

Molecular characterization of *Listeria monocytogenes* isolated from animal products in a city of Northern Brazil

Caracterização molecular de *Listeria monocytogenes* isolada de produtos de origem animal em uma cidade da região Norte do Brasil

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ABSTRACT

Listeria monocytogenes, a foodborne pathogen causes listeriosis, a fatal disease in about 30% of cases that affects mainly immunocompromised persons. The aim of this research was to characterize *L. monocytogenes* pulsed-field gel electrophoresis (PFGE) types isolated from meat products collected at public markets in Araguaína city, TO. Sixty samples of raw ground beef and frescal sausage were analyzed during the second half of 2008. Five out of 30 samples (16.7%) of raw ground beef tested positive for *L. monocytogenes*, three of which were classified as serotype 1/2b and two as serotype 4b. Among the 30 samples of sausage collected, two strains of *L. monocytogenes* were isolated (6.7%), one of them belonging to serotype 1/2a and the other belonging to serotype 1/2b. The restriction enzymes used were *ApaI* and *SmaI*. Similarities among the strains were determined by Dice coefficient. The macro restriction profile obtained by using *SmaI* enzyme allowed the distribution of seven strains in two clusters, two pulsotypes and two subtypes. The result indicates that *L. monocytogenes* isolates, belonging to serotype 4b, 1/2a and 1/2b, are strongly correlated within the same serotype group, and in some cases among different serotypes, suggesting that they have a common source.

Key words: pulsed field gel electrophoresis, ground beef, frescal sausage, *L. monocytogenes*.

RESUMO

Listeria monocytogenes é um patógeno de origem alimentar que causa a listeriose, doença fatal em aproximadamente 30% dos casos, e que afeta principalmente pessoas imunocomprometidas. O presente trabalho teve como objetivo analisar os perfis PFGE de cepas de *L. monocytogenes* isoladas de produtos de origem animal, obtidos em mercados públicos da cidade de Araguaína, TO. Foram analisadas 60 amostras de carne moída crua e de linguiça frescal, no segundo semestre de 2008. Cinco (16,7%) das 30 amostras de carne moída crua foram

positivas ao patógeno, sendo que três pertenciam ao sorotipo 1/2b e duas ao sorotipo 4b. Das 30 amostras de linguiça mista frescal, duas (6,7%) foram positivas para *L. monocytogenes*, sendo uma do sorotipo 1/2a e outra do 1/2b. Foram utilizadas as enzimas de restrição *ApaI* e *SmaI*. A similaridade entre eles foi determinada pelo coeficiente de Dice. A análise do perfil de macrorestrição com a enzima *SmaI* permitiu a distribuição dos sete isolados em dois clusters, dois pulsotipos e dois subtipos. Os resultados permitiram concluir que os isolados de *L. monocytogenes* sorotipos 4b, 1/2a e 1/2b foram fortemente correlacionados dentro dos mesmos sorotipos e em alguns casos entre diferentes sorotipos, sugerindo uma fonte comum.

Palavras-chave: eletroforese em gel de campo pulsado, carne moída, linguiça frescal.

INTRODUCTION

Listeria monocytogenes is a foodborne pathogen responsible for sporadic outbreaks of listeriosis that can cause septicemia, meningitis, meningoencephalitis and abortion. Listeriosis is fatal in about 30% of cases and mainly affects immunocompromised people, pregnant women, newborn babies and the elderly (PARIHAR et al., 2008; FILIOUSIS et al., 2009). This ubiquitous pathogen is particularly important because of its ability to multiply at low temperatures and under high salt concentrations. Moreover, it has the ability to form biofilms (GRAVES et al., 2005; SHEN et al., 2006).

The genus *Listeria* contains six species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshmeri*,

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L. seeligeri, and *L. grayi*. During the last two decades the group was divided as *L. monocytogenes* and nonpathogenic species (ROCCOURT, 1999). Although *Listeria monocytogenes* is an ubiquitous organism, genetic lineages among this species are detected, presenting a virulence heterogeneity which can also differ in their association with food and environmental sources (JACQUET et al., 2002; GRAY et al., 2004).

GRAVES et al. (1999) divided the *Listeria monocytogenes* subtyping methods into two categories: conventional (serotyping, phage typing) and molecular methods (Multilocus Enzyme Electrophoresis - MEE; Chromosomal DNA Restriction Endonuclease Analysis - REA, Restriction Fragment Length Polymorphism Analysis - RFLPs).

Conventional techniques by themselves cannot detect the source of listeriosis outbreaks; however, molecular subtyping methods are a useful tool in this determination. Among these methods, pulsed-field gel electrophoresis (PFGE) offers great sensitivity along with a high discriminatory power, and it is considered the gold standard method for *L. monocytogenes* subtyping (TENOVER et al. 1997; FUGETT et al., 2007; LEMES-MARQUES et al., 2007). PFGE can also be used in long-term studies both to determine the persistence of a species in an environment, or for determining external contamination into an environment (SENCZEK et al., 2000; MORETRO & LANGSRUD, 2004). Because of its specificity and sensitivity PFGE has been used for *Listeria* species typing replacing the serotyping technique (MELLO et al., 2008). Besides its good reproducibility this technique is highly discriminatory when compared with other molecular typing methods currently available (TENOVER et al. 1997; BASIM & BASIM, 2001).

In Brazil, there is a lack of information about listeriosis outbreaks, antimicrobial susceptibility of clinical strains and their phenotypic and genotypic characterization. Along with the lack of control and study of listeriosis in Brazil, LEMES-MARQUES et al. (2007) include the difficulties in tracing the bacterial types involved in the outbreaks, the lack of notification and the delay in obtaining final results in conventional medical tests.

The aim of this study was to characterize *L. monocytogenes* strains isolated from raw ground beef and fresh mixed sausage sold in Araguaina, northern Brazil, by means of PFGE analysis in order to investigate any relationships among the strains.

MATERIAL AND METHODS

Bacterial isolates

Listeria monocytogenes was isolated from 30 samples of raw ground beef and from 30 samples of fresh sausage, obtained from butcher shops in Araguaina city, from August to December 2008. The strains were serotyped by the Bacteriology Department at the Oswaldo Cruz Institute, Rio de Janeiro (FIOCRUZ). The techniques of isolation, selection, confirmation and identification were in accordance with the Normative nº. 62, from August 26, 2003 (BRASIL, 2003). After identification, the strains were frozen at -20°C in a mixture of 50% Luria Bertani broth (LB) and 50% glycerol.

Pulsed-field gel electrophoresis

The genetic profile of *L. monocytogenes* strains was determined by PFGE of DNA digested with the restriction enzymes *ApaI* and *SmaI*, and electrophoresed on a Chef DRII system (Bio-Rad Laboratories, Hercules, CA). PFGE patterns were classified as indistinguishable if the DNA fragment matched each other completely, as closely related if they differed by one to three bands, and as unrelated if they differed by more than three bands (TENOVER et al., 1995). Numbers were used to designate the cluster profiles. Closely related patterns were assigned an additional capital letter.

PFGE was performed according to the PulseNet protocol (CDC/PulseNet with modifications using the *ApaI* and *SmaI* restriction enzymes (GRAVES & SWAMINATHAN, 2001). Bacterial cells embedded in agarose (plugs) with lysozyme (10mg mL⁻¹) were lysed in lysis buffer [50mM Tris pH 8.0, 50mM EDTA pH 8.0, 1.0% sarcosil, 0.15mg mL⁻¹ proteinase K] for two hours at 54°C in a water bath with agitation. After that, the plugs were washed twice in type I water and four times in TE buffer (10mM Tris, 1mM EDTA pH 8.0).

The digestion of each DNA sample in the plugs was carried out by using two restriction enzymes, *ApaI* (40U, 30°C, overnight) and *SmaI* (20U, 25°C overnight). The pulsed field gel electrophoresis was performed in 1% agarose gel (agarose running gel, Sigma, USA) in Tris-boric acid-EDTA 0.5X [90mM Tris, 90mM boric acid pH 8.0 and 2mM EDTA] in the CHEF DRII system (Bio-Rad Laboratories) at 6V cm⁻¹, 14°C for 19h with switch times of 4s to 40s. The molecular weight lambda DNA PFGE ladder (New England Biolabs, Ipswich, MA) was positioned at the ends of each gel. To confirm the genetic profile of strains, the technique was performed in triplicate.

Images were obtained with a Bio-Rad Gel Doc XR system, using the software Quantity One 4.4.1 (Bio-Rad Laboratories), after staining with GelRed for 15 minutes. Comparison of the PFGE patterns was made using the BioNumerics software package (version 4.5; Applied Maths, Belgium). The similarity between the strains was determined by the Dice coefficient and dendrograms were created by using the unweighted pair group method with arithmetic averages - UPGMA. A cutoff value of 80% was set to determine the clusters similarity as performed by FRANCIOSA et al. (2005) and identified with Arabic numbers. The pulsotypes (PT) were used to identify strains with unique electrophoretic profiles, and subtypes (ST) with profiles up to three bands of difference were indicated by using capital letters and numbers, respectively.

RESULTS AND DISCUSSION

From the 30 samples of raw ground beef, five (16.7%) tested positive for *Listeria monocytogenes*, three of them belonging to serotype 1/2b and two of them belonging to serotype 4b. From the 30 samples of fresh mixed sausage evaluated, two strains (6.7%) of *L. monocytogenes* were isolated, one belonging to serotype 1/2a and the other to serotype 1/2b. According to KATHARIOU (2002), 1/2a, 1/2b and 4b are the serotypes most frequently involved in human listeriosis.

The profile analysis by PFGE macrorestriction and the results obtained with the restriction enzyme *ApaI* (Figure 1) allowed the distribution of the seven strains in one cluster (>80% similarity), two pulsotypes (A and B) and two subtypes (A1 and B1). The pulsotypes B comprised four samples, two from ground beef and two from frescal sausage. Three of these samples were classified

as serotype 1/2b and one as serotype 1/2a showing the genetic similarity between these strains.

The profile generated with the restriction enzyme *SmaI* was similar to the results obtained when using *ApaI* enzyme, except for clustering. After *SmaI* enzyme digestion the strains were grouped into two clusters (1 and 2, >80% similarity) (Figure 2). According to figures 1 and 2, the *SmaI* enzyme digestion gave a more discriminating result than the *ApaI* enzyme digestion.

Even though listeriosis can be caused by any of the 13 *L. monocytogenes* serotypes (LEMES-MARQUES et al., 2007), it is known that 1/2a, 1/2b and 4b serotypes are responsible for almost 90% of outbreaks and sporadic cases worldwide (LÓPEZ et al., 2006). In this study, it was found that the isolates of *L. monocytogenes* serotypes 4b, 1/2a and 1/2b were strongly correlated within the same serotypes and, in some cases, between the different serotypes, indicating a common origin. The same results were observed by other authors (PARIHAR, et al., 2008).

The discrimination of results obtained by using different enzymes can vary even in isolates of the same origin, as happened in this study between the enzymes *ApaI* and *SmaI*. Likewise, YDE & GENICOT (2004) explained that the discrimination of three strains sent to the Belgian Reference Center varied depending on which enzyme they were digested with: two of them showed an identical profile when digested with *AscI* enzyme, but were different with *ApaI* enzyme. Furthermore, SHEN et al. (2006) also found differences in the discrimination of bands between the enzymes *AscI* and *ApaI*.

There is a discussion about the relationship of the origin of two lineages found in *L. monocytogenes*, the lineage I represented by serovars 1/2b and 4b, and the lineage II represented by serovars 1/2a and 1/2c (PARIHAR et al., 2008). According to

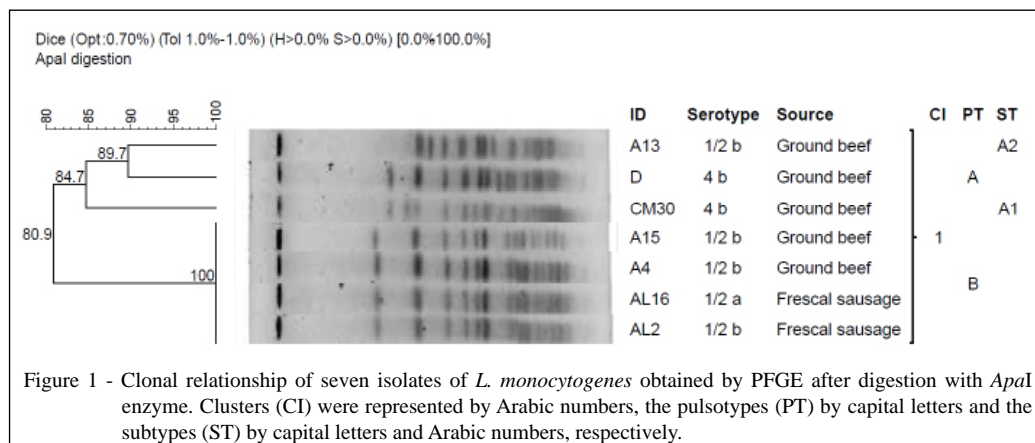
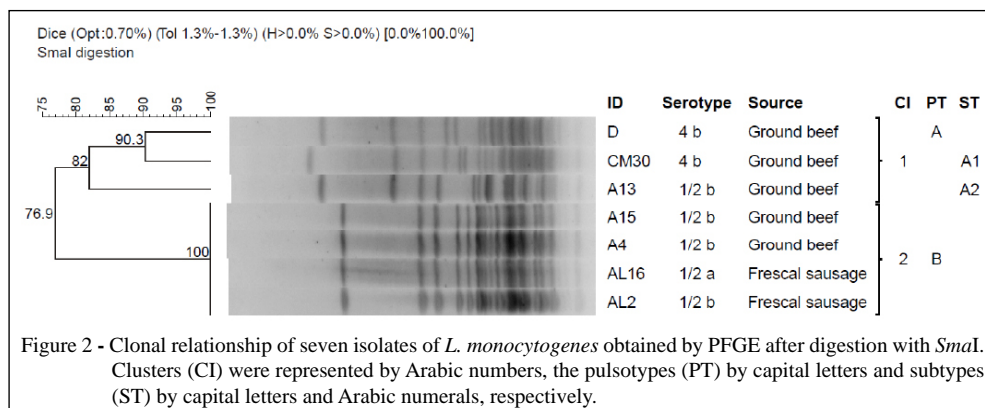


Figure 1 - Clonal relationship of seven isolates of *L. monocytogenes* obtained by PFGE after digestion with *ApaI* enzyme. Clusters (CI) were represented by Arabic numbers, the pulsotypes (PT) by capital letters and the subtypes (ST) by capital letters and Arabic numbers, respectively.



NIGHTINGALE et al. (2005), lineage I comprises the strains which have adapted to the human host, while lineage II comprises the strains which have been environmentally adapted.

In this sense, it is necessary to identify *L. monocytogenes* in different environments in order to associate them with the different pulsotypes described for human listeriosis. However in Brazil, data from both human and environment samples isolation has been scarce, especially in the second case. Therefore, in our country, a map of the geographical and temporal distribution of the occurrence of *L. monocytogenes* (and much less of their pulsotypes) does not yet exist (LEMES-MARQUES et al., 2007).

Considering the handmade preparation of frescal sausage, mostly from unknown origin, it was not possible to establish the persistence of *L. monocytogenes*, as suggested by LONCAREVIC et al. (1996) who reported the persistence of this *Listeria* in processing plants where the products may have been continuously contaminated. This was confirmed by VON LAER et al. (2009) who analyzed samples of frescal sausage from a processing line in Pelotas, Brazil. These authors observed no contamination by *L. monocytogenes* in the raw material, but the pathogen was detected in all the final product samples.

Sometimes the *L. monocytogenes* that persists in a production line for a long period of time may be regarded as a dominant strain (RORVIK et al., 2003). This fact emphasizes the importance of the environment as a source of food products contamination.

Thus, it is important to emphasize the disagreement related to the determination of the main sources of *L. monocytogenes* contamination. While some authors consider the importance of the equipment and the environment, others believe that the raw material would be the most common source of contamination.

CONCLUSION

The molecular analysis showed that the *L. monocytogenes* strains were identical or closely related even though they were obtained from products sold in different places and collected at different times. This would suggest the environment as a common source of the food products contamination.

In order to prevent outbreaks of human and animal listeriosis, molecular techniques such as PFGE are important tools that allow prevalence studies to identify and recall contaminated products. It is recommended a surveillance program in this matter from public health authorities once highly virulent listeriosis serotypes were detected in samples coming from Araguaina, northern Brazil.

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