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Characterization of *Listeria* spp. isolated from ready-to-eat products in Argentina using SDS–PAGE and restriction endonuclease

K.E. Pellicer [a], J.A. Copes [a], E.O. Nosetto [b], M.G. Echeverría [b,*]

[a] *Laboratory of Food Technology, Faculty of Veterinary Sciences, National University of La Plata (FCV-UNLP), CC 296, 60 and 118, 1900 La Plata, Argentina*
[b] *Laboratory of Virology, CONICET, Faculty of Veterinary Sciences, National University of La Plata (FCV-UNLP), CC 296, 60 and 118, 1900 La Plata, Argentina*

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Abstract

Listeria spp. are considered of interest in public health since their presence indicates the potencial existence of *L. monocytogenes*. Total cellular proteins and DNA from four strains of *L. monocytogenes* serotype 4, four strains of *L. monocytogenes* belonging to serotype 1, twelve strains of *L. innocua*, four strains of *L. seeligeri* and two strains of *L. welshimeri* isolated from ready-to-eat food were studied by SDS–PAGE and restriction endonuclease digestion. SDS–PAGE protein profiles obtained were species specific and could be evaluated by visual comparison. Enzyme for restriction endonuclease analysis was *Eco*RI, discriminating *L. monocytogenes* from other *Listeria* spp. These methodologies might be a helpful tool and a good alternative for epidemiological tracking of listeriosis in laboratories, where other methods are not available.
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1. Introduction

Listeriosis, a fatal infection (up to 30%) in human and animal species, presents severe consequences such as abortion, septicemia, meningitis and encephalitis (Seeliger & Jones, 1986).

Although *Listeria monocytogenes* is the major public health concern, the nonpathogenic species *L. innocua* and *L. welshimeri* are considered of interest in food microbiology since their presence indicates the potential presence of *L. monocytogenes* (Donnelly, 1994). *L. monocytogenes* has been isolated from raw and pasteurized milk, soft cheese, ice cream, raw meat and poultry, raw fish and shellfish, ready-to-eat products, fermented sausages, vegetables, food processing plants and soil (Gellin & Broome, 1989).

The presence of *Listeria* spp. in thermaly processed food suggests post-process contamination, or an insufficient thermal process (Holsinger, Rajkowski, & Stabel, 1997; Slutsker, Altekruse, & Swerdlow, 1998).

The Department of Food Control has adopted a zero tolerance policy for *L. monocytogenes* in ready-to-eat food products (Código Alimentario Argentino).

During recent years there has been an increase in consumption of ready-to-eat foods, and in the laboratory of Food Technology of the FCV-UNLP we have isolated several strains of *Listeria* spp. from different sources (Copes et al., 2000; Pellicer et al., 2002a, 2002b).

Several methods have been used for identification and classification of *Listeria* spp.: serotyping, phagetyping,

* Corresponding author. Fax: +54 221 489 6726.
E-mail address: gecheverria@fcv.unlp.edu.ar (M.G. Echeverría).

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biochemical characterization, plasmid profile and pulsed-field gel electrophoresis (PFGE) (Mclauchlin, Audurier, & Taylor, 1986; Pérez-Dıaz, Vicente, & Baquero, 1982; Seeliger & Hohne, 1979). DNA fingerprints are useful for epidemiological analysis of phenotypically indistinguishable *Listeria* spp. (Nocera, Bannerman, Rocourt, Jaton-Ogay, & Bille, 1990; Wesley, Wesley, Heisick, Harrell, & Wagner, 1990).

As would a mention in their work (Wesley & Ashton, 1991) the World Health Organization Expert Committee on Listeriosis has proposed as digestion of chromosomal DNA with specific endonucleases as an alternative method for visualizing genetic changes that may not be detected by protein-based analysis. There is evidence that cell-surface proteins of *Listeria* spp. are involved in their virulence (Galdiero, D'Isanto, & Aliberti, 1997). But there has not been much research on their characterization (Tabouret, De Rycke, & Dubray, 1992).

The detection of *Listeria* spp. is currently achieved by enrichment procedures in selective broth followed by identification using biochemical test that are time consuming. In Argentina, the reference laboratory uses serotyping for identification and classification of *Listeria* spp.

The purpose of this study was to characterize strains of *Listeria* spp. isolated from ready-to-eat products by restriction endonuclease analysis of total DNA and proteins profiling, in order to evaluate the utility of these methods for use in Argentina laboratories.

Table 1
List of *Listeria* strains isolated from ready-to-eat products used in this study

Strain designation	Species (serotype)	Origin
ATCC 19115	*L. monocytogenes* (4b)	Reference
355/98 (H 85)	*L. monocytogenes* (1)	Reference
3J	*L. monocytogenes* (4)	Salad
CT	*L. monocytogenes* (4)	Clinic case
A1	*L. monocytogenes* (4)	Soft cheese
A2	*L. monocytogenes* (4)	Soft cheese
A3	*L. monocytogenes* (4)	Soft cheese
5	*L. monocytogenes* (1)	"Chorizo"
8T	*L. monocytogenes* (1)	"Chorizo"
S1	*L. monocytogenes* (1)	Salami
S2	*L. monocytogenes* (1)	Sausage
ATCC 33091	*L. innocua*	Reference
6T	*L. innocua*	Salami
129	*L. innocua*	Salami
LS	*L. innocua*	Large sausage
CC	*L. innocua*	"Chorizo"
2T	*L. innocua*	Salami
3T	*L. innocua*	Salami
2T′	*L. innocua*	Salami
5T′	*L. innocua*	Salami
1T	*L. innocua*	"Chorizo"
2	*L. innocua*	"Chorizo"
4	*L. innocua*	"Chorizo"
SP	*L. innocua*	Salad
PF1T	*L. welshimeri*	Salami
PF2T	*L. welshimeri*	Salami
7	*L. seeligeri*	Large sausage
8	*L. seeligeri*	Large sausage
9	*L. seeligeri*	Large sausage
10	*L. seeligeri*	Large sausage

2. Materials and methods

2.1. Strains

From a total of 190 ready-to-eat products including 50 salads, 35 soft cheese, 50 salami, 10 "chorizo" (Spanish fermented sausage), 10 different sausages and 35 ice creams, a total of 26 strains were isolated. These isolated were from soft cheese (three isolates), salads (two isolates), salami (10 isolates), "chorizo" (six isolates), sausages (six isolates). Strains used throughout this study, including reference strains and their sources are listed in Table 1. *L. monocytogenes* from a bovine clinical case was included. The sampling isolation and identification methods for the *Listeria* spp. isolates from ready-to-eat products were describe earlier (Copes et al., 2000; Pellicer et al., 2002a, 2002b). Briefly, 25 g of sample was homogenized in 225 ml of Bleb (Oxoid CM862) and incubated at 28 °C for 24 h; then 1 ml of this broth was inoculated into 9 ml of Bleb and incubated for another 24 h more to complete 48 h at the same temperature (FDA). Finally, broth were inoculated in PALCAM (Oxoid CM877) and incubated at 37 °C for 48 h. After colony identification, biochemical procedures were carried out. The serological identification was carried out at the Instituto Nacional de Enfermedades Infecciosas (INEI/ANLIS) using commercial antisera for serotypes 1 and 4 (Pasteur Institute).

Reference strains *L. monocytogenes* type 1 355/98 (H 85), type 4b ATCC 19115 and *L. innocua* ATCC 33091 were obtained from the INEI/ANLIS.

2.2. SDS–PAGE

Each strain was inoculated into 25 ml brain heart infusion broth (BHI) in a 50 ml centrifuge tube and incubated at 37 °C for 24 h. The cells were then centrifuged (12,000*g*, 10 min) and resuspended in 1 ml 50 mM Tris–HCl, pH 7.5 containing 10 mg/ml of lysozyme and incubated during 3 h at 37 °C. Lysates were treated with 0.05 ml 20% SDS and heated in a microwave at 50 Hz (SANYO EM-526T (W) JAPAN), three times for 30 s each. Finally, the lysates were diluted 1:1 in distilled water, centrifuged at 20,000*g* 20 min. Supernatants were diluted in sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.002% bromophenol blue, 0.02 M Tris–HCl) and boiled for 3 min at 100 °C (Gómez-Zavaglia, Abraham, Giorgeri, & de Antoni, 1999). SDS–PAGE were performed according to the method of Laemmli (1970) in 12% polyacrylamide gels at 150 V in 170 × 190 × 1 mm, using Tris–glycine buffer (25 mM

Tris, 250 mM glycine, 0.1% SDS, pH 8.9) as the electrophoresis buffer.

The low molecular weight marker (Pharmacia: LMW Market Kit M_r range 14,400–94,000. Code No. 17-0446-01) was used. Following electrophoresis, gels were stained with Coomassie blue.

2.3. Chromosomal DNA

Genomic DNA was purified according to the method of Nocera et al. (1990), with some modifications. The strains were grown overnight at 37 °C in BHI broth. After centrifugation at 12,000*g* for 10 min, pellets were washed with TES buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8) and resuspended in 540 μl of 25 mM Tris–10 mM EDTA, 50 mM glucose, pH 8, and treated with 60 μl lysozyme at 10 mg/ml at 37 °C for 30 min. Proteinase K (2 μl of 20 mg/ml) and 6 μl of 10% SDS were then added, gently mixed and incubated at 50 °C for 60 min. DNA was extracted with 1 volume of phenol–chloroform–isoamylalcohol mixture (25:24:1). The aqueous phase of the lysate was separated and DNA was precipitated after addition of 0.1 vol of 3 M sodium acetate and 2.5 volume of cold ethanol. DNA was collected by centrifugation at 20,000*g* for 30 min, washed with 70% ethanol, dried and resuspended in TE buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8).

The restriction enzymes *Hin*dIII, *Eco*RI and *Hha*I (10 U/μl each) were used as recommended by the manufacturers. Digestions consisted of 5 μg DNA approximately, 10 U of each enzyme, and RNAse in a volume of 20 μl with overnight incubation at 37 °C. Electrophoresis was done in 0.6% agarose with TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) as running buffer at 20 V overnight. After electrophoresis gels were stained with ethidium bromide (10 mg/l) and photographed on a UV transilluminator using Polaroid film 665. Lambda DNA restriction fragments (*Hin*dIII + *Eco*RI) were employed as size standards.

3. Results

3.1. Proteins

SDS–PAGE showed approximately 30 bands with molecular weights ranging between 20 and 120 kDa. Total protein patterns were strictly specific for species (Figs. 1–4), and permits distinguish between pathogenic and non-pathogenic strains (Fig. 4). Only a few major heavily stained bands appeared common to all species and were mostly located between 30 and 120 kDa, except for a 40 kDa band that apparently had minor mobility in PF1T strain of *L. welshimeri*. Differences could be seen between protein patterns of *L. monocytogenes*, *L. innocua*, *L. seeligeri* and *L. welshimeri* by visual comparison because of the high resolution of gels. Apart from common bands (30, 40, 43, 64, 94 and 120 kDa) strains *L. monocytogenes* serotype 1 were characterized by high stained bands of 62, 38 and 25 kDa. *L. monocytogenes* presents high homogeneity between strains belonging to same serovar (Fig. 1). *L. seeligeri* is more similar to reference *L. monocytogenes* serotype 4b than 1. The two strains of *L. welshimeri* are different from *L. monocytogenes* serotype 1 and 4, and the two isolates are different each other (Fig. 3). *L. innocua* could be divided in two groups: one group is similar to the reference strain that comprise 10 isolates; the other group has a pattern characteristic which is different from the species analyzed – strains 4 and 5T′ – (Fig. 2).

3.2. DNA

*Eco*RI was shown to give the distinguishable pattern under the conditions used (Nocera et al., 1990). With *Eco*RI we obtained more than 50 bands. *Hin*dIII gave poor discrimination between isolates under the electrophoretic conditions used (data not shown). We did not obtain good results in our system with *Hha*I. A single band difference in the patterns was considered

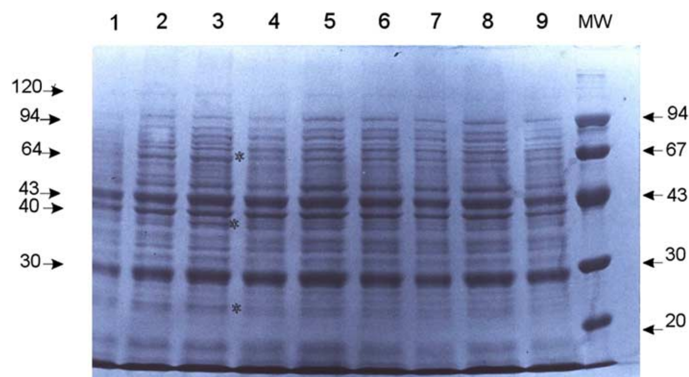

Fig. 1. Protein profiles of *L. monocytogenes* serotypes 1 and 4b. Lanes 1–3: *L. monocytogenes* serotype 1 S2 strain, S1 strain and reference strain 355/98 (H 85), respectively; lanes 4–8: *L. monocytogenes* serotype 4 CT strain, A3 strain, A2 strain, A1 strain and 3J strain, respectively; lane 9: *L. monocytogenes* serotype 4b reference strain ATCC 19115; MW: molecular weight marker expressed in kDa on the right. Molecular weight of common bands is indicated on the left of the figure. An asterisk denotes particular bands (62, 38 and 25 kDa).

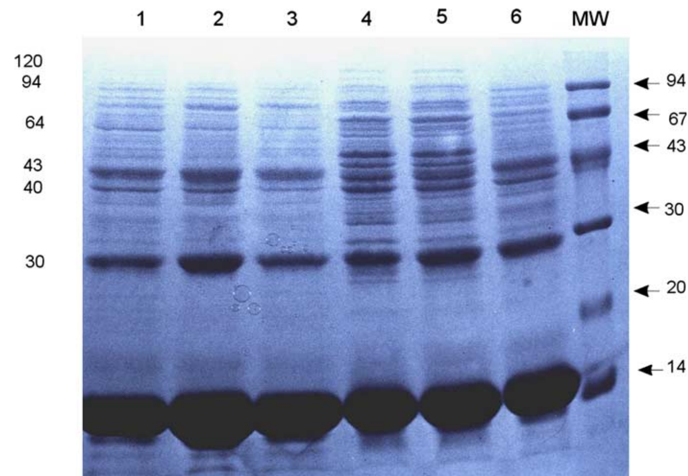

Fig. 2. Protein profiles of *L. innocua*. Lanes 1–6: CC strain, 1T strain, 2 strain, 4 strain, 5T′ strain and reference strain ATCC 33091, respectively; MW: molecular weight marker expressed in kDa on the right. Molecular weight of common bands is indicated on the left of the figure.

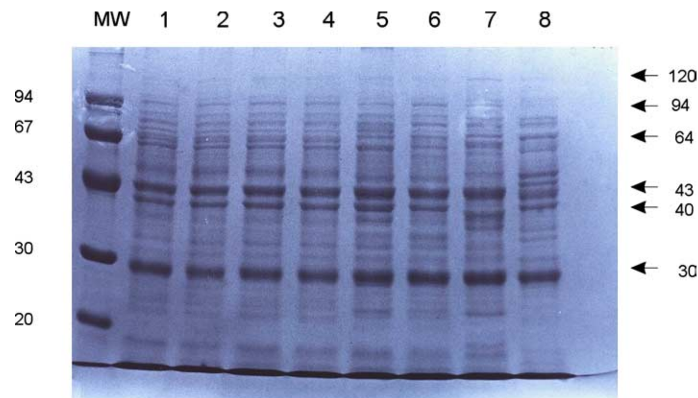

Fig. 3. Protein profiles of *L. monocytogenes*, *L. seeligeri* and *L. welshimeri*. MW: molecular weight marker expressed in kDa on the left. Molecular weight of common bands is indicated on the right. Lanes 1–4: *L. seeligeri* strains 7, 8, 9 and 10, respectively; lane 5: *L. monocytogenes* serotype 1 reference strain 355/98 (H 85); lane 6: *L. monocytogenes* serotype 4b reference strain ATCC 19115; lanes 7 and 8: *L. welshimeri* strains PF1T and PF2T, respectively.

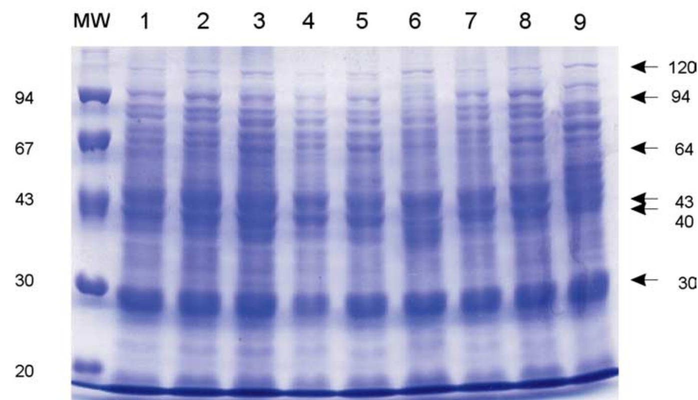

Fig. 4. Protein profiles of *L.* spp. MW: molecular weight marker expressed in kDa on the left. Molecular weight of common bands is indicated on the right. Lane 1: *L. monocytogenes* serotype 4b reference strain ATCC 19115; lane 2: *L. monocytogenes* serotype 4b A3 strain; lane 3: *L. monocytogenes* serotype 1 reference strain 355/98 (H 85); lane 4: *L. monocytogenes* serotype 1 S2 strain; lane 5: *L. seeligeri* 7 strain; lane 6: *L. welshimeri* PF1T strain; lane 7: *L. innocua* reference strain ATCC 33091; lane 8: *L. innocua* 2T strain; lane 9: *L. innocua* 5T′ strain.

significant and used to distinguish between serotypes. Strains isolated from different sources exhibited the same restriction pattern. The differences were clearly seen between 23 and 5 kb. Non-pathogenic *Listeria* gave Restriction profiles that were clearly distinct from those obtained by *L. monocytogenes*. The restriction fragment obtained from reference strains of: *L. monocytogenes* serotype 1 – 355/98 (H 85) and 4b – ATCC 19115, and *L. innocua* – ATCC 33091 matched the corresponding isolated strains (Figs. 5 and 6). We could not obtain differences between serotype 1 and 4b of *L. monocytogenes* with *Eco*RI (Fig. 5).

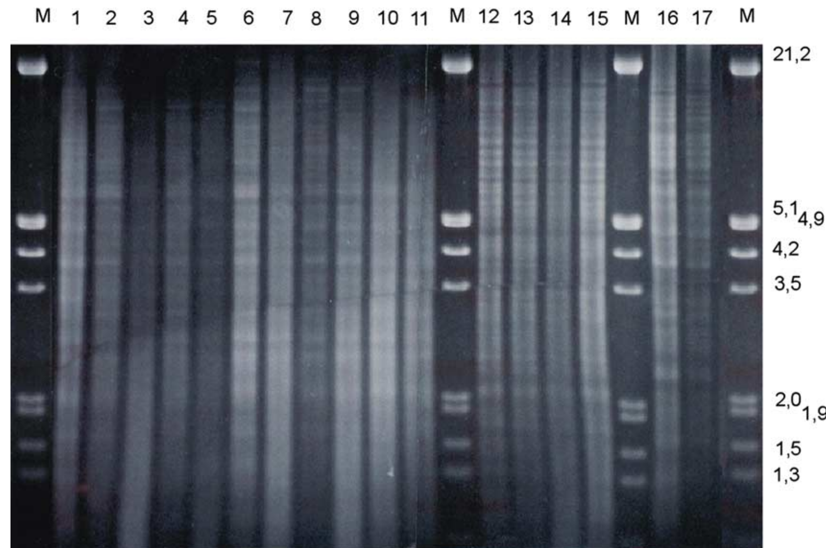

Fig. 5. *Eco*RI restriction fragments of DNA from *L. monocytogenes*, *L. seeligeri* and *L. welshimeri*. M: size marker (bacteriophage λ DNA *Eco*RI and*Hin*dIII digest) – indicated on the right in kb. Lane 1: *L. monocytogenes* serotype 1 reference strain 355/98 (H 85); lane 2: *L. monocytogenes* serotype 4b reference strain ATCC 19115; lanes 3–7: *L. monocytogenes* serotype 4 A1, A2, A3, 3J and CT strains, respectively. Lanes 8–11: strains of *L. monocytogenes* serotype 1, S1, S2, 5 and 8T, respectively. Lanes 12–15: *L. seeligeri* strains 7, 8, 9 and 10, respectively. Lane 16 and 17: *L. welshimeri* strains PF1T and PF2T, respectively.

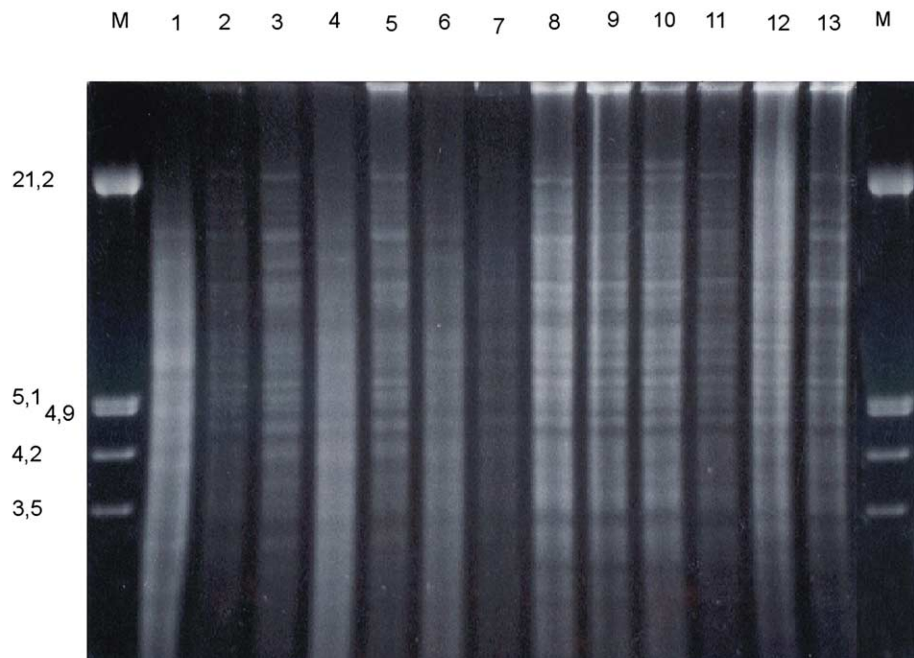

Fig. 6. *Eco*RI restriction fragments of DNA from *L. innocua*. M: size marker (bacteriophage λ DNA *Eco*RI and *Hin*dIII digest) – indicated on the left in kb. Lane 1: *L. innocua* reference strain ATCC 33091; lanes 2–13: *L. innocua* strains isolated 129, 6T, 2T, 3T, 2T′, 5T′, SP, LS, CC, 1T, 2 and 4, respectively.

Bands smaller than 4 kb could not be positively ascertained because the bands in this group were not completely resolved.

4. Discussion

In contrast with results obtained by others (Hof & Chatzipanagiotou, 1987; Lamont, Petrie, Melvin, & Postlethwaite, 1986; Paquet, Bitutsi, Espaze, & Pechere, 1986), electrophoretic analysis of whole *Listeria* spp. revealed differences between species, and within *L. monocytogenes*, between serovars 1 and 4b. Major bands of 30, 40, 43, 64, 94 and 120 kDa appeared common to the species studied, except for PF1T strain (*L. welshimeri*).

Other researchers studied only surface proteins (Tabouret et al., 1992). They considered as specific surface antigens for *L. monocytogenes* bands located between 64 and 68 kDa, and especially for serotype 4b bands between 76 and 78 kDa. In our work, we obtained same bands located at similar position not only for *L. monocytogenes*. These bands appeared highly conserved within pathogenic and non-pathogenic strains. We also confirmed that 4b constitute a very homogeneous group as was obtained by Tabouret et al. (1992).

Taking into account the difficult identification of species of *Listeria* spp. since different species can have the same O and H composition (Seeliger & Jones, 1986), our results can be useful for characterization of *Listeria* spp.

Although other authors mentioned enzymes such as *Hha*I, *Xho*I, *Hae*III, they choose the enzyme that offers the best discrimination among *L. monocytogenes* strains (Baloga & Harlander, 1991; Ericsson, Danielsson-Tham, Stalhandske, Tham, & Ursing, 1993; Gerner-Smidt, Boerlin, Ischer, & Schmidt, 1996; Nocera et al., 1990; Saito, Sawada, Tokumaru, & Hondo, 1997; Wesley & Ashton, 1991). We used *Eco*RI to characterize the 26 isolates of *Listeria* spp. by restriction enzyme analysis of total DNA.

As previously reported by Ericsson et al. (1993) and Gerner-Smidt et al. (1996), we could not see difference between serotypes of *L. monocytogenes* digested with *Eco*RI. However, this enzyme did allow us to discriminate between different species. Baloga and Harlander (1991) did not recommend *Eco*RI to differentiate between strains of *L. monocytogenes*, however we obtained good results to separate between different species of *Listeria* spp. with this enzyme.

A single band difference in the patterns was considered significant and used to distinguish between serotypes as well as was determined by Nocera et al. (1990). Because using *Eco*RI was enough for differentiation into *Listeria* spp., we considered not necessary to digest DNA with several enzymes according to our main objective. Moreover, separation of restriction bands obtained with *Eco*RI was best for fragments larger than 5 kb (Gerner-Smidt et al., 1996).

In our system we did not differentiate between *L. monocytogenes* serotype 1 and serotype 4, probably due to difficulties in avoiding a heavy DNA smear between the restriction bands, reported by others using enzymes with high frequency cutting sites (Baloga & Harlander, 1991; Ericsson et al., 1993).

In a case or outbreak of listeriosis, it is urgent to trace the origin of infection. By using restriction endonuclease analysis it is important, useful and necessary to characterize the isolates from both patients and suspected food to establish a possible epidemiological relationship.

Total protein profiles and DNA fingerprint analysis using restriction endonuclease analysis appear to be a helpful tool for epidemiological investigations of listeric infections in laboratories where serotyping or other methods are not available.

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