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Prevalence and serotype distribution of *Listeria monocytogenes* isolated from foods in Montevideo-Uruguay

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ABSTRACT

The aim of this work was to study the prevalence of *Listeria monocytogenes* in foods obtained in retail shops and food industries located in Montevideo-Uruguay, and to identify the serogroups of the obtained isolates. Three-thousand one-hundred and seventy-five food samples (frozen, deli meats, ready-to-eat and cheese) were analyzed. The obtained isolates were serogrouped by multiplex PCR and serotyped by conventional procedure. Genetic comparisons were performed using pulsed-field gel electrophoresis on a sub-set of isolates belonging to the same serotype successively recovered from the same establishment. *L. monocytogenes* was isolated from 11.2% of samples. The highest prevalence was observed in frozen foods (38%), followed by cheese (10%). 1/2b and 4b were the most frequently identified serotypes. In six of 236 analyzed establishments we successively recovered *L. monocytogenes* isolates belonging to the same serotype. Most of them corresponded to serotype 1/2b. Pulsed-field gel electrophoresis profiles suggest that at least 33% of *L. monocytogenes* 1/2b isolates are genetically related and that may remain viable for prolonged periods. The observed prevalence of *L. monocytogenes* was lower than reported in neighboring countries. Our findings highlight the role that frozen foods may play in the spread of this pathogen, and the relevance of serotypes 1/2b and 4b.

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Introduction

Listeria monocytogenes is a bacterial pathogen that causes a potentially severe disease both in people and animals, called listeriosis. Worldwide, it has been estimated that almost 90% of human cases occur after consumption of contaminated food. The most frequently implicated food-stuffs both in outbreaks and sporadic cases are soft cheese, frankfurters, unpasteurized milk, deli meats, smoked fish, dairy products, salads and refrigerated ready-to-eat products (RTE).^{1–5}

Listeriosis may occur as a mild febrile gastroenteritis or as a more severe invasive disease such as meningoencephalitis or sepsis. *L. monocytogenes* is capable of producing serious illness in pregnant women, newborns, elderly people and immunocompromised individuals (e.g. transplant recipients, patients receiving antineoplastic drugs or immunosuppressive therapy). In this high-risk group, listeriosis has a high lethality rate of 20–30%.^{4–7}

This bacterium is widely disseminated in different food production environments, including manufacturing and packing plants, distribution and storage facilities. It develops biofilms on equipment, walk-in freezers, drains, etc.^{8–10} This feature, added to its capacity for growth at cooling temperatures and survival through long freezing–thawing periods¹¹ are responsible for the increasing risks of food contamination with this bacterium.

Since 1980s, *L. monocytogenes* has emerged as a major public health burden. Nevertheless, its importance as a food-borne pathogen is not always recognized, due to a very long incubation period between consumption of the contaminated food and onset of illness as well as sporadic exposure. Nowadays *L. monocytogenes* represents a serious challenge to food safety and it is one of the microorganisms of significant concern the food industry.^{12–14}

While several phenotypic and genotypic methods have been described and applied to the study of *L. monocytogenes* isolates, the most frequently used are serotyping and DNA macro-restriction digest followed by pulsed-field gel electrophoresis (PFGE).^{15–23} PFGE remains as the gold standard method applied to the study of listeriosis outbreaks.^{19–22,24} Serotyping is an additional tool to characterize *L. monocytogenes* isolates.²³ To date, 13 serotypes have been defined for *L. monocytogenes*. More than 90% of the recovered strains from food, animal and human samples typically belong to serotypes 1/2a, 1/2b, 1/2c and 4b. The multiplex PCR assay previously described by Doumith et al.,²³ appears as practical screening procedure to identify these serotypes.

PFGE remain as the gold standard method applied to the study of listeriosis outbreaks.^{19–22,24}

The purposes of this work were to study the prevalence of *L. monocytogenes* contamination in various food samples obtained from retail store and food industries located in Montevideo city, Uruguay and to identify the main serotypes of the obtained isolates.

Materials and methods

Processing of food samples

Between October 2011 and August 2013, 3175 food samples were analyzed (220 were frozen products, both from animal and vegetable source; 2180 ready-to-eat foods, from animal and vegetable origin; 580 deli meats; and 195 cheese) at the Bromatological Laboratory (Intendencia de Montevideo). Samples were taken from 236 establishments (retail shops and food industries) located in Montevideo city, Uruguay as part of a food safety surveillance program.

For microbiological studies, pools of five products from the same establishment and food category were prepared (44 of frozen products, 436 of ready-to-eat foods, 116 of deli meats, and 39 of cheese, respectively) and were analyzed according to ISO 11290-1-1996 and ISO 11290-1:1996/Amd 1:2004 guide^{25,26} using a real-time PCR DuPont Qualicon BAX[®] System (DuPont Qualicon, Wilmington, DE, USA) following the manufacturer's instructions.

L. monocytogenes isolation and identification

Positive samples according to real-time PCR results were streaked onto two selective media plates: Oxford medium base with Modified Oxford Antimicrobial Supplement (BD – Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and BBL[™] CHROMagar[™] *Listeria* (BD), both incubated for 24–48 h at 35–37 °C. Suspect colonies (small, convex and surrounded by a black halo; blue colonies less than 3 mm in diameter and surrounded by a white halo, respectively) were identified using API *Listeria* kit (bioMérieux, Marcy l'Etoile, France) and additional standard biochemical assays that included: β -Hemolysis halo production on Trypticase Soy Agar (BD) supplemented with sheep blood (5%) plates, catalase reaction, bile esculin; and Christie-Atkins-Munch-Petersen (CAMP) tests with control strains of *Staphylococcus aureus* and *Rhodococcus equi*.²⁷

When food samples from retail shop or factory displayed positive results to *L. monocytogenes*, it was visited again and samples of the same food types were taken and analyzed according to the above described procedure.

Confirmed *L. monocytogenes* isolates were conserved in 10% reconstituted skim milk at –20 °C and also in 20% glycerol at –80 °C for further assays.

Serotyping

Multiplex PCR was used to determine *L. monocytogenes* serogroup as described previously.²³ Briefly, DNA was extracted from colonies grown overnight in Trypticase Soy Agar plates (BD) supplemented with sheep blood (5%). Colonies (4–5) were suspended in 50 μ L of lysis buffer (SDS 0.25%, NaOH 0.05 N) and boiled for 15 min. Then, 100 μ L of ultra-purified water was added to each tube and centrifuged at 15 000 $\times g$ for 15 min. Supernatant (2 μ L) was used in each PCR reaction.

Table 1 – Serotype distribution of *Listeria monocytogenes* in different analyzed food samples.

Serotype	Food category (number of analyzed pools)			
	Frozen (44)	Ready to eat (436)	Deli meat (116)	Cheese (39)
	Number of products (%) positive for a particular serotype			
1/2a	2 (11.76)	3 (7.14)	0 (0)	0 (0)
1/2b	6 (35.29)	19 (45.24)	6 (75)	2 (50)
1/2c	1 (5.88)	0 (0)	2 (25)	0 (0)
4b	8 (47.08)	20 (47.62)	0 (0)	2 (50)
Total	17 (100)	42 (100)	8 (100)	4 (100)

The amplification reactions were carried out in a Gene Amp PCR System 2700 thermocycler (Applied Biosystems®, Foster City, CA, USA). PCR products were run in 2% agarose gel and visualized by staining with ethidium bromide. *L. monocytogenes* serotype 1/2a (CIP 89381), *L. monocytogenes* serotype 1/2b (CIP 90602), *L. monocytogenes* serotype 1/2c (CIP 89756) and *L. monocytogenes* serotype 4b (CIP 88868) strains were used as positive controls and ultra purified water as negative control. Classical serotyping for *L. monocytogenes* 1 and 4 somatic antigens was carried out using commercially available *Listeria* Antisera (BD) according to manufacturer's instructions for strains belonging to PCR serogroups IIa, IIb, IIc and IVb.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed according to CDC procedure for *L. monocytogenes* using only the *ApaI* restriction enzyme.²⁸ This procedure was applied only to isolates belonging to the same serotype recovered from the same establishment. Briefly, bacterial suspensions were adjusted to an optical density of 1.4 at 610 nm, mixed with equal volume of 1.2% pulsed-field grade certified agarose (Bio-Rad, Hercules, CA, USA) solution pre-heated to 55 °C, and then placed into suitable molds. Plugs were washed six times with wash solution, cut in thick pieces (height ca. 2 mm) and digested for 2 h at 30 °C with 25 U of *ApaI* restriction enzyme (New England Biolabs Inc., Ipswich, MA, USA).

The separation of DNA fragments was performed by using a CHEF DR-II system (Bio-Rad). *Salmonella enterica* serotype Braenderup (CDCH9812) was included as molecular mass control. We also included one isolate belonging to serotype 1/2c, recovered in this study.

The bands were visualized by gel staining with 100 µg/mL ethidium bromide, and the image was captured with the FOTO/Analyst™ Investigator Eclipse FOTODYNE system (Thermo Fischer Scientific, Waltham, MA, US). PFGE patterns were analyzed using the BioNumerics software, 6.1 version (Applied Maths NV, Sint-Martens-Latem, Belgium). The Dice correlation coefficient was used to determine the similarity levels.²⁹ For the cluster patterns analysis the UPGMA (unweighted-pair group matching algorithm)^{24,30} was used with a tolerance and optimization of 1.5%. Strains that showed ≥80% similarity were considered as belonging to the same pulsogroup.¹³

Results

Occurrence of *L. monocytogenes* in different types of food

We isolated *L. monocytogenes* from 71 out of 635 (11.2%; 95% CI 8.75–13.65%) pooled food samples. The distribution of 71 *L. monocytogenes* isolates included frozen foods (17), ready-to-eat (42), deli meats (8), and cheese (4). The highest prevalence

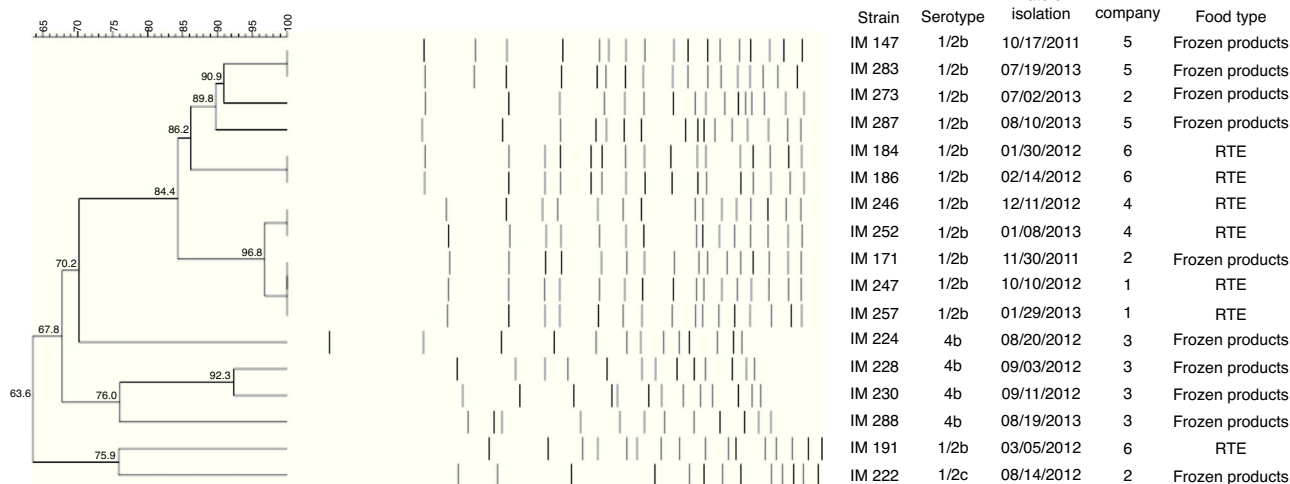


Fig. 1 – Dendrogram of *L. monocytogenes* isolates belonging to the same serotype and recovered from the same company. One isolate belonging to 1/2c serotype from this study is also included as control. Most 1/2b isolates analyzed by *ApaI* PFGE were in the same pulsogroup (similarity > 80%). RTE, ready-to-eat food (sandwiches, vegetable salads).

of *L. monocytogenes* contamination was observed in frozen foods (38%; 95% CI 24–52%), followed by cheese (10%; 95% CI 0.6–19.4%), RTE samples (9%; 95% CI 6.3–11.7%) and deli meat (7%; 95% CI 2.4–11.6).

Serotype determination and distribution in food

All isolates identified as serogroup IIa, IIb and IIc by multiplex PCR showed positive agglutination with serotype 1 antiserum; and were considered serotypes 1/2a, 1/2b and 1/2c, respectively. Because the serotypes 4d and 4e are rare both in food and human samples,²³ all the isolates that were PCR-positive for serogroup IVb were considered serotype 4b. In the present study 1/2b and 4b were the most frequently identified serotypes: 33 strains (45.83%) and 30 strains (41.67%), respectively; followed by 1/2a, 5 strains (6.94%) and 1/2c, 3 strains (4.17%). Table 1 shows the serotype distribution of *L. monocytogenes* isolated from food samples analyzed in this study.

PFGE profiles

In 41 out of 236 (17.4%) establishments we recovered *L. monocytogenes*, and in 27 (68%) of them this agent was again isolated in later visits. In only six cases (15%) we repeatedly recovered *L. monocytogenes* belonging to the same serotype (Fig. 1). Most of these isolates (12 out of 16) corresponded to serotype 1/2b and were recovered from food samples taken from five out of six establishments. In four out of five (#1, #4, #5 and #6) we identified 1/2b isolates with an identical PFGE pattern in successive visits (Fig. 1). The analyzed isolates from the sixth establishment (#3) corresponded to serotype 4b.

In four establishments (#1, #4, #5 and #6) we recovered successive 1/2b isolates that had an identical *ApaI* PFGE pattern to the first isolate (Fig. 1). Also, in two different companies (#1 and #2) we recovered two isolates (IM 257 and IM 171, respectively) belonging to serotype 1/2b that showed identical *ApaI* PFGE profile. In company #3, we detected four isolates belonging to serotype 4b in successive visits. Isolates IM 228 and IM 230 showed a 90% > similarity; whereas isolates IM 224 and IM 288, had a similarity of 63% (Fig. 1).

Discussion

The overall prevalence of *L. monocytogenes* contamination found in this set of analyzed foods (11.2%) is lower than that previously reported in Chile (25%)¹³ or in China (22%).³¹ Differences in prevalence may be due to the types of analyzed food in our investigation. We did not analyze pâté; instead, Montero et al. included 62 samples of this food category and they found that 55% were contaminated with *L. monocytogenes*.¹³ Likewise, prevalence figures lower than those found by us have been reported. In India, examining other food types involved in the spread of *L. monocytogenes* as milk, ice cream, fruit salad, meat and fish, Nayak et al. found an overall prevalence of 1.5%.³²

As was previously reported in China by Chen et al.,³¹ in the present study the highest prevalence of *L. monocytogenes* contamination was observed in frozen foods (38%) such as pizza, fish fillets, lasagna, ravioli, chicken, and meat; followed by cheese (10%) and RTE samples (9%) (sandwiches, vegetable

salads, chicken salads, Bolognese sauce, cheesecake and apple pie). These findings highlight the potential role that these food categories may play in the spread of this bacterium and alert to health personnel about the need to proper handling and cooking in individuals that have known high-risk of developing severe illness as pregnant women, elderly people and immunocompromised patients.^{4,5}

In our study, 1/2b and 4b were the most frequently recovered serotypes, followed by 1/2a and 1/2c. In Chile, Montero et al.,¹³ found a slightly different distribution; with 4b serotype (46%) being the most prevalent, followed by 1/2a (32%) and 1/2b (13%). The observed variation may be due to differences in the types of food included in each investigation. Likewise, in Colombia, Muñoz³³ found that the most prevalent serotypes isolated from 1424 food samples taken between 2000 and 2009 were 4b, 1/2b, 1/2a, with an evident predominance of strains belonging to 4b regarding the other serotypes (ratio of 5 to 1). In contrast, the most common serotypes isolated from RTE food (including natural cheese, meat products, seafood and pickled vegetables) in Japan were 1/2a (47.6%), 1/2b (20.6%) and 4b (14.3%).³⁴ Similar findings were reported in other countries of the northern hemisphere. In Poland, Korsak et al.,³⁵ analyzed 471 *L. monocytogenes* strains isolated from different foods and found the following serotype distribution: 1/2a, 54.4%; 1/2c, 25.5%; 1/2b, 12.5% and 4b 7.6%. In Ireland, O'Connor et al.¹⁴ found that the most common serotype present in different food categories was 1/2a followed by 4b. Also in China, Chen et al. found that the most prevalent isolates from raw foods corresponded to serotypes 1/2a and 1/2b.³¹ In the present study, 87% of the analyzed *L. monocytogenes* isolates belong to lineage I (1/2b and 4b); the remaining cultures belong to lineage II (1/2a and 1/2c). Both lineages include the most commonly recovered serotypes from human listeriosis cases. Our results contrast with other reports showed that strains belonging to lineage II are more prevalent in foods.^{15,36,37} These authors suggest that the higher genetic variation seen in lineage II strains is consistent with adaptation to more diverse environments.

As occurs in Chile, Colombia, Ireland, Japan, and other countries, serotypes recovered in the present study are responsible for human listeriosis cases.^{10,11,13,14,19,33,38,39} In Uruguay severe cases of listeriosis in people have been attributed to serotypes 1/2a, 1/2b and 4b isolates. In the same period in which this study was conducted, we received at the Department of Bacteriology and Virology laboratory 26 isolates of *L. monocytogenes* recovered from human samples; 14 of them belonged to serotype 1/2b, 9 to 4b and 3 to 1/2a (unpublished results).

Unlike what occurs in some northern hemisphere countries, *L. monocytogenes* belonging to serotype 4b appears to be prevalent in foods from several South American countries including Uruguay.^{13,33,40} Therefore, it would be interesting to perform other studies to test for a possible hemispheric correlation with *L. monocytogenes* serotypes. Especially taking into account the ability of this serotype to produce severe illnesses and also considering that some investigators suggest that 4b serotype strains may have a higher tendency to cause outbreaks when they are present in foods.^{33,41}

Apart from the deli meat samples, in the remaining analyzed food samples (frozen foods, ready-to-eat and cheese)

1/2b and 4b isolates accounted for over 90% of the isolates. This result suggest the role of these food categories in the spread of *L. monocytogenes* strains belonging to serotypes frequently recovered from human cases of listeriosis.

The PFGE results suggest that isolates belonging to serotype 1/2b have a capacity to survive for prolonged periods in the environment and/or food. In this regard, 19 months after the first visit at establishment #5 we recovered one isolate 1/2b that showed an identical *Apal* PFGE pattern to the first one. Similar observations were made at premises #1, #4 and #6, although in these cases the time elapsed between visits was lower (3 months, 27 and 15 days, respectively). These results agree with that previously suggested by Orsi et al. about the higher genetic stability of *L. monocytogenes* strains belonging to lineage I.³⁶

In two different companies (#1 and #2) and in different periods (10 month apart), we recovered two isolates (IM 257 and IM 171, respectively) belonging to serotype 1/2b with identical *Apal* PFGE profile. It could be because both establishments share product suppliers. On the other hand, in company #3, we recovered four isolates of serotype 4b from frozen food. Isolates IM 228 and IM 230 were recovered eight months apart and showed a similarity of 93%. Instead, isolates IM 224 and IM 288 were recovered one year apart and showed a 63% similarity. It is difficult to establish by PFGE whether these last pair it is the same isolate that suffered genetic modifications or they are two different isolates.⁴² We must analyze them by PFGE using another restriction enzyme or with other procedures such as MLST or WGS, among other typing methods, for proper characterization.^{16,43} They could even correspond to different serotypes, taking into account the aforementioned limitations of serotyping procedure used. The overall PFGE results obtained in the present study suggest that at least 11 out of 33 1/2b isolates recovered from different foods are part of a single pulsogroup (similarity $\geq 80\%$).

Conclusions

The present study provided the first data about food prevalence and serotype distribution of *L. monocytogenes* in foods in Uruguay. The highest prevalence was observed in frozen foods followed by cheese and RTE samples. Our results highlight the potential role that these food categories may play in the spread of this food-borne pathogen and alert to health personnel about the need to proper handling and cooking in individuals that have known high-risk of developing severe. 1/2b and 4b were the most frequently identified serotypes. The *Apal* PFGE results suggest that most 1/2b strains recovered from different foods are genetically related and that they may remain viable for prolonged periods in frozen food and/or food production, storage and distribution environments. These findings highlight the need to apply standardized sanitary and control practices to reduce the contamination with this pathogen in the food production chains.

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Conflicts of interest

The authors declare no conflict of interest.

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