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Original Article

Characterization of epidemic clones of *Listeria monocytogenes* serotype 4b isolated from humans and meat products in Brazil

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Abstract

Introduction: *Listeria monocytogenes* is an important foodborne pathogen and the 4b serotype is responsible for many cases of human listeriosis reported in Brazil. Several listeriosis outbreaks worldwide have involved a small number of well-defined clonal groups, designated as epidemic clones (ECs).

Methodology: We studied 71 strains of serotype 4b, including 25 isolates from human cases of listeriosis and 46 from meat-based foods, collected in Brazil between 1977 and 2010. The presence of ECs (I and II) markers and virulence genes (*inlA, inlB, ilnC, inlJ* and *actA*) were evaluated by PCR assay. The genetic relationship of ECs-positive strains was assessed by pulsed field gel electrophoresis.

Results: ECI and ECII markers were found both in human and food strains, with 19.7% positive for the ECI marker and 40.8% for ECII. Most strains (97.2%) were positive for the virulence genes that were studied. Nevertheless, the *actA* gene amplicons showed two distinct sizes, with all ECI positive strains exhibiting a 105bp deletion. Pulsed field gel electrophoresis (PFGE) analysis allowed the recognition of highly related strains, particularly from two outbreaks of neonatal listeriosis in São Paulo State occurred in 1992 and 1997, both ECII-positive; and two ECI strains from a human case (1982) and from bovine meat (2009).

Conclusions: The presence of ECs among clinical samples and beef isolates of serotype 4b from some regions of Brazil highlights the need for rigorous control of production procedures. Furthermore, the association of ECII with two nosocomial outbreaks suggests its ability to spread in these settings.

Key words: *Listeria monocytogenes*; serotype 4b; epidemic clones; virulence, epidemiology.

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Introduction

Listeria monocytogenes, a common environmental bacterium, has been recognized as an important foodborne pathogen. Due to its opportunistic nature, listeriosis constitutes a life-threatening disease in immunocompromised or elderly patients, and can also lead to serious outcomes in pregnant women. Two disease presentations can be observed: a non-invasive febrile gastroenteritis and a severe invasive form which may lead to sepsis, meningoencephalitis, abortion and still-birth [1]. In order to survive and multiply in the host cells, *L. monocytogenes* expresses a set of several virulence factors. The invasion of non phagocytic cells is induced by the attachment of bacterial surface proteins, called internalins, on specific receptors of the host cell [2-4]. After invading, *L. monocytogenes* escapes from the endocytic vacuole through the secretion of listeriolisin O (LLO) and phospholipases, and spreads by the cytoplasm, with the support of the ActA protein, an essential process for cell-to-cell movement and propagation in the host [2].

Outbreaks associated with the consumption of a broad range of foods can result when food-processing plants are persistently contaminated or when such food products are stored for a long time prior to consumption [5]. Several factors can influence the degree of contamination, including cross-contamination, lack of hygienic measures by food handlers, and processing parameters. *Listeria* is able to grow at low temperatures; thus, conditions at the retail level, transport, and home storage may allow the pathogen growth to hazardous levels. [6].

Most human infections are caused by serotypes 1/2a, 1/2b and 4b, with predominance of 4b in the majority of the outbreaks and sporadic cases [7,8]. Furthermore, a small number of well-defined clonal groups, designated as epidemic clones (ECs), is involved in different temporally and geographically unrelated outbreaks [9]. Three ECs were recognized among serotype 4b strains: ECI, a cosmopolitan clone associated with several outbreaks worldwide; ECII, that was first detected in two multistate outbreaks in the USA (1998-99 and 2002) and ECIV (previously identified as ECIa) linked to few other food outbreaks. Furthermore, ECIII, a common epidemic clone isolated in food, is usually related to 1/2a serotype [10,11].

A rapid and low cost multiplex PCR assay was developed for presumptive detection of major *L. monocytogenes* epidemic clones [12]. The clonal-specific associated genetic regions were defined after genome comparison among several EC strains, displaying a high sensitivity and specificity. However, it has been shown that within an EC, subpopulations associated with outbreaks exhibit genetic differences [13]. The use of methodologies capable of discriminating ECs subpopulations are of great value in screening for routes by which the clones are transmitted to food and, therefore, to individuals. Combined with epidemiological investigations, PFGE typing can be a valuable tool for recognizing common source outbreaks [8].

In Brazil listeriosis is not a disease of compulsory notification and there are few data on the epidemiology and prevalence of the species in our country. Nevertheless, previous studies described a high ocurrence of 4b serotype in human and foods specimens [14-18]. In an extensive study, Hofer *et al.* [14] observed the prevalence of the 4b serotype in meat products and fish from different sources in Brazil, followed by 1/2a and 1/2b serotypes. Due to the importance of the 4b serotype in the epidemiological scenario of listeriosis, the aim of this study was to evaluate the presence of major epidemic clones (ECI and ECII), virulence markers and genetic relatedness among a selected group of *L. monocytogenes* serotype 4b strains isolated from human listeriosis and meat-based foods in Brazil.

Methodology

Bacterial strains

The collection in this study consisted of 71 serotype 4b *L. monocytogenes* obtained from the *Listeria* sp. Collection (CLIST) from Instituto Oswaldo Cruz (Fiocruz, Brazil), 25 of them isolated from human infections between 1977 and 2010, and 46 recovered from meat products from 2006 to 2010 (Table 1). The strains came from different parts of Brazil, from public and private institutions, in order to establish a conclusive laboratory diagnosis, or were isolated by the Laboratório de Zoonoses Bacterianas (Instituto Oswaldo Cruz, Brazil) in the lab's various investigations. All strains underwent biochemical testing [19] and serotyping [20] for confirmation purposes. Antigenic characterization was performed through the use of somatic and flagellar antisera. All strains were maintained at -20°C in brain heart infusion (BHI) broth with 20% glycerol.

PCR and sequencing assays

Strains were streaked in tryptose agar plates and incubated for 18-24 hours at 37 °C. Total DNA was extracted using the DNeasy Blood & Tissuekit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The PCR assays in a final volume of 50 µl were performed using the following: 1X reaction buffer, $MgCl_2$ 1.5 mM, 0,4 mM of each dNTP, 0.6 pmol/µl of each primer and 0.5 U/µl of HotStarTaq polymerase (Qiagen, Hilden, Germany). The detection of ECs markers (ECI e ECII) and search

Table 1. Profile of *L. monocytogenes* strains of serotype 4b from human and food origin, isolated in Brazil.

Decade	Nº of strains / source									
	Human					Food				
	N	FAE	BLO	CSF	LYM	N	UBM	PBM	PPM	USM
1970	2	1	1	-	-	-	-	-	-	-
1980	6	-	4	1	-	-	-	-	-	-
1990	11	-	5	5	1	-	-	-	-	-
2000	4	-	1	3	-	44	26	13	3	2
2010	3	-	-	3	-	2	1	-	-	1
Total	25	1	11	12	1	46	27	13	3	3

Humans - FAE: faeces; BLO: blood; CSF: cerebrospinal fluid; LYM: lymphnode fluid; Food - UBM: unprocessed bovine meat; PBM: processed bovine meat; PPM: processed poultry meat; USM: unprocessed bovine meat.

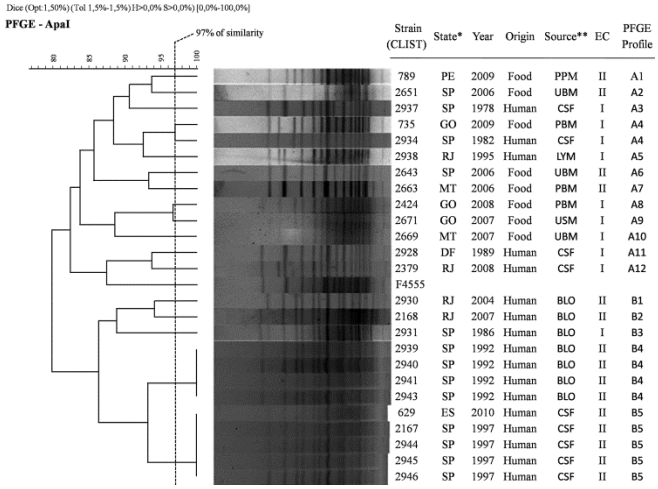
for virulence genes *actA, inlA, inlB*, *inlC* and *inlJ*, was performed as previously described and the set of primers is summarized in Table 2. *L. monocytogenes* serotype 4b strains ATCC19115 and CDCF4555 were used as positive controls for the virulence genes, and *L. innocua* ATCC 12612 strain as negative control.

Additionally, to confirm PCR specificity, the sequencing of the *actA* gene fragment was performed in a selected group of 29 strains, considering the year, origins, source of isolation and presence of ECs markers. The BigDye Terminator v3.1 Cycle Sequencing Kit, with the same primers as those of the PCR assay, was used according to the manufacturer's instructions. Sequencing was conducted in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA) automated sequencer at Centro de Pesquisa Aggeu Magalhães (CpqAM / Fiocruz, Pernambuco, Brazil).

Molecular characterization by PFGE

PFGE was performed only with isolates presenting the ECI and/or ECII markers. A total of 26 strains, 18 from human origin and eight from food (considering the year and region of isolation), were analyzed according to the *L. monocytogenes* Pulse Net protocol (http://www.cdc.gov/pulsenet) using *Apa*I and *Asc*I restriction enzymes (New England BioLabs, Beverly, USA). *Salmonella enterica* serotype Braenderup H9812 (Courtesy of Laboratório de Enterobactérias / Fiocruz, Rio de Janeiro, Brazil) digested with the *Xba*I enzyme (New England BioLabs, Beverly, MA) was used as a molecular-weight size marker. Cluster analysis and dendrogram construction were performed using BioNumerics software version 4.0 (Applied Maths, Belgium), using the UPGMA method and the Dice coefficient with a 1.5% tolerance index. A minimum of 97% similarity was established to define a particular clone profile, as proposed by Félix *et al*. [21]. The Simpson diversity index was used to evaluate the discriminatory ability of PFGE [22].

Figure 1. Dendrogram by UPGMA method with Dice coefficient and tolerance index of 1.0%. The PFGE profiles of 27 strains of *Listeria monocytogenes* obtained with the enzyme ApaI.



*State – DF: Distrito Federal; ES: Espírito Santo; GO: Goiás; MT: Mato Grosso; PE: Pernambuco; RJ: Rio de Janeiro; SP: São Paulo. **Source –FAE: faeces; BLO: blood; CSF: cerebrospinal fluid; LYM: lymphnode fluid;UBM: unprocessed bovine meat; PBM: processed bovine meat; PPM: processed poultry meat; USM: unprocessed bovine meat. ***Not performed.

Table 2. Primers, amplicon size and references of PCR assays to genetic markers searched in this study.

Target	Nucleotide sequence (5'→3')	Determinant	Size (bp)	Reference
ORF2110	F = AGTGGACAATTGATTGGTGAA R = CATCCATCCCTTACTTTGGAC	Serotype 4b	597	10
LMOf2365_2798	F = AATAGAAATAAGCGGAAGTGT R = TTATTTCCTGTCGGCTTAG	Epidemic clone I (ECI)	303	10
LMOh7858_0487.8	F = ATTATGCCAAGTGGTTACGGA R = ATCTGTTTGCGAGACCGTGTC	Epidemic clone II (ECII)	889	10
inlA	F = ACTATCTAGTAACACGATTAGTGA R = CAAATTTGTTAAAATCCCAAG TGG	Internalin A	250	20
inlB	F = AAGCACAACCCAAGAAGGAA R = AAAATTCCACTCATGCCCAC	Internalin B	1107	21
actA	F = GGTACGTGATAAAATCGACGA R = TAGTTATGTCACTTATCAGAGC	Inducing actin polymerization	724/619	35
inlC	F = AATTCCCACAGGACACAACC R = CGGGAATGCAATTTTTCACTA	Internalin C	517	22
inlJ	F = TGTAACCCCGCTTACACAGTT R = AGCGGCTTGGCAGTCTAATA	Internalin J	238	22

Table 3. A summary of epidemiological features of *L. monocytogenes* serotype 4b isolates positives for ECI and ECII markers.

Strains	Year	State*	Origin	Source**	EC	PFGE profile
2938	1995	RJ	Human	LYM	ECI	A1
735	2009	GO	Food	PBM	ECI	A2
789	2009	PE	Food	PPM	ECII	A3
2379	2008	RJ	Human	CSF	ECI	A4
2928	1989	DF	Human	CSF	ECI	A5
2424	2008	GO	Food	PBM	ECI	A6
2671	2007	GO	Food	USM	ECI	A7
2934	1982	SP	Human	CSF	ECI	A8
2651	2006	SP	Food	UBM	ECII	A9
2663	2006	MT	Food	PBM	ECII	A10
2643	2006	SP	Food	UBM	ECII	A11
2669	2007	MT	Food	UBM	ECI	A12
2937	1978	SP	Human	CSF	ECI	A13
2168	2007	RJ	Human	BLO	ECII	B1
2930	2004	RJ	Human	BLO	ECII	B2
2931	1986	SP	Human	BLO	ECI	B3
2939	1992	SP	Human	BLO	ECII	B4
2940	1992	SP	Human	BLO	ECII	B4
2941	1992	SP	Human	BLO	ECII	B4
2943	1992	SP	Human	BLO	ECII	B4
2167	1997	SP	Human	CSF	ECII	B5
2944	1997	SP	Human	CSF	ECII	B5
2945	1997	SP	Human	CSF	ECII	B5
2946	1997	SP	Human	CSF	ECII	B5
629	2010	ES	Human	CSF	ECII	B5
2641	2006	SP	Food	UBM	ECII	NP***
2642	2006	SP	Food	UBM	ECII	NP
2644	2006	SP	Food	UBM	ECII	NP
2645	2006	SP	Food	UBM	ECII	NP
2646	2006	SP	Food	UBM	ECII	NP
2647	2006	SP	Food	UBM	ECII	NP
2648	2006	SP	Food	UBM	ECII	NP
2649	2006	SP	Food	UBM	ECII	NP
2650	2006	SP	Food	UBM	ECII	NP
2652	2006	SP	Food	UBM	ECII	NP
2661	2006	MT	Food	PBM	ECII	NP
2662	2006	MT	Food	PBM	ECII	NP
2664	2006	MT	Food	PBM	ECII	NP
2668	2006	MT	Food	UBM	ECII	NP
2670	2007	GO	Food	USM	ECI	NP
2420	2008	GO	Food	PBM	ECI	NP
2422	2008	GO	Food	PBM	ECI	NP
2426	2008	GO	Food	PBM	ECI	NP

*State – DF: Distrito Federal; ES: Espírito Santo; GO: Goiás; MT: Mato Grosso; PE: Pernambuco; RJ: Rio de Janeiro; SP: São Paulo. **Source –FAE: faeces; BLO: blood; CSF: cerebrospinal fluid; LYM: lymphnode fluid;UBM: unprocessed bovine meat; PBM: processed bovine meat; PPM: processed poultry meat; USM: unprocessed bovine meat. ***Not performed.

Results

Detection of epidemic clones and virulence genes

The ECII marker prevailed both in strains from human (46%) and from food (39%) origin when compared to the ECI marker (27% and 17.5%, respectively). The distribution of epidemic clones by origin, source, region and year of isolation are shown in Table 3. In addition, most strains (97.2%) showed all virulence genes studied, except for two strains (CLIST 2651 and CLIST 2105) from bovine meat, negative for the *inlA* gene. All ECI positive strains showed the amplicon of the *actA* gene smaller than the remaining strains and sequencing revealed a 105bp deletion within these fragments.

PFGE analysis of the epidemic clones

PFGE typing with the *Apa*I enzyme revealed a genetic similarity of ≥80% among most isolates. The strains were grouped into two clusters (Figure 1). Cluster A grouped strains from human and food origins with 12 clonal profiles. On the other hand, cluster B, with five clonal profiles, grouped only human strains. The Simpson index showed a satisfactory discriminatory power (D = 0.950).

An ECII-positive group (CLIST2939, 2940, 2941, 2943) of neonatal outbreak strains isolated in the state of Sao Paulo in 1992 was found to belong to the same clone (profile B4). Furthermore, a group of human strains (CLIST 2167, 2944, 2945, 2946), isolated from another hospital in the state of São Paulo in 1997, and a human strain isolated from the state of Espírito Santo in 2010 (CLIST 629), presented the same profile (B5), and it was closely related (≅93% of similarity) to the B4 profile. Some other strains showed related restriction profiles (Figure 1); for example, the ECI human strain of 1982 from the state of São Paulo (CLIST 2934) and the ECI strain isolated in 2009 from processed beef in the state of Goiás (CLIST 735), showed the same A4 profile.

For confirmation, the related strains of profiles B4 and B5 were digested with the *Asc*I enzyme, showing an identical restriction profile, although a few bands were observed (data not shown).

Discussion

In this study, positive strains for epidemic clones I and II were detected in a group of *L. monocytogenes* of serotype 4b isolated from human and food sources in Brazil in the last four decades, suggesting that these clones are present in the Brazilian food distribution chain. These findings are reinforced by other studies, which describe the frequency and distribution of epidemic clones [25-29], indicating their wide distribution and importance in the epidemiology of *L. monocytogenes* in the world.

Hofer *et al*. [15], evaluated the distribution and frequency of *Listeria* species and serotypes from different areas of Brazil, isolated from various sources of infection and transmission vehicles during the period from 1971 to 1997, totaling 3,112 strains. The authors found that the occurence of *L. monocytogenes* serotypes was usually limited to 4b, 1/2a and 1/2b, with prevalence of 4b in isolates from human and meat/fish sources. Similar results were observed in a posterior study with human clinical specimens [18]. Recent studies also described the high occurence of 4b serotype in dairy products [17, 30], ready-to-eat vegetables [31] and meat products retailed [6] in Brazil. Although the wide distribution of serotype 4b has been described in Brazil, details about the occurrence of ECs and your association with sporadic cases or outbreaks is ignored.

A recent work evaluated the presence of major ECs in 4b serotype isolates from sporadic cases of listeriosis in the United States (2003 to 2008), identifying ECI, ECII and ECIa, in 32%, 17% and 7% of total isolates, respectively, with an overall prevalence of 57% of these clonal groups [32]. Similarly, another recent study in India identified ECI in most 4b-serotype isolates by multi-virulence-locus sequence typing technique (MVLST), a multi-locus sequence type (MLST) variation [33]. In comparison, our study showed a higher occurrence of ECII compared to ECI, both in human and food samples. These findings highlight the presence of ECs, but due the limited number of strains, its not possible to determine the prevalent epidemic clone in a particular region or food in Brazil. Extensive investigations are needed to elucidate the epidemiology of epidemic clones in our country.

The fact that most strains presented all virulence markers investigated, as expected, confirms the risk of spread by food contamination of this 4b serotype in Brazil. A previous study evaluated the virulence potential of 4b and 1/2b serotypes isolated from vegetable salad samples in São Paulo (Brazil), by searching for the *inlA*, *inlC* and *inlJ* genes, finding similar results [31].

The variation observed in *actA* gene has been described in previous studies, and its association with a decrease in virulence has been suggested [28,34,35]. This change leads to a deletion of 35 amino acids in the ActA protein, that represent two proline-rich regions (PRR) required for binding to the WASP

(Wiskott–Aldrich syndrome protein) and Mena proteins, essential steps to triggering actin-based cell mobility in the host cell cytoplasm. Nevertheless, *in vitro* and *in vivo* assays showed that the invasion and multiplication ability of bacteria apparently is not affected by this deletion [27,32,34].

Moreover, Chen *et al.* [27] recognized the alteration in seven strains of serotype 4b and observed that all ECI-positive strains presented this deletion. These results agree with our study, emphasizing that changes in the *actA* gene appear to be characteristic of ECI isolates and can be used as a marker in differentiating between other epidemic clones. Although this deletion causes a significant change in the structure of the protein product, it is evident that it does not interpose with its virulence, because ECI isolates are involved in many sporadic cases and outbreaks of human listeriosis in the world [36].

Some studies have also discussed the relevance of the analysis of polymorphism in the *actA* gene for typing of *L. monocytogenes* samples. Zhou *et al.* [37] characterized food isolates through the partial sequencing of the *actA* gene, separating them into two lineages (I and II), suggesting that the use of this polymorphic gene analysis may be an easier and alternative typing method than MLST and PFGE. Similar results were obtained by Bania *et al*. [38].

EC-positive strains presented different PFGE profiles, but showed a genetic similarity of $\geq 80\%$, although the ECI and ECII strains were not separated into distinct clusters, regardless of year or origin. In the present study, some strains were isolated more than 30 years ago and it is recognized that the occurrence of random genetic events over time can change the macrorestriction patterns. Thus, *L. monocytogenes* strains of the same epidemic clone sometimes do not exhibit an identical pulsotype [38,39]. However, we identified two related outbreaks of neonatal listeriosis associated to ECII, suggesting the ability of this clone to spread in nosocomial environments. Although nosocomial listeriosis has been described in neonates [40], the association with ECII is unknown.

Conclusion

We demonstrated the presence of ECs markers in several isolates of *L. monocytogenes* serotype 4b from human samples and meat-based food in some regions of Brazil. This finding alerts the risk of these vehicles for the transmission of bacteria and reinforces the relevance of PCR as a presumptive and rapid method for detection of these clones. Furthermore, the association of ECII with two hospital outbreaks suggest its ability to spread in nosocomial environments.

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