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Molecular serotyping and identification of the 85M fragment in different Colombian isolates of *Listeria monocytogenes* strains: A descriptive study

Serotipificación molecular e identificación del fragmento 85M de aislamientos colombianos de *Listeria monocytogenes*: Un estudio descriptivo

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Summary

Introduction: *Listeria monocytogenes* is a pathogen acquired through the consumption of contaminated foods. Thirteen serotypes have been reported, of which 1/2a, 1/2b, and 4b are responsible for 98% of human listeriosis cases. This study examines the association between serotypes and virulent clones, offering greater information and providing tools to prevent and control diseases caused by *L. monocytogenes* serotype 4b.

Objective: To identify the serotypes from *L. monocytogene* strains isolated from different samples by performing the molecular subtyping technique; to determine the 85M fragment that codifies for epidemic clone I.

Methods: 108 strains of *L. monocytogenes* were used, isolated from samples of animals, body fluids, foods, and food processing plant equipment and spaces. The samples were identified by following the Bacteriological Analytical Manual protocol described by the Food and Drug Administration (FDA). The strains were identified by Polymerase Chain Reaction (PCR) using primers and a standardized protocol from a previous research project. Serotype identification was performed by multiplex PCR. The determination of the 85M fragment of the SSCS cassette was done by following the protocol by Yildirim *et al.*

Results: Of the 108 *L. monocytogenes* strains analyzed, 60.2% (65 strains) belonged to the 4b-4d-4e serotype, 17.6% (19 strains) were identified as 1/2a-3a serotype, 14.8% (16 strains) were 4a-4c serotype, 3.7% (4 strains) belonged to the 1/2c-3c serotype, and (3.7%) corresponded to the 1/2b-3b-7 serotype. It was determined that the *L. monocytogenes* strains serotype 4b-4d-4e and 1/2a-3b have the 85M fragment of the SSCS cassette.

Conclusion: This study reports the predominant existence of *L. monocytogenes* strains serotype 4b-4d-4e in food, environmental, and clinical samples. The presence of an epidemic clone I region was also found in *L. monocytogenes* strains.

Keywords: *Listeria monocytogenes*; Listeriosis; Serotypes; 85M fragment; Isolation; Food.

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Resumen

Introducción: *Listeria monocytogenes* es un patógeno adquirido por el consumo de alimentos contaminados. Se han reportado 13 serotipos de los cuales 1/2a, 1/2b y 4b son responsables del 98% de las listeriosis humanas. Se ha

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estudiado la asociación entre serotipos y clones de virulencia, lo cual aporta información y brinda herramientas para la prevención y control de las enfermedades causadas por *L. monocytogenes* serotipo 4b.

Objetivo: Identificar los serotipos de *L. monocytogenes* aisladas de diferentes fuentes y determinar la presencia del fragmento 85M que codifica para clon epidémico I.

Métodos: Aislamiento de *L. monocytogenes* a partir de muestras animales, fluidos corporales, comestibles, equipos y ambientes en plantas de alimentos, según el protocolo FDA. Identificación por reacción en cadena de la polimerasa (PCR), serotipificación molecular por PCR multiplex y determinación del fragmento 85M según Yildirim *et al.*

Resultados: De las 108 cepas de *L. monocytogenes* serotipificadas molecularmente, 60.2% (65 cepas) pertenecen al serotipo 4b-4d-4e, el 17.6% (19 cepas) fueron 1/2a-3a, el 14.81% (16 cepas) son serotipo 4a-4c, el 3.7% (4 cepas) corresponden a 1/2c-3c y el 3.7% fueron aislamientos serotipo 1/2b-3b-7. Se determinó que todas las cepas serotipo 4b-4d-4e y 1/2a-13b contienen el fragmento 85M del clon epidémico I.

Conclusión: Este estudio reporta la existencia predominante de cepas de *L. monocytogenes* serotipo 4b-4d-4e en muestras de alimentos, ambientes y otras. Asimismo, evidencia la presencia de una región del fragmento 85M del clon epidémico I de *L. monocytogenes* en aislamientos pertenecientes al serotipo con más frecuencia relacionado con brotes de listeriosis en humanos (4b).

Palabras clave: *Listeria monocytogenes*; Listeriosis; Serotipos; Fragmento 85M; Serotipos; Aislamiento; Alimentos.

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The *Listeria* genus is found throughout nature: in soil, water, effluents, domestic animals, and a wide variety of foods (including dairy products, meats, and vegetables). Six species and two subspecies have been identified, of which *L. ivanovii* and *L. monocytogenes* are considered potentially pathogenic¹. Because of the latter's capability of contaminating multiple sources, people have historically believed all strains of this species cause diseases.

Listeria monocytogenes is an intracellular pathogen for humans and animals, which is acquired through

the consumption of contaminated foods. It causes listeriosis, an opportunistic invasive disease that can manifest itself in epidemic or sporadic cases. It leads to complications like meningitis, septicemia, miscarriage, and stillbirth^{2,3}.

To characterize the strains of this species that are associated with virulence, conventional serotyping has been used (based on O and H antigens) as a discriminatory method. This has led to identifying 12 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7^{4,5}. Given some restrictions of the serologic method itself (the presence of the same flagellin and somatic antigens in strains of distinct serotypes⁶ does not allow for 100% serotyping of isolates), the use of new techniques derived from molecular biology is becoming increasingly common.

Recent studies based on microarrays and catheters in three complete genomes of *L. monocytogenes* serotype 1/2a strain EGDe, *L. monocytogenes* serotype 4b strain CLIP 80459, and *L. innocua* serotype 6a strain CLIP 11262, allowed identifying specific genetic markers for the molecular determination of *Listeria* serotypes. These markers are: lmo0737 associated with serotypes 1/2a, 1/2c, 3a, and 3c, lmo1118 associated with serotypes 1/2c and 3c, ORF2819 for serotypes 1/2b, 3b, 4b, 4d, 4c, and the marker ORF2110 for serotypes 4b, 4d, and 4e⁵.

Serotypes 1/2a, 1/2b, 1/2c, and 4b are responsible for 98% of listeriosis cases in humans, where 4b is the most widely associated with epidemic outbreaks resulting from the consumption of contaminated foods. Serotypes 3a, 3b, 3c, 4a, 4c, 4e, 4d, and 7 are very infrequent in foods, which makes them rarely mentioned as causing human listeriosis. Serotypes 4a and 4c are associated with veterinary cases, and are rarely thought to be involved in outbreaks or human diseases^{4,7,8}.

The study of the molecular serotypes of the *L. monocytogenes* strains responsible for massive listeriosis epidemics has represented the foundation of the idea of virulence variability; this is because different techniques have demonstrated the existence of a small number of implicated serotypes and epidemic

clones⁹. The concept of an epidemic clone (EC) is used to refer to genetically related groups derived from the same ancestor, associated with outbreaks reported at different moments and geographic regions⁴. Until now, epidemic clones have been described (ECI, ECII, ECIII, and ECIV)^{4,10,11}, and although some studies report that clonal groups Ia and II play a vital epidemiological role¹², ECI is the most important and most studied, given that it has been involved in several listeriosis outbreaks in North America and Europe⁴.

The location of strains in the EC is done by detecting specific molecular markers. To group the *L. monocytogenes* strains in the ECI, the cassette of the 85M region (85M, 85R, and 85S) and specific GATC sites of the modification-restriction system (which according to Kocks in 2001 is specific for this clonal group¹³) are amplified. Previously attained results suggest that the strains of *L. monocytogenes* serotypes 4b and 1/2b are grouped in this clone (ECI); this makes it possible to infer that the *L. monocytogenes* strains that belong to one of the serotypes responsible for the highest number of human listeriosis caused by consuming contaminated foods belongs to ECI.

In Colombia, studies show that a high percentage of foods (cheeses, milk products, and meats) are contaminated with *L. monocytogenes*. A study carried out in Boyacá revealed the presence of this Gram positive bacteria in 26.9% of soft cheeses and in 25.9% of analyzed raw milk samples¹⁴. Another study demonstrated that it is possible to isolate this microorganism in 24% of ready-to-eat meat products sold in Bogotá's public markets¹⁵.

The pathogenicity of the *L. monocytogenes* strains that contaminate foods is influenced by factors like the population density of the microorganism at the moment of consumption and the type of contaminating strain (some clonal groups have greater pathogenicity than others). This suggests that only a fraction of *L. monocytogenes* strains are agents that cause diseases in humans¹⁶.

There are few epidemiological studies available in Colombia; this is because notification of the disease

is not obligatory¹⁷. However, the potential for *L. monocytogenes* to contaminate foods, spaces, and other sources, as well as its high mortality rate, reveals the risk consumers are being exposed to. This calls for the need to create policies for prevention and control in food processing plants, as well as the need to develop studies to determine the serotypes that circulate in our environment and that are grouped in the EC. All the information that can be gathered on this microorganism will contribute to the construction of the national panorama and provide tools to prevent and control diseases caused by *L. monocytogenes*. With this context in mind, the objective of this study was to determine the serotype of different *L. monocytogenes* strains isolated from humans, environments, and foods and to be able to associate them with the 85M cassette of the ECI.

Materials and methods

Bacterial isolates. This study used 108 strains of *L. monocytogenes* that were previously isolated and identified in the LEMA laboratory by using the protocol established by the Food and Drug Association (FDA) in its Bacteriological Analytical Manual (BAM) manual¹⁸. The confirmation of species was done through polymerase chain reaction (PCR) amplifying specific genes¹⁹. Strains were maintained at -70°C in Brain Heart Infusion broth (Oxoid, Basingstoke, United Kingdom) with glycerol at 1% up to the moment of analysis. The 108 studied strains were isolated from samples of animals, body fluids (clinical samples), foods, and food processing plant equipment and environments (Table 1).

As a positive control for the molecular serotyping and amplification of the 85M fragment, *L. monocytogenes* F2365 serotype 4b was used, donated by the University of North Carolina. *L. innocua* ATCC73016 was used as a negative control.

Molecular serotyping of *L. monocytogenes*. Oligonucleotides were used (Table 2), as well as the procedure established by Doumith *et al.*⁵ The final volume reaction was 100 µl: 1x of PCR

Table 1
Strains of *Listeria monocytogenes* analyzed through molecular serotyping*

Food	Serotype	N° isolates	Food	Serotype	N° isolates
Cheese	4b-4d-4e	11	Milk	4b-4d-4e	9
	1/2a-3a	3	Food processing	4b-4d-4e	18
	1/2c-3c	3	equipment and	1/2a-3 ^a	1
	4a-4c	1	environments	1/2b-3b	1
Meats (chicken, ham, sausage, bacon)	4b-4d-4e	10		4a-4c	13
	1/2a-3a	2		4b-4d-4e	12
	1/2b-3b	1	Clinical samples	1/2a-3 ^a	3
	4a-4c	2		1/2b-3b	1
Vegetables	4b-4d-4e	1	Animal samples	4b-4d-4e	4
	1/2a-3a	10		1/2c-3c	1
	1/2b-3b	1	Total	108	

* Strains donated by the National Health Institute

Table 2
Oligonucleotide sequences used for the molecular serotyping of *L. monocytogenes*

Target gene	Oligonucleotide sequence	Molecular weight	Amplified serotypes
lmo0737	F-AGGGCTTCAAGGACTTACCC R-ACGATTTCTGCTTGCCATT	691pb	1/2a, 1/2c, 3a, and 3c
lmo1118	F-AGGGGTCTTAAATCCTGGAA R-CGGCTTGTTTCGGCATACTTA	906pb	1/c and 3c
ORF2819	F-AGCAAAATGCCAAACTCGT R-CATCACTAAAGCCTCCCATTG	471pb	1/2b, 3b, 4b, 4d, and 4e
ORF2110	F-AGTGGACAATTGATTGGTGAA R-CATCCATCCCTTACTTTGGAC	597pb	4b, 4d, and 4e

F: Refers to the oligonucleotide (5'-3')

R: Refers to the oligonucleotide (3'-5')

GoTaq Green Master Mix (Promega Corporation, Madison, USA), 10 µM of each primer, nuclease-free water and 50 ng of DNA. *Salmonella enteritidis* ATCC 13076 was used as a negative control.

The PCR products were separated by electrophoresis in agarose gel at 2% during 60 min at 80 V and 400 mA. The molecular weight marker 100 pb-step

ladder (Promega Corporation, Madison, USA) was the standard to determine the molecular weight of the bands visualized in the ChemiDoc XRS System transilluminator (Bio-Rad Laboratories, Inc, USA).

PCR to identify the 85M fragment. A uniplex PCR was used to amplify the 85M fragment (product of the 471-pb amplification) that belongs to the SSCS

cassette of the epidemic clone I. The 85M-F indicators (AATATATTTCATGTTTGATGGT) and 85-R (GCTAATTCAATCCCTATTCT) were used, as well as the protocol established by Yildirim *et al.*¹⁶ The amplification reaction contained 12.5 µl of 2X PCR master mix (Promega corporation, USA), 1 µM of each primer, and 240 ng of DNA mold. The amplification was done by using a DNA denaturalization cycle at 94°C for 15 seconds, followed by 30 amplification cycles at 94°C for 3 seconds, hybridization at 53°C for one minute and 72°C for two minutes for each elongation. The final extension was done at 74°C for two minutes.

The amplification products were visualized in agarose gel at 2% dye with ethidium bromide, in the ChemiDoc XRS System transilluminator (Bio-Rad Laboratories, Inc, USA).

Results

The molecular weight of the multiplex PCR products obtained through electrophoresis in agarose gel was determined by using Quantity One 1-D analysis software, which uses the ChemiDoc XRS System transilluminator from Bio-Rad. The amplification of the positive control and the absence of

bands in the negative control demonstrated the technique's specificity (Figure 1).

A total of 60.2% (65) of the molecularly serotyped strains of *L. monocytogenes* are serotype 4b-4d-4e, of these, 47.7% (31 strains) correspond to milk, cheese, meat, and vegetable isolates. Some 27.7% (18) are from food processing environments (drains, walls, floors) or equipment (slicers, sealers). Another 18.5% (12) are from clinical samples, specifically *L. monocytogenes* isolated from cerebrospinal fluid and blood cultures. The remaining 6.2% (4) correspond to veterinary samples (uterus of pregnant cows).

Serotype 1/2b-3b was identified in 17.6% (19 strains) of isolates, and was particularly more frequent in food samples (78.9%, 15 of 19 strains) than in clinical samples (15.8%, 3 strains) or environmental samples (5.3%, 1 strain). There were no strains of this serotype found in *L. monocytogenes* isolates from veterinary samples.

Sixteen strains (14.8%) belong to serotype 4a-4c, which predominated in samples taken from food processing plant equipment (81.3%, 13 strains) and 18.8% (3 strains) corresponded to artisan jam sold in public markets.

For serotypes 1/2b-3b-7 and 1/2c-3c, the same number of *L. monocytogenes* strains (3.7%) was found

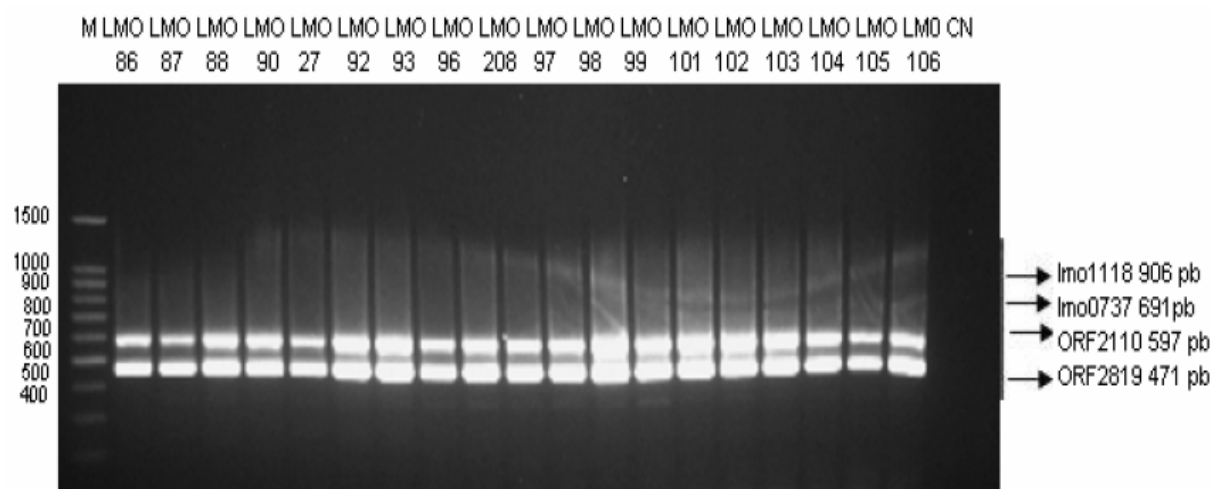


Figure 1. Multiplex PCR. Molecular serotyping of *L. monocytogenes*. Lane 1: M. Molecular weight marker (100 pb DNA step Ladder, Promega Corporation); Lanes 2-19: Environmental and *Listeria* food isolates. Lane 20: Negative control

for each (4). However, serotype 1/2b-3b-7 was identified in food (50%, 2 strains), food processing environments (25%, 1 strain), and clinical samples (25%, 1 strain), while serotype 1/2c-3c was found predominately in foods (75%, 3 strains) and was not found in other spaces.

In regards to amplifying the 85M region, the 471-pb fragment obtained for the *L. monocytogenes* F2365 strain (positive control) and the absence of contamination in the negative control (*L. ivannovii*) (Figure 2) demonstrated the specificity of this uniplex PCR.

Of the 108 *L. monocytogenes* strains evaluated, the presence of the 85M region of the cassette that identifies the ECI (Figure 2) was determined in 63.8% (69) of the strains. All the isolates that had the 471 pb band associated with the 85M fragment completely corresponded with *L. monocytogenes* serotype 4b-4c-4d. The amplification product was not obtained in any of the molecularly serotyped isolates.

Discussion

Over the last several years, researchers have looked for virulence markers specific for *L. monocytogenes* and

that can be used for epidemiological studies and the characterization of strains present in different environments¹³. In this descriptive study, the molecular serotyping technique was studied together with the amplification of a genetic marker that corresponds to the 85M fragment. The intention was to demonstrate that *L. monocytogenes* isolates identified as serotype 4b- 4d-4e could be associated with the presence of said fragment of the cassette that codifies for the ECI. Given the exploratory character of this research project, the presence of the three regions of the cassette was not determined, but an approximation was used to continue with the characterization of the serotyped Colombian isolates.

Although serotype 4a has been widely associated with animals^{20,21}, this study did not find it present in isolates from veterinary samples. This finding does not imply the absence of *L. monocytogenes* 4a-4c in this type of sample; on the contrary, it suggests that sample size should be increased to determine if this serotype is found in animals that produce foods or in food of animal origin.

With the exception of the samples of animal origin, at least one strain from each source evaluated in this study (foods, clinical samples, and food processing

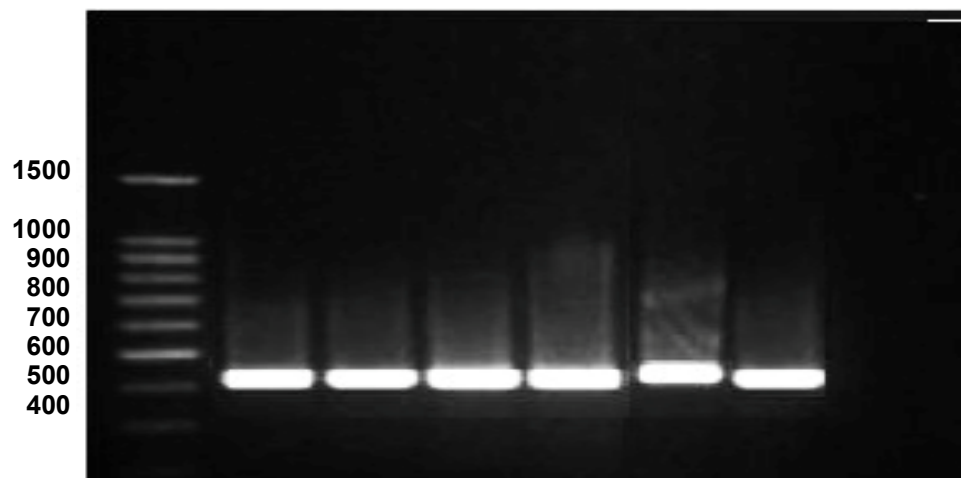


Figure 2. Uniplex PCR for the amplification of the 85M fragment in *L. monocytogene* strains that belong to different serovars. Lane 1: M. Marker of molecular weight (100 pb DNA step Ladder, Promega Corporation); Lanes 2-7: Environmental and Listeria food isolates serotype 4b. Lane 8: LMO 174, *L. monocytogenes* serotype 1/2a.

equipment and environments) was identified as belonging to the serotype 1/2a-3a, particularly, in vegetable and milk products. These results concur with previous studies, where this serotype is associated with outbreaks caused by ingesting contaminated foods²².

The results of this study show that all the strains belonging to the serotype 4b-4d-4e possess at least one of the fragments (85M) that make up the cassette used to identify the epidemic clone I. This relationship appears coherent and in accordance with previous studies where serotypes 4b-4d-4e was found grouped in clones I and II⁷. This information is of great importance to the food industry, public health, and risk-analysis studies; it reveals that potential highly virulent strains were found circulating in our environment, which is very significant for food safety. It is also important to highlight that most 4b-4d-4e serotypes found corresponded to food samples, coinciding with previous reports that demonstrate serotype 4b as the main cause of listeriosis cases^{4,23}. The presence of *L. monocytogenes* serotype 4b-4d-4e in food products ready for human consumption and in food processing equipment and spaces represents a health risk for consumers. The number of potentially affected consumers is currently unknown and difficult to determine because of limited data and the fact that notification is not obligatory for listeriosis cases in Colombia.

The use of the molecular marker 85M to identify ECI is useful as an initial method for screening and characterization of *L. monocytogenes* strains because it allows for the location of a specific clone depending on its genetic characteristics. It is necessary to continue conducting molecular studies that complement information on the genotypes of strains that have been identified as possibly belonging to ECI, as well as studies on the characterization of other virulent clones.

Conclusions

This study reports the predominant existence of serotype 4b-4d-4e strains in food, environmental,

and other samples. Furthermore, it reveals the presence of one region of the SSCS cassette of the *L. monocytogenes* epidemic clone I in isolates belonging to the serotype (4b) most frequently associated with human listeriosis outbreaks. Further studies should focus on identifying the other regions of the cassette (85F, 85R) to determine if strains belong to the epidemic clone I. Such information would contribute in advancing our understanding of the pathogenicity and diversity of this bacterium.

Conflict of interests. The authors declare having no conflict of interest whatsoever with this study's sponsor, the LEMA laboratory at Universidad de los Andes. Furthermore, the strains donated by the National Health Institute used in this study had the full consent of said Institution.

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