Molecular epidemiology of *Listeria monocytogenes* isolated from different sources in Brazil

Epidemiologia molecular de *Listeria monocytogenes* isoladas de diferentes fontes no Brasil

Andrea Micke MORENO [1]; Renata PAIXÃO[2]; Luisa Zanolli MORENO[4]; Débora Dirani Sena de GOBBI[1]; Daniele Cristine RAIMUNDO[1]; Thais Sebastiana Porfida FERREIRA[1]; Ernesto HOFER [3]; Maria Helena MATTE[4]; Marina MORENO[1]

[1]Departamento de Medicina Veterinária Preventiva e Saúde Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, São Paulo - SP, Brasil
[2]Faculdade de Medicina Veterinária da Faculdades Metropolitanas Unidas, São Paulo – SP, Brasil
[3]Laboratório de Zoonoses Bacterianas da Fundação Instituto Oswaldo Cruz, Rio de Janeiro – RJ, Brazil
[4] Laboratório de Saúde Pública da Faculdade de Saúde Pública da Universidade de São Paulo, São Paulo – SP, Brasil

Abstract

Listeria monocytogenes is an important foodborne pathogen that primarily affects pregnant women, neonates, the elderly and immune-compromised individuals, and it may cause abortion, septicemia, and meningitis. From the 13 capsular groups described, serotypes 4b, 1/2b and 1/2a are most closely related to human infection. For this reason, serotyping has limited value as an epidemiological tool; thus, improved discriminatory typing methods are required to enhance knowledge of *L. monocytogenes* contamination and infection. The aim of this study was to characterize the genetic diversity of *L. monocytogenes* isolates in the pork processing industry in Sao Paulo, Brazil and human infection isolates by ERIC-PCR and single enzyme AFLP. Serotypes 1/2c and 4b were frequent among isolates from pork and slaughterhouse/market environments, whereas serotypes 4b and 1/2a were observed among human isolates. ERIC-PCR and AFLP revealed 34 and 31 distinct profiles, respectively, which had tendencies of separation according to serogroup and isolate origin. The genetic profiles from slaughterhouse and market environments suggest the possibility of different sources of *Listeria* contamination in the environment, although in certain cases, continuous contamination caused by the persistence of clonal strains is also a possibility.

Keywords: *Listeria monocytogenes*. Public health. ERIC-PCR. AFLP.

Resumo

Listeria monocytogenes é um importante patógeno de origem alimentar que afeta principalmente grávidas, neonatos, idosos e indivíduos imunocomprometidos, e pode causar abortamento, septicemia e meningite. Dos 13 grupos capsulares descritos, os sorotipos 4b, 1/2b e 1/2a são os mais relacionados à infecção humana. Por esta razão, a sorotipagem possui valor limitado como ferramenta epidemiológica e, dessa forma, métodos mais discriminatórios são necessários para melhorar o conhecimento sobre a contaminação e a infecção por *L. monocytogenes*. O objetivo deste estudo foi caracterizar a diversidade genética de isolados de *L. monocytogenes* da indústria de processamento de carne suína no Estado de São Paulo, Brasil, e compará-los a isolados de casos de infecção humana através do ERIC-PCR e AFLP com uma única enzima. Os sorotipos 1/2c e 4b foram frequentes em carne suína e ambientes de abatedouros e mercados, enquanto os sorotipos 4b e 1/2a foram observados nos isolados de humanos. ERIC-PCR e AFLP resultaram em 34 e 31 perfis distintos, respectivamente, com uma tendência a separar de acordo com o sorogrupo e a origem do isolado. Os perfis genéticos de ambiente dos abatedouros e mercados sugerem a possibilidade de diferentes origens de contaminação por *Listeria* nos ambientes estudados, porém, em alguns casos, é possível que ocorra a persistência de cepas clonais causando contaminação contínua.

Palavras-chave: *Listeria monocytogenes*. Saúde pública. ERIC-PCR. AFLP.

Introduction

Listeria monocytogenes has been considered an important foodborne pathogen since the 1980s when the bacterium was suspected to be responsible for serious widespread outbreaks of human listeriosis in

Correspondence to:
Andrea Micke Moreno
Laboratório de Sanidade Suína e Virologia – FMVZ/USP
Av. Prof. Dr. Orlando Marques de Paiva, 87, Cidade Universitária
CEP: 05508 270 - São Paulo/ SP – Brazil.
e-mail: morenoam@usp.br

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North America and Europe[1,2]. *L. monocytogenes* is a non-sporulated, gram-positive rod that can infect humans and animals[3] and is considered a troublesome agent for the food industry due to its ability to grow at low temperatures, adhere to various contact surfaces, and adapt to disinfectants[4]. The major clinical symptoms of human listeriosis are abortion, septicemia, and meningitis, and it primarily affects pregnant women, neonates, the elderly and immune-compromised individuals[1].

Thirteen serotypes have been described for *L. monocytogenes* based on capsular antigens; serotypes 4b, 1/2b and 1/2a are most frequently related to human infection[1,5], with serotype 4b associated with epidemic outbreaks and serotypes 1/2a and 1/2b related to sporadic infections[6]. There have been reports of meningitis, abortion, neonatal infection and diarrhea caused by *L. monocytogenes* in different states of Brazil[7,8]. The serotypes isolated in the Brazilian territory from 1969 to 2000 were 4b, 1/2b, 1/2a, 4a, 1/2c and 3a[9].

Listeria monocytogenes has been isolated from foods such as milk, dairy products, vegetables, sliced vacuum-packaged meat products, chicken, pork, fish, raw and processed meat, as well as the environment of the food processing industry, from the slaughterhouse to the processing and packaging rooms[1,10,11,12]. In Europe, the epidemiologic link between environmental, animal, food and human samples has already been established using molecular typing methods to compare samples from different origins[10,11].

In Brazil, *L. monocytogenes* has been isolated from dairy products, sausages, salami, chicken, shrimp and animal carcasses[13,14,15,16,17]. In the last two decades, a few studies in Brazil have compared isolates from food, animals and the food processing environment, employing molecular typing methods to find a possible epidemiological relationship between the sources[16,18], although no study has compared these strains with human isolates. Therefore, the aim of this study was to characterize the genetic diversity of *L. monocytogenes* isolates from the pork processing industry in São Paulo, Brazil, and human infection isolates by Enterobacterial Repetitive Intergenic Consensus Sequences (ERIC-PCR) and single enzyme Amplified Fragment Length Polymorphism (AFLP).

Material and Methods

Bacterial strains

A total of 90 *L. monocytogenes* isolates were used in this study, 80 obtained from two slaughterhouse environments, pork pieces, and two meat markets located in São Paulo, Brazil, and 10 isolates from human infection. The environmental and pork samples originated from a total of 12 collections obtained between 2007 and 2008 in two lines of the pork processing industry (a slaughterhouse and its respective meat market) in São Paulo. Bacteria were isolated from swabs of slaughterhouse and market environment locations (wall, table, or floor) and pork fragments. The isolates were maintained at -80°C and stock medium was maintained at 24°C until analysis. The isolates were reactivated from the stock medium in ALOA® medium (Biolife – Milano, Italy) at 37°C for 48 hours. Colonies displaying characteristics of *L. monocytogenes* were maintained in BHI broth (Difco-BBL, Detroit, MI, USA) at 37°C for 48 hours for DNA extraction.

Serotyping

Serotyping was performed according to Seeliger and Höehne[19], based on the detection of somatic and flagellar polyclonal antigens by the Laboratory of Bacterial Zoonosis, Institute Oswaldo Cruz/ FIOCRUZ, Rio de Janeiro, Brazil.

DNA isolation and PCR amplification

Purified DNA was recovered using a DNA extraction protocol by Boom and Salimans[20] following

enzymatic treatment for 60 minutes at 37°C with 10 µg of lysozime (USBiological, Swampscott, MA, USA) and 400 µg of proteinase K (LGC Biotecnologia, São Paulo, Brazil); the purified DNA was subsequently stored at –20ºC. The strains were identified by PCR based on species-specific amplification of the *hly* gene, as described by Border et al.[21]. PCR was performed with 5 µl of extracted DNA, 50 pmol of each primer, 4 mM of $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 1 U of Taq DNA polymerase, 1X PCR buffer and water to a final volume of 25 µl. PCR was carried out with an initial denaturation at 94ºC for 4 min, followed by 35 cycles consisting of denaturation for 1.5 min at 94°C, annealing for 1.5 min at 50°C, and extension for 2 min at 72°C, and a final extension of 72ºC for 5 min. The amplified products were stained with BlueGreen® (LGC Biotecnologia, São Paulo, Brazil) and separated by electrophoresis in a 1.5% agarose gel. A 100 bp DNA ladder was used for molecular weight determinations.

Enterobacterial Repetitive Intergenic Consensus Sequences (ERIC-PCR)

ERIC-PCR was performed as previously described by Versalovic, Koeuth and Lupski[22] using 5 µl of the extracted DNA, 1.5 mM of $MgCl_2$, 10 pmol of ERIC1 (ATGTAAGCTCCTGGGGATTCAC) and ERIC2 (AAGTAAGTGACTGGGGTGAGCG) primers, 1.0 U of Taq DNA polymerase, 1X PCR buffer and water to a total volume of 50 µl. The PCR reaction was carried out for 35 cycles, consisting of denaturation for 4 min at 94°C, annealing for 60 sec at 50°C, and extension for 2.5 min at 72°C, with a final extension of 20 min at 72°C, as described by Rafiee et al.[23]. The amplification products were detected through electrophoresis at 57 V for 4 h in 2% UltraPure™ Agarose 1000 (Invitrogen Corporation, Carlsbad, CA, USA) gel, stained with Sybr Safe® (Invitrogen Corporation, Carlsbad, CA, USA). The 1 Kb Plus DNA Ladder® (Invitrogen Corporation, Carlsbad, CA, USA) was used as a molecular weight marker.

Amplified Fragment Length Polymorphism (AFLP)

Restriction endonuclease digestion and ligation was performed using a modification of a described method[24]. To 10 µL of the extracted DNA, 24 U of *Hind* III (Invitrogen Corporation, Carlsbad, CA, USA) was added and brought to a final volume of 20 µL with ultra-pure water; this reaction was incubated overnight at 37ºC. An aliquot of digested DNA (5 µL) was added to 0.2 µg of the adapter ADH1 and ADH2 oligonucleotides, along with 1 U of T4 DNA ligase (Invitrogen Corporation, Carlsbad, CA, USA) and ultra-pure water to a final volume of 20 µL; the reaction was incubated at room temperature for 3 hours. The ligated DNA was heated to 80ºC for 10 min, and 5 µL was used for each PCR reaction. The PCR reactions were performed in 50 µL volumes, each containing 5 µL of ligated DNA, 2.5 mM $MgCl_2$, 30 pmol of primer (HI-G), and 1 U of Taq polymerase in 1X PCR buffer. The mixture was subjected to an initial denaturing step of 94ºC for 4 min, followed by 35 cycles consisting of 1 min at 94ºC, 1 min at 60ºC, and 2.5 min at 72ºC. The base sequences of the adapter (ADH1 e ADH2) and selective primer (HI-G) were as follows: ADH1 - ACGGTATGCGACAG, ADH2 – AGCTCTGTCGCATA CCGTGAG, HI-G - GGTATGCGACAGAGCTTG. The electrophoresis was conducted on a 1.5% agarose gel at 22 V for 24 h. The amplified products were visualized with Blue Green® (LGC Biotecnologia, São Paulo, Brazil) staining and were compared to a 1 Kb Plus DNA Ladder® (Invitrogen Corporation, Carlsbad, CA, USA)

Data analysis

ERIC-PCR and AFLP results were analyzed using the Dice coefficient by means of Bionumerics 6.6 software (Applied Maths NV, Saint-Martens-Latem, Belgium) to generate the dendrogram. The discriminatory index was calculated according to the method described by Hunter and Gaston[25].

Results

Serotyping

Serotype 4b was the most commonly isolated (37.7%), followed by 1/2c (31.1%), 1/2b (20.0%) and 1/2a (11.1%). Among isolates from pork and slaughterhouse/market environments, serotypes 4b and 1/2c were the most present, and serotypes 4b and 1/2a were more common among human isolates (Table 1).

ERIC-PCR analysis

All 90 *L. monocytogenes* isolates were confirmed by PCR using primers for the *hly* gene by the presence of a 174 bp band fragment, as described by Border et al.[21]. All isolates were also examined and characterized with the ERIC-PCR technique. A visual comparison of the banding patterns revealed 34 distinct ERIC profiles, with DNA fragments ranging in sizes from 200 to 3000 bp. ERIC patterns usually consisted of six to nine DNA fragments. The dendrogram (Figure 1) resulted in two main groups, with 65% similarity. Group I comprised 37 isolates (profiles 1 to 12) from the market and slaughterhouses collected at different time points, and six human strains with serotypes 4b and 1/2b. Group II comprised 43 isolates, divided into 13 profiles, mainly with serotypes 1/2c, 1/2a, 1/2b and 4b, originating from human, market and slaughterhouse environments.

AFLP analysis

All *L. monocytogenes* isolates were examined and characterized using AFLP. A visual comparison of the banding patterns revealed 31 distinct AFLP patterns, generally consisting of 9 to 17 DNA fragments (Figure 2). The resulting dendrogram formed two main groups (I, II) with 39% similarity. Group I was composed of profiles 1 to 5, with isolates from slaughterhouses collected at different time points, mostly of serotype 1/2c. Group II can be divided into three subgroups. Subgroup A consisted of AFLP patterns 6 to 12 and was predominantly of the slaughterhouse origin and serotype 1/2c. Subgroup B consisted of AFLP patterns 13 to 22 and was predominantly serotype 1/2a and 1/2b, with strains from slaughterhouse, market and human environments. Subgroup C consisted of AFLP patterns 23 to 31; the most frequent serotype was 4b, followed by 1/2b, with strains from slaughterhouse, market and human environments. In both groups B and C, there were human isolates dispersed with more than 90% similarity to environmental isolates from the slaughterhouse and the market.

Discriminatory power

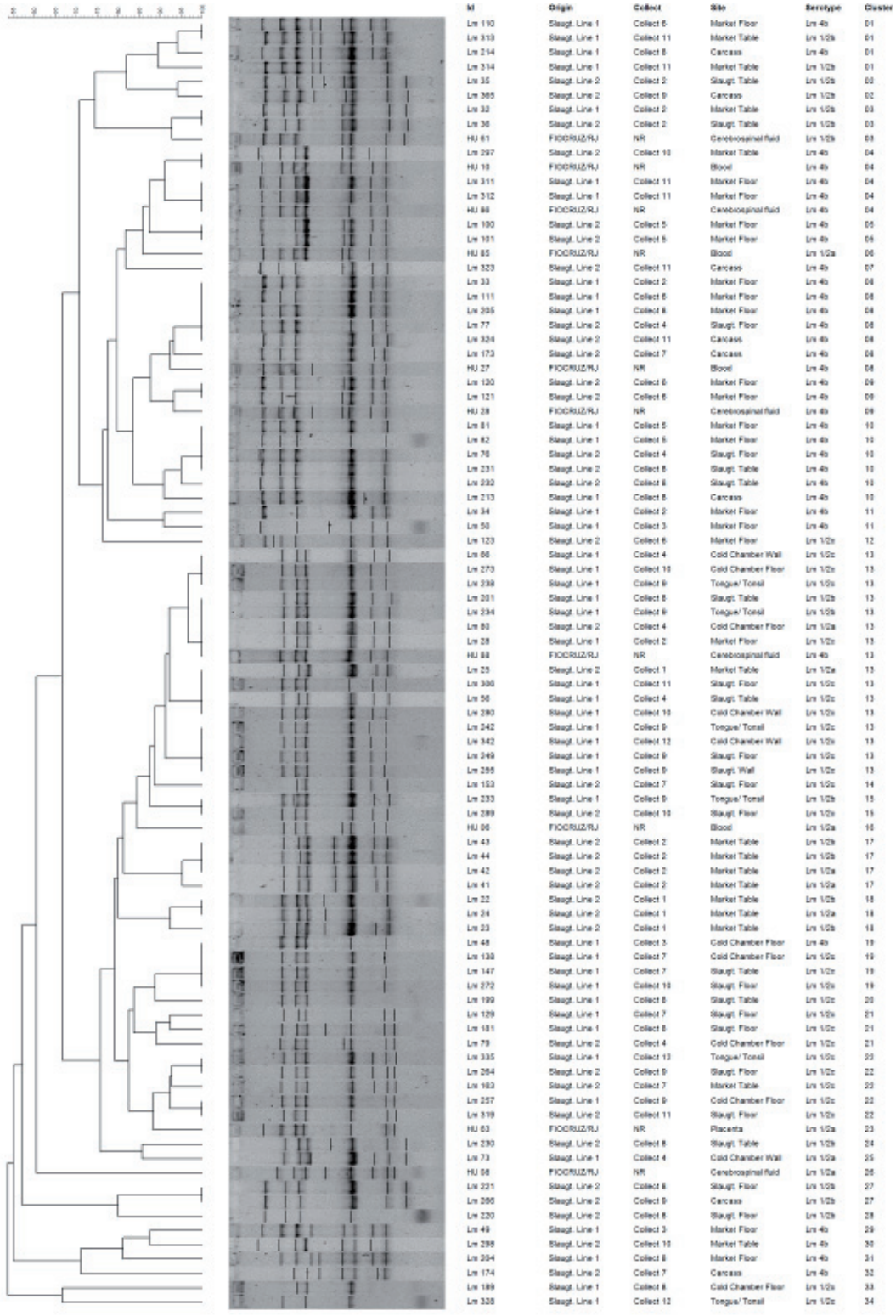
The discriminatory indexes obtained for serotyping, ERIC-PCR and single enzyme AFLP were 0.75, 0.94 and 0.95, respectively.

Discussion

All serotypes isolated in the present study (1/2a, 1/2b, 1/2c and 4b) are considered to be among the most prevalent *L. monocytogenes* serotypes isolated from clinical infection and meat processing environments[1,3]. For this reason, serotyping has limited value as an epidemiological tool, and the low discriminatory index computed supports this fact. Therefore, improved discriminatory typing methods are required to enhance knowledge regarding

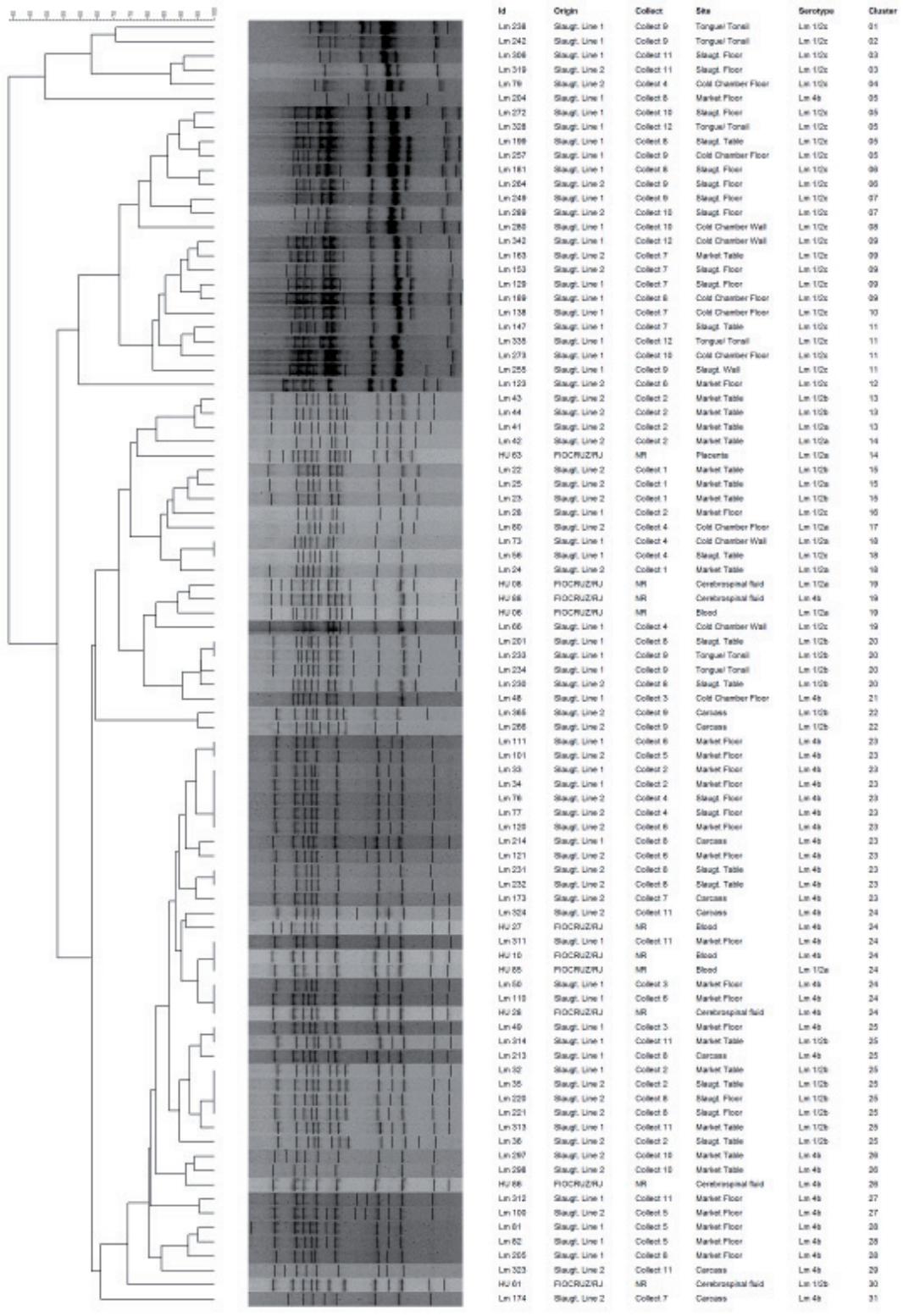
Table 1 - Frequency of *Listeria monocytogenes* serotypes in swine slaugtherhouses, pork samples and human cases – São Paulo - 2012

Sample	Serotype				
	1/2a	1/2b	1/2c	4b	Total
Environment/pork	6 (7.5%)	17 (21.25%)	28 (35%)	29 (36.25%)	80 (100%)
Human	4 (40.00%)	1 (10.00%)	-	5 (50.00%)	10 (100%)
Total	10 (11.1%)	18 (20%)	28 (31.1%)	34 (37.7%)	90 (100%)



Id	Origin	Collect	Site	Serotype	Cluster
Lm 110	Slaugt. Line 1	Collect 5	Market Floor	Lm 4b	01
Lm 313	Slaugt. Line 1	Collect 11	Market Table	Lm 1/2b	01
Lm 214	Slaugt. Line 1	Collect 8	Carcass	Lm 4b	01
Lm 314	Slaugt. Line 1	Collect 11	Market Table	Lm 1/2b	01
Lm 35	Slaugt. Line 2	Collect 2	Slaugt. Table	Lm 1/2b	02
Lm 365	Slaugt. Line 2	Collect 9	Carcass	Lm 1/2b	02
Lm 32	Slaugt. Line 1	Collect 2	Market Table	Lm 1/2b	03
Lm 36	Slaugt. Line 2	Collect 2	Slaugt. Table	Lm 1/2b	03
HU 61	FIOCRUZ/RJ	NR	Cerebrospinal fluid	Lm 1/2b	03
Lm 297	Slaugt. Line 2	Collect 10	Market Table	Lm 4b	04
HU 10	FIOCRUZ/RJ	NR	Blood	Lm 4b	04
Lm 311	Slaugt. Line 1	Collect 11	Market Floor	Lm 4b	04
Lm 312	Slaugt. Line 1	Collect 11	Market Floor	Lm 4b	04
HU 88	FIOCRUZ/RJ	NR	Cerebrospinal fluid	Lm 4b	04
Lm 100	Slaugt. Line 2	Collect 5	Market Floor	Lm 4b	05
Lm 101	Slaugt. Line 2	Collect 5	Market Floor	Lm 4b	05
HU 85	FIOCRUZ/RJ	NR	Blood	Lm 1/2a	06
Lm 323	Slaugt. Line 2	Collect 11	Carcass	Lm 4b	07
Lm 33	Slaugt. Line 1	Collect 2	Market Floor	Lm 4b	08
Lm 111	Slaugt. Line 1	Collect 5	Market Floor	Lm 4b	08
Lm 205	Slaugt. Line 1	Collect 8	Market Floor	Lm 4b	08
Lm 77	Slaugt. Line 2	Collect 4	Slaugt. Floor	Lm 4b	08
Lm 324	Slaugt. Line 2	Collect 11	Carcass	Lm 4b	08
Lm 173	Slaugt. Line 2	Collect 7	Carcass	Lm 4b	08
HU 27	FIOCRUZ/RJ	NR	Blood	Lm 4b	08
Lm 120	Slaugt. Line 2	Collect 6	Market Floor	Lm 4b	09
Lm 121	Slaugt. Line 2	Collect 6	Market Floor	Lm 4b	09
HU 28	FIOCRUZ/RJ	NR	Cerebrospinal fluid	Lm 4b	09
Lm 81	Slaugt. Line 1	Collect 5	Market Floor	Lm 4b	10
Lm 82	Slaugt. Line 1	Collect 5	Market Floor	Lm 4b	10
Lm 76	Slaugt. Line 2	Collect 4	Slaugt. Floor	Lm 4b	10
Lm 231	Slaugt. Line 2	Collect 8	Slaugt. Table	Lm 4b	10
Lm 232	Slaugt. Line 2	Collect 8	Slaugt. Table	Lm 4b	10
Lm 213	Slaugt. Line 1	Collect 8	Carcass	Lm 4b	10
Lm 34	Slaugt. Line 1	Collect 2	Market Floor	Lm 4b	11
Lm 50	Slaugt. Line 1	Collect 3	Market Floor	Lm 4b	11
Lm 123	Slaugt. Line 2	Collect 6	Market Floor	Lm 1/2c	12
Lm 66	Slaugt. Line 1	Collect 4	Cold Chamber Wall	Lm 1/2c	13
Lm 273	Slaugt. Line 1	Collect 10	Cold Chamber Floor	Lm 1/2c	13
Lm 238	Slaugt. Line 1	Collect 9	Tongue/ Tonsil	Lm 1/2c	13
Lm 201	Slaugt. Line 1	Collect 8	Slaugt. Table	Lm 1/2b	13
Lm 234	Slaugt. Line 1	Collect 9	Tongue/ Tonsil	Lm 1/2b	13
Lm 80	Slaugt. Line 2	Collect 4	Cold Chamber Floor	Lm 1/2a	13
Lm 28	Slaugt. Line 1	Collect 2	Market Floor	Lm 1/2c	13
HU 88	FIOCRUZ/RJ	NR	Cerebrospinal fluid	Lm 4b	13
Lm 25	Slaugt. Line 2	Collect 1	Market Table	Lm 1/2a	13
Lm 306	Slaugt. Line 1	Collect 11	Slaugt. Floor	Lm 1/2c	13
Lm 56	Slaugt. Line 1	Collect 4	Slaugt. Table	Lm 1/2c	13
Lm 280	Slaugt. Line 1	Collect 10	Cold Chamber Wall	Lm 1/2c	13
Lm 242	Slaugt. Line 1	Collect 9	Tongue/ Tonsil	Lm 1/2c	13
Lm 342	Slaugt. Line 1	Collect 12	Cold Chamber Wall	Lm 1/2c	13
Lm 249	Slaugt. Line 1	Collect 9	Slaugt. Floor	Lm 1/2c	13
Lm 255	Slaugt. Line 1	Collect 9	Slaugt. Wall	Lm 1/2c	13
Lm 153	Slaugt. Line 2	Collect 7	Slaugt. Floor	Lm 1/2c	14
Lm 233	Slaugt. Line 1	Collect 9	Tongue/ Tonsil	Lm 1/2b	15
Lm 289	Slaugt. Line 2	Collect 10	Slaugt. Floor	Lm 1/2c	15
HU 86	FIOCRUZ/RJ	NR	Blood	Lm 1/2a	16
Lm 43	Slaugt. Line 2	Collect 2	Market Table	Lm 1/2b	17
Lm 44	Slaugt. Line 2	Collect 2	Market Table	Lm 1/2b	17
Lm 42	Slaugt. Line 2	Collect 2	Market Table	Lm 1/2a	17
Lm 41	Slaugt. Line 2	Collect 2	Market Table	Lm 1/2a	17
Lm 22	Slaugt. Line 2	Collect 1	Market Table	Lm 1/2b	18
Lm 24	Slaugt. Line 2	Collect 1	Market Table	Lm 1/2a	18
Lm 23	Slaugt. Line 2	Collect 1	Market Table	Lm 1/2b	18
Lm 48	Slaugt. Line 1	Collect 3	Cold Chamber Floor	Lm 4b	19
Lm 138	Slaugt. Line 1	Collect 7	Cold Chamber Floor	Lm 1/2c	19
Lm 147	Slaugt. Line 1	Collect 7	Slaugt. Table	Lm 1/2c	19
Lm 272	Slaugt. Line 1	Collect 10	Slaugt. Floor	Lm 1/2c	19
Lm 199	Slaugt. Line 1	Collect 8	Slaugt. Table	Lm 1/2c	20
Lm 129	Slaugt. Line 1	Collect 7	Slaugt. Floor	Lm 1/2c	21
Lm 181	Slaugt. Line 1	Collect 8	Slaugt. Floor	Lm 1/2c	21
Lm 79	Slaugt. Line 2	Collect 4	Cold Chamber Floor	Lm 1/2c	21
Lm 335	Slaugt. Line 1	Collect 12	Tongue/ Tonsil	Lm 1/2c	22
Lm 264	Slaugt. Line 2	Collect 9	Slaugt. Floor	Lm 1/2c	22
Lm 163	Slaugt. Line 2	Collect 7	Market Table	Lm 1/2c	22
Lm 257	Slaugt. Line 1	Collect 9	Cold Chamber Floor	Lm 1/2c	22
Lm 319	Slaugt. Line 2	Collect 11	Slaugt. Floor	Lm 1/2c	22
HU 83	FIOCRUZ/RJ	NR	Placenta	Lm 1/2a	23
Lm 230	Slaugt. Line 2	Collect 8	Slaugt. Table	Lm 1/2b	24
Lm 73	Slaugt. Line 1	Collect 4	Cold Chamber Wall	Lm 1/2a	25
HU 58	FIOCRUZ/RJ	NR	Cerebrospinal fluid	Lm 1/2a	26
Lm 221	Slaugt. Line 2	Collect 8	Slaugt. Floor	Lm 1/2b	27
Lm 266	Slaugt. Line 2	Collect 9	Carcass	Lm 1/2b	27
Lm 220	Slaugt. Line 2	Collect 8	Slaugt. Floor	Lm 1/2b	28
Lm 49	Slaugt. Line 1	Collect 3	Market Floor	Lm 4b	29
Lm 298	Slaugt. Line 2	Collect 10	Market Table	Lm 4b	30
Lm 204	Slaugt. Line 1	Collect 8	Market Floor	Lm 4b	31
Lm 174	Slaugt. Line 2	Collect 7	Carcass	Lm 4b	32
Lm 189	Slaugt. Line 1	Collect 8	Cold Chamber Floor	Lm 1/2c	33
Lm 328	Slaugt. Line 1	Collect 12	Tongue/ Tonsil	Lm 1/2c	34

Figure 1 - Dendrogram showing comparison of *Listeria monocytogenes* strains from human cases and swine slaughterhouses through ERIC-PCR



Id	Origin	Collect	Site	Serotype	Cluster
Lm 238	Slaugt. Line 1	Collect 9	Tongue/ Tonsil	Lm 1/2c	01
Lm 242	Slaugt. Line 1	Collect 9	Tongue/ Tonsil	Lm 1/2c	02
Lm 366	Slaugt. Line 1	Collect 11	Slaugt. Floor	Lm 1/2c	03
Lm 319	Slaugt. Line 2	Collect 11	Slaugt. Floor	Lm 1/2c	03
Lm 76	Slaugt. Line 2	Collect 4	Cold Chamber Floor	Lm 1/2c	04
Lm 264	Slaugt. Line 1	Collect 8	Market Floor	Lm 4b	05
Lm 272	Slaugt. Line 1	Collect 10	Slaugt. Floor	Lm 1/2c	05
Lm 328	Slaugt. Line 1	Collect 12	Tongue/ Tonsil	Lm 1/2c	05
Lm 189	Slaugt. Line 1	Collect 8	Slaugt. Table	Lm 1/2c	05
Lm 257	Slaugt. Line 1	Collect 9	Cold Chamber Floor	Lm 1/2c	05
Lm 181	Slaugt. Line 1	Collect 8	Slaugt. Floor	Lm 1/2c	06
Lm 264	Slaugt. Line 2	Collect 9	Slaugt. Floor	Lm 1/2c	06
Lm 249	Slaugt. Line 1	Collect 9	Slaugt. Floor	Lm 1/2c	07
Lm 289	Slaugt. Line 2	Collect 10	Slaugt. Floor	Lm 1/2c	07
Lm 280	Slaugt. Line 1	Collect 10	Cold Chamber Wall	Lm 1/2c	08
Lm 342	Slaugt. Line 1	Collect 12	Cold Chamber Wall	Lm 1/2c	09
Lm 163	Slaugt. Line 2	Collect 7	Market Table	Lm 1/2c	09
Lm 153	Slaugt. Line 2	Collect 7	Slaugt. Floor	Lm 1/2c	09
Lm 129	Slaugt. Line 1	Collect 7	Slaugt. Floor	Lm 1/2c	09
Lm 169	Slaugt. Line 1	Collect 8	Cold Chamber Floor	Lm 1/2c	09
Lm 138	Slaugt. Line 1	Collect 7	Cold Chamber Floor	Lm 1/2c	10
Lm 147	Slaugt. Line 1	Collect 7	Slaugt. Table	Lm 1/2c	11
Lm 338	Slaugt. Line 1	Collect 12	Tongue/ Tonsil	Lm 1/2c	11
Lm 279	Slaugt. Line 1	Collect 10	Cold Chamber Floor	Lm 1/2c	11
Lm 255	Slaugt. Line 1	Collect 9	Slaugt. Wall	Lm 1/2c	11
Lm 123	Slaugt. Line 2	Collect 6	Market Floor	Lm 1/2c	12
Lm 43	Slaugt. Line 2	Collect 2	Market Table	Lm 1/2b	13
Lm 44	Slaugt. Line 2	Collect 2	Market Table	Lm 1/2b	13
Lm 41	Slaugt. Line 2	Collect 2	Market Table	Lm 1/2a	13
Lm 42	Slaugt. Line 2	Collect 2	Market Table	Lm 1/2a	14
HU 63	FIOCRUZ/RJ	MR	Placenta	Lm 1/2a	14
Lm 22	Slaugt. Line 2	Collect 1	Market Table	Lm 1/2b	15
Lm 25	Slaugt. Line 2	Collect 1	Market Table	Lm 1/2a	15
Lm 23	Slaugt. Line 2	Collect 1	Market Table	Lm 1/2b	15
Lm 28	Slaugt. Line 1	Collect 2	Market Floor	Lm 1/2c	16
Lm 60	Slaugt. Line 2	Collect 4	Cold Chamber Floor	Lm 1/2a	17
Lm 73	Slaugt. Line 1	Collect 4	Cold Chamber Wall	Lm 1/2a	18
Lm 56	Slaugt. Line 1	Collect 4	Slaugt. Table	Lm 1/2c	18
Lm 24	Slaugt. Line 2	Collect 1	Market Table	Lm 1/2a	19
HU 08	FIOCRUZ/RJ	MR	Cerebrospinal fluid	Lm 1/2a	19
HU 68	FIOCRUZ/RJ	MR	Cerebrospinal fluid	Lm 4b	19
HU 06	FIOCRUZ/RJ	MR	Blood	Lm 1/2a	19
Lm 66	Slaugt. Line 1	Collect 4	Cold Chamber Wall	Lm 1/2c	19
Lm 201	Slaugt. Line 1	Collect 8	Slaugt. Table	Lm 1/2b	20
Lm 233	Slaugt. Line 1	Collect 9	Tongue/ Tonsil	Lm 1/2b	20
Lm 234	Slaugt. Line 1	Collect 9	Tongue/ Tonsil	Lm 1/2b	20
Lm 238	Slaugt. Line 2	Collect 8	Slaugt. Table	Lm 1/2b	20
Lm 48	Slaugt. Line 1	Collect 3	Cold Chamber Floor	Lm 4b	21
Lm 365	Slaugt. Line 2	Collect 9	Carcass	Lm 1/2b	22
Lm 268	Slaugt. Line 2	Collect 9	Carcass	Lm 1/2b	22
Lm 111	Slaugt. Line 1	Collect 6	Market Floor	Lm 4b	23
Lm 101	Slaugt. Line 2	Collect 5	Market Floor	Lm 4b	23
Lm 33	Slaugt. Line 1	Collect 2	Market Floor	Lm 4b	23
Lm 34	Slaugt. Line 1	Collect 2	Market Floor	Lm 4b	23
Lm 76	Slaugt. Line 2	Collect 4	Slaugt. Floor	Lm 4b	23
Lm 77	Slaugt. Line 2	Collect 4	Slaugt. Floor	Lm 4b	23
Lm 128	Slaugt. Line 2	Collect 6	Market Floor	Lm 4b	23
Lm 214	Slaugt. Line 1	Collect 8	Carcass	Lm 4b	23
Lm 121	Slaugt. Line 2	Collect 6	Market Floor	Lm 4b	23
Lm 231	Slaugt. Line 2	Collect 8	Slaugt. Table	Lm 4b	23
Lm 232	Slaugt. Line 2	Collect 8	Slaugt. Table	Lm 4b	23
Lm 173	Slaugt. Line 2	Collect 7	Carcass	Lm 4b	23
Lm 324	Slaugt. Line 2	Collect 11	Carcass	Lm 4b	24
HU 27	FIOCRUZ/RJ	MR	Blood	Lm 4b	24
Lm 311	Slaugt. Line 1	Collect 11	Market Floor	Lm 4b	24
HU 10	FIOCRUZ/RJ	MR	Blood	Lm 4b	24
HU 65	FIOCRUZ/RJ	MR	Blood	Lm 1/2a	24
Lm 50	Slaugt. Line 1	Collect 3	Market Floor	Lm 4b	24
Lm 110	Slaugt. Line 1	Collect 6	Market Floor	Lm 4b	24
HU 28	FIOCRUZ/RJ	MR	Cerebrospinal fluid	Lm 4b	24
Lm 49	Slaugt. Line 1	Collect 3	Market Floor	Lm 4b	25
Lm 314	Slaugt. Line 1	Collect 11	Market Table	Lm 1/2b	25
Lm 213	Slaugt. Line 1	Collect 8	Carcass	Lm 4b	25
Lm 32	Slaugt. Line 1	Collect 2	Market Table	Lm 1/2b	25
Lm 35	Slaugt. Line 2	Collect 2	Slaugt. Table	Lm 1/2b	25
Lm 220	Slaugt. Line 2	Collect 8	Slaugt. Floor	Lm 1/2b	25
Lm 221	Slaugt. Line 2	Collect 8	Slaugt. Floor	Lm 1/2b	25
Lm 313	Slaugt. Line 1	Collect 11	Market Table	Lm 1/2b	25
Lm 36	Slaugt. Line 2	Collect 2	Slaugt. Table	Lm 1/2b	25
Lm 297	Slaugt. Line 2	Collect 10	Market Table	Lm 4b	26
Lm 298	Slaugt. Line 2	Collect 10	Market Table	Lm 4b	26
HU 66	FIOCRUZ/RJ	MR	Cerebrospinal fluid	Lm 4b	26
Lm 312	Slaugt. Line 1	Collect 11	Market Floor	Lm 4b	27
Lm 109	Slaugt. Line 2	Collect 5	Market Floor	Lm 4b	27
Lm 61	Slaugt. Line 1	Collect 5	Market Floor	Lm 4b	28
Lm 62	Slaugt. Line 1	Collect 5	Market Floor	Lm 4b	28
Lm 265	Slaugt. Line 1	Collect 8	Market Floor	Lm 4b	28
Lm 323	Slaugt. Line 2	Collect 11	Carcass	Lm 4b	29
HU 01	FIOCRUZ/RJ	MR	Cerebrospinal fluid	Lm 1/2b	30
Lm 174	Slaugt. Line 2	Collect 7	Carcass	Lm 4b	31

Figure 2 - Dendrogram showing comparison of Listeria monocytogenes strains from human cases and swine slaughterhouses through AFLP

L. monocytogenes contamination and infection. Molecular typing methods such as ribotyping, random amplification of polymorphic DNA (RAPD), multilocus enzyme electrophoresis (MEE), and pulsed-field electrophoresis have been applied to the epidemiologic study of *L. monocytogenes* from different origins, linking isolates from food to its processing environment[11,16,18,26].

The ERIC-PCR technique has been previously applied to the epidemiologic study of *L. monocytogenes* from human, animal and food origins[27,28]. Jersek et al.[28] found that ERIC-PCR discriminated samples from different serotypes and clustered them according to their origin (human or animal) from food isolates. The ability to discriminate serotypes was also discussed by Harvey, Norwood and Gilmoura[27], who described the possibility of using ERIC-PCR separate isolates into lineage I (serotypes 4b and 1/2b) and lineage II (serotypes 1/2a, 1/2c). However, the author had trouble with the interpretation of the ERIC profiles because of the occurrence of numerous faint bands. The AFLP technique has also been applied to the study of *L. monocytogenes* from different origins[26,29], presenting satisfactory results in the differentiation of isolates between serotypes and sample origin, with higher discriminatory power than serotyping. The single enzyme variation of this technique, described first by McLauchlin et al.[24], has been used for *Listeria* spp. genotyping, and has already been proven to be a useful tool for genotyping studies of many bacterial species, such as *Clostridium perfringens*, *Pasteurella multocida* and *Erysipelothrix rhusiopathiae*[24,30,31].

In this study, both AFLP and ERIC-PCR were used to characterize *L. monocytogenes* strains isolated from human cases of listeriosis at different time periods, isolates from two slaughterhouses and their respective market environment, as well as pork from São Paulo, Brazil. These techniques presented a tendency to separate slaughterhouse isolates from market and pork isolates, with human strains of *L. monocytogenes* dispersed between them. These techniques also presented the tendency to separate isolates according to serotype, but they were not fully capable of discriminating strains in serogroup 1/2 as profiles were found comprising isolates from serotypes 1/2a, 1/2b and 1/2c, with no discrimination of serogroups 1/2 and 4. The persistence of one profile among slaughterhouse environment isolates from successive time periods observed using ERIC and AFLP analysis suggests the possibility of persistent strains in the food processing environment, consistent with observations by Autio et al.[26]. The presence of similar profiles among isolates from slaughterhouses and their respective market environments and pork suggests that contamination with *L. monocytogenes* may start at the slaughterhouse and continue in the market, most likely carried by pork. On the other hand, different and exclusive profiles were also found according to isolate origins, thus suggesting different routes of contamination. These situations are observed among isolates from ERIC and AFLP patterns.

In European studies, the continuous contamination of food processing environments and final products (food) and subsequent human infection[6,10,11] are well-established because most of the food processing industry is primarily a single physical structure, a fact that facilitates environmental contamination. In Brazil, the food processing industry is usually separated between the slaughterhouse and the processing plant facility, and most markets offer food processing services such as cutting, slicing and preparing meats and vegetables. This enables different origins and routes of contamination of the environment and food by *L. monocytogenes,* and finally, human infection.

Conclusions

The present study determined that in the environments addressed, different contamination sources are present, although the possibility of

continuous contamination by persistent strains could not be excluded. More importantly, the existence of common ERIC and AFLP profiles among environmental and pork isolates and human infection strains demonstrates the high risk of listeriosis in the studied environment. Future studies are also required to enhance the knowledge of *L. monocytogenes* virulence and the ability of these environmental and food isolates to become pathogenic to humans.

Disclosure Statement

No competing financial interests exist.

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Ethics

The animal experiment was conducted with the approval of the Scholl of Veterinary Medicine and Animal Science- São Paulo University- Ethics Committee, project number 1103/2007.

Referências

1. FARBER, J. M.; PETERKIN, P. I. *Listeria monocytogenes*, a food-borne pathogen. **Microbiology and Molecular Biology Reviews**, v. 55, n. 3, p. 476-511, 1991.
2. SCHLECH, W. F.; LAVIGNE, P. M.; BOTOLUSSI, R. A.; ALLEN, A. C.; HALDANE, E. V.; WORT, A. J.; HIGHTOWER, A. W.; JOHNSON, S. E.; KING, S. H.; NICHOLLS, E. S.; BROOME, C. V. Epidemic listeriosis – evidence for transmission by food. **New England Journal of Medicine**, v. 308, n. 4, p. 203-206, 1983.
3. SEELIGER, H. P. R.; JONES, D. Genus *Listeria*. In: SNEATH, P. H. A.; MAIR, N. S.; SHARPE, M. E.; HOLT, J. G. (Ed.). **Bergey's manual of systematic bacteriology**. Baltimore: Williams & Wilkins. 1986. v. 2, p. 1235-1245.
4. LUNDÉN, J. M.; AUTIO, T. J.; SJÖBERG, A. M.; KORKEALA, H. J. Persistent and nonpersistent *Listeria monocytogenes* contamination in meat and poultry processing plants. **Journal of Food Protection**, v. 66, n. 11, p. 2062-2069, 2003.
5. LIU, D. Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. **Journal of Medical Microbiology**, v. 55, pt. 6, p. 645-659, 2006.
6. WIEDMANN, M. Molecular subtyping methods for *Listeria monocytogenes*. **The Journal of AOAC International**, v. 85, n. 2, p. 524-531, 2002.
7. ESPER, M. R. N. R.; PESSOA, G. V. A.; HOFER, E.; LEE, I. M. L.; MELLES, C. E. A.; SAKATA, E. E.; CALZADA, C. T. Meningite por *Listeria monocytogenes* em São Paulo, Brasil. **Revista do Instituto Adolfo Lutz**, v. 38, p. 37-41, 1978.
8. SCHWAB, J. P.; EDDELWEISS, M. I. A. Identificação de *Listeria monocytogenes* em placentas humanas e espécimes de aborto pela técnica de imunoistoquímica. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, v. 39, n. 2, p. 111-114, 2003.
9. HOFER, E.; REIS, C. M. F.; HOFER, C. B. Sorovares de *Listeria monocytogenes* e espécies relacionadas, isoladas de material clínico humano. **Revista da Sociedade Brasileira de Medicina Tropical**. v. 39, n. 1, p. 32-37, 2006.
10. GIOVANNACCI, I.; RAGIMBEAU, C.; QUEGUINER, S.; SALVAT, G.; VENDEUVRE, J. L.; CARLIER, V.; ERMEL, G. *Listeria monocytogenes* in pork slaughtering and cutting plants use of RAPD, PFGE and PCR-REA for tracing and molecular epidemiology. **International Journal of Food Microbiology**, v. 53, n. 2-3, p. 127-140, 1999.
11. BOERLIN, P.; PIFFARETTI, J. C. Typing of human, animal, food and environmental isolates of *Listeria monocytogenes* by multilocus enzyme electrophoresis. **Applied Environmental Microbiology**, v. 57, n. 6, p. 1624-1629, 1991.
12. RORVIK, L. M.; CAUGANT, D. A.; YNDESTAD, M. Contamination pattern of *Listeria monocytogenes* and other *Listeria* spp. in a salmon slaughterhouse and smoked salmon processing plant. **International Journal of Food Microbiology**, v. 25, n. 1, p. 19-27, 1995.
13. MELLO, J. F.; EINSFELDT, K.; FRAZZON, A. P. G.; COSTA, M.; FRAZZON, J. Molecular analysis of the *iap* gene of *Listeria monocytogenes* isolated from cheeses in Rio Grande do Sul, Brazil. **Brazilian Journal of Microbiology**, v. 39, n. 1, p. 169-172, 2008.
14. BORGES, M. F.; SIQUEIRA, R. S. de; BITTENCOURT, A. M.; VANETTI, M. C. D.; GOMIDE, L. A. M. Occurrence of *Listeria monocytogenes* in salami. **Revista de Microbiologia**, v. 30, n. 4, p. 362-364, 1999.
15. DEGENHARDT, R.; SANT'ANNA, E. S. Survival of *Listeria monocytogenes* in low acid Italian sausage produced under Brazilian conditions. **Brazilian Journal of Microbiology**, v. 38, n. 2, p. 309-314, 2007.
16. DESTRO, M. T.; LEITÃO, M. F.; FARBER, J. M. Use of molecular typing methods to trace the dissemination of *Listeria monocytogenes* in a shrimp processing plant. **Applied Environmental Microbiology**, v .62, n. 2, p. 705-711, 1996.
17. ANTONIOLLO, P. C.; BANDEIRA, F. S.; JANTZEN, M. M.; DUVAL, E. H.; SILVA, W. P. Prevalence of *Listeria* spp. in feces and carcasses at a lamb packing plant in Brazil. **Journal of Food Protection**, v. 66, n. 2, p. 328-330, 2003.
18. BARBALHO, T. C. F.; ALMEIDA, P. F.; ALMEIDA, R. C. C.; HOFER, E. Prevalence of *Listeria* spp. at a poultry processing plant in Brazil and a phage test for a rapid test confirmation of suspect colonies. **Food Control**, v. 16, n. 3, p. 211-216, 2005.
19. SEELIGER, H. P. R.; HÖEHNE, K. Serotyping of *Listeria monocytogenes* and related species. In: BERGAN, T.; NORRIS,

J. (Ed.). **Methods in microbiology**. New York: Academic Press. 1979. p. 33-48.

20. BOOM, R.; SOL, C. J. A.; SALIMANS, M. M. Rapid and simple method for purification of nucleic acids. **Journal of Clinical Microbiology**, v. 28, n. 3, p. 459-453, 1990.

21. BORDER, P. M.; HOWARD, J. J.; PLASTROW, G. S.; SIGGENS, K. W. Detection of *Listeria* species and *Listeria monocytogenes* using polymerase chain reaction. **Letters in Applied Microbiology**, v. 11, n. 3, p. 158- 162, 1990.

22. VERSALOVIC, J.; KOEUTH, T.; LUPSKI, J. R. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. **Nucleic Acids Research**, v. 19, n. 24, p. 6823-6831, 1991.

23. RAFIEE, M.; BARA, M.; STEPHENS, C. P.; BLACKALL, P. J. Application of ERIC-PCR for the comparison of isolates of *Haemophilus parasuis*. **Australian Veterinary Journal,** v. 78, n. 12, p. 846-849, 2000.

24. MCLAUCHLIN, J.; RIPABELLI, G.; BRETT, M. M.; THRELFALL, E. J. Amplified fragment length polymorphism (AFLP) analysis of *Clostridium perfringens* for epidemiological typing. **International Journal of Food Microbiology**, v. 56, n. 1, p. 21-28, 2000.

25. HUNTER, P. R.; GASTON, M. A. Numerical index of the discriminatory ability of typing systems: an application of Simpson´s index of diversity. **Journal of Clinical Microbiology**, v. 26, n. 11, p. 2465-2466, 1988.

26. AUTIO, T.; MARKKULA, A.; HELLSTRÖM, S.; NISKANEN, T.; LUNDÉN, J.; KORKEALA, H. Prevalence and genetic diversity of *Listeria monocytogenes* in the tonsils of pigs. **Journal of Food Protection**, v. 67, n. 4, p. 805-808, 2003.

27. HARVEY, J.; NORWOOD, D. E.; GILMOURA, A. Comparison of repetitive element sequence-based PCR with multilocus enzyme electrophoresis and pulsed field gel electrophoresis for typing *Listeria monocytogenes* food isolates. **Food Microbiology,** v. 21, n. 3, p. 305-312, 2004.

28. JERSEK, B.; GILOT, P.; GUBINA, M.; KLUN, N.; MEHLE, J.; TCHERNEVA, E.; RIJPENS, N.; HERMAN, L. Typing of *Listeria monocytogenes* strains by repetitive element sequence-based PCR. **Journal of Clinical Microbiology**, v. 37, n. 1, p. 103-109, 1999.

29. KETO-TIMONEN, R.; TOLVANEN, R.; LUNDÉN, J.; KORKEALA, H. An 8-year surveillance of the diversity and persistence of *Listeria monocytogenes* in a chilled food processing plant analyzed by amplified fragment length polymorphism. **Journal of Food Protection**, v. 70, n. 8, p. 1866-1873, 2007.

30. MORENO, A. M.; BACCARO, M. R.; FERREIRA, A. J.; PESTANA DE CASTRO, A. F. Use of Single-Enzyme Amplified Fragment Length Polymorphism for typing *Pasteurella multocida* subsp. *multocida* isolates from pigs. **Journal of Clinical Microbiology**, v. 41, n. 4, p. 1743-1746, 2003.

31. COUTINHO, T. A.; IMADA, Y.; DE BARCELLOS, D. E.; DE OLIVEIRA, S. J.; MORENO, A. M. Genotyping of Brazilian *Erysipelothrix* spp. strains by Amplified Fragment Length Polymorphism. **Journal of Microbiological Methods**, v. 84, n. 1, p. 27-32, 2011.