

PRESENCE OF *LISTERIA MONOCYTOGENES* IN RAW MILK FOR HUMAN CONSUMPTION*

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SUMMARY

Listeria monocytogenes is an important cause of zoonosis (listeriosis) and is responsible for food-related illness ranging from gastroenteritis to meningitis. Listeriosis is transmitted to humans through contaminated foods, such as raw milk. In Italy most of the milk consumed daily is heat treated, however since in 2007, the Ministry of Health authorized automatic vending machines delivering unpacked raw cow milk, the consumption of liquid raw milk increased. To date, there are 1032 dairy herds allowed to supply raw milk to vending machines (Integrated National Annual Report of the Italian Ministry of Health), 112 of them (about 10%) are registered in the Piedmont Region. In this study, the presence of *Listeria monocytogenes* in raw milk from bulk tanks in farms and from raw milk vending machines in North Western Italy (Piedmont Region) was investigated. Between July 2010 and July 2011, 407 samples of raw milk were collected from 112 vending machines or from the 93 farms supplying vending machines and were analyzed. *Listeria monocytogenes* was isolated from 7 out of 407 milk specimens (1.72%; CI95%: 0.7%-3.5%); 3/93 were isolated from farms (3.23%; CI95%: 0.7%-9.1%) and 4/112 from vending machines (3.6%; CI95%: 0.9%-8.9%). The serotypes most frequently isolated were 1/2 a and 4b/4e.

These results outline the importance of the advice « to be boiled before drinking » that Italian Ministry of Health made compulsory on the vending machines to inform consumers. Heat treatment of raw milk still remains the most effective tool to reduce the risk of listeriosis and other food-borne illness.

Keywords: *Listeria monocytogenes*, Bovine raw milk.

RÉSUMÉ

Listeria monocytogenes est une cause importante de zoonose (listériose) à transmission alimentaire allant de la gastro-entérite à la méningite. La listériose est transmise aux humains par des aliments contaminés, comme le lait cru. En Italie, la majorité du lait consommé quotidiennement est le lait traité thermiquement, mais depuis 2007, comme le Ministère de la Santé a autorisé les distributeurs automatiques de vente de lait cru, la consommation de ce produit a augmenté. Actuellement, en Italie, 1 032 troupeaux sont autorisés à fournir des distributeurs automatiques de lait et 112 (environ 10%) sont enregistrés dans la Région Piémont. Cette étude a voulu étudier la présence de *Listeria monocytogenes* dans le lait cru provenant des troupeaux et des distributeurs automatiques dans le Nord-Ouest de l'Italie (Région du Piémont). Entre juillet 2010 et juillet 2011, 407 échantillons de lait cru ont été recueillis à partir des 112 distributeurs automatiques ou des 93 fermes qui fournissent des distributeurs automatiques et ont été analysés. *Listeria monocytogenes* a été isolé à partir de 7 sur 407 échantillons de lait (1,72%, IC95% : 0,7% -3,5%); 3/93 ont été isolés dans les fermes (3,23%, IC95%: 0,7% -9,1%) et 4/112 chez les distributeurs automatiques (3,6%, IC95%: 0,9% -8,9%). Les sérotypes les plus fréquemment isolés étaient 1/2 a et 4b/4e.

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* Texte de la communication écrite présentée au cours des Journées scientifiques AEEMA, 31 mai-1er juin 2012

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Ces résultats décrivent l'importance de l'avis « de faire bouillir avant de le boire » que le ministère italien de la Santé a rendu obligatoire sur les machines automatiques de vente afin d'informer les consommateurs. Le traitement thermique du lait cru est toujours le plus efficace pour réduire le risque de listériose et d'autres maladies d'origine alimentaire.

Mots-clés : *Listeria monocytogenes*, lait cru de vache.



I - INTRODUCTION

Bacteria belonging to genus *Listeria* are widespread in nature and live naturally in plants and soil environments: their high adaptability enables to growth in a large kind of matrices. Within the genus, six species are nowadays known: *monocytogenes*, *innocua*, *seeligeri*, *welshimeri*, *ivanovii* and *grayi* [Donnelly 2001]: only *Listeria monocytogenes* is known to be pathogen for animals and humans.

Listeria monocytogenes is a gram-positive, facultative anaerobe and intracellular bacterium, motile via flagella at 30 °C but usually not at 37 °C. According to the presence of different antigens *Listeria monocytogenes* can be classified in 13 different serotypes named 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. *Listeria monocytogenes* can cause life-threatening infections in foetuses, newborns, and immunocompromised people. It also causes a severe flulike illness in pregnant women and self-limited gastrointestinal infections in immunocompetent people.

As these bacteria are recognized to be an important cause of zoonosis (listeriosis), *Listeria monocytogenes* is included in the list of agent of zoonoses to be monitored, according to Directive 2003/99. Furthermore, as the transmission to human through food is stated [Kozak, 1996] *Listeria monocytogenes* is also listed among microbiological criteria to be checked according to food safety regulation and in particular in line with Regulation (EC) 2073/2005. In compliance with this European regulation, the presence of *Listeria monocytogenes* must be checked in ready to eat foods in order to guarantee consumers protection.

Milk is recognized to be one of the food often responsible for foodborne listeriosis: animal can be infected through feed contaminated by *Listeria* spp. And, after an incubation period, they can spread bacteria though milk even in absence of symptoms [Kozak, 1996].

In the most cases, in Italy as in other industrialized countries, milk is consumed after heat treatment: pasteurization or UHT treatment are able to eliminate all viable *Listeria* cells, making milk safe for consumers. However, since 2007, as Italian Ministry of Health authorized automatic vending machines (VMs) for selling unpacked raw bovine milk, in Italy the consumption of raw milk has increased. Before this national law was enacted, traditionally, raw milk was used to produce artisan cheeses, in which long ripening periods chemically modify substrate. Indeed, the reduction of Aw and pH makes the survival of pathogen bacteria less probable and reduces their virulence: in the case of raw milk, consumed as bought at VMs, neither heat treatments nor chemical changes in the matrix can reduce the risk of presence of *Listeria*.

In the last 3 years in Italy the number of authorized VMs increased rapidly. Presently, there are 1032 dairy herds allowed to supply raw milk vending machines (Integrated National Annual Report of Italian Ministry of Health), 112 of them (about 10%) are registered in Piedmont Region. In this area the number of VMs grew from 109 in 2007 to 175 in 2011, about 4 VMs for each 100.000 inhabitants.

The success of these VMs is to be ascribed in the higher money intake for farmer, as milk is directly sold in farm; at the same time,

consumers appreciate this new way of distribution as they can have a better prize and an enhanced taste buying raw product.

According to consumer safety policy, Ministry of Health fixed criteria that must be respected for raw milk vending machines; food business operators have to guarantee that milk sold by VMs is daily supplied, kept in refrigeration temperature, both during transport and inside VMs, and microbiological own-checks analyses must be performed in order to maintain high hygienic level of the product. In addition to

these requirements, official control services collect milk samples in order to verify the compliance of milk to microbiological (*Listeria monocytogenes*, *Salmonella*, *E.coli* STEC, Coagulase positive *staphylococci*) and chemical (M1 aflatoxin) criteria.

This papers report the results of microbiological criterion *Listeria monocytogenes* obtained in the period from July 2010 to July 2011 in raw milk samples collected in Piedmont both from VMs and from bulk tanks of farm supplying VMs.

II - MATERIALS AND METHODS

In the Northwestern side (Piedmont Region) of Italy from July 2010 to July 2011, a total of 407 raw milk samples were collected and checked for the presence of *Listeria monocytogenes*. Each milk sample was constituted by 5 units forming the samples, for a total 2035 performed analyses. Out of 407 raw milk samples, 216 were collected at VMs and 191 from bulk tanks in farm supplying VMs; this sampling activity included 112 vending machines and 93 farms. Collection of samples was performed by Local Veterinary Service and milk was transported to the Food Control Laboratory (Laboratorio Controllo Alimenti) of Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta in Turin. According to ISO 6218:2007 requirements, milk samples and analyzed within 24 hours.

Qualitative analyses were performed in order to check the compliance of samples on the criterion *Listeria monocytogenes* (absence in 25 mL): biomolecular method (*Real-time PCR* - Biorad AFNOR BRD 07/10-04/05) was firstly performed for the detection of DNA of *Listeria monocytogenes*. In case of positive results, milk samples were re-tested by microbiological protocol according to ISO 11290:1996

Test portion of 25 mL was pre-enriched with 225 mL of Half-Fraser (Microbiol[®]) and incubated at 30°C for 24 hours. After incubation, a portion of this enriched broth was spread on ALOA (Microbiol[®]) and OXFORD (Liofilchem Srl[®]) Petri dishes and incubated at 37°C for 48 hours. A second portion of 1 mL was trasferred into 9 mL of Fraser (Meus Srl[®]) broth for incubation at 30°C for 48 hours and after incubation period a portion was spread on

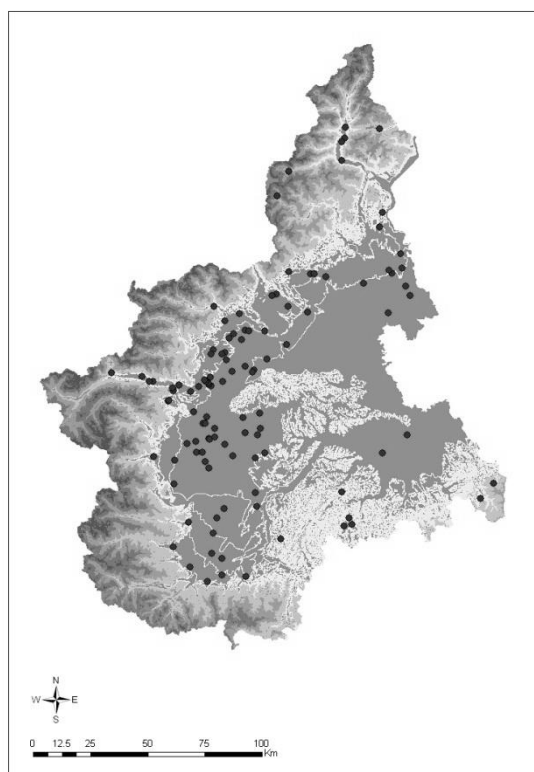
ALOA (Microbiol[®]) and OXFORD (Liofilchem Srl[®]) Petri dishes and incubated at 37°C for 48 hours. *Listeria monocytogenes* colonies appear blue with white aloe on ALOA agar, and black with deep center on OXFORD (Liofilchem Srl[®]) agar.

Viable colony suspected to be *Listeria monocytogenes* were biochemically confirmed by GP-VITEK[®] (BioMérieux).

Listeria monocytogenes strains were analyzed for the identification of serotypes with antisera provided by Denka Seiken Co, LTD[®]. For determination of the O-antigen a heat-inactivated bacterial culture was used in a slide agglutination protocol. For the determination of the H-antigen test tube method with bacteria cultured in liquid media was performed: in order to obtain clear results, as *L. monocytogenes* possesses only few flagella, mobility of testing organisms was raised by passing them through a semi-liquid agar medium (BHI –DIFCO[™] media containing 0.2% agar).

All statistical analyses were done with SAS[®] software v9.2 (SAS Institute Inc., Cary, NC, USA). Descriptive statistics were performed for all variables measured. Frequency data (percentages) were calculated to determine the occurrence of *Listeria monocytogenes* and 95% interval confidence were estimated. The association between occurrence of *Listeria monocytogenes* and the following factors (distance between dairy herds and vending machine, number of heads in the herd, previous occurrence of pathogens, daily average temperature, months and seasons) was evaluated by Pearson χ^2 test.

Figure 3
Map of milk sampling collection (herds and VM) distributions in Piedmont region



III - RESULTS

Listeria monocytogenes was isolated from 7 out of 407 milk samples (1.72%; CI95%:0.7%-3.5%).

Three strains were isolated from milk collected at farms: 3.23% (CI 95%: 0.7%-9.1%); four *L. monocytogenes* were isolated in milk at vending machines: 3.6% (CI95%: 0.9%-8.9%).

As from two samples were possible to isolate respectively two *Listeria monocytogenes* strains, a total of 9 strains were serotyped.

The most frequently isolated serotypes were 1/2a (6 strains) and 4b/4e (3 strains).

The strains were isolated from a total of 5 farms responsible for the positive results: three of them were settled in Turin province, one in Cuneo Province and the last in the Verbania

Area.

The farm settled in Cuneo area was found positive for *Listeria monocytogenes* three times during this study: twice at vending machine and once at tank level. Every single time, the serotype involved in this context was 1/2a. On the contrary, in a milk sample collected from the bulk tank in a Turin area farm, two different serotypes (1/2a and 4b/4e) were identified.

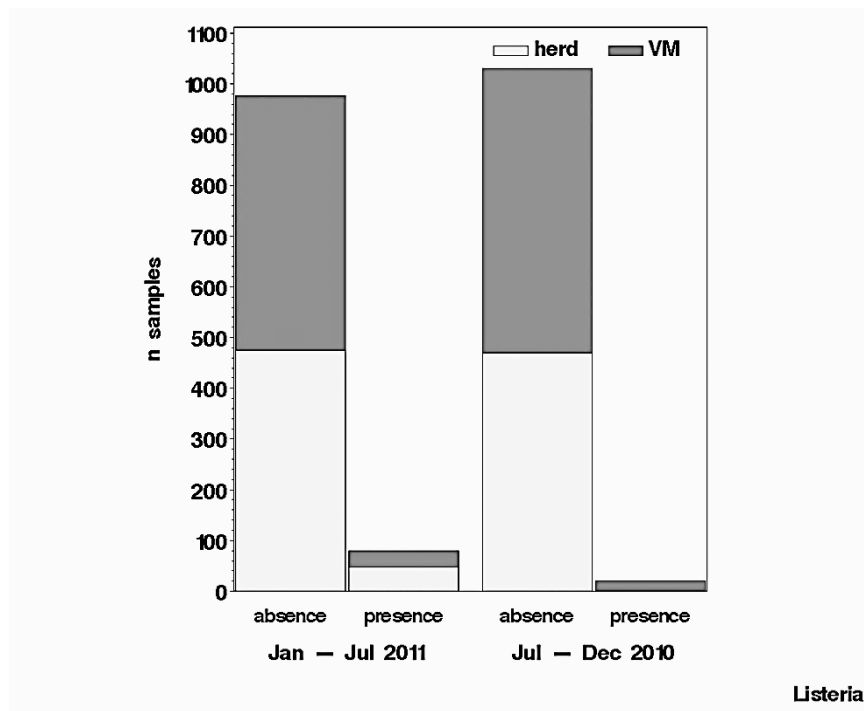
Only occurrence of pathogens in the past was related to presence of *Listeria monocytogenes* and statistically significant at bivariate analysis. (Table 1). Hence, the presence of *Listeria monocytogenes* in samples was detected at herd and at VM from November to June, quite uniformly in the study period.

Table 1
Bivariate analysis calculated by Pearson χ^2 test

FACTOR	Category	% nc	χ^2	p-value
Season	Winter	0.0%	2.1241	0.5471
	Spring	2.2%		
	Autun	0.8%		
	Summer	2.7%		
Daily temperature average	$\leq 4.1^\circ$	0.0%	2.9355	0.4017
	4.1° - 10°	2.3%		
	10.1° - 18°	2.9%		
	$>18^\circ$	0.8%		
N° of dairy cattle	≤ 66	3.9%	4.8678	0.1817
	67-126	1.8%		
	127-196	1.1%		
	> 196	0.0%		
Sampling collection	Herd	1.6%	0.0474	0.8276
	VM	1.5%		
Wending machine position	into farm	1.5%	2.6208	0.1055
	market	3.3%		
Occurrence of pathogens in the past	yes	25.0%	39.6441	$<.0001$
	no	1.0%		

Figure 4

Number of samples analyzed to detect *Listeria monocytogenes* at herd and at VM



IV - DISCUSSION AND CONCLUSION

Other authors report various prevalences of *Listeria monocytogenes* in bulk tank milk: previous studies describe values ranging from 1% to 12% [Oliver *et al.*, 2005]. In this study, *Listeria monocytogenes* was isolated in 7 milk samples (1.72%). These bacteria are widespread in nature and their high adaptability enables to growth in refrigerated raw milk and their presence in this kind of food does not surprise, as dairy-related outbreaks of listeriosis are worldwide reported [Allerberger *et al.*, 2010].

Among the different serotypes known, the ones identified in this study were 1/2 a and 4b/4e: this result is also in line with the finding of other author [Oliver *et al.*, 2005] [Jayarao *et al.*, 2006].

According to scientific literature 1/2 a and 4b/4e are considered in the strains often responsible for food-borne outbreaks: possible mechanisms for the apparent prevalence of certain serotypes in human foodborne illness remain unidentified. The propensity of certain strain lineages (epidemic clones) to be implicated in common-source outbreaks and the prevalence of serotype 4b among epidemic-associated strains also remain poorly understood [Kathariou, 2002].

Results of bivariate analysis showed no effects of seasonality as the presence of *Listeria monocytogenes* in raw milk were found in

winter, spring, summer and autumn. This result was expected [Franciosi *et al.*, 2009]. Also sampling collection (at herd or vending machine) demonstrated no association with occurrence of pathogen. *Listeria* can be found indifferently in dairy herds as in VM.

In conclusion, this study confirms that unpasteurized milk can be vehicle of *Listeria monocytogenes*: its multiplication capacity at refrigeration temperature and the long persistence in food make these bacteria an important threat for the population health status, especially if consumers are younger, elderly or exhausted people.

The presence of pathogen bacteria in milk can be due both to the contact with contaminated sources in the farm and to excretion from infected animals and can be an important source of food-borne outbreak. For this reason, high level of hygienic condition in farm management is required in particular when raw milk is sold throughout vending machine. As pasteurization and other Heat treatment of raw milk still remains the most effective tool to reduce the risk of listeriosis and other food-borne illness, consumers must be informed about potential risk linked to raw milk consumption Furthermore, results obtained in this study outline the importance of the advice "to be boiled before drinking" that Italian Ministry of Health made compulsory on the vending machines to inform consumers.

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