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Molecular Identification and Sequence Based Typing of *Legionella pneumophila* in Environmental and Clinical Samples in Palestine

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Dedication

To my mother and father…

To my dear wife, Alya'…

To my daughter, Reem…

To my family…

To my friends…

To my teachers…

To all the people who supported, and encouraged me.

Mahmod Jameel Ishaq Amro

Declaration:

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

Signed

Mahmod Amro

Date: ...8/5/2017

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Abstract

Legionellae are gram-negative bacteria, rod shaped, strictly aerobic and nutritionally fastidious. *Legionella* species are implicated in two clinical syndromes: Legionnaires' Disease (LD), and Pontiac fever, which are collectively known as legionellosis. Among the 56 species and 70 serogroups of *Legionella* species, *Legionella 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 that the main source for LD is the potable water systems in large buildings like hospitals and hotels. The contamination of hospitals' water systems with *Legionella* is high risk for patients with various diseases, especially immunocompromised and those who may stay hospitalized for long period of time. LD is acquired by inhalation of aerosols contaminated with *Legionella spp*. or less commonly by aspiration of contaminated drinking water.

Previous work in the Microbiology Research Laboratory at AQU has shown high prevalence of *Legionella spp.* in the water and biofilm samples collected from eight hospitals in the West Bank over a two-year period December 2012- December 2014, by using culture method and 16S rRNA-based PCR. Moreover study of the prevalence of *L. pneumophila* in 195 respiratory samples (sputum or Broncho alveolar lavage (BAL)) by culture yielded only one positive. However, by PCR, 23% (44/195) of the respiratory samples were positive for *L. pneumophila*. BAL presented a higher percentage 35% (26/74) than sputum 15% (18 /121).

Molecular diagnosis of *L. pneumophila* is well established and adopted worldwide especially that culture methods are time consuming and less efficient. Furthermore, genotyping of *L. pneumophila* is important for epidemiological investigation and control of legionellosis

outbreaks. The current gold standard for *L. pneumophila* genotyping is Nested PCR Sequence Based Typing (NPSBT), based on the sequence of seven loci (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA* and *neuA*). NPSBT allows the Sequence Typing (ST) *of L. pneumophila* in the absence of isolates. This high-resolution molecular typing tool is recommended by the European Working Group for Legionella Infections (EWGLI).

The previous results in the Microbiology Research Laboratory entitled the use of NPSBT to be able to do epidemiological typing of the respiratory samples in the absence of isolates and to relate the ST's of environmental samples previously collected from the same hospital with the ST of the respiratory samples to evaluate possible nosocomial infection.

The overall goal of this study was to determine the Sequence types (ST's) of the PCR positive respiratory samples by NPSBT method. Also to determine the ST's of the environmental samples obtained from the same hospital ward.

Our sample study included a subset (34 samples) out of the 44 respiratory samples previously tested positive by PCR targeting 16S rRNA for *L. pneumophila*. These thirty-four positive samples were further subtyped by NPSBT method. Also DNA previously extracted from 15 biofilm samples previously collected from Makassed hospital wards and tested positive for *L. pneumophila* was also analyzed by NPSBT.

Analysis of the seven allele profiles for the NPSBT of the 34 selected respiratory samples showed a full 7-allele profile for 3 /34 (8.8 %) specimens, a further 18/34 (52.9%) gave 5- or 6-allele profiles (sufficient to identify the strain as one or two sequence types (ST's), 6/34 (17.6%) gave 3- or 4-allele profiles (usually enough to differentiate different profiles), and 5 /34 (14.7%) gave 1- or 2-allele profiles (sufficient to distinguish strains). Overall, 24/34 (70%) samples gave ≥ 4 alleles profiles (4, 5, 6 and 7 alleles). However, 10 samples gave < 4 alleles profiles, these samples were excluded from sequencing in order to identify the ST. Analysis of the seven alleles' products of the selected fifteen environmental samples revealed fourteen samples positive for six to seven alleles and one sample was positive for one allele, this sample was excluded from sequencing in order to identify the ST

Sequence analysis showed the following ST's in the 24 respiratory samples: ST1 (29.1%, 7/24), ST 461 (25%, 6/24), ST 1037 (4%, 1/24), and (41.9%, 10/24) gave incomplete profile.

On the other hand, 14 environmental samples typing showed: ST 1(28.6%, 4/14), ST 187 (21.4%, 3/14), one sample of each ST 2070, ST 461 and ST 187 (7.1 %, 1/14), while the rest of samples (28.5%, 4/14) were unspecified Sequence Types.

Thus ST1 is the most prevalent sequence type in both the respiratory samples and the environmental samples representing 29.1% and 28.5% respectively. The other ST's were unique to each type of sample. ST1 is also the most prevalent ST worldwide in clinical and environmental samples.

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AE	Elution buffer
AP-PCR	arbitrarily primed PCR
AFLP	Amplified fragment length polymorphism
asd	aspartate-semi aldehyde dehydrogenase gene
ATL	animal tissue lysis buffer
AW1	wash buffers 1
AW2	wash buffers 2
BAL	Bronchial alveolar lavage
BCYE	Buffered charcoal yeast extract
C	Celsius
CAP	Community acquired pneumonia
CDC	Centers for Disease Control and Prevention
CO_2	Carbon dioxide
DFA	Direct fluorescent antibody assay
DNA	Deoxyribonucleic acid
DW	Distilled water
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylene diamine tetra acetic acid
ELB	Enzymatic lyses buffer
ELISA	Enzyme linked immunosorbent assay
EtOH	Ethanol
EWGLI	The European Working Group for *Legionella* Infections
FDA	Fluorescent direct antibody

flaA	Flagellin A gene
ftsZ	Filamenting **t**emperature-**s**ensitive mutant Z gene
FISH	Fluorescent in situ hybridization
GVPC	Glycine, Vancomycin, Polymyxin B, Cycloheximide
HCl	Hydrochloric acid
HZI	Helmholtz Center for Infection Research
IFA	Immuno fluorescence assay
Ig	Immunoglobulins
ISO	International organization for standardization
KCl	Potassium chloride
KOH	Potassium hydroxide
L	liter
LD	Legionnaires' disease
M	Molar
m	meter
MAb	Monoclonal antibody
mg/ml	milligram per milliliter
min	minute
Mip	Macrophage infectivity potentiator
ml	milliliter
MLVA	Multi Locus Variable number of tandem repeat Assay
um	micrometer
mompS	Major outer membrane protein gene
NAATs	Nucleic Acid Amplification Tests
NaCl	Sodium chloride
neuA	N-Acylneuraminate Cytidyltransferase Gene
ng	Nano gram

NPSBT	Nested PCR Sequence Based Typing
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
pilE	pilus biogenesis gene
proA	Gamma-glutamyl phosphate reductase gene
PWA	The Palestinian Water Authority
rDNA	Ribosomal Deoxyribonucleic acid
rep-PCR	repetitive element PCR
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Round per minute
rRNA	Ribosomal Ribonucleic acid
RT-PCR	Real time PCR
SATs	Slide agglutination tests
SBT	Sequence based typing
Sg	Serogroup
sidA	siderophore biosynthetic gene
Spp	Species
ssrA	Small stable RNA gene
TEA	Tris-acetate buffer
UAT	Urinary antigen test
USA	United States of America
VAMC	Veterans' Administration Medical Center
VBNC	Viable but non culturable
WHO	World health organization

Chapter One

1.1 Introduction

The year 2016 marked forty years since legionellosis has been discovered back in 1976. Legionellosis is a collection of infections that are caused by *Legionella* spp. bacteria, ranging from a self-limited febrile illness (Pontiac fever) to a severe and fatal form of pneumonia Legionnaires' disease (LD). LD can affect any individual but usually affects those who are more susceptible due to immunosuppression, illness, age, and other risk factors, such as smoking (Phin et al., 2014).

Legionellae are gram-negative bacteria, rod shaped, strictly aerobic and nutritionally fastidious (Napoli et al., 2010). Among the 56 species and 70 serogroups (Sgs) of *Legionella* species (Tijet et al., 2010), *Legionella pneumophila* is the major cause of sporadic and outbreak legionellosis (91.5%), and serogroup 1 is the predominant serotype (84.2%) (Nathalie Tai et al., 2012).

L. pneumophila is the second most common pathogen in industrialized countries related with cases of community-acquired pneumonia that requires patient admission to intensive care units (Tijet et al., 2010).

Legionella spp. are ubiquitous in freshwater habitats, including natural occurring and man-made water, such as rivers, lakes, streams, ponds, hot springs, and subsurface waters, and are naturally part of microbial ecosystems (Fliermans et al., 1981; Fliermans, 1996b; Qin et al., 2013).

The potable water systems in large buildings like hospitals and hotels are considered the main source for LD (Fraser, et al., 1977), and the contamination of hospitals' water systems with *Legionella* is high risk for patients with various diseases, who may stay hospitalized for long period of time. It is well known that LD is an important cause of hospital-acquired pneumonia (Fraser et al., 1977; Kool et al., 1999).

L. pneumophila is the most frequently isolated *Legionella species* from water distribution networks and is also the one predominantly associated with disease (Fields et al., 2002).

1.2 History

In the summer of 1976, the Centers for Disease Control and Prevention (CDC) in Atlanta, responded to a sudden, explosive epidemic of febrile illness with pneumonia among attendees of the American Legion conference in Philadelphia, Pennsylvania (Sharrar et al., 2002).

The cause of the Philadelphia outbreak was unknown for months in spite of determined laboratory examination for heavy metals. More than 77 known pathogens and culture on more than 25 different media for bacteria were examined without success (McDade et al., 1977). An epidemiologic analysis determined that the disease most likely was airborne and primarily focused at one convention hotel, which later had to be closed because of adverse publicity (Fraser et al., 1977). After six months, Joseph McDade and co-worker announced that they had discovered the etiologic agent, a fastidious Gram-negative bacillus namely *L. pneumophila.* (McDade et al., 1977).

The source of infection was believed to be the air conditioning system at the conference hotel. However, no clear proof of the mode of transmission was obtained. Thirty-four (15%) individuals died from 221 who were infected. After the isolation of *Legionella* bacteria the epidemiological scientists tried to solve earlier outbreaks with the same symptoms (McDade et al., 1977), the first was in Washington, DC, in 1965, where 14 of 81 infected individuals died (McDade et al., 1977; Thacker et al., 1978), and the second was a non- pneumonic outbreak that occurred in Pontiac, MI, in 1968, where no deaths were reported among 144 cases (McDade et al., 1977; Glick et al., 1978).

Over the years several new serogroups of *L. pneumophila* and other *Legionella* spp. have been discovered (Travis et al., 2012; Pravinkumar et al., 2010; Casati et al., 2009; Currie et al., 2014). Currently, there are more than 56 known species comprising 70 distinct Sgs (Tijet et al., 2010). Fifteen Sgs of *L. pneumophila* have been described (Pearce et al., 2012; Muder et al., 2002).

After these outbreaks the surveillance systems and control were evaluated and improved to manage any future outbreak in many countries including USA, Japan, Europe, Australia, New Zealand (Phin et al., 2014). However there is no surveillance for *Legionella spp.* in most Arabic country including Palestine.

1.3 Bacterial characteristics

Legionellae are Gram-negative, aerobic, non-spore-forming and motile rods belonging to the gamma- *proteobacteria* (Molofsky and Swanson, 2004), They are not related to other bacteria except for *Coxiella burnetii* (Swanson and Hammer, 2000).

The bacterium is considered as pleomorphic in shape, influenced by temperature, available nutrients or metabolites, growth environment (e.g., inside amoebae), and medium type. *Legionellae* appear as coccoid or bacillary (0.3- to 0.6-m by 3-m), and/or long filamentous (8- to 50-m) forms (Rowbotham, 1986; Greub and Raoult, 2003; Katz et al., 1984).

The documentation of *Legionella* species and serogroups (Sgs) increased after the first outbreak. *Legionella* species and serogroups associated with human disease are shown in (Table 1.1) (Benson and Fields, 1998;Fields et al., 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

Legionellae are urease-negative, catalase-positive, heterotrophic, aerobic, chemo-organ trophic, and transitionally motile. When motile, they have one or more straight or curved polar or lateral flagella. *Legionellae* have different biphasic life cycle that alternates between a non-motile, replicative phase and a virulent, flagellated, trans-missive phase (Garrity et al., 2005; Rowbotham, 1980). When cultured in the laboratory, *Legionellae* normally have one polar flagellum in the stationary phase (Swanson and Hammer, 2000).

Different from most bacterial species, *Legionellae* use protein for energy source rather than carbohydrates, several amino acids are metabolized, especially serine and threonine, which are the main sources of energy (George et al., 1980; Tesh et al., 1983). *Legionellae* utilize amino acids for energy and carbon, do not oxidize or ferment carbohydrates, and require L-cysteine-HCl and iron salts for growth amongst other nutrients (Garrity et al., 2005). Sodium chloride (NaCl) is inhibitory to planktonic *Legionellae* in water and stationary phase cells recovered from samples (Swanson and Hammer, 2000; Garrity et al., 2005).

Legionellae can grow on different types of artificial media such as buffered charcoal yeast extract medium (BCYEα), pH 6.9. It is the medium of choice for culturing *L. pneumophila*,

although it might not be the most favorable for some non-*L. pneumophila* species (Edelstein, 1981a; Edelstein, 1983b).

Legionellae are strict aerobes. When *Legionella pneumophila is* cultured on laboratory BCYEα media it grows equally well with air and in a microaerophilic environment. Reduced O_2 tension has however been shown to be favorable under some conditions (Mauchline et al., 1992; Dennis et al., 1984). However, the addition of CO_2 in the medium does not stimulate growth, except possibly for some non-*L. pneumophila* species (Edelstein, 1983). The optimum temperature is 35 - 37ºC, but some growth can also be observed in the laboratory at 42°- 43ºC (Dennis et al., 1984; Kusnetsov et al., 1996; Ohno et al., 2003).

Since *Legionellae* are able to grow and retain metabolic activity in high temperatures over 50ºC, it is conceivable that they might also multiply at temperatures higher than 43ºC in natural environments, e.g. in biofilms and intracellularly in amoebae (Ohno et al., 2003).

The cell wall of *Legionellae* unlike most gram-negative bacteria contains long, branched hydroxyl fatty acids that are unique for the family. The difficulty of cell staining is influenced by ubiquinone's with side chains of 9-14 isoprene units in fatty acid chain in the cell wall (Garrity et al., 2005). Also the polysaccharide epitopes of the lipopolysaccharide in the cell wall are specific and can be used for serological grouping (Helbig et al., 1996).

L. pneumophila is the most *Legionella* spp. studied because; approximately 90% of all Philadelphia outbreak cases of LD were caused by *L. pneumophila* Sg 1 (Benin et al., 2002; Yu et al., 2002). *L. pneumophila* Sg 1 accounts approximately 90% of American and European *Legionella* isolates. However in Australia and New Zealand, *L. pneumophila* Sg 1 accounts for only 50% of the cases of community acquired legionellosis, whereas *L. longbeachae* accounts for approximately 30% of the cases (Yu et al., 2002).

1.4 Environmental investigations

Legionellae bacteria are aquatic organisms that live in freshwater environments (Fliermans et al., 1981). *L. pneumophila* has been cultured and recovered from both natural and human-made

habitats, from lakes and streams to air-conditioning cooling towers, fountains, and spa baths (Lettinga et al., 2002; Molmeret et al., 2004; Morris et al., 1979). But *L. longbeachae* are the exception and differ from the ecological niche of *L. pneumophila* which resides primarily in soil, and infection is often associated with exposure to soil (Steele et al., 1990). *Legionella* bacteria are not free-living aquatic bacteria; rather, this bacterium needs an environmental host to live within as a parasite or form a commensal relationship with free-living, freshwater, and soil amoebae (Steinert et al., 1997).

Legionella species multiply intracellularly in many types of protozoa, and this relationship is central to the ecology of the organism in both aquatic and soil environments. While providing a niche for *Legionella* replication, amoebae also protect *Legionella* from hard environmental conditions. This relationship between *Legionella* and amoeba can increase the resistance *to* biocides, antibiotics, acid, osmotic and temperature stress (Cirillo et al., 1994a; Cirillo et al., 1999b), Furthermore, some amoeba species expel biocide-resistant vesicles containing large numbers of *L. pneumophila* bacteria, which may act as airborne agents for the transmission of the bacteria (Berk et al., 1998).

In human-made water systems, such as in buildings and hospitals water systems, *Legionella* bacteria are found almost exclusively within complex biofilms (Rogers, et al., 1994). Also in this ecosystem the characterization of *Legionella* is difficult, but biofilm systems have demonstrated that the replication of *L. pneumophila* within this biofilm depends on the presence of an amoeba host (Murga et al., 2001).

LD is most strongly associated with human-made aquatic environments that contain water at high temperatures. Instead, many disease outbreaks are linked to cooling towers and evaporative condensers, which can produce water droplet contaminated with *L. pneumophila* that are inhaled (Newton et al., 2010).

The increased presence of *Legionella* reservoir such as human-made aquatic reservoirs has likely led to the increased exposure of humans and unfortunately to increased incidence of *Legionella* infection in the latter half of the 20th century.

The pathogenesis of LD is largely due to the ability of *L. pneumophila* to invade and grow within macrophages in the lungs (Rowbotham, 1986). Therefore, the evolution of virulence

traits in *L. pneumophila* has resulted largely from the organism's need to replicate in an intracellular host and also avoid predation by environmental protozoa (Newton et al., 2010).

Protozoa are considered its natural host in water environments. *Legionella* are able to survive phagocytosis and multiply inside protozoan cells. Infections result with lysis of the protozoan host and the release of *Legionella* into the media, where it can be found in its free-living form or associated with biofilm. Elevated temperature, inorganic and organic contents of the water and the presence of host protozoa, play key roles in *Legionella* growth and spread (Fliermans et al., 1981).

1.5 Ecology

Legionella spp. are ubiquitous in freshwater habitats, including rivers, lakes, streams, ponds, hot springs, and subsurface waters, and are naturally part of microbial ecosystems (Fliermans et al., 1981; Qin et al., 2015; Ortiz-Roque and Hazen, 1987). However, potting mixes and soil are considered an important source for infection with *L. longbeachae* which was recovered from potting mixes in Australia, United States, and the United Kingdom and in the soil of Thai farmland (Travis et al., 2012; Pravinkumar et al., 2010; Casati et al., 2009).

Many studies have proved the ability of these bacteria to infect and multiply within amoebae in their environment (Declerck, 2010; Declerck et al., 2007; Sheehan et al., 2005). Today it has become clear that *Legionella are* adapted to live, proliferate and replicate within different protozoan genera, including *Acanthamoeba*, *Naegleria*, *Hartmannella*, and *Tetrahymena* (Taylor et al., 2009). This is very important in *Legionella* life cycles to induce virulent bacterial traits, assist in distribution, and provide protection from harsh or bactericidal environmental conditions, such as excessive heat and chlorine (Berk et al.,2008; Hojo et al., 2012; Neumeister et al., 2000; Bigot et al., 2013).

Legionellae spp. can live in different water temperatures in cold and hot water at (25°C to 37°C) but may replicate and proliferate at temperatures above and below this range and may even survive at growth-restrictive temperatures of 20°C and 55°C (Arvand et al., 2011).

Also, they are found growing in the biofilm that lines the inside of pipes (Fields et al., 2002). Additionally, 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).

1.6 Mode of transmission

Different modes have been identified for transmission of *Legionella* to humans; Legionellosis is transmitted via the airborne route when aerosols are inhaled from a water source contaminated with the bacteria, there is evidence for aerosolization, aspiration, or even instillation into the lung during respiratory tract manipulation (Stout and Yu, 2003). Legionellosis may be very rarely transmitted from person to person (Amodeo et al., 2009; Lindsay et al., 2012).

LD is a respiratory illness caused by inhalation of *Legionella*-containing aerosols generated by showers, faucets, air conditioning cooling towers, whirlpool spas, and fountains (García-Fulgueiras et al., 2003; Bennett et al., 2014), and other aerosols formation devices. Aspiration of water containing *Legionella* has also been suggested to be a common transmission route (Yu, 2001). Figure (1.1).

Many studies on the mode of transmission including aspiration have been suggested by some authors to be more important than inhalation (Yu, 1993; Pedro-Botet et al., 2002). Susceptibility to disease is associated with smoking, older age, chronic cardiovascular or respiratory disease, diabetes, alcohol misuse, cancer especially profound monocytopenia as seen in hairy cell leukemia, and immunosuppression (Boer et al.,2006), especially, in higher risk individuals such as hospitalized patients and immunocompromised. If the tap water contains *L. pneumophila*, the bacteria could possibly be instilled directly into the lung of a patient (Tablan et al., 2003).

In numerous studies, the risk of LD disease was significantly greater for patients who underwent endotracheal tube placement more often or had a significantly longer duration of intubation than for patients who had other causes of pneumonia. Use of sterile water for all

nasogastric suspensions, for humidifiers in breathing circuits of mechanical ventilators, and for flushing tubes has been recommended to prevent *Legionella* infection (Fields et al., 2002).

LD due to *L. longbeachae* is thought to have a different route of transmission, which is yet to be fully identified, but exposure to potting compost or soil, or gardening activities is regarded as a risk factor (Amodeo et al., 2009; Lindsay et al., 2012). Poor hand washing practices after gardening, long-term smoking, and being near dripping hanging flowerpots have also been associated with a greater risk (O'Connor et al., 2007).

Inhalation or micro-aspiration of amoebae could be a potential risk, since one single amoeba might harbor >1000 legionella cells (Rowbotham, 1980). Furthermore, intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances the virulence of *L. pneumophila*. Thus, it is conceivable that infection in humans may require the presence of both *Legionellae* and an amoeba host (Swanson and Hammer, 2000). This could explain why the attack rate in LD outbreaks is low, despite the presence of *Legionellae* in the plumbing system. In one study occurrence of LD cases was related to the presence of both protozoa and *L. pneumophila* (Breiman et al., 1990).

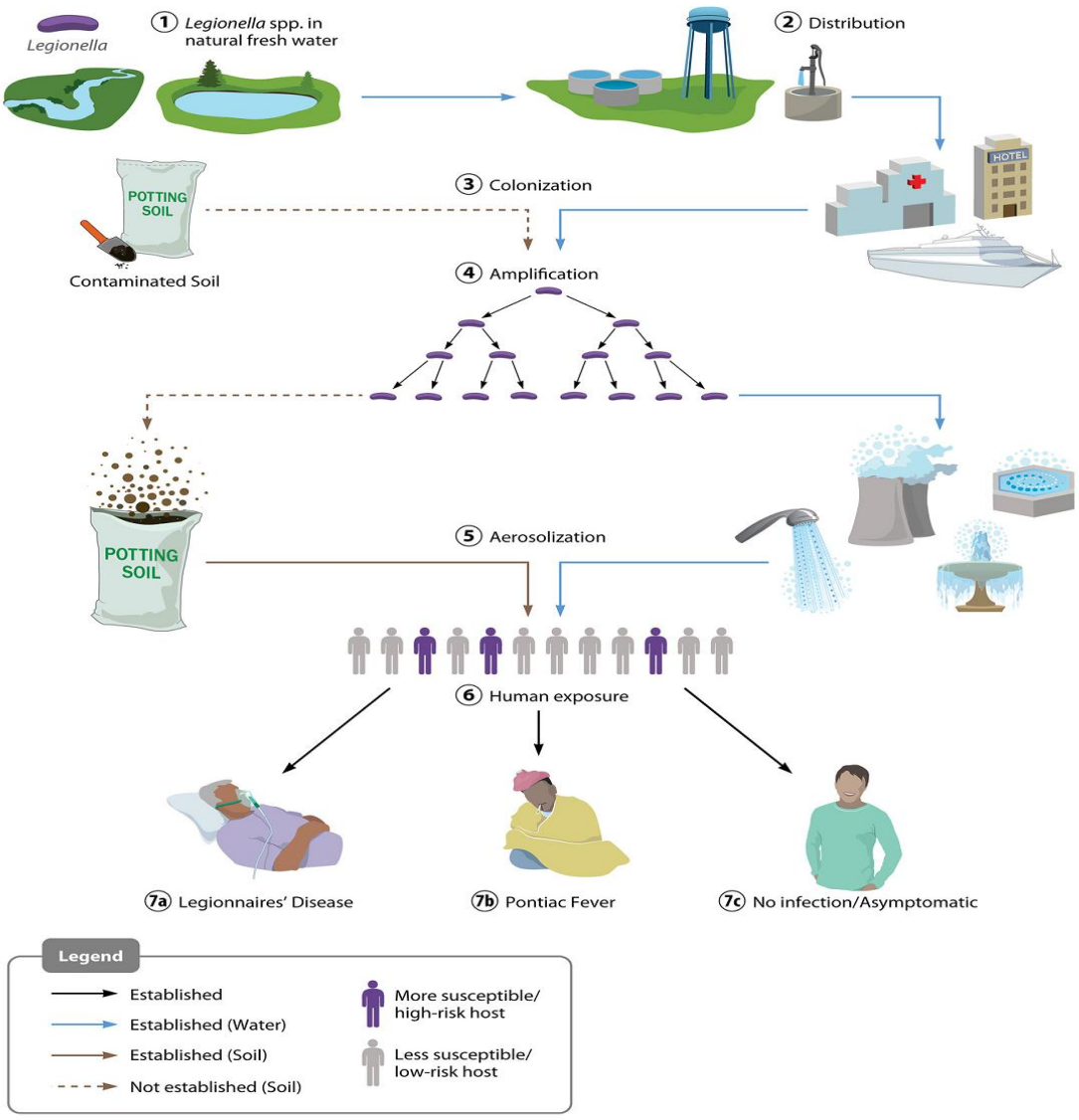

Figure 1.1: Modes of Legionella dissemination from natural waters to development of Legionnaires' disease and/or Pontiac fever. *Legionella* from freshwater sources (1) is distributed at low concentrations from points of water purification (2) to colonize downstream local plumbing networks and cooling 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).*

At the same time, the problem is particularly important in hospitals, where the medical equipment can also be a potential source of infection (Endoscopes, devices for artificial respiration and oxygen therapy, dental devices, etc.) (van Heijnsbergen et al., 2015).

1.7 Clinical features

It is clear that LD classically presents as two distinct clinical entities, pneumonia with severe multisystem disease, and Pontiac fever, a self-limited flulike illness.

The symptoms of Legionnaires' are similar to those of other types of pneumonia. LD does not have specific, defining clinical features because it presents as a range of clinical manifestations and symptoms, undetected cases of LD end up being classified merely as pneumonia and are effectively treated with a broad spectrum antibiotic (Viasus et al., 2013; Levy et al., 2010; Cunha, 2008).

Most of the LD patients present with fever, non-productive cough, headache, myalgia and dyspnea. Clinical syndromes may include diarrhea, nausea, vomiting, liver and kidney dysfunction, thrombocytopenia, hyponatremia and neurological disorders (Fields et al., 2002; Zarogoulidis et al., 2011), and also may include: fever with organ-specific symptoms and signs, such as diarrhea or confusion, or both; fever with multisystem disease including rhabdomyolysis with renal failure; pneumonia with extra pulmonary features; and severe fulminant disease (Chidiac et al., 2012)

LD pneumonia has no particular clinical feature that clearly distinguishes it from other types of pneumonia (De Jong et al., 2013), and laboratory investigations must be carried out to confirm the diagnosis for appropriate and successful management of the disease. It normally takes between 2 and 10 days to develop symptoms (Den Boer et al., 2002). Patients usually start with a dry cough, fever, headache and sometimes diarrhea and many develop pneumonia. People over the age of 50 years are at higher risk than younger people, and males are at higher risk than females (Phin et al., 2014).

The incubation period of Legionnaires' ranges from 2–10 days (average 6–7 days); but also may be longer or shorter (Den Boer et al., 2002; Greig et al., 2004), However, in some cases the incubation period of 19 days was noted in one outbreak (Den Boer et al., 2002).

Conversely, a mild, self-limiting, and non-pneumonic form known as Pontiac fever has also been associated with exposure to aerosols containing *Legionella spp*. The incubation period of Pontiac fever is short (between 5 and 66 h but usually 24–48 h) and duration (2–5 days), and is more common in younger people (Tossa et al., 2006).

A mortality rate is typical in most people ranging from 8–12% but might be higher in people who are at higher risk including: elderly, have preexisting medical conditions, smokers, are nosocomial cases, or have a delay in diagnosis and treatment of their disease (Dominguez et al., 2009). The average case-fatality rate is 10% in Europe (range 0–27% in countries reporting ≥30 cases) and 8% in the USA. Also the case-fatality rate in nosocomial cases is higher and ranges between 15% and 34% (Beauté et al., 2013; Control, C. f. D., and Prevention, 2011).
Unfortunately the high mortality and morbidity associated with untreated LD was noted and reported (Stout and Yu, 2003).

1.8 Diagnosis

LD disease is perceived as rare because most cases are never detected, and not all detected cases are reported to public health authorities and most of the cases occur sporadically. Since the clinical signs and symptoms for LD are not specific, prompt and accurate laboratory diagnosis is crucial, for the successful management and treatment (Phin et al., 2015).

A case of LD will go undetected unless special laboratory tests are performed. Unfortunately, these tests are not routinely available in many countries. In the USA some hospitals have recognized cases of Legionnaires' disease only after increased testing of patients with pneumonia. Likewise, in hospitals where only one to three cases of LD were identified over several months, numerous additional cases were recognized after surveillance was intensified. Studies of community-acquired pneumonia have also indicated that increased surveillance

leads to better diagnoses (Fields et al., 2002).

The methods for detection of *Legionella* were developed year by year. Confirmed methods used to test this bacterium include specific culture, serology, urinary antigen test and direct fluorescent antibody screening. Some of these methods are too slow for clinical use and or inadequately sensitive or specific (Ginevra et al., 2009). Thus molecular methods were evaluated to increase the specificity and sensitivity in clinical diagnosis of LD and early detection and management (Figure 1.2).

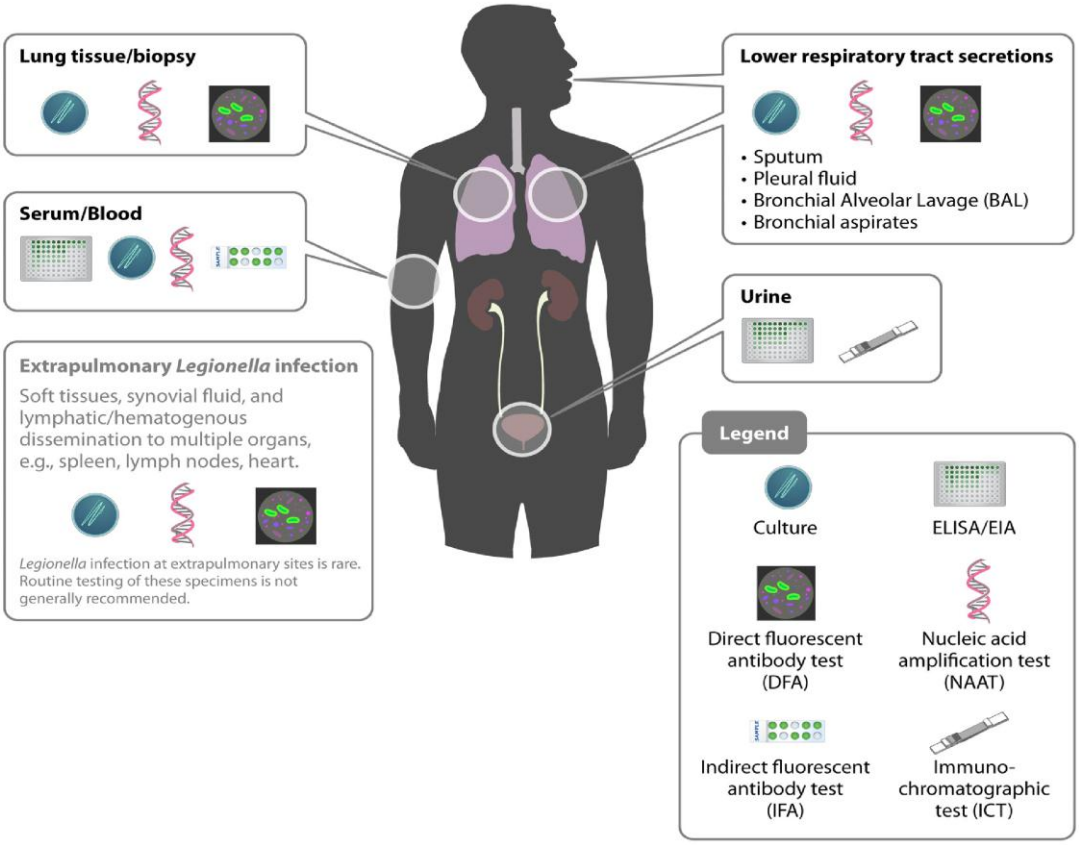

Figure 1.2: Samples and diagnostic methods : In this figure we can see many of the samples and diagnostic tests for detection of *Legionella pneumophila*. Some assays are applicable to multiple specimen types, such as culture and nucleic acid amplification. In general, the success of detecting *Legionella* is dependent on the severity of the disease, specimen integrity, technical proficiency of the laboratory, and particular test characteristics. (*Adapted from Mercante and Winchell, 2015*)

For epidemiological investigation of LD, the identification of *Legionella* spp. and serogroups is not sufficient. It is important to use subtyping methods, which discriminate between the strains at the molecular level

1.8.1 Microbiological culture

Culture and isolation remain to this time the "gold standard" for *Legionella* identification and LD diagnosis. Using specimens from the lower respiratory tract, such as sputum, pleural fluid, bronchial aspirates, and bronchial alveolar lavage (BAL) fluid (Fields et al., 2002). However, due to the specific nutrient requirements of *Legionella*, it requires some special growth factors and special media.

Legionellae can grow on different types of artificial media; but, the most successful medium is buffered charcoal yeast extract (BCYE) agar containing 0.1% α ketoglutarate with L-cysteine incubated at 35°C in a humidified, 2.5% CO_2 atmosphere (Feeley et al., 1979).

The sensitivity of detection of *Legionella* by culturing of clinical specimens is highly variable, ranging from >10% to 80%, and recovery is dependent on the sample type as well as the experience and technical proficiency of laboratory personnel (Reller et al., 2003). Most isolates demonstrate growth in 3 to 5 days, but non-*L. pneumophila Legionella* species and occasionally primary-specimen isolates may require considerably longer incubation times, sometimes up to 2 weeks (Control, C. f. D., and Prevention report, 2005). Culture yield depends on the severity of illness, with the lowest yield from 15% to 25% in mild pneumonia and the highest yield more than 90% for severe pneumonia causing respiratory failure (Reller et al., 2003).

Although for enhancing *Legionella* recovery, one can use semi-selective procedures in the presence of competing bacteria (from clinical and environmental samples), including brief acid and heat exposure and/or the addition of glycine, Polymyxin B, Cycloheximide, and vancomycin to the growth media, to which *Legionellae* are naturally resistant. Most *Legionellae* are cysteine auxotroph's (the exceptions being *L. oakridgensis*, *L. jordanis*, and *L. nagasakiensis*, all of which may adapt to cysteine-deficient media after serial passage (Orrison, et al., 1983; Yang et al., 2012).

1.8.2 Urinary antigen test

Legionella urinary antigen tests (UAT) are significantly quicker than culture and have a high sensitivity and specificity, showing a positive detection of >90% of cases in under 15 minutes of incubation .The antigen can become present in the urine in as little as 1 to 3 days after symptomatic onset, representing 82% and 97% of the diagnostic tools used for LD confirmation in Europe and the United States, respectively (Control, C. f. D., and Prevention report, 2011).

The popularity and ubiquity of the UAT are attributed to its speed, relatively low cost, uncomplicated procedure, and ease of sample collection. *Legionella* specific urinary antigens can be detected in the majority of *L. pneumophila* infections shortly after clinical symptoms appear (2 to 3 days) and may be excreted for several days to 10 months, even during antibiotic treatment and after disease resolution (Jarraud et al., 2015).

The most significant problem with the UAT is that it is specific only to *L. pneumophila* serogroup 1, therefore a positive test indicates LD, but a negative test cannot rule it out.

1.8.3 Serological and antibody-based assays

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

In Europe the use of serology for LD diagnosis and confirmation decreased from 61% to 6% on average in the period from 1995 to 2010. According to the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO), displaced by the

more fast, less technically demanding UAT (Beauté et al., 2013; World Health Organization report, 1997).

There are different reasons for this change; the serological methods indirect fluorescent antibody test and Enzyme linked Immunosorbent Assay (ELISA) for detecting patient seroconversion depend on a 4-fold increase in anti-body titer (to 1:128) between acute- and severe-phase serum samples taken 4 to 8 weeks apart, which means that the window for treatment has long passed (Edelstein, 1987).

Other tests such Direct fluorescent antibody test (DFA) assays, slide agglutination tests (SATs), and Monoclonal Antibody (MAb) screens are antibody based but the patient serum is not directly tested. For the SATs and MAb test, a pure-culture isolate is needed. DFA assays can be performed on cultures, patient tissues, or fluid secretions. However, their use for *Legionella* respiratory antigen detection in clinical laboratory was decreased to minimal, from a rate of 1% in 1996 to 1/10 of 1% in 2010 (Beauté et al., 2013).

DFA assays, SATs, and MAb blotting are useful for qualitative *Legionella* identification and for *Legionella* typing at the species and serogroup levels. The tests benefit from being relatively rapid, inexpensive, and reliable, allowing strain comparisons across time with commercially available reagents (Edelstein, 1987), but similar to the Indirect Fluorescent Antibody (IFA) assay, they require a moderate-to-high level of laboratory expertise

1.8.4 Nucleic acid-based molecular diagnostics

Since *Legionella* are difficult to culture and the sensitivity of the culture is variable and highly dependent on the laboratory technical skills, molecular methods that are 100 percent sensitive and rapid were sought. In the mid-1980s Nucleic acid-based research for *Legionella* detection, diagnostic and typing began (Mercante and Winchell, 2015).

The first use of Polymerase Chain Reaction (PCR) as a tool for *Legionella* detection came 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 environmental samples, also evaluated and validated this powerful new method in a variety of matrices, including water from cooling towers, rivers, and hot tubs as well as sputum, BAL fluid, serum, and urine (Yamamoto et al., 1993; Fry et al., 1991; Jaulhac et al., 1992). In the early 2000s Real-time PCR was used for quantitative and monitoring of *L. pneumophila.*

The benefits of Nucleic Acid Amplification Tests (NAATs) are high sensitivity and specificity, and rapid turnaround time. NAATs such as conventional PCR, and real-time PCR (single and multiplex) protocols have been developed for *Legionella* detection and characterization. Most NAAT-based assays are highly specific (close to 100%), and the growing consensus is that the sensitivity of PCR (both conventional and real time) is equal to or greater than that of culture-based detection using specimens from the lower respiratory tract or environmental water samples (Jespersen et al., 2009). Detection may be superior for diagnosing milder LD cases or detecting prior exposure. The most common targets include a conserved segment of the rRNA genes for the 5S and 16S subunits, the 16S-23S spacer, and/or the macrophage inhibitor protein *mip*, found primarily in the genus *Legionella* and highly conserved in all *L. pneumophila* isolates. Also the *ssrA* gene is target (for all *Legionella* species), and *wzm for L. pneumophila* serogroup *1*(for Lp1) genes ((Mercante and Winchell, 2015).

In addition to detection and diagnosis, NAATs are commonly used for *Legionella* typing, mainly in conjunction with traditional MAb or serology

1.9 Typing of *Legionella*

In epidemiological studies the detection and identification of *Legionella* bacteria is not enough, therefore scientists developed methods with more discrete discrimination for further subtyping

Many subtyping methods can be used on *Legionella 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* species- and strain specific genes, including *ftsZ* and *sidA* , among others (Gilmour et al., 2007; Gomez-Lus et al., 1993; Ratcliff, 2013).

Subtyping methods of *L. pneumophila* strains are considered the most discriminatory methods for typing and subtyping however they require 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).

In the past few years, epidemiological techniques have been developed to be used directly with clinical samples by PCR-based typing methods such as; Multiple locus variable tandem-repeat (MLVA) assays are based on the separation and sizing of short to long tandem repeated sequences. Sequence Based Typing (SBT) is a powerful method based on the sequencing of seven gene loci and is recognized as the new EWGLI (European Working Group for *Legionella* Infections) gold standard tool for *L. pneumophila* typing (Fields et al., 2002; Pancer, 2013).

1.9.1 Nested PCR Sequence Based Typing (NPSBT)

SBT methods are considered the gold-standard methods for *L. pneumophila* genotyping for epidemiological studies and investigations based on multi locus sequence typing (Gaia et al., 2003a; Gaia et al., 2005b; Ratzow et al., 2007). SBT-based strain discrimination depending on the sequences of seven-gene named (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*), with the option of including a *neuA* homologue (*neuAh*) if the standard SBT primers fail to amplify the target in non-sg1 strains. Direct sequence comparison is very important advantage for SBT methods, which eliminates the interpretational subjectivity of non-sequence-based methods such as PFGE, which are prone to banding ambiguities over time or between laboratories (Scaturro et al., 2005).

The European Society for Clinical Microbiology Study Group on *Legionella* Infections (ESGLI) (formerly the European Working Group on *Legionella* Infections [EWGLI]) maintains an allele database (currently version 3.0) that allows querying of large sets of raw sequence data, delivering both an allelic profile and a final combined sequence type (ST) for each isolate. The database is dynamic and continually updated with the addition of new allele sequences and STs.

SBT is performed on DNA extracted from culture isolates; although, many studies have demonstrated some success when SBT was performed directly on nucleic acids extracted from clinical samples such as BAL or Sputum (Ginevra et al., 2009; Coscollá and González-Candelas, 2009), however, SBT when used on culture-independent samples varies widely with sample origin (e.g., sputum versus BAL fluid) and quality and also is typically much lower than that of pure isolate extractions.

Laboratory expertise and the use of high-quality media can maximize bacterial growth from otherwise low-quality samples, yet if an isolate is not obtained because of prior antibiotic therapy or suboptimal shipping and storage, culture-independent SBT offers a potentially viable alternative (Mercante and Winchell, 2015).

DNA sequence based molecular typing techniques are becoming more and more popular. Sequence based typing have several advantages such as the speed of data generation from DNA (i.e., not living strains but DNA can be sent to reference laboratories), unambiguous and highly reproducible nature of data, additive nature of data (data for "eternity"), highly portable data (electronic intra- and inter-laboratory exchange), easy storage of data in searchable databases, easy usage of a standardized nomenclature (central expanding databases) and straight-forward phylogenetic reconstructions from data (Mercante and Winchell, 2015).

Recently, Ginevra et al., (2009), applied SBT methods directly on 63 clinical samples and the efficiency of SBT, was very poor and provided only 3.6% the complete sequencing data. To solve this problem and to increase the sensitivity of SBT when directly performed on clinical samples this group newly designed sets of seven primers that are external to the seven existing ones that are already used for *Legionella SBT*. This new SBT named as Nested-PCR-based

SBT (NPSBT) technique has improved the sensitivity of SBT when applied directly to the same respiratory samples. The results showed that NPSBT significantly improved epidemiological typing 17– fold compared to the initial SBT when applied directly to clinical samples. NPSBT gives rapid and robust discriminatory epidemiological data, in particular when no isolates are available.

NPSBT represents an excellent tool for *L. pneumophila* subtyping, in particular when no isolates are available. NPSBT has an important advantage over SBT for *Legionella* epidemiological investigations because the rapid identification of the source of infection, can allow prompt implementation of environmental control measures to prevent further legionellosis cases (Scaturro et al., 2011).

1.10 Literature review

Legionella is an important cause of mostly adult pneumonia and it must be actively considered in both community – acquired and nosocomial pneumonia (Touray et al., 2014). LD was named after the 1976 outbreak in Philadelphia, Pennsylvania (McDade et al., 1977), after this first outbreak the attention and the awareness has increased. Surveillance schemes for LD was placed in some countries such as USA, Canada, Europe, Japan, Australia and New Zealand, However in most countries LD is under recognized because of lack of awareness of medical personnel and lack of routine microbiological testing for *Legionella* in the hospital bacteriological laboratories (Phin et al., 2014).

LD outbreaks occur each year in the United States and affect 10,000 to 20,000 cases based on The Centers for Disease Control and Prevention (CDC) (Arora et al., 2012).

Large outbreaks of LD have been associated with contaminated cooling towers, hot and cold water systems, and whirlpool spas. Indeed Cruise ships can be sources of *Legionella* for similar reasons and have been associated with outbreaks of LD (Phin et al. 2014).

Although sporadic outbreaks of the disease occur throughout the year, most of the epidemics of *Legionella* infection occur in late summer and autumn, presumably because the organism

proliferates in water reservoirs during the warm months. A large outbreak at a flower show in the Netherlands was traced to the whirlpool spas, which were used in the exhibits (Arora et al., 2012).

Furthermore, beside the community acquired LD, LD has been recognized as an important cause of hospital-acquired pneumonia, Micro-aspiration is the major mode of transmission in hospital-acquired LD (Sabria and Victor, 2002).

Nosocomial LD has become increasingly common, contributing up to 30 percent of hospital-acquired pneumonias in some institutions. LD usually is acquired by inhalation or aspiration of *Legionellae* from contaminated environmental sources (Borella et al., 1999). Potable water is an important source of both nosocomial and community acquired *Legionella* infections (Fields et al., 2002). Nosocomial LD is an important problem in some hospitals in the world. It has been estimated that 20–30% of legionellosis are nosocomial infections and that they are associated with a contamination of the hospital's water distribution system (Tai et al., 2012).

Thacker et al., (1975) reported the first outbreak of hospital-acquired LD in a psychiatric hospital in Washington DC in 1965, 81 patients were reported with pneumonia, with 15 deaths. Retrospective studies on stored serum samples showed antibody seroconversion for *L. pneumophila* in 85% of the patients

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

The largest outbreak of hospital-acquired LD occurred at the Wadsworth Veterans' Administration Medical Center (VAMC) in Los Angeles, from 1982 to 1985, and the potable water supply was the actual source of hospital acquired LD (Sabria and Victor, 2002).

Now, it is well known that the potable water system is a major source of infection by *Legionella* in hospitals; *Wellinghausen et al*., (2001) studied the contamination of hospital water systems with *Legionella* at three different hospitals belonging to the University of Ulm in Germany between October 2000 and February 2001. A total of 77 potable water samples were collected. The rates of detection of *Legionella* were 70.1% (54 of 77).

In Palestine the hospitals water systems are also contaminated with *L. pneumophila* based on a previous study by *Shareef et al.,* (2008) and a more recent study in our laboratory in which a two-year (December 2012 – December 2014) proactive environmental surveillance of *L. pneumophila* in the water distribution systems of eight hospitals in the West Bank was carried out. All the water sources tested positive for the presence of *Legionella* with 8.3% prevalence for the water samples (n=72) by culture and the prevalence rate increased to 50% (n=72) by PCR. Testing biofilms, the prevalence of *L. pneumophila* was much more prevalent being 16.8% (n= 1136) by culture and increasing to 61.3% (n=225) by PCR.

For the conformation of nosocomial acquired LD in hospitals, different methods can be used, but when no isolates are available as pure colonies the molecular methods such as SBT and Nested-SBT are considered as a gold standard method. *Luck et al*., (2008) reported a LD case for a 66-year-old man who was hospitalized at the University Hospital of Berlin in 2004. Eighteen days later, he was readmitted to the intensive care unit of the same hospital with a 3-day history of fever, also the patient had received a cadaveric kidney transplant 4 months earlier. A Broncho alveolar lavage fluid (BAL) collected on day 2 after admission was positive for *L. pneumophila*-species antigen by direct immunofluorescence mAb staining. Four months after developing *Legionella* pneumonia he died of multi-organ failure. Environmental samples were taken from the hospital and home water supplies, the results of AFLP and SBT typing confirmed that the clinical strain and environmental isolates from the patient's home were indistinguishable. This is a strong argument for the transmission from the patient's home water supply.

1.11 Problem statement

Since water environments (naturally occurring and man- made water) are the major reservoirs for *L. pneumophila*, this is a huge problem when the *Legionella* bacteria are present in hospital water systems. The serious health risk to hospital staff and the patients is a well-known risk factor for hospital-acquired pneumonia (Benin et al., 2002).

The Microbiology Research Laboratory at Al-Quds University investigated the prevalence of *Legionella* spp. in environmental samples, and showed high prevalence of *Legionella* spp. in the Palestinian water environment collected from eight hospitals by using culturing methods and PCR. Regarding the clinical prevalence of *L. pneumophila*, 121 sputum and 74 Broncho alveolar lavage specimens (n=195) collected from suspected pneumonia patients in the West Bank, from September 2014 till June 2016, were cultured for *L. pneumophila* and genomic DNA was extracted and tested by PCR amplification for *L. pneumophila 16S rRNA* gene. Only one out of 195 cultured respiratory specimens was positive for *L. pneumophila.* The very low yield by culture maybe very much related to the heavy antibiotic use in Palestine prior to hospital admission. By PCR, 44/195 (23%) of the respiratory samples were positive for *L. pneumophila*. Bronchial lavage presented a higher percentage 35%, (26/74) than sputum, which revealed 15% (18/121) positive.

These results are the first evidence for the presence of *L. pneumophila* in clinical samples collected from suspected pneumonia patients from Makassed Hospital in Palestine. In this study we took the research project a step further analyzing the *L. pneumophila* PCR positive BAL and sputum samples by NPSBT in order to identify the ST's in the respiratory samples and to compare these ST's with the ST's of *L. pneumophila* PCR positive environmental DNA samples obtained from the same hospital.

1.12 Goals

The main goal of this study is SBT of *L. pneumophila* by using Nested PCR sequenced based typing method. NPSBT is the best method available to determine the sequence type of

Legionella directly from clinical samples in the absence of isolates and to compare the sequence types in the study sample with those in the EWGLI data base. Finally to try to determine possible nosocomial infection by comparing the sequence types from clinical samples with the sequence types of environmental isolates obtained from patient's wards in Makassed hospital.

1.13 Questions

1. How do the ST's of *L. pneumophila* in Palestine compare to other countries?
2. How do the Sequence types of the clinical samples compare to the Sequence types of the environmental samples.
3. What is the possible source of infection?

1.14 Hypothesis

L. pneumophila is present in water systems in Palestinian hospitals, and it is very important to take action regarding surveillance of water systems for *Legionella* spp.

Legionella are opportunistic bacteria that cause a fatal form of an atypical pneumonia for hospitalized and immunocompromised patients, as well as community acquired pneumonia. *L. pneumophila* is the cause of up to 90% of clinical cases and serogroup 1 is the cause of 84% of those cases (Yu, 2001a; Yu et al., 2002b).

Previous work in the laboratory tested 195 clinical specimens obtained from suspected pneumonia patients by culture methods and by molecular methods (PCR). The identification by culture yield was very low, one positive sample out of 195 samples tested, most likely due to the overuse of antibiotics in Palestine. By PCR the yield was much higher (23%). However in order to do an epidemiological study we need to do subtyping at the DNA level. Since Nested PCR-derived SBT method is highly recommended and can be applied to respiratory samples from patients with legionellosis directly (without the need for an isolate) we set out to test and

analyze the clinical samples (sputum or BAL) collected from suspected pneumonia patients from Makassed hospital for *L. pneumophila*. Specimens that tested positive by PCR for *L. pneumophila* were subtyped by using Nested-PCR-SBT. This typing is based on the sequences of seven gene loci (*flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA*), and comparing those sequences with the allele database of EWGLI (Ginevra et al., 2009).

Chapter Two

2. Methodology

2.1 Inclusion criteria

The clinical samples were collected from suspected pneumonia patients presenting to Makassed Hospital in Jerusalem in the period between September 2014 and August 2015, after signing a written informed consent form (see Appendix A) and ethical approval from Al-Quds University and the Hospital's Ethical Committee and the Palestinian Health Research Council/ Helsinki Committee (PHRC/HC/211/17).

2.2 Research place

The research was carried out at the Microbiology Research Lab, Al-Quds University, towers building B, 6th floor, Abu Dies, Jerusalem.

2.3 Sample collection

2.3.1 Clinical samples

Broncho Alveolar Lavage (BAL) samples (n=74) and sputum samples (n=121) were collected at Makassed hospital from suspected pneumonia patients. The samples were stored at 4°C until received by the Microbiology Research Lab at Al-Quds University within 48 hours.

2.3.2 Environmental samples

The environmental samples (water from (faucet and shower) and biofilm) had been collected from Makassed Hospital previously, and the microbiological (culture) and molecular (PCR) analyses for *L. pneumophila* had been performed in the Microbiology Research laboratory at Al-Quds University (Zayed, 2013).

2.4 Sample preparation

2.4.1 Culture method

Total samples (Sputum and BAL samples) were treated thermally (56°C for 10 min) and then inoculated onto GVPC (Glycine-Vancomycin-Polymyxin-Cycloheximide; BD, United States) *Legionella* selective media. Plates were incubated at 37 ºC for 7 days. Five colonies of each positive sample were selected and re-isolated on GVPC plates. Identification as *Legionella spp*. was confirmed by the ability to grow on GVPC and inability to grow on blood agar (M073, HI media, India) (L-cysteine free) plates. Positive samples were re streaked on BCYE and further identified by an agglutination test using (*Legionella* Latex Test, Oxoid DR0800M, England) according to manufacturer's instruction. The test allows a separate identification of *L. pneumophila* serogroup 1, serogroups 2-14 and of detection of seven *Legionella* species (*L. non- pneumophila*).

2.4.2 Molecular method

2.4.2.1 DNA extraction from sputum and BAL samples

The BAL or sputum samples were stored at 4°C until processed. Each sample was vortexed at room temperature for 10 seconds and 1-2 ml of each sample was transferred to a fresh sterile tube. Genomic DNA was extracted from each BAL and sputum sample by using DNA extraction kit (Qiagen, 69506,Germany).

Briefly, 2 ml of BAL and 1 ml of sputum was centrifuged at 17000xg for 10 minutes then the pellet was suspended in 250 ul of sputum 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) with 250 ul of molecular grade water (Promega, USA), then incubated at 37^{o}C for 30 minutes (with shaking at 1500 rpm). The samples were centrifuged at 17000x g for 15 minutes, and then the supernatant was discarded. 180ul of ATL buffer was added with 20 ul of proteinase K (15mg/ml) and incubated at 56 ^{o}C for 1 hour (with shaking at 1500 rpm), then 200ul of AL buffer was added to the sample and incubated at 70^{o}C for 10 min, then 200 mml of ethanol (96-100%) was added and mixed by vortexing, the samples were loaded onto the spin column and centrifuged at 17000x g for 1 minute, then 500ul of AW1 buffer was added and centrifuged at 6000x g for 1 min, then 500 ul of AW2 buffer was added then centrifuged at 17000x g for 3 minutes, then the spin column was placed in a new 2 ml collection tube and 100 ul of AE buffer or distilled water was added then centrifuged at 6000x g. Extracted DNA was stored at -80^{o}C

2.4.2.2 Environmental samples

We used extracted DNA from the 15 environmental samples (water (cold and hot), and biofilm), which were collected from faucets and showers from different departments at Makassed Hospital, and processed previously by the Microbiology Research Lab, Al-Quds University (Zayed, 2013).

2.5 Positive controls

DNA of *Legionella* as shown in (Table 2.1) kindly provided by (Prof. Manfred Höfle, HZI, Braunschweig, Germany) was used as DNA control in this study.

Table 2.1: *Legionella* reference strains

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

2.6 Negative control

Molecular grade water (Promega, USA) was used as negative control in 16S rRNA PCR and the Nested Sequence Based Typing PCR.

2.7 Polymerase Chain Reaction

2.7.1 PCR for respiratory samples

PCR was used for identification of *L. pneumophila* species in BAL and sputum samples. PCR *L. pneumophila* species (L1) primers were purchased from (hylabs, Park Tamar, 76326 Rehovot, Israel) to amplify PCR product of 544bp. The primer sequences are shown in (Table 2.2). Their location on the complete genome of 16s rRNA of *L. pneumophila subsp.pneumophila* ATCC 43290 is shown in (Fig. 2.1). Some of the clinical samples were

previously analyzed in Microbiology Research laboratory at Al-Quds University, for identification of *L. pneumophila* by PCR.

AccuPower ® HotStart PCR Premix was used for PCR amplification; to each AccuPower ® Hot Start PCR Premix tube, a mixture of 0.8ul (10mmol) forward primer (L1F), 0.8ul (10mmol) reverse primer (L1R), and 4ul (100ug/ml) DNA template was distributed into the each Premix and 14.4 ul molecular grade water (Promega, USA), were added. PCR amplification was done on thermal cycler (1861096, Bio-Rad, USA) per the following conditions: predenaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 60 seconds, elongation at 72°C for 45 seconds and final elongation at 72°C for 5 minutes. DNA of *L. pneumophila* (AUG2013 (4pg) was used as positive control and Molecular grade water (Promega, USA) was used as negative control. PCR products of 544bp were analyzed by agarose gel electrophoresis.

2.7.2 PCR for environmental samples

The detection of *L. pneumophila in* environmental samples was done previously in the Microbiology Research laboratory (Zayed, 2013).

Table 2.2: Primers used in the study

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

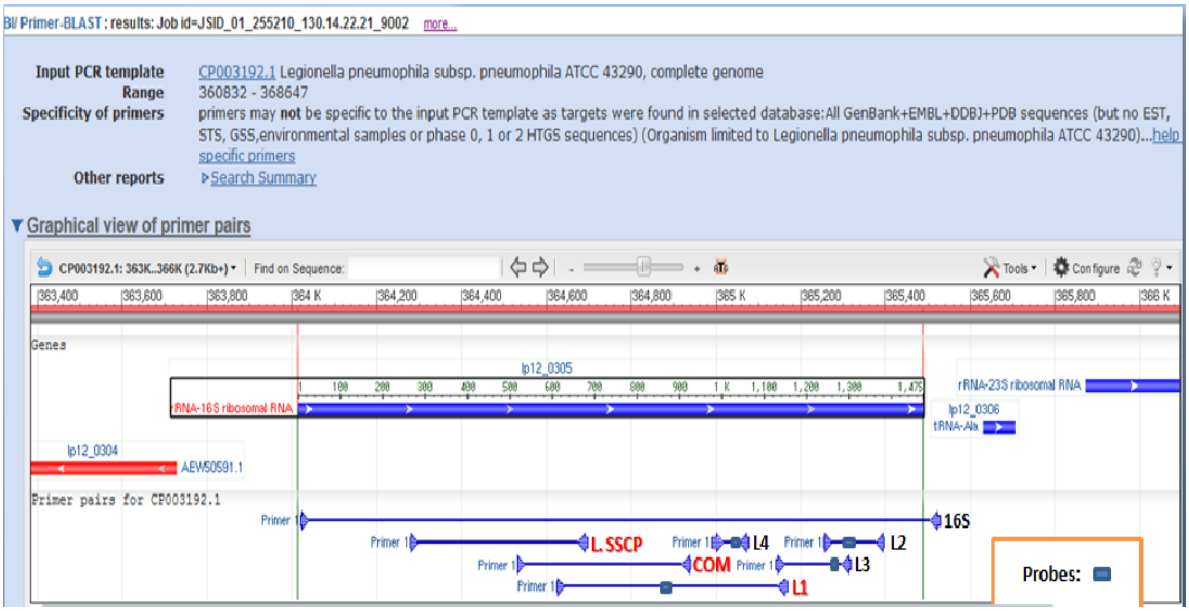

Figure 2.1: *L. pneumophila* whole genome and primers location (Dr. Manfred Höfle,, HZI)

2.8 Molecular typing methods

2.8.1 Nested PCR Sequence Based Typing

NPSBT was used to obtain typing data for *L. pneumophila* from BAL and sputum samples. Typically DNA extracts from respiratory samples previously demonstrated to be positive for the presence of *L. pneumophila* by PCR targeting 16S rRNA were used as the starting material.

The procedure according to European Working Group for *Legionella* Infections (EWGLI) , Nested -SBT (version 2.0) was applied (Mentasti and Fry, 2012).

(http://www.hpabioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php).The NPSBT method includes two rounds of PCR; the first round of PCR is carried out with 7 primer pairs *(flaA, pilE, asd, mip, mompS, proA, neuA)* (Table 2.3), which bind externally to those used in the second round PCR for another 7 primer pairs (Table 2.4)

Table2.3 : First round NPSBT amplification primers (*Adapted from Mentasti and Fry, 2012)*

First Round NESTED SBT amplification primers:

Gene	Primer name	Primer sequence (5'-3')	Annealing temperature
flaA	flaA-L-N [1]	TAT GCG TGA GCT TTC CGT TC	50 °C
	flaA-960R [2]	CCA TTA ATC GTT AAG TTG TAG G	
pilE	pilE-L-N [1]	CGT TGG AAT CGG CTT GTC	50 °C
	pilE-R-N [1]	CGC ATT GGC AGA GGA ATC TA	
asd	asd-1-N [1]	CCC TGG AAG TGA ATC CTC AT	50 °C
	asd-2-N [1]	TTG CAG TAT TTC AGC GAT CTG T	
mip	mip-1-N [1]	TGA AGA TGA AAT TGG TGA CTG C	50 °C
	mip-2-N [1]	AAT AGG TCC GCC AAC GCT AC	
mompS	mompS-450F [2]	TTG ACC ATG AGT GGG ATT GG	50 °C
	mompS-R-N [1]	TGG ATA AAT TAT CCA GCC GGA CTT C	
proA	proA-L-N [1]	CCG CTT CTC CAA CCA ATG A	50 °C
	proA-R-N [1]	CAC TCA ACA TAC CGC AAC CA	
neuA	neuA-F-N [1]	CCT TGC AGT CGT CTT GTT GT	50 °C
	neuA-R-N [1]	TTT CTG TTA GAG CCC AAT CG	

Table 2.4 : Second NPSBT amplification primers *(Adapted from Mentasti and Fry, 2012)*

Second round NESTED SBT PCR amplification primers:

Gene	Primer name	Primer sequence (5'-3')	Annealing temperature
flaA	flaA-587F [2]	GCG TAT TGC TCA AAA TAC TG	55 °C
	flaA-R-N [1]	GGT ATC ACC TGC GGT TCC A	
pilE	pilE-35F [2]	CAC AAT CGG ATG GAA CAC AAA CTA	55 °C
	pilE-453R [2]	GCT GGC GCA CTC GGT ATC T	
asd	asd-511F [2]	CCC TAA TTG CTC TAC CAT TCA GAT G	62°C
	asd-1039R [2]	CGA ATG TTA TCT GCG ACT ATC CAC	
mip	mip-74F [2]	GCT GCA ACC GAT GCC AC	60 °C
	mip-595R [2]	CAT ATG CAA GAC CTG AGG GAA C	
mompS	mompS-509F [2]	GAC ATC AAT GTG AAC TGG	55 °C
	mompS-1015R [2]	CAG AAG CTG CGA AAT CAG	
proA	proA-1107F [2]	GAT CGC CAA TGC AAT TAG	55 °C
	proA-1553R [2]	ACC ATA ACA TCA AAA GCC	
neuA	neuA-196F [2]	CCG TTC AAT ATG GGG CTT CAG	55 °C
	neuA-634R [2]	CGA TGT CGA TGG ATT CAC TAA TAC	

2.8.2 First round PCR reaction

In the first round PCR, DNA samples which were positive by 16S *rRNA* targeting *L. pneumophila* were used as starting material. PCR amplification was performed in a total volume of 20 µl. PCR plate was placed on a cold block, PCR mix was started by adding 4 µl of Taq DNA polymerase Mix to each PCR tube (Table 2.5), then 11 µl of the Master Mix was added (Table 2.6), finally the DNA template (5 µl) was added. The negative control received (5 µl of nuclease free water (Promega, USA) and the positive control received 5 ul of *L. pneumophila* DNA. The Pipetting of solutions was done in the following order; test samples, negative control, and lastly positive control (Mentasti and Fry, 2012).

Table 2.5: Taq polymerase mix

Nuclease-free water	3.4 µl
PCR buffer (x10)	0.4 µl
Taq DNA polymerse (5U/µl)	0.2 µl
Volume/reaction	4.0 µl

Table 2.6: Reaction Master Mix

Reagents	**Final concentration**	**Volume/reaction (µl)**
Nuclease-free water		7.1 µl
PCR buffer	1 X	1.1µl
MgCl2	2.5 mM	0.4 µl
Nested Primer F	10 pmoles	1 µl
Nested Primer R	10 pmoles	1 µl
DNTPs	200µM	0.4 µl
Volume/reaction		11.0 µl

2.8.3 Thermal cycler parameters

PCR amplification was performed using thermal cycler (1861096,Biorad, USA) according to the following conditions: initial denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, elongation at 72°C for 60 seconds and final elongation at 72°C for 10 minutes (Table 2.7) (Mentasti and Fry, 2012).

Table 2.7: Thermal cycler conditions for first round.

Step	Temperature (°C)	Time		No. cycles
1	95	5 min	Initial denaturation	1
2	95	30 sec	Denaturation	40
	50	30 sec	Annealing	
	72	60 sec	Extension	
3	72	10 min	Final Extension	1
4	12	∞	Hold	

2.8.4 Second round PCR reaction

The PCR products from the first round PCR were used as starting material for the second round.

PCR amplification was performed in a total volume of 20 µl, PCR plate was placed on a cold block. PCR mix was started by adding 4 µl of Taq DNA polymerase Mix to each PCR tube (Table 2.5), Then 11 µl of Master Mix was added to each tube (Table 2.6), with using second specific primers. Finally 5 µl from each of the completed PCR reaction mix from the first round was used as starting template. The tubes/plates were spun briefly in a centrifuge before placing them in a thermocycler heating block. The pipetting of solutions

was done in the following order, test samples, negative control and lastly positive control.

2.8.5 Thermal cycler parameters for second round

PCR amplification was performed using thermal cycler (1861096,Biorad, USA) according to the following conditions: initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 60 seconds and final elongation at 72°C for 10 minutes (Table 2.8) (Mentasti and Fry, 2012).

Table 2.8: Thermal cycler conditions for the second round

Step	Temperature (°C)	Time		No. cycles
1	95	5 min	Initial denaturation	1
2	95	30 sec	Denaturation	35
	55	30 sec	Annealing	
	72	60 sec	Extension	
3	72	10 min	Final Extension	1
4	12	∞	Hold	

2.9 Agarose gel preparation and electrophoresis

The 1.5 % agarose gel was prepared by dissolving 3g agarose (A9539, Sigma Aldrich, Germany) in 200 ml 1X TAE electrophoresis buffer (0.04 M Tris-Acetate, pH 8.0 and 0.001M EDTA), boiled in the microwave, and when it cooled to 50°C, 5µl of ethidium bromide (1 µg/ml) (hylabs, Israel) was added. The PCR products were analyzed on horizontal gel electrophoresis (Multisub, Biocom, Germany). The applied voltage was 120 volts for one hour

2.10 DNA Sequencing

After performing gel electrophoresis on the PCR products from the second round, the positive Nested PCR products were sent to hylabs, Israel, for purification and sequencing

and the second round primers were used as sequencing primers. Sequencing results were submitted to the online *Legionella* SBT Quality Tool www.hpa-bioinformatics.org.uk/cgibin/legionella/sbt/seq_assemble_legionella1.cgi. which was used to assign individual allele numbers. For each isolate, the combination of seven alleles was defined as a seven-digit allelic profile by using the predetermined order *flaA, pilE, asd, mip, mompS, proA,* and *neuA* (e.g., 1-4-3-1-1-1-1) and a sequence type (ST) represented by a number (e.g., ST1).

Chapter Three

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3. Results

3.1 Samples

3.1.1 Sample study (Inclusion criteria)

This study included respiratory samples (n=195); 74 BAL and 121 sputum samples collected from suspected pneumonia patients that were hospitalized at Makassed Hospital, in the period between September 2014 and August 2015.

3.1.2 Patients' data

The study included 75 female patients, twenty two of them (29.3%) were positive for *L. pneumophila* by PCR targeting the *16SrRNA* and 120 of male patients, twenty-two of them (18.3%) were positive for *L. pneumophila* by PCR targeting the *16SrRNA*. The age of the patients ranged between 3 months and 86 years. Patients were hospitalized in different departments; internal medicine, pediatric, intensive care unit (ICU), ICU pediatric, cardiac care unit (CCU), surgery and gynecology (see Appendix B)

3.2 Detection of *L. pneumophila* in respiratory samples

3.2.1 Cultivation dependent analysis

A total of 195 respiratory samples (74 BAL and 121 sputum samples) were tested by cultivation dependent analysis by plating on GVPC *Legionella* selective media (Glycine – Vancomycin – Polymyxin, Cycloheximide; BD, United States). Only one of the total 195 samples was positive for *L. pneumophila by* routine bacteriological culture method. This very low yield may be explained due to the regimen of antibiotics heavily used by patients prior to hospitalization.

3.2.2 Cultivation independent analysis

3.2.2.1 Detection of *L. pneumophila* in respiratory samples by PCR using 16*S* rRNA (L1 primer)

DNA extracted from 195 respiratory samples, 121 sputum and 74 BAL samples collected from suspected pneumonia patients presenting to Makassed hospital in Eastern Jerusalem were screened for the presence of *L. pneumophila* using (L1 primer) *16S rRNA* PCR. L1 primer gives PCR product 544bp as shown in figure (3.1)

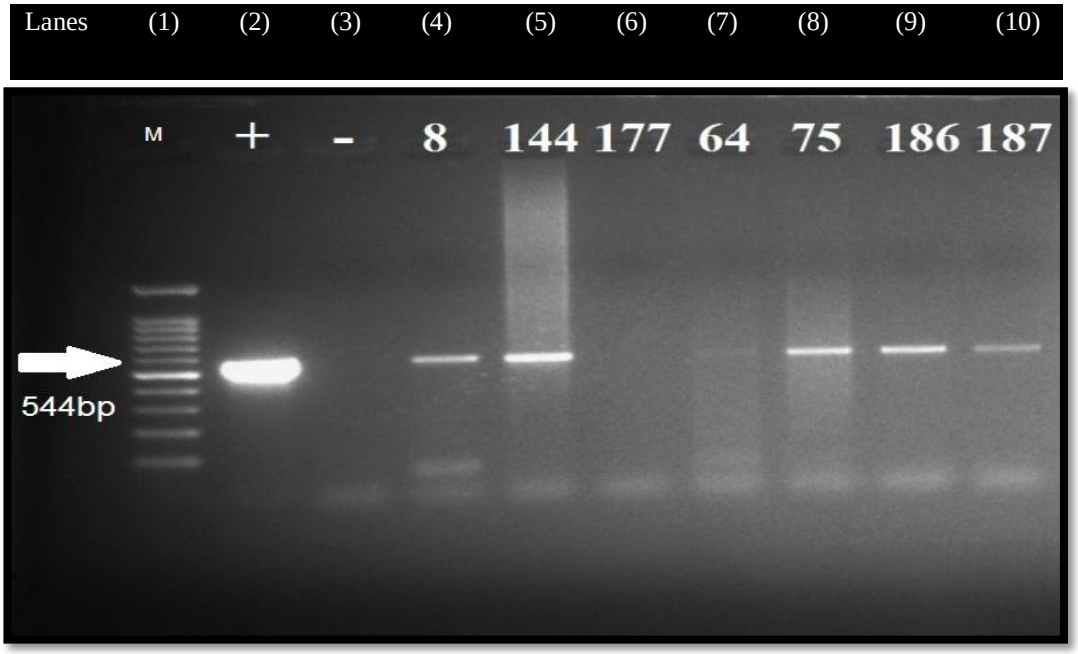

Figure 3.1: Representative *16S rRNA* PCR using L1 primer of DNA extracted from the respiratory samples; Lane 1 represents 100bp ladder (M). Lane 2 represents positive control (P) DNA of cultured *L. pneumophila*. Lane 3 (N) represents negative control (Molecular grade water (Promega, USA). Lanes 4 to 10 represent clinical respiratory samples, lane 4, 5, 7, 8, 9 and10 represent positive samples showing a 544bp band.

3.2.2.2.1 The prevalence of *L. pneumophila* in respiratory samples

PCR analysis of 195 respiratory samples for *L. pneumophila* revealed (44/195) 23% positive and (151/195) 77% negative in all (sputum and BAL) samples targeting *16S rRNA* specific for *L. pneumophila* (Figure 3.2).

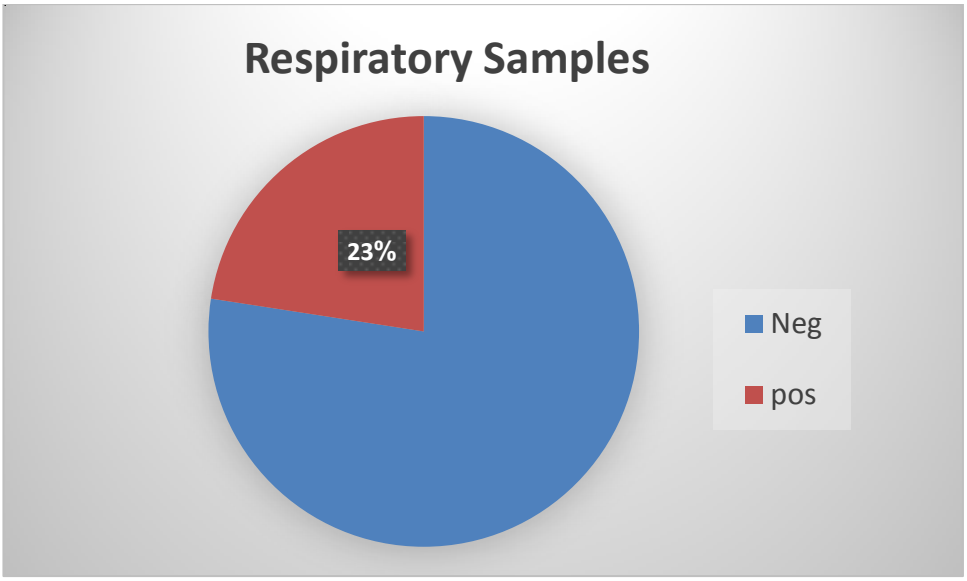

Figure (3.2): Prevalence of *L. pneumophila* in respiratory samples by PCR targeting *16S rRNA* (L1 primer).

3.2.2.2.2 The prevalence of *L. pneumophila* in sputum Vs. BAL samples

PCR analysis of sputum samples (n=121) for *L. pneumophila* (L1 primer), showed 18 (15%) positive and 103 (85%) negative samples, In contrast, the PCR analysis of BAL samples (n=74) for *L. pneumophila* (L1 primer), showed 26 (35%) positive and 48 (65%) negative samples (Figure 3.3).

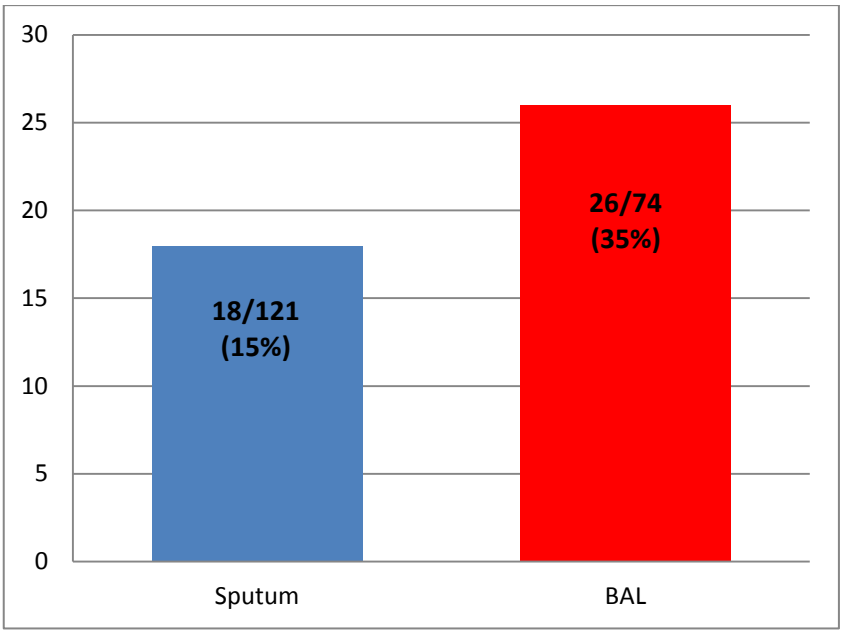

Figure 3.3: The prevalence of *L. pneumophila* in sputum vs. BAL samples using PCR targeting *16S rRNA* (L1 primer).

3.2.2.2.3 The distribution of *L. pneumophila* positive samples from pneumonia patients according to hospital department

The largest number of positive samples was in patients hospitalized in, internal medicine ward (36.3%), followed by patients from pediatric wards each (25%), followed by patients from ICU ward with (22.7%), followed by surgery (9%), ICU pediatric (4.5%) and gynecology (2.3%) wards. (Figure 3.5).

3.2.2.2.4 The distribution of LD patients according to age

The largest number of patients with legionellosis was under 20 years of age with 15 cases (34 %), 6 cases (13.6%) were between 20-40 years, 10 cases were between 41-60 years (22.7%), and 13 cases above 60 years (29.5 %) Figure (3.6).

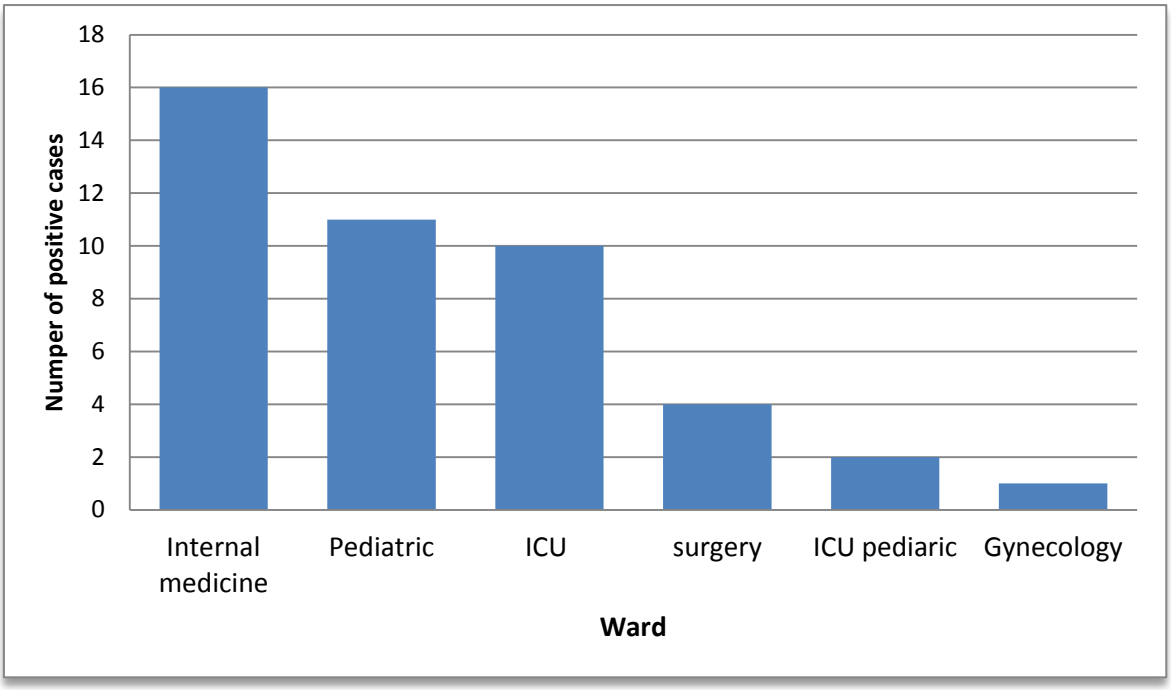

Figure 3.5: The distribution of *L. pneumophila* positive samples (n=44) by PCR according to hospital department

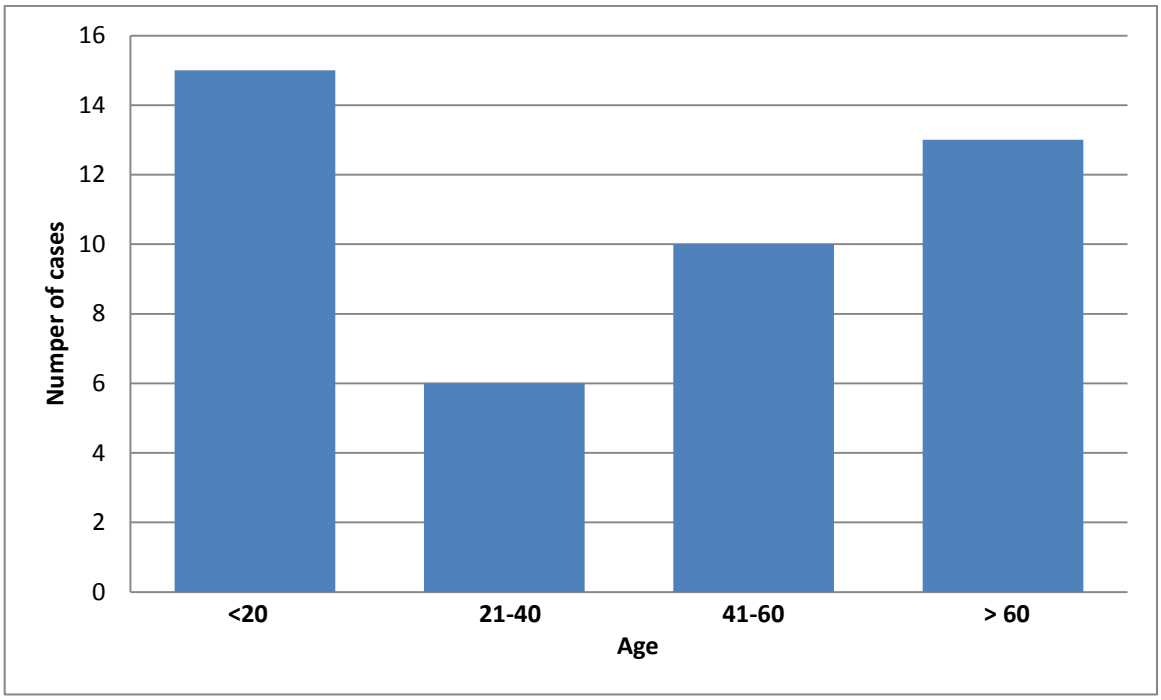

Figure 3.6: The distribution of *L. pneumophila* positive samples (n=44) by PCR, from pneumonia patients according to age.

3.2.2.2.5 The distribution of patients with legionellosis according to Gender

Twenty two males were positive for *L. pneumophila* by PCR out of 120 total males with (18.3%), in contrast, twenty two females were positive out of 75 total females with (29.3%).

Table (3.1): The distribution of *L. pneumophila* positive samples (n=44) by PCR according to gender.

Gender		Negative	Positive	Total
F	Count	53	22	75
	% within Gender	70.7%	29.3%	100.0%
	% within Conventional PCR	35.1%	50.0%	38.3%
M	Count	98	22	120
	% within Gender	81.7%	18.3%	100.0%
	% within Conventional PCR	64.9%	50.0%	61.2%
Total	Count	151	44	195
	% within Gender	77.43%	22.56%	100.0%
	% within Conventional PCR	100.0%	100.0%	100.0%

P value < 0.05

3.3 Molecular typing of *L. pneumophila* in respiratory samples by Nested PCR sequence Based Typing (NPSBT)

Out of 44 samples positive for *L. pneumophila* by 16S rRNA PCR, 34 clinical samples which contains sufficient quantity were selected and processed for Nested PCR Sequence Based Typing method according to EWGLI standard scheme as explained in materials and methods.

Seven PCR tubes (one for each targeted gene) were prepared for each sample. In the first round, PCR primers flanking the targeted gene of interest (*flaA, pilE, asd, mip, momps, proA and neuA)* were used. The PCR products from the first round were used as starting material for the second PCR set, in which primers targeting the genes (*flaA, pile, asd, mip, momps, proA and neuA)* were used. PCR products of second round were analyzed by gel electrophoresis, Figure (3.8).

In addition, 15 environmental samples obtained from the different wards of Makassed hospital which were collected and analyzed previously by the Microbiology Research laboratory were also typed by Nested PCR Sequence Based Typing molecular method. The aim is to compare the Sequence types obtained for the respiratory samples, with the Sequence types of the environmental samples to find any nosocomial correlation.

PCR products of both clinical and environmental samples were purified and sequenced in Hy Laboratories Ltd (hylabs), sequencing results were submitted to the "Sequence Quality Tool"

(www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi).

which was used to assign individual allele numbers. For each isolate, the combination of seven alleles was defined as a seven-digit allelic profile by using the predetermined order *flaA, pilE, asd, mip, mompS, proA,* and *neuA* (e.g., 1-4-3-1-1-1-1) and a sequence type (ST) represented by a number (e.g., ST1).

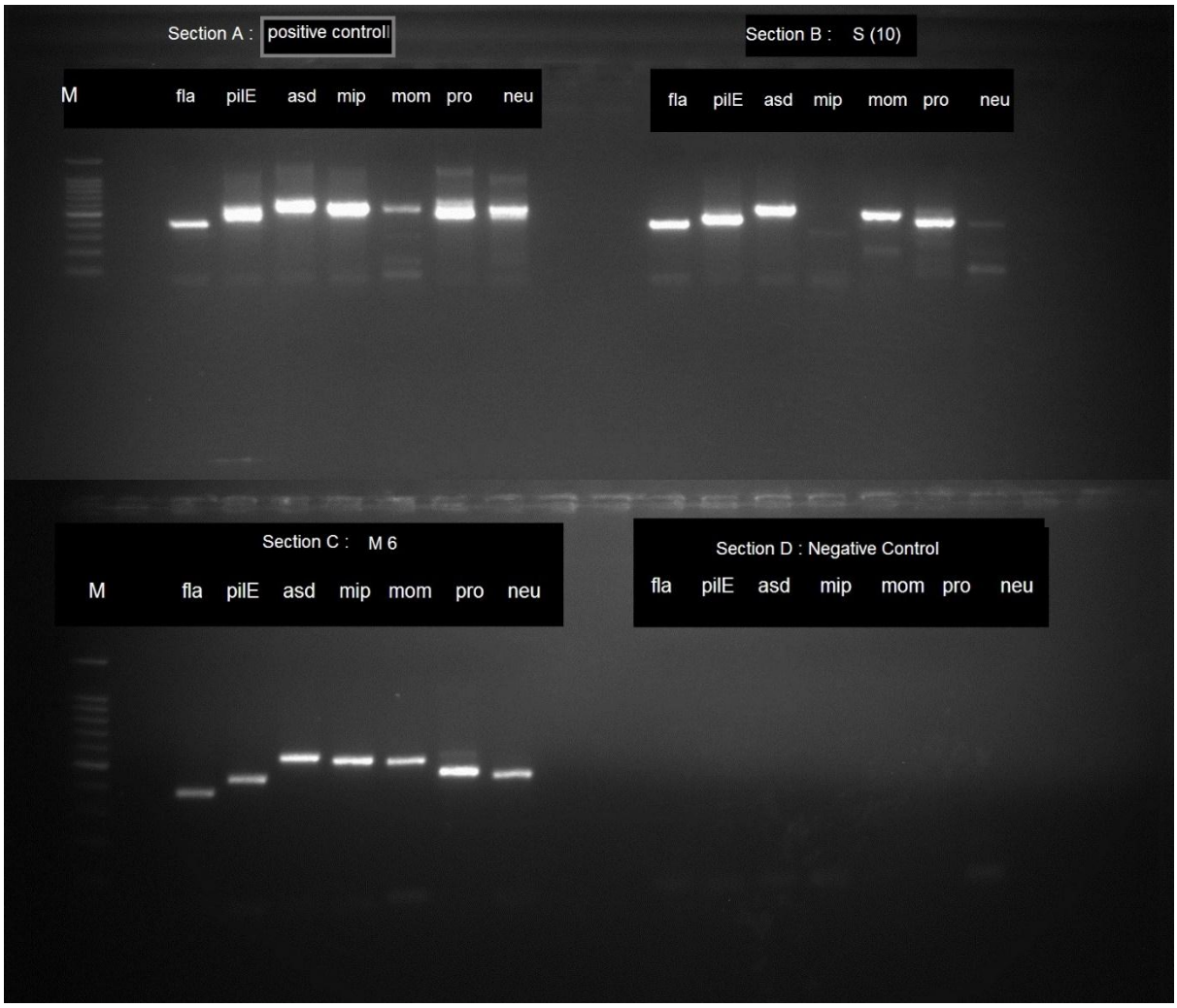

Figure 3.8: Gel electrophoresis for seven alleles' products of the second round PCR; M is 100pb ladder, section A is the positive control for the seven alleles *flaA, pilE, asd, mip, mompS, proA, and neuA*, section B represents BAL specimen (S10), section C represents BAL specimen (M6,) section D represents the negative control for this experiment.

3.3.1 Nested PCR for respiratory samples

Analysis of the allele profile (number of positive bands seen in the gel) for the nested PCR of 34 selected samples showed 24 positive with ≥ 4 positive bands (4, 5, 6 and 7 bands), however, 10 samples gave < 4 positive bands, these samples were excluded from sequencing, (Figure 3.1). (see Appendix C).

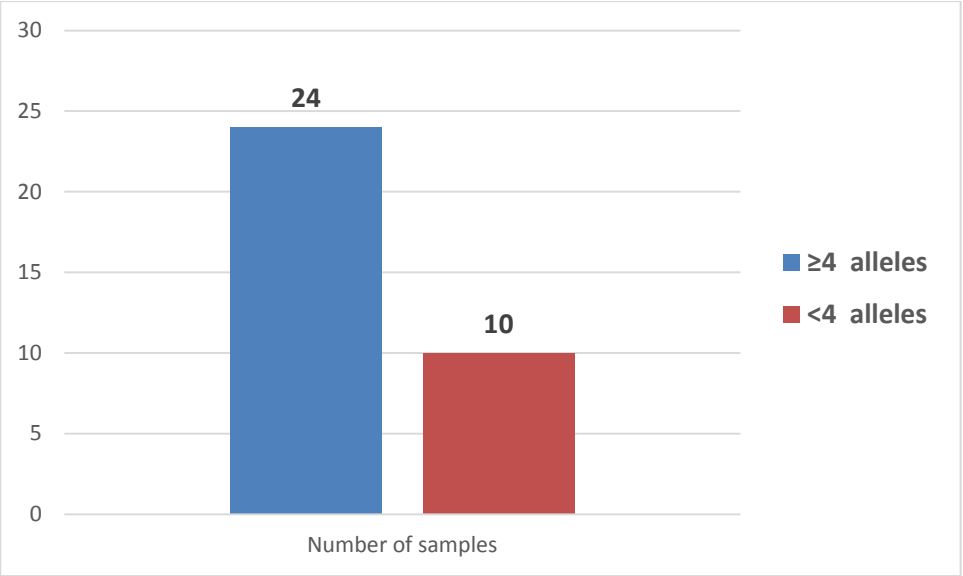

Figure 3.9: The number of samples with ≥ 4 bands (alleles) shown in gels of second round PCR for respiratory samples.

Table (3.1) illustrates the percentage of positive alleles amplified by Nested PCR in respiratory samples *pilE* allele was the highest amplified allele with 85.2% of the thirty four samples, followed by *flaA* and *mompS* with 61.7%, then *mip* allele (58.8%), *asd* and *neu* with (55.8%), and finally the lowest amplified allele was *proA* with 50% of the 34 samples.

Table (3.2) Number of positive alleles and percentage in respiratory samples

Allele	Number of alleles amplified	Number of alleles not amplified	% positive
flaA	**21/34**	**13/34**	**61.7%**
pilE	**29/34**	**5/34**	**85.2%**
Asd	**19/34**	**15/34**	**55.8%**
Mip	**20/34**	**14/34**	**58.8%**
mompS	**21/34**	**13/34**	**61.7%**
proA	**17/34**	**17/34**	**50 %**
neuA	**19/34**	**15/34**	**55.8%**

3.3.2 Nested PCR for environmental samples

Analysis of gel electrophoresis for seven alleles' products of the second round PCR for the selected fifteen environmental samples revealed fourteen samples were positive for six to seven alleles (bands), and one sample was positive for one allele, this sample was excluded from sequencing. (Figure 3.10). (see Appendix c).

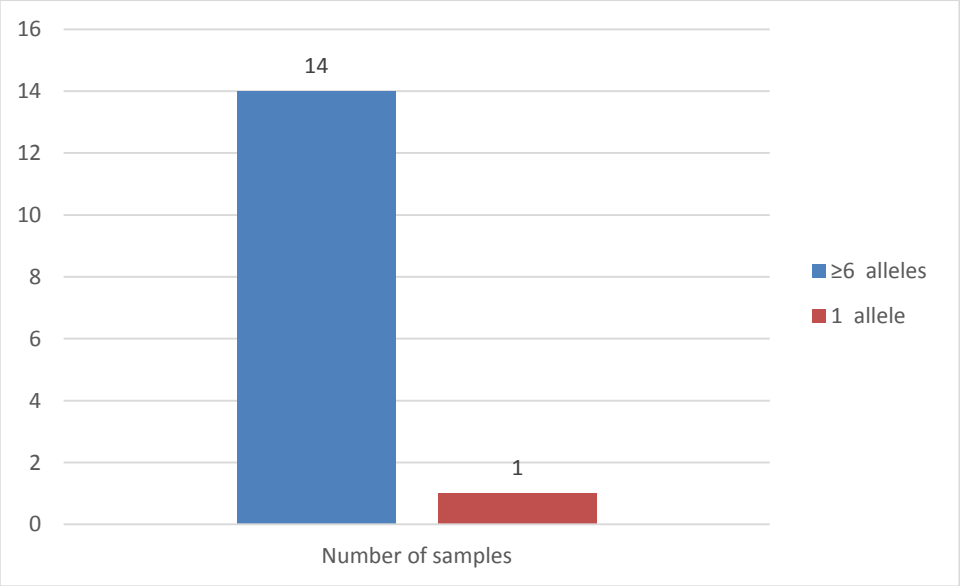

Figure 3.10: The number of samples with ≥ 6 bands (alleles) shown in gels of second round PCR for environmental samples.

Table (3.2) illustrates the percentage of positive alleles, which were amplified by Nested-PCR in the environmental samples. The alleles' amplification in Nested-PCR of the environmental samples were higher than the amplification in clinical samples. The percentage of amplification of *flaA*, *pilE*, *asd*, *mip*, and *mompS* alleles was (93.3%) of all fifteen samples, followed by *proA* and *neu* with (86.6%) amplified.

Table (3.3) Number of positive alleles and percentage in the environmental samples

Allele	Number of alleles amplified	Number of alleles not amplified	% positive
FlaA	**14/15**	**1/15**	**93.3%**
PilE	**14/15**	**1/15**	**93.3%**
Asd	**14/15**	**1/15**	**93.3%**
Mip	**14/15**	**1/15**	**93.3%**
Momps	**14/15**	**1/15**	**93.3%**
ProA	**13/15**	**2/15**	**86.6%**
Neu	**13/15**	**2/15**	**86.6%**

3.4 Sequence type distribution:

3.4.1 Sequence type distribution in respiratory samples

Twenty-four BAL samples that were positive for four, five, six or seven alleles by Nested PCR were sequenced, the sequence files were submitted to (www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi). Website to get the Sequence Types, (Figure 3.12).

After submission of data files, the sequence types were; ST1 (29.1%), ST 461 (25%), ST 1037 (4.7%), and 41.9% incomplete profile. ST 1 was the most predominant sequence type in clinical samples (Figure 3.11). (see Appendix D).

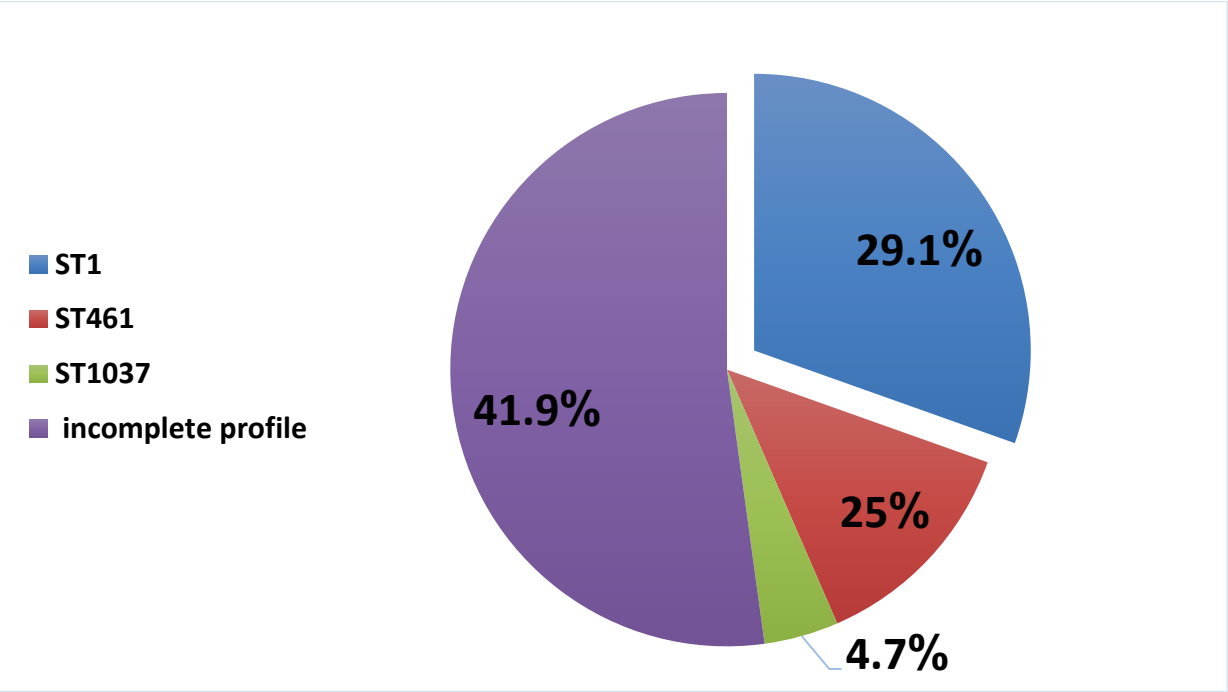

Figure 3.11: The distribution of Sequence Types (STs) for respiratory samples.

3.4.2 Sequence type distribution in environmental samples

Fourteen environmental samples were sequenced after Nested PCR analysis the sequence files for the alleles were submitted to (www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi). to determine the Sequence Types.

The results of Sequence Types of environmental samples resulted in five different sequence types; ST 1(28.5%, 4/14), ST 187 (21.4%, 3/14), one sample of ST 2070, ST 461 and ST 187 (7.1 %, 1/14), while the rest of samples (28.5%, 4/14) were unspecified Sequence types. The most prevalent ST in the environmental samples is ST1, Figure 3.8. (See Appendix D).

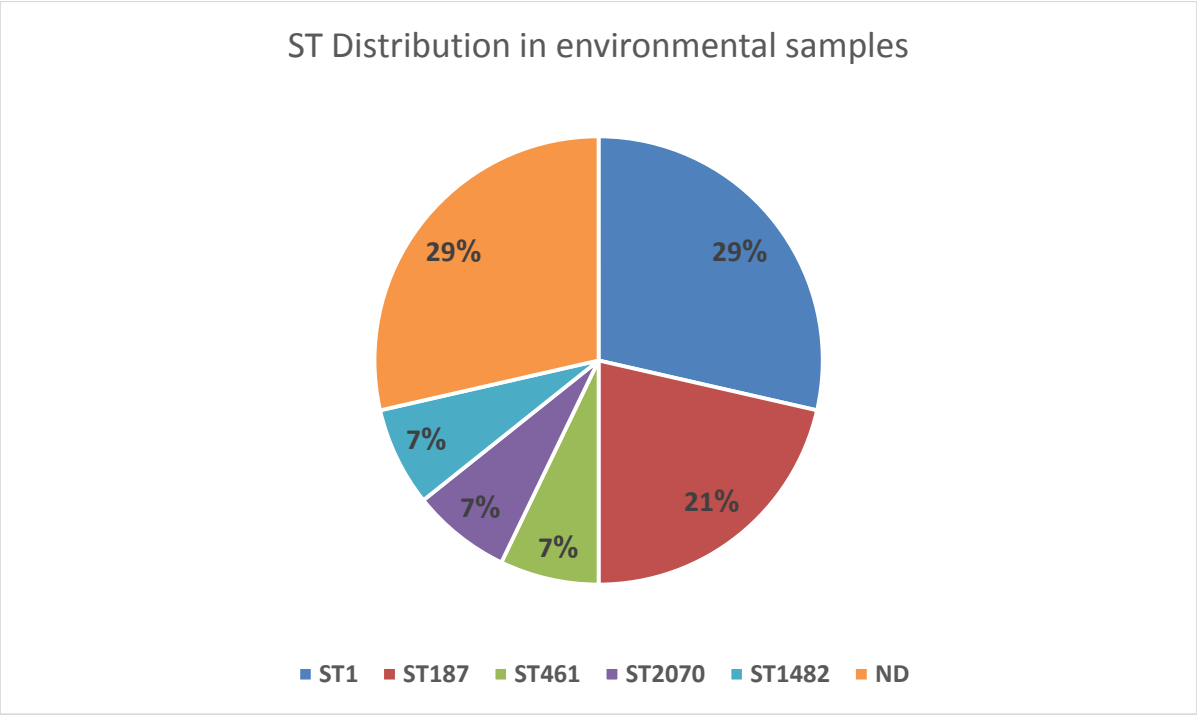

Figure 3.12: The distribution of Sequence Types (STs) for environmental samples from Makassed Hospital.

Chapter Four

4.1 Discussion

This study reports the results of the first attempt to identify *L. pneumophila* in respiratory samples obtained from pneumonia patients, thus diagnosing legionellosis in Palestinian patients from Makassed hospital. The singularity of the current study is that it used high specific and sensitive molecular methods to identify *Legionella* in BAL and sputum samples and NPSBT applied directly to clinical specimens for Sequence typing in the absence of isolates.

NPSBT allows the epidemiological typing and comparison with EWGLI Sequence-Based Typing (SBT) database for *Legionella pneumophila* (Mentasti and Fry, 2012; Fiore et al., 1998).

The need for such a study was eminent especially that previous work in the Microbiology Research Laboratory at AQU, showed the abundant prevalence of *L. pneumophila* in water systems in eight hospitals including Makassed hospital in the West Bank, Palestine. Microbiological culture as well as molecular testing for *Legionella ssp*. was performed on both water and biofilm samples. We found 8.3% (6/72) prevalence of *L. pneumophila* in the water samples by culture analysis, this percentage increased to 50% (18/36) by PCR. As for biofilms, the prevalence of *L. pneumophila* was higher, 16.8% (192/1136) by culture versus 61.3% (84/138) by PCR analysis (Zayed, 2013).

The Presence of *Legionella* bacteria in water distribution systems is a serious health risk to hospital staff and patients (Fiore et al., 1998), where very high-risk individuals such as organ transplant, intensive care (IC), cancer, infant patients and sites that commonly care for chronically ill or immunocompromised patients are found (Mercante and Winchell, 2015).

In Palestine there are no specific guidelines for protecting patients from exposure to *Legionella* and no routine microbiological testing for *Legionella*. Thus *L. pneumophila* infections have not been recognized in Palestinian patients prior to this study. *Legionella* identification is hampered by the viable but non- culturable (VBNC) state compounded by limited clinical awareness of the disease and unavailable trained personnel in the diagnostic laboratories. However, it is well known that LD identification in pneumonia cases generally is underestimated due to the failure to diagnose LD in routine practice (Steinert et al., 2002).

Since 2004, respiratory infections are considered as the largest human disease category worldwide and one of the leading causes of death. *Legionella* is among the top non-zoonotic atypical agents of severe respiratory illness, and successful disease resolution requires quick treatment together with rapid diagnosis for informed and accurate antibiotic management and epidemiological awareness (Mercante and Winchell, 2015).

We further continued our enquiry to test the prevalence of *Legionella* in respiratory tract secretions in pneumonia patients. A one-year prospective study from September 2014 to August 2015 was performed. Sputum and BAL samples were collected from Makassed hospital in East Jerusalem since it is the only center that performs bronchoscopy with Broncho alveolar lavage (BAL) in the West Bank; patients from all over the West Bank, suspected of pulmonary infections who needed bronchoscopy had their BAL samples extracted and examined in this medical center, so we considered this center to be representative for all the West Bank hospitals. The study was approved by the hospital's ethical committee, and subjects were provided with written informed consent (Appendix A).

Furthermore, even though isolation of *L. pneumophila* is considered the "gold standard" for the diagnosis of LD (Gaia et al., 2003a; Gaia et al., 2005b), Collected samples (*n*=195) were cultured on GVPC *Legionella* selective media before DNA extraction. However, the culture of 195 respiratory specimens obtained from suspected pneumonia patients seen at Makassed yielded only one isolate (previous results at the Microbiology Research laboratory at AQU). This result is similar to study in Israel by Mizrahi et al (2015), in which was *L. pneumophila* isolated from only one respiratory sample out of 133 samples, This is due to the sensitivity of detection of *Legionella* by culturing of respiratory

specimens which is highly variable, ranging from >10% to 80%, and recovery is dependent on the sample type as well as the antibiotic therapy before processing of the samples (Feeley et al., 1979). In Palestine the heavy use of antibiotics without prescription is the most likely explanation for the very low yield of *Legionella* by culture.

Cultivation independent analysis was performed using 16S rRNA PCR for *L. pneumophila* identification. The use of PCR-based culture-independent methods for the *Legionella* detection is considered necessary and complementary to the traditional culture-dependent techniques (Fields et al., 2002). PCR techniques using specific primers for the identification of *Legionella*, has the potential to detect infections caused by any *Legionella* species with greater sensitivity than the culture method and provides results within a short time (Kahlisch et al., 2010). Thus, in this study, culture-independent methods for *Legionella* detection were used in parallel with the isolation of *Legionella* by culture.

Our results show that 44 out of 195 samples (23%) of respiratory tract samples were positive for *L. pneumophila*, Figure 2. This indicates an annual incidence rate of 0.76 in 100,000, coincides with many European countries, where Legionnaires' disease has an overall incidence of 1.16 per 100,000 EU population (Phin et al., 2014), and the highest incident in Europe was in Spain 2.07 per 100,000, followed by France 1.9 per 100,000 in 2012 (Euro surveillance epidemiological report (2013). On the other hand. the reported crude incidence of LD in the USA increased from 0.39 to 1.15 per 100,000 between 2000 and 2009, with higher notification rates in the northeastern states than in other states (Phin et al., 2014), In Israel, a crude incidence 0.67 cases/ 100 000 was reported (Moran-Gilad et al., 2014).

When comparing the identification of *L. pneumophila* in the sputum versus the BAL samples, the BAL samples were more sensitive and showed higher percentage of positive PCR results (26 out of 74, 35%) whereas sputum samples showed less sensitivity, (18 out of 121, 15%), Figure 3.4 and 3.5. Among all potential specimens for culture and analysis, sputum is generally most commonly sought, but in significant LD patients the sputum production is little or no sputum for analysis (Mercante and Winchell, 2015), in contrast BAL sample allow recovery of both cellular and non-cellular components of the epithelial surface of the lower respiratory tract (Goldstein et al., 1990).

Meyer et al (2007) reported the BAL's widespread acceptance as a clinical technique for sampling of respiratory secretions with its leukocytes, and also other cellular components such as bacteria, and acellular components such as cytokines, viral particles, and microbial signatures (e.g., proteins and nucleic acids), For this the BAL sample could be more representative with this component than sputum.

Department distribution of hospitalized pneumonia patients that were found to be *Legionella* positive by molecular methods (Figure 3.5) shows, the predominant positive cases were in Internal Medicine ward with 16 cases (36.3%), then the Pediatric and ICU wards with 11 and 10 respectively, in these wards the patients are more susceptible to disease. Susceptibility to disease is associated with smoking, older age, chronic cardiovascular or respiratory disease, diabetes, alcohol misuse, cancer (especially profound monocytopenia as seen in hairy cell leukemia), and immunosuppression (Phin et al., 2014).

In Figure (3.6) the majority of *L. pneumophila* positive patients were under 20 years (15/44 cases 34%), about 20% of the cases were under 10 years, although we reported 6 cases (31.6%) were between 20-40 years, 10 cases (22.7%) were between 41-60 years, and 9 cases (20.4%) above 60 years. Our results are different from studies which reported the incidence of LD in children is rare and most of the cases occur in older people (74–91%) patients ≥50 years) (Phin et al., 2014). Moran-Gilad et al (2014) reported the vast majority (86.4%) of cases of LD in Israel occurred amongst patients over 45 years of age, and 51.7% of cases occurred amongst the 65+ year age group, this difference is due to the most of cases under the age of 10 years with recurrent lung infection and immunodeficiency (data not shown).

Our results showed in table (3.3) the distribution of the *L. pneumophila* positive samples according to gender, twenty two males were positive out of 120 male patients with 18.3 %, in contrast, twenty two females were positive out of 75 total females with 29.3%, this results showed the incident in females was more than male. In contrast, Globally the gender distribution of cases is similar between countries and cases are predominantly in men (1·4–4·3 male patients for every female patient) (Mercante and Winchell, 2015), In Israel the incidence of LD in men was 2.16 per women (Moran-Gilad et al., 2014), Faradonbeh et al (2015), reported no statistically significant differences in the incidence of *L. pneumophila* between males and females in Iranian patients.

In addition to *Legionella* detection in clinical material, typing of *Legionella* strains is of considerable importance in determining sources of nosocomially-acquired legionellosis. Thus NPSBT was the method to follow in order to be able to perform epidemiological typing in the absence of isolates and to try to link the ST's of the clinical samples with ST's of the environmental samples (Schousboe et al., 2013).

NPSBT is a discriminatory method to genotype *L. pneumophila* and can also be used to prove correlation between patients and environmental samples. Many studies used NPSBT to confirm nosocomial infection caused by *Legionella*. A study in Geneva, Switzerland confirmed two nosocomial infections in 1999 and 2006 by testing three patient samples and environmental samples from the same hospital by using SBT method. The SBT profiles from clinical and environmental samples were the same in two cases (Schousboe et al., 2013). Also in Canada, a 74-year-old woman was admitted to the hospital due to myocardial infarction. She was intubated in the emergency room (ER) and then transferred to the intensive care unit (ICU), after 10 days she developed pneumonia, and from her BAL specimen *L. pneumophila* was isolated, and she died on day 24 of cardiac complications and *L. pneumophila* was isolated from potable water in the ER and ICU in the hospital. The infection was defined as nosocomial, based on the onset and the incubation period. But to confirm nosocomial infection SBT was used on patient sample and environmental samples from (ER and ICU) and the SBT profile was the same as the patient's SBT, confirming nosocomial infection (Wong et al., 2006).

Therefore, we adopted a highly discriminatory typing method, NPSBT, to provide useful information about the sources of infection particularly because it could be applied *in situ* as no *Legionella* isolates could be obtained. In this study, we applied NPSBT on clinical and presumptively linked environmental samples, in order to look for any correlation and to know the sequence type of *Legionella* trying to demonstrate the source of infection. The genetic variability of *L. pneumophila* has been assessed for countries such as the United Kingdom, Belgium, Portugal, Italy, United State, Spain and other countries (Wong et al., 2006), but there is no similar information available for Palestine.

Thirty-four *L. pneumophila* PCR-positive specimens were tested by nested PCR SBT using a protocol described previously (Mercante and Winchell, 2015),

After submission of data files to the website:
(www.hpa-bioinformatics.org.uk/cgi-bin/legionella/sbt/seq_assemble_legionella1.cgi)

A full 7-allele profile from the DNA of respiratory tract samples was obtained form 3/34 (8.8%) specimens, a further 18/34 (53%) specimens gave 5- or 6-allele profiles usually sufficient to identify the strain as belonging to one or two sequence types (STs)], 6/34 (17·6%) gave 3- or 4-allele profiles (usually sufficient to differentiate one profile from another without determining their ST, and 7/34 (20.5%) gave 1- or 2-allele profiles for which we couldn't determine the ST (Appendix D). these results are similar a recent to study performed in China by Qin et al (2016), where by 44/71 (62%) of samples gave a full profile, (33/41 BAL samples, and 11/30 from sputum), and 13 /71 (18.3%) gave 5 to 6 alleles.

Four sequence types were identified in the 24 respiratory samples; ST1 (29.1%, 7/21), ST 461 (25%, 4/21), ST 1037 (4.7% 1/21), and 41.9% incomplete profile. ST 1 was the most predominant sequence type in clinical samples, (Figure3.11). According to several studies it appears that the most prevalent ST among clinical and environmental populations of Lp1 is ST1. However in Belgium and the United Kingdom, the most frequently detected ST among clinical isolates is ST47. Also the most prevalent genotype among clinical Lp1 isolates in France, the Netherlands (Kozak-Muiznieks et al., 2014), and Italy is ST23 (Sánchez-Busó et al., 2015).

The results of Sequence Types of environmental samples revealed five different sequence types (ST 1 (28.5%, 4/14), ST 187 (21.4%, 3/14), one sample of each ST 2070, ST 461 and ST 1482 (7.1 %, 1/14), while the rest of samples (21.3%, 3/14) were unspecified Sequence types. The most predominant ST in the selected environmental samples is also ST1. This result is in correlation with our previous study which included eight hospitals in the West Bank, Multiple-Locus Variable number of Tandem Repeats (VNTR) Assays (MLVA)) was adopted for genotyping, the most prevalent MLVA8 genotype for this hospital as well as the total of hospitals included in the survey, was genotype 4 which corresponds to ST1 (Zayed, 2013).

Worldwide, ST1 is the most predominant ST of *L. pneumophila*, Kozak-Muiznieks et al (2014), reported the most prevalent ST for both clinical sporadic and environmental isolates was ST1 and it is the most widespread ST in the world.

According to the studies of the distribution of Lp1 STs in different regions of the world, it appears that the most prevalent ST among clinical and environmental populations of Lp1 is ST1 (Kozak-Muiznieks et al., 2014), Moran-Gilad et al (2014) reported that the ST1 accounts for 39% of ST1s in the global *Legionella* database.

The closely related ST35, ST36, and ST37 are widely distributed in the world and were responsible for multiple sporadic cases and outbreaks in the past, including the first described outbreak in Philadelphia, Pennsylvania, in 1976 caused by ST36. In contrast, ST222 is an emerging strain identified only 14 years ago, which now may be expanding from North America to other continents as was hypothesized previously (Kozak-Muiznieks et al., 2014).

ST1 was the cause of multiple outbreaks in Canada and Europe (Kozak-Muiznieks et al., 2014), and in many regions of the world. In a recent study in Spain ST1 was the most frequently found ST in environmental samples, with 203/643 (31.6%) strains having this genetic profile, in agreement with data from previous reports from other areas, however ST578 was found to be the second most frequently reported type (65/643; 10.1%) (Sánchez-Busó et al., 2015).

The alleles' amplification in Nested-PCR of the environmental samples was higher than the amplification in clinical samples. This is mostly related to the genomic copies present in the sample, where *L. pneumophila* DNA content is higher in the environmental samples than in the respiratory samples. The *pilE* gene was the gene mostly amplified in the respiratory samples, whereas five genes *flaA, pilE, asd, mip,* and *mompS* were equally amplified in the environmental samples including *pilE* gene.

This study demonstrates the advantage of PCR for sensitive detection and identification of *L. pneumophila* in clinical samples by PCR than by culture, particularly, in a country where most patients use antibiotics prior to hospital admission, making it difficult to isolate *L. pneumophila* strains from clinical specimens. Another important finding is the nosocomial health risk to susceptible patients, since ST 1 was the most predominant ST in

both clinical and environmental samples, though we could not verify the source of infection with full certainty, the nosocomial threat persists, and sustainable monitoring of hospital water systems is demanded.

The monitoring of hospital water systems, by setting a guideline to detect and prevent colonization in hospital water system will decrease legionellosis risk in Palestinian hospitals. Two studies from Spain show that *Legionella* colonization was extensive in Barcelona hospitals, and that environmental monitoring followed by intensive clinical surveillance identified previously unrecognized cases of hospital-acquired LD. After this action the effect of this approach recently was evaluated and the results showed a significant decrease in the number of health care-associated cases of LD after the preventive guideline was in place (Sabrià et al 2004; Squier et al., 2005).

In conclusion, ST1 was the predominant ST in clinical samples, and the most frequent ST in the hospital environmental samples. ST461 was the second most frequent ST in the clinical samples and was also present in the hospital environmental samples. This may indicate possible nosocomial infection.

Regular monitoring and surveillance of hospital water systems for colonization by *Legionella species* should be implemented to lower the risk of nosocomial infections.

4.2 Conclusions

1. *L. pneumophila* serogroup 2-14 was isolated from one out of 195 cultured respiratory samples.

2. By using a molecular method (PCR) for detection of *Legionella 44/195* (23%) of respiratory samples were positive for *L. pneumophila*, showing that the molecular method is much more sensitive than the culture method. The identity of the positive samples was confirmed as *L. pneumophila* by sequencing.

3. BAL samples gave a higher number of positive identification of *L. pneumophila* by PCR than sputum samples. BAL samples are better representation of the microbial content of the lung.

4. NPSBT is a high-resolution genotyping method that is very useful when no isolates are available, the case that is present in PALESTINE most likely because of the heavy use of antibiotics prior to hospitalization.

5. Four sequence types were identified in the respiratory samples ,with ST 1 being the most prevalent.

6. Five sequence types were identified in the environmental samples ,with ST 1 being the most prevalent.

7. There is high likelihood of nosocomial infection of the patients.

4.3 Recommendations

1. Raising awareness regarding the prevalence of *Legionella* spp. in the water systems and in respiratory samples amongst hospital staff and medical teams.

2. Regular surveillance and monitoring of water systems in the hospitals for *Legionella* identification and quantification and management of high prevalence when found.

3. The implementation of PCR for the identification of *Legionella* rather than culture

4. In hospitals hot water should be maintained above 50°C for reduction of *Legionella* count.

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Zayed, R, A., (2013), *The molecular identification of Legionella species from water samples in Palestine* (Unpublished Master's thesis), Al-Quds university , Palestine.

Appendix A

MAKASSED ISLAMIC CHARITABLE HOSPITAL - JERUSALEM

Ref: 3/2/245
Date: 13, May 2014

Dr. Dina Bitar
Associate prof. of Microbiology
Microbiology Department/ Faculty of Medicine
Al-Quds University
Abu Deis
Fax: 2796110

Greetings,

Following the meeting of the Ethics Committee at Makassed Hospital and your meeting with Dr. Izzedin Hussein/ Head of Ethics Committee, I am glad to inform you that the committee approved your research program which you are performing in the Microbiology Research Laboratory in collaboration with Makassed Hospital.

We wish you and your team success and progress in your research, we are confidant that your work will contribute to enrich the educational process and the health service of our patients.

Many thanks….

Dr. Izzedin Hussein
Head of Internal Medicine Department
Head of Ethics Committee

Dr. Bassam Abu Libdeh
Medical Director

Copies:
- Head of Internal Medicine Department
- Head of Pediatrics Department
- Director of Medical Laboratory

المجلس الفلسطيني للبحث الصحي
Palestinian Health Research Council
تعزيز النظام الصحي الفلسطيني من خلال مأسسة استخدام المعلومات البحثية في صنع القرار
Developing the Palestinian health system through institutionalizing the use of information in decision making

Helsinki Committee
For Ethical Approval

Date: 2017/04/03 **Number:** PHRC/HC/211/17

Name: DINA M. BITAR الاسم:

We would like to inform you that the committee had discussed the proposal of your study about: نفيدكم علماً بأن اللجنة قد ناقشت مقترح دراستكم حول:

"Molecular identification of Legionella pneumophila from sputum or bronchoalveolar lavage from pneumonia patients".

The committee has decided to approve the above mentioned research. Approval number PHRC/HC/211/17 in its meeting on 2017/04/03 و قد قررت الموافقة على البحث المذكور عاليه بالرقم والتاريخ المذكوران عاليه

Signature

Member **Member**

Chairman 2017

Genral Conditions:-
1. Valid for 2 years from the date of approval.
2. It is necessary to notify the committee of any change in the approved study protocol.
3. The committee appreciates receiving a copy of your final research when completed.

Specific Conditions:-

E-Mail:pal.phrc@gmail.com
Gaza - Palestine غزة - فلسطين
شارع النصر - مفترق العيون

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

جامعة القدس
كلية الطب
أبوديس – القدس

Dear Participant,

The following is information regarding the research project and a patient consent form.

Information Leaflet for Participation
The scientific goal of this research project is to look at the prevalence of *Legionella pneumophila* pneumonia in Palestine.
In order to enable us to perform this study, we ask for your consent to collect a sample of

- **Sputum or**
- **Bronchoalveolar lavage (BAL)**

We intend to culture the sample on special bacteriologic medium to allow for the growth of Legionella.
Also we will extract DNA (genetic material) from the sample to be able to do molecular identification of *Legionella pneumophila.*

Risk for sputum collection
There is no risk in sputum collection
For collection of BAL: A suction catheter, preferably a protected BAL catheter to minimize contamination, is passed down the endotracheal tube until resistance is met. An aliquot of sterile saline is injected and then aspirated. This method provides a lower respiratory tract sample without the need for bronchoscopy and without the attendant risks of transtracheal aspiration.

Data storage:
Your personal data (last name, first name, address, date of birth) will be stored separately from the sample data. The samples are given a code. Any data linked to the samples can only be retrieved using this code. To enable us to conduct our research we also request your permission to access the medical records in the hospital that is relevant for our research.
Publication of data:
It is possible that the research data made completely anonymous, will be published in scientific journals.
Cancellation:
Participation in this research study is voluntary. You can cancel your consent at any timer. This will not result in any disadvantages for you.

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

ص. ب ٢٠٠٠٢
هاتف ٠٢٢٧٩٩٢٠٣ فاكس ٠٢٢٧٩٦١١٠

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

جامعة القدس
كلية الطب
أبوديس – القدس

INFORMED CONSENT FORM

LAST NAME:

FIRST NAME:

BIRTH NAME IF APPLICABLE:

DATE OF BIRTH:

STREET:

RESIDENCE:

PLACE OF BIRTH:

COUNTRY OF BIRTH:

Please read the following text carefully and then sign at the bottom of the page.
I have received the information leaflet for this consent form and I was handed copies of the two documents for my files. I have read both documents and understand them. I have received detailed information about the study; purpose, procedure, risks of my participation and my rights and duties in connection with this study.
I will participate in the above mentioned study on "Molecular identification of *Legionella pneumophila* in pneumonia patients in Palestine".
I give my consent for collection of sputum or BAL. processing, storage and transmission of data obtained in this connection to the researchers.
I authorize the publication of my anonymized medical data for scientific purposes.
I was given sufficient time and opportunity to make my decision. I understand that no disadvantages whatsoever will arise should I not participate in the study. I realize that I can cancel my consent at any time and that such cancellation will not result in any disadvantages for me.
I hereby authorize the responsible medical investigator to access my medical records kept in the hospital and release my attending physicians from their obligation concern medical confidentiality.

Signature: **Place & Date:**

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

ص. ب ٢٠٠٠٢
هاتف ٠٢٢٧٩٩٢٠٣ فاكس ٠٢٢٧٩٦١١٠

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

جامعة القدس
كلية الطب
أبوديس – القدس

عزيزي المشترك،

فيما يلي معلومات تتعلق بمشروع البحث و نموذج موافقة على المشاركة.

معلومات المشاركة
الهدف العلمي من مشروع البحث هو النظر الى مدى انتشار التهاب الرئة الناتج عن بكتيريا *Legionella pneumophila* في فلسطين.
حتى نتمكن من القيام بهذه الدراسة، نطلب موافقتك على تزويدنا عينة
- بصاق
- غسل شعبي

نحن نرغب بزراعة العينة على وسط بيولوجي خاص يسمح بنمو بكتيريا ال *Legionella*
سنقوم أيضاً بفصل المادة الوراثية DNA من العينة حتى نتمكن من اجراء تعريف جزيئي ل *Legionella pneumophila*

لا يوجد اي خطر في جمع عينات البصاق.
فيما يتعلق بجمع عينات الغسل الشعبي: سيتم استخدام انبوب قسطرة للشفط، ويفضل أن يكون انبوب قسطرة خاص بالغسل الشعبي لتقليل احتمالية التلوث، يتم تمريره في الأنبوب الرغامي حتى تتحقق مقاومة. يتم حقن كمية صغيرة من سائل ملحي معقم و من ثم استنشاقه. يوفر هذا الأسلوب عينة من السائل التنفسي السفلي دون الحاجة لتنظير القصبات ودون المخاطر المصاحبة لاستنشاق القصبة الرغامية..

تخزين البيانات
سيتم حفظ بياناتك الشخصية (اسم العائلة، الاسم الأول، العنوان و تاريخ الولادة) بمعزل عن بيانات العينة. تعطى العينات رمز. أي بيانات مرتبطة بالعينة يمكن استرجاعها فقط باستخدام هذا الرمز. حتى نستطيع القيام بهذا البحث سنطلب منك إذن للوصول إلى السجلات الطبية في المستشفى فيما له علاقة ببحثنا.

نشر البيانات
من الممكن أن يتم نشر بيانات البحث المخفية تماما في مجلة علمية.

الإلغاء
المشاركة في هذا البحث هو اختياري. يمكنك إلغاء موافقتك عل الاشتراك في أي وقت. هذا لن يتسبب بأي مضار لك.

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

ص. ب ٢٠٠٠٢
هاتف ٠٢٢٧٩٩٢٠٣ فاكس ٠٢٢٧٩٦١١٠

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

جامعة القدس
كلية الطب
أبوديس – القدس

نموذج الموافقة

اسم العائلة

الاسم الشخصي

الاسم عند الولادة(ان امكن)

تاريخ الولادة

مكان الولادة

بلد الولادة

مكان الإقامة

العنوان

التلفون

الرجاء قراءة النص التالي بعناية و من ثم التوقيع في أسفل الصفحة.
لقد تلقيت منشور المعلومات لنموذج الموافقة على العلاج و قد أعطيت نسخة لملفي. لقد قرأت المستندين الاثنين و فهمتهما. لقد تلقيت معلومات مفصلة حول الدراسة؛ الهدف، العملية، مخاطر مشاركتي و حقوقي وواجباتي فيما يتعلق بهذه الدراسة.
سوف أشارك في الدراسة المذكور أعلاه (التعريف الجزيئي لبكتيريا *Legionella pneumophila* في مرضى التهاب الرئة في فلسطين).
سوف أعطي موافقتي لجمع عينات البصق أو الغسل الشعيبي، المعالجة، التخزين و نقل المادة التي تم الحصول عليها في هذا المجال للباحثين.
أنا أسمح بنشر أي معلومات طبية مكتومة لأهداف علمية.
تم اعطائي الوقت الكافي و الفرصة لأخذ القرار. انا أفهم انه لا يوجد أي مضار قد تظهر و تمنع مشاركتي بالدراسة. أدرك بأنه بامكاني أن ألغي موافقتي في أي وقت و أن هذا الالغاء لن يتسبب بأي مضار لي.
و عليه أنا أصادق الباحث الطبي المسؤول أن يتمكن من الوصول لملفاتي الطبية الموجودة في المستشفى و إعفاء الاطباء المشرفين على حالتي من قلق التزام السرية الطبية.

التوقيع: المكان و التاريخ:

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

ص. ب ٢٠٠٠٢
هاتف ٠٢٢٧٩٩٢٠٣، فاكس ٠٢٢٧٩٦١١٠

نم الشارة: ٢٤٥/٢/٣ Ref. .....

تاريخ: ١٣ أيار ٢٠١٤ Date: .....

حضرة الدكتورة دينا البيطار المحترمة
أستاذ مشارك في الأحياء الدقيقة
دائرة الأحياء الدقيقة / كليـــة الطب
جامعة القـــدس
أبـــو ديـــــس
فاكس : ٢٧٩٦١١٠

تحية طيبة وبعد ،

بعد إجتماع لجنة الأخلاقيات في مستشفى المقاصد وبعد الإجتماع الذي تم بينك وبين الدكتور عز الدين حسين / رئيس لجنة الأخلاقيات ، يسعدني أن أبلغك بأن اللجنة قد وافقت على برنامج الأبحاث الذي تقومين به في مختبر الأحياء الدقيقة بالتعاون مع مستشفى المقاصد .

لذا نتمنى لك والفريق العامل معك التوفيق والتقدم في أبحاثكم ، ونحن على ثقة بأن عملكم سوف يسهم في إثراء العملية التعليمية والخدمة الصحية لمرضانا .

ولكـــم جزيـــل الشـــكر ،،،

الدكتور بسام أبو لبدة
المديـــر الطبـــي

الدكتور عز الدين حسين
رئيس قسم الباطني
رئيس لجنة الأخلاقيات

نسخة :
- رئيس قسم الباطني
- رئيس قسم الأطفال
- مدير المختبر

Al-Quds University
Jerusalem
Deanship of Scientific Research

بسم الله الرحمن الرحيم

جامعة القدس
القدس
عمادة البحث العلمي

Dr. Dina M. Bitar
Associate professor of Microbiology
Al-Quds University
Faculty of Medicine
Microbiology & Immunology Dept.

2 September 2013

Dear Dr Bitar,

Thanks very much for submitting your Research Ethics application regarding your research proposal "Molecular identification of *Legionella pneumophila* from sputum or bronchoalveolar lavage from pneumonia patients" in connection to your project proposal "Understanding the ecology and Virulence of Legionella spp.population in the fresh water system in Germany, Palestine and Israel". A specialized members of REC reviewed your research proposal and attachments as per Alquds University's research ethics guidelines, and recommended to grant you approval for conducting your research. Therefore, I would like in the name of the Research Ethics Committee to inform you of our approval to conduct your research. Please let us know if there will be any changes or amendments in your research methodology or objectives. We wish you all the best in carrying out your important research work.

Regards

Dr Mohammed Shaheen, Chair
Research Ethics Committee
Al-Quds University
Palestine

Appendix B

Sample ID	Sampling date	Age	Gender	Department
6	20/09/2014	61 years	Female	ICU
7	27/09/2014	40 years	Female	Internal medicine
8	27/09/2014	70 years	Male	ICU
9	27/09/2014	34 months	Male	Pediatric
10	27/09/2014	10 years	Male	Pediatric
11	27/09/2014	7 months	Male	Pediatric
14	27/09/2014	60 years	Female	ICU
15	27/09/2014	45 years	Male	Internal medicine
16	27/09/2014	6 years	Male	Pediatric
17	27/09/2014	65 years	Female	Internal medicine
18	27/09/2014	32 years	Male	Internal medicine
44	15-30/10/2014	16 years	Male	Pediatric
64	17/11/2014	62 years	Male	ICU
75	01/12/2014	55 years	Male	Internal medicine
109	15/12/2014	70 years	Female	ICU
113	29/12/2014	59 years	Female	ICU
116	3/1/2015	36 years	Female	Gynecology
117	20/1/2015	49 years	Male	Internal medicine
119	02/02/2015	78 years	Male	Surgery
125	10/02/2015	16 years	Male	Internal medicine
127	15/02/2015	13 years	Female	Pediatric
128	15/02/2015	2 years	Male	Pediatric
129	15/02/2015	1 years	Female	ICU pediatrics
130	09/02/2015	86 years	Male	Internal medicine
131	08/02/2015	15 years	Female	Pediatrics
133	29/01/2015	1 years	Female	ICU pediatrics

142	27/02/2015	31 years	Female	ICU
144	09/03/2015	3 years	Female	Pediatrics
145	09/03/2015	66 years	Female	Internal medicine
146	09/03/2015	64 years	Female	Internal medicine
148	09/03/2015	45 years	Female	Internal medicine
149	09/03/2015	46 years	Female	Internal medicine
151	09/03/2015	8 years	Female	Surgery
160	15/03/2015	49 years	Male	ICU
161	21/04/2015	39 years	Female	ICU
171	10/05/2015	34 years	Male	Surgery
172	10/05/2015	6 years	Male	Pediatric
176	31/05/2015	61 years	Female	Internal medicine
177	31/05/2015	13 years	Male	Pediatrics
186	16/06/2015	48 years	Male	Internal medicine
187	11/05/2015	52 years	Male	Internal medicine
191	10/06/2015	76 years	Female	Internal medicine
192	10/08/2015	68 years	Male	Surgery
195	15/08/2015	38 years	Female	ICU

APPENDIX C

Number of positive alleles in respiratory samples								
sample #	band #	Flax	pile	asd	mip	momp	pro	neu
s (218)	7	×	×	×	×	×	×	×
S1 (125)	2		×		×			
S2 (145)	5	×	×		×		×	×
S3 (146)	5	×	×			×	×	×
S4 (147)	1		×					
S5 (117)	3		×			×		×
S 6 (144)	7	×	×	×	×	×	×	×
S7 (109)	2		×			×		×
S8 (142)	5	×	×	×		×		
S9 (148)	5	×	×	×		×		×
S10 (149)	5		×	×	×	×		×
S11 (171)	3		×			×		×
S12 (172)	4		×	×		×		
S13 (176)	1				×			
S14 (177)	1				×			
S15 (195)	4		×	×	×	×		
S16 (160)	4	×	×	×				×
S17 (161)	0							
S18 (130)	6	×	×	×		×	×	×
S19 (131)	5	×	×	×	×			×
S20 (75)	6	×	×	×		×	×	×
S21(145)	5	×	×		×		×	×
S22(186)	6	×	×		×	×	×	×
S23(187)	3				×	×	×	
S24(44)	6	×	×	×	×	×	×	
S25(133)	0							
S26(10)	6	×	×	×		×	×	×
S27(151)	5	×	×		×		×	×
S28 (11)	5	×	×	×	×	×		
S29(14)	6	×	×	×	×	×	×	
S30(15)	5	×	×	×	×		×	
S31(16)	6	×	×	×	×	×	×	
S32 (17)	7	×	×	×	×	×	×	×
S33(18)	6	×	×	×	×		×	×

Number of positive alleles in environmental samples									
Sample	collection	band #	fla	pilE	asd	mip	momps	pro	neu
M 1	8 th	7	×	×	×	×	×	×	×
M 3	8th	7	×	×	×	×	×	×	×
M 4	8th	7	×	×	×	×	×	×	×
M 5	8th	7	×	×	×	×	×	×	×
M 7	8th	7	×	×	×	×	×	×	×
M H	8th	7	×	×	×	×	×	×	×
M 6	9th	7	×	×	×	×	×	×	×
M 8	9th	7	×	×	×	×	×	×	×
M 13	9th	6	×	×	×	×	×	×	
M 5	9th	7	×	×	×	×	×	×	×
M 1	7th	0							
M 3	7th	6	×	×	×	×	×		×
M 4	7th	7	×	×	×	×	×	×	×
M 7	7th	7	×	×	×	×	×	×	×
M C	7th	7	×	×	×	×	×	×	×

Appendix D

NPSBT alleles and sequence types (respiratory samples)								
Sample	*Fla*	*pilE*	*asd*	*mip*	*momps*	*proA*	*neuA*	*ST*
S0	1	4	3	1	1	1	1	ST 1
S2	6	0	0	28	0	14	0	ST 461
S3	6	0	0	0	21	4	9	ST 1037
S6	6	10	14	30	21	1	1	ST 461
S8	1	0	0	0	1	0	0	ST 1
S9	6	0	11	0	1	0	9	ST461
S10	0	0	3	28	0	0	1	N/D
S12	0	0	0	0	1	0	0	N/D
S15	0	0	3	0	1	0	0	N/D
S16	4	7	14	0	0	0	0	N/D
S18	1	4	3	0	1	1	1	ST 1
S19	1	4	3	1	0	0	0	ST 1
S20	1	10	14	0	1	1	1	N/D
S21	6	0	0	1	0	14	9	ST 461
S22	1	0	0	1	1	1	1	N/D
S24	1	4	3	1	1	1	0	ST 1
S26	1	10	3	0	1	1	1	N/D
S27	6	4	0	28	0	1	1	ST 461
S28	6	0	3	1	21	0	0	N/D
S29	1	4	14	1	21	1	0	N/D
S30	1	4	3	1	0	1	0	ST 1
S31	1	10	3	0	21	1	0	N/D
S32	1	4	3	1	1	1	1	ST 1
S33	6	0	3	1	0	14	9	ST 461

NPSBT alleles and sequence types (environmental samples)										
Sample ID	Collection Number	Ward	*flaA*	*pilE*	*asd*	*mip*	*mompS*	*proA*	*neu*	ST
M 1	7th		0	0	0	0	0	0	0	
M 3	7th	Internal medicine	3	10	1	0	0	0	0	ST187
M 4	7th	ICU Pediatric	1	4	1	1	0	0	0	ST 1
M 7	7th	Neonate	1	4	11	1	0	1	0	N/D
M C	7th	General cold water	1	7	5	1	0	9	0	N/D
M1	8th	ICU	1	4	3	1	0	1	0	ST 1
M 3	8th	Pediatrics	1	4	3	1	0	1	0	ST 1
M 4	8th	Internal medicine	0	10	0	0	0	1	0	ST 2070
M 5	8th	Internal medicine	2	4	0	28	0	1	0	N/D
M 7	8th	Surgery	3	10	1	0	0	1	0	ST 187
M H	8th	General hot water	1	4	0	0	0	0	0	ST 1
M 5	9th	Internal medicine	3	10	1	0	0	0	0	ST 187
M 6	9th	ICU	7	10	0	0	0	0	0	ST 1482
M 8	9th	ICU Pediatric	6	10	14	0	0	0	0	ST 461
M 13	9th	Arthroscopy								N/D

Appendix E

Sequencing of Seven alleles for sample S0 (218)

> Seq0-flaA-F

GGTAANATTCTCGATGGCTCTTTCTCTGGTGCAAGCTTCCAGGTTGGAGCGAATTCAAACCAAACCATCAAT
TTCAGTATCGGCAGCATAAAAGCTTCTTCCATTGGTGGTATTGCCACGGCAACAGGAACAGAAGTAGCTGG
TGCAGCAGCGACAGATATCACTATCGCAATTGGAGGAGGAGCAGCAACCAGTATTAACTCTTCTGCCAATT
TTACCGGGGCACTAAACGGGCAAGATGCTACGTCTGCCTATGCCAAAGCAGCGGCTATCAACGATGCCGG
GATAGGAGGACTATCAGTTACAGCATCTACCAGCGGCACACAAGCAGTTGGCGCAATAGGTGGAACCAGC
AGGTGATACCA

> seq0-flaA-R

TGNNCNTGCTTGTGTGCCGCTGGTAGATGCTGTAACTGATAGTCCTCCTATCCCGGCATCGTTGATAGCCG
CTGCTTTGGCATAGGCAGACGTAGCATCTTGCCCGTTTAGTGCCCCGGTAAAATTGGCAGAAGAGTTAATA
CTGGTTGCTGCTCCTCCTCCAATTGCGATAGTGATATCTGTCGCTGCTGCACCAGCTACTTCTGTTCCTGTTG
CCGTGGCAATACCACCAATGGAAGAAGCTTTTATGCTGCCGATACTGAAATTGATGGTTTGGTTTGAATTC
GCTCCAACCTGGAAGCTTGCACCAGAGAAAGAGCCATCGAGAATTCTTTGACCATTGAATTCAGTATTTTG
AGCAATACGCAC

>seq0-pilE-F

GNTTCATCGCTTCCNACATNACGCCAGNAAAATAGGCTTGGTTGGCAAATTTCAATAGAGGATACCCAAAT
GCTCAGTCATGTACATTTTTATGAAGAATAGCCGTATGAAACAATCCGCGTTTACCCTGGTTGAAGTTCTGA
TCAGCATGGTCATTATGGGCATTCTGGTTTCAATTGCCTATCCATCCTATTTACAATATATCCAAAAATCCCG
TCGTGCCGATGCTCACGCCACATTGACACAAGATCAAATTATTTTAGAACGCTGTTATTCACAGAATTTTTCT
TATGCTGCGGCGTGTGGCGCCTTACCAGCATTTCCTCAAACAACGCCGAACGGGTACTATACTATCAATATT
TCAAACCTGACAGCCACAACGTATACCTTAACTGCAACCCCTGTTGGAACTCAAGCCAAAGATACCGAGTG
CGCCACCATTGTCTTTTAACCGGGCCAATGTACCACCCCCTGTAGATTCCTCGGCAAGGCCG

>seq0-PilE-R

TANTCAGNAACAACGGGGCCGCAGTCAAGGTATACGACGCGGCAGTCGGTCTGAAATATTGATAGTATAG
TACCCGTACGGCGTTGTTTGAGGAAATGCTGGTAAGGCGCCACACGCCGCAGCATAAGAAAAATTCTGTG
AATAACAGCGTTCTAAAATAATTTGATCTTGTGTCAATGTGGCGTGAGCATCGGCACGACGGGATTTTTGG
ATATATTGTAAATAGGATGGATAGGCAATTGAAACCAGAATGCCCATAATGACCATGCTGATCAGAACTTC

AACCAGGGTAAACGCGGATTGTTTCATACGGCTATTCTTCATAAAATGTACATGACTGAGCATTTGGGTATC
CTCTATTGAATTTGCCACCAGCCTATTTTTCTTGGCGTAATGTTAGGAGCGATAACAGTAGTTTGTGTTCCAT
CCGATTGTGAGGAAGTAACCAGNTTAATTCCNTCCCTGCCGGNCN

>seq0-asd-F

CCCNNCGCATATAGCAATCCANGACGCCGTCGGCATAAGATCGTATCAAAGCGGCGACTAACCAGCCTGT
TTCCGGGACTGGCAAAAAAGCCATCAGCGAACTGGTCGCTCAGGTAGGCGATCTTTTAAATGGGAGACCA
GCTAATGTTCAAGCCATCCTCAGCAAATCGCTTTTAATGCGCTTCCTCATATTGATCAGTTCGAAGACAATG
GTTATACCCGGGAAGAGATGAAGATGGTCTGGGAAACCCGCAAGATTATGGAAGATGACAGCATTATGGT
TAACCCTACAGCTGTCAGGGTTCCCGTTATTTATGGGCATTCTGAAGCGGTTCATCTGGAATTAAAAAAGCC
TTTGACGGCTGACGATGCTCGCGCGCTTTTGGCAAAGGCACCCGGCGTTACTGTAGTGGATAATCTTTCTA
AAGCAAGTTATCCCACAGCAATTAAGAATGCAGTTGGGCATGATGATGTTTTTGTAGGGCGCATAAGACAG
GATATTTCTCATCCTTGTGGACTAAATTTGTGGATAGTCGCAGATAACATTCGA

>seq0-asd-R

AAAANCACAAGGAAGAGAAATCCTGTCTAATGCGCCCTACAAAAACATCATCATGCCCAACAGCATACTAA
ATAGCTGTGGGATAACTAGCTTTAGAAAGATTATCCACTACAGTAACGCCGGGTGCCTTTGCCAAAAGCGC
GCGAGCATCGTCAGCCGTCAAAGGCTTTTTTAATTCCAGATGAACCGCTTCAGAATGCCCATAAATAACGG
GAACCCTGACAGCTGTAGGGTTAACCATAATGCTGTCATCTTCCATAATCTTGCGGGTTTCCCAGACCATCT
TCATCTCTTCCCGGGTATAACCATTGTCTTCGAACTGATCAATATGAGGAAGCGCATTAAAAGCAATTTGCT
GAGGATAGACTTGAACATTAGCTGGTCTCCCATTTAAAAGATCGCCTACCTGAGCAACCAGTTCGCTGATG
GCTTTTTTGCCAGTCCCGGAAACAGACTGGTAAGTCGCCACATTAATACGACTAATACCAACAGCGTCATA
GATTGGCTTTAGAGCAACGACCATCTGANNNNTAGAGCAATTAGGG

>seq0-mip-F

NACANNAGATAGGAAAGACGNCAAAAAGCACAGGAGCCGATACGGGGAAGAAACAAAAAATAAGGCAT
AGAAGATAATCCGGAAGCAATGGCTAAAGGCATGCAAGACGCTATGAGAGGCGCTCAATAGGCTTTAACC
GAACAGCAAATGAAAGACGATCTTAACAAGATTCAGAAAGATTTGATGGCTAAGCGTACTGCTGAATTCAA
TAAGAAAGCGGATGAAAATAAAGTAAAAGGGGAAGCCTTTTTAACTGAAAACAAAAACAAGCCAGGCGTT
GTTGTATTGCCAAGTGGTTTGCAATACAAAGTAATCAATTCTGGAAATGGTGTTAAACCCGGAAAATCGGA
TACAGTCACTGTCGAATATACTGGTCGTCTGATTGATGGTACCGTTTTTGACAGTACCGAAAAAACTGGTAA
GCCAGCAACGTTCCAGGTTTCACAAGTTATCCCTGGATGGACAGAAGCTTTGCAATTGATGCCAGCTGGAT
CAACTTGGGAAATTTATGTTCCCTCAGGTCTTGCATATGAATGA

>seq0-mip-R

CAANNAGANAGAACAGCCGGCATAACCGCAAAGCAACCGACCCCCAGGGAAACCCGCGAAACCCGGAAC
GACGCCGGCAAACCAGAAAAAACGGAACCGACAAAAACGGAACCAACAAACAGACGACCAGAAAACACG
ACAGCGACAGCATCCGATAACCCGGGCCTAACACCATCACCAGAATAGATCACTCTGTATCGCAAACCACT
CGGCAATACAACAACGCCTGGCTTGTTTTTGTTTTCAGTTAAAAAGGCTTCCCCTTTTACTTTATTTTCATCCG
CTTTCTTATTGAATTCAGCAGTACGCTTAGCCATCAAATCTTTCTGAAACTTGTTAAGAACGTCTTTCATTTG
CTGTTCGGTTAAAGCCAATTGAGCGCCACTCATAGCGTCTTGCATGCCTTTAGCCATTGCTTCCGGATTAAC
ATCTATGCCTTGATTTTTAAAATTCTTCCCCAAATCGGCACCAATGCTATAAGACAACTTATCCTTGTCTGTA
GCTAATGATGTGGCATCGGTGGCAGCA

>seq0-mompS-F

GTANGACGTTGANANATGGGCATGANTTTTGCTAACTTGGCACAACTACAACAACAAGAGGGATGCTGTT
AATGCTGAATAAGGTCAATACGTAGATTTCAGCGCTAACAAGAAAATGCGATTCCACGGCGGTGTTCAATA
CGCTCGCATTGAAGCTGATGTGAACCGTTATTTCAATAACTTTGCCTTTAACGGGTTCAACTCTAAGTTCAAT
GGCTTTGGTCCTCGCACTGGTTTAGACATGAACTATGTATTTGGCAATGGCTTTGGTGTTTATGCTAAAGGC
GCTGCTGCTATTCTGGTTGGTACCAGCGATTTCTACGATGGAATCAACTTCATTACTGGTTCTAAAAATGCT
ATCGTTCCTGAGTTGGAAGCTAAGCTTGGTGCTGATTACACTTACGCAATGGCTCAAGGCGATTTGACTTTA
GACGTTGGTTACATGTGGTTTAACTACTTCAACGCTATGCACAATACTGGCGTATTTAATGGATTTGAAACT
GATTTCGCAGCTTCTGGTCTG

>seq0-mompS-R

TTANCTTANAACGCCGTTATCTGTGCATAGCGTTGAAGTAGTTAAACCACATGTAACCAACGTCTAAAGTCA
AATCGCCTTGAGCCATAGCGTAAGTGTAATCAGCACCAAGCTTAGCTTCCAACTCAGGAACGATAGCATTTT
TAGAACCAGTAATGAAGTTGATTCCATCGTAGAAATCGCTGGTACCAACCAGAATAGCAGCAGCGCCTTTA
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A

الكشف الجزيئي Molecular Identification والتصنيف القائم على التسلسل الجيني Sequence Based Typingلبكتيريا الليجيونيلا نيوموفيلا *Legionella pneumophila* في العينات البيئية والسريرية في فلسطين

اعداد : محمود جميل عمرو

اشراف: الدكتورة دينا البيطار

اليجيونيلا هي البكتيريا سالبة الجرام، تكون اسطوانية الشكل , زد على ذلك ان اللجيونيلا من البكتيريا التي تصعب زراعتها فهي بطيئة النمو (fastidious) و تسبب أنواع الليجيونيلا في متلازمين سريريين: مرض الالتهاب الرئوي الليجونيلي (legionnares' disease)، وحمى بونتياك (Pontiac fever)، التي تعرف مجتمعة باسم الفيلق (legionellosis).
ومن بين 52 نوعا و 70 فصيلة من أنواع الليجيونيلا، فإن الليجيونيلا نيوموفيلا *Legionella pneumophila* هي المسبب الرئيسي لداء الالتهاب الرئوي بنسبه (91.5٪) من ضمن جميع الانواع، والمجموعة المصلية 1 هي النمط المصلي السائد بنسبه (84.2٪).

وقد أظهرت العديد من الدراسات أن المصدر الرئيسي لهذه البكتيريا هو أنظمة المياه الصالحة للشرب في المباني الكبيرة مثل المستشفيات والفنادق. تلوث شبكات المياه بالمستشفيات مع الليجيونيلا هو خطر كبير للمرضى الذين يعانون من أمراض مختلفة، وخاصة المرضى الذين يعانون من نقص في المناعة ومن أولئك الذين قد يقيمون في المستشفى لفترة طويلة من الزمن. يتم الحصول على الالتهاب الرئوي الليجونيلي من خلال استنشاق قطرات صغيرة من الماء الملوثة مع الليجيونيلا .

وقد أظهرت دراسة سابقة في مختبر أبحاث الميكروبيولوجي في جامعة القدس أن انتشارا كبيرا لبكتيريا اليجيونيلا في عينات المياه التي تم جمعها من ثمانية مستشفيات في الضفة الغربية على مدى فترة سنتين من ديسمبر 2012 إلى ديسمبر 2014، وذلك باستخدام أسلوب التقليدي (culture dependent method و cultivation independent analysis(باستخدام التفاعل التسلسلي (PCR(للحمض النووي والذي يستهدف جين *16S rRNA* علاوة على ذلك دراسة وكشف عن بكتيريا الليجيونيلا نيوموفيلا في 195 من عينات الجهاز التنفسي من خلال استخدام الطرق التقليدية (culture dependent method والتي اعطت عن عينة إيجابية واحدة من ضمن 195 عينه في حين أظهرت النتائج ارتفاع معدل انتشار ووجود هذه البكتيريا في عينات الجهاز التنفسي إيجابية 23٪ (195/44) باستخدام طرق الكشف الجزيئية Molecular techniquesعن طريق استخدام التفاعل التسلسلي PCR التي تستهدف جين *16S rRNA*

التشخيص عن بكتيريا الليجيونيلا باستخدام طرق الكشف الجزيئية تطورت في أنحاء العالم لأن طرق الكشف التقليدية تحتاج وقت كبير وقليلة الكفاءة. ومن ناحيه أخرى التصنيف الجيني genotypic لليجيونيلا نيوموفيلا مهم بشكل كبير من أجل الدراسات الوبائية و التحكم واحتواء انتشار مرض الفيلق.

والأساليب المعتمدة حاليا في التصنيف الجيني لهذه البكتيريا مبني على التفاعل التسلسلي والتصنيف من خلال تسلسل جينات (NPSBT)الذي يقوم على أساس معرفه تسلسل سبعه من الجينات وهذه الطريقة تمكن من معرفه النوع التسلسلي لبكتيريا اليجيونيلا نيوموفيلا في حاله عدم وجود عزلات بكتيرية. هذه الأداة عالية الدقة في التصنيف الجزيئية موصى بها من قبل مجموعه العمل الأوروبية لعدوى الليجيونيلا EWGLI

وكان الهدف العام من هذه الدراسة هو تحديد ومعرفة النوع التسلسلي لبكتيريا اليجيونيلا .بنيوموفيلا في عينات الجهاز التنفسي من المرضى المصابين بالالتهاب الرئوي الليجونيلي، باستخدام NPSBT. وأيضا لتحديد النوع التسلسلي (ST) من عينات المياه و الغلاف الخلوي Biofilm التي تم الحصول عليها من اجنحة المستشفى نفسه.

وشملت الدراسة على (34 عينة) من أصل 44 عينات تنفسية تم اختبارها وتحليلها وكانت إيجابية لوجود هذه البكتيريا باستخدام التفاعل التسلسلي (PCR) الذي يستهدف جين *16S rRNA*. وقد تم تقسيم وتصنيف هذه العينات الأربعة والثلاثين بشكل إضافي بواسطة طريقة NPSBT. وتم ادخال واضافه 15 عينه من عينات مياه وغلاف خلوي من الدراسة السابقة التي تم جمعها سابقا من أجنحة مستشفى المقاصد وكانت إيجابية لليجونيلا نيوموفيلا وتم تحليلها أيضا من خلال NPSBT

من خلال تحليل 34 من عينات الجهاز التنفسي باستخدام التفاعل التسلسلي للتصنيف المبني على الترتيب التسلسلي NPSBT, حصلنا من 34/3 (8.8٪) من العينات على 7 اليل (Allele) كامله ، و من 34/18 أخرى (52.9٪) حصلنا على 5 أو 6 أليل (Allele)(كافية لتحديد الصنف) وايضا حصلنا من (34/6) (17.6٪) على 3 أو 4 أليل (Allele) (عادة ما يكفي للتمييز بين التشكيلات المختلفة)، و 34/5 (14.7٪) أعطى 1- أو 2 اليل.

من مجموع العينات حصلنا على اكثر من 4 أليل (Allele) (4، 5، 6 و 7 أليلات في (70٪) من العينات)، ومع ذلك، أعطت 10 عينات اقل من اربع أليلز ، تم استبعاد هذه العينات من فحص تسلسل الحمض النووي (PCR).

ومن تحليل الأليلات (Alleles) السبعة "من عينات المياه ومسحات الغلاف الخلوي الخمسة عشر المختارة كشفت أربعة عشر عينة إيجابية لستة إلى سبعة أليلات وكانت عينة واحدة إيجابية لأليل واحد، تم استبعاد هذه العينة من تسلسل الحمض النووي.

وأظهر تحليل تسلسل الحمض النووي ل 24 عينه من عينات جهاز التنفسي الانواع التسلسلية التالية : النوع التسلسلي 1 (ST1) ظهر في 24/7 بنسبه 29.1% ،و النوع التسلسلي 461 (ST461) ظهر في 24/6 من العينات بنسبة 25% , وايضا النوع التسلسلي 1037 (ST1037) ظهر في عينه واحده بنسبة 4%, و 41.6٪ من العينات اعطت انواع تسلسلية غير مكتملة.

من ناحية أخرى، أظهرت 14 عينة بيئية :حصلنا على النوع التسلسلي 1 (ST1 **) في 14/4 من العينات بنسبه 28.4 %, والنوع التسلسلي 187 (**ST187 **) في 14/3 من العينات بنسبة 21.4% , وفي عينة واحدة حصلنا على النوع التسلسلي 2070(** ST2070 **) ، والنوع 461 (**ST461 **) و النوع 1482 (**ST1482**) بنسبة (7.1٪)، في حين أن بقية العينات (28.5٪، 14/4) لم تكن محددة الأنواع التسلسلية.**

وبالتالي فإن النوع التسلسلي 1 (ST1) **هو النوع الأكثر انتشارا في كل من عينات الجهاز التنفسي والعينات البيئية التي تمثل 33٪ و 28.5٪ على التوالي. ويعتبر هذا النوع التسلسلي هو الاكثر انتشارا على مستوى العالم.**