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Molecular Typing by Pulsed-Field Gel Electrophoresis of Spanish Animal and Human *Listeria monocytogenes* Isolates

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A total of 153 strains of *Listeria monocytogenes* isolated from different sources (72 from sheep, 12 from cattle, 18 from feedstuffs, and 51 from humans) in Spain from 1989 to 2000 were characterized by pulsed-field gel electrophoresis. The strains of *L. monocytogenes* displayed 55 pulsotypes. The 84 animal, 51 human, and 18 feedstuff strains displayed 31, 29, and 7 different pulsotypes, respectively, indicating a great genetic diversity among the Spanish *L. monocytogenes* isolates studied. *L. monocytogenes* isolates from clinical samples and feedstuffs consumed by the diseased animals were analyzed in 21 flocks. In most cases, clinical strains from different animals of the same flock had identical pulsotypes, confirming the existence of a listeriosis outbreak. *L. monocytogenes* strains with pulsotypes identical to those of clinical strains were isolated from silage, potatoes, and maize stalks. This is the first study wherein potatoes and maize stalks are epidemiologically linked with clinical listeriosis.

Listeria monocytogenes is the only *Listeria* species pathogenic for humans and animals. In ruminants it is responsible for different clinical manifestations, such as septicemia, meningitis, abortions, or mastitis (10). Animal listeriosis is associated with the consumption of contaminated feedstuffs, mainly poor-quality silage (4, 22, 24). This epidemiological link has been confirmed by different techniques such as serotyping, phage typing, ribotyping, or random amplified polymorphism (22, 24, 25). Pulsed-field gel electrophoresis (PFGE) is a molecular technique that has been successfully used for the epidemiological characterization of both human clinical isolates and foodstuff strains of *L. monocytogenes* involved in sporadic cases and outbreaks of human listeriosis (7, 11, 12). However, this typing method has scarcely been used for the molecular characterization of animal isolates. There is only one study on clinical isolates of *L. ivanovii* from meningoencephalitis in sheep (18), but no similar studies have been performed on *L. monocytogenes* in animal clinical cases. Moreover, *L. monocytogenes* is a zoonotic microorganism, but only few studies have specifically compared strains of *L. monocytogenes* isolated from animal clinical cases and those responsible for infection in humans (1, 9). Several epidemiological studies of human and animal listeriosis in Spain have been published during the last decade (3, 13, 21, 22), but molecular characterization was not performed. Thus, the purpose of this study was to extend the knowledge about the diversity of the Spanish isolates of *L. monocytogenes* from animal and human infections as well as feedstuffs.

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In this study we analyzed 153 strains of *L. monocytogenes* isolated from different sources during the period from 1989 to 2000 (Table 1). Of these, 84 strains were isolated from animal clinical sources (74 from the brains of animals with meningoencephalitis, 9 from vaginal swabs of animals with abortions, 1 from the milk of a cow with mastitis). Some of the animal strains were isolated from different animals of the same flock (Table 2). A total of 18 strains were isolated from feedstuffs (1 from maize stalks, 3 from potatoes, and 14 from silage) in 12 farms in which clinical cases of listeriosis had been diagnosed (Table 2). An additional 51 human clinical isolates of *L. monocytogenes* (20 strains from meningitis, 25 from septicemia, 4 from abortions, 1 from cirrhosis, and 1 from ascites) were also included. From animal clinical samples, *L. monocytogenes* was isolated by direct plating on Modified *Listeria* Selective agar (4). From feedstuffs, 100 μl of a 10-fold dilution of the blended sample was plated on this selective agar. Five colonies from each feedstuff sample were biochemically identified and serotyped as described below. In all of the samples, the respective five colonies were indistinguishable by biochemical and serological analysis and were considered a single strain. One colony from the different samples was further molecularly characterized. The human strains were isolated in different hospitals and submitted to the Servicio de Bacteriología of the Centro Nacional de Microbiología-Instituto de Salud Carlos III in Majadahonda, Madrid, Spain.

All strains were biochemically identified by using the commercial API *Listeria* system (bioMérieux España S.A.) and the CAMP test with *Rhodococcus equi* (5). The serotype was determined as described by Seeliger and Höhne (20). All strains were phenotypically typical of, and were confirmed as *L. monocytogenes*. All of the *L. monocytogenes* strains, except S_{11}21-93

TABLE 1. Molecular characterization by PFGE of Spanish *L. monocytogenes* strains

Strain(s)-yr of isolation (geographical region)[a]	Sero-type	REDP		Pulso-type	No. of strains
		*Apa*I	*Sma*I		
$S_1$3-92, $S_1$4-92, $S_1$5-92 (Aragón); C_{25}2-96, C_{27}4-94, C_{47}11-93, C_{47}12-93, F_{27}7-97, F_{36}10-93, F_{36}11-93, F_{38}13-93 (País Vasco)	4b	1	3	I	11
S_{34}44-96 (País Vasco)	4b	1	9	II	1
C_{38}9-93 (País Vasco)	4b	1	15	III	1
H30-96 (Navarra)	4ab	1	16	IV	1
S_{29}39-95 (País Vasco)	4b	2	25	V	1
$F_4$2-93 (Aragón)	4ab	3	11	VI	1
S_{52}63-92, S_{52}64-92, S_{52}65-92, S_{52}66-92, F_{52}14-92, F_{52}15-92, F_{52}16-92 (Galicia)	4b	4	3	VII	7
$S_1$6-92 (Aragón)	4ab	5	1	VIII	1
H22-94 (Andalucía); $S_1$1-92, $S_1$2-92 (Aragón)	4b	5	3	IX	3
H35-97 (Madrid); S_{12}22-93, S_{17}28-95, S_{12}30-95, S_{12}32-95; F_{12}5-95 (Aragón)	4b	6	3	X	6
H3-92, H6-92, H8-92, H9-92, H11-93, H13-93, H14-93 (Canarias); S_{41}49-93, S_{41}50-93 (Castilla-León); S_{43}53-93, S_{45}55-93, S_{45}56-93, S_{45}57-93, S_{45}58-93, S_{50}61-91 (País Vasco); S_{13}24-94, S_{54}70-00 (Aragón)	4b	7	3	XI	17
H41-97 (Cataluña)	4b	7	6	XII	1
H17-94, H18-94, H19-94 (Madrid); $S_8$18-93, $S_8$23-94, $F_8$4-94 (Aragón); S_{39}47-94, S_{43}52-93 (País Vasco); S_{42}51-93 (Castilla-León)	4b	7	15	XIII	9
H46-97 (Castilla-León)	4b	8	3	XIV	1
H40-97 (Navarra); H44-97 (Castilla La Mancha)	4b	8	6	XV	2
H28-96, H29-96 (Cataluña); H47-98 (Extremadura)	4b	8	14	XVI	3
S_{28}38-95, S_{35}46-94, S_{48}59-93, C_{37}7-93, C_{37}8-93, F_{37}12-93 (País Vasco); S_{55}71-00, S_{55}72-00 (Extremadura)	4b	8	15	XVII	8
H37-97 (Castilla-León); H43-97, H45-97 (Castilla La Mancha); H38-97 (Comunidad Valenciana); $S_2$7-93, $S_2$8-93, $S_4$11-93, $S_6$13-93, S_{18}31-95, $F_2$1-93 (Aragón)	4b	9	3	XVIII	10
H23-94 (Castilla-León)	4b	9	6	XIX	1
$S_3$9-93, $S_3$10-93 (Aragón)	4b	10	3	XX	2
F_{27}6-97 (País Vasco)	4b	10	27	XXI	1
S_{15}26-94 (Aragón)	4b	11	3	XXII	1
C_{24}1-96 (Aragón)	4b	11	12	XXIII	1
$S_5$12-93, $S_7$14-93, $S_7$15-93, $S_7$16-93, $S_7$17-93, S_{10}20-93, $F_5$3-93 (Aragón)	4b	12	3	XXIV	7
H50-98 (Madrid); $S_9$19-93 (Aragón)	4b	12	15	XXV	2
H10-93 (Canarias)	4b	13	25	XXVI	1
H16-94 (Madrid)	4b	14	3	XXVII	1
H42-97 (Castilla La Mancha)	4b	14	16	XXVIII	1
H24-95, H25-95, H51-92 (Canarias); H21-94 (Andalucía); S_{19}33-96, S_{20}34-96 (Aragón); F_{35}9-94 (País Vasco)	4b	15	3	XXIX	7
C_{26}3-97 (País Vasco)	4b	15	8	XXX	1
H33-96 (La Rioja)	4b	15	10	XXXI	1
H2-89, S_{16}27-94 (Aragón); H4-92 (Galicia); H12-93 (Canarias); S_{40}48-94 (País Vasco)	4b	15	15	XXXII	5
S_{14}25-94 (Aragón)	NT	15	17	XXXIII	1
S_{51}62-98 (Cantabria)	4b	15	20	XXXIV	1
H36-97 (Castilla-León); S_{22}36-98 (Aragón); S_{23}37-95, S_{31}41-95, C_{36}6-93, C_{46}10-93, F_{23}8-95 (País Vasco)	4b	16	15	XXXV	7
H39-97 (Castilla-León)	4b	17	15	XXXVI	1
S_{17}29-95 (Aragón)	4ab	18	15	XXXVII	1
S_{44}54-93 (País Vasco)	4b	19	15	XXXVIII	1
H15-94 (Madrid); H32-96 (La Rioja)	1/2a	20	5	XXXIX	2
S_{33}43-97 (País Vasco)	1/2a	20	13	XL	1
S_{30}40-95, S_{35}45-94, C_{35}5-94 (País Vasco)	1/2a	20	18	XLI	3
S_{49}60-93 (País Vasco); F_{52}17-92 (Galicia)	1/2a	20	28	XLII	2
S_{21}35-97 (Aragón)	1/2b	21	22	XLIII	1
S_{53}67-92, S_{53}68-92, S_{53}69-92 (Galicia)	1/2b	22	2	XLIV	3
S_{32}42-95 (País Vasco)	1/2a	23	26	XLV	1
F_{52}18-92 (Galicia)	1/2b	24	2	XLVI	1
H31-96 (Navarra)	1/2a	25	23	XLVII	1
H26-96 (Castilla-León)	1/2a	26	4	XLVIII	1
S_{11}21-93 (Aragón)	NT	27	21	XLIX	1
H27-96 (Madrid); H48-98 (Castilla-León)	1/2a	28	18	L	2
H1-96 (Navarra)	1/2a	28	24	LI	1
H34-96 (Castilla La Mancha)	1/2a	29	18	LII	1
H49-98 (Madrid)	1/2a	29	19	LIII	1
H5-92, H7-92 (Canarias)	1/2a	29	28	LIV	2
H20-94 (Madrid)	1/2a	30	7	LV	1

[a] S, C, H, and F, strains isolated from sheep, cattle, human, and feedstuffs, respectively. Subscripts indicate the flock in which the strain was isolated. Different strains with the same subscript indicate that they were isolated from different animals of the same flock. All ovine and bovine strains were isolated from animals with meningoencephalithis, except strains S65, S66, C2, C4, C6, C7, and C9 (isolated from abortions) and strain C5 (isolated from mastitis). The feedstuff strains were isolated from silage, except for strains F1, F2, and F3 (isolated from potatoes) and F5 (isolated from maize stalks). Strains H35 and H46 were isolated from cases of cirrhosis and ascites, respectively. Strains H2 to H6, H8 to H17, H19, H20, H23, H29 to H31, H40, and H41 were isolated from patients with septicemia, and strains H7, H24, H25, and H51 were isolated from abortions. The other human strains were isolated from cephaloraquid fluid in patients with meningitis. NT, not typeable by serotyping.

TABLE 2. Pulsotypes of the animal and feedstuff *L. monocytogenes* strains isolated from flocks with clinical listeriosis

Flock	Strain(s)[a]	Pulsotype
1	S1-92, S2-92	IX
	S3-92, S4-92, S5-92	I
	S6-92	VIII
2	S7-93, S8-93, F1-93	XVIII
3	S9-93, S10-93	XX
4	S11-93	XVIII
	F2-93	VI
5	S12-93, F3-93	XXIV
7	S14-93, S15-93, S16-93, S17-93	XXIV
8	S18-93, S23-94, F4-94	XIII
12	S22-93, S30-95, S32-95, F5-95	X
17	S28-95	X
	S29-95	XXXVII
23	S37-95, F8-95	XXXV
27	C4-97, F7-97	I
	F6-97	XXI
35	S45-94, C5-94	XLI
	S46-94	XVII
	F9-94	XXIX
36	C6-93	XXXV
	F10-93, F11-93	I
37	C7-93, C8-93, F12-93	XVII
38	C9-93	III
	F13-93	I
41	S49-93, S50-93	XI
45	S55-93, S56-93, S57-93, S58-93	XI
47	C11-93, C12-93	I
52	S63-92, S64-92, S65-92, S66-92, F14-92, F15-92, F16-92	VII
	F17-92	XLII
	F18-92	XLVI
53	S67-92, S68-92, S69-92	XLIV
55	S71-00, S72-00	XVII

[a] See footnote of Table 1 for explanation of abbreviations.

and S_{14}25-94, were serotyped (Table 1). Most of the isolates (81.7%) were serotyped as 4b, 19 (12.4%) belonged to serovar 1/2a, 5 (3.3%) were serotyped as 1/2b, and 4 (2.6%) were serotyped as 4ab.

For PFGE, DNA was isolated and restricted with *Apa*I (Promega) and *Sma*I (MBI Fermentas), respectively, as described by Vela et al. (23). PFGE was performed as described previously (23) except that the pulsed times were linearly ramped from 0.1 to 40 s. Gels were stained with ethidium bromide (0.5 μg/ml) for 15 min, destained in distilled water, and photographed under UV light. Lambda ladder PFGE marker (Boehringer Mannheim) was used for molecular weight size determination. Although several methods have been used for typing *L. monocytogenes* (2, 11, 12, 16, 25), in the present study PFGE was used because of its great discriminatory power (12, 18). All isolates displayed *Apa*I and *Sma*I restriction endonuclease digestion profiles (REDPs). The REDP patterns of the same isolate generated for each of the two restriction endonucleases were found to be stable and reproducible in at least two separate trials (data not shown). The *Apa*I restriction enzyme generated 12 to 17 major fragments over a size ranging from ca. 20 to 557.2 kb, while *Sma*I-digested DNA generated REDPs with 11 to 18 major fragments over a size ranging from ca. 18.2 to 339.5 kb (data not shown). Visual comparison of macrorestriction patterns generated with the *Apa*I enzyme revealed 30 different DNA fragment profiles (Table 1). Profile 7 was the most common among isolates (27 strains; 17.6%), followed by profiles 15 (15 isolates; 9.8%) and 1 and 8 (14 isolates each; 9.1%). The *Sma*I enzyme generated 28 different DNA fragment profiles (Table 1). The most common profile was number 3 with 82 strains (53.6%). Although this enzyme was slightly less discriminative than *Apa*I, some strains with identical *Apa*I profile (e.g., profile 1; Table 1) displayed different *Sma*I profiles. These results support previous results indicating that the discriminative power of this technique is improved by the combination of both enzymes (2, 16).

The REDPs were used to examine whether individual or group of pulsotypes were more frequently associated with animal and/or human listeriosis in Spain. After composite profiling, the strains of *L. monocytogenes* were divided into 55 pulsotypes, most (62%) of which were represented by a single isolate (Table 1). The 84 animal, 51 human, and 18 feedstuff strains displayed 31, 29, and 7 different pulsotypes, respectively. The 12 cattle strains were characterized by 7 different pulsotypes, while the 72 ovine isolates displayed 28 pulsotypes. The results of this study indicate a great genetic diversity amongst the strains of *L. monocytogenes* studied, a finding which is consistent with previous reports for this species (1, 12). Despite this significant number of pulsotypes encountered among the Spanish *L. monocytogenes* isolates, five pulsotypes (I, XI, XIII, XVII, and XVIII) included 37 and 27% of the animal and human *L. monocytogenes* strains, respectively. This fact may be related either to a wider environmental distribution or to a higher pathogenic potential of these pulsotypes (14). Although recent studies suggest the existence of differences in the pathogenic potential among *L. monocytogenes* strains (26), most of the molecular and experimental pathogenicity studies have not been able to find the existence of strain-specific differences in terms of epidemiogenicity in the virulence of *L. monocytogenes* (6, 24). Therefore, although no studies about the virulence of these pulsotypes were carried out, the simultaneous isolation of these pulsotypes in different parts of Spain and in different years, together with their isolation from 39% of the feedstuff samples, would be consistent with the idea of a wider environmental distribution.

L. monocytogenes isolates from clinical samples and feedstuffs consumed by the diseased animals were analyzed in 21 flocks. *L. monocytogenes* was isolated from feedstuff samples in 12 of the 21 flocks examined (Table 2). Modified *Listeria* Selective agar has been successfully used for the isolation of *L. monocytogenes* from silage (22). However, if *L. monocytogenes* is present in low numbers, there may be overgrowth by the normal microflora of silage, explaining why in nine flocks it was not possible to recover *L. monocytogenes* from the feedstuffs. The isolation of different pulsotypes among the clinical isolates of the same flock (flocks 1, 17, and 35; Table 2) agrees with previous reports (24). This fact could be related to the great genetic diversity of this microorganism observed in this study and/or to the widespread distribution of *L. monocytogenes* in the farm environment (25), all of which favor the exposure of animals to multiple *L. monocytogenes* strains. Similarly, the diversity that can exist in the population of *L. monocytogenes* in silage (19, 25) can explain the isolation in flock 52 of *L. monocytogenes* of different pulsotypes from silage samples of different bales (Table 2). Contamination of the farm environment can occur with the manure of diseased or carrier animals (10).

Thus, the existence of fecal carriers and the ability of *L. monocytogenes* to survive in the farm environment over long periods of time (17) could also explain the persistence of a particular strain of *L. monocytogenes* in a farm, as suggested by repeated isolation for several years of the same pulsotype from different animals in flocks 8 and 12 (Table 2).

Clinical strains with identical pulsotypes were isolated from different animals of the same flock, indicating that strain as being responsible for the clinical cases, as well as the existence of a common source of infection. *L. monocytogenes* with a pulsotype identical to that of clinical strains isolated from silage (flocks 8, 23, 27, 37, and 52), potato (flocks 2 and 5), and maize stalk (flock 12) samples (Table 2) therefore incriminated these feedstuffs as the source of the disease. These results are consistent with the idea that improperly fermented silage is the main source of listeriosis in ruminants (22, 25). *L. monocytogenes* has been isolated from potatoes (8). However, as far as we know this is the first description in which this product, as well as maize stalks, is epidemiologically linked with clinical cases of listeriosis.

The silage samples were collected after listeriosis was diagnosed, and it is possible that the silage analyzed may not be the same as that consumed by animals at the time of infection, which is likely if the silage is stored in separate bales. In these instances, the strain of *L. monocytogenes* isolated from the silage sample may not match the respective clinical strain. This fact could explain the isolation in flocks 4, 35, 36, and 38 of *L. monocytogenes* strains with pulsotypes different from those of the silage and clinical samples (24). In addition, *L. monocytogenes* isolates with identical pulsotypes that were also different from those of the respective clinical samples were isolated from the same silage sample (flock 27; Table 2). The isolation of distinct unrelated *L. monocytogenes* strains in a silage sample implicated in listeriosis agree with previous results (24), and the infection with one of the strains present in the silage could have occurred by chance or could have been due to a higher virulence of the strain recovered from the clinical samples (15).

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