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Characterization of *Listeria monocytogenes* isolates from cattle and ground beef by pulsed-field gel electrophoresis

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ABSTRACT

The aims of this study were to determine the occurrence of *Listeria monocytogenes* in cattle feces and ground beef, to characterize these strains by pulsed-field gel electrophoresis and to compare them to three listeria strains found in humans. Cattle from different origins (n = 250) and ground beef obtained from supermarkets (n = 40) were sampled. The results show low occurrence in cattle feces (0.4 %) but a higher presence in ground beef (37 %). An important part of the ground beef strains (80 %) had > 95 % similarity with a strain isolated from a human sporadic case and the ATCC 19115 used as control. The strain isolated from cattle feces had 93 % similarity to clone 009, previously associated with a listeriosis outbreak related to cheese. Cattle and ground beef can harbor virulent *L. monocytogenes* strains. Further studies in animals and animal products are needed to improve listeriosis control.

Key words: *Listeria monocytogenes*, cattle ground beef, pulsed-field gel electrophoresis, Chile

RESUMEN

Caracterización de aislamientos de *Listeria monocytogenes* obtenidos de ganado y de carne molida de vacuno por electroforesis de campo pulsado. Los objetivos de este estudio fueron determinar la presencia de *Listeria monocytogenes* en el ganado y en la carne molida de vacuno comercializada en Chile, caracterizar los aislados mediante electroforesis de campo pulsado y compararlos con los obtenidos en tres cepas que han producido listeriosis en humanos, en ese país. Se tomaron muestras de heces de bovinos (n = 250) y de carne molida obtenida en supermercados (n = 40). Se encontró una baja incidencia de este patógeno en las heces de bovinos (0.4 %; un solo animal), pero mayores porcentajes en la carne molida (37 %). Gran parte de las cepas encontradas en la carne molida (80 %) mostraron una similitud mayor del 95 % con un caso esporádico de listeriosis y con la cepa de referencia ATCC 19115. La cepa aislada de bovino tuvo un 93 % de similitud con el clon 009, responsable de un brote asociado al consumo de queso, ocurrido en 2008. Se concluye que el ganado y la carne molida pueden albergar cepas virulentas de *L. monocytogenes*. Se necesita un mayor número de estudios en animales y en los productos que se obtienen de ellos para mejorar el control de la listeriosis.

Palabras clave: *Listeria monocytogenes*, carne molida de vacuno, electroforesis de campo pulsado, Chile

*Listeria monocytogenes* is a foodborne pathogen that can cause listeriosis, a severe disease that can lead to septicemia, meningitis, meningoencephalitis and spontaneous abortion or stillbirth, and affects mainly the elderly, pregnant women, newborns and immunocompromised adults (8).

Studies have implicated contaminated foods of animal origin such as cheese, milk and beef in the transmission of the bacteria to humans. The prevalence of *L. monocytogenes* contamination of raw and processed meat products can be high (from 1 to 70 %) and also high percentages (11 to 52 %) of farm animals are healthy fecal carriers of the pathogen (8). Evidence links strains of human listeriosis cases with healthy (4) as well as sick cattle (7). Since there is a high prevalence of *L. monocytogenes* in cattle and raw meat, it is recommendable to study the possible links between them and listeriosis.

In Chile there were only sporadic cases of the disease until 2007, with low and stable rates (3 cases per million). This trend changed when a big outbreak of listeriosis that included 165 cases (10 cases per million) and caused 14 deaths was reported in 2008. The foods involved were two types of cheese. A pulsed-field gel electrophoresis (PFGE) based study, conducted by the Public Health Institute of Chile (ISP: Instituto de Salud Pública, in Spanish), revealed that the pathogen involved was a single *L. monocytogenes* clone (clone 009). During 2009, another listeriosis outbreak, this time associated to sausages and other...
meat by-products, was detected. The findings of the ISP linked this outbreak to a PFGE-strain designated as clone 001. In this case, there were 73 listeriosis cases (4.5 cases per million) with a lethality rate of 25 %. During 2010, there were 68 sporadic cases reported (4.2 cases per million) with a lethality rate of 22 % (http://epi.minsal.cl/epi/html/boletos/reportes/Listeriosis/Informe%20Listeria%202010.pdf). Considering such frequency and lethality rates, it was most important to study the origin of the food contamination with *L. monocytogenes* in order to establish improved prevention systems and to further our knowledge on the probable origin of listeriosis in Chile. PFGE was selected as the subtyping method because it has shown accurate, discriminatory and reproducible results (4).

The objectives of this study were: i) to determine the occurrence of *Listeria monocytogenes* in cattle of diverse origins and in ground beef ready for sale in different supermarkets, both data presently not available in Chile; ii) to characterize the isolates by PFGE; and iii) to compare these strains with 3 strains that had caused previous human listeriosis cases in Chile.

A total of 250 bovines from different origins, sex and ages were sampled during 2009 for requested studies (Table 1). The feces samples were obtained directly from the rectum of the cattle using sterile cotton swabs. Slaughterhouse samples were taken after the animal was sacrificed and dairy samples were taken at the time of the cow’s reproductive control. Each sterile swab that was transported in a sterile tube with 2 ml Cary-Blair medium. Samples were identified and stored in clean coolers with ice packs and sent to the laboratory. Samples were processed within 2 hours of collection. The swabs were plated directly on Oxford and Palcam agars (Oxoid Ltd., Cambridge, UK) and were incubated for 24-48 h at 37 °C. Subsequently, the swabs were incubated in Listeria Enrichment broth (Oxoid) for 4 h. After that time, the following selective agents were added: acriflavine (7.5 mg/500 ml) and nalidixic acid (20 mg/ml). The culture was incubated at 30 °C for 44 h, and then the swabs were plated on Oxford and Palcam agars and incubated for 24-48 h at 37 °C. The typical colonies were identified by macroscopic observation, Gram staining and biochemical tests (production of hemolysins and catalase, growth at 25 °C and motility). Additionally, the identification of *L. monocytogenes* was confirmed by PCR with primers derived from the iap gen (MonoA 5’-CAAACCGCTACAACAGCTACT3’ and MonoB 5’-GCACTTGAATGCAGCATTG3’) using the PCR protocols described by Bubert et al. (1).

On the other hand, a total of 40 trays of 10 % fat ground beef were sampled from 6 supermarkets (named A to F) of the Metropolitan Region, Chile, during November and December 2008. The samples

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
<th>Sample</th>
<th>Cattle origin (Region)</th>
<th>Cattle origin (City)</th>
<th>Young&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Adult&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Old&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Total</th>
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<td>Osorno</td>
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<td>Puerto Montt</td>
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<td>5</td>
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<td>RM&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Colina</td>
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<td>Calera Tango</td>
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<td>7</td>
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</table>

<sup>1</sup> < 4 years old; <sup>2</sup> 4 to 8 years old; <sup>3</sup> > 8 years old; <sup>4</sup> Metropolitan Region

Table 1. Cattle sampled during 2009 from a Metropolitan Region slaughterhouse and three dairies
were transported separately in clean coolers with ice packs for transit to the laboratory. Samples were processed within 2 hours of collection. *Listeria* spp. were detected by the BAX® System (DuPont). The positive BAX-*Listeria* spp. ground beef were processed according to the FDA Bacteriological Analytical Manual (BAM: http://www.fda.gov/food/scienceresearch /laboratorymethods/bacteriologicalanalyticalman ualbam/ucm071400.htm).

Typical *Listeria* spp. colonies were identified by macroscopic observation, Gram staining, and biochemical tests (production of hemolysins and catalase, growth at 25 °C and motility). The isolates were confirmed as *L. monocytogenes* by PCR as stated above.

Confirmed *L. monocytogenes* isolates were compared with 3 strains from human listeriosis cases available at the Microbiology and Probiotics laboratory of the Food Technology and Nutrition Institute (INTA: Instituto de Nutrición y Tecnología de los Alimentos in Spanish), University of Chile. The strains were: clone 001, clone 009 and one strain that came to the laboratory from a sporadic case of listeriosis in 2008. Furthermore, the isolates were compared with 2 *L. monocytogenes* strains isolated from ground beef bought in 2011 at one of the supermarkets of the study, in order to determine the possible persistence of the strain in this environment.

PFGE was carried out following the Centers for Disease Control and Prevention (CDC) standardized PulseNet protocol for *L. monocytogenes* (http://www.cdc.gov/pulsenet/protocols/pulsenet_listeria_protocol%20.pdf), with *Ascl* (New England Biolabs, Massachusetts, USA) and *ApaI* (Promega Corporation, Wisconsin, USA) as the restriction endonucleases. The PFGE patterns were analyzed using the Gel ComparII Software (Bionumerics © 2011 Applied Maths NV), that uses the standard strain *Salmonella enterica* serovar Braenderup H9812 loaded in three lanes in each gel to normalize the images. Also, the reference strain *L. monocytogenes* ATCC 19115 was used as control. Matching and dendogram UPGMA (unweighted pair group method with averages) analysis of the PFGE patterns was performed using the Dice coefficient with a 1.5 % tolerance window.

Of the 250 bovine sampled (Table 1), only one was positive for *L. monocytogenes* (occurrence of 0.4 %). The positive animal was an old cow from a farm of the X Region of Chile. Of the 40 ground beef sampled, 15 were positive for *L. monocytogenes* (occurrence of 37.5 %), being present in 5 of the 6 supermarkets sampled. The supermarket designated as "C" had 67 % of the total positive samples (Figure 1).

The analysis of macrorestriction patterns obtained by PFGE-*ApaI* showed 68 % similarity of the 22 strains studied, distributed in 2 clusters: I and II as shown in Figure 1a. Closely related isolates (greater than 90 % similarity in banding patterns) were assigned a PFGE group. Cluster I had 3 isolates and included clone 001 and a ground beef strain (BS78) with a similarity of 83 %. Cluster II had 19 strains and included the PFGE-*ApaI* group 1 that grouped 15 strains with 92 % similarity. This group contained 80 % (12/15) of the ground beef strains, the strain isolated in the human sporadic case of 2008 (77-3), the ATCC 19115 and a ground beef isolate recovered in 2011 from one of the supermarkets (AL 112). All 5 supermarkets positive for *L. monocytogenes* had this group. The strain isolated from cattle feces had a 93 % similarity to clone 009 associated with a listeriosis outbreak in 2008, both strains grouped in PGFE-*ApaI* group 2 (Figure 1a). The results of the PFGE patterns obtained with *AscI* enzyme are shown in Figure 1b. All strains showed 48 % similarity distributed in 3 clusters (I, II and III). Cluster I included 18 isolates, 14 of which had > 91 % similarity and were grouped in PFGE-*Ascl* group 1, as it had the same strains of PFGE-*ApaI* group 1. The exceptions were: strain AL113, present in PFGE-*Ascl* group 1 with an indistinguishable profile compared with the sporadic case and other 2 ground beef strains; AL112 and BS75 were absent in PFGE-*Ascl* group 1. With this enzyme, clone 009 and the strain isolated from cattle feces had a similarity of 89 %. Cluster II had 2 strains, clone 001 and BS78 with a similarity of 87 %. Cluster III also had 2 strains having a similarity of 75 % (Figure 1b).

The occurrence of *L. monocytogenes* in apparently healthy cattle was relatively low with respect to international data that had found a prevalence of 3.1 % to 46 % of *L. monocytogenes* in animal feces (2, 6). We believe that this result could be slanted towards a lower value, because it was based on specific prevalence studies. Guided sampling can lead to a high error value that has to be considered. However, these results can serve as a starting point for conducting further studies involving a higher number of samples and fully random methods. Moreover, the great variability among regions, including productive methods and feeding system, should be considered, as it is known that the infection of animals with *L. monocytogenes* is associated to the ingestion of silage harboring high counts of this pathogen (6). More
information about the presence of this pathogen in cattle and other animals are urgently needed to study the transmission dynamics and ecology of *L. monocytogenes* in the Chilean primary food production system.

The only strain isolated from cattle in this study had 93% similarity to clone 009. Although in this case the contamination was associated with the processing plant, we can hypothesize that the origin of this virulent strain could be traced back to an animal whose biofilm became adapted to the food processing facilities. The involved company had goats
and other animals near the processing plant that could have provided the primary contamination.

The occurrence of *L. monocytogenes* obtained in ground beef is consistent with other studies conducted in raw meat. For example Samelis and Metaxopoulos (9), and Gudbjörnsdóttir et al. (5) detected 51% and 15.6%, respectively of *L. monocytogenes* in raw beef. Although listeriosis is mainly associated with the consumption of ready-to-eat foods, it is important to establish that disease can result from intentional raw consumption, undercooking or cross-contamination of meat (11). In fact, there are associations of sporadic listeriosis with the consumption of insufficiently cooked chicken (10) and undercooked hamburgers. Intentional raw ground beef consumption is rare in most countries but it is relatively widespread in continental Europe, especially Belgium, France and Holland, where it is consumed as “tartare” also known as “filet americain” (11).

Based on the results obtained in the PFGE, we could link one profile of *L. monocytogenes* in most of the ground-beef sampled in different supermarkets. It appears that the origin of the contamination with *L. monocytogenes* came from a common origin (meat from slaughterhouse or wholesale distributor) that was distributed to 5 out of the 6 supermarkets sampled. In one attempt to confirm the persistence of this strain in the supermarket environment, 2011 ground-beef samples were taken. Results showed that the 2 isolates found in these samples had high similarity with the isolates previously found during 2008. PFGE group 1 also included a strain from cerebrospinal fluid obtained from a 49-year-old woman with ophthalmoplegia. This sporadic listeriosis case that occurred in 2008 had no relation to that year’s outbreak. Further studies of our laboratory also link PFGE group 1 with *L. monocytogenes* isolated from ground turkey packaged in the processing plant (3), suggesting a widespread distribution of this strain in Chile.

The finding of a common PFGE type between ground beef and a sporadic case of listeriosis can lead us to think that raw animal products can be the cause of sporadic cases if they are not eaten or manipulated properly. In this regard, it seems important to define if this group is also linked to other food matrices. This task could be facilitated as PFGE group 1 includes ATCC 19115 that can be used as *L. monocytogenes* PFGE control.

The results presented here suggest that cattle and ground beef can be contaminated with virulent *L. monocytogenes*. Based on this fact, it seems necessary to include this pathogen among the Hazard Analysis and Critical Control Point (HACCP) plans of this matrix. The risk assessment of *L. monocytogenes* in meat products should be a valuable tool to evaluate the real impact of the consumption of meat and meat by-products regarding listeriosis cases and also to devise better prevention plans to control human listeriosis.

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