Listeria monocytogenes persistence in ready-to-eat sausages and in processing plants

Anna Mureddu, Roberta Mazza, Federica Fois, Domenico Meloni, Roberto Bacciu, Francesca Piras, Rina Mazzette

Settore di Ispezione degli Alimenti di Origine Animale, Dipartimento di Medicina Veterinaria, Università di Sassari, Italy

Abstract

Listeria monocytogenes is of major concern in the fermented meat products and is able to persist in their processing environments. The aim of the present work was to evaluate the virulence profile and the persistence capacity of *L. monocytogenes* strains isolated in Sardinian fermented sausages processing plants. Food (ground meat, sausages at the end of acidification and ripening stage) and environmental samples (a total of n. 385), collected from 4 meat processing plants located in Sardinia (Italy), were examined to detect *L. monocytogenes* presence. All the *L. monocytogenes* isolates were identified by polymerase chain reaction (PCR) method. A subset of strains was also characterised by multiplex PCR-based serogrouping, using the *lmo0737*, *lmo1118*, *ORF2819* and *ORF2110* genes. Three different multiplex PCRs were used to obtain the virulence profiles by the *rrn*, *hlyA*, *actA*, *prfA*, *inlA*, *inlB*, *iap*, *plcA*, *plcB* and *mpl* marker genes. Furthermore, *in vitro* biofilm forming ability and resistance to disinfectants were carried out on microtiter plate. The overall prevalence was 31.5% in food, and 68.5% in environmental samples. The prevalent serotype resulted 1/2c (43%), followed by 1/2a (40%), 4b (8.6%), and 1/2b (8.6%). The amplification products of the virulence genes were found in all the isolates with the following prevalence: 77.1% *hlyA*; 100% *rrn*; 100% *prfA*; 97.1% *iap*; 65.7% *inlB*; 88.6% *inlA*; 100% *plcA*; 100% *plcB* and 74.3% *mpl*. As for biofilm forming ability, 37.1% of the strains were positive and resulted weak producer, but all the isolates were sensible to disinfectants showing a reduction of *L. monocytogenes* growth after each incubation time. More appropriate technologies and application of measures of hygienic control should be implemented to prevent the *L. monocytogenes* growth and cross-contamination in *salsiccia sarda* processing plants.

Introduction

Listeria monocytogenes environmental persistent colonisation of ready-to-eat meat processing plants has been reported by several authors (Gram *et al.*, 2007). The pathogen may survive in meat processing plants because its capability to multiply at low temperatures, adapt to disinfectants and adhere to various surfaces; furthermore, the biofilm forming ability is an important cause for such persistence (Arevalos-Sánchez *et al.*, 2012; Cruz *et al.*, 2012; Fonnesbech Vogel *et al.*, 2001). It is not still clear if the persistence ability of *L. monocytogenes* is the result of adaption of certain subtypes, of poor cleaning and disinfection procedures or tolerance to some of the used disinfectants (Gram *et al.*, 2007). Efficiency in cleaning and disinfection process is essential in preventing *L. monocytogenes* contamination of meat products. However, the presence of organic debris may inactivate the disinfectant, hence, the bacteria may be exposed to only sublethal concentrations and survives (Cruz *et al.*, 2012).

In Sardinian fermented sausages and their facilities *L. monocytogenes* was detected (Mazzette *et al.*, 2006), and serotypes involved in sporadic outbreaks of listeriosis (1/2a, 1/2B, 1/2c and 4b) were reported. Longitudinal studies were previously carried out to evaluate the occurrence in this traditional ready to eat meat product, and to trace the sources and the routes in the processing plants. Moreover, the persistence ability of *L. monocytogenes* strains was demonstrated by molecular typing methods (Meloni *et al.*, 2007, 2012a, 2012b).

The aim of the present work was to evaluate the virulence profile and the persistence capacity of *L. monocytogenes* strains isolated in Sardinian fermented sausages processing plants, during a short period of time, and to study the effectiveness of disinfecting products against the strains, in order to define more appropriate control strategies of the environment contamination.

Materials and Methods

A total of n. 385 samples taken from 4 Sardinian sausage processing plants, from 2 production batches, were examined. The samples were from: ground meat, sausages at the end of acidification and ripening period (n. 165), and surfaces (n. 220) with and without contact with meat.

Detection and enumeration of *L. monocytogenes* were carried out by using the ISO 11290-1:1996 and 11290-2:1998 (ISO, 1996, 1998) protocols, respectively. All the isolates (n. 173) had been previously identified and characterised by

molecular tests (Mureddu *et al.*, 2011). Among these, a subset of n. 35 *L. monocytogenes* strains, representative of the positive samples, was selected from various sources. The different plants, and, within these, the environmental and food samples, and the sampling sessions (I, II), were considered. The subset of *L. monocytogenes* strains were submitted to the biomolecular and phenotypic investigations described in the following.

Serotyping and molecular pathogenic profile

Serotyping was performed as described by Doumith *et al.* (2004). The marker genes selected for the multiplex polymerase chain reaction (PCR) assay were *lmo0737*, *lmo1118*, *ORF2819* and *ORF2110*. Moreover, three different multiplex PCRs were performed in order to detect ten virulence-associated genes using the protocols of Border *et al.* (1990) and Jaradat *et al.* (2002), with some modifications. *Multiplex PCR 1* amplified the *rrn*, *hlyA*, *actA* and *prfA* genes, *multiplex PCR 2* the *inlA*, *inlB* and *iap* genes and *multiplex PCR 3* the *plcA*, *plcB* and *mpl* genes. The GoTaq Hot Start Master Mix (Promega Corp., Madison, WI, USA) was used for amplification of the target DNA fragments, as indicated in the manufacturer's instructions. Primers sequences used are set in Table 1.

In vitro biofilm forming ability and resistance to disinfectants

The quantitative *in vitro* biofilm forming ability (BFA) was carried out on sterile 96-well polystyrene microtiter plates, using the method described by Stepanovic *et al.* (2004), modified. Each strain was inoculated into two

different microplates in order to assess biofilm formation after incubation at 30°C for 20 and 40 h. The optical density (OD) was evaluated using a Sunrise RC absorbance reader (Tecan, Maennedorf, Switzerland). In relation to the OD values, the strains of *L. monocytogenes* have been classified into 4 categories (Meloni *et al.*, 2012a, 2012b).

A qualitative *in vitro* evaluation of the resistance to disinfectants (chlore and quaternary ammonium compounds), commonly used in the plants included in this survey, was performed, according to Fox *et al.* (2004) method, modified. For each strain, 5 mL of the overnight *L. monocytogenes* culture (37°C) on TSB was obtained [approximately 10^8 colony forming units (CFU)/mL^{-1}]. The day of the experiment, starting from the disinfectant concentration used in each plant (stock concentration), two serial dilutions (10^{-1}, 10^{-2}) were prepared. A sterile microtiter ninety-six well plate, containing 150 µL of the disinfectants (stock, 10^{-1}, 10^{-2} concentration) was inoculated with 10 µL of a 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} fold diluted overnight culture. A positive and reagent control (without inoculum) were also used. Each plate was then placed in a Sunrise RC absorbance reader (Tecan,

Mannedorf, Switzerland), and reduction of *L. monocytogenes* concentration was monitored by measuring the OD at 620 nm, after four incubation time, for 0 up to 3 days (72 h).

Results

L. monocytogenes prevalence resulted as follows: 31.5% in food samples, 17.4% in *surface in contact*, and 11.4% in *surface not in contacts* with meat. In particular, during the process, prevalence increased until the end of the acidification stage (60%), then decreased in the ripening period (20% in finished products), but the contamination levels were always below 100 CFU/g, as recommended in EC Reg. 2073/2005 (European Commission, 2005) for the food safety criteria in the RTE foods.

Serotyping and molecular pathogenic profile

The 1/2c was the prevalent serotype (43%) in all the plants, except A, followed by 1/2a (40%), that was detected in all the samples (surfaces and food), 1/2b (8.6%) and 4b (8.6%) The molecular analysis of the virulence factors

(Table 2) showed that the amplification products of the analysed virulence genes were found in all the *Listeria monocytogenes* strains, with the following prevalence: 77.1% *hlyA*; 100% *rrn*; 100% *prfA*; 97.1% *iap*; 65.7% *inlB*; 88.6% *inlA*; 100% *plcA*; 100% *plcB* and 74.3% *mpl* (Table 2). Primers used for the detection of *actA* allowed the amplification of two different products (268 and 385 bp), showing a polymorphism for this gene. The 268 bp amplification band was detected in only 8.6% of isolates, while the 68.6% showed a 385 bp fragment.

In vitro biofilm forming ability and resistance to disinfectants

The 37.1% of the strains resulted able to *in vitro* biofilm formation after 40 h of incubation. All these strains belonged to the weak producer (WP) category, while none showed moderate (MP) or strong (SP) ability. The 50% of the strains able to produce biofilm belonged to serotype 1/2c, the 41.6% to 1/2a and the 8.3% to 4b.

The results of the *in vitro* evaluation of the resistance to disinfectants is expressed in Table 3 as a reduction (+) and not reduction (-) of *L. monocytogenes* growth, on the basis of the OD value at 620 nm. All the strains showed

Table 1. Sequence, concentration and size of the primers used to detect virulence genes.

PCR	Primers		Sequence (5'-3')	Primer concentration (µM)	Product size (bp)
1	*rrn*	F	CAGCAGCCGCGGTAATAC	0.9	938
		R	CTCCATAAAGGTGACCCT		
	hlyA	F	CCTAAGACGCCAATCGAA		702
		R	AAGCGCTTGCAACTGCTC		
	actA	F	GACGAAAATCCCGAAGTGAA		268/385
		R	CTAGCGAAGGTGCTGTTTCC		
	prfA	F	CTGTTGGAGCTCTTCTTGGTGAAGCAATCG		1060
		R	AGCAACCTCGGAACCATATACTAACTC		
2	*inlA*	F	CCTAGCAGGTCTAACCGCAC	0.9	255
		R	TCGCTAATTTGGTTATGCCC		
	inlB	F	AAAGCACGATTTCATGGGAG		146
		R	ACATAGCCTTGTTTGGTCGG		
	iap	F	ACAAGCTGCACCTGTTGCAG		131
		R	TGACAGCGTGTGTAGTAGCA		
3	*plcA*	F	CGAGCAAAACAGCAACGATA	0.9	129
		R	CCGCGGACATCTTTTAATGT		
	plcB	F	GGGAAATTTGACACAGCGTT		261
		R	ATTTTCGGGTAGTCCGCTTT		
	mpl	F	TTGTTCTGGAATTGAGGATG		502
		R	TTAAAAAGGAGCGGTGAAAT		

PCR, polymerase chain reaction.

Table 2. Virulence genes prevalence (%) of the *L. monocytogenes* strains (n. 35) in relation to the plants.

| Plant | Strains (n.) | Virulence genes prevalence (%) | | | | | | | | | |
| | | Multiplex 1 | | | | Multiplex 2 | | | Multiplex 3 | | |
		actaA	*hlyA*	*rrn*	*prfA*	*iap*	*inlB*	*inlA*	*plcA*	*plcB*	*mLp*
A	9	100	100	100	100	100	89	100	100	100	89
B	10	100	90	100	100	100	90	70	100	100	70
C	10	80	70	100	100	90	30	90	100	100	70
D	6	100	100	100	100	100	67	100	100	100	100

a reduction of *L. monocytogenes* growth after each incubation time (24, 48 and 72 h). The *L. monocytogenes* isolates were from environmental (n. 16) and products (n. 19) samples. Most of the strains belonged to 1/2c (n. 15) and 1/2a (n. 14) serogroups, while only 6 to 1/2b (n. 3) and 4b (n. 3).

In Table 4 the results of serotyping, biofilm forming ability and resistance to disinfectants of *L. monocytogenes* strains are summarised in relation to the plant, batches and source. In plant A, *L. monocytogenes* was detected both in food (except for the sausage at the end of ripening) and in environmental samples; all the isolates belonged to 1/2b and 1/2c serovars, and resulted NP biofilm and sensitive to disinfectants. In plant B, the strains were also isolated in food and in environmental sites, from both production batches. The 4b serovar profile (n. 1 strain) was isolated only in this plant from GM samples. Five strains isolated from food and environmental samples belonged to 1/2a resulted NP, while two strains isolated from the SWC were WP. In plant C, two strains from GM samples (batch I), belonging to 1/2a and 1/2c serovars, were WP; WP strains belonging to 1/2a serotype were also detected in AS, and both serotypes in surface *in contact* samples. The plant C had the higher production capacity. In plant D, four strains (batch I) belonged to 1/2c serovars, and isolated from food samples, were WP, in particular three were from GM and one from AS.

Discussion and Conclusions

The results underline the overall presence of *L. monocytogenes* in different samples of Sardinian fermented sausages and environmental niches. In addition to the primary contamination by raw meat, cross contamination by surfaces in contact with meat should be considered as source of *L. monocytogenes*. An inhomogeneous decrease of the pathogen prevalence was observed in ripened sausages (29.1%), highlighting, in agreement with other authors (Thévenot *et al.*, 2005), the presence of strains able to survive during sausages fermentation. Even if the contamination level was always low, the presence of *L. monocytogenes* in the ripened products confirms that the hurdles of microbial growth (mainly the a_w decrease) should properly be applied during the ripening in order to prevent the *L. monocytogenes* growth.

The evaluation of biofilm production showed a low short-time persistence (3-4 months) capacity of the *L. monocytogenes* strains included in the study. Most of the isolates were unable, and only 37.1% showed a weak *in vitro* ability to biofilm formation, in particular strains from food (61.5%) and, less

frequently, from surfaces with (trolleys) and without (floor drains) contact with meat. Harborage niches, as the floor drains, can be critical sites for the processing plant environment and food product contamination (Tompkin, 2002). Decontaminating floor drains is essential to prevent the *L. monocytogenes* biofilm formation, and a potential resistance against available disinfectants and treatments (Gram *et al.*, 2007). About this topics, disinfectant resistance has been studied among groups of persistent and non persistent strains, but no clear link between persistence

and increased disinfectant resistance was seen (Kastbjerg *et al.*, 2009; Holah *et al.*, 2002). Some authors showed that a reorganisation or regeneration mechanism of the lipidic membrane may occur (Fox *et al.*, 2011), and others highlight that no strains show unique properties that lead to persistence, but harborage sites in food industry premises and equipment can persist (Carpentier *et al.*, 2011). In our study all the strains resulted sensitive to all the tested disinfectants. Other authors had previously reported that the recommended concentrations of commercial sanitisers are higher

Table 3. *In vitro* qualitative reduction evaluation of *L. monocytogenes* by disinfectant products.

Disinfectant	Plant	Concentration (%)	Temperature (°C)	Incubation time (h)
Chlore substances	1-5%	37	24	(+)
			48	(+)
			72	(+)
Quaternary ammonium compounds	2%	37	24	(+)
			48	(+)
			72	(+)

Table 4. Serotype, biofilm forming ability and resistance to disinfectants of *L. monocytogenes* strains, in relation to the plants, batches and sources.

Plant	Source	Batch I	Batch II	Serotype	BFA	Disinfectant resistance
A	GM	■		1/2b (1)	NP	(-)
	AS	■		1/2b (2)	NP	(-)
				1/2c (1)	NP	(-)
	RS	■		1/2c (1)	NP	(-)
	SC	■		1/2c (3)	NP	(-)
	SWC	■		1/2c (1)	NP	(-)
Total n. 9						
B	GM	■	■	1/2a (2) batch I	NP	(-)
				4b (2) batch II	WP (1)	(-)
	AS		■	4b (1)	NP	(-)
	RS					
	SC	■	■	1/2a (1) batch I	NP	(-)
				1/2a (2) batch II	NP	(-)
	SWC	■		1/2a (2)	WP	(-)
Total n. 10						
C	GM		■	1/2a (2)	NP (1) WP (1)	(-)
				1/2c (1)	WP	(-)
	AS	■		1/2a (1)	WP	(-)
	RS	■		1/2a (1)	NP	(-)
		■	■	1/2a (2) batch I	WP	(-)
	SC			1/2c (1) batch I	WP	(-)
				1/2c (2) batch II	NP	(-)
	SWC					
Total n. 10						
D	GM	■		1/2c (3)	WP	(-)
	AS					
	RS	■		1/2c (1)	WP	(-)
	SC	■		1/2c (1)	NP	(-)
	SWC	■		1/2a (1)	NP	(-)
Total n. 6						

BFA, biofilm forming ability; GM, ground meat; AS, sausage at the end of acidification; RS, sausage at the end of ripening; SC, surface in contact; SWC, surface without contact.

than required (Cruz *et al.*, 2012). Although the results are preliminary and related to a small number of strains, the microtiter plate assay seems to be useful as an indirect way of assess the disinfectant resistence of *L. monocytogenes* strains.

The molecular findings revealed the presence of all the considered virulence genes, suggesting that all the isolates could be potentially dangerous for public health. No correlation between serotype and virulence profile was observed, neither between serotypes and plants. This is not in agreement with previous studies, where some *L. monocytogenes* serotypes appeared to be unique for a processing plant and should be considered as plant-specific (Fugett *et al.*, 2007).

The fermented pork meat RTE products, as dry and semi-dry sausages, have been rarely implicated in food-borne disease. However, more risks should be linked to the consumption of these products, mainly in the manufacturing of traditional products, where an empirical application of the hurdles technology and lack of measures of hygienic control often occurs.

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