Article/Artigo

Antimicrobial resistance and investigation of the molecular epidemiology of *Listeria monocytogenes* in dairy products

A resistência antimicrobiana e investigação de epidemiologia molecular de *Listeria monocytogenes* em produtos lácteos

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ABSTRACT

Introduction: *Listeria monocytogenes* is a ubiquitous microorganism in nature and is responsible for listeriosis, an infectious disease caused by consumption of contaminated food. **Methods:** Molecular characterization was performed on 19 strains of *Listeria monocytogenes* (serovars 1/2a, 1/2b, 4b and 4c), isolated from dairy products in Rio Grande do Sul, Brazil. The molecular techniques applied were random amplification of polymorphic DNA and restriction enzyme analysis. In addition to the molecular analysis, the antimicrobial resistance profile was determined. **Results:** The strains studied showed a low degree of diversity. In relation to the antimicrobial resistance profile of those microorganisms from the samples analyzed, all of them were susceptible to the antimicrobials tested. **Conclusions:** The molecular techniques that were used presented good discriminatory power for the strains studied. Furthermore, all of the samples that were analyzed were susceptible to the antimicrobials tested.

Key-words: *Listeria monocytogenes*. PCR-REA. RAPD. Dairy products.

RESUMO

Introdução: *Listeria monocytogenes* é um microrganismo que se encontra disseminado na natureza, sendo responsável por causar listeriose, uma doença infecciosa causada pelo consumo de alimentos contaminados. **Métodos:** A análise molecular de 19 linhagens de *Listeria monocytogenes*, sorovares 1/2a, 1/2b, 4b, 4c, isoladas de produtos lácteos do Rio Grande do Sul, Brasil. As técnicas moleculares aplicadas foram: Amplificação Randômica do DNA Polimórfico e Análise por Enzimas de Restrição. Além da análise molecular foi realizado o perfil de resistência antimicrobiana. **Resultados:** As linhagens estudadas mostraram baixo grau de diversidade, em relação ao perfil de resistência antimicrobiana desses microrganismos das amostras analisadas todas foram susceptíveis aos antimicrobianos testados. **Conclusões:** As técnicas moleculares estudadas apresentaram um bom poder de discriminação para as linhagens estudadas. Além disso, todas as amostras analisadas foram susceptíveis aos antimicrobianos analisados.

Palavras-chaves: *Listeria monocytogenes*. PCR-REA. RAPD. Produtos lácteos.

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INTRODUCTION

The genus *Listeria* is formed by six species, among which *Listeria monocytogenes* is known as one of the main pathogenic species in human beings. They consist of Gram-positive rods that are capable of growing under adverse conditions such as low temperatures, acidic pH, high salt concentrations and the procedures that are applied by the food industry to halt the growth of pathogenic microorganisms. Due to their ubiquitous nature, these bacteria are constantly isolated from decomposing material, soil and water, and in this way, they can contaminate raw foods through cross-contamination[1].

In general, most strains of *L. monocytogenes* isolated from clinical samples, food and the environment are susceptible to active antimicrobials against Gram-positive bacteria, and the treatment selected is a combination of ampicillin with an aminoglycoside, normally gentamicin[2]. Although all the 13 serovars of *L. monocytogenes* are capable of causing listeriosis, nearly 95% of the infection cases in humans are caused by 3 serotypes: 1/2a, 1/2b and 4b. In Brazil, one study has shown that serovar 1/2a is predominant in dairy products[3].

The aim of the present study was to determine the genotype profile for *L. monocytogenes* that is predominant in southern Brazil, using molecular biology tools such as random amplification of polymorphic DNA (RAPD) and restriction endonuclease analysis (PCR-REA), on 19 strains isolated from dairy products. Furthermore, the antimicrobial resistance profile of these microorganisms against several antimicrobials that were tested was observed.

METHODS

Bacterial strains and DNA extraction

The nineteen strains used in the present study were isolated from dairy products and were provided by the National Agricultural and Livestock-Rearing Laboratory in Porto Alegre (LANAGRO/RS).

These 19 strains included eight strains of serovar 1/2b (A32, A33, A34, A35, A36, A37, A38 and A39), eight strains of 4b (A40, A42, A43, A45, A47, A48, A49 and A50), two strains of 1/2a (A55 and A54) and one strain of serovar 4c (A46). The serovars of the strains were determined by the Oswaldo Cruz Institute, Rio de Janeiro, Brazil. ATCC 7645 (serovar 1/2a) and strains isolated in Canada A51 (serovar 4b) and A56 (serovar 1/2a) were used as controls. In order to analyze the morphology and confirm the colony purity, cells were grown in listeria enrichment broth (LEB; Acumedia) for 18 hours at 37°C in a shaker and then isolated on solid media (LEB, 1.5% agar-agar). The cells were stored at -70°C in glycerol. The chromosomal DNA was extracted from the strains as described by Torres et al[4].

Antimicrobial susceptibility test

All antimicrobial susceptibility tests were carried out using the standard disk diffusion method recommended by the NCCLS/CLSI guidelines, using ampicillin, gentamicin, vancomycin, ciprofloxacin, erythromycin, tetracycline, chloramphenicol and imipenem. None of the other antimicrobial agents tested had a standardized breakpoint for *Listeria* sp, so the breakpoints established for *Staphylococcus* sp were used[5].

PCR-REA analysis

The procedure described was based on the method by Ericsson et al[6], which consists of amplification of a 2,916bp fragment containing parts of the *inlA* and *inlB* genes, which have been correlated with virulence of *L. monocytogenes*, and on further cleavage of the DNA fragment using the restriction endonuclease *Alu*I. PCR was performed using the primers FD (5'CGACAACATTTAGTGAACCGTG3') and FN (5' GCTGCTTTCGTCCAACCAATGAA 3'), synthesized by Invitrogen Brazil, Ltd. The samples were analyzed by means of gel electrophoresis in 2% agarose stained with ethidium bromide (0.5µg/ml). They were then viewed under UV transillumination and photographed using Kodak Digital Science™ DC120.

RAPD analysis

The two primers used in this study (UBC127 and UBC155) were designed in accordance with Farber & Addison[7]. All PCR amplification was performed in a final volume of 25µl containing 1.5mM of $MgCl_2$, 0.2mM of each dNTP, 1mM of each primer and 1.25U of *Taq* DNA polymerase. A thermal cycler (MJ Research, Inc.; PTC-100) was used for the PCR reaction. The cycling parameters used were: one cycle at 94°C for 2 min followed by 35 cycles at 94°C for 1 min, 35°C for 1 min and 72°C for 2.5 min, with a final extension of 5 min at 72°C. The PCR products were analyzed and photographed as described above. The reproduction of RAPD was assessed by carrying out least three independent trials.

Data analysis

The results obtained from the RAPD analysis were examined using the SPSS software. The similarity between isolates was calculated by means of the simple association coefficient and the grouping analysis by means of UPGMA (unweighted pair group method using average). The presence or absence of bands generated by RAPD was considered to be an alternative characteristic, and it was coded as 1 or 0, respectively.

RESULTS

Antibiotic susceptibility test

None of the 19 *L. monocytogenes* strains analyzed were resistant to the antimicrobials tested and only one of the isolates (A49) showed reduced susceptibility to ciprofloxacin antimicrobials.

PCR-REA analysis

The DNA fragment of 2,916bp obtained from amplification of the genome of *L. monocytogenes* using the primers FD/FN that correspond the internal region of genes *inlAB* was digested using the restriction endonuclease *Alu*I. The product from DNA cleavage made it possible to divide the strains analyzed into three different profiles. Most of the strains[8] presented a p1 profile including the control strains A51 and A56[8]. The p2 profile appeared in only one of the strains studied (A45) and the p3 profile was observed in the control strain ATCC7645 and strain A46 (**Figure 1**).

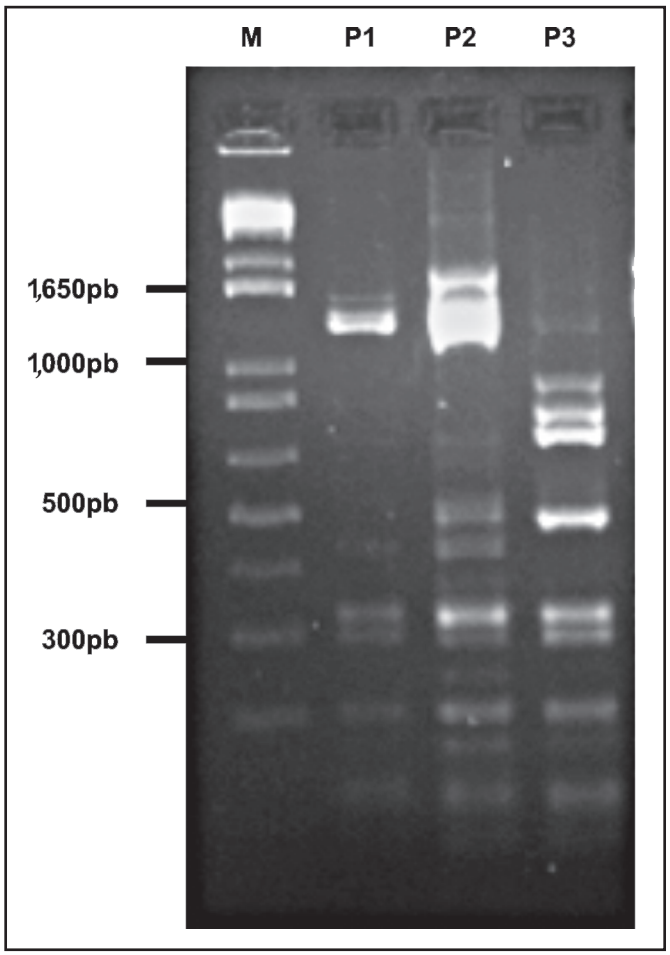

FIGURE 1 - PCR-REA profiles of *L. monocytogenes* from cleavage with restriction endonuclease *Alu*I. M: molecular weight - Kb ladder, p1: profile 1, p2: profile 2, p3: profile 3.

RAPD analysis

The UBC 127 primer produced three to five DNA bands with a molecular size between 450bp and 2500bp (**Figure 2**). The profiles of the UBC 127 primer are shown in a dendrogram (**Figure 3**). The strains of *L. monocytogenes* that were studied could be divided into three main clusters, with a similarity level around 0.74. The first cluster included all the strains with serovars 1/2b, 4c and 4b, which were divided into three subgroups. The second one included only the two strains of *L. monocytogenes* isolated from dairy products with serovar 1/2a (A54 and A55). The third cluster separated the two control strains ATCC 7645 and A56, both with serovar 1/2a. It was also observed that the band for the highest molecular size (2.5Kb) was reproduced in all the isolates from dairy products except in strain A39, while the molecular band of 1.5Kb size was reproduced

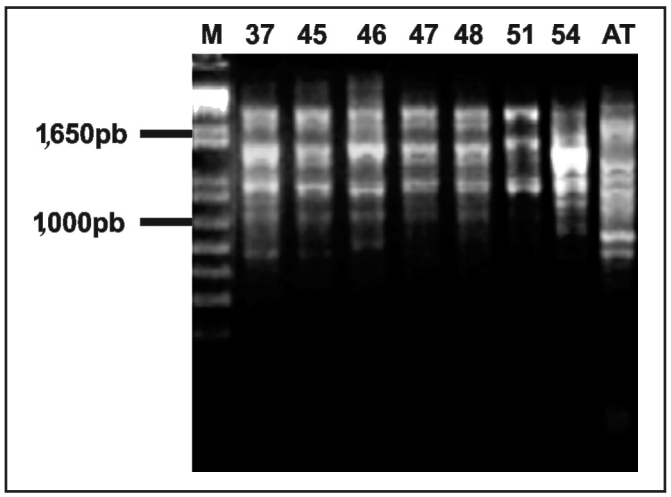

FIGURE 2 - RAPD typing of the *L. monocytogenes* strains, using the primer UBC 127. M: molecular weight - Kb ladder, 37: (A37), 45: (A45), 46: (A46), 47: (A47), 48: (A48), 51: (A51), 54: (A54) and AT: reference strain ATCC 7,645.

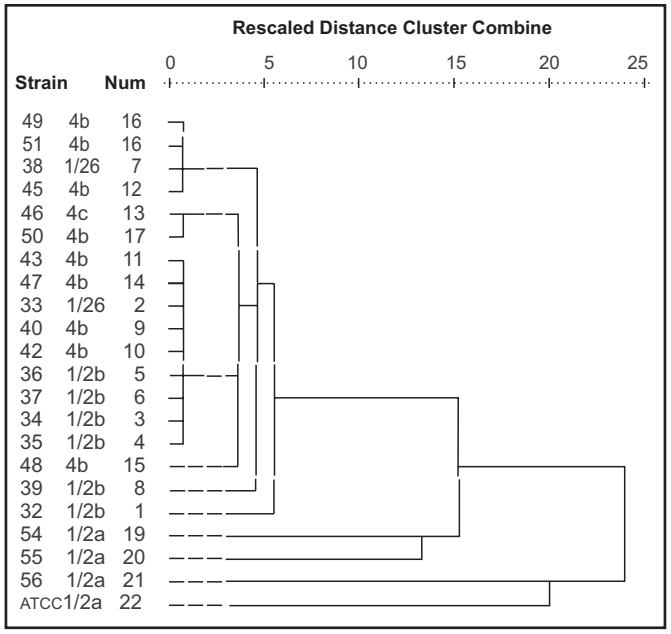

FIGURE 3 - Dendrogram of *Listeria monocytogenes* RAPD for strains isolated in southern Brazil and strains A51, A56 and ATCC 7,645, using the primer UBC127 and serovar discrimination.

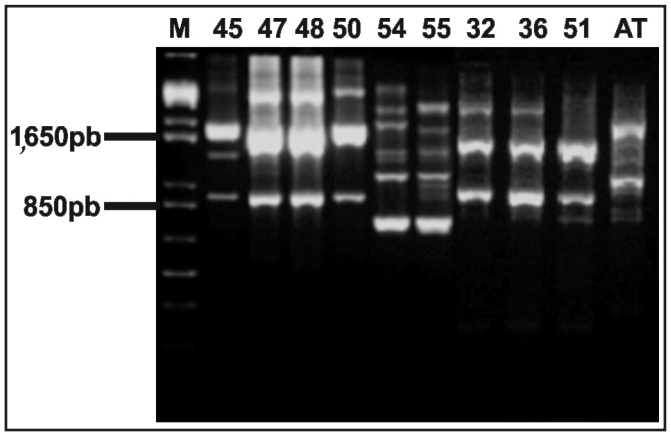

FIGURE 4 - RAPD typing of the *L. monocytogenes* strains, using the primer UBC 155. M: molecular weight - Kb ladder, 45: (A45), 47: (A47), 48: (A48), 50: (A50), 54: (A54), 55: (A55), 32: (A32), 36: (A36), 51: (A51), and AT; reference strain ATCC 7,645.

in most of the strains of dairy products that were isolated, except in 1/2a serovars. The UBC 155 primer produced three to six DNA bands with molecular sizes of 250bp to 4,250bp (**Figure 4**). The profiles of the UBC 155 primer are shown in a dendrogram (**Figure 5**). The strains analyzed were divided into 3 main groups with a similarity level around 0.75. The first cluster involved all the strains of *L. monocytogenes* serovars 1/2b, 4b and 4c; this cluster could be further divided into four subgroups. The second group was formed by strains A54 and A55 of serovar 1/2a, isolated from dairy products in southern Brazil, and strain A56 isolated in Canada. ATCC 7,645 represented the third cluster.

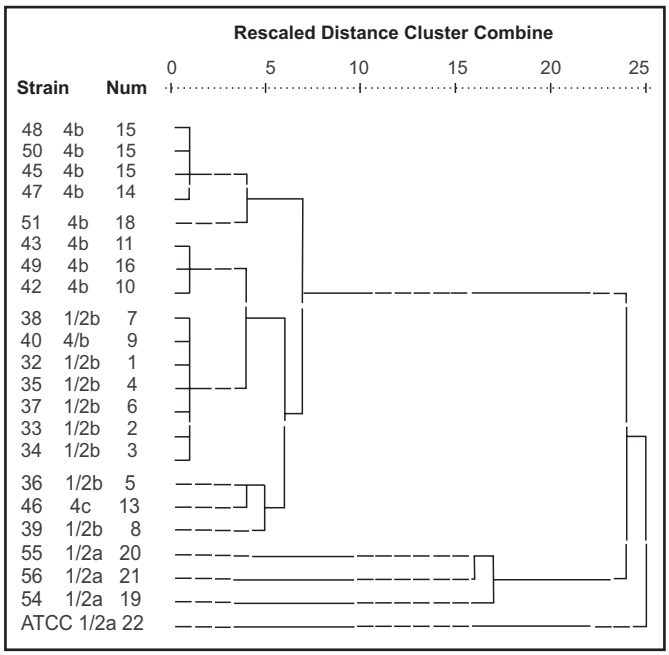

FIGURE 5 - Dendrogram of *Listeria monocytogenes* RAPD for strains isolated in southern Brazil and strains A51, A55 and ATCC 7,645, using the primer UBC 155 and serovar discrimination.

DISCUSSION

Listeriosis is an important and severe disease caused by the *L. monocytogenes* microorganism, in which the main source of disease transmission is ingestion of contaminated food, especially dairy products, which have been responsible for outbreaks of listeriosis[1]. In Brazil, there is a lack of information on listeriosis, particularly because there are no official statistics about the disease, given that its notification is not compulsory. There is no reporting of outbreaks; rather, only isolated cases are noted[9].

The treatment chosen for listeriosis is the administration of penicillin or ampicillin associated with an aminoglycoside, usually gentamicin. For patients who are allergic to penicillin, the treatment can be administered successfully using a combination of vancomycin and an aminoglycoside or sulfamethoxazole-trimethoprim (TMP-SMX), in association with rifampicin. The use of cephalosporin is not suggested because strains of *L. monocytogenes* are generally resistant to those antimicrobials[10]. The incidence of resistance among isolates from clinical samples and food continues to be low. However, the emergence of resistant strains is being described in many studies[11,12]. In 1988, the first strain of multi-resistant *L. monocytogenes* was isolated from human clinical material in France. The resistance was the result of plasmidial transfer, which was believed to have originated from

enterococci or streptococci[13]. In Brazil, a study conducted on 13 samples of *L. monocytogenes* that had been isolated from 12 clinical cases of listeriosis in the south-western region of São Paulo, between January 1995 and May 2005, showed that none of the clinical isolates presented resistance to the antimicrobial that was tested, except for seven strains that presented reduced sensitivity to sulfamethoxazole[14]. In the present study, resistance was hardly ever found among the *L. monocytogenes* strains isolated from dairy products: only the strain A49 presented intermediate susceptibility to ciprofloxacin antimicrobials. The results presented in this study show that the strains isolated in this region are still susceptible to the antibiotics commonly used in hospitals for treating listeriosis.

The *inlA* and *inlB* genes have an essential role in the virulence of *L. monocytogenes*, through coding for two proteins found on the cell surface that are related to the invasion of epithelial cells[15]. Different authors have discriminated between *L. monocytogenes* strains, even in the same serovar, through using PCR-REA, based on the polymorphism that exists within the *inlA* and *inlB* genes, using the restriction enzyme *Alu*I. In a study carried out on 133 strains of *L. monocytogenes* serovar 4b, isolated from food, animals, humans and the environment, it was possible to categorize the strains into two groups, group I containing 37 strains and group II containing 96 strains[6]. Another analysis applying the same technique to 287 isolates of serovars 1/2a, 3a, 1/2c and 3c put the isolates into two clusters[16]. In the present study, using the abovementioned technique to analyze 19 strains isolated from dairy products, three profiles were found through using restriction endonuclease *Alu*I: p1, containing 17 strains of serovars 1/2a, 1/2b and 4b; p2, containing one strain (A45) of serovar 4b; and p3, containing one strain (A46) of serovar 4c and ATCC 7645. This result shows the low degree of polymorphism in the *inlA* and *inlB* regions, which has already been shown in previous studies[6,16,17]. This has also already been observed in other genic regions associated with virulence of *L. monocytogenes*, such as the *mpl*, *prfA* and *iap* genes[8,18].

RAPD is considered to be an easy and quick intra-species technique, with a good capacity for differentiation[7,19]. RAPD has been used in several pieces of research to characterize strains of *L. monocytogenes*, observing their origins, contamination focus and epidemiology[16,17,20]. The primers used in the present study (UBC127 and UBC 155) presented good power of discrimination among the strains that were isolated. It was observed that the strains were separated into three main clusters: cluster I, containing serovars 1/2b, 4b and 4c; and clusters II and III, containing serovar 1/2a (A54, A55) as well as the strain control. Since RAPD analysis is based on the use of random-sequence primers, these results point towards a high level of polymorphism, particularly among serovars 1/2a and 1/2b. This had already been observed in research that used the primers UBC155, UBC156 and UBC157 in the RAPD technique, to analyze 39 strains of *L. monocytogenes* that were isolated from raw milk, smoked meat and chicken carcasses, belonging to serovars 1/2a, 4a, 1/2b, 3b and 4b[17]. The primers used in the present study had already been applied[19] to trace *L. monocytogenes* contamination in a shrimp-processing factory, from which it was concluded that this technique could be used to trace the contamination by *L. monocytogenes* in food-processing factories.

In conclusion, the results presented in this study show that strains isolated in this region remain susceptible to antibiotics commonly used in hospitals for treating listeriosis. The diverse serovars presented in strains of *L. monocytogenes* isolated from dairy products in southern Brazil showed a low degree of molecular diversity among the strains analyzed, and the main differences occurred between the strains belonging to serovar 1/2a and the other serovars studied.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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