AMERICAN UNIVERSITY OF BEIRUT

MOLECULAR CHARACTERIZATION OF *LISTERIA MONOCYTOGENES*

by
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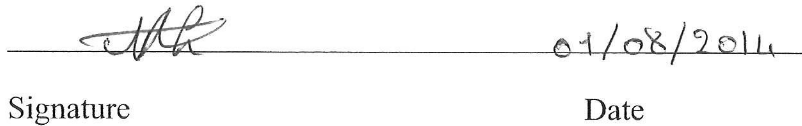

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AN ABSTRACT OF THE THESIS OF

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Title: Molecular Characterization of *Listeria monocytogenes*

Background: *Listeria monocytogenes* is an opportunistic intracellular pathogen that can survive harsh conditions, invade and spread into host cells. It is widespread in nature and has acquired resistance to many commonly used antimicrobials. This bacterium is the agent of listeriosis, a foodborne disease which is life threatening for immunocompromised patients and pregnant women. It harbors a number of genes encoding for virulence factors important in its pathogenesis and has the potential of producing biofilms rendering the organism resistant to antimicrobial agents. *L.monocytogenes* is not routinely screened for in Lebanon and there is lack of data about the prevalent strains and their potential pathogenicity. To that purpose, this study was undertaken in order to characterize *L. monocytogenes* from various food products, by evaluating resistance to commonly used antimicrobial agents, assessing the in vitro biofilm forming ability, detecting their virulence potential, and characterizing them at the strain level.
Methods: Fifty-nine isolates were obtained from the Lebanese Agriculture Research Institute (LARI). These isolates were collected in 2012-2013 from local and imported food products in the Lebanese market. *L. monocytogenes* strains from the Centers for Disease Control and Prevention (CDC) were used as controls. Antimicrobial susceptibility testing was done by the disc diffusion technique and biofilm formation was measured using the Microtiter Plate Assay. Polymerase Chain Reaction (PCR) analysis was performed to detect the presence of the three main virulence genes; *hly*, *actA*, and *inlB*. Pulsed Field Gel Electrophoresis (PFGE) and BIONUMERICS analysis was carried out on all isolates to determine genomic relatedness.
Results: Antimicrobial Susceptibility testing (AST) demonstrated resistance to oxacillin (n= 59; 100%), ampicillin (n= 15; 25.43%), penicillin (n= 30; 50.85%), and clindamycin (n= 39; 66.1%). Lebanese isolates from cheese and raw meat showed higher biofilm formation than imported and Lebanese seafood isolates. PCR amplification of the virulence encoding genes for *hly* and *actA* genes was positive in all (100%) tested isolates while for *inlB* gene PCR was positive in 58 of 59 (98.3%) of the tested isolates. PFGE analysis demonstrated the prevalence of 13 different subtypes with 100% similarity among each subtype. Detected subtypes were grouped into 6 clusters of 90% genomic similarity. Clustered subtypes were particular to the country of origin.
Conclusion: This study highlights the presence of *L. monocytogenes* in the Lebanese food market with high pathogenic potential and antimicrobial resistance, which stresses the importance of enhanced surveillance and the implementation of strict regulations on local and imported food. Future work needs to be done on a larger scale and a more representative selection from different food samples, as well as on clinical specimens when available.

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CHAPTER I

INTRODUCTION

Listeriosis, a serious foodborne disease that can lead to abortion, meningoencephalitis and septicemia, has become a public health concern for the elderly, infants, immunocompromised patients, and pregnant women. Even though its incidence is low in comparison to other foodborne diseases, it has one of the highest mortality rates (20-30%) among them.

Listeria monocytogenes is the agent of listeriosis. It is transmitted to humans by ingestion of contaminated raw and ready-to-eat food and can cause both invasive and non-invasive gastrointestinal infections. This bacterium is not routinely screened for as it only causes mild symptoms in immunocompetent individuals. However, it can lead to many serious complications for the high-risk groups such as immunocompromised persons and pregnant women. *L. monocytogenes* is widespread in nature, can survive various harsh conditions, and has developed resistance to many antimicrobials, and this could be attributed to biofilm formation as well as to resistance encoding genes. Additionally, this opportunistic intracellular pathogen has a genetic composition encoding for various virulence factors, mainly Internalin B (*inlB*), listeriolysin O (*hly)* and Actin-assembly inducing protein precursor (*actA)* genes, that confer to the organism the ability to attach, invade and spread into host cells.

In Lebanon, *L. monocytogenes* is being detected in food products and this poses a potential threat on health especially on high-risk group individuals leading to listeriosis and

serious complications. Determination of prevalent clones, with their virulence potential and antimicrobial resistance is of primordial importance.

We aimed in this study at collecting *L. monocytogenes* isolates from the Lebanese Agricultural Research Institute (LARI) isolated from food samples at the Lebanese market (imported and local) in order to:

- Evaluate their resistance to commonly used antimicrobial agents
- Measure their ability to produce biofilms *in vitro*
- Assess their virulence potential by detecting the most important virulence encoding genes implicated in the pathogenesis: Internalin B (*inlB*), listeriolysin O (*hly)* and Actin-assembly inducing protein precursor (*actA)* genes
- Characterize the isolates at the strain level by determining their genomic relatedness and clonality, using Pulsed Field Gel Electrophoresis (PFGE) analysis

CHAPTER II

LITERATURE REVIEW

A. History

Despite the progress in food production technology, quality control systems and the raised awareness, foodborne diseases are still a public health concern and a recurring topic for the media especially with the emergence of antimicrobial drug resistance.

According to the Centers for Disease Control and Prevention (CDC), in the United States alone, foodborne diseases cause 48 million cases of sickness each year of which 128,000 are hospitalized and 3,000 die (1). There are more than 250 known foodborne diseases that can be caused by bacteria, viruses, parasites, as well as chemicals in food products (2). The most common foodborne pathogens according to U.S. Food and Drug Administration (FDA) are: *Campylobacter jejuni*, *Clostridium botulinum*, *Clostridium perfringens,* Pathogenic *Escherichia coli, Listeria monocytogenes, Norovirus, Salmonella* Enteritidis, *Salmonella* Typhimurium, *Shigella, Staphylococcus aureus, Vibrio cholera, Vibrio parahaemolyticus, Vibrio vulnificus,* and *Yersinia enterocolitica* (3). Symptoms vary depending on the causative agent but are mostly gastrointestinal such as diarrhea, nausea and vomiting, in addition to fever, malaise, headache and dizziness (4).

L. monocytogenes, one of the emerging bacterial foodborne pathogens, was first isolated in 1926 by Murray and colleagues during an epidemic in animal care house. It was called *Bacterium monocytogenes* because it caused monocytosis in laboratory rodents. In the following year, a similar microorganism was isolated in Johannesburg and was named *Listerella hepatolytica* in honor of Joseph Lister. *Bacterium monocytogenes* and *Listerella*

hepatolytica were later found to be the same bacterium and the name "*Listeria monocytogenes"* was assigned in 1940 (5, 6). Nevertheless, it was not until 1979 for *L. monocytogenes* to be linked to serious listeriosis although it was involved in human illness since 1920 (7). In 1981, foodborne transmission of listeriosis was documented for the first time after an outbreak in Canada that was associated with coleslaw (8). And since then, many outbreaks have been reported worldwide, highlighting the significant role of food as primary route of transmission for *L. monocytogenes* (9).

B. Genus *Listeria*

The genus *Listeria* consists of ten closely related species (*L. monocytogenes*, *L. ivanovii, L. innocua, L. seeligeri, L. welshimeri, L. grayi, L. marthii, L. rocourtiae, L. fleischmannii* and *L. weihenstephanensis*), but only two are known to be pathogenic; *L. ivanovii* which can infect cattle and sheep, and *L. monocytogenes* which can infect both humans and animals (5). Furthermore, *Listeria* is divided into at least 16 serovars of which 13 are for *L. monocytogenes*. Serotype 4b is known to be the main cause of endemic listeriosis (49%) (10-11). *Listeria* species are distributed ubiquitously and are of varied pathogenicity. Given their close morphological and biochemical similarities, it has been difficult to accurately and rapidly distinguish between the pathogenic and the non-pathogenic species. But the development and application of various molecular techniques has led to the quick and precise determination of *Listeria* organisms (5).

C. Characteristics of *Listeria monocytogenes*

1. General properties

L. monocytogenes is a gram-positive rod that is capable of growing at a broad temperature range (1-45°C). It is a non-spore-forming facultative anaerobe, motile at room temperature and is of psychrotrophic nature; it has the ability to replicate at 4°C, leading to very high concentrations of bacteria in contaminated refrigerated food (12-14). In addition to that, *L. monocytogenes* can tolerate a high concentration of sodium chloride and low pH giving it the ability to survive in some types of processed food. When gram-stained, *L. monocytogenes* appears as short, rounded-end rods (0.4 to 0.5 x 0.5 to 2µm), that can be single or arranged in pairs and short chains. It can be confused under the microscope with *Streptococcus pneumoniae* and *Enterococcus* that also cause meningitis (14). Moreover, when grown on Blood agar, *L. monocytogenes* exhibits 0.5 to 1.5 mm in diameter, smooth translucent colonies, with a weak β-hemolysis. These colonies look like those of Group B *Streptococci* (14, 15). Since the hemolysis is weak and cannot be always seen, it can be enhanced by growing β-hemolytic *Staphylococcus aureus* perpendicularly to it on a sheep blood agar plate and incubating it at 35°C for 24 hours. This technique is known as the CAMP test (16).

2. Virulence factors

This opportunistic intracellular pathogen can adhere and penetrate cells, escape the phagolysosome, multiply and form listeriopods, which help it in invading adjacent cells. It can replicate and move within cells, avoiding by that antibody-mediated clearance and diminishing the role of humoral immunity in managing *L. monocytogenes* infections. *L.*

monocytogenes can also be carried within macrophages to the liver and spleen, leading to a disseminated form of the disease (14). It can also overpass three tight physiological lines of defense: intestinal, blood-brain, and feto-placental barrier (17).

When *L. monocytogenes* is ingested with contaminated food, stress response genes allow the bacteria to survive degradations caused by the acidity of the stomach, the action of bile salts and the proteolytic enzymes (18). After that, it adheres to and penetrates host cells with the help of surface proteins called internalins (19). Internalins A (*InlA*) and B (*inlB*) are two major invasion proteins that mediate the uptake of *L. monocytogenes* into non-phagocytic cells (20). InlA interacts with its host cell receptor E-cadherin leading to cytoskeletal reorganization, which facilitates *L. monocytogenes* entry into epithelial cells, whereas InlB recognizes a variety of receptors; gC1qR, c-Met and glycosaminoglycans, allowing by that the entry of *L. monocytogenes* into a wider range of host cell types such as hepatocytes in the liver, fibroblasts and epithelioid cells (6, 12). Following the penetration of the host cell, *L. monocytogenes* is trapped in a phagocytic vacuole. But with the help of the pore-forming toxin Listeriolysin O, working in synergy with phosphatidylinositol-specific and broad-range phospholipases (C, plcA and plcB), it is capable of lysing this vacuole and escaping to the cytoplasm where it becomes free to undergo intracellular growth and multiplication. Successively, spreading of *L. monocytogenes* requires another protein; ActA, involved in recruiting and polymerizing actin filaments at one pole of the bacteria. These filaments resemble a tail and enable the movement of *L. monocytogenes* from cell to cell by propulsion (6). ActA also facilitate the penetration of *L. monocytogenes* that do not produce internalins (20). Finally, when *L. monocytogenes* reaches the host cell membrane, it becomes enveloped in filopodium-like structures that are recognized by

neighboring cells. This will lead to its engulfment in a secondary double-membraned vacuole that will be lysed later when PC-PLC is activated. And a new infection cycle begins (21).

Most of the genes encoding the virulence-associated proteins can be found on the same cluster, which is mainly regulated by *PrfA*, a pleiotropic regulator that activates the transcription of these virulence-associated genes (12, 22). In addition to these virulence associated genes and proteins, several other proteins are found in *L. monocytogenes,* giving it its ability to invade, survive and spread:

- Surface protein p104 that aids in the adhesion to intestinal cells.
- Metalloprotease (*Mpl*) that activates phospholipases by cleaving off a portion of the precursor.
- Clp proteases and ATPases, which are proteolytic enzymes and chaperones.
- Protein p60, a cell surface murein hydrolase enzyme, which plays a role in cell division of *L. monocytogenes* (20).

3. Biofilm formation

Bacteria can be found either in a planktonic form or in complex microbial communities encased in an extracellular polysaccharide matrix they produced and called Biofilm (23, 24). These biofilms can be found on biotic and abiotic surfaces if there is sufficient moisture, and they provide protection to the bacteria from environmental factors, antimicrobials and used disinfectants (25-30).

Listeria monocytogenes can form biofilms with populations reaching 104–107 CFU/cm^2 on many types of surfaces; such as plastic, propylene, marble, quartz, rubber, stainless steel, glass, granite, and on the interface of two different materials (31-36). Furthermore, it grows preferentially in the industrial system through producing biofilms and persisting on food processing equipment, on conveyor belts, in drains, and on other parts of the production machinery (37, 38). Some studies show that *L. monocytogenes* biofilms can stay on surfaces for months, while others proclaim it can persist for years (38). In addition to that, *L. monocytogenes* exists in mixed biofilms with other species such as *Pseudomonas* spp. (39-41). This association can increase the resistance to antibiotics and enhance the attachment of other bacteria to this surface (42).

Moreover, catheters provide a potential abiotic surface for *L. monocytogenes* to form biofilms and affect hospitalized patients (43). In fact, there have been many incidences of nosocomial infections due to *L. monocytogenes,* such as the listeriosis outbreak in 2010 in Texas that was caused by contaminated diced celery and led to 10 infected people, of which 5 died (44-48).

D. Listeriosis

1. Overview of the disease

L. monocytogenes, the agent of listeriosis, is an opportunistic pathogen that mostly affects a specific high-risk group. The incidence of listeriosis is considered low compared to other foodborne illnesses but has a very high mortality rate (20-30%) (9). Furthermore, *L. monocytogenes* ranks 3rd in pathogens contributing to domestically acquired foodborne

illnesses resulting in death (49).

Listeriosis sporadic cases and outbreaks are mainly due to ready-to-eat foods. Consequently, a higher incidence is observed in industrialized countries. A very high number of outbreaks occurred in the United States in the last few years although it has zero tolerance policy for *L. monocytogenes* according to the FDA (50-51). One of the largest recent listeriosis outbreaks occurred in 2011 and was associated with cantaloupe melons. It affected 147 persons in the United States and caused 33 cases of death. The persons who died during this outbreak had a median age of 81 years old, hence belonging to the elderly risk group (50). Furthermore, It is not known yet if the differences in incidence rates between developed and developing countries are due to dissimilarities in food habits and geographical location, or because of differences in diagnostic and reporting practices (9).

2. Sources of infection

Listeria monocytogenes is highly widespread in nature; it can be found in soil and water, and carried in the gastrointestinal tract of asymptomatic animals leading to the contamination of their meat and dairy products. This bacterium can infect humans via the oral route through the uptake of certain foods like unpasteurized milk and dairy products, raw or smoked seafood, and raw meat, which are of high risk since *L. monocytogenes* can grow and survive in them (52). However, there are also some food that were considered of low risk until they were linked to listeriosis outbreaks, such as the one that was reported in Italy and affected more than 1500 person following the consumption of corn (53). Infection can also be transmitted from mother to fetus during pregnancy (54).

3. Risk groups

The population at high risk of the severe form of listeriosis and prone to a high mortality rate consists of pregnant women, people with an impaired immune system, infants and elderly (55).

a. Pregnant women

Pregnant women are 10 times more likely than other infected people to acquire listeriosis. In fact 14% of listeriosis incidents happen during pregnancy, which can cause fetal loss in 20% of the cases, preterm labor, and illness of the newborn or even his death (56). Furthermore, around two-thirds of the newborns that survive, develop clinical neonatal listeriosis (56, 57).

b. Elderly

Adults above 65 years old are four times more likely than the general population to get listeriosis. In addition, they constitute 58% of the total people infected with *L. monocytogenes* (56).

c. People with impaired immune system

People within this group have certain medical conditions, cancer or any chronic disease that can affect their immune status. It also includes patients under immunosuppressive therapy (undergoing chemotherapy, radiation or taking certain mediations), malnourished, lacking

physical fitness, and alcoholics (9, 56, 58, 59). Notably, patients with HIV/AIDS contract *Listeria* meningitis at a rate that is 60 times higher than that of general population (60).

4. *Symptoms*

There are two forms of listeriosis; invasive and non-invasive depending on the immune status of the host.

- The non-invasive form is the most common; it usually occurs in immunocompetent individuals and is characterized by flu-like symptoms. It manifests as muscle ache, fever, diarrhea and other gastrointestinal problems that start to appear within 12 hours or more (6, 61).

- The invasive form that has an onset time of few days to 3 weeks and can cause septicemia or meningoencephalitis in high-risk groups (6, 61). Symptoms such as headache, confusion, stiff neck, loss of balance and convulsions might occur in case of invasion of the nervous system (62). Moreover, meningitis is the most common form of *Listeria* infection in adults, causing a mortality rate of 20-50% and significant neurologic consequences for survivors (14).

During pregnancy, listeriosis can be life threatening for both the fetus and the mother. It can cause neonatal disease in two ways:

- Early-onset neonatal disease, acquired transplacentally, leading to miscarriage, stillbirth or premature birth (14, 56). In cases of severe listeriosis, it can also cause Granulomatosis infantiseptia, which is characterized by disseminated abscesses and granulomas in many organs of the body, as well as a high mortality rate (14).
- Late-onset neonatal disease, which occurs few weeks after delivery, causes neonatal

central nervous system disease with symptoms similar to those induced by Group B streptococcal disease (14).

5. Treatment and antimicrobial resistance

Non-invasive listeriosis that affects immunocompetent patients does not require administration of antibiotics, since it usually manifests as febrile gastroenteritis and resolves in two to three days prior to identifying the causing organism (63). Nevertheless, for the invasive form of the disease, the treatment of choice is ampicillin or penicillin in combination with an aminoglycoside (64). This combination has demonstrated synergistic effect in high-risk patients with infections of the central nervous system and endocarditis (65-70). Trimethoprim with sulfamethoxazole is the second-choice therapy for individuals who are allergic to penicillin (64, 71). In case of pregnancy, both vancomycin and erythromycin are administered to the mother (64). Moreover, the emergence of antimicrobial resistance is enabling *L. monocytogenes* to counteract the effect of these agents. Correspondingly, there is an increasing resistance to the antibiotics that are commonly used to treat listeriosis (penicillin, ampicillin, gentamicin) (71-75).

The first *L. monocytogenes* strain that showed resistance was reported in 1988. And since then, many resistant strains were isolated from humans, animals and food (73, 76). Antimicrobial resistance can be due to the uncontrolled prescription of antibiotics and their use as growth promoters in animal feed (77). This resistance is attributed to a mutation in an intrinsic chromosomal gene or to the acquisition of exogenous genetic material throughout conjugative plasmids and transposons, transformation or transduction (64, 78-

80). These genetic exchanges are possible between unrelated bacterial species, and the resistance genes can be even transferred from saprophytic or commensal bacteria in ingested food to the pathogenic species within the gastrointestinal tract (81, 82). The conjugative transfer of resistance genes to *L. monocytogenes* is mainly from *enterococci* and *streptococci*. However, it can be also acquired from other gram-positive and gram-negative bacteria (83-85). Furthermore, there is evidence of multiresistant *L. monocytogenes* strains from various sources, which raises the issue of reaching an era where all the used antibiotics will become ineffective (61, 86).

E. Identification and detection methods

Listeria species share some distinctive biochemical features; they are known to be catalase positive, indole and oxidase negative. Additionally, they can hydrolyze aesculin, but fail to do so with urea.

As for species-specific identification, there are significant variations in exhibiting hemolysis of horse or sheep red blood cells and acid production in fermentation of certain sugars; *L. monocytogenes* can ferment L-Rhamnose and α-Methylmannoside, but not D-Xylose, Ribose nor D-Mannitol (87, 88).

The identification of *Listeria* species by biochemical methods used to be laborious and time consuming (up to 6 days) (12). However, nowadays all these tests can be performed using a single API strip, making the process easier and quicker since results can be obtained within 18 to 24 hours (89). Nonetheless, this type of tests measures the phenotypic characteristics of the bacteria, so their performance can be influenced by uncontrolled

external factors that might affect the growth and metabolic mechanisms of *Listeria* spp., making the accuracy of the results questionable (12). Therefore, Polymerase Chain Reaction (PCR), which is a reliable and reproducible technique based on the nucleic acid composition instead of the phenotypic expression, is being used for the identification of *Listeria* spp. and for the differentiation of *L. monocytogenes* from other *Listeria* spp. using specific primers targeting genes of virulence factors (90, 91).

Reverse transcription (RT)-PCR and Real-time PCR are also used to detect *L. monocytogenes,* with the latter being quantitative (91).

F. Molecular subtyping

Molecular methods are reliable techniques that differentiate and subtype species at the strain level. They are based on the genomic content and are not influenced by the environment, contrarily to phenotype expression (92).

Molecular subtyping methods are valuable tools with various levels of discriminatory power to assess the genomic relatedness between isolates and to provide information about the strains responsible for a certain outbreak so that more effective strategies can be planned to control them. The most important subtyping methods used for foodborne pathogens are Multilocus Sequence Typing and Pulsed Field Gel Electrophoresis, with the latter being the gold standard molecular typing method used for typing foodborne pathogens such as *Salmonella*, *E.coli*, *Campylobacter*, *Yersinia*, *Vibrio* and *Listeria* (93).

PFGE is known for its high discriminatory power and epidemiological relevance. Its regular use on *L. monocytogenes* isolates has led to improved detection and control of

human listeriosis outbreaks. In fact, A protocol developed by PulseNet at the CDC (Atlanta, GA, USA) is being largely used at the international level and has shown some notable success in relation to the identification and tracking the source of food-borne listeriosis outbreaks (93-95). However, PFGE uses relatedness as a guide instead of true phylogenetic measure (94).

Another tool for molecular subtyping is Multilocus Sequence Typing (MLST) which is being increasingly applied nowadays as a routine typing tool for international comparison of isolates. It is used when there is an association of particular genotypes with virulence or antigenic factors, in cases of emergence of antimicrobial-resistant variants, and when global spread of disease is caused by a novel variant (96). MLST can improve the differentiation of *L. monocytogenes* strains related to listeriosis outbreaks and tracing of these strains to the source and can help us determining the evolutionary relatedness among *L. monocytogenes* strains (97). Nevertheless, low sequence diversity might make it less useful in accurately assessing the genomic relatedness between isolates and distinguishing between them (98). On the other hand, PFGE and MLST have some limitations; they are time consuming and laborious, require highly trained personnel, and need specialized equipment and expensive reagents. They also cannot type all the strains (94, 98).

There are many other methods that can be used for typing *L. monocytogenes* such as Ribotyping, which is based on the variations in ribosomal genes or proteins, and Random Amplified Polymorphic DNA (RAPD), which is a PCR-based approach that uses random primers to amplify DNA fragments randomly (91). The whole genome sequencing may recently be the subtyping method of choice with a high discriminatory power (99).

CHAPTER III

MATERIALS AND METHODS

A. Source of *Listeria monocytogenes* isolates

Fifty-nine isolates that originate from food products (imported and local) present in the Lebanese market were obtained from the Lebanese Agricultural Research Institute (L.A.R.I). Information about each isolate is presented in table 1.

All isolates were cultured immediately after being received and then stored in Brucella broth (Becton, Dickinson & Co., Sparks USA) with 10% glycerol (Sigma Chemical Co., St. Louis, MO) at -20ºC. For short-term storage, the samples were cultured on Brain Heart Infusion (BHI) (LAB M Limited, United Kingdom) agar plates and stored at 4ºC for two weeks, after which they were re-cultured. They were also kept on slants at room temperature for three months.

Eight strains of *L. monocytogenes* were obtained from the Centers of Disease Control and Prevention (CDC) and used as controls in the performed experiments. Information about each control strain is presented in table 2.

B. Identification using API kit

The fifty-nine isolates and the CDC reference strains were confirmed using API Listeria kit (bioMérieux, France).

1. *Materials needed (provided by the kit)*

- API Listeria strips that have ten microtubes containing dehydrated substrates to perform the reactions.

- Suspension medium
- ZYM B reagent
- Incubation boxes
- Result sheets

2. *Procedure*

- Incubation boxes were prepared and 3 ml of sterile distilled water were added into the wells of the tray to provide a humid atmosphere.
- The strips were removed from their individual packaging and placed in the incubation box.
- Few isolated colonies of each isolate were added into a suspension medium ampule using a sterile cotton swab. The colonies should be from a fresh culture (18-24 hours old).
- Turbidity of the suspensions was adjusted to 1 McFarland using the (Densimat, Biomerieux, France) turbidimeter. The suspensions should be used immediately after regulating them.
- With a pipette, the tubes of each strip were filled with 50 µl of the corresponding suspension except for DIM microtube that was filled with 100 µl. The strip was tilted slightly forward and the pipette was placed against the side of the tube while filling it to avoid the formation of bubbles.
- The isolate number was written on the elongated flap of the tray then the incubation box was closed and incubated at 36°C ± 2°C for 18-24 hours in aerobic conditions.
- After the incubation time is over, ZYM B reagent was added to the DIM microtube.
- The results were read within three minutes and recorded as positive or negative on the provided sheet to be analyzed later by “Apilab” software.

- The same procedure was carried out on the CDC control strains to be able to use them as a reference when reading the results.

C. Antimicrobial Susceptibility Test (Disc Diffusion)

Antimicrobial resistance was analyzed using the Kirby-Bauer disk diffusion method, in accordance with The Clinical and Laboratory Standards Institute (CLSI) guidelines (2011), and was performed with standard discs (Oxoid).

Listeria monocytogenes isolated colonies were inoculated into BHI broth. The tubes were incubated at 37ºC for 24 hours. A sterile swab was then used to evenly spread 1ml of cell suspension on the surface of Muller-Hinton agar supplemented with 5% Horse blood. antibiotic discs; ampicillin (10 µg), erythromycin (15 µg), penicillin (10 u), tetracycline (30 µg), sulfamethoxazole-trimethoprim (1.25/23.75 µg), gentamicin (10 µg), clindamycin (2 µg), oxacillin (1 µg) and vancomycin (30 µg) were then placed onto the agar with a disc dispenser (Oxoid, United Kingdom) and the plates were incubated at 37ºC for 24 hours. Standard strains of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used to maintain quality control of performance and reliability of the results. Since there are still no specific guidelines for disc diffusion method for *L. monocytogenes,* the size of the inhibition zone was measured according to CLSI guidelines (2011) for *Staphylococcus* spp. (100).

As for susceptibility to vancomycin, it was determined using the criteria established for *Listeria* spp. by Soussy et al (2005) (101).

D. DNA extraction

DNA was extracted from the isolates and controls according to a standardized protocol combining a CDC procedure for "Enzyme lysis for clinical specimens of unknown etiology or known gram-positive cell suspensions (*S. pneumoniae*)" and the illustra bacteria genomic Prep Mini Spin kit (GE Healthcare, UK) protocol for purification of genomic DNA from gram positive bacteria.

1. *Materials needed*

- Lysis buffer type 4 (provided by the kit)
- Elution buffer type 5 (provided by the kit)
- Wash buffer type 6 (provided by the kit)
- Mini Spin columns and collection tubes (provided by the kit)
- Proteinase K (20 mg/ml; Thermo Scientific Inc., USA)
- Lysozyme (USB, USA)
- TE buffer (Amresco, USA)
- 10% Sodium Dodecyl Sulfate (SDS) (Biorad, USA)
- 0.5 M EDTA pH 8.0 (Amresco, USA)
- Sterile, Nuclease-Free water (Amresco, USA)
- Mutanolysin (Sigma Chemical Co., St. Louis, MO) stock solution (The whole bottle

was reconstituted with sterile distilled water for a concentration of 2,500 U/ml and aliquots of 60 µl were prepared and stored at -20°C)

2. *Procedure*

a. Preparation of the bacterial suspension

- Few colonies from a fresh culture of each isolate grown on BHI agar plates were inoculated in BHI broth (LAB M Limited, United Kingdom) tubes and incubated overnight at 37ºC.

After the incubation time was over, 1 ml of each bacterial suspension was added in a microcentrifuge tube and spun at 16000 × g for 30 seconds. Then, the supernatant was removed by aspiration without disturbing the cell pellet at the bottom of the tube.

b. Lysis

- Digestion buffer was prepared by adding 80mg of lyophilized lysozyme and 60 µl of stock mutanolysin (2500 U/ml) to 2 ml TE buffer. This solution should be used immediately and cannot be stored for more than 15 minutes.
- 100 µl of the digestion buffer were added to each microcentrifuge tube containing the bacterial suspension, vortexed for 10 seconds, and then incubated for one hour at 37ºC.
- Lysis buffer was prepared by mixing 40 µl of 10% SDS with 2 ml of 0.5 M EDTA pH 8.0 and adding sterile distilled water to obtain a final volume of 100 ml. this buffer was stored at room temperature.
- 200 µl of the cell lysis buffer and 10 µl of proteinase K were added to the microcentrifuge tubes which were then inverted for mixing.
- The microcentrifuge tubes were incubated at 37ºC for 15 minutes, then at 55ºC for 30 minutes.

c. Purification

- Each microcentrifuge tube was then vortexed and the samples were transferred to the Mini Spin columns placed inside the collection tubes.
- The tubes were centrifuged at 11000 × g for one minute. Consecutively, the flowthrough was discarded by emptying the collection tubes then placing the columns back inside them.

d. Washing and drying

- 500 µl of lysis buffer type 4 were added to each column.
- The tubes were centrifuged for one minute at 11000 × g, the flowthrough was discarded and the columns were placed back inside each corresponding collection tube.
- 500 µl of wash buffer type 6 were added to each column and they were spun for three minutes at 16000 × g.
- The flowthrough was discarded and each column was transferred to a fresh DNase free 1.5 ml labeled microcentifuge tube.

e. Elution

- 200 µl of elution buffer type 5, preheated at 70ºC, were added directly to the top of glass fiber matrix in each column. Pipette tips were changed for each sample to avoid any variation in volume.
- The samples were incubated at room temperature for one minute then centrifuged at 11000 × g to recover the genomic DNA as flowthrough and collect it.

f. DNA concentration measurement and storage

- 20 µl of each DNA sample was diluted in 480 µl of sterile nuclease free water and then the concentration of each sample was measured by spectrophotometry at a wavelength of 260 nm.
- Aliquots of 10 µl of the DNA samples were then prepared and stored at-20ºC.

E. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed in order to determine if the virulence genes (*actA*, *hly*, and *inlB*) were present in the isolates. The amplicon size of these genes is 839, 1590, and 1893 base pair (bp) respectively.

1. *Materials needed*

- 5 U/µl Taq DNA polymerase (Fermentas, USA)
- 10x Taq PCR buffer with $(NH4)_2SO_4$ (Fermentas, USA)
- 2 mM Magnesium chloride ($MgCl_2$) (Fermentas, USA)
- Sterile, Nuclease-Free water (Amresco, USA)
- 2 mM dNTPs prepared by mixing:

- 10 µl of 100 mM dATP (Fermentas, USA)
- 10 µl of 100 mM dGTP (Fermentas, USA)
- 10 µl of 100 mM dTTP (Fermentas, USA)
- 10 µl of 100 mM dCTP (Fermentas, USA)
- 460 µl nuclease free water

- The extracted DNA of each isolate (10 µg/ml)

- Primers:

- Forward *actA* primer (Thermo Scientific Inc., USA)

Sequence: 5'-CGC CGC GGA AAT TAA AAA AAGA-3'

- Reverse *actA* primer (Thermo Scientific Inc., USA)

Sequence: 5'-ACG AAG GAA CCG GGC TGC TAG-3'

- Forward *hly* primer (Thermo Scientific Inc., USA)

Sequence: 5'-ATG AAA AAA ATA ATG CTA GT-3'

- Reverse *hly* primer (Thermo Scientific Inc., USA)

Sequence: 5'-TTA TTC GAT TGG ATT ATC TA-3'

- Forward *inlB* primer (Thermo Scientific Inc., USA)

Sequence: 5'-ATG AAA GAA AAG CAC AAC CC-3'

- Reverse *inlB* primer (Thermo Scientific Inc., USA)

Sequence: 5'-TTA TTT CTG TGC CCT TAA AT-3'

The lyophilized primers were reconstituted with 1x TE buffer (Amresco, USA) according to the manufacturer instructions to obtain a concentration of 100 µM for each one.

10 µl aliquots of 10 µM were prepared and stored at -20ºC.

2. *Procedure*

25 µl of PCR Master Mix was prepared for each sample, in addition to the positive and negative controls.

This mix contained 2.5 µl dNTPs (2 mM), 2 µl $MgCl_2$ (2 mM), 2.5 µl 10x Taq PCR buffer with $(NH4)_2SO_4$, 0.2 µl Taq DNA polymerase (1 unit), 10.3 µl nuclease free water, 2.5 µl forward primer (10 µM), 2.5 µl reverse primer (10 µM), in addition to 2.5 µl of the 10 µg/ml diluted DNA sample.

The same procedure was carried out for the three genes, but for each one the corresponding primers were used.

After distributing the Master Mix into 1 ml microcentrifuge tubes, they were placed in a thermal cycler (PCR Sprint Thermal Cycler, Thermo Fisher Scientific, Waltham, MA, USA) with a programmed cycling sequence for each primer:

- The cycling program for the *inlB* and *hly* primers was as follows:

1 cycle of initial denaturation at 94ºC for four minutes, then amplified for 35 cycles consisting of denaturation at 94ºC for 45 seconds followed by annealing at 50ºC for one minute and elongation at 72ºC for one minute 30 seconds, and finally a five minutes final extension at 72ºC followed by a holding temperature of 4ºC.

- The cycling program for the *actA* primer was as follows:

1 cycle of initial denaturation at 95ºC for five minutes, then amplified for 30 cycles consisting of denaturation at 95ºC for 45 seconds followed by annealing at 60ºC for 45 seconds and elongation at 72ºC for 45 seconds, and finally an eight minutes final extension at 72ºC followed by a holding temperature of 4ºC.

F. Gel Electrophoresis

1. *Materials needed*

- 10x TBE (prepared by mixing 108g Tris Base, 55g Boric acid and 9.3g disodium EDTA with 1 liter of distilled water and then autoclaved) (Amresco, USA)
- Seakem LE Agarose Powder (Lonza, USA)
- Ethidium bromide (0.625 mg/ml) (Amresco, USA)
- 100 bp DNA ladder (Fermentas, USA)

- 6x Loading dye (Fermentas, USA)

2. *Procedure*

100 ml of 1x TBE (diluted from 10x TBE) was added to 1.5 g of Seakem Agarose Powder to prepare a 1.5% agarose gel. Afterward, the gel was dissolved by heating the mixture in the microwave for an initial 30 seconds while stirring it, then repeating the heating/stirring process for two or three times until the solution became clear.

Two drops of ethidium bromide were added to the gel, after which it was poured into the gel-casting tray with the combs in place.

The gel was allowed to cool for approximately 30 minutes until it solidified completely.

Then the combs were removed and 1x TBE was added to the electrophoretic chamber until the gel was submerged.

The first well in the gel was loaded with a 100 bp ladder. Then, the others were loaded with the samples, negative and positive controls.

The loading mixtures were prepared as follows:

- The ladder: 2 µl of 100 bp ladder with 2 µl of 6x loading dye and 8 µl of 1x TBE
- PCR samples: 2 µl of 6x of loading dye with 10 µl of the PCR products

The gel was run at 120 V for approximately 45 minutes.

Ultraviolet (UV) transilluminator (Haake buchler Instruments Inc., USA) was used to visualize the bands, while Olympus digital camera using the Digi-Doc Program was used for photographing them. DNA extraction, PCR and gel electrophoresis were repeated three times for confirmation in case of negative results.

G. Pulsed Field Gel Electrophoresis (PFGE)

PFGE was carried out on the fifty-nine isolates to determine their genomic relatedness according to a standardized PulseNet protocol for *L. monocytogenes* (102).

1. *Materials needed*

- 10× TBE (Tris-Boric Acid-EDTA)

For a volume of 500 ml, the following mixture was prepared and then autoclaved:

- 60.55 g Tris Base (Amresco, USA)
- 30.99 g Boric Acid (Amresco, USA)
- 1.85 g disodium EDTA (Amresco, USA)
- 500 ml distilled water

- Cell Lysis Buffer (CLB)

For a volume of 500 ml:

- 50 ml of 10% Sarcosyl (N-Lauroylsarcosine sodium salt) (Sigma Chemical Co., St. Louis, MO).
- 25 ml 1M Tris pH 8 (Amresco, USA)
- 50 ml 0.5 M EDTA pH 8 (Amresco, USA)
- Completed to a volume of 500 ml with sterile distilled water and stored in an autoclaved flask.

- TE buffer (for plugs preparation, cell suspension, and plugs washing)

For a volume of 1000 ml:

- 10 ml 1M Tris, pH 8 (Amresco, USA)
- 2 ml 0.5 M EDTA, pH 8 (Amresco, USA)

- Completed to a volume of 1000 ml with sterile distilled water and stored in an autoclaved flask.

- Seakem Gold (SKG) Agarose for PFGE (Lonza, USA)
- 10% Sodium Dodecyl Sulfate (SDS) (Biorad, USA)
- Mutanolysin (2500 U/ml) (Sigma Chemical Co., St. Louis, MO)
- Ladder: *Salmonella* ser. Braenderup (BAA 664)
- Autoclaved distilled water
- Sterile, Nuclease-Free water (Amresco, USA)
- Proteinase K (20 mg/ml; Thermo Scientific Inc., USA)
- Pre-restriction incubation mix (200 µl) for each plug:

- 20 µl of 10× Tango buffer (Fermentas, USA)
- 180 µl nuclease free water

- Restriction mix (200 µl) for each plug:

- 175.5 µl nuclease free water
- 20 µl Corresponding Restriction buffer (10× Tango buffer)
- 2 µl Bovine Serum Albumin (BSA) (10mg/ml) (Amresco, USA)
- 2.5 µl *AscI* (10 U/ml) enzyme (Fermentas, USA) for *L. monocytogenes* plugs or 2.5 µl *XbaI* enzyme (Fermentas, USA) for BAA plugs

- Lysozyme stock solution (20 mg/ml stock was prepared by mixing 100 mg Lysozyme (USB, USA) with 5 ml TE buffer and was stored in 250 µl aliquots at -20ºC)
- Ethidium bromide (Amresco, USA)

2. *Procedure*

Four days were needed to carry out PFGE procedure for 12 *L. monocytogenes* isolates at each time.

i. Day one:

- *L. monocytogenes* isolates were grown on BHI agar plates and incubated overnight at 37ºC.
- The ladder was grown on MacConkey agar plate and incubated overnight at 37ºC.
- TE, CLB and 10× TBE buffers were prepared. And needed glassware was autoclaved.

ii. Day two:

- Preparation of 1% Seakem Gold agarose for plugs:

- 10% SDS solution was put into a water bath to warm at 55-60ºC.
- 0.25 SKG were weighed and mixed with 23.75 ml TE buffer into a 250 flask.
- The flask was loosely covered with clear film and heated for 30 seconds in the microwave, mixed gently, then heated again for 10 seconds intervals until the agarose was completely dissolved.
- 1.25 ml of the warmed 10% SDS solution was added to the flask and was mixed by swirling.
- The flask was placed in the water bath at 55-60ºC and left to equilibrate the temperature for 15 minutes or until ready to use.

- Preparation of cell suspensions:

- 2 ml of TE buffer were distributed into labeled autoclaved tubes.
- Few colonies were taken from each culture plate with a sterile cotton swab and suspended in the corresponding tube.

- The suspensions were vortexed and the concentration of the samples was adjusted to 2 McFarland, while that of the ladder was adjusted to 2.5 McFarland.

- Casting of plugs:

- 400 µl of the adjusted cell suspensions was transferred to labeled sterile 1.5 ml microcentrifuge tubes

- 10 µl of thawed lysozyme solution was added to each microcentrifuge tube and mixed gently by pipetting up and down a few times. Then the tubes were placed into a 55-60ºCwater bath for 10-20 minutes. Unused thawed lysozyme was discarded.

- 20 µl proteinase K (20 mg/ml) and 5 µl mutanolysin (2500 U/ml) were added to each tube and mixed gently.

- 400 µl of the prepared melted agarose was added to each tube and mixed gently by pipetting up and down a few times, and then the mixture was immediately dispensed into the appropriate well of reusable plug mold.

- The wells of the mold were carefully filled to avoid the formation of air bubbles or contamination. The mold was then incubated at 4ºC for 10 minutes until the plugs solidified.

- Lysis of cells in agarose plugs:

- 50 ml sterile falcon tubes were labeled and each one was filled with 5ml CLB and 25 µl Proteinase K (20 mg/ml)

- The solidified plugs were carefully removed from the plug mold and transferred into their corresponding falcon tube, making sure they were completely immersed in the buffer.

- The falcon tubes were incubated at 54ºC in a shaker incubator with constant vigorous agitation (150-175 rpm) for two hours.

- A flask containing TE buffer and one containing autoclaved distilled water were placed in the same incubator with the falcon tubes to be heated and used later in the washing step.

- Washing of plugs after cell lysis:

- After the tubes were removed from the incubator, the lysis buffer was poured off carefully from each falcon tube and 5 ml of pre-heated (54-55ºC) autoclaved distilled water were added to each one.

- The tubes were subsequently incubated in the shaker incubator for 15 minutes at 54ºC, then the water was discarded and the washing step with autoclaved distilled water was repeated one more time.

- After washing the plugs with water, 5 ml preheated TE buffer were added to each falcon tube. The tubes were incubated in the shaker incubator for 15 minutes at 54ºC, after which the TE was poured off. The washing step with TE was repeated three more times.

- After the washing step is over, 5 ml of TE were added to each falcon tube which were subsequently stored overnight at 4ºC.

iii. *Day three:*

- Cutting the plugs:

- The falcon tubes were removed from the refrigerator and the plugs were cut into 2 mm × 6 mm using a blade. Three slices were cut for the ladder.

- Each cut plug was put into the corresponding labeled 1.5 ml microcentrifuge tube containing 200 µl of pre-restriction incubation mix using a spatula.

- The microcentrifuge tubes were incubated at 37ºC for 5-10 minutes.

- Restriction digestion of DNA in agarose plugs:

- After incubation, the mix was discarded from the tubes using a pipette. This had to be done carefully not to damage the plugs.
- 200 µl of restriction buffer was added into each tube then incubated at 37ºC for two hours.

- Casting agarose gel:

- An hour before the incubation time is over, agarose gel was prepared by mixing 1 g of SKG with 100 ml of 0.5 × TBE (diluted from 10 × TBE) into a 250 ml flask. The flask was loosely covered with clear film and heated for 30 seconds in the microwave, mixed gently, then heated again for 10 seconds intervals until the agarose was completely dissolved.
- 2-5 ml of melted agarose were taken from the flask and kept in a tube to be used later for sealing the wells. The flask and the recapped tube were placed in the water bath at 55-60ºC and left to equilibrate the temperature for 15 minutes or until ready to use.
- After cooling, the gel was poured into the cast with 15-tooth comb and left to solidify at room temperature for 30-45 minutes.

- Loading the plugs into the wells:

- After the two hours incubation is over, the restricted plugs were removed from the incubator and the mix was discarded from the tubes using a pipette. This should be done carefully not to damage the plugs.
- 200 µl of 0.5 × TBE were added to each tube and they were incubated at room temperature for five minutes.
- The comb was pulled out of the cast and wells were formed.
- The plugs were removed from each tube using a spatula and each one was loaded into a well. The ladder plugs were loaded into wells 1, 8, and 15.

- 50 µl of the melted agarose kept earlier in a capped tube was used to cover each wells and trap the plugs inside.
- The gel was allowed to harden for three to five minutes.

- Running the gel:

- 2200 ml of 0.5 × TBE were poured into the electrophoresis chamber (Bio-rad, USA). The cooling module was turned on and the buffer was left to cool down to 14℃.
- Afterwards, the cast was unscrewed and the gel was removed and placed inside the electrophoresis chamber.
- The following program for *L. monocytogenes* was inserted on the CHEF MAPPER^{TM}(Bio-rad, USA):

. Auto Algorthim

. 49 kb - low MW

. 450 kb - high MW

. Default values were selected except where noted by pressing "enter"

. Initial switch time = 4.0 s

. Final switch time = 40.0 s

. Run Time of 18 hours was selected

After entering the program, the run was carried out overnight.

iv. *Day four:*

- Staining the gel:

- On the next day, after the run was over, the machine was turned off and the gel was put in a covered container filled with 400 ml of distilled water and 8 drops of ethidium bromide.

- The container was placed on a rocker machine for 20 minutes.

- Destaining the gel:

- The water containing ethidium bromide was discarded in a designated jar for hazardous waste and the container was filled with 500 ml distilled water and placed back on the rocker machine for 20 minutes. The destaining procedure was repeated twice.

- After the destaining step, Gel Doc XR + system Machine (Bio-rad, USA) and "Quality one" software were used to visualize the bands and capture a picture of the gel. Bionumerics (Applied Maths, TX) software was used later to analyze the results.

H. Assessment of Biofilm Formation using the Microtiter Plate Assay

1. *Materials needed*

- Polysterene microtiter plate (Costar 3788, Corning Incorporated, NY)
- 95% ethanol
- 1% Crystal Violet (CV) solution (100 ml were prepared by mixing 2g of CV powder dissolved in 20 ml 99% ethyl alcohol, with 0.8g ammonium oxalate dissolved in 80 ml distilled water)

2. *Procedure*

After analyzing the results of PFGE, one isolate from each group of identical profiles (100% similarity) was chosen as a representative of the group. The total was 13 representative isolates and eight CDC controls.

Four days were needed to assess biofilm formation of *L. monocytogenes* isolates using the following protocol:

v. *Day one:*

L. monocytogenes isolates were grown on BHI agar plates and incubated overnight at 37ºC.

vi. *Day two:*

Few colonies of each isolate were inoculated in BHI broth tubes and incubated overnight at 37ºC.

vii. *Day three:*

- Turbidity of the suspensions was adjusted to 0.5 McFarland with BHI broth.
- 20 µl of each culture were distributed into triplicate wells of 96-well rounded-bottomed microplate containing 230 µl of BHI broth
- Triplicate wells containing only BHI broth added individually served as negative controls.
- The wells at the corners of the plate were filled with distilled water to maintain the humid atmosphere in the plate.
- The plate was incubated for 20 hours at 37ºC.

viii. *Day four:*

- The wells were washed three times with sterile distilled water using the multichannel pipette to remove excess of planktonic cells. This was done with care in order not to disturb the biofilm formed on the side of each well.
- 150 µl of the 1% CV solution was added to each well and the plate was incubated for 15 minutes at room temperature followed by washing five times with sterile distilled water using the multichannel pipette.
- The plate was left few minutes to air dry, then 200 µl of 95% ethanol were added to each well and left at room temperature for 20 minutes to solubilize the CV stained biofilms

- 125 µl were transferred from each well to a new plate and the absorbance was measured with BIO-TEK ELx800 Automated Microplate Reader at 630nm.

The assessment of biofilm formation was done by the calculation of the mean average for the three wells of each isolate.

Standard deviations were also calculated to determine statistically significant results relying on the Unpaired t-test using "GraphPad Software". Results were considered significant when p-value calculated was < 0.05.

CHAPTER IV

RESULTS

A. API Identification

API test was done for confirmation of the 59 isolates identity. All the isolates except number 1 had the same API profile. They showed positive results in microtubes containing Esculin Ferric Citrate, 4-nitrophenyl-αD-mannopyranoside, D-ArabitoL, L-Rhamnose, Methyl- αDglucopyranoside, and negative results in microtubes containing enzymatic substrate, D-Xylose, D-Ribose, Glucose-1-phosphate, and D-Tagatose. The code was entered into the "Apilab" software and it confirmed 58 isolates were *L. monocytogenes* with a probability of 98.6%. As for isolate number 1, it differed by its ability to ferment D-Xylose. Its probability of being *L. monocytogenes* was 20% (80% for being *Listeria welshimeri*).

B. Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing was performed on the 59 isolates and the results are shown in Table 1. All the isolates were found to be resistant to one or more antimicrobials. The isolates were resistant to ampicillin (25.43%), penicillin (50.85%), clindamycin (66.1%), and oxacillin (100%). However, they were all susceptible to erythromycin, trimethoprim-sulfamethoxazole, tetracycline, gentamicin and vancomycin (Figure 1). Antimicrobials to which Lebanese isolates demonstrated resistance were oxacillin (100%), penicillin (64.29%), ampicillin (42.96%) and clindamycin (35.71%). While antimicrobials to which Vietnamese isolates demonstrated resistance were oxacillin (100%) followed by clindamycin (75%), penicillin (47.5%) and ampicillin (22.5%) (Table 3). Resistance to

oxacillin was common among all the food products, followed by penicillin which was common in Vietnamese fish filet, Lebanese cheese and raw meat (Table 4).

Additionally, six different antimicrobial resistance patterns were found, the highest diversity of these patterns was found in fish filet from Vietnam (Figure 7).

C. Polymerase Chain Reaction

PCR amplification of the virulence encoding genes showed that *L. monocytogenes* isolates and CDC controls were all positive for *hly* gene, and the amplification products were of the expected size 1590 bp. As for the *inlB* gene, all isolates were PCR positive with the expected size 1893 bp except isolate number 51 from Vietnam that was PCR negative. All the isolates were positive for the *actA* gene, however, there were two different sizes of amplification products; isolates number 13, 31, 32 and four of the CDC controls (C1, C2, C6, and C7) showed a product size of 839 bp, while all the other isolates showed a product size of around 950 bp. Both amplification products indicate the presence of *actA* gene (103). Figures 2, 3 and 4 show the amplicons of the virulence-associated genes *actA*, *hly*, and *inlB* respectively.

Moreover, isolate number 1 that had a probability of 20% for being *L. monocytogenes* according to API identification was found PCR positive for these specific 3 virulence genes and was hence confirmed to be *L. monocytogenes.*

D. Pulsed Field Gel Electrophoresis

PFGE analysis showed that there were 13 different subtypes with 100% similarity (Figure 6). These 13 subtypes formed 6 distinct clusters of 90% relative clonal relatedness (Figure 7). The most predominant clusters were E (including subtypes GX6A16.0008,

GX6A16.0009 and GX6A16.0010) comprising 33 isolates, followed by cluster B (including subtypes GX6A16.0000, GX6A16.0001, GX6A16.0002 and GX6A16.0003) comprising 12 isolates and cluster D (including subtypes GX6A16.0006 and GX6A16.0007) with 10 isolates (Table 5). One of the PFGE gels of *L. monocytogenes* restricted with *AscI* enzyme is shown in figure 5.

Table 5 demonstrates that cluster B comprises isolates from Lebanese products only (cheese and raw meat), while clusters D and E consisted mainly of Vietnamese fish filet, except for isolate number 56 in cluster E that was from a Lebanese seafood sample. It was notable that two of the CDC controls were strongly related to some of the isolates; J0095 had 85.7% genetic similarity with isolate number 12 (Lebanese salmon fish from cluster A). Additionally, 2009L-1181 had 89.4% genetic similarity with isolates of cluster B (Lebanese cheese and raw meat) (figure 7).

E. Assessment of Biofilm Formation

After PFGE analysis was done, assessment of biofilm formation was performed on the 13 subtypes by measuring the absorbance at 630 nm, all results were found to be statistically significant (p-value < 0.05) using the Unpaired Student's t-test.

Table 6 demonstrates the average absorbance of biofilm for each of the 13 subtypes and 8 CDC controls. It shows that subtypes belonging to the same cluster on the dendrogram have close absorbance results. Additionally, subtypes with the highest absorbance (GX6A16.0000, GX6A16.0003, GX6A16.0002, GX6A16.0001 respectively) are all from cluster B, while subtypes that weakly formed a biofilm, belong to clusters A, D, E and F. As for cluster C, it ranks second after cluster B. Furthermore; subtypes from cluster B demonstrated stronger biofilm formation. J0095 (85.7% genomic similarity to

cluster A) had almost identical absorbance results with cluster A. Nevertheless, 2009L-1181 (89.4% genomic similarity with cluster B) had a lower absorbance value compared with cluster B.

Table 1: Antimicrobial susceptibility of the 59 *L. monocytogenes* isolates obtained from L.A.R.I. and the subtypes assigned to genetically identical isolates after PFGE analysis.
(R: Resistant; I: Intermediate; S: Susceptible; P: penicillin; DA: clindamycin; AMP: ampicillin; CN: gentamicin; OX: oxacillin; VA: vancomycin; E: erythromycin; TE: tetracycline; SXT: trimethoprim-sulfamethoxazole)

Isolate #	Information		Antimicrobial Susceptibility zone diameter (mm)									PFGE Subtype
	Food Source	Year	P (10 U)	DA (2 µg)	AMP (10 µg)	CN (10 µg)	OX (1µg)	VA (30 µg)	E (15 µg)	TE (30 µg)	SXT (1.25/ 23.75 µg)	
1	Fish filet - Vietnam	2012	31 (S)	14 (R)	33 (S)	30 (S)	6 (R)	22 (S)	33 (S)	30 (S)	36 (S)	GX6A16.0008
2	Fish filet - Vietnam	2012	25 (R)	20 (I)	28 (R)	27 (S)	6 (R)	24 (S)	30 (S)	26 (S)	33 (S)	GX6A16.0010
3	Fish filet - Vietnam	2012	31 (S)	14 (R)	34 (S)	30 (S)	6 (R)	24 (S)	33 (S)	30 (S)	38 (S)	GX6A16.0010
4	Fish filet - Vietnam	2012	32 (S)	14 (R)	32 (S)	30 (S)	6 (R)	23 (S)	33 (S)	30 (S)	37 (S)	GX6A16.0010
5	Fish filet - Vietnam	2012	31 (S)	14 (R)	31 (S)	31 (S)	6 (R)	23 (S)	34 (S)	31 (S)	36 (S)	GX6A16.0010
6	Fish filet - Vietnam	2012	31 (S)	14 (R)	31 (S)	31 (S)	6 (R)	23 (S)	34 (S)	31 (S)	39 (S)	GX6A16.0010
7	Cheese baladi - Lebanon	2012	30 (S)	24 (S)	32 (S)	31 (S)	6 (R)	22 (S)	33 (S)	31 (S)	37 (S)	GX6A16.0000
8	Fish filet - Vietnam	2012	30 (S)	14 (R)	33 (S)	33 (S)	6 (R)	23 (S)	34 (S)	31 (S)	37 (S)	GX6A16.0010
9	Fish filet - Vietnam	2012	31 (S)	14 (R)	32 (S)	33 (S)	6 (R)	23 (S)	34 (S)	32 (S)	39 (S)	GX6A16.0008
10	Unknown	-	28 (R)	12 (R)	34 (S)	27 (S)	6 (R)	23 (S)	31 (S)	30 (S)	36 (S)	GX6A16.0004
11	Unknown	-	30 (S)	22 (S)	33 (S)	32 (S)	6 (R)	25 (S)	36 (S)	32 (S)	37 (S)	GX6A16.0000
12	Salmon fish – Lebanon	2012	34 (S)	16 (I)	34 (S)	33 (S)	6 (R)	24 (S)	34 (S)	30 (S)	39 (S)	GX6A16.0011
13	Unknown	-	31 (S)	14 (R)	31 (S)	30 (S)	6 (R)	23 (S)	33 (S)	30 (S)	37 (S)	GX6A16.0006
14	Unknown	-	25 (R)	12 (R)	30 (S)	28 (S)	6 (R)	29 (S)	30 (S)	25 (S)	30 (S)	GX6A16.0008
15	Unknown	-	29 (S)	14 (R)	32 (S)	31 (S)	6 (R)	23 (S)	34 (S)	30 (S)	38 (S)	GX6A16.0008
16	Fish filet - Vietnam	2013	29 (S)	15 (I)	30 (S)	31 (S)	6 (R)	22 (S)	33 (S)	27 (S)	37 (S)	GX6A16.0008
17	Fish filet - Vietnam	2013	31 (S)	14 (R)	31 (S)	33 (S)	6 (R)	24 (S)	33 (S)	30 (S)	38 (S)	GX6A16.0008
18	Fish filet - Vietnam	2013	32 (S)	13 (R)	33 (S)	32 (S)	6 (R)	23 (S)	33 (S)	29 (S)	38 (S)	GX6A16.0008
19	Fish filet - Vietnam	2013	31 (S)	14 (R)	34 (S)	32 (S)	6 (R)	23 (S)	34 (S)	31 (S)	37 (S)	GX6A16.0008
20	Fish filet - Vietnam	2013	31 (S)	17 (I)	32 (S)	31 (S)	6 (R)	24 (S)	34 (S)	31 (S)	37 (S)	GX6A16.0008
21	Fish filet - Vietnam	2013	31 (S)	14 (R)	32 (S)	32 (S)	6 (R)	23 (S)	33 (S)	31 (S)	37 (S)	GX6A16.0008
22	Fish filet - Vietnam	2013	31 (S)	14 (R)	32 (S)	32 (S)	6 (R)	23 (S)	34 (S)	31 (S)	38 (S)	GX6A16.0008
23	Fish filet - Vietnam	2013	32 (S)	14 (R)	33 (S)	32 (S)	6 (R)	23 (S)	34 (S)	31 (S)	39 (S)	GX6A16.0008
24	Fish filet - Vietnam	2013	29 (S)	15 (I)	35 (S)	33 (S)	6 (R)	23 (S)	35 (S)	31 (S)	38 (S)	GX6A16.0008
25	Fish filet - Vietnam	2013	26 (R)	18 (I)	26 (S)	23 (S)	6 (R)	17 (S)	28 (S)	20 (S)	28 (S)	GX6A16.0008
26	Cheese baladi - Lebanon	2013	27 (R)	14 (R)	27 (R)	28 (S)	6 (R)	21 (S)	32 (S)	28 (S)	36 (S)	GX6A16.0003
27	Cheese baladi - Lebanon	2013	27 (R)	14 (R)	29 (S)	28 (S)	6 (R)	22 (S)	32 (S)	31 (S)	37 (S)	GX6A16.0002
28	Cheese baladi - Lebanon	2013	26 (R)	14 (R)	28 (R)	29 (S)	6 (R)	22 (S)	32 (S)	29 (S)	35 (S)	GX6A16.0000
29	Cheese baladi - Lebanon	2013	25 (R)	20 (S)	28 (R)	26 (S)	6 (R)	20 (S)	30 (S)	30 (S)	35 (S)	GX6A16.0000
30	Cheese baladi - Lebanon	2013	25 (R)	16 (I)	26 (R)	26 (S)	6 (R)	19 (S)	38 (S)	26 (S)	31 (S)	GX6A16.0003

Isolate #	Information		Antimicrobial Susceptibility zone diameter (mm)									PFGE Subtype
	Food Source	Year	P (10 U)	DA (2 µg)	AMP (10 µg)	CN (10 µg)	OX (1µg)	VA (30 µg)	E (15 µg)	TE (30 µg)	SXT (1.25/ 23.75 µg)	
31	Cheese baladi - Lebanon	2013	26 (R)	13 (R)	28 (R)	27 (S)	6 (R)	21 (S)	20 (I)	29.5 (S)	32 (S)	GX6A16.0005
32	Cheese baladi - Lebanon	2013	26 (R)	14 (R)	27 (R)	29 (S)	6 (R)	20 (S)	30 (S)	25 (S)	32 (S)	GX6A16.0001
33	Cheese baladi - Lebanon	2013	20 (R)	25 (S)	30 (S)	30 (S)	6 (R)	22 (S)	31 (S)	30 (S)	38 (S)	GX6A16.0002
34	Fish filet - Vietnam	2013	25 (R)	15 (I)	35 (S)	25 (S)	6 (R)	29.5 (S)	31 (S)	25 (S)	35 (S)	GX6A16.0006
35	Fish filet - Vietnam	2013	30 (S)	11 (R)	30 (S)	30 (S)	6 (R)	25 (S)	31 (S)	30 (S)	32 (S)	GX6A16.0010
36	Fish filet - Vietnam	2013	25 (R)	12 (R)	28 (R)	30 (S)	6 (R)	25 (S)	33 (S)	26 (S)	36 (S)	GX6A16.0007
37	Fish filet - Vietnam	2013	30 (S)	12 (R)	30 (S)	28 (S)	6 (R)	20 (S)	30 (S)	25 (S)	38 (S)	GX6A16.0010
38	Fish filet - Vietnam	2013	25 (R)	12 (R)	30 (S)	30 (S)	6 (R)	24 (S)	30 (S)	26 (S)	30 (S)	GX6A16.0007
39	Fish filet - Vietnam	2013	26 (R)	13 (R)	25 (R)	26 (S)	6 (R)	20 (S)	30 (S)	23 (S)	30 (S)	GX6A16.0008
40	Fish filet - Vietnam	2013	25 (R)	12 (R)	30 (S)	31 (S)	6 (R)	25 (S)	32 (S)	26 (S)	32 (S)	GX6A16.0007
41	Fish filet - Vietnam	2013	25 (R)	12 (R)	25 (R)	30 (S)	6 (R)	24 (S)	31 (S)	25 (S)	32 (S)	GX6A16.0007
42	Fish filet - Vietnam	2013	25 (R)	20 (I)	28 (R)	29 (S)	6 (R)	23 (S)	31 (S)	26 (S)	33 (S)	GX6A16.0007
43	Fish filet - Vietnam	2013	23 (R)	13 (R)	27 (R)	29 (S)	6 (R)	23 (S)	31 (S)	26 (S)	31 (S)	GX6A16.0007
44	Fish filet - Vietnam	2013	25 (R)	12 (R)	27 (R)	30 (S)	6 (R)	25 (S)	30 (S)	26 (S)	35 (S)	GX6A16.0007
45	Fish filet - Vietnam	2013	26 (R)	11 (R)	30 (S)	30 (S)	6 (R)	25 (S)	31 (S)	25 (S)	35 (S)	GX6A16.0008
46	Fish filet - Vietnam	2013	26 (R)	12 (R)	26 (R)	30 (S)	6 (R)	20 (S)	30 (S)	25 (S)	35 (S)	GX6A16.0008
47	Fish filet - Vietnam	2013	25 (R)	11 (R)	30 (S)	30 (S)	6 (R)	20 (S)	30 (S)	26 (S)	35 (S)	GX6A16.0007
48	Fish filet - Vietnam	2013	30 (S)	10 (R)	30 (S)	30 (S)	6 (R)	20 (S)	31 (S)	27 (S)	38 (S)	GX6A16.0008
49	Fish filet - Vietnam	2013	28 (R)	22 (S)	30 (S)	30 (S)	6 (R)	20 (S)	30 (S)	25 (S)	34 (S)	GX6A16.0008
50	Fish filet - Vietnam	2013	25 (R)	20 (I)	30 (S)	26 (S)	6 (R)	20 (S)	28 (S)	25 (S)	31 (S)	GX6A16.0008
51	Fish filet - Vietnam	2013	33 (S)	25 (S)	34 (S)	32 (S)	6 (R)	22 (S)	34 (S)	31 (S)	35 (S)	GX6A16.0012
52	Fish filet - Vietnam	2013	28 (R)	12 (R)	30 (S)	30 (S)	6 (R)	20 (S)	30 (S)	25 (S)	35 (S)	GX6A16.0008
53	Fish filet - Vietnam	2013	26 (R)	13 (R)	28 (R)	28 (S)	6 (R)	21 (S)	30 (S)	25 (S)	31 (S)	GX6A16.0008
54	Fish filet - Vietnam	2013	31 (S)	12 (R)	33 (S)	30 (S)	6 (R)	20 (S)	31 (S)	26 (S)	38 (S)	GX6A16.0008
55	Akkawi cheese – Lebanon	2013	30 (S)	25 (S)	30 (S)	30 (S)	6 (R)	22 (S)	35 (S)	30 (S)	40 (S)	GX6A16.0003
56	Seafood – Lebanon	2013	30 (S)	20 (I)	33 (S)	28 (S)	6 (R)	25 (S)	31 (S)	28 (S)	35 (S)	GX6A16.0009
58	Fish filet - Vietnam	2013	26 (R)	12 (R)	32 (S)	27 (S)	6 (R)	22 (S)	30 (S)	28 (S)	35 (S)	GX6A16.0008
59	Raw meat – Lebanon	2013	30 (S)	25 (S)	30 (S)	28 (S)	6 (R)	20 (S)	32 (S)	28 (S)	38 (S)	GX6A16.0000
60	Raw meat – Lebanon	2013	26 (R)	21 (S)	34 (S)	25 (S)	6 (R)	20 (S)	32 (S)	25 (S)	35 (S)	GX6A16.0000

Table 2: *L. monocytogenes* CDC control strains

CDC strain #	Source	Serotype	Country	Year	Outbreak
2009L-1023	Human blood	1/2a	USA	2009	Mexican-style cheese
2009L-1181	Human blood	1/2b	USA	2009	-
2010L-1846	Human blood	1/2a	USA	2010	Hog head cheese (a meat product)
F2365	Food/ cheese	4b	USA	1985	Mexican style cheese outbreak
H7858	Food	4b	USA	1998	Hot dog outbreak
2012L-5227	Human blood	4c	USA	2012	-
J00097	Human	4b	Germany	2000	Palumbo et al. J Clin Micro 2003, 41(2), 564-571
J00095	Food/pie	3a	Germany	2000	Palumbo et al. J Clin Micro 2003, 41(2), 564-571

Table 3: Antimicrobial resistance of the 59 *L. monocytogenes* isolates from Lebanese and Vietnamese sources.

(P: penicillin; DA: clindamycin; AMP: ampicillin; CN: gentamicin; OX: oxacillin; VA: vancomycin; E: erythromycin; TE: tetracycline; SXT: trimethoprim-sulfamethoxazole)

	Number of resistant isolates to the antimicrobial agents used (%)								
Country	AMP	E	P	SXT	TE	CN	DA	OX	VA
Lebanon	6 (42.96%)	0	9 (64.29%)	0	0	0	5 (35.71%)	14 (100%)	0
Vietnam	9 (22.5%)	0	19 (47.5%)	0	0	0	30 (75%)	40 (100%)	0
Unknown	0	0	2 (40%)	0	0	0	4 (80%)	5 (100%)	0

Table 4: Antimicrobial resistance of the 59 *L. monocytogenes* isolates from various food types.

(P: penicillin; DA: clindamycin; AMP: ampicillin; CN: gentamicin; OX: oxacillin; VA: vancomycin; E: erythromycin; TE: tetracycline; SXT: trimethoprim-sulfamethoxazole)

	Number of resistant isolates to the antimicrobial agents used (%)								
Food type	AMP	E	P	SXT	TE	CN	DA	OX	VA
Fish filet Vietnam	9 (22.5%)	0	19 (47.5%)	0	0	0	30 (75%)	40 (100%)	0
Cheese Lebanon	6 (60%)	0	8 (80%)	0	0	0	5 (50%)	10 (100%)	0
Raw meat Lebanon	0	0	1 (50%)	0	0	0	0	2 (100%)	0
Salmon fish Lebanon	0	0	0	0	0	0	0	1 (100%)	0
Seafood Lebanon	0	0	0	0	0	0	0	1 (100%)	0
Unknown	0	0	2 (40%)	0	0	0	4 (80%)	5 (100%)	0

Table 5: PFGE subtypes obtained by BioNumerics analysis in relation to food type.

Clusters (90% genomic similarity)	Subtypes (100% genomic similarity)	Food type	Number of isolates per food type	Total
A	GX6A16.0011	Salmon fish Lebanon	1	1
B	GX6A16.0000	Cheese baladi - Lebanon	3	12
		Raw meat – Lebanon	2	
		Unknown	1	
	GX6A16.0001	Cheese baladi - Lebanon	1	
	GX6A16.0002	Cheese baladi - Lebanon	2	
	GX6A16.0003	Cheese baladi - Lebanon	2	
		Akkawi cheese – Lebanon	1	
C	GX6A16.0004	Unknown	1	2
	GX6A16.0005	Cheese baladi - Lebanon	1	
D	GX6A16.0006	Unknown	1	10
		Fish filet - Vietnam	1	
	GX6A16.0007	Fish filet - Vietnam	8	
E	GX6A16.0008	Fish filet - Vietnam	22	33
		Unknown	2	
	GX6A16.0009	Seafood - Lebanon	1	
	GX6A16.0010	Fish filet - Vietnam	8	
F	GX6A16.0012	Fish filet - Vietnam	1	1

Table 6: Average absorbance of biofilm formation at 630 nm for each subtype and CDC control and the respective P-value.
(* : statistically significant)

Sample	Average absorbance at 630 nm	P-value
Negative control	0.047	-
GX6A16.0000	0.102	0.0013 *
GX6A16.0001	0.086	0.0001 *
GX6A16.0002	0.088	0.0001 *
GX6A16.0003	0.099	0.0001 *
GX6A16.0004	0.082	0.0001 *
GX6A16.0005	0.077	0.0006 *
GX6A16.0006	0.062	0.0072 *
GX6A16.0007	0.061	0.0001 *
GX6A16.0008	0.059	0.0001 *
GX6A16.0009	0.072	0.0074 *
GX6A16.0010	0.059	0.0135 *
GX6A16.0011	0.067	0.0160 *
GX6A16.0012	0.068	0.0063 *
CDC Control J00097	0.057	0.0270 *
CDC Control J00095	0.066	0.0012 *
CDC Control 2009L-1023	0.089	0.0003 *
CDC Control 2012L-5227	0.122	0.0001 *
CDC Control H7858	0.065	0.0216 *
CDC Control F2365	0.055	0.0030 *
CDC Control 2009L-1181	0.076	0.0001 *
CDC Control 2010L - 1846	0.083	0.0014 *

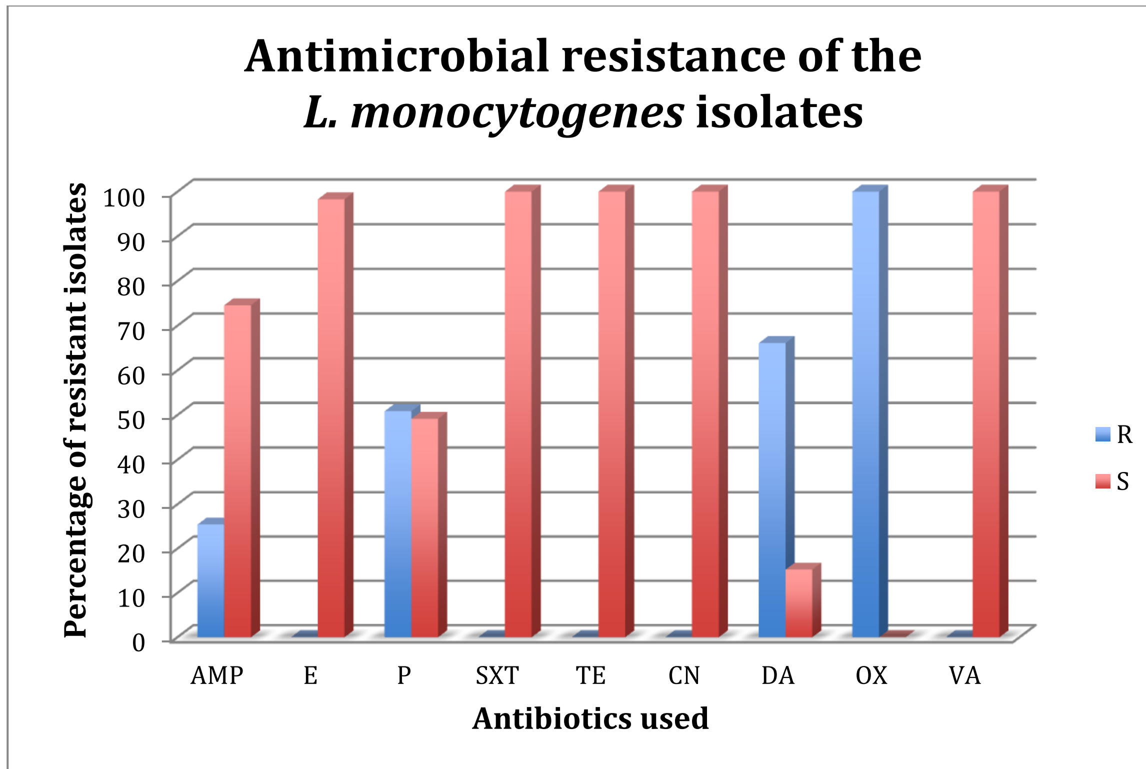

Figure 1: The percentage of antimicrobial resistance among the 59 *L. monocytogenes* isolates.
(R: Resistant; S: Susceptible; P: penicillin; DA: clindamycin; AMP: ampicillin; CN: gentamicin; OX: oxacillin; VA: vancomycin; E: erythromycin; TE: tetracycline; SXT: trimethoprim-sulfamethoxazole)

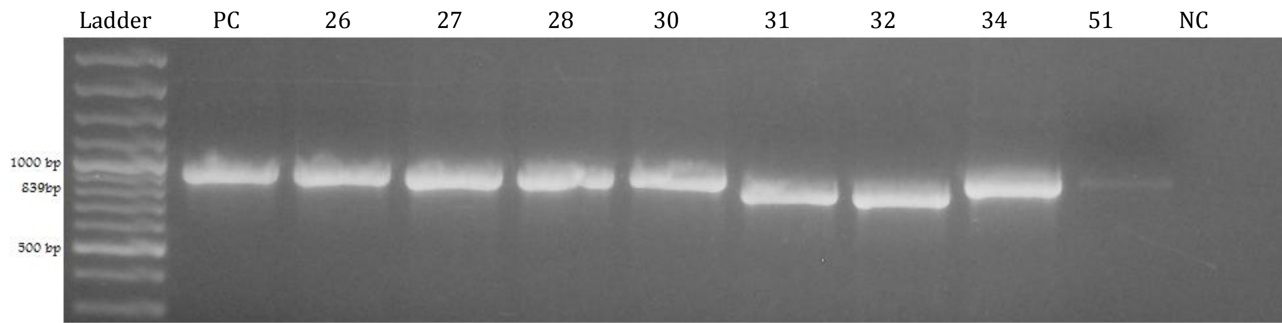

Figure 2: PCR results for the detection of the *actA* gene with expected size of 839bp.
(NC: negative control; PC: positive control)

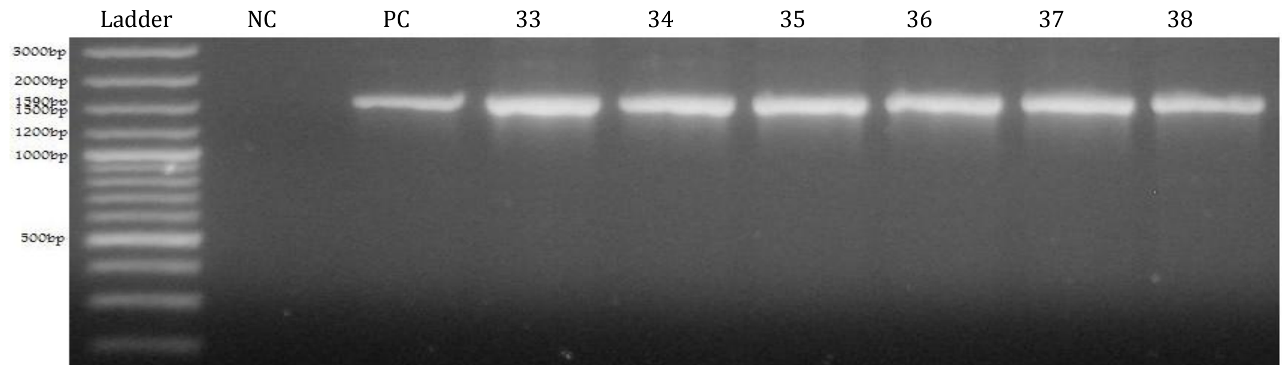

Figure 3: PCR results for the detection of the *hly* gene with expected size of 1590bp.
(NC: negative control; PC: positive control)

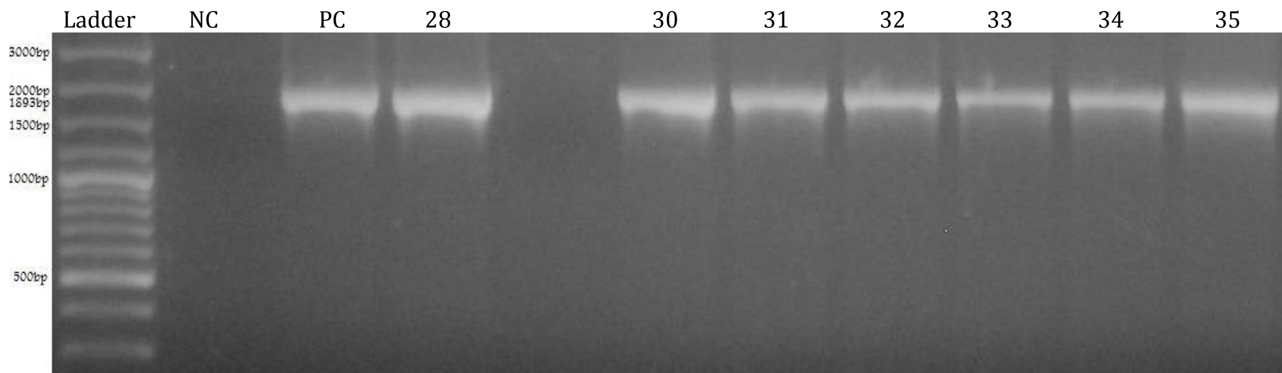

Figure 4: PCR results for the detection of the *inlB* gene with expected size of 1893bp.
(NC: negative control; PC: positive control)

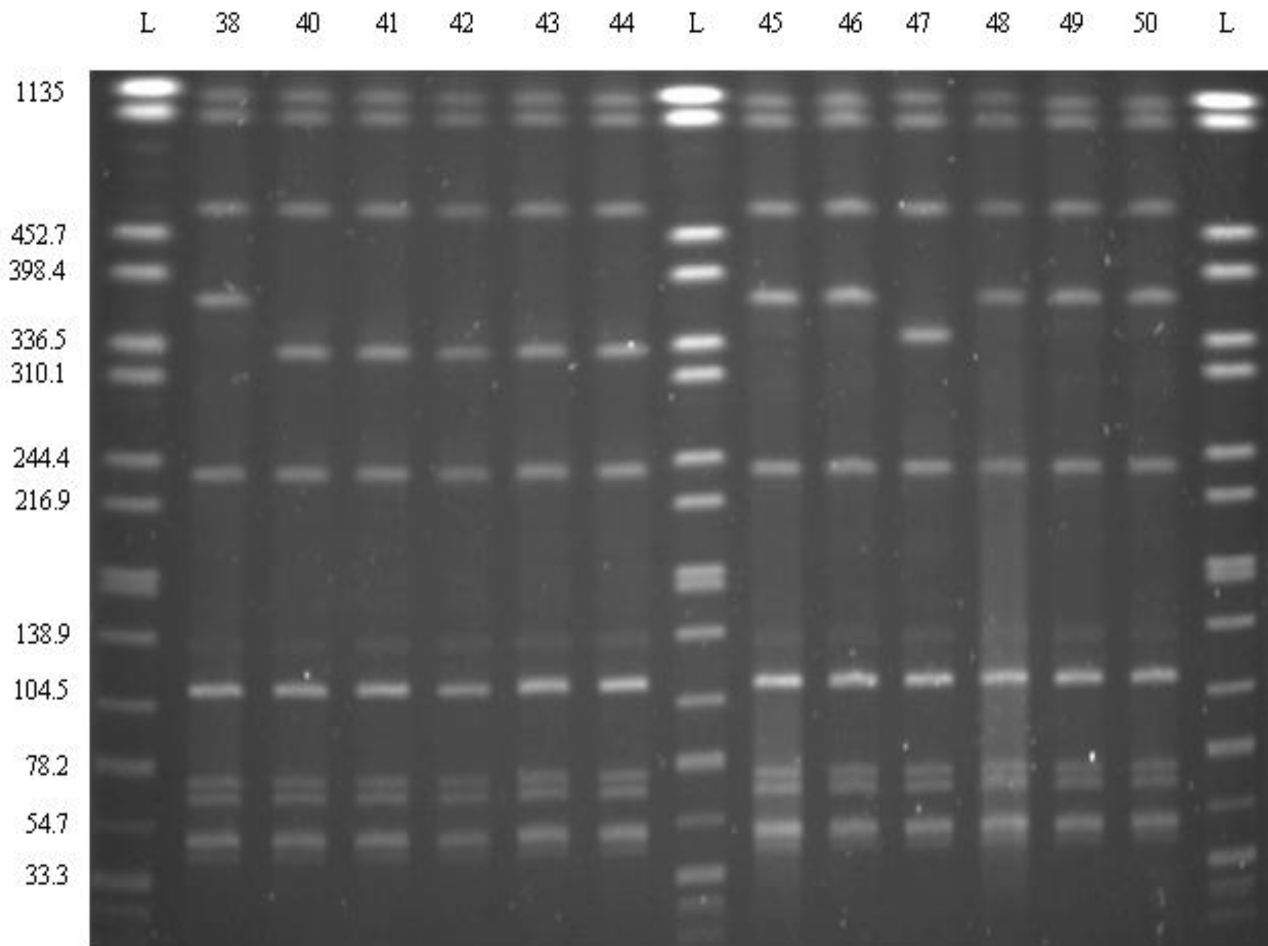

Figure 5: PFGE analysis of *L. monocytogenes* restricted with *AscI* enzyme; lanes labeled with 38, 40-50 represent the molecular patterns generated for different isolates, lanes labeled with L represent the ladder used (*Salmonella* ser. Braenderup BAA 664)

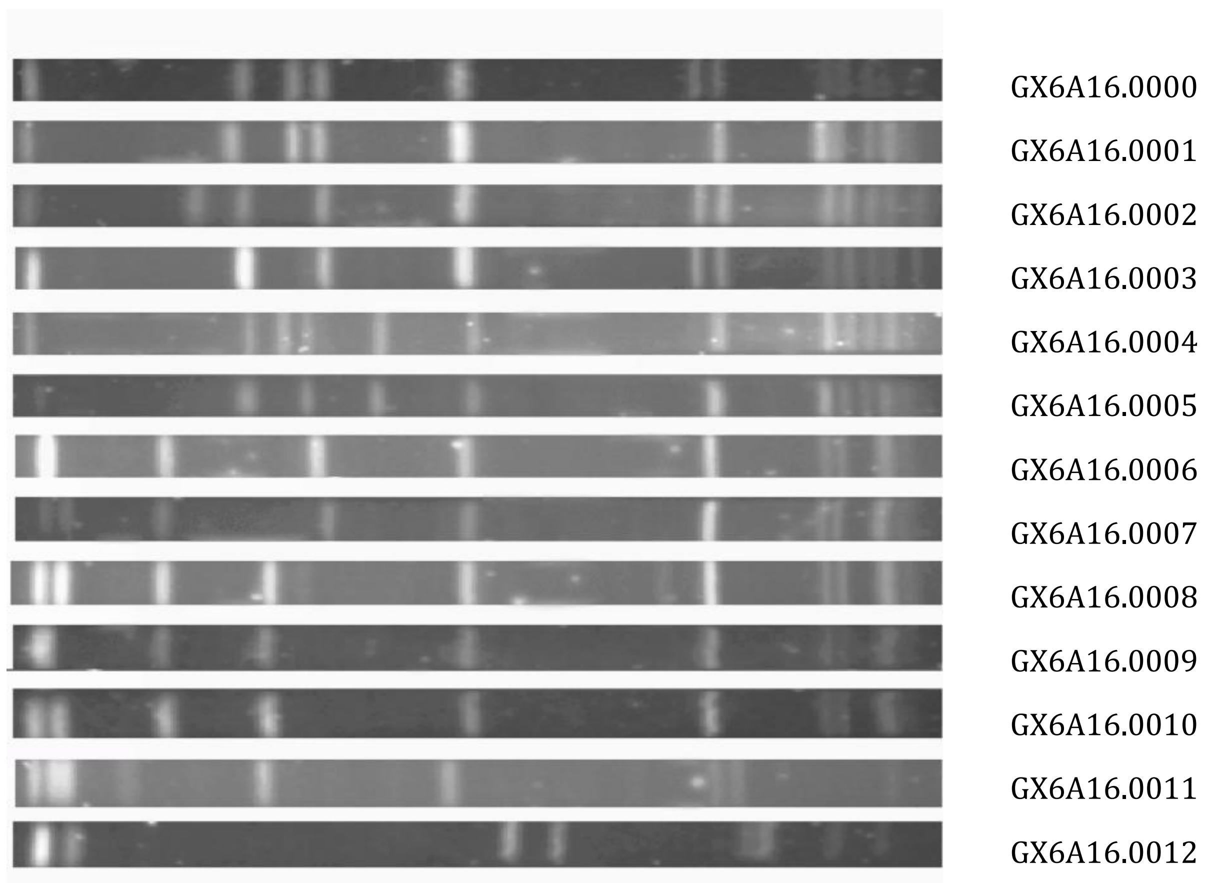

Figure 6: Representative molecular patterns for the different subtypes of *L. monocytogenes* after restriction with *AscI* enzyme

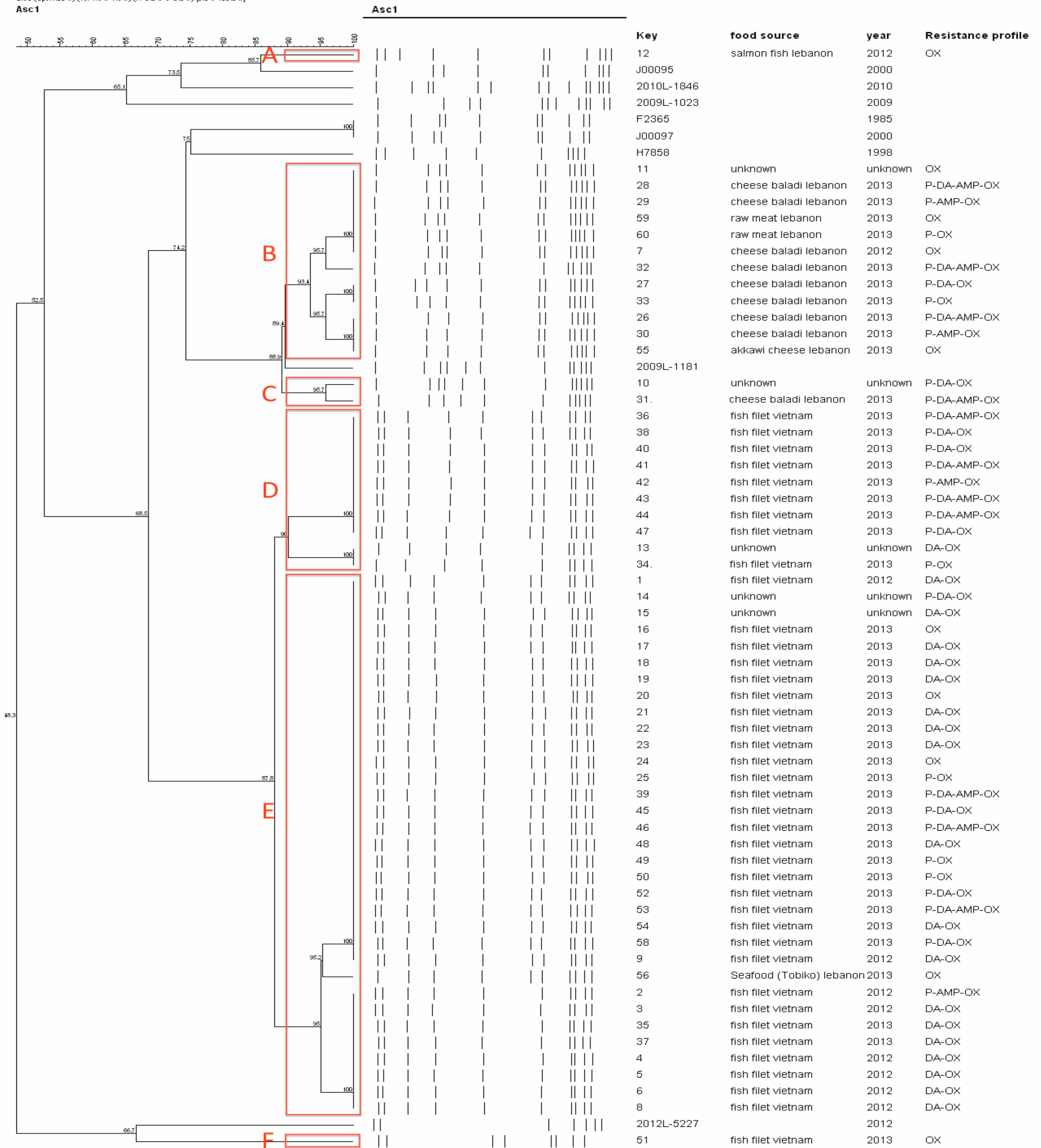

Figure 7: Dendrogram of the PFGE patterns of the 59 *L. monocytogenes* isolates and the 8 CDC controls. *AscI* macrorestriction patterns were analyzed using the Dice coefficient and visualized by unweighted-pair group method, using average linkages with 1% tolerance and 1.5% optimization settings. Clusters are assigned to isolates with 90% relative genomic similarity. The assigned number, the food source, the year of isolation and the antimicrobial susceptibility are included.

CHAPTER V

DISCUSSION

Despite all the developments in food production techniques and the quality control standards practiced worldwide, foodborne diseases are still considered a growing public health concern (104).

According to the Ministry of Public Health Surveillance Unit, the rate of food and water borne diseases in Lebanon is consistently elevated (2579 reported cases in 2013) (105). This can be attributed to several factors such as improper practices and poor hygiene in food production facilities, contaminated raw materials, non-strict microbiological regulations on imported food, and even the improper handling of food by the end consumer (38, 106). Nevertheless, there is a lack of information about the prevalence of *L. monocytogenes* and the occurrence of listeriosis cases in Lebanon. Moreover, this microorganism manifests as mild symptoms in immunocompetent individuals, thus it is not routinely screened for and cases are not reported. However, it can be life threatening for immunocompromised patients; it can cause 20-30% mortality rate and serious complications for pregnant women and their fetuses (9, 57). Consequently, monitoring the prevalence of this microorganism, studying its virulence potential, antimicrobial resistance profiles and prevalence of a single or multiple clonal spread are essential to identify the sources of human infection, understand its pathogenic potential and decide about possible treatment options.

Therefore, to assess the antimicrobial susceptibility, the confirmed *L. monocytogenes* isolates were tested against nine commonly used antimicrobials. All isolates were resistant

to oxacillin, and a relatively high percentage showed resistance to both penicillin and ampicillin. This resistance was more prominent in the Lebanese samples which can be associated with the indiscriminate use and uncontrolled prescription of antimicrobials (107). Antimicrobial resistance against clindamycin was also detected, especially in Vietnamese samples. Resistance to clindamycin in dairy and meat might be attributed to using this antimicrobial in veterinary practices as a growth promoter. Besides, it has been also shown that antimicrobial resistance can be transmitted in fish ponds since animal manure is used as fertilizers in fish farming ponds, the manure shed is usually taken from livestock which was given antimicrobial agents to induce weight gain (108, 109). Antimicrobial resistance has been shown to be acquired by conjugative plasmids and transposons, as well as by mutations in intrinsic chromosomal genes (64, 78-80). Similarly, the antimicrobial resistance of *L. monocytogenes* isolates from dairy products in Lebanon was also highlighted in a previous study showing antimicrobial resistance against penicillin, oxacillin, ampicillin, tetracycline, gentamycin, erythromycin, chloramphenicol and clindamycin (110).

Furthermore, the detection of virulence-associated genes revealed that 58 out of the 59 isolates were positive for all three tested genes, implying their high pathogenic potential and their ability to invade the host cells, survive and spread into adjacent cells.
There was a variation in the band size obtained for the *actA* gene; 94.91% of isolates showed an amplification product of around 950bp instead of the expected 839bp. In fact, a study done by Alho using the same sequence for the *actA* primer showed a similar amplification product and the band size was determined to be 944bp (110). This can be explained by genetic polymorphism for the *actA* gene (111, 112).

In this study, PFGE analysis was performed to assess the clonal relatedness of the *L. monocytogenes* isolates. The results showed that the majority of isolates from the same country assembled in the same cluster at 90% similarity. Indeed, isolates from Lebanese samples, both cheese and raw meat, assembled together in cluster B. While clusters D, E and F were mainly isolates from Vietnamese fish filet. One exception is isolate number 56 which was from Lebanese seafood but showed 95.2% genomic similarity to Vietnamese isolates in cluster E; this can be due to contamination in the seafood plant in Lebanon. This can occur during processing or packaging of the end product as demonstrated by a study done by Autio et al. in Finland (113). Furthermore, raw meat and some of the white (Baladi) cheese isolates showed 100% clonal relatedness and belonged to the same subtype, which might be explained by cross-contamination between dairy and meat products in cattle farms (114). Interestingly, two of the CDC controls used showed a high genomic similarity with the tested isolates. Isolate number 12 from Lebanese salmon fish showed 85.7% similarity with CDC control J0095 that was isolated in 2000 from pies in Germany. In fact, a study done by Chenal-Francisque et al. demonstrated the prevalence of a few frequent clones of *L. monocytogenes* on a worldwide level, which clarifies the high similarity between isolate number 12 from Lebanon and the CDC control from Germany and it also explains why most of the isolates we tested had a high percentage of genetic resemblance (115). Likewise, Control 2009L-1181 of *L. monocytogenes*, which was isolated in 2009 from human blood in USA and causing systemic listeriosis, showed 88.9 - 89.4% genomic similarity to all the isolates from Lebanese cheese and raw meat. This genomic similarity between the *L. monocytogenes* isolate obtained from human blood and the Lebanese cheese and raw meat isolates, emphasizes the possible virulence potential of the latter.

L. monocytogenes is known for its ability to form biofilms on food production machinery which allows it to persist for a long period of time and become a source of contamination (50). Our results showed that isolates from cheese and raw meat were stronger biofilm formers than isolates obtained from fish. This highlights the issue of biofilm formation on utensils and equipment used for dairy and meat processing, especially on stainless steel surfaces (38). Moreover, subtypes from the same cluster had a close biofilm forming ability, which suggests a possible association between the genetic subtype and the amount of biofilm formed. There has been few studies that contradicted this theory but the relationship between biofilm formation and subtypes should be further investigated (116).

The results of this study exposed the virulence ability of *L. monocytogenes* strains present in our Lebanese market and emphasized the need of additional studies and enhanced surveillance to determine the pathogenicity, epidemic potential and the sensitivity to therapeutic agents. In addition, it raises concern about the importance of implementing strict regulations on the microbiological quality and hygiene practices during food processing and better inspection on imported food.

Future work must be done on a larger scale and a more representative selection from different food, as well as on clinical specimens when available.

Besides from the non diversity of samples, another limitation was the restraint on the data related to the samples obtained; information such as in what country were the fish packaged or which samples were brought from the same farm are critical to understand the possible source of contamination.

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