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smcL as a novel diagnostic marker for quantitative detection of *Listeria ivanovii* in biological samples

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Abstract

Aims: To develop a novel molecular tool for the quantitative detection of the ruminant pathogen *Listeria ivanovii* in different biological matrices.

Methods and Results: A real-time PCR (RTi-PCR) for the quantitative and species-specific identification of *L. ivanovii* was designed to target the region of the *smcL* gene. The assay includes an internal amplification control (IAC) to avoid false-negative results. The *smcL*-IAC RTi-PCR assay was 100% selective and allowed the detection of as little as one genome equivalent in 45% of reactions. The quantification accuracy was excellent, as demonstrated by its high linearity ($R^2 > 0.9989$) and PCR efficiency ($E > 0.984$) over a 6-log dynamic range, down to 10 genome equivalents. Finally, the applicability of this assay was evaluated with artificially contaminated biological matrices implicated in the transmission of this bacterium such as sheep raw milk, blood and amniotic fluid. The *smcL*-IAC RTi-PCR assay allowed the detection of as few as 50 colony forming unit numbers (CFUs) per 25 ml of raw milk, 43 CFUs per 1 ml of blood or 50 CFUs per 1 ml of amniotic fluid.

Conclusions: This method can be an adequate alternative for the identification of *L. ivanovii* and for complete diagnosis of animal and human listeriosis.

Significance and Impact of the Study: We present an alternative for the detection of another pathogenic member of *Listeria* genus, which can help to distinguish from *Listeria monocytogene*s and therefore facilitates the establishment of preventive and prophylactic measures in food and farm environments.

Introduction

The Gram-positive bacterial genus *Listeria* includes two pathogenic species: *Listeria monocytogenes* and *Listeria ivanovii* (Seeliger and Jones 1986). Both are facultative intracellular parasites, although they present different species and tissue tropisms (González-Zorn *et al.* 1999, 2000; Vázquez-Boland *et al.* 2001; Domínguez-Bernal *et al.* 2006). Listeriosis, the infectious disease caused by these bacteria, is produced almost exclusively by *L. monocytogenes* in humans, *L. ivanovii* infection being unusual (Elischerová *et al.* 1990; Cummins *et al.* 1994; Lessing *et al.* 1994; Snapir *et al.* 2006) and principally circumscribed to abortions, stillbirths and neonatal septicaemia in ruminants (Dennis 1975; Elischerová *et al.* 1990; Sergeant *et al.* 1991; Alexander *et al.* 1992; Gill *et al.*

1997; Chand and Sadana 1999; Ramage *et al.* 1999; Wesley 1999; Sahin and Beytut 2006). For these reasons, the epidemiological and diagnostic studies of human and animal listeriosis have mainly been restricted to *L. monocytogenes*. However, *L. ivanovii* is an important ruminant pathogen, being responsible for 15% of all animal listeriosis (McLauchlin 1987) and related to abortion outbreaks in sheep and cattle (Elischerová *et al.* 1990; Sergeant *et al.* 1991; Alexander *et al.* 1992; Gill *et al.* 1997; Chand and Sadana 1999).

The conventional diagnostic approach for *Listeria* spp. involves pre- and enrichment in selective broths (such as Half-Fraser or Fraser) with subsequent isolation on selective media that provide a presumptive detection based on the media composition (Donnelly 2002; Allerberger 2003). Subsequent identification is usually based on a

range of biochemical, haemolysis (CAMP-Christie, Atkins, Munch–Petersen test) and serological tests. However, it is a laborious and time-consuming process, and it has been noted that confirmatory tests have not always produced the expected results and can generate equivocal profiles (McLauchlin 1997). A promising alternative to overcome these disadvantages is the use of molecular methods, and more specifically the real-time (RTi)-PCR. This molecular technique is taking its place in food and clinical microbiology diagnostics (Rodríguez-Lázaro *et al.* 2007) and can be a helpful choice for the quantitative detection and/or identification of this bacterium. In this scenario, several PCR-based methods have been described for the identification of different *Listeria* species, mainly based on the detection of *L. monocytogenes* (Nogva *et al.* 2000; Hein *et al.* 2001; Hough *et al.* 2002; Koo and Jaykus 2003; Rodríguez-Lázaro *et al.* 2004a), but other *Listeria* species have also been targeted (Liu *et al.* 2003, 2004a,b,c, 2005; Rodríguez-Lázaro *et al.* 2004a) as well as the *Listeria* genus (Manzano *et al.* 1996; Graham *et al.* 1997; Rodríguez-Lázaro *et al.* 2004b). Two conventional PCR methods have been described for the specific detection of *L. ivanovii* based on regions of putative *N*-acetylmuramidase and 16S rRNA genes (Wang *et al.* 1993; Liu *et al.* 2004b). However, there is no currently available RTi-PCR-based method for the detection of this bacterium. Although PCR is a simple, versatile, sensitive, specific and reproducible assay (Malorny *et al.* 2003; Rodríguez-Lázaro *et al.* 2007) in its conventional format, this technique does not allow for a quantification of the initial bacterial load.

In this work, we describe the first RTi-PCR method for the quantitative and species-specific identification of *L. ivanovii*. It targets to a specific region of the *smcL* gene, which is included in a *L. ivanovii*-specific pathogenicity island (LIPI-2), and encodes a sphingomyelinase C that plays a critical role in the pathogenesis of this bacterium. The RTi-PCR assay described here also incudes an internal amplification control (IAC) which can help to assess the PCR performance of the reaction and to identify false-negative results because of a failure in the PCR (Rodríguez-Lázaro *et al.* 2004d, 2005c, 2007). Finally, we evaluated the applicability of the assay in biological matrices that play an important role in the transmission of this agent between animals – and potentially to humans – such as sheep blood, amniotic fluid and raw milk.

Materials and methods

Bacterial species and strains

Bacterial strains used in this study included 56 *Listeria* strains (13 *L. ivanovii*, 21 *L. monocytogenes*, 6 *Listeria seeligeri*, 6 *Listeria welshimeri*, 3 *Listeria grayi* and 7

Listeria innocua,) from different sources and localizations and 84 non-*Listeria* strains including bacterial species phylogenetically or environmentally related to *Listeria* spp. (Tables S1 and S2). All the strains were maintained at −80°C in suitable broth supplemented with 16% glycerol (v/v). *Listeria* strains were grown in brain heart infusion medium (Difco-BD, Detroit, MI, USA) at 37°C. The non-*Listeria* strains were grown following the recommendations of the Spanish Type Culture Collection (CECT). The type strain *L. ivanovii* CECT 913 was used for the development of the RTi-PCR assay.

DNA isolation and quantification

Isolation of the bacterial genomic DNA was carried out from early stationary phase cultures using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The quantity of the DNA was measured with the ND-100 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The calculation of the ratios of optical density at 260 nm to that at 280 nm and at 260 nm to that at 230 nm allowed us to determine the quality of the DNA samples.

Oligonucleotides

The diagnostic primers and probes used in this study were designed to amplify a *L. ivanovii*-specific region of the sphingomyelinase C (*smcL*) gene (GenBank accession no. Y09477) (González-Zorn *et al.* 1999) using the Primer Express™ version 2.0 software (Applied Biosystems, Foster City, CA, USA) (Table 1). Oligonucleotides were purchased from MWG-Biotech AG (Ebensburg, Germany) except the probes that were acquired from Applied Biosystems (Warrington, UK). The *LivQP* and *IACLivQP* probes were labelled with FAM (6-carboxyfluorescein) and with VIC™ reporter dyes at the 5′ end, respectively, and with a nonfluorescence quencher dye at the 3′ end.

IAC construction

IAC consists of a 124-bp chimeric DNA that contains a *L. ivanovii* nonrelated internal region (a *Clostridium tyrobutyricum* flagellin-*fla*-gene region-López-Enríquez *et al.* 2007-) flanked by the specific *L. ivanovii* primers. The IAC was constructed for the assay as previously described (Rodríguez-Lázaro *et al.* 2005c; López-Enríquez *et al.* 2007; Oravcová *et al.* 2009). Briefly, 1 ng of *C. tyrobutyricum* genomic DNA was used as a PCR template to amplify a specific 83-bp *fla* gene fragment (López-Enríquez *et al.* 2007). Subsequently, the purified and 1 : 1000 diluted PCR product was used as a PCR template using the *IACLivQF/R* primers (Table 1). These primers contain the complete

Table 1 Oligonucleotides used in this study

Target	Name	Type	Sequence
Listeria ivanovii smcL gene	*LivQF*	Forward primer	5′-CGGTCATGCACGTCCACAT- 3′
	LivQR	Reverse primer	5′-CCACTGTGGTGACTTGGTATGC-3′
	LivQP	TaqMan MGB probe	5′-FAM-ATGGCATAACAAAGTC-MGB-3′
IAC chimeric DNA	*IACLivQ*	TaqMan probe	5′-VIC-CGGCAGGAGCTACATTTGCTTCAACAG-TAMRA-3′
Clostridium tyrobutyricum fla gene	*IACLivF*	Forward primer	5′-CGGTCATGCACGTCCACATCAGTTACAATTACGA-3′
	IACLivR	Reverse primer	5′ -CCACTGTGGTGACTTGGTATGCTGTACCACCAACTAA-3′

sequence of the *L. ivanovii smcL-specific* RTi-PCR primers at the 5′ end followed by a fragment of the *C. tyrobutyricum fla*-specific primers. The final PCR product was purified and cloned, and the plasmid including the IAC insert was transformed in One Shot MAX Efficiency DH5α-T1 competent *Escherichia coli* (Invitrogen, Carlsbad, CA, USA). Finally, the purified plasmid DNA was quantified and diluted in 10 mmol l^{-1} Tris-HCl in presence of 0·1 mg ml^{-1} acetylated bovine serum albumin as a blocking agent to minimize the binding of IAC DNA to the wall of the microtubes. With the exception of its target sequence in the *C. tyrobutyricum fla* gene (nucleotide positions 539–621), the IAC did not display significant similarity to any DNA sequence deposited in public databases, as determined by BlastN searches (National Centre for Biotechnology Information, Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov). In addition, the 124-bp IAC amplicon was longer than the *smcL*-specific amplicon (61 bp), facilitating the differentiation of the two PCR products by gel electrophoresis.

RTi-PCR

RTi-PCRs were carried out in a final volume of 20 μl containing 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nmol l^{-1} of specific primers, 200 nmol l^{-1} of *LivQP* probe, 100 nmol l^{-1} of *IACLivQP* probe, 2 μl of IAC and 5 μl of the target DNA solution. The RTi-PCRs were run on an 7500 Real-Time PCR System Platform (Applied Biosystems) for 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 63°C. The analyses of the results were performed using Sequence Detection System software version 1.2.3 (Applied Biosystems). Unless otherwise stated, all reactions were performed in triplicate and included a nontemplate control.

Artificial contamination challenge of *Listeria ivanovii* in biological samples

As *L. ivanovii* is principally a small ruminant pathogen causing abortion, and raw sheep milk, blood and amni-

otic fluid were selected as biological samples of interest for the detection of this pathogenic bacterium in this study. The samples were collected from a local farm (Valladolid, Spain) and stored at −20°C until used. They were analysed by standard methods to confirm the absence of *L. ivanovii*. Serial 10-fold dilutions of an overnight culture of *L. ivanovii* strain CECT 913 were used to inoculate the three biological matrices in the artificial contamination challenges. All the artificial contamination assays were carried out in triplicate and included a noncontaminated sample (blank). In parallel, the serial dilutions were also plated on ALOA agar (Agar Listeria selon Ottaviani & Agosti, AES Labortories, Einbourg, France) (Bauwens *et al.* 2003) to estimate the colony forming unit numbers (CFUs) per ml.

Raw milk

One millilitre of *L. ivanovii* serial 10-fold dilutions containing approx. $5\cdot3 \times 10^7$, $5\cdot3 \times 10^6$, $5\cdot3 \times 10^5$, $5\cdot3 \times 10^4$, $5\cdot3 \times 10^3$, 530, 53 and 10 *L. ivanovii* CFUs per ml was transferred to 50-ml centrifuge tubes containing 25 ml of raw sheep milk. Bacterial DNA was isolated as described previously (López-Enríquez *et al.* 2007) and eluted in 50 μl of 10 mmol l^{-1} Tris-HCl, pH 8·0.

Blood and amniotic fluid

One hundred microlitres of *L. ivanovii* serial 10-fold dilutions containing approx. $4\cdot3 \times 10^7$, $4\cdot3 \times 10^6$, $4\cdot3 \times 10^5$, $4\cdot3 \times 10^4$, 4300, 430, 43 and 10 *L. ivanovii* CFUs for the blood, or approx. 5×10^7, 5×10^6, 5×10^5, 5×10^4, 5000, 500, 50 and 10 *L. ivanovii* CFUs for the amniotic fluid, was added to 2-ml microcentrifuge tubes containing 900 μl of sheep blood or amniotic fluid. The bacterial DNA was isolated as follows: one volume of buffer TT-12% Triton X-100 (v/v) (Sigma, St Louis, MO, USA); 1% Trypsin (w/v) (Sigma) was added, and the suspension was mixed vigorously. The samples were incubated at 37°C, for 1 h, at 1000 rev min^{-1}. Subsequently, the samples were centrifuged at 15 700 *g* for 15 min, and the pellet was washed with 1 × PBS. Then, DNA was extracted

using a CTAB (cetyl trimethyl ammonium bromide)-based protocol (Rodríguez-Lázaro *et al.* 2006). The DNA was resuspended in 100 or 50 μl Tris-HCl 10 mmol l^{-1} pH 8·0, for blood or amniotic fluid, respectively.

Results

Design and optimization of the *Listeria ivanovii*-specific RTi-PCR assay

We selected the sphingomyelinase C (*smcL*) gene (González-Zorn *et al.* 1999) as a target for the specific detection of *L. ivanovii*. The *smcL* gene is a single copy gene that encodes a species-specific sphingomyelinase that mediates bacterial escape from the phagocytic vacuole during the intracellular proliferation of this bacterium (González-Zorn *et al.* 1999). Consequently, it represents an excellent molecular marker for the PCR-based detection of this bacterium. Using standard search parameters, nucleotide BLAST using the gene sequence (accession no. Y09477) detects no significant similarity in all GenBank + EMBL + DDBJ + PDB sequences. The *LivQF* and *LivQF R* primers amplify a 100% 61-bp fragment from the coding sequence of the *L. ivanovii smcL* gene corresponding to position 864–925 (accession no. Y09477, González-Zorn *et al.* 1999). The BLASTN tool version 2.2.14 (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) was used to confirm *in silico* that the primers and the PCR amplicons did not recognize any other related microbial DNA sequence in GenBank. Primers and *L. ivanovii*-specific probe concentrations in the RTi-PCR system (described in Materials and methods) were optimized according to general guidelines (Anonymous 1999).

Optimization of duplex *smcL*-IAC RTi-PCR assay

The optimal concentration of IAC probe was determined experimentally, by carrying out a RTi-PCR assay that included 10 000 genome equivalents of IAC per reaction as template, 200 nmol l^{-1} of *LivQP* TaqMan MGB probe and different concentrations of IAC probe (from 25 to 250 nmol l^{-1}). Genomic DNA of *L. ivanovii* was not included in the assay. The minimum probe concentration not resulting in an increase in C_T values was 150 nmol l^{-1}. Furthermore, the optimal number of IAC genome equivalents per PCR was determined examining the amplification of decreasing amounts of *L. ivanovii* DNA (100, 10 and 1 genome equivalents per reaction) close to the limits of quantification and detection of the RTi-PCR assay in the presence of increasing amounts of IAC (1, 10, 100 and 1000 genome equivalents per reaction). The maximum amount of IAC without affecting

the detection of the *L. ivanovii*-specific PCR assay was 100.

Selectivity of the *smcL*-IAC assay

To determine the inclusivity and exclusivity of the *smcL*-IAC RTi-PCR system, 13 *L. ivanovii* strains, 43 *Listeria* spp. and 84 non-*Listeria* species were tested using 1 ng of bacterial genomic DNA as template. All the *L. ivanovii* strains showed positive *smcL* amplification, i.e. the RTi-PCR system was 100% inclusive, whereas the remaining 127 nontarget bacterial strains did not show positive *smcL* amplification, i.e. the RTi-PCR system was 100% exclusive. Furthermore, all reactions generated a positive IAC (VIC) signal, excluding the possibility that the absence of a *smcL* (FAM) signal observed in non-*L. ivanovii* isolates was attributed to PCR failure.

Analytical sensitivity and quantification capacity of the *smcL*-IAC RTi-PCR assay

Bacterial genomic DNA isolated from an overnight culture of *L. ivanovii* CECT 913 was used to determine the analytical sensitivity and quantification range of *smcL*-IAC RTi-PCR. Two independent PCR experiments were performed with an ample range of dilution series of *L. ivanovii* DNA equivalent to approx. to 1×10^6, 1×10^5, 1×10^4, 1×10^3, 100, 10 and 1 genome equivalents per reaction. This experimental approach was followed including or not including the IAC in each reaction, to determine whether the simultaneous coamplification of the IAC could affect the detection limit of the assay. Table 2 shows the mean C_T values of a total of 10 PCR replicates (20 replicates for 10, and 1 genome equivalents) from two independent experiments. Positive amplifications in all the replicates were obtained when the concentration of the target in the PCR was 10 genome equivalents or more and 1 genomic equivalent was detected with a 45 and 30% probability, in the absence or presence of IAC, respectively. This difference in the probability of detection was analysed statistically, and no significant differences ($P < 0.05$) were found.

The main parameters determining the quantification capacity of a PCR assay are the linearity, the PCR efficiency (E), the quantification range and the limit of quantification (LOQ) (Rodríguez-Lázaro *et al.* 2005c, 2007). Linearity is the ability of the method to generate results proportional to the amount of analyte present in the sample and is represented by the regression coefficient (R^2 value). PCR efficiency is the capacity of the PCR to duplicate the amplicon molecules in each cycle and is calculated from the slope of the linear regression curve (s) from the equation $E = 10^{-1/s} - 1$. The *smcL* and *smcL*-IAC linear

Table 2 Determination of the detection and quantification limits of the RTi-PCR assay with genomic DNA from *Listeria ivanovii* strain CECT 913*

Approx. genome equivalents/reaction	Confidence interval limit†		Signal ratio‡	C_T§
	Lower	Upper		
smcL				
1×10^6	997693	1004372	10/10	17·98 ± 0·04
1×10^5	99643	100358	10/10	21·03 ± 0·03
1×10^4	9887	10113	10/10	24·32 ± 0·03
1×10^3	964	1036	10/10	27·77 ± 0·06
1×10^2	89	111	10/10	31·21 ± 0·19
10	7	14	20/20	34·27 ± 0·12
1	0	2	9/20	36·97 ± 0·14
smcL-IAC				
1×10^6	997693	1004372	10/10	18·14 ± 0·01
1×10^5	99643	100358	10/10	21·24 ± 0·02
1×10^4	9887	10113	10/10	24·43 ± 0·03
1×10^3	964	1036	10/10	27·94 ± 0·02
1×10^2	89	111	10/10	31·24 ± 0·03
10	7	14	20/20	34·97 ± 0·19
1	0	2	6/20	38·08 ± 0·26

*Nontemplate controls (NTC) for both RTi-PCR systems were negative (C_T values of 50 in all the replicates). The overall slope (*s*) of the regression curve was −3·2258 and −3·3609 for the *smcL* and *smcL*-IAC RTi-PCR systems, respectively, indicating the PCR efficiency (*E*) of 1·042 and 0·984, respectively, and the regression coefficients (R^2) were 0·9989 and 0·9995, respectively.
†Calculated for the expected number of template molecules at each dilution, at the 95% confidence level.
‡Positive results out of 10 reactions, 20 reactions for those containing 10 and 1 genome equivalents per reaction.
§Cycle number at which fluorescence intensity equals a fixed threshold. Mean value ± standard error of the mean. The experimental results were statistically significant ($P < 0.01$), taking into account the unavoidable error associated with serial dilutions.

regression curves were constructed by plotting the mean C_T values obtained for each dilution against the logarithms of the number of genome equivalents per reaction. The calculated R^2 values (*smcL*, 0·9989 and *smcL*-IAC, 0·9995) were close to 1, showing a high linearity over a dynamic quantification range of at least 6 logs (from 1×10^6 to 10 genome equivalents). The slope values (*smcL*, −3·2258 and *smcL*-IAC, −3·3609) correspond to *E* values of 1·042 and 0·984 for *smcL* and *smcL*-IAC, respectively. They were very close to the theoretical values ($s = -3.3219$ and $E = 1$) showing an excellent efficiency. The LOQ was established as the lowest sample dilution in which the 99% confidence interval does not overlap with that of the next dilution. A statistical analysis (ANOVA and Tukey *post-hoc* method, $\alpha = 0.01$) determined the absence of overlapping C_T values, and therefore confirmed reliable *L. ivanovii* quantification was possible down to 10 genome equivalents of this bacterium.

Quantitative detection of *Listeria ivanovii* in biological samples

The *smcL*-IAC RTi-PCR assay was used for the identification and quantification of *L. ivanovii* in three different artificially contaminated biological matrices; i.e. raw sheep milk, blood and amniotic fluid. The RTi-PCR assays yielded similar results in the different matrices in terms of absolute detection values (Tables 3, 4 and 5). It was possible to detect as few as 53 CFUs per 25 ml of artificially contaminated raw sheep milk samples with 66% probability (Table 3), and as few as 43 CFUs with 77% probability (Table 4) and 50 CFUs with 100% probability (Table 5) per ml of artificially contaminated blood and amniotic fluid samples, respectively. Assuming 100% DNA isolation efficiency and taking into account that 50 μl of DNA was obtained from processing of 25 ml of contaminated raw milk, and that only 5 μl was used as template for the RTi-PCR assay, the detection limit of 53 CFUs per 25 ml of raw sheep milk corresponds to

Table 3 Quantitative detection of *Listeria ivanovii* in artificially contaminated raw sheep milk*

Approx. *L. ivanovii* CFUs 25 ml^{-1}	Approx. *L. ivanovii* genome equivalent/ reaction†	Signal ratio‡	C_T value§	Relative accuracy¶
$5·3 \times 10^7$	$5·3 \times 10^6$	9/9	14·92 ± 0·11	96·82
$5·3 \times 10^6$	$5·3 \times 10^5$	9/9	17·47 ± 0·06	101·18
$5·3 \times 10^5$	$5·3 \times 10^4$	9/9	21·07 ± 0·16	101·01
$5·3 \times 10^4$	$5·3 \times 10^3$	9/9	24·27 ± 0·10	103·92
$5·3 \times 10^3$	$5·3 \times 10^2$	9/9	27·54 ± 0·38	108·10
$5·3 \times 10^2$	53	9/9	32·30 ± 0·39	92·84
53	5	6/9	35·91 ± 0·57	NA**
10	1	5/9	37·44 ± 0·25	NA
0††	0	0/9	>50	NA

*Raw milk collected directly from a sheep farm. Results of three independent experiments, with three PCR replicates used in each. The overall efficiency was 0·9543, and the linearity (R^2) was 0·9928.
†Estimated number of *L. ivanovii* genome equivalents in each PCR run, assuming 100% DNA extraction efficiency (each reaction contained 5 μl of a DNA preparation of 50 μl extracted from 25 ml milk).
‡Positive results out of nine reactions.
§Cycle number at which fluorescence intensity equals a fixed threshold. Mean value ± standard error of the mean. The experimental results were statistically significant ($P < 0.01$), taking into account unavoidable error associated with serial dilutions.
¶Degree of correspondence between the results obtained with the standard plating technique (log *L. ivanovii* CFUs ml^{-1}) and those obtained with the *smCL-IAC* RTi-PCR method (Log *L. ivanovii* genome equivalents/reaction).
**Not applicable.
††Noncontaminated sample.

Table 4 Quantitative detection of *Listeria ivanovii* in artificially contaminated sheep blood*

Approx. *L. ivanovii* CFUs ml^{-1}	Approx. *L. ivanovii* genome equivalent/ reaction†	Signal ratio‡	C_T value§	Relative accuracy¶
4.3×10^7	2.15×10^6	9/9	16.31 ± 0.03	100.72
4.3×10^6	2.15×10^5	9/9	20.13 ± 0.07	98.89
4.3×10^5	2.15×10^4	9/9	23.50 ± 0.14	99.17
4.3×10^4	2.15×10^3	9/9	26.68 ± 0.08	101.39
4.3×10^3	2.15×10^2	9/9	30.21 ± 0.06	101.02
4.3×10^2	21	9/9	33.82 ± 0.10	98.45
43	2	7/9	37.46 ± 0.43	NA**
0††	0	0/9	>50	NA

*Blood collected directly from a healthy sheep. Results of three independent experiments, with three PCR replicates used in each. The overall efficiency was 0.9469, and the linearity (R^2) was 0.9994.

†Estimated number of *L. ivanovii* genome equivalents in each PCR run, assuming 100% DNA extraction efficiency (each reaction contained 5 μl of a DNA preparation of 100 μl extracted from 1 ml sheep blood).

‡Positive results out of nine reactions.

§Cycle number at which fluorescence intensity equals a fixed threshold. Mean value ± standard error of the mean. The experimental results were statistically significant ($P < 0.01$), taking into account unavoidable error associated with serial dilutions.

¶Degree of correspondence between the results obtained with the standard plating technique (log *L. ivanovii* CFUs ml^{-1}) and those obtained with the *smcL-IAC* RTi-PCR method (Log *L. ivanovii* genome equivalents mL^{-1}).

**Not applicable.

††Noncontaminated sample.

Table 5 Quantitative detection of *Listeria ivanovii* in artificially contaminated sheep amniotic fluid*

Approx. *L. ivanovii* CFUs ml^{-1}	Approx. *L. ivanovii* genome equivalent/ reaction†	Signal ratio‡	C_T value§	Relative accuracy¶
5×10^7	5×10^6	9/9	14.82 ± 0.02	100.59
5×10^6	5×10^5	9/9	18.58 ± 0.07	99.08
5×10^5	5×10^4	9/9	21.68 ± 0.04	100.98
5×10^4	5×10^3	9/9	25.55 ± 0.05	97.82
5×10^3	5×10^2	9/9	28.51 ± 0.08	102.25
5×10^2	50	9/9	32.30 ± 0.16	97.56
50	5	9/9	35.50 ± 0.34	103.94
10	1	3/9	37.10 ± 0.22	NA**
0††	0	0/9	>50	NA

*Amniotic fluid collected directly from a healthy sheep. Results of three independent experiments, with three PCR replicates used in each. The overall efficiency was 0.9528, and the linearity (R^2) was 0.9993.

†Estimated number of *L. ivanovii* genome equivalents in each PCR run, assuming 100% DNA extraction efficiency (each reaction contained 5 μl of a DNA preparation of 50 μl extracted from 1 ml sheep amniotic fluid).

‡Positive results out of nine reactions.

§Cycle number at which fluorescence intensity equals a fixed threshold. Mean value ± standard error of the mean. The experimental results were statistically significant ($P < 0.01$), taking into account unavoidable error associated with serial dilutions.

¶Degree of correspondence between the results obtained with the standard plating technique (Log *L. ivanovii* CFUs ml^{-1}) and those obtained with the *smCL-IAC* RTi-PCR method (Log *L. ivanovii* genome equivalents ml^{-1}).

**Not applicable.

††Noncontaminated sample.

approx. five genomic equivalents per reaction. Similarly, the detection limit of 43 and 50 CFUs per 1 ml of sheep blood and amniotic fluid corresponds to approx. two and five genome equivalents per reaction, respectively. These results are consistent with those obtained when pure *L. ivanovii* genomic DNA was used (Table 2) and also similar to those published previously for other foodborne and animal pathogens (Rodríguez-Lázaro *et al.* 2004a,b,c, 2005a,b,c, 2006; López-Enríquez *et al.* 2007; Oravcová *et al.* 2009). In addition, the R^2 values (0.9928, 0.9994 and 0.9993 for raw sheep milk, blood and amniotic fluid, respectively) obtained showed a high linearity over a range of six logs (from approx. 10^6 to 10 genome equivalents per reaction) (Tables 3–5). Similarly, the PCR efficiencies (0.9543, 0.9469 and 0.9528 for raw sheep milk, blood and amniotic fluid, respectively) demonstrated that the performance of the RTi-PCR assay was also excellent.

The C_T values obtained from the three different types of samples artificially contaminated with *L. ivanovii* were extrapolated to the corresponding standard regression curves, and the resulting theoretical numbers of *L. ivano-*

vii CFUs were compared with those obtained with standard plating techniques (Tables 3–5). Relative accuracy values ranged between 92.84 and 108.10%, indicating a high degree of correspondence between the quantitative results obtained by the reference method (number of *L. ivanovii* CFUs ml^{-1} as determined by standard plating) and the results obtained by the *smcL*-IAC RTi-PCR method (Tables 3–5).

Discussion

In this study, we developed a RTi-PCR assay for the quantitative species-specific detection of *L. ivanovii* targeting a conserved region of the sphingomyelinase C (*smcL*) gene, a membrane-damaging virulence factor in *L. ivanovii* (González-Zorn *et al.* 1999, 2000). Current diagnostic methods for the detection of this bacterium are based on conventional culture methods, requiring selective enrichment and/or isolation of presumptive bacteria on

selective solid media and final confirmation using bio-chemical, CAMP or molecular tests, and they are there-fore laborious and time-consuming. Consequently, rapid detection alternatives, such as RTi-PCR-based methods, can improve the detection and quantification of this pathogenic bacterium. Reliable bacterial PCR detection systems depend on the amplification of specific fragments, preferably located in the coding regions of specific chro-mosomal genes. The *L. ivanovii smcL* gene is a good can-didate target for the species-specific identification of this bacterium by PCR-based detection methods as it has described as specific for *L. ivanovii* and plays a critical role in its pathogenicity (González-Zorn *et al.* 1999, 2000; Vázquez-Boland *et al.* 2001; Domínguez-Bernal *et al.* 2006).

One of the most important barriers to the effective implementation of PCR-based methods in diagnostic microbiology laboratories is the common occurrence of false-negative results because of the presence of PCR inhibitors in the sample. This is of particular interest in the PCR analysis of *L. ivanovii*, because the typical biolog-ical samples under analysis (e.g. raw milk or blood) con-tain PCR inhibitors and can therefore generate an underestimation of the *L. ivanovii* load or even the com-plete suppression of the amplification reaction (Al-Soud and Radstrom 2001). To minimize this situation, we have followed a bacterial DNA extraction protocol that has given excellent results in similar matrices (López-Enríquez *et al.* 2007). In addition, the RTi-PCR assay described here was designed to assess the inhibition during the amplification reaction, using an IAC. This IAC consists of a nontarget chimeric DNA that is coamplified with the target sequence (Hoorfar *et al.* 2004; Rodríguez-Lázaro *et al.* 2005c, 2007). When a negative signal is obtained for the target *smcL* signal, the absence of a positive IAC signal indicates that amplification has failed (Rodríguez-Lázaro *et al.* 2005c, 2007). We constructed an IAC, by fusing the forward and reverse *smcL* target sequences to either end of an unrelated DNA fragment, to which a second fluorescent probe (the IAC probe) hybridized. IAC coamplification and detection in duplex format made possible the simultaneous quantitative detection of target DNA and assessment of PCR efficiency. The inclusion of an IAC had no significant impact on the performance of the *smcL* RTi-PCR assay (Table 2) and allowed us to exclude the possibility of false-negative results during evaluation of the panel of nontarget isolates (Table S2).

One of the principal parameters to evaluate the analyti-cal performance of a diagnostic method is its capacity to discriminate between target and nontarget bacteria. In our hands, the *smcL*-IAC RTi-PCR assay was 100% inclu-sive as it correctly detected all *L. ivanovii* strains, and 100% exclusive as it did not detect any of the 127 non-

L. ivanovii strains (Tables S1 and S2). Accordingly, our RTi-PCR assay in combination with already available PCR methods for another *Listeria* species (Rodríguez-Lázaro *et al.* 2004a,b) could contribute to the easy identi-fication of closely related *Listeria* species which could be present in clinical, food and environmental samples.

The achievement of low detection and quantification limits is a critical aspect in the design of molecular diag-nostic methods for microbial contaminants. With purified genomic *L. ivanovii* DNA, the *smcL* RTi-PCR assay was able to detect approx. one target genome equivalent in at least 30% of the replicates and 10 genome equivalents in all cases (Table 2). The capacity of a RTi-PCR assay to determine accurately the number of targets present in the sample depends on the linearity and efficiency of the PCR (Rodríguez-Lázaro *et al.* 2005c). In our method, the line-arity (R^2 values above 0·9989) and the PCR efficiency (*E* values above 0·984) demonstrated an accurate quantifi-cation down to approx. 10 genome equivalents per reac-tion, in a dynamic range of six logs (from 1×10^6 down to 10). These results were consistent with other RTi-PCR methods design to detect other bacteria and eukaryotic organisms (Hernández *et al.* 2003, 2004; Rodríguez-Lázaro *et al.* 2004a,b,c, 2005a,b,c, 2006; López-Enríquez *et al.* 2007; Oravcová *et al.* 2009).

Interestingly, the level of performance was similar when the technique was applied to the counting of *L. ivanovii* in biological samples. As *L. ivanovii* is a ruminant patho-gen causing abortions, raw sheep milk, blood and amni-otic fluid were selected as biological samples of interest for the study as they are specimens commonly used in the clinical diagnosis of small ruminant listeriosis. In addition, they possess a very different nature and com-plexity and can serve as ideal biological samples to evalu-ate the applicability of our *smcL*-IAC RTi-PCR method in a real scenario. Owing to the DNA extraction protocol used – which involved processing of initial sample; 25 ml of raw whole milk samples or 1 ml of blood or amniotic fluid – and the inclusion in each reaction of a fraction of DNA extract (5 of 50 μl in the case of milk and amniotic fluid samples and 5 of 100 μl for blood), the practical quantification and detection limits for *L. ivanovii* were around 50 CFUs (or around five genome equivalents per reaction), and 10 CFUs (or one genome equivalent per reaction), in at least 33·33% of the replicates, respectively (Tables 3–5).

The effective implementation of any alternative method in microbiology laboratories would require a demonstra-tion of its equivalence to the accepted reference method in terms of relative accuracy (Rodríguez-Lázaro *et al.* 2004c,d, 2005a). Relative accuracy is defined as the close-ness of agreement between the results obtained by an accepted method and those obtained by an alternative

method (Anonymous 2003). A relative accuracy of 100% indicates total agreement between the alternative and reference methods. The relative quantitative accuracy values obtained in this study, ranging between 92·84 and 108·10%, clearly indicate perfect or almost perfect correspondence between the quantitative results obtained with our *smcL*-IAC RTi-PCR method and those obtained by standard plating, over a wide (at least six logs) dynamic range. The assay described here therefore provides a promising alternative for the quantitative detection of *L. ivanovii* in an ample range of biological samples.

In conclusion, we describe here the first reliable and sensitive real-time PCR-based method for quantifying *L. ivanovii* in an ample range of biological matrices, incorporating an IAC to assist in the interpretation of the results obtained. It has an excellent quantification capacity, as defined by its wide dynamic quantification range (at least six orders of magnitude) linearity ($R^2 > 0.9989$), PCR efficiency ($E = 0.984$) and quantification limit (down to 10 *L. ivanovii* genome equivalents). When combined with a simple DNA extraction procedure, it provides a highly efficient quantitative analysis of different biological matrices such as raw sheep milk, blood and amniotic fluid with detection down to 10 CFUs, with an excellent relative accuracy compared to the reference microbiological method. This method therefore provides a promising alternative to traditional microbiological methods for the rapid, sensitive detection of *L. ivanovii* that could be useful in both medical and veterinary diagnostic laboratories.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 *Listeria* strains used in this study.

Table S2 Non-*Listeria* strains used in this study.

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