	1	1	1	1	2.02.2018	10:30 AM
Tayser Alnana	Nablus	T. N	1	1	1	1	4.02.208	11:00 AM
Mohammed Altammam	Nablus	M. T	1	1	1	1	4.02.2018	11:30 AM
Fadi Daghlas	Nablus	F. D	1	1	1	1	2.02.2018	10:00 AM
Saed Habash	Nablus	S. H	1	1	1	1	4.02.2108	8:30 AM
Mosab jorishi	Nablus	M. SH	1	1	1	1	8.02.2018	10:00 AM
Amera Mesleh Sartawi	Nablus	A. Sar	1	1	1	1	8.02.2018	10:30 AM
Ammar Halaweh	Nablus	A. Haw	1	1	1	1	27.08.2018	9:00 AM
Arafat Alwazani	Nablus	A. W	1	1	1	1	2.09.2018	9:15 AM
Alaa Yasen	Nablus	A. Y	1	1	1	1	2.09.2018	9:30 AM
Omar Alzaghah	Nablus	O. Z	1	1	1	1	2.09.2018	9:00 AM
Yahya Zanoun	Nablus	Y. Z		1		1		
Adib Halob	Tulkarem	A. H	1	1	1	1	12.02.2018	9:15 AM
Ala Alsheikh	Tulkarem	A. SH	1	1	1	1	12.02.2018	9:30am
Moath Abu Baker	Tulkarem	M. AB	1	1	1	1	12.02.2018	9:45 AM
Nurshams Health center	Tulkarem	N. H. C	1	1	1	1	9.10.2018	8:00 AM
Monia Sabbah	Tulkarem	M. Sab	1	1	1	1	22.09.2018	8;30 am
Mohammed Hafi	Tulkarem	M. haf	1	1	1	1	22.09.2018	9:00 AM
Tulkarem Health center	Tulkarem	T. H .C	1	1	1	1	17.08.2018	8:30 AM
Abd alkarem abu sharkh	Hebron	AB. SH	1	1	1	1	10.02.2018	9:00 AM

Usama Zallom	Hebron	U. Z	1	1	1	1	10.02.2018	9:30 AM
AQU Surgery	AQU Abu Deis	QUF1	1	1	1	1	18.02.2018	9:15 AM
AQU Surgery	AQU Abu Deis	QUF1	1	1	1	1	30.9.2018	8:30 AM
AQU periodontology	AQU Abu Deis	QUF2	1	1	1	1	19.02.2018	9:15 AM
AQU periodontology	AQU Abu Deis	QUF2	1	1	1	1	19.02.2018	9:30 AM
a	AQU Abu Deis	QUF2	1	1	1	1	30.9.2018	8:40 AM
AQU Neurosurgery	AQU Abu Deis	QUF3	1	1	1	1	30.9.2018	9:00 AM
AQU Neurosurgery	AQU Abu Deis	QUF3	1	1	1	1	24.02.2018	8:30 AM
AQU Neurosurgery	AQU Abu Deis	QUF3	1	1	1	1	24.02.2018	8:45 AM
AQU pediatric	AQU Abu Deis	Pedo 1	1	1		1	30.9.2018	9:00 AM
AQU pediatric	AQU Abu Deis	Pedo 2+3		1		1	30.9.2018	9:00 AM
AQU pediatric	AQU Abu Deis	Pedo 4+5		1		1	30.9.2018	9:00 AM
AQU pediatric	AQU Abu Deis	Pedo 6		1		1	30.9.2018	9:00 AM
AQU pediatric	AQU Abu Deis	Pedo 7		1		1	30.9.2018	9:00 AM
AQU pediatric	AQU Abu Deis	Pedo 8		1		1	30.9.2018	9:00 AM
AAUP G Clinics	Jenin	G 1	1	1	1	1	28.04.2018	8:00 AM
AAUP G Clinics	Jenin	G 2	1	1	1	1	28.04.2018	8:15 AM
AAUP G Clinics	Jenin	G 9	1	1	1	1	28.04.2018	8:30 AM
AAUP G Clinics	Jenin	G 10	1	1	1	1	28.04.2018	8:45 AM
AAUP G Clinics	Jenin	G 11	1	1	1	1	29.04.2018	8:30 AM
AAUP G Clinics	Jenin	G 12	1	1	1	1	29.04.2018	8:45 AM
AAUP G Clinics	Jenin	G 13	1	1	1	1	29.04.2018	9:00 AM
AAUP G Clinics	Jenin	G 14	1	1	1	1	29.04.2018	9:15 AM
AAUP G Clinics	Jenin	G 1	1	1	1	1	15.07.2018	8:30 AM
AAUP G Clinics	Jenin	G 2	1	1	1	1	15.07.2018	8:40 AM
AAUP G Clinics	Jenin	G 9	1	1	1	1	15.07.2018	8:50 AM
AAUP G Clinics	Jenin	G 10	1	1	1	1	15.07.2018	9:00 AM
AAUP G Clinics	Jenin	G 11	1	1	1	1	16.07.2018	8:30 AM
AAUP G Clinics	Jenin	G 12	1	1	1	1	16.07.2018	8:45 AM
AAUP G Clinics	Jenin	G 13	1	1	1	1	16.07.2018	9:00 AM
AAUP G Clinics	Jenin	G 14	1	1	1	1	16.07.2018	9:15 AM
Total			45			51		

Appendix D

Tab water Culture				Culture	Serogroups
Zaid Atabeh	Nablus	Z.A TW	Tap water	(-)	
Tayser Alnana	Nablus	T.N TW	Tap water	(-)	
Mohammed Altammam	Nablus	M.T TW	Tap water	(-)	
Fadi Daghlas	Nablus	F. D TW	Tap water	(-)	
Saed Habash	Nablus	S.H TW	Tap water	(-)	
Saed Habash	Nablus	S.H TW	Tap water	(-)	
Mosab jorishi	Nablus	M.SH TW	Tap water	(-)	
Amera Mesleh	Nablus	A.M TW	Tap water	(-)	
Abd Alkarem Abu Sharkh	Hebron	AK.SH TW	Tap water	(-)	
Usama Zallom	Hebron	U.Z TW	Tap water	(-)	
Adib Haloub	Tulkarem	Z.H TW	Tap water	(-)	
Alaa Alsheikh	Tulkarem	A.SH TW	Tap water	(-)	
Moath Abu Baker	Tulkarem	M,AB TW	Tap water	(-)	
AQU Surgery	Abu Deis	QU F1 TW	Tap water	(+)	Sg 1
AQU Surgery	Abu Deis	QU F1 TW	Tap water	(-)	
AQU Periodontology	Abu Deis	QU F2 TW	Tap water	(-)	
AQU Periodontology	Abu Deis	QU F2 TW	Tap water	(-)	
AQU Periodontology	Abu Deis	QU F2 TW	Tap water	(-)	
AQU Periodontology	Abu Deis	QU F2 TW	Tap water	(+)	Sg 1
AQU Neurosurgery	Abu Deis	QU F3 TW	Tap water	(-)	
AQU Neurosurgery	Abu Deis	QU F3 TW	Tap water	(-)	
AQU Neurosurgery	Abu Deis	QU F3 TW	Tap water	(+)	Sg 1
AAUP Jenin	Jenin	G14 TW	Tap water	(-)	
AAUP Jenin	Jenin	G13TW	Tap Water	(-)	
AAUP Jenin	Jenin	G12 TW	TapWater	(-)	
AAUP Jenin	Jenin	G11 TW	Tap Water	(-)	
AAUP Jenin	Jenin	G10 TW	Tap Water	(-)	
AAUP Jenin	Jenin	G9 TW	Tap Water	(+)	Sg 1
AAUP Jenin	Jenin	G2 TW	Tap Water	(-)	
AAUP Jenin	Jenin	G1TW	Tap Water	(-)	
Tulkarem Health Center	Tulkarem	THC .TW	Tap water	(+)	Sg 1
Tulkarem Health Center	Tulkarem	THC .TW	Tap water	(-)	
Ammar Halaweh	Nablus	A.H .TW	Tap water	(+)	Sg 2-14
Ammar Halaweh	Nablus	A.H .TW	Tap water	(-)	
Arafat Alwazani	Nablus	A.W TW	Tap water	(-)	
Nurshams Health center	Tulkarem	NHC	Tap water	(+)	Sg 1
Monia Sabbah	Tulkarem	M.S Tw	Tap Water	(-)	
Monia Sabbah	Tulkarem	M.S TW	Tap Water	(-)	
Mohd Hafi	Tulkarem	M.H .Tw	Tap water	(-)	

Appendix D

DUWL water Culture				Culture	Serogroups
Zaid Atabeh	Nablus	Z.A DW	D U Water	(-)	
Tayser Alnana	Nablus	T.N DW	D U Water	(-)	
Mohammed Altammam	Nablus	M.T DW	D U Water	(-)	
Fadi Daghlas	Nablus	F.D DW	D U Water	(-)	
Saed Habash	Nablus	S.H DW	D U Water	(-)	
Saed Habash	Nablus	S.H DW	D U Water	(-)	
Mosab jorishi	Nablus	M.SH DW	D U Water	(-)	
Amera Mesleh	Nablus	A.M DW	D U Water	(-)	
Abd Alkarem Abu Sharkh	Hebron	AK.SH DW	D U Water	(-)	
Usama Zallom	Hebron	U.Z DW	D U Water	(-)	
Adib Haloub	Tulkarem	Z.H DW	D U Water	(-)	
Alaa Al-sheikh	Tulkarem	A.SH DW	D U Water	(-)	
Moath Abu Baker	Tulkarem	M.AB DW	D U Water	(-)	
AQU Surgery	Abu Deis	QU F1DW	D U Water	(-)	
AQU Surgery	Abu Deis	QU F1DW	D U Water	(-)	
AQU Periodontology	Abu Deis	QU F2 DW	D U Water	(-)	
AQU Periodontology	Abu Deis	QU F2 DW	D U Water	(-)	
AQU Periodontology	Abu Deis	QU F2 DW	D U Water	(-)	
AQU Neurosurgery	Abu Deis	QU F3 DW	D U Water	(-)	
AQU Neurosurgery	Abu Deis	QU F3 DW	D U Water	(-)	
AQU Neurosurgery	Abu Deis	QU F3 DW	D U Water	(-)	
AQU Neurosurgery	Abu Deis	QU F3 DW	D U Water	(-)	
AAM U Jenin	Jenin	G14 DW	D U Water	(-)	
AAM U Jenin	Jenin	G13 DW	D U Water	(-)	
AAM U Jenin	Jenin	G12 DW	D U Water	(-)	
AAM U Jenin	Jenin	G11 DW	D U Water	(-)	
AAM U Jenin	Jenin	G10 DW	D U Water	(+)	Sg 1
AAM U Jenin	Jenin	G9 DW	D U Water	(-)	
AAM U Jenin	Jenin	G1 DW	D U Water	(-)	
AAM U Jenin	Jenin	G2 DW	D U Water	(-)	
Tulkarem Health Center	Tulkarem	THC .DW	D U Water	(+)	Sg 1
Tulkarem Health Center	Tulkarem	THC .DW	D U Water	(+)	Sg 2-14
Ammar Halaweh	Nablus	A.H .DW	DU water	(-)	
Ammar Halaweh	Nablus	A.H .DW	DU water	(-)	
Arafat Alwazani	Nablus	A.W DW	DU water	(-)	
Mohd Hafi	Tulkarem	M.H .DW	DU water	(-)	
Monia Sabbah	Tulkarem	M.S DW	DU Water	(-)	
Nurshams Health center	Tulkarem	NHC	DU water	(-)	
AQU surgery	Abu Deis	Q1F1 D	D.U Water	(-)	
AQU periodontology	Abu Deis	Q2F2 D	D.U Water	(-)	
AQU neurosurgery	Abu Deis	Q3F3 D	D.U Water	(+)	Sg 1
AQU pediatric	Abu Deis	Q4F4 D	D.U Water	(+)	Sg 1
AAM U Jenin	Jenin	G14 DW	D U Water	(-)	
AAM U Jenin	Jenin	G13 DW	D U Water	(-)	
AAM U Jenin	Jenin	G12 DW	D U Water	(-)	
AAM U Jenin	Jenin	G11 DW	D U Water	(-)	
AAM U Jenin	Jenin	G10 DW	D U Water	(-)	
AAM U Jenin	Jenin	G9 DW	D U Water	(+)	Sg 1
AAM U Jenin	Jenin	G2 DW	D U Water	(-)	
AAM U Jenin	Jenin	G1 DW	D U Water	(-)	

Appendix D

Tab Biofilm Culture				Culture	Serogroups
Zaid Atabeh	Nablus	Z.A TB	Tap biofilm	(-)	
Tayser Alnana	Nablus	T.N TB	Tap biofilm	(-)	
Mohammed Altammam	Nablus	M.T TB	Tap biofilm	(-)	
Fadi Daghlas	Nablus	F.D TB	Tap biofilm	(-)	
Saed Habash	Nablus	S.H TB	Tap biofilm	(-)	
Mosab jorishi	Nablus	M.SH TB	Tap biofilm	(-)	
Amera Mesleh	Nablus	A.M TB	Tap biofilm	(-)	
Abd Alkarem Abu Sharkh	Hebron	AK.SH TB	Tap biofilm	(-)	
Usama Zallom	Hebron	U.Z TB	Tap biofilm	(-)	
Adib Haloub	Tulkarem	Z.H TB	Tap biofilm	(-)	
Alaa Al-sheikh	Tulkarem	A.SH TB	Tap biofilm	(-)	
Moath Abu Baker	Tulkarem	M.AB TB	Tap biofilm	(-)	
AQU Surgery	Abu Deis	QU F1 TB	Tap biofilm	(-)	
AQU Surgery	Abu Deis	QU F1 TB	Tap biofilm	(-)	
AQU Neurosurgery	Abu Deis	QU F3 TB	Tap biofilm	(-)	
AQU Neurosurgery	Abu Deis	QU F3TB	Tap biofilm	(-)	
AQU Neurosurgery	Abu Deis	QU F3 TB	Tap biofilm	(-)	
AAUP Jenin	Jenin	G14 TB	Tap biofilm	(-)	
AAUP Jenin	Jenin	G13 TB	Tap biofilm	**(-)**	
AAUP Jenin	Jenin	G12 TB	Tap biofilm	(-)	
AAUP Jenin	Jenin	G11 TB	Tap biofilm	(-)	
AAUP Jenin	Jenin	G10 TB	Tap biofilm	(+)	Sg 2-14
AAUP Jenin	Jenin	G9 TB	Tap biofilm	(-)	
AAUP Jenin	Jenin	G1 TB	Tap biofilm	(-)	
AAUP Jenin	Jenin	G2 TB	Tap biofilm	(+)	Sg 1
Nurshams Health center	Tulkarem	NHC TB	Tap biofilm	(-)	
Tulkarem Health Center	Tulkarem	THC .TB	Tap biofilm	(+)	Sg 1
Ammar Halaweh	Nablus	A.H. TB	Tap biofilm	(-)	
Omar Alzaghah	Nablus	O.Z TB	Tap biofilm	(+)	Sg 1
Alaa Yasen	Nablus	A.Y TB	Tap biofilm	(+)	Sg 1
Yahya Alzanoun	Nablus	Y.Z TB	Tap biofilm	(+)	Sg 1
Tayser Nana	Nablus	T.N TB	Tap biofilm	(-)	
Monia Sabbah	Tulkarem	M.S DW	Tap Biofilm	(+)	Sg 1
Mohd Hafi	Tulkarem	M.H .TB	Tap biofilm	(-)	
AQU periodontology	Abu Deis	QU F2 TB1	Tap biofilm	(-)	
AQU periodontology	Abu Deis	QU F2 TB3	Tap biofilm	(+)	Sg 1
AQU periodontology	Abu Deis	QU F2 TB5	Tap biofilm	(-)	
AAUP Jenin	Jenin	G14 TB	Tap biofilm	(-)	
AAUP Jenin	Jenin	G13 TB	Tap biofilm	**(-)**	
AAUP Jenin	Jenin	G12 TB	Tap biofilm	(-)	
AAUP Jenin	Jenin	G11 TB	Tap biofilm	(+)	Sg 1
AAUP Jenin	Jenin	G10 TB	Tap biofilm	(-)	
AAUP Jenin	Jenin	G9 TB	Tap biofilm	(-)	
AAUP Jenin	Jenin	G1 TB	Tap biofilm	(-)	
AAUP Jenin	Jenin	G2 TB	Tap biofilm	(-)	

Appendix D

DUWL biofilm Culture				Culture	Serogroups
Zaid Atabeh	Nablus	Z.A DB	D U Biofilm	(-)	
Tayser Alnana	Nablus	T.N DB	D U Biofilm	(-)	
Mohammed Altammam	Nablus	M.T DB	D U Biofilm	(-)	
Fadi DBghlas	Nablus	F.D DB	D U Biofilm	(-)	
Saed Habash	Nablus	S.H DB	D U Biofilm	(-)	
Mosab jorishi	Nablus	M.SH DB	D U Biofilm	(-)	
Amera Mesleh	Nablus	A.M DB	D U Biofilm	(-)	
Abd Alkarem abu Sharkh	Hebron	Ak.SH DB	D U Biofilm	(-)	
Usama Zallom	Hebron	U.Z DB	D U Biofilm	(-)	
Adib Haloub	Tulkarem	Z.H DB	D U Biofilm	(-)	
Alaa Al-sheikh	Tulkarem	A.SH DB	D U Biofilm	(-)	
Moath Abu Baker	Tulkarem	M.AB DB	D U Biofilm	(-)	
AQU surgery	Abu Deis	QU F1 DB	D U Biofilm	(-)	
AQU surgery	Abu Deis	QU F1 DB	DU Biofilm	(-)	
AQU periodontology	Abu Deis	QU F2 DB	DU Biofilm	(-)	
AQU periodontology	Abu Deis	QU F2 DB	D U Biofilm	(-)	
AQU periodontology	Abu Deis	QU F2 DB	D U Biofilm	(-)	
AQU neurosurgery	Abu Deis	QU F3 DB	D U Biofilm	(-)	
AQU neurosurgery	Abu Deis	QU F3 DB	D U Biofilm	(-)	
AAUP Jenin	Jenin	G14 DB	D U Biofilm	(-)	
AAUP Jenin	Jenin	G13 DB	D U Biofilm	(-)	
AAUP Jenin	Jenin	G12 DB	D U Biofilm	(-)	
AAUP Jenin	Jenin	G11 DB	D U Biofilm	(-)	
AAUP Jenin	Jenin	G10 DB	D U Biofilm	(+)	Sg 2-14
AAUP Jenin	Jenin	G9 DB	D U Biofilm	(-)	
AAUP Jenin	Jenin	G1 DB	D U Biofilm	(-)	
AAUP Jenin	Jenin	G2 DB	D U Biofilm	(-)	
Tulkarem Health Center	Tulkarem	THC .DB	D U Biofilm	(-)	
Omar Alzaghah	Nablus	O.Z DB	DU Biofilm	(+)	Sg 1
Arafat Alwazani	Nablus	A.W DB	DU Biofilm	(-)	

Alaa Yasen	Nablus	A.Y .DB	DU Biofilm	(-)	
Yahya Alzanoun	Nablus	Y.Z DB	DU Biofilm	(-)	
Tayser Nana	Nablus	T.N DB	DU Biofilm	(-)	
Mohd Hafi	Tulkarem	M.H DB	DU Biofilm	(-)	
Monia Sabbah	Tulkarem	M.S DW	DU Biofilm	(-)	
AQU pediatric	Abu Deis	QU F4 TB PEDO 1	DU Biofilm	(-)	
AQU pediatric	Abu Deis	QU F4 TB PEDO 1	DU Biofilm	(-)	
AQU pediatric	Abu Deis	QU F4 TB PEDO 2+3	DU Biofilm	(-)	
AQU pediatric	Abu Deis	QU F4TS PEDO 4+5	DU Biofilm	(+)	Sg 2-14
AQU pediatric	Abu Deis	QU F4TB PEDO 6	DU Biofilm	(-)	
AQU pediatric	Abu Deis	QU F4TB PEDO 7	DU Biofilm	(-)	
AQU pediatric	Abu Deis	QU F4TB PEDO 8	DU Biofilm	(-)	
Nurshams Health center	Tulkarem	NHC	D.U Swap	(+)	Sg 1
AAUP Jenin	Jenin	G14 DB	D U Biofilm	(-)	
AAUP Jenin	Jenin	G13 DB	D U Biofilm	(+)	Sg 1
AAUP Jenin	Jenin	G12 DB	D U Biofilm	(-)	
AAUP Jenin	Jenin	G11 DB	D U Biofilm	(-)	
AAUP Jenin	Jenin	G10 DB	D U Biofilm	(-)	
AAUP Jenin	Jenin	G9 DB	D U Biofilm	(+)	Sg 1
AAUP Jenin	Jenin	G1 DB	D U Biofilm	(-)	
AAUP Jenin	Jenin	G2 DB	D U Biofilm	(-)	

Appendix E

Dr .name	Site of isolation	Sample designation	sample type	16S rRNA PCR (Primers Name)			Culture
				Com	Lgsp	L1	
Zaid Atabeh	Nablus	Z.A TW	Tap water	(+)	(+)	(+)	(-)
Tayser Alnana	Nablus	T.N TW	Tap water	(+)	(+)	(+)	(-)
Mohammed Altammam	Nablus	M.T TW	Tap water	(+)	(+)	(+)	(-)
Fadi Daghlas	Nablus	F. D TW	Tap water	(+)	(+)	(+)	(-)
Saed Habash	Nablus	S.H TW	Tap water	(+)	(+)	(+)	(-)
Saed Habash	Nablus	S.H TW	Tap water	(+)	(+)	(+)	(-)
Mosab jorishi	Nablus	M.SH TW	Tap water	(+)	(+)	(+)	(-)
Amera Mesleh	Nablus	A.M TW	Tap water	(+)	(+)	(+)	(-)
Abd Alkarem abu Sharkh	Hebron	AK.SH TW	Tap water	(+)	(+)	(+)	(-)

Usama Zallom	Hebron	U.Z TW	Tap water	(+)	(+)	(-)	(-)
Adib Haloub	Tulkarem	Z.H TW	Tap water	(+)	(+)	(-)	(-)
Alaa Alsheikh	Tulkarem	A.SH TW	Tap water	(+)	(+)	(+)	(-)
Moath Abu Baker	Tulkarem	M,AB TW	Tap water	(+)	(+)	(-)	(-)
AQU Surgery	Abu Deis	QU F1 TW	Tap water	(+)	(+)	(+)	(+)
AQU Surgery	Abu Deis	QU F1 TW	Tap water	(+)	(-)	(-)	(-)
AQU Periodontology	Abu Deis	QU F2 TW	Tap water	(+)	(+)	(+)	(-)
AQU Periodontology	Abu Deis	QU F2 TW	Tap water	(+)	(+)	(+)	(-)
AQU Periodontology	Abu Deis	QU F2 TW	Tap water	(+)	(-)	(-)	(-)
AQU Periodontology	Abu Deis	QU F2 TW	Tap water	(+)	(+)	(+)	(+)
AQU Neurosurgery	Abu Deis	QU F3 TW	Tap water	(+)	(+)	(+)	(-)
AQU Neurosurgery	Abu Deis	QU F3 TW	Tap water	(+)	(-)	(-)	(-)
AQU Neurosurgery	Abu Deis	QU F3 TW	Tap water	(+)	(+)	(+)	(+)
AAUP Jenin	Jenin	G14 TW	Tap water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G13TW	Tap Water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G12 TW	TapWater	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G11 TW	Tap Water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G10 TW	Tap Water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G9 TW	Tap Water	(+)	(+)	(+)	(+)
AAUP Jenin	Jenin	G2 TW	Tap Water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G1TW	Tap Water	(+)	(-)	(-)	(-)
Tulkarem Health Center	Tulkarem	THC .TW	Tap waer	(+)	(+)	(+)	(+)
Tulkarem Health Center	Tulkarem	THC .TW	Tap waer	(+)	(+)	(+)	(-)
Ammar Halaweh	Nablus	A.H .TW	Tap water	(+)	(+)	(+)	(+)
Ammar Halaweh	Nablus	A.H .TW	Tap water	(+)	(+)	(+)	(-)
Arafat Alwazani	Nablus	A.W TW	Tap water	(+)	(+)	(+)	(-)
Nurshams Health center	Tulkarem	NHC	Tap water	(+)	(+)	(+)	(+)
Monia Sabbah	Tulkarem	M.S Tw	Tap Water	(+)	(+)	(+)	(-)
Monia Sabbah	Tulkarem	M.S TW	Tap Water	(+)	(+)	(+)	(-)
Mohd Hafi	Tulkarem	M.H .Tw	Tap water	(+)	(+)	(+)	(-)
Zaid Atabeh	Nablus	Z.A DW	D U Water	(+)	(-)	(-)	(-)
Tayser Alnana	Nablus	T.N DW	D U Water	(+)	(+)	(+)	(-)
Mohammed Altammam	Nablus	M.T DW	D U Water	(+)	(+)	(+)	(-)
Fadi Daghlas	Nablus	F.D DW	D U Water	(+)	(-)	(-)	(-)
Saed Habash	Nablus	S.H DW	D U Water	(+)	(-)	(-)	(-)
Saed Habash	Nablus	S.H DW	D U Water	(+)	(-)	(-)	(-)
Mosab jorishi	Nablus	M.SH DW	D U Water	(+)	(+)	(+)	(-)
Amera Mesleh	Nablus	A.M DW	D U Water	(+)	(+)	(+)	(-)
Abd Alkarem abu Sharkh	Hebron	AK.SH DW	D U Water	(+)	(-)	(-)	(-)
Usama Zallom	Hebron	U.Z DW	D U Water	(+)	(-)	(-)	(-)
Adib Haloub	Tulkarem	Z.H DW	D U Water	(+)	(-)	(-)	(-)
Alaa Al-sheikh	Tulkarem	A.SH DW	D U Water	(+)	(+)	(+)	(-)
Moath Abu Baker	Tulkarem	M.AB DW	D U Water	(+)	(-)	(-)	(-)
AQU Surgery	Abu Deis	QU F1DW	D U Water	(+)	(-)	(-)	(-)

AQU Surgery	Abu Deis	QU F1DW	D U Water	(+)	(+)	(+)	(-)
AQU Periodontology	Abu Deis	QU F2 DW	D U Water	(+)	(+)	(+)	(-)
AQU Periodontology	Abu Deis	QU F2 DW	D U Water	(+)	(+)	(+)	(-)
AQU Periodontology	Abu Deis	QU F2 DW	D U Water	(-)	(-)	(-)	(-)
AQU Neurosurgery	Abu Deis	QU F3 DW	D U Water	(-)	(-)	(-)	(-)
AQU Neurosurgery	Abu Deis	QU F3 DW	D U Water	(+)	(-)	(-)	(-)
AQU Neurosurgery	Abu Deis	QU F3 DW	D U Water	(+)	(+)	(+)	(-)
AQU Neurosurgery	Abu Deis	QU F3 DW	D U Water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G14 DW	D U Water	(+)	(-)	(-)	(-)
AAUP Jenin	Jenin	G13 DW	D U Water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G12 DW	D U Water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G11 DW	D U Water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G10 DW	D U Water	(+)	(+)	(+)	(+)
AAUP Jenin	Jenin	G9 DW	D U Water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G1 DW	D U Water	(+)	(-)	(-)	(-)
AAUP Jenin	Jenin	G2 DW	D U Water	(+)	(+)	(+)	(-)
Tulkarem Health Center	Tulkarem	THC .DW	D U Water	(+)	(+)	(+)	(+)
Tulkarem Health Center	Tulkarem	THC .DW	D U Water	(+)	(+)	(+)	(+)
Ammar Halaweh	Nablus	A.H .DW	DU water	(+)	(-)	(-)	(-)
Ammar Halaweh	Nablus	A.H .DW	DU water	(+)	(-)	(-)	(-)
Arafat Alwazani	Nablus	A.W DW	DU water	(+)	(-)	(-)	(-)
Mohd Hafi	Tulkarem	M.H .DW	DU water	(+)	(+)	(+)	(-)
Monia Sabbah	Tulkarem	M.S DW	DU Water	(+)	(+)	(+)	(-)
Nurshams Health center	Tulkarem	NHC	DU water	(+)	(+)	(+)	(-)
AQU surgery	Abu Deis	Q1F1 D	D.U Water	(+)	(-)	(-)	(-)
AQU Periodontology	Abu Deis	Q2F2 D	D.U Water	(+)	(-)	(-)	(-)
AQU Neurosurgery	Abu Deis	Q3F3 D	D.U Water	(+)	(+)	(+)	(+)
AQU Pediatric	Abu Deis	Q4F4 D	D.U Water	(+)	(+)	(+)	(+)
AAUP Jenin	Jenin	G14 DW	D U Water	(+)	(-)	(-)	(-)
AAUP Jenin	Jenin	G13 DW	D U Water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G12 DW	D U Water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G11 DW	D U Water	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G10 DW	D U Water	(+)	(-)	(-)	(-)
AAUP Jenin	Jenin	G9 DW	D U Water	(+)	(+)	(+)	(+)
AAUP Jenin	Jenin	G2 DW	D U Water	(+)	(-)	(-)	(-)
AAUP Jenin	Jenin	G1 DW	D U Water	(+)	(-)	(-)	(-)
Zaid Atabeh	Nablus	Z.A TB	Tap biofilm	(+)	(+)	(+)	(-)
Tayser Alnana	Nablus	T.N TB	Tap biofilm	(+)	(+)	(+)	(-)
Mohammed Altammam	Nablus	M.T TB	Tap biofilm	(+)	(+)	(+)	(-)
Fadi Daghlas	Nablus	F.D TB	Tap biofilm	(+)	(+)	(+)	(-)
Saed Habash	Nablus	S.H TB	Tap biofilm	(+)	(+)	(+)	(-)
Mosab jorishi	Nablus	M.SH TB	Tap biofilm	(+)	(+)	(+)	(-)

Amera Mesleh	Nablus	A.M TB	Tap biofilm	(+)	(+)	(+)	(-)
Abd Alkarem abu Sharkh	Hebron	AK.SH TB	Tap biofilm	(+)	(+)	(+)	(-)
Usama Zallom	Hebron	U.Z TB	Tap biofilm	(+)	(+)	(+)	(-)
Adib Haloub	Tulkarem	Z.H TB	Tap biofilm	(+)	(+)	(+)	(-)
Alaa Al-sheikh	Tulkarem	A.SH TB	Tap biofilm	(+)	(+)	(+)	(-)
Moath Abu Baker	Tulkarem	M.AB TB	Tap biofilm	(+)	(+)	(+)	(-)
AQU Surgery	Abu Deis	QU F1 TB	Tap biofilm	(+)	(+)	(+)	(-)
AQU Surgery	Abu Deis	QU F1 TB	Tap biofilm	(+)	(+)	(+)	(-)
AQU Neurosurgery	Abu Deis	QU F3 TB	Tap biofilm	(+)	(+)	(+)	(-)
AQU Neurosurgery	Abu Deis	QU F3TB	Tap biofilm	(+)	(+)	(+)	(-)
AQU Neurosurgery	Abu Deis	QU F3 TB	Tap biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G14 TB	Tap biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G13 TB	Tap biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G12 TB	Tap biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G11 TB	Tap biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G10 TB	Tap biofilm	(+)	(+)	(+)	(+)
AAUP Jenin	Jenin	G9 TB	Tap biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G1 TB	Tap biofilm	(+)	(-)	(-)	(-)
AAUP Jenin	Jenin	G2 TB	Tap biofilm	(+)	(+)	(+)	(+)
Nurshams Health center	Tulkarem	NHC TB	Tap biofilm	(+)	(+)	(+)	(-)
Tulkarem Health Center	Tulkarem	THC .TB	Tap biofilm	(+)	(+)	(+)	(+)
Ammar Halaweh	Nablus	A.H. TB	Tap biofilm	(+)	(+)	(+)	(-)
Omar Alzaghah	Nablus	O.Z TB	Tap biofilm	(+)	(+)	(+)	(+)
Alaa Yasen	Nablus	A.Y TB	Tap biofilm	(+)	(+)	(+)	(+)
Yahya Alzanoun	Nablus	Y.Z TB	Tap biofilm	(+)	(+)	(+)	(+)
Tayser Nana	Nablus	T.N TB	Tap biofilm	(+)	(+)	(+)	(-)
Monia Sabbah	Tulkarem	M.S DW	Tap Biofilm	(+)	(+)	(+)	(+)
Mohd Hafi	Tulkarem	M.H .TB	Tap biofilm	(+)	(+)	(+)	(-)
AQU Periodontology	Abu Deis	QU F2 TB1	Tap biofilm	(+)	(+)	(+)	(-)
AQU Periodontology	Abu Deis	QU F2 TB3	Tap biofilm	(+)	(+)	(+)	(+)
AQU Periodontology	Abu Deis	QU F2 TB5	Tap biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G14 TB	Tap biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G13 TB	Tap biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G12 TB	Tap biofilm	(+)	(+)	(+)	(-)

AAUP Jenin	Jenin	G11 TB	Tap biofilm	(+)	(+)	(+)	(+)
AAUP Jenin	Jenin	G10 TB	Tap biofilm	(+)	(-)	(-)	(-)
AAUP Jenin	Jenin	G9 TB	Tap biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G1 TB	Tap biofilm	(+)	(-)	(-)	(-)
AAUP Jenin	Jenin	G2 TB	Tap biofilm	(+)	(-)	(-)	(-)
Zaid Atabeh	Nablus	Z.A DB	D U Biofilm	(+)	(-)	(-)	(-)
Tayser Alnana	Nablus	T.N DB	D U Biofilm	(+)	(+)	(+)	(-)
Mohammed Altammam	Nablus	M.T DB	D U Biofilm	(+)	(+)	(+)	(-)
Fadi DBghlas	Nablus	F.D DB	D U Biofilm	(+)	(+)	(+)	(-)
Saed Habash	Nablus	S.H DB	D U Biofilm	(+)	(+)	(+)	(-)
Mosab jorishi	Nablus	M.SH DB	D U Biofilm	(+)	(-)	(-)	(-)
Amera Mesleh	Nablus	A.M DB	D U Biofilm	(+)	(+)	(+)	(-)
Abd Alkarem abu Sharkh	Hebron	Ak.SH DB	D U Biofilm	(+)	(-)	(-)	(-)
Usama Zallom	Hebron	U.Z DB	D U Biofilm	(+)	(-)	(-)	(-)
Adib Haloub	Tulkarem	Z.H DB	D U Biofilm	(+)	(+)	(-)	(-)
Alaa Al-sheikh	Tulkarem	A.SH DB	D U Biofilm	(+)	(+)	(+)	(-)
Moath Abu Baker	Tulkarem	M.AB DB	D U Biofilm	(+)	(+)	(+)	(-)
AQU surgery	Abu Deis	QU F1 DB	D U Biofilm	(+)	(+)	(+)	(-)
AQU surgery	Abu Deis	QU F1 DB	DU Biofilm	(+)	(+)	(+)	(-)
AQU Periodontology	Abu Deis	QU F2 DB	DU Biofilm	(+)	(+)	(+)	(-)
AQU Periodontology	Abu Deis	QU F2 DB	D U Biofilm	(+)	(+)	(+)	(-)
AQU Periodontology	Abu Deis	QU F2 DB	D U Biofilm	(+)	(+)	(+)	(-)
AQU Neurosurgery	Abu Deis	QU F3 DB	DU Biofilm	(+)	(-)	(-)	(-)
AQU Neurosurgery	Abu Deis	QU F3 DB	D U Biofilm	(+)	(+)	(+)	(-)
AQU Neurosurgery	Abu Deis	QU F3 DB	D U Biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G14 DB	D U Biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G13 DB	D U Biofilm	(+)	(+)	(+)	(+)
AAUP Jenin	Jenin	G12 DB	D U Biofilm	(+)	(-)	(-)	(-)
AAUP Jenin	Jenin	G11 DB	D U Biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G10 DB	D U Biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G9 DB	D U Biofilm	(+)	(+)	(+)	(+)
AAUP Jenin	Jenin	G1 DB	D U Biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G2 DB	D U Biofilm	(+)	(+)	(+)	(+)
Tulkarem Health Center	Tulkarem	THC .DB	D U Biofilm	(+)	(+)	(+)	(-)

Omar Alzaghah	Nablus	O.Z DB	DU Biofilm	(+)	(+)	(+)	(-)
Arafat Alwazani	Nablus	A.W DB	DU Biofilm	(+)	(+)	(-)	(-)
Alaa Yasen	Nablus	A.Y .DB	DU Biofilm	(+)	(+)	(+)	(-)
Yahya Alzanoun	Nablus	Y.Z DB	DU Biofilm	(+)	(+)	(+)	(-)
Tayser Nana	Nablus	T.N DB	DU Biofilm	(+)	(+)	(+)	(-)
Mohd Hafi	Tulkarem	M.H DB	DU Biofilm	(+)	(+)	(+)	(-)
Monia Sabbah	Tulkarem	M.S DW	DU Biofilm	(+)	(+)	(+)	(-)
AQU Pediatric	Abu Deis	QU F4 TB PEDO 1	DU Biofilm	(+)	(+)	(+)	(-)
AQU Pediatric	Abu Deis	QU F4 TB PEDO 2+3	DU Biofilm	(+)	(+)	(+)	(-)
AQU Pediatric	Abu Deis	QU F4TS PEDO 4+5	DU Biofilm	(+)	(+)	(+)	(-)
AQU Pediatric	Abu Deis	QU F4TB PEDO 6	DU Biofilm	(+)	(+)	(+)	(-)
AQU Pediatric	Abu Deis	QU F4TB PEDO 7	DU Biofilm	(+)	(+)	(+)	(-)
AQU Pediatric	Abu Deis	QU F4TB PEDO 8	DU Biofilm	(+)	(+)	(+)	(-)
Nurshams Health center	Tulkarem	NHC	D.U Biofilm	(+)	(+)	(+)	(+)
AAUP Jenin	Jenin	G14 DB	D U Biofilm	(+)	(-)	(-)	(-)
AAUP Jenin	Jenin	G13 DB	D U Biofilm	(+)	(+)	(+)	(+)
AAUP Jenin	Jenin	G12 DB	D U Biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G11 DB	D U Biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G10 DB	D U Biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G9 DB	D U Biofilm	(+)	(+)	(+)	(+)
AAUP Jenin	Jenin	G1 DB	D U Biofilm	(+)	(+)	(+)	(-)
AAUP Jenin	Jenin	G2 DB	D U Biofilm	(+)	(+)	(+)	(-)

Appendix F

Dr .name	Site of isolation	Sample des	sample type	temp	pH	conductivit	total iron	chlorine	nitrate	nitrite	ammonia	copper	phosphate	zinc	carbonate	total hardn
Zaid Atabeh	Nablus	ZA Tap water		21	7.9	683	0 mg	0.1 mg	10	0	0 mg	0 mg	0	<2	20 d	20 d
Zaid Atabeh	Nablus	Z.A DW	D U Water	22	8.8	59	0 mg	0	10	0	0	0	0	<2	10 d	20 d
Tayser Alnana	Nablus	T.N TW	Tap water	19	8.4	409	0	0	10	0	0	0	0	<2	5 d	15 d
Tayser Alnana	Nablus	T.N DW	D U Water	19	8.4	430	0	0	10	0	0	0	0	<2	5 d	15 d
Mohammed Altammar	Nablus	M.T TW	Tap water	23	8.7	616	0 mg	0 mg	10	0	0.5 mg	0	0	<2	10 d	20 d
Mohammed Altammar	Nablus	M.T DW	D U Water	24	8.8	614	0 mg	0 mg	10	0	0	0	0	<2	10 d	20 d
Fadi Daghlas	Nablus	F. D TW	Tap water	21	8.3	480	2 mg	0.1	10	0	0	0	0	0	10 d	15 d
Fadi Daghlas	Nablus	F.D DW	D U Water	22	8.3	741	2 mg	0.1	10	0	0	0	0	0	10 d	20 D
Saed Habash	Nablus	S.H TW	Tap water	22	8.6	601	2 MG	0.1	10	0	0	0	0	0	20 d	10 d
Saed Habash	Nablus	S.H DW	D U Water	22	8.7	609	2 mg	0	10	0	0	0	0	0	20 d	20 d
Mosab jorishi	Nablus	M.SH TW	Tap water	19	8.5	506	2 mg	0	10	0	0	0	0	<2	10 d	20 d
Mosab jorishi	Nablus	M.SH DW	D U Water	18	5.5	551	2 mg	0	10	0	0	0	0	<2	10 d	20 d
Amera Mesleh	Nablus	A.M TW	Tap water	24	8.8	453	2 mg	0.1	10	0	0	0	0	<2	5 d	20 d
Amera Mesleh	Nablus	A.M DW	D U Water	24	8.7	613	2 mg	0.1	10	0	0	0	0	<2	5 d	20 d
Abd Alkarem abu Shark	Hebron	AK.SH TW	Tap water	15	8.9	249	0 mg	0	10	0	0	0	0	<2	5 d	20 d
Abd Alkarem abu Shark	Hebron	AK.SH DW	D U Water	17	8.7	236	0 mg	0	10	0	0	0	0	<2	5 d	20 d
Usama Zallom	Hebron	U.Z TW	Tap water	18	8.6	534	0 mg	0	10	0	0	0	0	<2	10 d	20 d
Usama Zallom	Hebron	U.Z DW	D U Water	20	8.7	544	0 mg	0	10	0	0	0	0	<2	10 d	20 d
Adib Haloub	Tulkarem	Z.H TW	Tap water	24	8.8	819	2 mg	0.5	25	0	0	0	0	0	15 d	20 d
Adib Haloub	Tulkarem	Z.H DW	D U Water	24	8.7	984	2 mg	0.5	25	0	0	0	0	0	20 d	20 d
Alaa Alsheikh	Tulkarem	A.SH TW	Tap water	23	8.8	830	0	0.5	25	0	0	0	0	0	15 d	20 d
Alaa Alsheikh	Tulkarem	A.SH DW	D U Water	23	8.9	1020	0	0.5	25	0	0	0	0	0	15 d	20 d
Moath Abu Baker	Tulkarem	M,AB TW	Tap water	23	8.9	876	2 mg	0.5	25	0	0	0	0	0	10 d	20 d
Moath Abu Baker	Tulkarem	M.AB DW	D U Water	22	8.9	946	2 mg	0.5	25	0	0	0	0	0	15 d	20 d
AQU Surgery	Abu Deis	QU F1 TW	Tap water	17	9.1	508	0	0	0	0	0	0	0	0	10 d	15 d
AQU Surgery	Abu Deis	QU F1DW	D U Water	18	9.2	546	0	0	0	0	0	0	0	0	5 d	15 d
AQU Periodentology	Abu Deis	QU F2 TW	Tap water	17	9.1	590	0	0	0	0	0	0	0	0	5 d	15 d
AQU Periodentology	Abu Deis	QU F2 DW	D U Water	18	9	529	0	0	0	0	0	0	0	0	10 d	15 d
AQU Periodentology	Abu Deis	QU F2 TW	Tap water	17	9	507	0	0	0	0	0	0	0	0	10 d	20 d
AQU Periodentology	Abu Deis	QU F2 DW	D U Water	18	9	514	0	0	0	0	0	0	0	0	5 d	20 d
AQU Neurosurgery	Abu Deis	QU F3 TW	Tap water	17	8.8	509	2	0.1	0	0	0	0	0	0	10 d	20 d
AQU Neurosurgery	Abu Deis	QU F3 DW	D U Water	18	8.9	501	2	0.1	0	0	0	0	0	0	5 d	20 d
AQU Neurosurgery	Abu Deis	QU F3 TW	Tap water	18	8.9	507	0	0.1	0	0	0	0	0	0	5 d	20 d
AQU Neurosurgery	Abu Deis	QU F3 DW	D U Water	17	8.9	506	0	0	0	0	0	0	0	0	5 d	20 d
AAUP	Jenin	G14 TW	Tap water	10	8.5	1078	0	0	0	0	0	0	0	0	15 d	20 d
AAUP	Jenin	G14 DW	D U Water	11	8.6	998	0	0	0	0	0	0	0	0	15 d	20 d
AAUP	Jenin	G13 DW	D U Water	12	8.7	997	0	0	0	0	0	0	0	0	5 d	20 d
AAUP	Jenin	G12 DW	D U Water	14	8.8	1024	2 mg	0	0	0	0	0	0	0	10 d	20 d
AAUP	Jenin	G11 DW	D U Water	13	9	956	0	0	0	0	0	0	0	0	20 d	20 d
AAUP	Jenin	G10 DW	D U Water	13	9.1	952	0	0	0	0	0	0	0	0	10 d	20 d
AAUP	Jenin	G9 DW	D U Water	14	8.8	987	0	0	0	0	0	0	0	0	20 d	15 d
AAUP	Jenin	G1 DW	D U Water	13	8.8	952	0	0	10	0	0.5 mg	0	0	0	10 d	20 d
AAUP	Jenin	G2 DW	D U Water	14	9	983	2	0.1	10	0	0	0	0	0	10 d	20 d
Tulkarem Health Cente	Tulkarem	THC .TW	Tap waer	17	8.8	1047	2	0.1	10	0	0	0	0	0	20 d	20 d
Tulkarem Health Cente	Tulkarem	THC .DW	D U Water	25	8.1	680	2	0.1	0	0	0	0	0	0	20 d	20 d
Arafat Alwazani	Nablus	A.W TW	Tap water	25	8.4	430	0	0	10	0	0	0	0	0	10 d	20d
Monia Sabbah	Tulkarem	M.S DW	DU Water	23	8.2	750	0	0.5	25	0	0	0	0	0	15 d	20 d
AQU Surgery	Abu Deis	Q1F1	D.U Water	25	8.9	503	0	0	0	0	0	0	0	0	10 d	20 d
AQU Periodentology	Abu Deis	Q2F2	D.U Water	23	8.8	498	0	0	0	0	0	0	0	0	10 d	20 d
AQU Neurosurgery	Abu Deis	Q3F3	D.U Water	24	8.9	481	0	0.1	0	0	0.5 mg	0	0	0	10 d	20 d
AQU Peiatric	Abu Deis	Q4F4	D.U Water	25	8.8	455	0	0	0	0	0	0	0	0	5 d	20 d

Appendix G

G10.Tap biofilm –

TGACYGTCMCWRGCWAAGGGTTGCGCTCGTTACGGGACTTAACCCCAACATC
TCACGACACGAGCTGACGACAGCCATGCAGCACCTGTATCAGTGTTCCCGAAG
GCACTAATGCATCTCTGCAAAATTCACTGTATGTCAAGGGTAGGTAAGGTTCTT
CGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCA
ATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCAACTTATCGC
GTTTGCTGCGCCACTAATTATTTTCATATAACCAACAGCTAGTTGACATCGTTT
ACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCC
TCAGTGTCAGTATTAGGCCAGGTAGCCGCCTTCGCCACTGGTGTTCCTTCCGAT
CTCTACGCATTTCACCGCTACACCGGAAATTCCACTACCCTCTCCCATACTCGA
GTCAACCAGTATTATCTGACCGTCCCAGGTTAAGCCCCMSGSAGGA

M.S-Tap biofilm --

TGACTGCMCARGCWAAGGGTTGCGCTCGTTACGGGACTTAACCCCAACATCTC
ACGACACGAGCTGACGACAGCCATGCAGCACCTGTATCAGTGTTCCCGAAGGC
ACTAATGCATCTCTGCAAAATTCACTGTATGTCAAGGGTAGGTAAGGTTCTTCG
CGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATT
CCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCAACTTATCGCGTT
TGCTGCGCCACTAATTATTTTCATATAACCAACAGCTAGTTGACATCGTTTACA
GCGTGGACTACCASGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAS
TGTCARTATTATGCCTGGTATCCGCCTTCCCGACTGKGGTGCTGCCCGATCTCT
ATCCRCTTCGTGTCCTGACAGGAGAKTTAACTCCCCTCTCCCAYAGWCKCCWT
TAACCYTATTAGGAAGASAAGKGACGGTGGGTGSAACCAGAA

N.SH.C – Tap water

GRCTWCCTGGTTGMCTCGRTATGGGAGAGGTAGTGGAATTTCCGGTGTAGCGG
TGAAATGCGTAGAGATCGGAAGGAACACCAGTGGCGAAGGCGGCTACCTGGC
CTAATACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCTGTAAACGATGTCAACTAGCTGTTGGTTATATGAAAAT
AATTAGTGGCGCAGCAAACGCGATAAGTTGACCGCCTGGGGAGTACGGTCGCA
AGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGATGCAACGCGAAGAACCTTACCTACCCTTGACATACAGTGAA
TTTTGCAGARATGCATTAGTGCCTTCGGGAACACTGATACAGGTGCTGCATGG
CTGTCGTCAGCTCGTGTCGYGAGATGTTGGGTTAARTCACSYWMCCASCGCAA
CCCKTATCCCTARWTTASCKGAYGTGATGGKGGTGAGCACCCCACAGAAT

O.Z-DUWL. biofilm--

CGACYGCMCWRGCWAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTC
ACGACACGAGCTGACGACAGCCATGCAGCACCTGTATCAGTGTTCCCGAAGGC
ACTAATGCATCTCTGCAAAATTCACTGTATGTCAAGGGTAGGTAAGGTTCTTCG
CGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATT
CCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCAACTTATCGCGTT
TGCTGCGCCACTAATTATTTTCATATAACCAACAGCTAGTTGACATCGTTTACA
GCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCA
GTGTCAGTATTAGGCCAGGTAGCCGCCTTCGCCACTGGTGTTCCTTCCGATCTC
TACGCATTTCACCGCTACACCGGAAATTCCACTACCCTCTCCCATACTCGAGTC
AACCAGTATTATCTGACCGTCCCAGGTTAAGCCCAGGACG

QF1-TAP WATER

TACTATCTGGTTGMCTCGRTATGGGAGAGGGTAGTGGAATTTCCGGTGTAGCG
GTGAAATGCGTAGAGATCGGAAGGAACACCAGTGGCGAAGGCGGCTACCTGG
CCTAATACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCTGTAAACGATGTCAACTAGCTGTTGGTTATATGAAAAT
AATTAGTGGCGCAGCAAACGCGATAAGTTGACCGCCTGGGGAGTACGGTCGCA
AGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAARCGGTGGAKCATGT
GGKTTAAKTCCAAGCAAGGCRAAWAAACCTAYCTACCCCCTYACGCCTTTSGG
GCTTMKGGGAAATRCTAAAGCCGCTTTCGGMCCTMCYGAAYGGGGGGTCCTT
CGAATKTCWYCMRWTTTWRCCGKKAAAGGGAATTTCMKTCACGTCYCCCRTG
CWCSATTMACCCAWACTAAGMKGAMGGCYCAGGKKKARAGCCCCAGAATAG
GGTTGCGCTCGTTACGGGACTTAACCAACTCTWMGACCGAGCTGAASGACGGC
ATGCGCACTGTATCAGTGTTYCGAAGCACTATGMTCTTGCAMATTMMMTGTA
TGTCAGGGGTAGGTAGGCTCTCGGTKSAWCGAATAACRGGCCACGSGTKAGM
AGCCGTMWCTCYTTGARAGTKAATCCTG

T.H.C-DUWL WATER

AGTRCTWCCTGGTTGMCTCGRTATGGGAGGGGTAGTGGAATTTCCGGTGTAGC
GGTGAAATGCGTAGAGATCGGAAGGAACACCAGTGGCGAAGGCGGCTACCTG
GCCTAATACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCTGTAAACGATGTCAACTAGCTGTTGGTTATATGAAA
ATAATTAGWGGCGCARCAAACGCGATAAGTTGACCGCCTGGGGAGTACGGYC
GCAWTATTAAAACTCAMAGGAATTGACGGGGGCCCGCACAAACGGT

Y.Z-Tap biofilm

TGACTACMCWRGCWAAGGGTTGCGCTCGTTACGGGACTTAACCCCAACATCT
CACGACMCGAGCTGACGACAGCCATGCAGCACCTGTATCAGTGTTCCCGAAGG
CACTAATGCATCTCTGCAAAATTCACTGTATGTCAAGGGTAGGTAAGGTTCTTC
GCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAAT
TCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCAACTTATCGCGT
TTGCTGCGCCACTAATTATTTTCATATAACCAACAGCTAGTTGACATCGTTTAC
AGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTC
AGTGTCAGTATTAGGCCAGGTAGCCGCCTTCGCCACTGGTGTTCCTTCCGATCT
CTACGCATTTCACCGCTACACCGGAAATTCCACTACCCTCTCCCATACTCGAGT
CAACCAGTATTATCTGACCGTCCCAGGTTAAGCCCAGGAGGA

أدلة وجود بكتيريا الفيلقية المُسْتَرْوِحَة (*Legionella pneumophila*) بطرق الزراعة والطرق الجزيئية في خطوط المياه لوحدات الأسنان في الضفة الغربية/ فلسطين

إعداد: معتصم زهير حلمي برغال

اشراف: د. دينا بيطار

ملخص:

الفيلقية *Legionella* بكتيريا عصوية سالبة جرام، هوائية قليلاً تتبع رتبة الفيلقيات، واسعة الانتشار في الأوساط المائية من ضمنها خطوط المياه في وحدات الاسنان (DUWL) وهذه البكتيريا بطيئة النمو تواجه منافسة شديدة من انواع البكتيريا المائية الاخرى التي تنمو بسرعة، أشهرها الفيلقية المُسْتَرْوِحَة *(Legionella pneumophila)* تسبب الفيلقية المُسْتَرْوِحَة نوعين من المرض الأول مرض الفيالقة (Legionnaires' Disease) الأشد خطورة وهو عبارة عن التهاب حاد في الرئتين والثاني حمى بونتياك (Pontiac fever) وهو شبية بالانفلونزا.

من بين 59 نوعا من الفيلقيات و70 مجموعة مصلية منها، تعد الفيلقية المستروحة المسبب الرئيسي لداء الفيلقيات بسنبة 91.5% والمجموعة المصلية 1 النمط الغالب بنسبة 84.2%

وقد اظهرت العديد من الدراسات السابقة التلوث البكتيري في خطوط المياه لوحدات الاسنان ومنها الفيلقية المستروحة، وعند دخول الفيلقية الى خطوط المياه وعند ركود المياه تقوم البكتيريا في بناء الاغشية الحيوية التي تحميها من الظروف البيئية القاسية. تحدث الإصابة بالفيلقية المُسْتَرْوِحَة عبر استنشاق الرذاذ الملوث بالبكتيريا الناتج من استخدام التوربين او القبضة اليدوية سواء للمريض او طبيب الاسنان نفسه او عبر بلع الماء الملوث. وتلوث مياه خطوط الاسنان يشكل خطرا كبيرا على المرضى كبار السن والامراض المزمنة ومرضى نقص المناعة.

استكملت الدراسة السابقة في مختبر الاحياء الدقيقة للأبحاث مدة ثلاث سنوات (2012-2015) في الكشف عن الفيلقية المُسْتَرْوِحَة في انظمة شبكات المياه لثمان مستشفيات في الضفة الغربية، واستخدمت الدراسة طريقة الزراعة الجرثومية والطرق الجزيئية، وقد أظهرت نتائج الدراسة وجود الفيلقية المُسْتَرْوِحَة والمجموعة المصلية 1 بمعدل مرتفع. بنسبة 8.3% لعينات المياه بطرق الزراعة الجرثومية وازدادت هذه النسبة الى 50% باستخدام الطرق الجزيئية. اما بالنسبة الى عينات الاغشية الحيوية كانت النسبة اعلى بنسبة 16.8 % بطرق الزراعة بالمقابل 61% بالطرق الجزيئية.

الهدف من هذه الدراسة تأكيد وجود الفيلقية المُسْتَرْوِحَة في خطوط مياه الاسنان باستخدام طرق الزراعة الجرثومية والطرق الجزيئية في عيادات كليات طب الاسنان التعليمية في جامعة القدس أبو ديس والجامعة العربية الامريكية في جنين وعيادات اسنان مختلفة في مدن الضفة الغربية: نابلس، طولكرم والخليل. شملت عينات الدراسة 185 عينة [89 (48 ٪) عينات مياه من الصنبور ومن خطوط الأسنان و96 (52 ٪) مسحات غشاء حيوي] تم تحليلها عن طريق الزراعة الجرثومية وتم تحليل نفس العينات من خلال الطرق الجزيئية (التقنيات الجزيئية).

لتاكيد نتائج طرق الزراعة الجرثومية تم استخدام فحوصات مصلية على عزلات الفيلقية المُسْتَرْوِحَة للتعرف على الانماط المصلية، النمط المصلي 1 او الانماط المصلية 2 –14 . وللطرق الجزيئية تم استخراج الحمض النووي من العينات والكشف عن جين (16S rRNA) بثلاث مراحل: عن البكتيريا بشكل عام، وعن جنس الفيلقية ثم نوع الفيلقية المُسْتَرْوِحَة . تم ارسال سبع عينات من عزلات الفيلقية المُسْتَرْوِحَة الى المستشفى الاستشاري في را م الله لضمان تحديد هوية الفيلقية المُسْتَرْوِحَة عن طريق تسلسل جين (16S rRNA).

وكذلك فحص الخصائص الفيزيائية والكيميائية للمياه. ولإضفاء الطابع التقني على الدراسة، لقد تم جمع ومعالجة وتحليل العينات وفقا للإجراءات القياسية الدولية رقم ISO11731, ISO 11731-2، تم عزل 28 عزلة من الفيلقية المُسْتَرْوِحَة (15 ٪) من أصل 185 عينة باستخدام الزراعة و142 (77 ٪) من 185 عينة باستخدام الطرق الجزيئية. الطرق الجزيئية أكثر دقة و نتائجها خمسة اضعاف طرق الزراعة الجرثومية بسبب حاله خاصه لهذه البكتيريا تدعى (VBNC) اي انها قابلة للحياه لكن غير قابله للزراعة.

اما بالنسبة الى الانماط المصلية 82 % من العزلات كانت من النمط المصلي 1 و18% كانت تنتمي الى الانماط المصلية 2-14. اظهرت نتائج الدراسة أن الخطوط المائية لوحدات عيادات طب الأسنان (DUWL) ملوثة بالفيلقية المُسْتَرْوِحَة في جامعة القدس والعربية الامريكية وعيادات الأسنان في المدن الفلسطينية. نابلس وطولكرم والخليل. هذه النتائج تشير الى خطرا محتملا على الصحة للمرضى الذين يعانون من نقص المناعة والامراض المزمنة و على أطباء الأسنان.

يجب على وزارة الصحة و سلطة المياه الفلسطينية وضع قيود وإرشادات لجودة المياه والرصد الميكروبيولوجي ومتابعة غسل وحدات الاسنان بالمطهرات مثل الكلورهيكسيدين غلوكونات (CHX) أو الماء النقي واستخدام فلتر المنقي والتحقق منه دوريًا.