

MOLECULAR ANALYSIS OF THE *IAP* GENE OF *LISTERIA MONOCYTOGENES* ISOLATED FROM CHEESES IN RIO GRANDE DO SUL, BRAZIL

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ABSTRACT

The polymorphic region sequences in the *iap* gene were analyzed in 25 strains of *Listeria monocytogenes* isolated from cheeses in the state of Rio Grande do Sul, and compared with reference strains. This investigation distinguished two clusters of *L. monocytogenes*: I (20 strains) and II (5 strains).

Key words: *Listeria monocytogenes*; *iap* gene; cheese; TN amino acids.

Listeria monocytogenes is an important foodborne pathogen responsible for causing outbreaks of listeriosis in humans and many animal species (10). The major clinical symptoms of *L. monocytogenes* infections in humans are abortion, septicemia, and meningitis (4). *Listeria monocytogenes* has been isolated from foods such as milk, dairy products, vegetables, sliced vacuum-packaged meat products, chickens, and meat (3,12).

Outbreaks of listeriosis show a correlation between infection and the ingestion of food, mainly dairy products contaminated with *L. monocytogenes* (5). The three serovars 1/2a, 1/2b, and 4b are related to most of the infections caused by this bacterium (8), and are the most prevalent in dairy products in Brazil (6).

The mechanism of pathogenicity in *L. monocytogenes* is related with the presence of the invasion-associated protein p60 encoded by the *iap* gene. The region encoding a central domain of protein p60 is characterized by the presence of a tandem repeat sequence (TRS) of ACAAAT, which correspond to the amino acids threonine and asparagine (TN). This domain has been used to determine the molecular variation between strains of *L. monocytogenes*. The *hly* gene has an important role in intracellular parasitism only in *L. monocytogenes*, because of the production of Listeriolysin O (LLO), a pore-forming exotoxin with hemolytic activity (15). The aim of the present

study was to draw a genotypic profile of *L. monocytogenes* isolated from cheeses in the state of Rio Grande do Sul, Brazil, by the nucleotide variations in the central domain of the *iap* gene compared with the reference strains F2365 and EGD-e.

L. monocytogenes strains isolated from cheeses and provided by the National Laboratory of Farming and Animal Husbandry in Porto Alegre (LANAGRO/RS) and the Department of Microbiology of the Federal University of Rio Grande do Sul (ICBS-UFRGS) were used in the present study (Table 1). The serovars were determined by the Oswaldo Cruz Institute, Rio de Janeiro, Brazil, using the methods described by Seeliger and Höhne (16). In order to analyze the morphology and confirm the culture purity, strains were grown in Listeria Enrichment Broth (LEB; Acumedia) for 18 hours at 37°C on a shaker and isolated on solid media (LEB, 1.5% agar-agar). Strains were stored at -70°C in glycerol. The chromosomal DNA was extracted as described by Agersborg and cols (1). The *L. monocytogenes* strains previously isolated were re-confirmed for genus and species by the PCR technique. All isolates were initially subjected to PCR with consensus primers GR/GF (5'-GCAACTATCGCGGCTACAGC-3' and 5'-CCAAGTTGCCCTAACAGATTTTC-3') to confirm the genus *Listeria* by amplification of 250 bp from the N-terminal domain of the *iap* gene (in this study). Each PCR reaction mixture (25 µL) contained: 2 mM MgCl₂, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.0 U

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Table 1. *Listeria monocytogenes* strains isolated from cheeses used in this study.

Strain	Serovar	TN	Substitution ^a	Deletion ^a	Insertion ^a	Genotype	Provider
EGD-e	1/2a	19	n.d.	n.d.	n.d.	I	Reference Strain
A54	1/2a	17	2	12	0	I	LANAGRO ^b
A55	1/2a	20	0	0	6	I	LANAGRO ^b
A5	1/2b	16	4	18	0	I	ICBS-UFRGS ^c
A11	1/2b	16	4	18	0	I	ICBS-UFRGS ^c
A20	1/2b	16	6	18	0	I	ICBS-UFRGS ^c
F2365	4b	15	n.d.	n.d.	n.d.	II	Reference Strain
A40	4b	15	0	0	0	II	LANAGRO ^b
A42	4b	15	0	0	0	II	LANAGRO ^b
A44	4b	15	0	0	0	II	LANAGRO ^b
A47	4b	15	0	0	0	II	LANAGRO ^b
A49	4b	15	0	0	0	II	LANAGRO ^b
A50	4b	15	0	0	0	II	LANAGRO ^b
A43	4b	15	0	0	0	II	LANAGRO ^b
A48	4b	15	0	0	0	II	LANAGRO ^b
A70	4b	15	0	0	0	II	ICBS-UFRGS ^c
A45	4b	15	1	0	0	II	LANAGRO ^b
A65	4b	15	0	0	0	II	ICBS-UFRGS ^c
qA69	4b	15	0	0	0	II	ICBS-UFRGS ^c
A32	1/2b	15	3	0	0	II	LANAGRO ^a
A33	1/2b	15	1	0	0	II	LANAGRO ^b
A34	1/2b	15	1	0	0	II	LANAGRO ^b
A35	1/2b	15	1	0	0	II	LANAGRO ^b
A36	1/2b	15	2	0	0	II	LANAGRO ^b
A37	1/2b	15	3	0	0	II	LANAGRO ^b
A38	1/2b	15	1	0	0	II	LANAGRO ^b
A39	1/2b	15	1	0	0	II	LANAGRO ^b

TN: threonine-asparagine amino acid within the tandem repeat sequence of the *iap* gene; ^a- number of nucleotides; ^b-National Laboratory of Farming and Animal Husbandry, Brazilian Department of Agriculture; ^c Microbiology Department, Federal University of Rio Grande do Sul; n.d.: not determined.

of *Taq* polymerase, and 25 ng of template DNA. A Thermal Cycler (MJ Research, Inc. PTC-100) was utilized to carry out the PCR reaction. The cycling parameters used were: 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 45°C and 1 min at 72°C, and a 5 min final extension at 72°C. PCR products were analyzed by gel electrophoresis in 1.5% agarose stained with ethidium bromide (0.5 µg mL⁻¹), observed in UV transillumination and photographed using Kodak Digital Science™ DC120. Negative controls included all reagents except DNA. *L. monocytogenes* ATCC number 7644 was used as a positive control.

To determine whether the genus primers were specific for the genus *Listeria*, template DNA from *Salmonella enteritidis*, *Enterococcus faecium*, *Escherichia coli*, *Bacillus circulans*, and *Staphylococcus aureus* were used, and any amplification was observed. All twenty-five positive strains were submitted to PCR

with a set of species-specific primers LR/LF to *L. monocytogenes* by amplification of 750 bp from the *hly* gene (2).

In order to illustrate the molecular variation among isolates, a DNA fragment of 550 bp amplified from primers SI4AD/SI4BD (11) which correspond to the central domain of the *iap* gene in *L. monocytogenes* were used to determine and analyze the nucleotide variation of protein p60. The amplified DNA fragment was purified using the Purification Kit (Pure Link, Invitrogen Life Technologies, São Paulo, Brazil) and sequenced by fluorescent dye-labeled dideoxynucleotides systems (BigDye Terminator V3.1 Cycle Sequencing Kit -Applied Biosystems) according to the manufacturer's protocol. The nucleotide sequences determined in this study were compared with reference strains EGD-e serovar 1/2a (GenBank accession number X52268) and F2365 serovar 4b (GenBank accession number NC_002973). A comparative analysis was carried out

using Molecular Toolkit for translation of the nucleotides and the public databases European Bioinformatics Institute CLUSTALW (<http://www.ebi.ac.uk/clustalw>) for multiple-alignment. Dendrogram was performed using the CLUSTALX Package (<http://www.uk.plbio.kvl.dk/bioinfo>).

The analysis of the *iap* DNA sequence and the comparison of reference strains EGD-e and F2365 allowed the distribution of the 25 strains in two clusters: I and II, as shown in Fig. 1. Cluster I, was subdivided in A and B, where the cluster IA was formed with the strains belonging to serovar 4b and cluster IB comprised eight strains of *L. monocytogenes* belonging to serovar 1/2b, all provided by LANAGRO. Cluster II comprised five *L. monocytogenes* serovars 1/2a and 1/2b alignment with reference strain EGD-e1/2a. In the reference strain EGD-e the tandem repeat DNA sequence was located at position 1373 to 1507 (7) and strains A54, A5, A11 and A20 showed a nucleotide substitution in position A1396G; strain A54 showed a substitution at A1402T. These same substitutions were previously reported by Ueda *et al.* (17) in *L. monocytogenes*

serovar 1/2b isolated from meat and patients. No nucleotide substitutions were detected in strain A55. Deletion in the tandem repeat DNA sequence was detected, and the predominant profile included all three strains of serovar 1/2b that displayed a gap of 18 nucleotides in the tandem repeat between positions 1408-1426. Strain A54 serovar 1/2a showed a deletion of 12 nucleotides at position 1414-1426. Another observation was that all serovars 1/2b had an A to G substitution at position 1549, and strain A42 showed nucleotide substitution at 5 positions; all these mutations were located out of the TRS. The tandem repeat in the reference strain F2365 is located at position 1373 to 1475 and in all the 20 strains substitutions, insertion or deletion of nucleotides was not detected. The dendrogram derived from the amino-acid sequence, in contrast to the DNA-sequence analysis, could not distinguish *L. monocytogenes* serovars (data not shown). Based on the protein alignment, a difference in length of 20 to 14 amino acids in the TN repeat was detected between the *L. monocytogenes* strains. Using the same parameter proposed by Schmidt *et al.* (13) to analyze *L.*

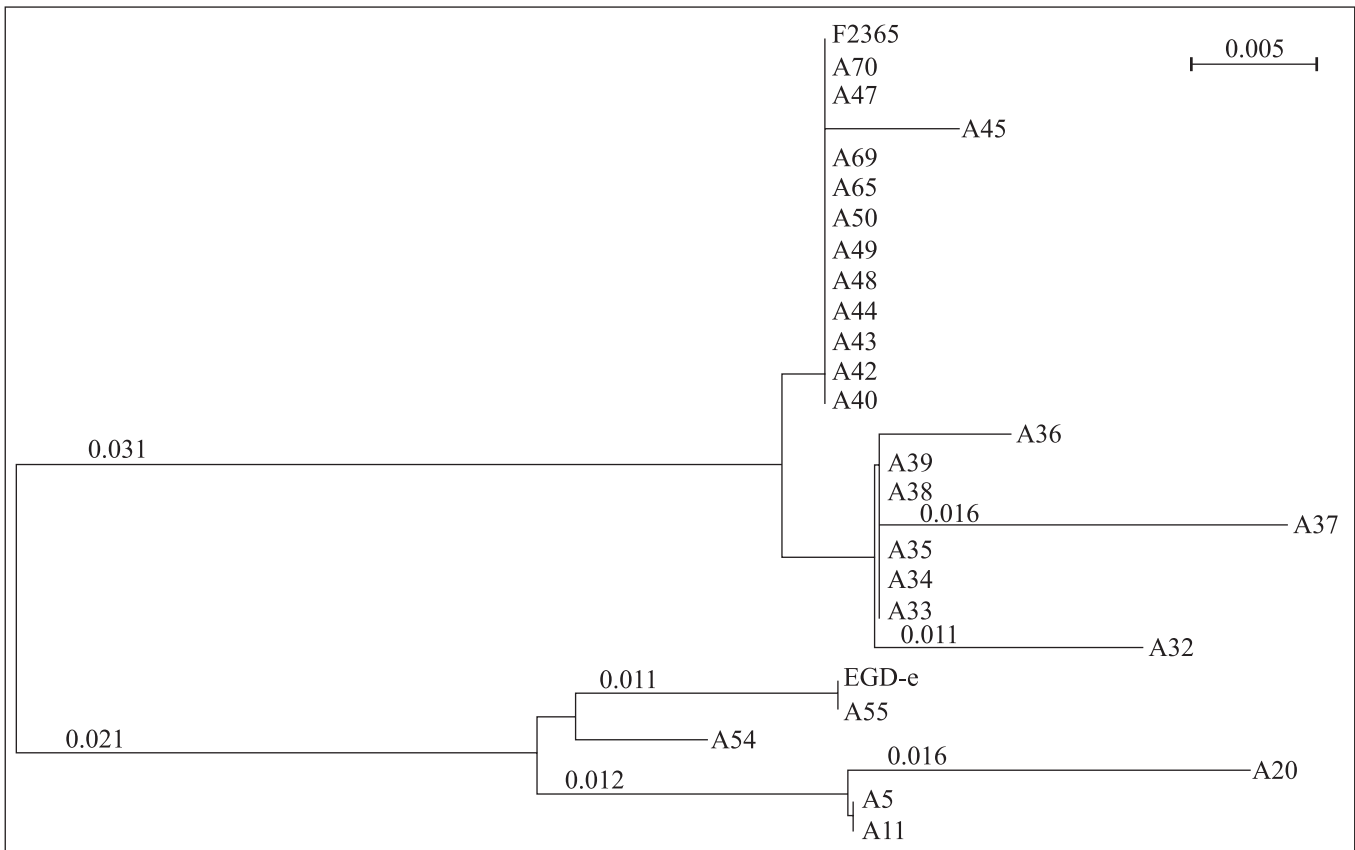


Figure 1. Dendrogram showing cluster analysis (unweighted pair group cluster method with arithmetic mean) of the 25 *Listeria monocytogenes* strains tested and the references strains *L. monocytogenes* F2365 serovar 4b and EGD-e serovar 1/2a. Cluster I and II, respectively.

monocytogenes from environmental sources, the strains were divided by the number of TN repeats present in the *iap* gene. We allocated strains into two genotypes: genotype I, with 20-16 TN number, and genotype II, with a 15 TN repeat (Table 1).

The common point in these strains is that they were isolated from the same geographical area in the state of Rio Grande do Sul. It is possible that the cheeses were contaminated by the same strain; however, samples were collected from different cheeses in different periods of the year. On the other hand, the antigen distribution occurring frequently among various *L. monocytogenes* serovars is the same; for example, 1/2b and 4b all have H antigens A, B and C; and 1/2a and 1/2b both contain the H antigens A and B. It can be a challenge to conclusively determine the serovar of some *L. monocytogenes* strains (14). Like biochemical methods, serotyping can give occasional discrepant results because of its dependence on the phenotypic characteristics of bacteria. For these reasons, serotyping methods have been superseded by molecular techniques that are more specific and sensitive for the identification and differentiation of *Listeria* species (9).

The deduced protein sequence resulted in alteration in the primary structure of the central domain of protein p60 in some strains. The major alterations were observed between the serovar 1/2a and also with three strains of serovar 1/2b that aligned with reference strain EGD-e. The complete analysis suggested that two genotype clusters of *L. monocytogenes* isolates are predominantly associated with cheeses in Rio Grande do Sul. One belongs to reference strain *L. monocytogenes* EGD-e serovar 1/2a, and the other to F2365 serovar 4b.

These results typify a molecular profile of *L. monocytogenes* in southern Brazil. Analysis using the central portion of the *iap* gene was shown to be efficient. Investigations of the *iap* region may contribute epidemiological, geographical, and molecular knowledge about this important human pathogen. Further investigations that include PFGE must be performed to extend our understanding of the predominant strains in Rio Grande do Sul and the possible current source of infection in this region.

RESUMO

Análise molecular do gene *iap* de *Listeria monocytogenes* isoladas de queijos no Estado do Rio Grande do Sul, Brasil

A seqüência da região polimórfica do gene *iap* foi analisada em 25 cepas de *Listeria monocytogenes* isoladas de queijo no Estado do Rio Grande do Sul e comparadas com cepas referências. Esta investigação distinguiu *L. monocytogenes* em dois grupos: I (20 cepas) e II (5 cepas).

Palavras-chave: *Listeria monocytogenes*; gene *iap*; queijo, aminoácidos TN

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