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Detection of virulence-associated genes and epidemic clone markers in *Listeria monocytogenes* isolates from PDO Gorgonzola cheese

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Highlights

► Applications of PCR for routine screening of *L. monocytogenes* strains.

► 98.1% of *L. monocytogenes* strains had all seven tested virulence-associated genes.

► High prevalence of presumptive ECV *L. monocytogenes* strains in dairy products.

► Detection of ECV-specific markers in 88% of serotype 1/2a *L. monocytogenes* isolates.

► Identification of an atypical L. innocua strain.

Keywords *Listeria* monocytogenes; Gorgonzola; Virulence genes; Epidemic clones; Pathogenic potential

Abstract

Fifty-three *Listeria monocytogenes* isolates obtained from Gorgonzola cheese and previously characterized with biochemical typing, serotyping and pulsed field gel electrophoresis (PFGE), were analyzed in this study. Seven virulence-associated genes were selected (*actA*, *inlC*, *inlJ*, *plcA*, *prfA*, *hlyA* and *iap*) and their presence was investigated using PCR. All virulence-associated genes were detected in 51 isolates. One isolate did not show amplification of the *inlC* gene and one other isolate, previously mis-identified as *L. monocytogenes* probably due to atypical phenotypes, resulted negative by PCR for all virulence genes and was identified as *Listeria innocua* by 16 S rRNA gene sequencing analysis. A multiplex PCR assay was used to evaluate the presence of markers specific for epidemic clones (ECs) of *L. monocytogenes*. The marker specific for the recently identified epidemic clone V (ECV) was detected in 38 of 43 (88%) of serotype 1/2a isolates. These findings suggest that Gorgonzola cheese can represent a significant source of *L. monocytogenes* and potential health risk for listeriosis as almost all isolates (94%) could be potentially virulent and that 38 (~ 72%) were presumptive positive ECV. PCR screening for both virulence-associated genes and EC markers may be useful for rapidly evaluating the epidemic potential and public health risk posed by *L. monocytogenes* in PDO Gorgonzola cheese and other dairy products.

1. Introduction

Listeria monocytogenes is a pathogenic bacterium responsible for listeriosis, which although has a low incidence, remains a major public health concern considering its high mortality rate. Additionally, *L. monocytogenes* can survive in stressful environmental conditions and therefore is found in many food manufacturing plants and foods (Swaminathan and Gerner-Smidt, 2007).

Gorgonzola, a neutral pH blue veined cheese, awarded with the Protected Designation of Origin (PDO) from the European Commission (EU Regulation, 1996), represents an optimal food substrate for the growth of L. monocytogenes. Gorgonzola is made from pasteurized cow's milk, but can become contaminated with *L. monocytogenes* as a result of post-pasteurization contamination from the ripening environment or during handling (Carminati et al., 2004). Consumption of soft fresh cheeses and surface-ripened soft cheeses has been responsible for numerous outbreaks and sporadic cases of listeriosis (Farber et al., 1990, Gaulin et al., 2003, Gianfranceschi et al., 2006, Linnan et al., 1988, Lundén et al., 2004 and Pagotto et al., 2006). A case of listeriosis associated with consumption of Gorgonzola cheese was reported in Italy in 2006. *L. monocytogenes* was detected both from the patient and packaged/unpackaged cheese samples. Molecular subtyping later confirmed that all isolates were indistinguishable and highlighted the persistence of this subtype of *L. monocytogenes* in the Gorgonzola production plant for five months (Gianfranceschi et al., 2006). Considering the worldwide commercialization of Gorgonzola PDO cheese and its economic relevance (CPGC, 2007), it is very important to better understand and characterize *L. monocytogenes* isolates from this environment. In a recent survey, 94.7% of isolates from Gorgonzola belonged to serotypes predominately associated with human listeriosis cases (Kathariou, 2002, Lomonaco et al., 2009 and Swaminathan and Gerner-Smidt, 2007).

In the past 30 years a small number of closely related clones have caused many different outbreaks of *L. monocytogenes* worldwide (Chen et al., 2007 and Kathariou, 2002). Epidemic clones (ECs) of *L. monocytogenes* have been defined as genetically related isolates of a presumably common ancestor, involved in different, temporally and geographically unrelated outbreaks (Chen et al., 2007, Cheng et al.,

2008 and Kathariou, 2002). Five ECs have currently been identified: three in serotype 4b (ECI, ECII, ECIV) and two in serotype1/2a (ECIII and ECV) (Chen et al., 2007, Kathariou, 2002 and Knabel et al., 2012) and EC-specific markers have repeatedly been observed worldwide (Chen et al., 2007, Franciosa et al., 2007, Kathariou, 2002, Lomonaco et al., 2011, Mammina et al., 2009 and Neves et al., 2008). Whether or not ECs have a greater pathogenic potential is still a controversial issue (Kathariou, 2002), as even ECs strains can show attenuated virulence in vitro (Roberts et al., 2009). Possibly, most outbreaks of human listeriosis are linked to EC strains, not because they are more virulent than other *L. monocytogenes* strains, but because they have a greater ability to persist in the environment and to replicate in foods (Roberts et al., 2009). Multi-virulence-locus sequence typing (MVLST), can be considered the gold standard in the molecular subtyping of EC strains. However, MVLST can be expensive, time-consuming and laborious and alternative methods for rapid screening of EC strains have been developed based on multiplex PCR or multiplex SNP-typing (Chen and Knabel, 2007 and Lomonaco et al., 2011).

Differentiation of virulent and epidemic strains of *L. monocytogenes* would be useful in evaluating the risk associated with strains isolated from dairy products and also in developing more effective intervention strategies for their control in dairy products like Gorgonzola. PCR may represent a rapid and easy way to screen for virulence- and EC- specific genes in order to i) determine the potential pathogenic profile and relative risk of *L. monocytogenes* isolates, ii) differentiate potential virulent and avirulent isolates, and iii) enhance the epidemiologic investigation and prevention of listeriosis outbreaks. Therefore, the purpose of this study was to evaluate the potential public health risk posed by *L. monocytogenes* isolates obtained from PDO Gorgonzola cheese by determining their virulence-gene profile and by testing for the presence of EC-specific markers.

2. Materials and methods

2.1. Isolates

In this study we analyzed 53 *L. monocytogenes* isolates (Table 1), previously characterized with conventional biochemical and molecular assays and selected from a larger set of 95 isolates obtained from 22 PDO Gorgonzola cheese plants from 2004 to 2007 (Lomonaco et al., 2009). Isolates were chosen to represent different sources, serotypes and different pulsed-field gel electrophoresis (PFGE) profiles: 24 isolates showing a unique PFGE profile (pulsotype) and 29 other isolates selected from profiles A (1), B (1), C (18), D (6) and E (3) (Lomonaco et al., 2009).

Twenty-five strains were used as positive reference strains divided as follows: 20 strains from the ILSI *Listeria monocytogenes* strain collection (Fugett et al., 2006); five human clinical sporadic strains isolated in the Piedmont Region of Italy in the last ten years and one strain of *L. innocua* as negative control (Supplemental Table 1). Each strain was incubated at 37 °C overnight in 5 ml of Tryptone Soy Broth (Oxoid). DNA was extracted by boiling and stored at − 20 °C before use.

2.2. PCR detection of virulence-associated genes

Seven genes (Table 2) were chosen based on available literature and on their importance in *L. monocytogenes* pathogenesis. The presence of a truncated form of the *prfA* gene or its absence has been linked to non-hemolytic and avirulent strains. The *iap* and *actA* genes are required for invasiveness and strains lacking the *hlyA* gene have been shown to be avirulent (Vázquez-Boland et al., 2001). The *plcA* gene

is required for primary vacuole lysis and finally, two internalin genes (*inlC* and *inlJ*) are involved in post-intestinal infection (Bierne et al., 2007). *PlcA* and *actA* primers were designed using available sequences on GenBank after alignment with MEGA 5.0 software (Tamura et al., 2011). All primers (Table 2) were synthesized by Sigma-Aldrich (Milan, Italy). PCRs were carried out as described by the authors with some modifications (Table 2). Amplicons were electrophoresed on 2% agarose gel, stained with ethidium bromide (0.5 μg/ml) and visualized by a UV transilluminator (Bio Rad UV-gentm).

2.3. PCR detection of EC markers

Isolates of serotype 1/2a were evaluated for the presence of markers specific for the ECs associated with this serotype (ECIII and ECV). A multiplex PCR reaction was used (Chen and Knabel, 2007), modified by the incorporation of ECV primers (Knabel et al., 2012). PCR products were visualized as described above.

3. Results and discussion

Different subtypes of *L. monocytogenes* diverge in their pathogenicity for humans and/or in their ability to transmit to humans (Cheng et al., 2008). Lineage I strains of *L. monocytogenes* have been predominantly associated worldwide with human listeriosis and are also considered more likely to cause human disease compared to lineage II strains (Cheng et al., 2008 and Kathariou, 2002). Many studies (reviewed by Vázquez-Boland et al., 2001) have identified several virulence genes used to study the pathogenic potential of *L. monocytogenes*. The presence of the *prfA*, *plcA*, *hlyA*, *actA* and *iap* genes was evaluated in *L. monocytogenes* strains isolated from patients with spontaneous abortions (Kaur et al., 2007); the occurrence of *inlA*, *inlC* and *inlJ* was investigated in strains isolated from human cases, food and environment (Liu et al., 2007). Several previous studies detected multiple distinct mutations leading to premature stop codons (PMSCs) in the *inlA* gene, resulting in expression of truncated forms of internalin A and consequent reduced virulence (Jacquet et al., 2004, Nightingale et al., 2007, Nightingale et al., 2008 and Van Stelten et al., 2010).

Virulence of *L. monocytogenes* strains isolated from dairy products remains poorly studied in Italy as literature has primarily focused on fish and ready-to-eat (RTE) meat products and their processing environments (Conter et al., 2007 and Mazzette et al., 2007). Therefore we deemed it important to evaluate the presence of virulence-associated and EC-specific genes in *L. monocytogenes* isolates from PDO Gorgonzola cheese to enhance our knowledge of the potential health risk posed by this product.

Although testing of different protocols and amplification conditions was carried out, a multiplex PCR could not be developed and thus a PCR simplex for each virulence-associated gene was used. Specificity of the two primers sets designed in the current study (*actA* and *plcA*) was assessed and amplification was observed only with *L. monocytogenes* isolates (Supplemental Table 1).

Concerning Gorgonzola-associated *L. monocytogenes* isolates, only one isolate (ID = 11) lacked all seven genes, thus likely representing an avirulent strain of *L. monocytogenes*, or a false-positive *L. monocytogenes*. Although previously characterized with several different pheno- and genotypic methods (Lomonaco et al., 2009), 16S rRNA gene sequencing was performed on isolate 11 to confirm its identification. This isolate was identified as *L. innocua* (data not shown), thus potentially representing an atypical *L. innocua* strain, as also previously observed (Johnson et al., 2004 and Moreno et al., 2012). Overall, results from the current study showed that 98.1% of the 52 analyzed *L. monocytogenes* isolates

had all virulence genes examined, as only isolate ID = 18 lacked the *inlC* gene (Table 1). Of note, the *inlJ* gene was detected in all isolates and thus they may potentially be virulent, as *inlJ* has been found to be present and fully operational only in strains showing full pathogenic potential (Liu et al., 2007). In general, no association with a particular serotype or pulsotype was observed, and this may be related to virulent strains showing non-functional major virulence genes or low virulence strains still carrying all virulence genes (Jiang et al., 2006 and Roche et al., 2009). Further studies will be aimed at clarifying whether these isolates are indeed virulent, also considering that both reduced invasion efficiency and attenuated virulence have been linked to atypical PMSCs in the *inlB* and *inlA* genes (Jacquet et al., 2004, Nightingale et al., 2007, Nightingale et al., 2008 and Van Stelten et al., 2010). *L. monocytogenes* strains with PMSCs were frequently isolated from RTE foods in the US, although *inlA* PMSCs were markedly underrepresented among ECs (Van Stelten et al., 2010). This is consistent with the frequent involvement of these strains in listeriosis cases worldwide (Kathariou, 2002). Notably, the presence of all seven virulence-associated genes does not mean a particular strain is virulent, as these genes are normally present in *L. monocytogenes* (virulent, less virulent and avirulent) but if strains do not have these genes, they are likely to be avirulent.

Therefore, screening for ECs can provide data critical for implementing intervention strategies to enhance food safety. Our findings showed that the ECIII-specific marker was never detected and 38 out of 43 (88%) of serotype 1/2a isolates were positive for ECV (Table 1). This EC was recently defined and linked to the 2008 nationwide Canadian outbreak, responsible for at least 57 cases with a 40% mortality rate (Gilmour et al., 2010 and Knabel et al., 2012). Specific ECs continue to be associated with sporadic cases around the world (Franciosa et al., 2007, Lomonaco et al., 2011, Mammina et al., 2009 and Neves et al., 2008). In Italy, EC-specific markers were detected in 27% to 38% of sporadic clinical isolates (Franciosa et al., 2007) and in three strains obtained from human sporadic cases occurred in Northern Italy (Lomonaco et al., 2011). Given the repeated and widespread incidences of listeriosis due to ECs in the past, ECs will likely again be involved in listeriosis cases in the future. Remarkably, *L. monocytogenes* EC strains may significantly vary in invasion efficiency and virulence phenotypes are not exclusively responsible for the association between EC strains and listeriosis outbreaks (Roberts et al., 2009). However, the high occurrence of multiple virulence-associated genes and ECV-specific markers in *L. monocytogenes* isolates obtained from PDO Gorgonzola cheese and related processing environments could pose a significant health risk as these isolates can be pathogenic and potentially capable of causing an epidemic. Further research is warranted to determine and compare expression profiles and virulence potentials of the isolates analyzed herein.

Notwithstanding, the proposed PCR screening protocol can be useful for surveillance, detection, and epidemiologic investigation of *L. monocytogenes*, and can also be employed to conduct preliminary screening prior to in vitro or in situ gene expression studies. Finally, findings from this study, one of the first reports of screening for EC-specific markers in isolates from dairy products and related processing environments, may be useful in risk assessment evaluations required by EU Regulation 178/2002 (EU Regulation, 2002).

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Table 1. List of the 52 Gorgonzola-associated *L. monocytogenes* isolates characterized in this study. One 4b/4e isolate (ID = 11, PFGE profile D) was initially considered as *L. monocytogenes* but was later identified as *L. innocua* by 16 S rRNA gene sequencing analysis, and thus is not listed in this table. Samples are indicated with E (environment) and C (cheese) in relation to the source of isolation. Positive and negative amplification for the eight genetic markers listed are indicated with + and −, respectively.

ID/Source	Previous ID/profile[a]	Serotype	Year	ECV	iap	plcA	hlyA	actA	prfA	inlJ	inlC
1-E	45/D	1/2a	2005	+	+	+	+	+	+	+	+
2-C	48/C	1/2a	2005	+	+	+	+	+	+	+	+
3-E	46/U[b]	1/2a	2005	+	+	+	+	+	+	+	+
4-E	47/C	1/2a	2005	+	+	+	+	+	+	+	+
5-E	9/U	1/2a	2005	−	+	+	+	+	+	+	+
6-E	10/U	1/2a	2005	−	+	+	+	+	+	+	+
7-E	49/U	1/2a	2005	+	+	+	+	+	+	+	+
8-E	50/C	3a	2005	/[c]	+	+	+	+	+	+	+
9-E	27/U	3a	2005	/	+	+	+	+	+	+	+
10-E	51/D	1/2a	2005	+	+	+	+	+	+	+	+
12-C	11/U	1/2a	2005	−	+	+	+	+	+	+	+
13-C	15/C	1/2a	2005	+	+	+	+	+	+	+	+
14-C	18/C	1/2a	2005	+	+	+	+	+	+	+	+
15-C	68/U	1/2a	2005	−	+	+	+	+	+	+	+
16-C	2/U	1/2a	2005	+	+	+	+	+	+	+	+
17-C	3/U	1/2a	2005	+	+	+	+	+	+	+	+
18-C	4/U	1/2a	2005	+	+	+	+	+	+	+	−
19-C	5/U	1/2a	2005	+	+	+	+	+	+	+	+
20-C	12/U	1/2a	2005	+	+	+	+	+	+	+	+
21-E	20/B	1/2a	2005	+	+	+	+	+	+	+	+
22-E	13/U	1/2a	2005	+	+	+	+	+	+	+	+
23-E	21/C	1/2a	2005	+	+	+	+	+	+	+	+
24-E	22/C	1/2a	2005	+	+	+	+	+	+	+	+
25-E	23/C	1/2a	2005	+	+	+	+	+	+	+	+
26-E	24/C	1/2a	2005	+	+	+	+	+	+	+	+
27-C	40/U	1/2a	2005	+	+	+	+	+	+	+	+
28-E	42/E	1/2a	2005	+	+	+	+	+	+	+	+
29-E	43/C	1/2a	2005	+	+	+	+	+	+	+	+
30-E	68/U	1/2b	2004	/	+	+	+	+	+	+	+
31-E	69/C	1/2a	2004	+	+	+	+	+	+	+	+
32-E	71/C	1/2a	2004	+	+	+	+	+	+	+	+
33-E	81/C	1/2a	2004	+	+	+	+	+	+	+	+
34-C	82/C	1/2a	2004	+	+	+	+	+	+	+	+
35-E	84/C	1/2a	2004	+	+	+	+	+	+	+	+
36-E	26/A	1/2a	2004	+	+	+	+	+	+	+	+
37-E	87/E	1/2a	2004	+	+	+	+	+	+	+	+
38-E	88/E	1/2a	2004	+	+	+	+	+	+	+	+
39-E	6/C	1/2a	2004	+	+	+	+	+	+	+	+
40-E	7/C	1/2a	2004	+	+	+	+	+	+	+	+
41-C	92/U	1/2b	2007	/	+	+	+	+	+	+	+
42-C	93/U	1/2b	2007	/	+	+	+	+	+	+	+
43-C	28/U	1/2a	2007	+	+	+	+	+	+	+	+
44-E	30/C	3a	2007	/	+	+	+	+	+	+	+
45-E	31/D	3a	2007	/	+	+	+	+	+	+	+
46-E	34/D	1/2a	2007	+	+	+	+	+	+	+	+
47-C	37/U	1/2a	2007	+	+	+	+	+	+	+	+
48-C	94/U	1/2b	2007	/	+	+	+	+	+	+	+
49-E	95/U	1/2b	2007	/	+	+	+	+	+	+	+
50-E	32/U	1/2a	2007	+	+	+	+	+	+	+	+
51-E	33/D	1/2a	2007	+	+	+	+	+	+	+	+
52-E	38/U	1/2a	2007	−	+	+	+	+	+	+	+
53-E	38/U	1/2a	2007	+	+	+	+	+	+	+	+

[a] Previous ID and PFGE profile (A, B, C, D, E or unique) as highlighted in Lomonaco et al. (2009).
[b] U, unique PFGE pulsotype.
[c] /, PCR screening test was not carried out on these samples.

Table 2. Target genes and PCR protocols used for *L. monocytogenes* virulence-associated gene detection.

Target gene	Encoded protein	Primer sequences	Amplicon size (bp)	Primer (cycling conditions)
iap [a]	Invasion associated protein	F 5′-ACAAGCTGCACCTGTTGCAG-3′ R 5′-TGACAGCGTGTGTAGTAGCA-3′	131	Kaur et al. (2007)
plcA [a]	Phospholipase C protein (PI-PLC)	F 5′-CTCGGACCATTGTAGTCATCTT-3′ R 5′-CACTTTCAGGCGTATTAGAAACGA-3′	326	This study (Kaur et al., 2007)
hlyA [a]	Listeriolisin O (LLO)	F 5′-GCAGTTGCAAGCCTTGGAGTGTGAA-3′ R 5′-GCAACGTATCCTTCCAGAGTGATCG-3′	456	Kaur et al. (2007)
prfA	Central virulence regulator	F 5′-ACCAATGGGATCCACAAGA-3′ R 5′-CAGCTGAGCTATGTGCGAT-3′	467	Bubert et al. (1999)
actA [a]	Actin-polymerizing protein	F 5′-CCAAGCGAGGTAAATACGGGA-3′ R 5′-GTCCGAAGCATTTACCTCTTC-3′	650	This study (Kaur et al., 2007)
inlJ	Internalin J	F 5′-TGTAACCCCGCTTACACAGTT-3′ R 5′-AGCGGCTTGGCAGTCTAATA-3′	238	Liu et al. (2007)
inlC	Internalin C	F 5′-AATTCCCACAGGACACAACC-3′ R 5′-CGGGAATGCAATTTTTCACTA-3′	517	Liu et al. (2007)

[a] For these genes, the following PCR conditions were used: 75 mM Tris-HCl (pH 8.8); 0.2 mM for each dNTPs; 5 pmol each for forward and reverse primer; 1,5 mM (*hlyA*) or 2.5mM (*iap, plcA, actA*) $MgCl_{2;}$ 1 unit of recombinant *Taq* DNA polymerase (Invitrogen, USA) and 25-50 ng DNA concentration in a final volume of 50 μl.

Supplemental Table 1. Reference strains used in the present study. Samples are indicated with H (human), F (food), E (environmental), and A (animal) in relation to the source of isolation. Strains were isolated either from epidemics or sporadic cases, indicated with Epi and Spor, respectively. Positive and negative amplification for the seven virulence-associated genes listed are indicated with + and -, respectively.

Species (serotype)	Strain	Source	Info	*iap* 131bp	*plcA* 326bp	*hlyA* 456bp	*actA* 650bp	*prfA* 467bp	*inlJ* 238bp	*inlC* 517bp
L. monocytogenes (1/2a)	R2-603[a]	F	Epi	+	+	+	+	+	+	+
L. monocytogenes (4b)	R2-764[a]	F	Epi	+	+	+	+	+	+	+
L. monocytogenes (4b)	N1-225[a]	H	Epi	+	+	+	+	+	+	+
L. monocytogenes (4b)	N1-227[a]	F	Epi	+	+	+	+	+	+	+
L. monocytogenes (4b)	J1-110[a]	F	Epi	+	+	+	+	+	+	+
L. monocytogenes (4b)	J1-003[a]	F	Epi	+	+	+	+	+	+	+
L. monocytogenes (4b)	J1-225[a]	H	Epi	+	+	+	+	+	+	+
L. monocytogenes (4b)	J1-220[a]	H	Epi	+	+	+	+	+	+	+
L. monocytogenes (4b)	J1-116[a]	H	Epi	+	+	+	+	+	+	+
L. monocytogenes (4b)	J1-123[a]	H	Epi	+	+	+	+	+	+	+
L. monocytogenes (3c)	J1-049[a]	H	Spor	+	+	+	+	+	+	+
L. monocytogenes (4b)	C1-122[a]	H	Spor	+	+	+	+	+	+	+
L. monocytogenes (1/2a)	C1-056[a]	H	Spor	+	-	+	+	+	+	+
L. monocytogenes (3a)	C1-115[a]	H	Spor	-	+	+	+	+	+	+
L. monocytogenes (1/2a)	J1-031[a]	F	Spor	-	-	+	+	+	+	+
L. monocytogenes (3b)	J1-169[a]	H	Spor	-	+	+	+	+	+	+
L. monocytogenes (1/2b)	J1-177[a]	H	Spor	-	+	+	+	+	+	+
L. monocytogenes (1/2c)	J1-094[a]	H	Spor	-	+	+	+	+	+	+
L. monocytogenes (N/A)	M1-004[a]	H	Spor	-	+	+	+	+	+	+
L. monocytogenes (4a)	J1-168[a]	H	Spor	+	+	+	+	+	+	+
L. monocytogenes (4b/4e)	U1[b]	H	Spor	+	+	+	+	+	+	+
L. monocytogenes (1/2b)	U2[b]	H	Spor	+	+	+	+	+	+	+
L. monocytogenes (4d)	U3[b]	H	Spor	+	+	+	+	+	+	+
L. monocytogenes (1/2a)	U4[b]	H	Spor	+	+	+	+	+	+	+
L. monocytogenes (4b)	U5[b]	H	Spor	+	+	+	+	+	+	+
L. innocua	33090[c]	H	na[d]	-	-	-	-	-	-	-
L. grayi	20601[e]	E	na	/[f]	/	/	-	/	/	/
L. welshimeri	20650[e]	E	na	/	/	/	-	/	/	/
L. seeligeri	20751[g]	E	na	/	/	/	-	/	/	/
L. ivanovii	20750[g]	A	na	/	/	/	-	/	/	/
Lactobacillus plantarum	Our collect.	F	na	/	/	/	-	/	/	/
Brochotrix termosphacta	Our collect.	F	na	/	/	/	-	/	/	/

[a] ILSI *Listeria monocytogenes* strains collection (Ithaca, NY, USA);
[b] Istituto Zooprofilattico della Liguria, Piemonte e Valle d'Aosta;
[c] American Type Culture Collection, (Teddington, Middlesex, UK);
[d] na, not available
[e] National Collection of Type Cultures (London, UK)
[f] /, PCR screening test was not carried out on these samples
[g] Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany)