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Original Article

Genotypic and virulence characteristics of *Listeria monocytogenes* recovered from food items in Lebanon

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Abstract

Introduction: *Listeria monocytogenes* is the agent of listeriosis, a life threatening foodborne disease for immunocompromised patients and pregnant women. This bacterium 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 to characterize *L. monocytogenes* from various food products, by assessing the *in vitro* biofilm forming ability, detecting their virulence potential, and characterizing them at the strain level.
Methodology: Fifty-nine isolates were obtained from the Lebanese Agriculture Research Institute (LARI). They were collected in 2012-2013 from local and imported food products in the Lebanese market. Biofilm formation was measured using the Microtiter Plate Assay. PCR amplification was performed for three main virulence genes; *hly*, *actA*, and *inlB*. Pulsed field gel electrophoresis (PFGE) and BIONUMERICS analysis were carried out.
Results: Lebanese isolates from cheese and raw meat showed higher biofilm formation than imported and Lebanese seafood isolates. A total of 100% of the isolates were PCR positive for *hly* and *actA* genes and 98.3% for *inlB* gene. PFGE analysis demonstrated the prevalence of 13 different subtypes with 100% similarity. 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 stresses the importance of enhanced surveillance and the implementation of strict regulations on local and imported food. Future investigations may be conducted on a larger food selection.

Key words: *Listeria monocytogenes*; virulence; molecular characterization; genotyping; foodborne disease.

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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 [1-4]. Even though its incidence is low in comparison to other foodborne diseases, it has one of the highest mortality rates (20-30%) [5].

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 [1,6]. This bacterium is not routinely screened for as it only causes mild symptoms in immunocompetent individual [5,7]. *L. monocytogenes* is widespread in nature, it can survive various harsh conditions, and has developed resistance to many antimicrobials. This could be attributed to biofilm formation as well as to resistance encoding genes [8-13]. Additionally, this opportunistic intracellular pathogen has a genetic composition encoding for various virulence factors, such as 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 [1,14].

Since *L. monocytogenes* is being detected in food products in Lebanon, posing a potential health threat on high-risk groups, the determination of prevalent clones with their virulence potential is of primordial importance.

The aim of this study was to collect *L. monocytogenes* isolates from the Lebanese Agricultural Research Institute (LARI) isolated from food samples at the Lebanese market in order to assess their virulence

potential by detecting the most important virulence encoding genes implicated in the pathogenesis (*inlB*, *hly* and *actA*), as well as to characterize the isolates at the strain level by determining their genomic relatedness and clonality using pulsed field gel electrophoresis (PFGE) analysis, and measure their ability to produce biofilms *in vitro*.

Methodology

Source and identification of L. monocytogenes isolates

Fifty-nine isolates that originate from food products (imported and local) collected during the period of 2012-2013 from the Lebanese market were obtained from the Lebanese Agricultural Research Institute (LARI) and eight control strains of *L. monocytogenes* from different serotypes and sources were obtained from the Centers of Disease Control and Prevention (CDC) (Table 1).

The Ministry of Public Health Epidemiological Surveillance Unit (ESUMOH) served as a link between LARI and our laboratory for provision of the isolates.

The origin and year of isolation of each isolate is indicated in Figure 1.

These isolates were cultured on Brain-Heart infusion agar (LAB M Limited, Bury, United Kingdom) after being received and then stored in Brucella broth (Becton, Dickinson & Co., Sparks USA) with 10% glycerol (Sigma Chemical Co., St. Louis, USA) at -20ºC.

The isolates were confirmed using API Listeria kit (bioMérieux, Marcy L'Etoile, France) and results were analyzed by "Apilab" software (bioMérieux, Marcy L'Etoile, France).

Determination of virulence determinants

DNA was extracted from the isolates and the eight CDC positive controls according to a modified protocol combining a CDC procedure for "Enzyme lysis for clinical specimens of unknown etiology or known Gram-positive cell suspensions (*S. pneumoniae*)" [15] and the illustra bacteria genomic Prep Mini Spin kit (GE Healthcare, Amersham, UK) protocol for purification of genomic DNA from Gram-positive bacteria. Polymerase chain reaction (PCR) for the virulence encoding genes *actA*, *hly*, and *inlB* (Table 2) with respective amplicon sizes of 839, 1590, and 1893 base pair (bp) was performed on the extracted DNA of each isolate.

Pulsed field gel electrophoresis (PFGE)

PFGE was performed according to a modified PulseNet protocol for *L. monocytogenes* [16] on the 59 isolates as well as on the eight CDC controls, with *AscI* as restriction endonuclease (Fermentas, Waltham, USA). Gel Doc XR+ system Machine (Bio-rad,

Table 1. *Listeria monocytogenes* CDC standard 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 2. Oligonucleotide primer sequences for amplification of virulence-associated genes of *L. monocytogenes* isolates.

Gene	Product	Primer	Sequence (5' to 3')	Annealing Temperature (°C)	Size (bp)	Reference
inlB	Internalin B	inlB-F	ATG AAA GAA AAG CAC AAC CC	50	1893	[30]
		inlB-R	TTA TTT CTG TGC CCT TAA AT			
hly	Listeriolysin O	Hly-F	ATG AAA AAA ATA ATG CTA GT	50	1590	[30]
		Hly-R	TTA TTC GAT TGG ATT ATC TA			
actA	Actin-assembly inducing protein precursor	ActA-F	CGC CGC GGA AAT TAA AAA AAGA	60	839	[31]
		ActA-R	ACG AAG GAA CCG GGC TGC TAG			

Hercules, 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 to generate a dendrogram.

Assessment of biofilm formation using the microtiter plate assay

One isolate from each group of identical profiles (100% similarity) according to PFGE results was chosen as a representative for the microtiter plate assay.

One colony of each *L. monocytogenes* isolate was inoculated in BHI broth (LAB M Limited, UK) and incubated overnight at 37ºC. Turbidity of the suspensions was adjusted to 0.5 McFarland using Densimat (Biomerieux, France) and 20 µl of each suspension was diluted with 230 µl of BHI broth and transferred into a 96-well polysterene microplate (Costar 3788, Corning Incorporated, NY) in triplicates. The plate was incubated for 20 hours at 37ºC then the wells were washed three times with sterile distilled water. Afterwards, subsequent steps of staining with Crystal Violet solution and dissolving in ethanol were done according to a previous protocol [17]. Absorbance was measured with BIO-TEK ELx800 Automated Microplate Reader at 630nm. An Unpaired t-test using GraphPad Software (GraphPad Software, Inc., California) was used to determine statistically significant results and p-values were calculated.

The eight CDC strains were used as positive controls, while triplicate wells containing only BHI broth added individually served as negative controls.

Figure 1. 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 and the year of isolation are included.

Results

API identification and polymerase chain reaction

All isolates were identified as *L. monocytogenes*. PCR amplification of the virulence encoding genes showed that *L. monocytogenes* isolates and CDC controls were all positive for *hly* gene. As for the *inlB* gene, all isolates were PCR positive except isolate number 51 from Vietnam. All the isolates were positive for the *actA* gene, however, there were two different sizes of amplification products; some isolates showed a product size of 839 bp (isolates 13, 31, 32), while all the other isolates showed a product size of around 950 bp. Both amplification products indicate the presence of *actA* gene [18].

Pulsed Field Gel Electrophoresis

The PFGE dendrogram generated by the BIONUMERICS analysis consisted of 13 different subtypes. These subtypes constituted 6 distinct clusters determined at a 90% clonal relatedness cut off point (Figure 1). 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. The dendrogram 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

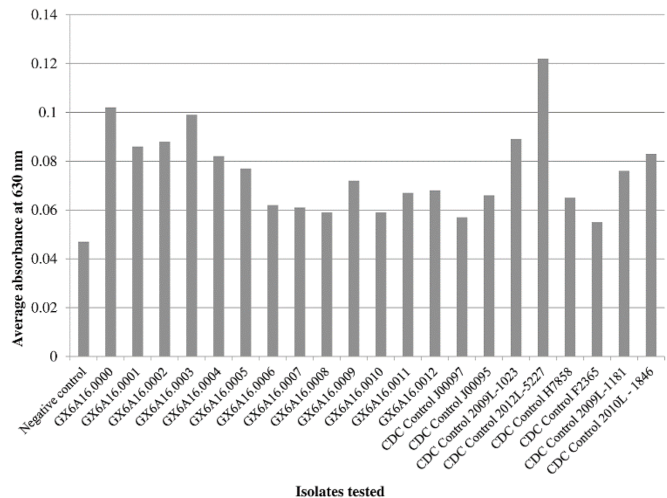
12 (Lebanese salmon fish from cluster A). Additionally, 2009L-1181 had 89.4% genetic similarity with isolates of cluster B (Lebanese cheese and raw meat) (Figure 1).

Assessment of biofilm formation

Figure 2 demonstrates the average absorbance of biofilm for each of the 13 subtypes and 8 CDC controls. Subtypes belonging to the same cluster on the dendrogram had 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.

Results were all found to be statistically significant (p-value < 0.05) using the Unpaired t-test.

Figure 2. Average absorbance of biofilm formation at 630 nm for each of the 13 subtypes and 8 CDC positive controls. All values are statistically significant with p-value < 0.05



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 [19].

According to ESUMOH, the rate of food and water borne diseases in Lebanon is unacceptably high (2579 reported cases in 2013) [20]. 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 [21,22]. 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 [5,7]. Consequently, monitoring the prevalence of this microorganism, studying its virulence potential, and prevalence of a single or multiple clonal spread are of primordial importance.

The detection of virulence-associated genes revealed that 58 out of the 59 isolates harbored all three tested genes. This may imply that if expressed the genes will contribute to the pathogenesis of the isolates by enhancing 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 using the same sequence for the *actA* primer showed a similar amplification product and the band size was determined to be 944bp [18]. This can be explained by genetic polymorphism for the *actA* gene [23,24].

In this study, PFGE analysis was performed to assess the clonal relatedness of the *L. monocytogenes* isolates. The results are interpreted with caution since only 10-12 bands are generated by A*scl*. The dendrogram 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 in Finland [25]. 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 [26]. 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*. [27] 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. 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 [28]. 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 [21]. 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 have been few studies that contradicted this theory but the relationship between biofilm formation and subtypes should be further investigated [29].

Conclusion

The results of this study highlights the virulence ability of *L. monocytogenes* strains present in the Lebanese market and emphasize the need of additional studies and enhanced surveillance to determine the pathogenicity and epidemic potential. In addition, it raises concerns about the importance of implementing strict regulations on the microbiological quality and hygienic 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.

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