Detection of premature stop codons leading to truncated Internalin A among food and clinical strains of *Listeria monocytogenes*

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Introduction

Listeria monocytogenes is a major intracellular bacterial foodborne pathogen capable of causing listeriosis, a severe invasive disease. The elderly, immunocompromised, pregnant women and neonates are risk groups for listeriosis [1]. In developed countries, the globalization of the food industry and the increased consumption of ready-to-eat, particularly chilled foods, has increased listeriosis outbreaks in the last 30 years [2]. Listeriosis was the most frequent cause of death due to the consumption of contaminated food in Europe (2008-2011) [3], and the third foodborne infection concerning illness cost and quality life losses in the USA [4]; mortality is one of the highest among bacterial infections (> 30%) [3]. Although an active surveillance program for listeriosis doesn't exist in Portugal, our research group demonstrated that listeriosis occurs at levels similar to those encountered in other countries [5, 6].

Although 13 serotypes have been identified in *L. monocytogenes*, 95% of the strains isolated from foods and human clinical cases belong to serotypes 1/2a, 1/2b, 1/2c, and 4b [7]. Strains of *L. monocytogenes* can be also distinguished into four genetic lineages (I to IV). The majority of human listeriosis outbreaks have been linked to lineage I serotype 4b isolates, even though some outbreaks have been caused by lineage I serotype 1/2b and lineage II serotype 1/2a isolates [8]. The majority of studies involving clinical isolates characterization have been performed by France and USA research groups, and point out that lineage I isolates are overrepresented among isolates from human listeriosis cases (predominantly serotype 4b), while lineage II isolates appear to be overrepresented among food isolates [9]. As overrepresentation of lineage I strains among human listeriosis cases cannot be attributed to more frequent exposure to these strains it has been hypothesized that lineage I isolates have a higher pathogenicity in comparison to isolates of lineage II.

After the ingestion of contaminated food, *L. monocytogenes* has the ability to induce its own entry into mammalian cells such as macrophages, epithelial cells and endothelial cells of the gastrointestinal tract [10]. Internalin A protein (InlA) encoded by *inlA* has a key role in *L. monocytogenes* infection mechanism. InlA facilitates uptake of *L. monocytogenes* by host cells expressing certain isoforms of E-cadherin, making the interaction between InlA and E-cadherin a critical first step for crossing the intestinal barrier during the initial stages of infection, in a process called internalization. A previous study characterized a set of paired isogenic mutants with and without an *inlA* premature stop codons (PMSC) using an intragastric guinea pig infection model demonstrate that inlA PMSC mutations appear to be causally associated with attenuated mammalian virulence [11]. *L. monocytogenes* isolates that carry a PMSC in *inlA* produce a truncated form of InlA that is secreted rather than anchored to the bacterial cell wall and demonstrate attenuated invasion of Caco-2 human intestinal epithelial cells. [12, 13]. A number of studies suggests that lineages differ in their virulence characteristics; lineage II in particular including a considerable proportion of virulence-attenuated isolates (>30%) due to premature stop codons in *inlA*, while serotypes 4b and 1/2b isolates typically harbor a full length intact InlA [8, 9].

While there is substantial information on *L. monocytogenes* genetic diversity and epidemic potential, additional studies may reveal additional diversity and contribute to understanding on how *L. monocytogenes* subtypes differ in their virulence potential. This study aimed to characterize 22 *L. monocytogenes* strains, collected in Portugal, from human clinical cases (n=11) and different food products (n=11) in terms of invasion efficiency in Caco-2 cell line and detection of virulence-attenuated mutations in *inlA* gene.

Methods

1. *Listeria monocytogenes* strains

For this study a set of 22 bacterial isolates from food and clinical origin collected in Portugal between were selected from the *L. monocytogenes* culture collection of Escola Superior de Biotecnologia.

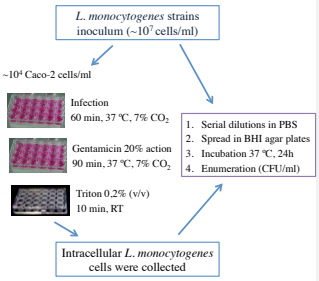
Table 1. Description of *L. monocytogenes* strains

Strain Code	Origin	Sample	Geno-Serogroup[a]
747	Food	Cheese	IIb
842	Food	Plant environment	IIc
854	Food	Lettuce salad	IVb
903	Food	Roast chicken	IVb
925/3	Food	Goat cheese	IIb
930/1	Food	Goat milk	IIb
994/1	Food	Goat milk	IIa
1044	Food	Meal	IVb
1216	Food	Raw chicken	IIb
1305	Food	Sausage	IIc
2652	Food	Cheese	IVb
1001	Clinical	Blood	IVb
1547	Clinical	Blood	IIb
1761	Clinical	CSF	IIb
1891	Clinical	CSF	IIb
2065	Clinical	Blood	IIb
2074	Clinical	Blood	IVb
2086	Clinical	Blood	IIa
2092	Clinical	Blood	IVb
2103	Clinical	Blood	IIa
2562	Clinical	Blood	IVb
2666	Clinical	Blood	IVb

[a] Detection of serotype-specific marker genes was performed using a multiplex-PCR assay previously reported by Doumith et al. [13]. This assay differentiates isolates in five major subtypes, which each represents more than one serotype, including (i) geno-serogroup IIa (which includes serotypes 1/2a and 3a); (ii) geno-serogroup IIb (serotypes 1/2b, 3b, and 7); (iii) genoserogroup IIc (serotypes 1/2c and 3c); and (iv) genoserogroup IVb (serotypes 4b, 4d, and 4e).

2. *Caco-2* invasion assays

For invasion assays in the human intestinal epithelial caco-2 cell line a laboratory control strain presenting a full-length InlA (FSL F8-146) and a strain presenting an *inlA* PMSC (FSL F8-144) (previoully reported by Ferreira et al. [15]) were included as controls in each invasion assay. At least three independent invasion assays were performed for each isolate. Results were reported as percent invasion efficiency (i.e., bacterial numbers recovered/bacterial numbers inoculated), normalized to the values obtained for the positive control strain (set as 100%).



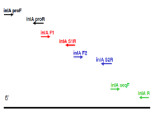
3. *inlA* sequencing

L. monocytogenes strains were screened for the presence of PMSCs in *inlA*, which encodes a protein critical for invasion of Caco-2 cells. Primer sequences, PCR conditions, and sequencing strategies have previously been detailed [16].

DNA extraction

1.Grow of 1 colony on BHI agar, at 37 ºC, overnight
2.Centrifugation (10.000 g, 10 min) of 250 μL of bacterial cells
3.Pellet resuspention in 95 μL of 1xPCR buffer and 4 μL of 50 mg/mL lysozyme solution
4.Incubation at RT, 15 min
5.Addiction of 1 μL of 20 mg/mL proteinase K, 58 ºC, 60 min
6.Enzymes heat inactivation , 95 ºC, 10 min

PCR amplification of *inlA*



Four pairs of primers:
- *inlA* proF/*inlA* proR,
- *inlA* F1/*inlA* S1R
- *inlA* F2/*inlA* S2R
- *inlA* seqF/*inlA* R

KAPA HiFi DNA Polymerase

PCR product Purification - EzWay PCR Clean-Up Kit

DNA Sequencing - Macrogen Inc., Netherlands

Sequence analysis - Geneious 4.8.2 program; Mega 5.05.

Results

Prevalence of PMSC in *L. monocytogenes* strains

Table 2 shows the 3 different mutation types leading to PMSC detected in five *L. monocytogenes* strains recovered from food and clinical sources, belonging to different geno-serogroups. The remaining seventeen strains tested did not show PMSC in *inlA*.

Table 2. Characterization of *L. monocytogenes* strains with PMSC.

Strains	Origin	Geno-serogroup	Mutation type [b]	Predicted truncated InlA (aa)	Invasion efficiency (%)
1216	Food	1/2b	6	492	0.9
1761	Clinical	1/2a	6	492	9.5
1305	Food	1/2c	11	685	1.4
747	Food	1/2b	11	685	54.3
842	Food	1/2c	12	576	1.4

[b] Seventeen mutations leading to PMSC in *inlA* have been detected between 1998 and 2010. In this study mutations have been classified according to categorization proposed by Van Stelten et al. [18].

Invasion efficiency in *L. monocytogenes* strains with PMSC mutations in inlA show reduced invasion efficiency in Caco-2

The invasion efficiency in Caco-2 cell of the strains with PMSC were evaluated and compared with the positive control (FSL F8-146), 100% efficient.

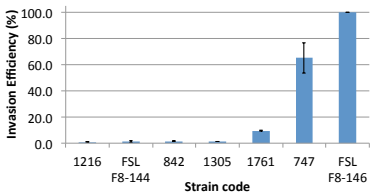


Figure 1. Caco-2 average invasion efficiency for selected *L. monocytogenes* strains, normalized to that of the control strain FSL F8-146 (full length *inlA* strain).

From the 22 *L. monocytogenes* strains characterized only five presented PMSC in *inlA*, namely one clinical isolate (geno-serogroup IIa, lineage II), and four food isolates (two genoserogroup IIb isolates, lineage I, and two geno-serogroup IIc, lineage II). These mutation types have been previously described in isolates from France, Portugal and USA [12, 15, 17].

Phenotypically, it was possible to relate the presence of PMSCs with invasion efficiencies below that of the positive control FSL F8-146, that shows a full-length InlA.

Conclusions

Mutations leading to a PMSC in *inlA* are responsible for attenuated mammalian virulence. In this study, as previously observed by other authors, PMSC mutations in *inlA* were more common in food isolates (4 /11) and underrepresented in clinical isolates (1/11), despite the relatively low number of strains analyzed. Previous molecular epidemiology studies reported that a significant proportion of *L. monocytogenes* strains isolated from food products carry PMSC mutations in *inlA*, indicating that there is a significant subpopulation of *L. monocytogenes* strains in the food supply with a limited ability to cause human disease. Taken together, in addition to the significance of these findings for food safety and public health, these data contribute to our understanding of the ecology and evolution of *L. monocytogenes*. Future efforts to elucidate the selective advantages associated with the production of a truncated and secreted InlA protein may provide a better understanding of the function of *L. monocytogenes* internalins in different hosts and nonhost environments.

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