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Genotypic profile of *Listeria monocytogenes* isolated in refrigerated chickens in southern Rio Grande do Sul, Brazil

Perfil genotípico de *Listeria monocytogenes* isolada em frangos refrigerados comercializados na região sul do Rio Grande do Sul, Brasil

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ABSTRACT

Listeria monocytogenes *is of notable concern to the food industry, due to its ubiquitous nature and ability to grow in adverse conditions. This study aimed to determine the genotypic profile of* ***L. monocytogenes*** *strains isolated from refrigerated chickens marketed in the southern part of Rio Grande do Sul, Brazil. The strains of* ***L. monocytogenes*** *isolated were characterized by serotyping and Pulsed Field Gel Electrophoresis (PFGE). Three different serotypes (1/2a, 1/2b and 4e) were evaluated by PFGE, and the macrorestriction patterns utilizing enzymes AscI and ApaI, revealed five different pulsotypes. The presence of such varied genotypic profiles demonstrates the prevalence of* ***L. monocytogenes*** *contamination of chicken processing environments, which combined with ineffective cleaning procedures, allowing the survival, adaptation and proliferation of these pathogens, not only in the processing environment, but also in local grocery stores.*

Key words: *contamination, pathogen, prevalence, serotyping.*

RESUMO

Listeria monocytogenes *é uma notável preocupação para a indústria de alimentos, devido à sua natureza ubíqua e a capacidade de se multiplicar em condições adversas. Este estudo objetivou determinar o perfil genotípico de* ***L. monocytogenes*** *isolada a partir de frangos refrigerados comercializados na região sul do Rio Grande do Sul, Brasil. As cepas de* ***L. monocytogenes*** *foram selecionadas e caracterizadas por sorotipagem e Eletroforese em Gel de Campo Pulsado (PFGE). Três sorotipos diferentes (1/2a, 1/2b e 4e) foram avaliados por PFGE, e a combinação dos padrões de macrorestrição utilizando as enzimas AscI e ApaI revelou cinco diferentes pulsotipos. A presença de diferentes perfis genotípicos demonstra a importância da contaminação no ambiente de processamento de frangos, o qual, juntamente com procedimentos de limpeza ineficazes, permitem a sobrevivência, adaptação e proliferação desses patógenos, não somente no ambiente de processamento, mas também no local de comercialização destes produtos.*

Palavras-chave: *contaminação, patógeno, prevalência, sorotipagem.*

INTRODUCTION

The bacterium ***L. monocytogenes*** is a public health concern, causing listeriosis. Infection with ***L. monocytogenes*** is potentially life threatening, producing a wide spectrum of disease states as enteritis, encephalitis, septicemia, meningitis or abortion (SCALLAN et al., 2011). The primary route of infection is through consumption of contaminated food, particularly, ready to eat products. Listeriosis has a high mortality rate, which occurs in well-defined risk groups, including immunocompromised individuals, elderly, children and pregnant women (FARBER & PETERKIN, 1991).

Due to its ubiquitous nature, and ability to proliferate under refrigeration conditions, ***L. monocytogenes*** has been isolated from a wide

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variety of foods, particularly dairy and meat products (CARPENTIER & CERF, 2011). In Brazil, there have not been any documented outbreaks of listeriosis associated with the consumption of contaminated food. However, ***L. monocytogenes*** has been successfully isolated from different food products (NALERIO et al., 2009; BUENO et al., 2010; GALVÃO et al., 2012).

In order to monitor the dissemination of ***L. monocytogenes*** in food, several typification methods have been developed based on the size of DNA fragments generated by enzymatic digestion (LOMONACO et al., 2009). Among the molecular methods currently available, PFGE has proven to be effective and invaluable for epidemiological typing of ***L. monocytogenes***. This technique has assisted in tracking listeriosis outbreaks, as well as, determining the routes of food contamination (WIEDMANN, 2002). Moreover, PFGE is a sensitive technique, with good discriminatory power that generates highly reproducible data (GRAVES & SWAMINATHAN, 2001).

The various stages involved in the production, processing/storage, and distribution/ commercialization of chickens, are all potential sources of contamination by ***L. monocytogenes*** (BARBALHO et al., 2005). Furthermore, it is important to investigate and understand the presence of ***L. monocytogenes*** in various food products, to establish appropriate strategies for its control. Therefore, the present study focused on determining the genotypic profile of samples obtained by PFGE. ***Listeria monocytogenes*** strains isolated and investigated in this study were obtained from raw refrigerated chickens marketed in Southern Rio Grande do Sul, Brazil.

MATERIALS AND METHODS

Sampling and isolation

A total of 45 samples of refrigerated chicken were evaluated. The samples were from two different brands of chicken, acquired in the original packaging, in supermarkets from the city of Pelotas, Brazil. Sampling was carried out according to the protocol established by RYSER & DONNELLY (2001). To collect microbial samples from the surface of the chicken a superficial washing technique was employed. Each sample wash was performed in sterile plastic bags containing 225mL of 0.1% peptone water. Aliquots of 25mL of this liquid was inoculated into 225mL of Listeria Enrichment Broth (LEB, Oxoid®, Basingstoke, United Kingdom), enriched with the supplement SR141E (Oxoid), and incubated at 30°C for 24 hours.

Identification

The isolation of ***Listeria*** as well as its phenotypic and biochemical identification (at the species level) was carried out according to the protocol described by FARBER et al. (1994). Briefly, after the primary enrichment in LEB broth (Oxoid), a second enrichment was performed in Fraser Broth (Oxoid) and further inoculated in agars Oxford (Oxoid) and Palcam (Oxoid), in order to obtain typical ***Listeria*** colonies. Genus and species confirmation were performed through the motility test at 25°C (Motility Test Medium, Difco®, Detroit, USA), catalase (3% hydrogen peroxide), β-hemolysin (Horse Blood Agar 5%) and sugar fermentation test with dextrose, rhamnose, xylose and mannitol (Vetec, Rio de Janeiro, Brazil). After, all isolates of ***L. monocytogenes*** were grown in eppendorf tubes containing Tryptone Soy Agar (TSA, Acumedia®, Baltimore, USA), enriched with 0.6% Yeast Extract (TSA-YE) incubated at 35-37ºC for 24 hours, and stored for subsequent evaluation.

Serotyping

Strains of ***L. monocytogenes*** were characterized by serotyping by the Laboratório de Zoonoses Bacterianas, Departamento de Bacteriologia, Instituto Osvaldo Cruz (FIOCRUZ), Manguinhos, Rio de Janeiro (RJ).

PFGE

PFGE was performed following the PulseNet standardized protocol for subtyping of ***L. monocytogenes*** (GRAVES & SWAMINATHAN, 2001). Briefly, genomic DNA was prepared by mixing 240µl of a standardized cell suspension and 60µl of 10mg.mL^{-1} lysozyme solution (Sigma-Aldrich, St. Louis, USA), followed by incubation at 37°C for 10min. Subsequently, ***L. monocytogenes*** DNA cleavage was achieved by restriction with *Asc*I (25U µL^{-1}) and *Apa*I (160U µL^{-1}) (New England BioLabs, Beverly, USA). Additionally, the restriction enzyme *Xba*I (Fermentas, Maryland, USA) was used for restriction of ***Salmonella enterica*** serotype Braenderup H9812 and functioned as the reference standard (HUNTER et al., 2005). Following restriction digestion, PFGE for ***L. monocytogenes*** and ***Salmonella*** was performed with the CHEF-DR II system (Bio-Rad Laboratories, Richmond, USA), in a 1% agarose gel and 0.5 x TBE buffer at 6V cm^{-1} for 23h at 14°C, with initial and final switch times of 4.0 and 40.0s, respectively. The gel was stained with ethidium bromide and the gel image was visualized with High Performance UV Transilluminator (UVP Inc., Upland, USA). The images were captured and digitized by Kodak 2200 system (Rochester, USA).

RESULTS AND DISCUSSION

Of the 45 chicken samples analyzed, 15 (33.3%) tested positive for ***L. monocytogenes*** (Table 1). All of the samples evaluated were taken from the package in which they are usually sold, indicating that the contamination with this microorganism occurred at the processing plant. This is in agreement with other studies that demonstrated contamination of chicken by ***L. monocytogenes*** while in processing plants. Consistent with this study, NALERIO et al. (2009) found that 11.7% of samples analyzed in a poultry slaughterhouse, were contaminated by this pathogen. Likewise, MIETTINEN et al. (2001) evaluated the presence of this bacterium in chicken from the Finnish local market, and found 62% of their chicken was contaminated by ***L. monocytogenes***. In Spain, VITAS et al. (2004), observed the presence of ***L. monocytogenes*** in 36.1% of the samples, also showing high prevalence of the pathogen in the products from the market, reflecting the wide distribution of this bacterium within the poultry processing plants.

Following the phenotypic identification of ***L. monocytogenes*** strains, the serotyping was performed. This technique consisted in a classical tool used to promote the differentiation of isolates based on the fact that different isolates of the same bacterial species, differ regarding the antigens presentation on the cell surface (WIEDMANN, 2002). In this study, six isolates used for genetic characterization have been characterized serologically, of which 33.3% (2) belonging to serotype 1/2a; 33.3% (2) to serotype 1/2b, and 33.3% (2) to serotype 4e. The single isolate belonging to serotype 1/2c was untyped, since it is different from the others. The serotypes distribution can be observed in table 1.

There was a prevalence of serogroup 1/2 (66.6%), in agreement with the results obtained in other studies, which also showed that serotypes 1/2a, 1/2b, 1/2c, belonging to this serogroup are most frequently isolated from food and food processing environments (LEITE et al., 2006; TAMBURRO et al., 2010). LAWRENCE & GILMOUR (1995), suggest that the recurring presence of isolates of serogroup 1/2 in a wide variety of food and processing plants, may be related to surface antigens present in these isolates, potentially facilitating their ability to colonize equipment in the processing environment. Moreover, it is interesting to note that serotypes 1/2a and 1/2b, were found in the samples evaluated in this study. Serotypes 1/2a and 1/2b are the most commonly associated serotypes observed with both individual cases and outbreaks of listeriosis worldwide, in addition to serotype 4b (ZHANG et al., 2013).

Table 1 - Isolation and serotyping of ***L. monocytogenes*** strains in refrigerated chickens marketed in Southern Rio Grande do Sul.

Source	Samples*	Isolates*	Serotypes
			4e (6)
Refrigerated chicken	33 (A)	11 (A)	1/2a (5)
	12 (B)	4 (B)	1/2b (3)
			1/2c (1)
Total	45	15	15

(A): Brand A; (B): Brand B; *number of samples and isolates.

Although important, serotyping has low discriminatory power, particularly for ***L. monocytogenes***, as it has only 13 serotypes, limiting epidemiological application. Therefore, stronger molecular techniques are required. PFGE has been widely used for discrimination between serotypes of ***L. monocytogenes*** (THÉVENOT et al., 2006; VON LAER et al., 2009). Due to its sensitivity, high discriminatory power, standardization and reproducibility, PFGE is recognized as the most important typification technique for ***L. monocytogenes*** (WIEDMANN, 2002). The restriction enzymes commonly used are *Asc*I and *Apa*I, when combined, allows accurate discrimination of this microorganism (GRAVES & SWAMINATHAN, 2001; LÓPEZ et al., 2008). Additionally, the protocol recommended by PulseNet (Centers for Disease Control and Prevention - CDC), for typification of ***L. monocytogenes,*** suggests the use of two enzymes, as the band patterns generated by these enzymes, are in the same size range, requiring only a single reference pattern (GRAVES & SWAMINATHAN, 2001).

Among the fifteen strains of ***L. monocytogenes*** obtained, six (isolates 5, 6, 9, 10, 23, 26) from three different serotypes, were selected for PFGE. With the exception of isolate 5, originating from final product of Brand B, all the other were obtained from the Brand A products (Table 2). The PFGE genotyping of the strains with the enzymes *Asc*I and *Apa*I, revealed five genotypic profiles for each enzyme individually, which assigned numbers and letters, respectively (Table 2).

The data obtained, allowed us to verify that the strain 5, serotype 1/2a, obtained from a product of the Brand B, showed pulsotype 9L. The strain 6, also serotype 1/2a was obtained from a sample of Brand A, and composed the pulsotype 8J. Strain 23, serotype

Table 2 - Genotypic and serologic profile of ***L. monocytogenes*** isolated in refrigerated chicken marketed in Southern Rio Grande do Sul.

Strains*	Brand	Serotype	Digestion profile by endonuclease	
			AscI	ApaI
5	B	1/2a	9	L
6	A	1/2a	8	J
9	A	4e	7	H
10	A	4e	7	H
23	A	1/2b	14	P
26	A	1/2b	12	N

*Identification number.

1/2b, a product of Brand A, showed pulsotype 14M, while the strain 26, also serotype 1/2b, with the same origin, had different genotypic profile, pulsotype 12N. Figure 1 demonstrates the different genotypic profiles obtained with the restriction of endonucleases *Asc*I and *Apa*I, for ***L. monocytogenes*** strains from refrigerated chickens marketed in southern Rio Grande do Sul.

Strains 9 and 10, both serotype 4e showed the same genotypic profile, pulsotype 7H (Table 2). It is interesting to note that these strains were isolated from samples collected at different local grocery stores, on different days, although the samples have been obtained from refrigerated chickens that were processed in the same slaughterhouse (Brand A). The prevalence of ***L. monocytogenes*** in refrigerated chickens from local grocery stores, along with the diversity of serotypes and the genotypic profiles found, reflect the wide distribution of this pathogen in poultry slaughterhouses, and the difficulty of their elimination from the environment of processing plants. Moreover, the inefficiency of the cleaning and sanitization procedures allow the adaptation and survival of ***L. monocytogenes*** strains in food processing environments, resulting in persistence and contamination of the final product (MENDONÇA et al., 2012; LEONG et al., 2014).

CONCLUSION

There is presence of different groups of ***L. monocytogenes*** in refrigerated chickens from the local grocery stores of southern Rio Grande do Sul. Since the samples were acquired from the primary packaging, these results emphasize the importance of contamination during the food processing.

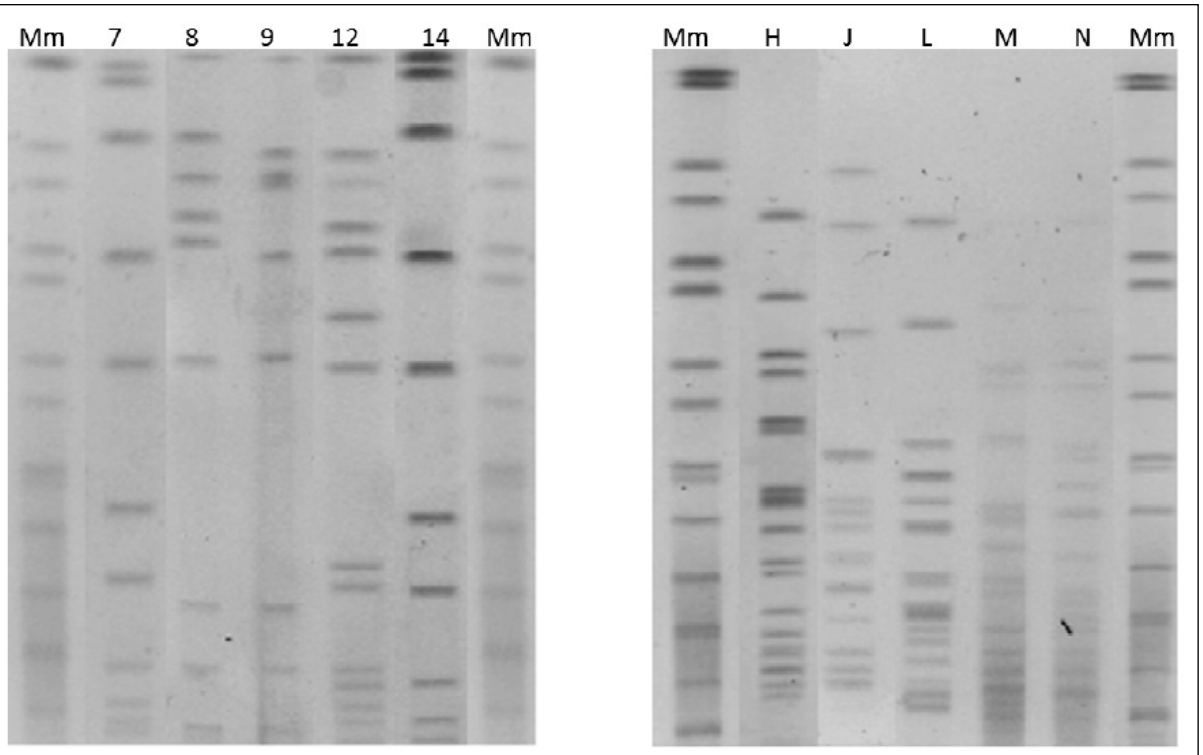

Figure 1 - Genotypic profiles by PFGE of genomic DNA isolated from ***L. monocytogenes***, obtained with the *Asc*I and *Apa*I enzymes. Mm: Molecular Marker ***Salmonella enterica*** serotype Braenderup strain H9812.

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