Journal of Food Protection, Vol. 76, No. 11, 2013, Pages 1863–1867
doi:10.4315/0362-028X.JFP-12-505

Listeria monocytogenes in Five Sardinian Swine Slaughterhouses: Prevalence, Serotype, and Genotype Characterization

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MS 12-505: Received 9 November 2012/Accepted 25 June 2013

ABSTRACT

In a 3-year study (2008 to 2011) to estimate the prevalence and the contamination sources of *Listeria monocytogenes* in pork meat in Sardinia, Italy, 211 samples were collected from five Sardinian swine slaughterhouses: 171 samples from slaughtered pigs and 40 from the slaughterhouse environment. Fifty *L. monocytogenes* isolates were characterized by PCR-based serotyping, presence of virulence-associated genes, and pulsed-field gel electrophoresis restriction analysis. The overall prevalence of *L. monocytogenes* was 33% in swine carcasses, 7% in cecal material, 23% on meat contact surfaces, and 25% on noncontact surfaces. Only two serotypes were detected: 1/2c (78%) and 1/2a (22%). In all, based on the presence of virulence-associated genes, eight pathogenic profiles were detected. Only 42% of all isolates carried the full complement of virulence-associated genes and were allotted to profile 1. Six pulsed-field gel electrophoresis profiles persisted in the slaughterhouses; restriction profiles appeared to be specific to each plant.

Pork carcasses are at risk of contamination by zoonotic bacteria during swine slaughter, spread from other slaughtered pigs (feces, pharynges) and from the environment *(4)*. Potentially pathogenic microorganisms such as *Listeria monocytogenes, Salmonella enterica,* and *Yersinia enterocolitica* are spread to the carcass mainly from the carrier animals *(2, 5, 7, 22)*. *L. monocytogenes* has been detected in pigs and pork meat products and in every stage during pork processing *(19, 20, 26)*. *L. monocytogenes* has occasionally been isolated from feces and from the skin of healthy carriers with intestinal colonization *(25)*. Generally, the prevalence of *L. monocytogenes* in feces ranges from 0 to 50% *(3)*. In the slaughterhouse, the role of live animals as a source of contamination of processing environments and, consequently, pig carcasses has been demonstrated. The prevalence of pig carcass contamination during the evisceration step can be as high as 65% *(19, 27)*, highlighting the importance of good hygiene practices and clean equipment *(4)*. Other authors *(1, 17)* have correlated the contamination of equipment at the slaughterhouse and, consequently, of the carcasses with the presence of *L. monocytogenes* in other niches, such as the swine tongue (14%) and tonsils (7 to 12%). Raw pork meat has rarely been implicated in foodborne illness, and the level of contamination increases along the pork supply chain *(19)*. Generally, the highest prevalence is found in samples of pork meat intended for processing (45 to 50%) compared with the muscles of freshly slaughtered pigs (0 to 2%). This highlights the important role of the after-slaughter phases, such as cooling and cutting, in the increase of pork meat contamination. In these processing phases, the prevalence of *L. monocytogenes* ranges from 70 to 100% *(17)*. Pigs slaughtered in Sardinia may be of local origin or may have been imported from other Italian regions and European Union countries *(22)*. Sardinia produces only 50% of the pork meat it consumes; as a consequence of this low self-sufficiency rate, more pig meat and pork products are imported. There is a lack of data describing *L. monocytogenes* prevalence and epidemiology in Sardinia at both the farm level and at the slaughterhouse. The aim of the present study was to evaluate *L. monocytogenes* prevalence in five swine slaughterhouses during a 3-year interval (2008 to 2011) by the detection of the pathogen in slaughter pigs and environmental samples. *L. monocytogenes* isolates were serotyped, characterized by presence of virulence genes, and profiled by pulsed-field gel electrophoresis (PFGE) in order to investigate their distribution in the slaughterhouses.

MATERIALS AND METHODS

Slaughterhouse description and sampling plan. From 2008 to 2011, a survey was carried out in five European Community swine slaughterhouses (SA to SE) in Sardinia, in which pigs from Sardinia or those imported from other Italian regions and European Union countries are slaughtered. The five slaughterhouses had comparable slaughter procedures and capacities (300 swine per

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hour). In order to evaluate the persistence of the pathogen in the slaughterhouse environment, from 2 to 4 sampling days were scheduled in every slaughterhouse early in the week, usually on Mondays: SA, SB, and SE were visited twice, SD three times, and SC four times, for a total of 13 sampling visits. A total of 211 samples were collected from 85 slaughtered pigs (100 to 120 kg weight) and from the slaughterhouse environment.

Sample collection. During every sampling day in the slaughterhouse, from 5 to 10 pigs were randomly selected (n = 85). After evisceration and before chilling, cecal and swab samples (n = 170) were taken from each pig.

For cecal material samples (n = 85), intestines were collected in plastic bags in a separate room near the slaughter line, and 25 g of cecal material was aseptically collected. Sterile scalpels and spoons were used to scoop out contents and place them in separate sterile Ziploc bags (SC Johnson Italy, Arese, Italy).

For carcass swab samples (n = 85), pork carcasses were sampled by swabbing animals with two sterile gauze pads rehydrated with 10 ml of neutralizing buffer (Solar-Cult sampling kit, Biogenetics, Padua, Italy). Carcass swabs were taken from the upper inner part of both hind legs, including the skin and the pelvic entrance (first swab), and from the cut surface area of the abdomen and chest, including the skin surface (second swab). A sterile template was used to delineate a 100-cm^2 swabbed area. The two swabs collected from each carcass were pooled prior to testing and were identified with a univocal code for each sample.

For environmental sampling, swabbing was performed in each of the five abattoirs at the end of the sampling day, before cleaning and disinfection. A total of 41 samples were collected with sterile gauze pads rehydrated with 10 ml of neutralizing buffer (Solar-Cult sampling kit, Biogenetics). Sampling locations were chosen to represent those most likely to harbor *L. monocytogenes*: product contact surfaces (n = 17), e.g., dehairing equipment, knives, carcass splitter; and product noncontact surfaces (n = 16), e.g., floor drains and walls in the dirty zone (stunning and bleeding area). The area of environmental sampling varied depending on the sampling location: on the walls of the dirty zone, a sterile template was used to delineate a 100-cm^2 swabbed area. Samples of scalding water (n = 8) were taken: 25-ml aliquots were collected in sterile collection tubes. All the specimens, identified with a univocal code, were transported at 4°C to the laboratory and analyzed on the same day.

Detection and enumeration of *L. monocytogenes*. The detection and enumeration of *L. monocytogenes* were performed according to international standard methods using ISO 11290-1:1996 *(14)* and 11290-2:1998 *(15)*, respectively. Samples of cecal material and swab samples were homogenized 1/10 with Fraser broth base (Biolife, Milan, Italy) in a Stomacher Lab Blender 400 (Seward Medical, London, UK) for 2 min. For enumeration of *L. monocytogenes,* 1-ml samples of each inoculum were directly streaked onto three Agar Listeria Ottaviani & Agosti (ALOA) 90-mm plates (Biolife) using a sterile spreader and were incubated (37°C for 48 h). For primary enrichments, the homogenates were supplemented by Fraser half selective supplement (Biolife) and were incubated (30°C for 24 h). For secondary enrichment, 0.1-ml aliquots of the primary enrichment were inoculated into 10-ml tubes containing Fraser broth (Biolife) supplemented by Fraser selective supplement (Biolife) and were incubated (37°C for 48 h). Aliquots of 0.1 ml from primary and secondary enrichments were streaked onto Oxford (Oxoid, Milan, Italy) and ALOA plates and incubated (30 and 37°C for 48 h, respectively). On Oxford medium, *L. monocytogenes* hydrolyzes esculin, producing black zones around the colonies due to the formation of black iron phenolic compounds derived from the aglucon. Presumptive *L. monocytogenes* colonies grown on ALOA medium appeared as bluish colonies surrounded by a distinctive opaque halo. From each positive sample plate of the primary and secondary enrichment, five colonies presumed to be *Listeria* spp. were streaked on tryptone soya yeast extract agar (TSYEA) plates (Biolife) and incubated (37°C for 24 h). Colonies were selected for typical appearance on TSYEA (dense, white to iridescent white appearing as crushed glass) and were submitted to Gram stain, catalase, and oxidase tests. Hemolytic activity and CAMP tests on sheep blood agar were performed for confirmation of *L. monocytogenes* isolates. Biochemical characterization of all the isolates was performed using the API Listeria identification system (bioMérieux, Marcy l'Etoile, France). After identification, strains were stored at −80 °C in brain heart infusion broth (Biolife) with glycerol (15%, vol/vol).

Multiplex PCR analysis of virulence-associated genes. *L. monocytogenes* isolates were characterized by previously described multiplex PCR methods *(6, 16)* to detect 10 virulence-associated genes. These were chosen according to their importance in *L. monocytogenes* pathogenesis: *rrn*-16SrRNA (confirmation of *L. monocytogenes*), *hlyA* (hemolysin, essential for virulence), *actA* (actin A, required for invasiveness), *prfA* (positive regulatory factor A), *inlA*, *inlB* (internalin A and internalin B, both required for entry into normally susceptible nonphagocytic cells), *iap* (invasion-associated protein, required for invasiveness), *plcA* (phospholipase A required for primary vacuole lysis), *plcB* (phospholipase B required for secondary phagosomes lysis), and *mpl* (metalloprotease involved in production of phosphatidylcholine-specific phospholipase C).

Multiplex PCR-based serotyping. *L. monocytogenes* isolates were subjected to a multiplex PCR-based serotyping assay targeting genes *lmo0737*, *lmo1118*, ORF2819, ORF2110, and *prs*, as previously described *(8)*.

DNA macrorestriction and PFGE. *L. monocytogenes* isolates were subjected to DNA macrorestriction with *Apa*I and *Asc*I (New England Biolabs, Beverly, MA). PFGE was performed according to the PFGE-PulseNet protocol *(13)* by using a CHEF Mapper XA system (Bio-Rad, Hercules, CA). *Asc*I and *Apa*I macrorestriction patterns were analyzed using GelCompar software (Applied Maths, Sint-Martens-Platen, Belgium). The similarity between restriction patterns, based on band position, was expressed as a Dice coefficient correlation. The position tolerance was optimal when set at 1.5%. Clustering and construction of dendrogram were performed through the unweighted pair group method using arithmetic averages, which combined both *Asc*I and *Apa*I macrorestriction patterns into one unique PFGE profile.

RESULTS

Detection and enumeration of *L. monocytogenes*. A summary of the prevalence of *Listeria* spp. and *L. monocytogenes* in the five slaughterhouses is reported in Table 1. As seen in Table 1, during a 3-year interval (2008 to 2011), *Listeria* spp. were found in all five slaughterhouses (SA to SE) in samples of cecal material (26 to 91%) and in swabs (33 to 84%). *L. monocytogenes* was found in three slaughterhouses (SB, SC, and SD), with an overall average prevalence of 33% in carcasses and 7% in samples of cecal material. Contamination levels were below 100

TABLE 1. *Prevalence of* Listeria monocytogenes *and* Listeria *spp. in pig carcass and environmental samples from five European Community abattoirs*

	Carcass swabs		Cecal material		Product contact surfaces		Product noncontact surfaces		Scalding water	
EC swine slaughterhouses	Positive/ total	%	Positive/ total	%	Positive/ total	%	Positive/ total	%	Positive/ total	%
SA										
L. monocytogenes	0/15[a]	0	0/15[a]	0	NA[b]	NA	NA	NA	NA	NA
Listeria spp.	5/15	33	4/15	26	NA	NA	NA	NA	NA	NA
SB										
L. monocytogenes	7/12[a]	58	1/12[a]	8	1/2	50	2/2	100	0/1	0
Listeria spp.	5/12	41	11/12	91	2/2	100	2/2	100	0/1	0
SC										
L. monocytogenes	20/25[a]	80	2/25[a]	8	2/7	28	1/6	16	0/3	0
Listeria spp.	21/25	84	13/25	52	4/7	57	3/6	50	0/3	0
SD										
L. monocytogenes	1/20[a]	5	3/20[a]	15	1/4	25	1/4	25	0/2	0
Listeria spp.	7/20	35	12/20	60	1/4	25	1/4	25	0/2	0
SE										
L. monocytogenes	0/13	0	0/13	0	0/4	0	0/4	0	0/2	0
Listeria spp.	5/13	38	4/13	30	0/4	0	1/4	25	0/2	0
Total										
L. monocytogenes	28/85[a]	33	6/85[a]	7	4/17	23	4/16	25	0/8	0
Listeria spp.	43/85	51	44/85	51	7/17	41	7/16	44	0/8	0

[a] Contamination levels were always below 100 CFU/g.
[b] NA, not analyzed.

CFU/g in the samples enumerated. In environmental samples, *L. monocytogenes* was found on product contact surfaces (23%), such as dehairing equipment, knives, and carcass splitters, as well as on product noncontact surfaces (25%), such as floor drains. In all, 170 isolates of *Listeria* spp. were recovered: *L. welshimeri* (39%), *L. monocytogenes* (29%), *L. innocua* (21%), *L. grayi* (10%), *L. ivanovii* (4%), and *L. seeligeri* (3%). The 50 *L. monocytogenes* isolates ($n = 7$ from SB, $n = 36$ from SC, and $n = 7$ from SD) were characterized by PCR-based serotyping, determination of the virulence potential, and macrorestriction enzyme analysis.

Multiplex PCR analysis of virulence-associated genes. Multiplex-PCR products of the 10 virulence-associated genes were obtained from the majority of the *L. monocytogenes* strains included in this study. Genotyping yielded five different pathogenic profiles (Table 2): the most prevalent were profile 3 (44%, nine virulence-associated genes, lack of *inlB*) and profile 1 (42%, complete

TABLE 2. *Pathogenic profile of* Listeria monocytogenes *isolates from pig carcass and environmental samples from five European Community abattoirs*

Samples	No. of isolates ($n = 50$)	Pathogenic profile	Serotypes (no. of isolates)	Virulence-associated genes
Carcass swabs	24	1	1/2c (7)	*prfA, hlyA, rrn, inlA, inlB, iap, plcA, plcB, actA, mpl*
		2	1/2c (1)	*prfA, hlyA, rrn, inlA, inlB, iap, plcA, plcB, actA*
		3	1/2c (11); 1/2a (4)	*prfA, hlyA, rrn, inlA, iap, plcA, plcB, actA, mpl*
		4	1/2c (1)	*prfA, hlyA, rrn, inlA, iap, plcA, plcB, actA*
Cecal material	5	1	1/2c (1)	*prfA, hlyA, rrn, inlA, inlB, iap, plcA, plcB, actA, mpl*
		3	1/2a (2)	*prfA, hlyA, rrn, inlA, iap, plcA, plcB, actA, mpl*
		5	1/2c (2)	*prfA, hlyA, rrn, plcA, plcB, actA, mpl*
Product contact surfaces	12	1	1/2c (8)	*prfA, hlyA, rrn, inlA, inlB, iap, plcA, plcB, actA, mpl*
		3	1/2c (3)	*prfA, hlyA, rrn, inlA, iap, plcA, plcB, actA, mpl*
		4	1/2a (1)	*prfA, hlyA, rrn, inlA, iap, plcA, plcB, actA*
Product noncontact surfaces	9	1	1/2c (5)	*prfA, hlyA, rrn, inlA, inlB, iap, plcA, plcB, actA, mpl*
		3	1/2a (2)	*prfA, hlyA, rrn, inlA, iap, plcA, plcB, actA, mpl*
		4	1/2a (2)	*prfA, hlyA, rrn, inlA, iap, plcA, plcB, actA*

TABLE 3. *Distribution of* Listeria monocytogenes *PFGE profiles (P1 to P6) recovered in the three positive European Community swine slaughterhouses*[a]

Samples	SB		SC				SD		
	sd 1	sd 2	sd 1	sd 2	sd 3	sd 4	sd 1	sd 2	sd 3
Carcass swabs	0	P6	0	P1	P1	P1	P2	0	P2
Cecal material	P4	0	0	0	P1	0	P2	0	P2
Product contact surfaces	0	P4	P1	P1	0	P1	0	0	P2–P3
Product noncontact surfaces	0	P4–P5	0	P1	0	0	0	0	P2

[a] sd 1 to 4, sampling days 1 to 4.

virulence profile). The prevalence of the other three pathogenic profiles ranged from 2 to 8%: profile 4, 8% (eight virulence-associated genes, lack of *mpl* and *inlB*); profile 5, 4% (seven virulence-associated genes, lack of *iap, mpl,* and *inlB*); and profile 2, 2% (nine virulence-associated genes, lack of *mpl*). In general, PCR products of the virulence-associated genes did not show size polymorphism except for the *actA* gene: 56% showed the predicted 385-bp amplicon, whereas 44% showed the 268-bp amplicon.

Multiplex PCR-based serotyping. Two predominant serotypes (1/2c and 1/2a) were detected (Table 2), representing 78 and 22% of the isolates, respectively.

DNA macrorestriction and PFGE. The application of DNA macrorestriction with *Apa*I and *Asc*I yielded six pulsotypes by both enzymes (P1 to P6), revealing a low genetic diversity within the subset of *L. monocytogenes* isolates. Based on the limited data, none of the PFGE profiles was shared between the slaughterhouses (Table 3), and they appeared to be plant-specific, in particular in SC.

DISCUSSION

The results of this 3-year survey demonstrate a high prevalence of *L. monocytogenes* in slaughtered pigs in Sardinia (33% on carcasses, 7% in cecal contents) and suggest that many *L. monocytogenes* isolates were introduced into the slaughterhouse by the delivered pigs. Differences in *L. monocytogenes* prevalence were observed among slaughterhouses, perhaps due to the origin of the animals and the varied application of slaughtering and hygiene practices by the operators. *L. monocytogenes* prevalence in Sardinia, based on cecal samples (7%) and pork carcasses (33%), was higher than that reported in Italy *(4, 21),* in Spain *(19),* and in the United States *(28).* However, the prevalence of *L. monocytogenes* reported in the literature varies widely and is influenced by husbandry practices, diet of the pigs, season, or by the sampling method and the part of the carcass chosen for the surface sampling *(18).* Slaughtered carrier pigs with positive cecal content could have been infected on the farm, during transport, or during the waiting period in highly contaminated lairage areas, which can be considered the main source for *L. monocytogenes* infections prior to slaughter *(1).* A similarly high prevalence was found on carcass surfaces in slaughterhouses with high environmental contamination *(1),* in particular on contact surfaces, such as knives, carcass splitters, or dehairing equipment. As reported also in our study, the prevalence of *L. monocytogenes* on this equipment confirmed its potential role in cross contamination of carcasses. The high prevalence of *L. monocytogenes* on product noncontact surfaces (25%) such as floor drains was probably correlated with inadequate application of cleaning procedures, which allowed the persistence of resident ''house strains.'' Although contaminated floor drain water is not considered a critical control point, carcass contamination can occur if contaminated water from the floor drains becomes airborne during cleaning procedures, e.g., high-pressure spraying, causing aerosol formation.

The application of multiplex PCR gave some unexpected results and highlighted that only 42% of all *L. monocytogenes* isolates carry the full complement of virulence genes that are typically present in *L. monocytogenes*. The absence of one or more PCR products is probably due to the fact that the primers may not hybridize sufficiently to one or more allelic variants of a gene, in particular *inlB,* which has previously been shown to be highly polymorphic *(9, 24).* Because absence of *inlB* from a large proportion of *L. monocytogenes* isolates has not been previously reported, further experiments need to be conducted to confirm *inlB* absence in the isolates that did not yield an *inlB* product.

According to previous studies, multiplex PCR–based serotyping highlighted the presence of two predominant serotypes: 1/2c and 1/2a *(26).* Despite the low prevalence rate in this survey, serotype 1/2a is increasing in importance in the epidemiology of listeriosis *(10).* In the last 11 years, Italy has reported an increase of cases due to serotype 1/2a and a decline in cases due to serotype 4b *(12, 23).*

Macrorestriction enzyme analysis showed a low genetic diversity (six different PFGE profiles) and provided additional strain information to enhance the traceability of *L. monocytogenes* in Sardinian swine slaughterhouses. The monitoring over time of the contamination of the five slaughterhouses highlighted the emergence of new subtypes (P3, P5, P6), as well as the persistence of predominant PFGE profiles (P1, P2, P4). Although reintroduction from an upstream point source (e.g., a farm) cannot be completely excluded, predominant PFGE profiles were recovered in SB, SC, and SD during the 3-year survey. The role of live animals as a source of new PFGE profiles (SB and SD) and of some environmental niches as a source of cross contamination of carcasses (SC and SD) was highlighted

(Table 3). According to previous studies *(11, 19)* some subtypes of *L. monocytogenes* (P1) can persist in the same ecological niches in the slaughterhouse environment for months or years, becoming endemic and plant-specific.

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