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# Simultaneous quantitative detection of *Listeria* spp. and *Listeria monocytogenes* using a duplex real-time PCR-based assay

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**Abstract**

We report a duplex real-time PCR-based assay for the simultaneous quantitative detection of *Listeria* spp. and the food-borne pathogen *Listeria monocytogenes*. The targets of this single tube reaction were the 23S rDNA and *hly* genes of *Listeria* spp. and *L. monocytogenes*, respectively. Our assay was efficient, 100% selective (i.e., it allowed accurate simultaneous identification of 52 *L. monocytogenes* and 120 *Listeria* spp. strains through the FAM-labelled *hly* and the VIC-labelled 23S rDNA probes, respectively); and had a detection limit of one target molecule in 100% (23S rDNA) and 56% (*hly*) of the reactions. Simultaneous quantification was possible along a 5-log dynamic range, with an upper limit of 30 target molecules and $R^2$ values >0.995 in both cases. Our results indicate that this assay based on the amplification of the 23S rDNA gene can accurately quantify any mixture of *Listeria* species and simultaneously unambiguously quantify *L. monocytogenes*.

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*Keywords:* *Listeria* spp; *Listeria monocytogenes*; Real-time PCR; Multiplex; Food-borne pathogen detection

## 1. Introduction

*Listeria monocytogenes* has caused several outbreaks of severe listeriosis [1]. This infection is acquired via the consumption of contaminated, most often ready-to-eat, food [2–4]. *L. monocytogenes* usually co-exists with other species of this genus such as the frequent and generally non-pathogenic *Listeria innocua*, which can be used as an indicator of the possible presence of *L. monocytogenes* in food [5–7], or *Listeria ivanovii* and *Listeria seeligeri*, which occasionally cause human infections [8,9]. The food safety regulations of most countries tolerate no *L. monocytogenes* in ready-to-eat food [10], although the minimal infectious dose is generally thought to be higher than 100 viable cells [11]. Thus, a quick assay capable of quantifying both *Listeria* spp. and *L. monocytogenes* in food would improve risk assessment.

Molecular-based methods, such as the polymerase chain reaction (PCR), can rapidly detect and identify food-borne bacterial pathogens [12–14]. Real-time (RTi-) PCR assays also allow the precise quantification of target DNA, which is correlated with the size of the bacterial population present in the sample. The absence of any essential post-PCR steps simplifies RTi-PCR, allowing high throughput and automation [15]. However, the use of PCR can be limited by cost or sample volume [16]. To overcome these limitations and to increase the diagnostic capacity of PCR, multiplex PCR was developed [16]. Multiplex PCR allows the simultaneous amplification of more than one target sequence in a single reaction. This saves considerable time and effort, and decreases the number of reactions that need to be performed to detect food-borne pathogens in a food sample [5,14–16]. Several conventional PCR assays targeting the *Listeria* genus and *L. monocytogenes* have been reported [5,17–20]. Furthermore, single RTi-PCR assays for the detection of

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*L. monocytogenes* have been described [21–25]. However, no RTi-PCR assay is currently available for the simultaneous quantitative detection of *Listeria* spp. and *L. monocytogenes*.

A specific RTi-PCR assay for the quantitative detection of *L. monocytogenes* has already been developed [25]. This assay targets the *hly* gene [26] (GenBank Accession No. M24199, positions 1602–1665), which codes for the virulence factor listeriolysin O. It is highly selective, sensitive and suitable for the accurate quantification of all strains of *L. monocytogenes*. Here, we describe a duplex RTi-PCR assay that allows the simultaneous quantitative detection of members of the *Listeria* genus and *L. monocytogenes*. This assay combines the *L. monocytogenes*-specific *hly*-based assay and the amplification of the highly conserved 23S rDNA gene of *Listeria* spp.

## 2. Materials and methods

### 2.1. Bacterial strains, culture media and growth conditions

This study included 120 *Listeria* strains (52 *L. monocytogenes*, 20 *L. innocua*, 7 *Listeria grayi*, 14 *Listeria seeligeri*, 9 *Listeria welshimeri* and 18 *L. ivanovii* strains) and 53 non-*Listeria* strains (Tables 1 and 2, respectively). They were maintained at −80 °C in Luria Bertani (LB) or MRS (lactic acid bacteria) broth supplemented with 15% (v/v) glycerol. *Listeria* spp. strains were grown in brain heart infusion (BHI) broth at 37 °C and non-*Listeria* strains in MRS broth or tryptone soya broth at 30 °C. For plate cultures, 1.5% (w/v) agar was added to these media. All media were purchased from Oxoid (Hampshire, UK).

### 2.2. DNA isolation and quantification

Genomic DNA was isolated from overnight bacterial cultures by using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) according to the manufacturer's recommendations. The concentration of the resulting DNA was determined by using PicoGreen® specific DNA dye (Molecular Probes, Inc., Eugene, OR, USA) in a Luminescence Spectrometer LS50B (Perkin–Elmer Corp., Norwalk, CT, USA). Concentrations were further checked by 1% agarose gel electrophoresis and 0.5 μg/mL ethidium bromide staining. UV fluorescence emission was recorded and quantified by using Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA).

### 2.3. Oligonucleotides

The primers and probes used in this study (Table 3) were designed using the Primer Express™ 2.0 software (Applied Biosystems, Foster City, CA, USA) and the coding sequences of the *L. monocytogenes hly* [25] and *Listeria* spp. *23S rDNA* genes. The BLAST-N [27] Tool (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) was used to *in silico* confirm the specificity of the oligonucleotides. All oligonucleotides were purchased from MWG-Biotech AG (Ebensburg, Germany) except for the 23S rDNA-specific probe, which was acquired from Applied Biosystems (Warrington, UK).

### 2.4. RTi-PCR

Reactions were performed using TaqMan® PCR Core Reagents kit (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, NJ, Germany) in a 20-μl reaction volume containing PCR TaqMan® buffer A (including ROX as a passive reference dye), 6 mM $MgCl_2$, 200 μM each dATP, dCTP, dGTP, 400 μM dUTP, 1 unit of AmpliTaq Gold® DNA polymerase, 0.2 units of AmpErase® uracil *N*-glycosylase (UNG) and 1 μl of the target DNA solution. The following concentrations of the appropriate primers and probe(s) were added to each reaction (optimised according to Perkin–Elmer Applied Biosystems User Bulletin 2 [ABI PRISM 7700 Sequence Detection System], 1997 and [25]): 300 nM *L23SQF/R* and *Lin23SQR* primers; 50 nM each *hlyQF/R* primer; 100 nM *L23QP* (VIC and TAMRA double labelled) and *hlyQP* (FAM and TAMRA double labelled; Rodríguez-Lázaro et al. [25]) probes. Duplex RTi-PCR assay combined in a single reaction tube the *Listeria* spp.-specific 23S rDNA oligonucleotides with those specific for *L. monocytogenes* previously described [25]. 23S rDNA- and *hly*-specific amplifications were independently analysed during the PCR by using two probes that were labelled with different reporter dyes (VIC and FAM, respectively).

Unless otherwise stated, three replicates of each reaction were performed. Reactions were run on an ABI PRISM® 7700 Sequence Detection System device (Applied Biosystems division of Perkin–Elmer Corp., Foster City, CA, USA) using the following conditions: 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 results were analysed using the Sequence Detection System software v1.7 (Applied Biosystems). Samples were quantified by comparison with a standard regression curve of $C_T$ values generated from samples of known concentrations. If the $C_T$ value was 50 or above, we considered that no amplification had occurred.

### 2.5. Specificity of the duplex RTi-PCR assay

The capacity of the duplex PCR assay to distinguish between target and non-target bacteria was confirmed using as template 1 ng of genomic DNA from 120 dif-

Table 1
*Listeria* strains used in this study

| Species | Strain | Other designations | Serotype | Source | PCR[a] | |
|---|---|---|---|---|---|---|
| | | | | | *hly* | 23S |
| *Listeria monocytogenes* | ATCC[b] 5577* | | 1/2c | Collection | + | + |
| *Listeria monocytogenes* | CECT[c] 911 | | 1/2c | Collection | + | + |
| *Listeria monocytogenes* | CECT 932 | | 1/2a | Collection | + | + |
| *Listeria monocytogenes* | CECT 933 | | 3a | Collection | + | + |
| *Listeria monocytogenes* | CECT 934 | | 4a | Collection | + | + |
| *Listeria monocytogenes* | CECT 935 | | 4b | Collection | + | + |
| *Listeria monocytogenes* | CECT 936 | | 1/2b | Collection | + | + |
| *Listeria monocytogenes* | CECT 937 | | 3b | Collection | + | + |
| *Listeria monocytogenes* | CECT 938 | | 3c | Collection | + | + |
| *Listeria monocytogenes* | CECT 940 | | 4d | Collection | + | + |
| *Listeria monocytogenes* | CECT 4031[T] | ATCC 15313[T] | 1/2a | Collection | + | + |
| *Listeria monocytogenes* | CECT 4032 | | 4b | Collection | + | + |
| *Listeria monocytogenes* | CECT 5725 | | 6 | Collection | + | + |
| *Listeria monocytogenes* | CECT 5366 | | 4b | Collection | + | + |
| *Listeria monocytogenes* | UdG[d] 1010 | CTC[i] 1010 | 1/2c | Food plant, meat | + | + |
| *Listeria monocytogenes* | UdG 1011 | CTC 1011 | 1/2c | Food plant, meat | + | + |
| *Listeria monocytogenes* | UdG 1034 | CTC 1034 | 4b | Food plant, meat | + | + |
| *Listeria monocytogenes* | NC[e] Mi-3 | | 3b | Cheese | + | + |
| *Listeria monocytogenes* | NC Fe-2 | | | Chicken | + | + |
| *Listeria monocytogenes* | NC Fe-4 | | | Pate | + | + |
| *Listeria monocytogenes* | NC Fi-2 | | 1/2b | Trout | + | + |
| *Listeria monocytogenes* | NC Fi-4 | | | Smoked salmon | + | + |
| *Listeria monocytogenes* | NC U-3 | | | Environment | + | + |
| *Listeria monocytogenes* | NC U-4 | | | Environment | + | + |
| *Listeria monocytogenes* | NC Me-3 | | | Clinical, human | + | + |
| *Listeria monocytogenes* | NC Me-4 | | | Clinical, human | + | + |
| *Listeria monocytogenes* | NC Ve-1 | | | Clinical, human | + | + |
| *Listeria monocytogenes* | NC Ve-3 | | | Milk | + | + |
| *Listeria monocytogenes* | NC Re-9 | NCTC[j] 11994 | 4b | Collection | + | + |
| *Listeria monocytogenes* | NC Re-10 | ATCC 5579 | 4c | Collection | + | + |
| *Listeria monocytogenes* | PAM[f] 35 | NCTC 7973, SLCC[k] 2371 | 1/2a | Collection | + | + |
| *Listeria monocytogenes* | PAM 484 | SLCC 2755 | 1/2b | Collection | + | + |
| *Listeria monocytogenes* | PAM 485 | NCTC 5348, SLCC 2373 | 1/2c | Collection | + | + |
| *Listeria monocytogenes* | PAM 486 | ATCC 19113, SLCC 2373 | 3a | Collection | + | + |
| *Listeria monocytogenes* | PAM 487 | SLCC 2540 | 3b | Collection | + | + |
| *Listeria monocytogenes* | PAM 489 | NCTC 5214, SLCC 2374 | 4a | Collection | + | + |
| *Listeria monocytogenes* | PAM 491 | NCTC 10527, SLCC 2375 | 4b | Collection | + | + |
| *Listeria monocytogenes* | PAM 493 | ATCC 19116, SLCC 2376 | 4c | Collection | + | + |
| *Listeria monocytogenes* | PAM 494 | NCTC 10888, SLCC 2377 | 4d | Collection | + | + |
| *Listeria monocytogenes* | PAM 495 | SLCC 2482 | 7 | Collection | + | + |
| *Listeria monocytogenes* | PAM 358 | EGD | 1/2a | Collection | + | + |
| *Listeria monocytogenes* | PAM 61 | | 1/2a | Cheese | + | + |
| *Listeria monocytogenes* | PAM 62 | | 1/2b | Cheese | + | + |
| *Listeria monocytogenes* | PAM 70 | | 4b | Cheese | + | + |
| *Listeria monocytogenes* | PAM 75 | | 3b | Cheese | + | + |
| *Listeria monocytogenes* | PAM 68 | | 1/2c | Environment | + | + |
| *Listeria monocytogenes* | PAM 80 | | 3c | Environment | + | + |
| *Listeria monocytogenes* | PAM 9 | | 4b | Clinical, ovine | + | + |
| *Listeria monocytogenes* | PAM 51 | | 1/2c | Clinical, human | + | + |
| *Listeria monocytogenes* | PAM 348 | | 1/2b | Clinical, human | + | + |
| *Listeria monocytogenes* | PAM 349 | | 4b | Clinical, human | + | + |
| *Listeria monocytogenes* | PAM 602 | | 1/2a | | + | + |
| *Listeria innocua* | DSMZ[g] 20649* | | 6a | Collection | – | + |
| *Listeria innocua* | CECT 910 | | 6a | Collection | – | + |
| *Listeria innocua* | CECT 4030 | | | Collection | – | + |
| *Listeria innocua* | CECT 5376 | | | Collection | – | + |
| *Listeria innocua* | CECT 5377 | | | Collection | – | + |
| *Listeria innocua* | CECT 5378 | | | Collection | – | + |
| *Listeria innocua* | UdG 1012 | CTC 1012 | | Food plant, meat | – | + |
| *Listeria innocua* | UdG 1014 | CTC 1014 | | Food plant, meat | – | + |
| *Listeria innocua* | NC IN-1 | | | Shrimps | – | + |

Table 1 (*continued*)

| Species | Strain | Other designations | Serotype | Source | PCR[a] | |
|---|---|---|---|---|---|---|
| | | | | | *hly* | 23S |
| *Listeria innocua* | NC IN-2 | | | Ham | – | + |
| *Listeria innocua* | NC IN-12 | ATCC 5578 | | Collection | – | + |
| *Listeria innocua* | NC IN-17 | | | Cheese | – | + |
| *Listeria innocua* | PAM 152 | ATCC 33091, SLCC 3423 | 6b | Collection | – | + |
| *Listeria innocua* | PAM 153 | ATCC 33090 | 6a | Collection | – | + |
| *Listeria innocua* | PAM 154 | SLCC 3379 | 6a | Collection | – | + |
| *Listeria innocua* | PAM 443 | | | | – | + |
| *Listeria innocua* | PAM 490 | NCTC 10528, SLCC 4951 | 4ab | Collection | – | + |
| *Listeria innocua* | PAM 550 | | 6b | | – | + |
| *Listeria innocua* | PAM 569 | | 6b | Meat | – | + |
| *Listeria innocua* | PAM 583 | | 6b | Milk | – | + |
| *Listeria grayi* | CECT 931[*] | | | Collection | – | + |
| *Listeria grayi* | CECT 942 | | | Collection | – | + |
| *Listeria grayi* | CECT 4181 | | | Collection | – | + |
| *Listeria grayi* | NC GR-1 | | | Milk | – | + |
| *Listeria grayi* | NC GR-3 | DSM 20601 | | Collection | – | + |
| *Listeria grayi* | PAM 450 | SLCC 3322 | | Collection | – | + |
| *Listeria grayi* | PAM 466 | SLCC 4425 | | Collection | – | + |
| *Listeria seeligeri* | CECT 917[T*] | | | Collection | – | + |
| *Listeria seeligeri* | CECT 939[h] | | 1/2b | Collection | – | + |
| *Listeria seeligeri* | CECT 941[h] | | | Collection | – | + |
| *Listeria seeligeri* | CECT 5339 | | 6b | Collection | – | + |
| *Listeria seeligeri* | CECT 5340 | | | Collection | – | + |
| *Listeria seeligeri* | CECT 5341 | | | Collection | – | + |
| | CECT 5342 | | | Collection | – | + |
| *Listeria seeligeri* | NC SE-1 | | | Collection | – | + |
| *Listeria seeligeri* | NC SE-3 | | | Salad | – | + |
| *Listeria seeligeri* | PAM 600 | DSM 20751 | 1/2b | Collection | – | + |
| *Listeria seeligeri* | PAM 498 | SLCC 5921 | 1/2b | Collection | – | + |
| *Listeria seeligeri* | PAM 499 | SLCC 3954, CIP[l]100100 | 1/2b | Collection | – | + |
| *Listeria seeligeri* | PAM 606 | | 1/2b | | – | + |
| *Listeria seeligeri* | UdG 1024 | CTC 1024 | | Food plant, meat | | + |
| *Listeria welshimeri* | PAM 497[T*] | SLCC 5334, CIP 8149[T] | 6a | Collection | – | + |
| *Listeria welshimeri* | CECT 919[T] | | 6a | Collection | – | + |
| *Listeria welshimeri* | CECT 5370 | | 1/2b | Collection | – | + |
| *Listeria welshimeri* | CECT 5371 | | 6a | Collection | – | + |
| *Listeria welshimeri* | CECT 5372 | | | Collection | – | + |
| *Listeria welshimeri* | CECT 5380 | | | Collection | – | + |
| *Listeria welshimeri* | UdG 1013 | CTC 1013 | | Food plant, meat | – | + |
| *Listeria welshimeri* | NC We-1 | DSM 20650 | | Collection | – | + |
| *Listeria welshimeri* | NC We-3 | | | Salami | – | + |
| *Listeria ivanovii* | PAM 424[*] | ATCC 19119 | 5 | Collection | – | + |
| *Listeria ivanovii* | PAM 55 | | 5 | Clinical, ovine | – | + |
| *Listeria ivanovii* | CECT 913 | | 5 | Collection | – | + |
| *Listeria ivanovii* | CECT 5368 | | 5 | Collection | – | + |
| *Listeria ivanovii* | CECT 5369 | | | Collection | – | + |
| *Listeria ivanovii* | CECT 5373 | | 5 | Collection | – | + |
| *Listeria ivanovii* | CECT 5374 | | | Collection | – | + |
| *Listeria ivanovii* | CECT 5375 | | | Collection | – | + |
| *Listeria ivanovii* | CECT 5379 | | | Collection | – | + |
| *Listeria ivanovii* | UdG 2001 | | 5 | Clinical, caprine | – | + |
| *Listeria ivanovii* | UdG 2002 | | 5 | Clinical, caprine | – | + |
| *Listeria ivanovii* | UdG 2003 | | 5 | Clinical, caprine | – | + |
| *Listeria ivanovii* | UdG 2004 | | 5 | Clinical, ovine | – | + |
| *Listeria ivanovii* | UdG 2005 | | 5 | Clinical, ovine | – | + |
| *Listeria ivanovii* | UdG 2006 | | 5 | Clinical, ovine | – | + |
| *Listeria ivanovii* | UdG 2007 | | 5 | Clinical, ovine | – | + |
| *Listeria ivanovii* | NC Iv-1 | | 5 | Milk | – | + |
| *Listeria ivanovii* | NC Iv-3 | DSM 20750 | 5 | Collection | – | + |

Table 1 (*continued*)

The strains marked with an asterisk were used to check the specificity of the 23S rDNA RTi-PCR assay in uniplex format.
[a] Qualitative results of duplex RTi-PCR assay.
[b] American Type Culture Collection.
[c] Spanish Type Culture Collection, Valencia, Spain.
[d] Collection of Food Microbiology Department of the University of Girona, Spain.
[e] Kindly provided by M. D'Agostino. Central Science Laboratory, Sand Hutton, York, UK.
[f] Collection of the Microbial Pathogenesis Group, Veterinary Molecular Microbiology Section, University of Bristol, UK.
[g] German Collection of Microorganisms.
[h] Recently assigned to the *L. seeligeri* species.
[i] Collection of Food Microbiology Unit, Meat Technology Center, Monells (Girona), Spain.
[j] National Type Culture Collection, UK.
[k] H.P.R. Seeliger's Special *Listeria* Culture Collection.
[l] Institut Pasteur Collection, France.
[T] Type strain.

ferent *Listeria* species and serovars and 53 non-*Listeria* strains (Tables 1 and 2). According to the mean genome size of the different microorganisms used (www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html), 1 ng of genomic DNA corresponded to at least $3 \times 10^5$cells.

### 2.6. Standard curves

Purified genomic DNA was used for quantification and to determine the detection (LOD) and quantification (LOQ) limits. Genomic DNA isolated from overnight cultures of *L. monocytogenes* strain ATCC 5577 and *L. innocua* DSMZ 20649 was diluted to final concentrations of $3 \times 10^5$, $3 \times 10^4$, $3 \times 10^3$, $3 \times 10^2$, 30, 15, 3 and 1 molecules per μl. According to the published sizes of the whole *L. monocytogenes* and *L. innocua* genomes [28] one genomic DNA molecule corresponds to 2.94 and 3.18 fg, respectively. For every serial dilution, the 95% confidence interval was calculated according to a binomial distribution as reported [29] using the SPSS software for Windows v11.5 (SPSS Inc., Chicago, Ill., USA). In addition, to test the possibility of directly quantifying *Listeria* spp. and *L. monocytogenes* in cell suspensions, duplex RTi-PCR assays were performed using as template 10-fold dilutions of a *L. monocytogenes* ATCC 5577 overnight culture. Ten-fold dilutions of the same overnight culture were simultaneously plated out for quantification [30].

### 2.7. Relative accuracy of the 23S rDNA and hly duplex RTi-PCR assay

We assessed the relative accuracy of the 23S rDNA and *hly* duplex RTi-PCR assay, i.e., the closeness of the agreement between the results obtained with our duplex RTi-PCR assay and those obtained with the DNA quantification method based on the PicoGreen® fluorescent dye. We extracted DNA from six *Listeria* spp. overnight cultures and the resulting DNA solutions were quantified by PicoGreen® and by RTi-PCR using a standard curve built with *L. monocytogenes* DNA. The results obtained by each method were compared and expressed as percentage.

## 3. Results and discussion

### 3.1. Selection of Listeria spp.-specific DNA sequences

The 16S rDNA and 23S rDNA sequences are phylogenetic markers widely used for taxonomic purposes [31,32]. Analysis of the *Listeria* spp. 23S rDNA sequences in public databases (that include sequences of the type strains of *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*. *L. welshimeri* and *L. grayi*) revealed a consensus region of 86 nucleotides suitable for the design of genus-specific primers and probes (i.e., nt 1484–1560 of *L. monocytogenes* EGD-e 23S rDNA gene; Accession No. LMO10046 in http://genolist.pasteur.fr/ListiList/). Primer pair *L23SQF/R* produced a PCR amplicon of 77-bp. None of the selected oligonucleotides recognised any registered DNA sequences other than the target. The sequence of the probe was conserved in all *Listeria* spp. sequences published to date (www.ncbi.nlm.nih.gov and www.ebi.ac.uk). Two unique mismatches were identified in all *L. grayi* and *L. innocua* sequences corresponding to the forward and reverse primer, respectively. Thus, after the *in silico* analyses, *L23SQF/R/P* are potential candidates for use in *Listeria* spp. specific RTi-PCR assays; but special care should be taken to overcome the possible consequences of the *L. grayi* and *L. innocua* mismatches described.

### 3.2. Optimisation of 23S rDNA RTi-PCR assay for the specific detection of Listeria genus

We optimised the concentrations of the primers and probe for the uniplex 23S rDNA-specific RTi-PCR assay using the same buffer and the same cycling conditions as for the previously developed *L. monocytogenes*-specific RTi-PCR assay [25] using 100 pg of genomic DNA from *L. monocytogenes* ATCC 5577 (equivalent to

Table 2
Non-*Listeria* strains used in this study

| Species | Strain | Other designations | Source | PCR[a] | |
|---|---|---|---|---|---|
| | | | | *hly* | 23S |
| *Bacillus subtilis* | PAM[b]870 | NCTC[e] 10400 | Collection | – | – |
| *Bacillus cereus* | PAM 871 | NCTC 7464 | Collection | – | – |
| *Brochothrix thermosphacta* | UdG[c]1510* | CTC 1510 | Food plant, meat | – | – |
| *Brochothrix thermosphacta* | PAM 873 | | Collection | – | – |
| *Citrobacter freundii* | PAM 878 | ATCC[f] 8090 | Collection | – | – |
| *Enterobacter aerogenes* | PAM 863 | NCTC 10006 | Collection | – | – |
| *Enterococcus faecalis* | UdG 2708* | CTC[g] 2708 | Food plant, meat | – | – |
| *Enterococcus faecalis* | PAM 872 | NCTC 775 | Collection | – | – |
| *Enterococcus faecium* | UdG 492* | CTC 492 | Food plant, meat | – | – |
| *Enterococcus malodoratus* | UdG 7007 | | Food plant, meat | – | – |
| *Enterococcus malodoratus* | UdG 7008 | | Food plant, meat | – | – |
| *Enterococcus malodoratus* | UdG 7009 | | Food plant, meat | – | – |
| *Klebsiella aerogenes* | PAM 862 | NCTC 9528 | Collection | – | – |
| *Escherichia coli* | CECT[d] 515[T] | | Collection | – | – |
| *Escherichia coli* | CECT 533 | | Collection | – | – |
| *Kurthia gibsonii* | PAM 876 | | Collection | – | – |
| *Kurthia zopfii* | PAM 875 | ATCC 6900 | Collection | – | – |
| *Lactobacillus curvatus* | UdG 742* | CTC 742 | Food plant, meat | – | – |
| *Lactobacillus curvatus* | UdG 759* | CTC 759 | Food plant, meat | – | – |
| *Lactobacillus curvatus* | UdG 1174* | CTC 1174 | Food plant, meat | – | – |
| *Lactobacillus murinus* | UdG 7004 | | Food plant, meat | – | – |
| *Lactobacillus murinus* | UdG 7005 | | Food plant, meat | – | – |
| *Lactobacillus murinus* | UdG 7006 | | Food plant, meat | – | – |
| *Lactobacillus plantarum* | UdG 305* | CTC 305 | Food plant, meat | – | – |
| *Lactobacillus reuteri* | UdG 7010 | | Food plant, meat | – | – |
| *Lactobacillus reuteri* | UdG 7011 | | Food plant, meat | – | – |
| *Lactobacillus reuteri* | UdG 7012 | | Food plant, meat | – | – |
| *Lactobacillus reuteri* | UdG 7013 | | Food plant, meat | – | – |
| *Lactobacillus sakei* | UdG 746* | CTC 746 | Food plant, meat | – | – |
| *Lactobacillus sakei* | UdG 748* | CTC 748 | Food plant, meat | – | – |
| *Lactobacillus sakei* | UdG 756* | CTC 756 | Food plant, meat | – | – |
| *Lactobacillus sakei* | UdG 757* | CTC 757 | Food plant, meat | – | – |
| *Lactococcus garvieae* | UdG 7001 | | Food plant, meat | – | – |
| *Lactococcus garvieae* | UdG 7002 | | Food plant, meat | – | – |
| *Lactococcus garvieae* | UdG 7003 | | Food plant, meat | – | – |
| *Lactococcus lactis* | UdG 122* | CTC 122 | Food plant, meat | – | – |
| *Leuconostoc carnosum* | UdG 747* | CTC 747 | Food plant, meat | – | – |
| *Pediococcus pentosaceus* | UdG 745* | CTC 745 | Food plant, meat | – | – |
| *Micrococcus luteus* | CECT 51[T] | | Collection | – | – |
| *Pediococcus acidolactici* | UdG 771* | CTC 771 | Food plant, meat | – | – |
| *Pseudomonas aeruginosa* | PAM 860 | | Collection | – | – |
| *Rhodococcus equi* | CECT[f] 555[T] | | Collection | – | – |
| *Pseudomonas aeruginosa* | CECT 108 | | Collection | – | – |
| *Salmonella cholerasuis* | CECT 915[T] | | Collection | – | – |
| *Salmonella enteriditis* | CECT 4300 | | Collection | – | – |
| *Salmonella typhimurium* | CECT 4594 | | Collection | – | – |
| *Staphylococcus aureus* | CECT 4520[T] | | Collection | – | – |
| *Staphylococcus aureus* | PAM 868 | CTC 868 | Collection | – | – |
| *Staphylococcus aureus* | CECT 435 | | Collection | – | – |
| *Staphylococcus aureus* | CECT 828 | | Collection | – | – |
| *Staphylococcus epidermidis* | PAM 869 | CTC 869 | Collection | – | – |
| *Streptococcus faecalis* | PAM 879 | CTC 879 | Collection | – | – |
| *Streptococcus pyogenes* | PAM 880 | CTC 880 | Collection | – | – |

The strains marked with an asterisk were used to test the specificity of the 23S RTi-PCR assay in uniplex format.
[a] Qualitative results of conventional PCR and Rti-PCR.
[b] Collection of the Microbial Pathogenesis Group, Veterinary Molecular Microbiology Section, University of Bristol, UK.
[c] Collection of Food Microbiology Department of the University of Girona, Spain.
[d] Spanish Type Culture Collection, Valencia, Spain.
[e] National Type Culture Collection, UK.
[f] American Type Culture Collection.
[g] Collection of Food Microbiology Unit, Meat Technology Center, Monells (Girona), Spain.
[T] Type strain.

Table 3
Oligonucleotides used in the RTi-PCR assays for *Listeria monocytogenes* and *Listeria* spp

| Name | Target species | Target gene | Type | Sequence |
|---|---|---|---|---|
| *hlyQF* | *Listeria monocytogenes* | *hly* | Forward primer | 5′-CAT GGC ACC ACC AGC ATC T-3′ |
| *hlyQR* | | | Reverse primer | 5′-ATC CGC GTG TTT CTT TTC GA-3′ |
| *hlyQP* | | | TaqMan® Probe | 5′-*FAM*-CGC CTG CAA GTC CTA AGA CGC CA-*TAMRA*-3′ |
| *L23SQF* | *Listeria* spp. | *23S rDNA* | Forward primer | 5′-AGG ATA GGG AAT CGC ACG AA-3′ |
| *L23SQR* | | | Reverse primer | 5′-TTC GCG AGA AGC GGA TTT-3′ |
| *L23SQP* | | | TaqMan® probe | 5′-*VIC*-TCT CAC ACT CAC TGC TTG GAC GC-*TAMRA*-3′ |
| *Lin23SQFR* | *Listeria innocua* | | Reverse primer | 5′-TTC GCA AGA AGC GGA TTT G-3′ |

approximately 30,000 cfu) [28]. The optimal conditions, which are given in the Materials and Methods, were those showing the lowest value for the threshold cycle ($C_T$) with the lowest primer and probe concentrations. The concentration of *L23SQR* was critical, with slight reductions resulting in a strong increase in $C_T$. Conversely, the concentration of *L23SQF* could vary between 25 and 900 nM without critically affecting the performance of the reaction (data not shown). These data suggests that the *L. innocua* point mutation (i.e., within the *L23SQR* target sequence) might have stronger repercussion than the one of *L. grayi* (i.e., within the *L23SQF* target sequence) on the RTi-PCR.

The variability of DNA sequences among bacterial targets is critical as the performance of the reaction and, in consequence, both the LOD and capacity for quantification depend on the particular sequence [25]. Consequently, to assess the impact of the two mismatches described above on the performance of the reaction and, thus, on the capacity of the assay to detect all *Listeria* species, we performed RTi-PCR using 1 ng of genomic DNA from a representative set of *Listeria* strains (Table 1). The primers and probe designed according to the sequence of the *L. monocytogenes* LMO10046 gene showed 100% identity to the published sequences for *L. ivanovii*, *L. seeligeri* and *L. welshimeri*. Thus, as expected, they resulted in very efficient amplification and similar low $C_T$ values (overall, $15.97 \pm 0.31$) in all tested strains from these species. The *L. grayi* type strain (harbouring a mismatch within the *L23SQF* primer) was amplified equally as efficiently (overall $C_T$ $16.25 \pm 0.12$). Conversely, *L. innocua* strains (including a mismatch within the *L23SQR* primer), were amplified around 10-fold less efficiently (overall $C_T$, $19.51 \pm 0.17$).

To optimise the 23S rDNA-specific RTi-PCR for *L. innocua* samples, we designed a second reverse primer matching the *L. innocua* strain CLIP 11262 23S rDNA sequence (*Lin23SQR*, positions 1542–1570 Accession No. LIN 10003 in http://genolist.pasteur.fr/ListiList/). This primer contains an additional nucleotide so that its melting temperature was similar to that of *L23SQR* (Table 3). The optimised reaction amplified all *Listeria* species with similar efficiencies (overall $C_T$ $15.99 \pm 0.31$).

To estimate the specificity of the 23S rDNA RTi-PCR assay, we checked for cross-reactivity with 1 ng of DNA extracted from 15 bacterial species phylogenetically related to *Listeria* spp. (Table 2). No amplification was detected.

### 3.3. 23S rDNA and hly duplex RTi-PCR assay

The performance of the duplex assay was evaluated on decreasing amounts of *L. monocytogenes* ATCC 5577 genomic DNA (equivalent to approximately $3 \times 10^5$, $3 \times 10^4$, $3 \times 10^3$, $3 \times 10^2$, 30, 15, 3 and 1 cells per reaction). The *hly* gene was amplified in all nine replicates down to three *L. monocytogenes* genome copies and in 56% of the replicates containing one cell (Table 4). In the same duplex reactions, 23S rDNA was amplified in every replicate (Table 4), giving an LOD of 1 target genome copy. A single *L. monocytogenes* genome DNA molecule could only be consistently detected by RTi-PCR targeting the multicopy gene 23S rDNA that is present in six copies in the *L. monocytogenes* and *L. innocua* genomes [28,33]; therefore improving the results obtained with single-copy genes such as *hly* in this study and in previous related works [22–25].

### 3.4. Specificity of the duplex RTi-PCR assay and suitability for quantification of all Listeria species

The fluorescence of the VIC reporter allowed unambiguous identification of all strains belonging to the genus *Listeria*, whereas the fluorescence values of the FAM reporter unequivocally distinguished *L. monocytogenes* strains (Tables 1 and 2). These results are in agreement with those based on uniplex *hly*- or 23S rDNA-specific PCR (see above; [25]) and prove that our duplex assay is highly specific.

If our quantitative duplex assay is to be successfully applied, it is crucial that the *Listeria* spp.-specific reaction is equally efficient with genomic DNA extracted from all *Listeria* species. Analysis of the VIC fluorescence profiles obtained in the specificity assays demonstrated that the 23S rDNA $C_T$ values obtained with the duplex reactions were the same as those obtained with the uniplex reactions and were independent of the particular *Listeria* species

Table 4
RTi-PCR detection and quantification limits obtained with (a) *Listeria monocytogenes* strain ATTC 5577 and (b) *Listeria innocua* strain DMSZ 20649 genomic DNA

| Approximate number of genomic DNA molecules | | $3 \times 10^5$ | $3 \times 10^4$ | $3 \times 10^3$ | $3 \times 10^2$ | 30 | 15 | 3 | 1 |
|---|---|---|---|---|---|---|---|---|---|
| (a) *Listeria monocytogenes* strain ATCC 5577 | | | | | | | | | |
| Upper limit CI | | 298,928 | 29,661 | 2,893 | 267 | 20 | 8 | 1 | 0 |
| Lower limit CI | | 301,073 | 30,340 | 3,108 | 334 | 41 | 23 | 8 | 3 |
| *Duplex* | | | | | | | | | |
| 23S rDNA Signal ratio | $R^2 = 0.997$ | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 |
| 23S rDNA Mean $C_T$ values | $s = -3.326$ | 16.04 | 18.58 | 22.05 | 25.55 | 28.95 | 30.55 | 31.92 | 33.81 |
| 23S rDNA SD $C_T$ values | | 0.05 | 0.05 | 0.19 | 0.10 | 0.14 | 0.20 | 0.64 | 2.90 |
| *Hly* signal ratio | $R^2 = 0.995$ | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 | 5/9 |
| *hly* Mean $C_T$ values | $s = -3.485$ | 18.28 | 20.73 | 24.67 | 28.23 | 32.02 | 33.58 | 34.34 | 36.45 |
| *Hly* sd $c_t$ values | | 0.09 | 0.12 | 0.17 | 0.11 | 0.09 | 0.21 | 0.25 | 1.56 |
| *Uniplex* | | | | | | | | | |
| 23S rDNA Signal ratio | $R^2 = 0.995$ | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 |
| 23S rDNA Mean $C_T$ values | $s = -3.315$ | 16.10 | 18.40 | 22.15 | 25.7 | 29.00 | 30.20 | 32.10 | 34.05 |
| 23S rDNA SD $C_T$ values | | 0.07 | 0.10 | 0.12 | 0.12 | 0.08 | 0.15 | 0.65 | 2.50 |
| (b) *Listeria innocua* strain DMSZ 20649 | | | | | | | | | |
| *Duplex* | | | | | | | | | |
| 23S rDNA Signal ratio | $R^2 = 0.999$ | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 | 9/9 |
| 23S rDNA Mean $C_T$ values | $s = -3.331$ | 16.17 | 19.07 | 22.18 | 26.02 | 29.73 | 30.28 | 31.96 | 33.82 |
| 23S rDNA SD $C_T$ values | | 0.05 | 0.05 | 0.07 | 0.12 | 0.07 | 0.04 | 1.48 | 1.52 |

Lower and upper limit 95% confidence intervals were calculated for the expected template molecules in each dilution, taking into account the experimental design. Signal ratio corresponds to the positive/total reactions performed in three independent experiments. $C_T$ refers to threshold cycle value, SD, the standard deviation, CI, confidence interval. Correlation coefficients ($R^2$) and slopes of the regression curves (s) are included.

(i.e., mean $C_T$ values of $16.18 \pm 0.29$). We further compared the standard curves generated using *L. monocytogenes* ATCC 5577 (Table 4; Fig. 1) and *L. innocua* DSMZ 20649 (Table 4) genomic DNA, as these species harbour the most divergent 23S rDNA target sequences within the *Listeria* genus. The slopes of the two regression curves were virtually the same, giving efficiencies of 99.8% and 99.6%, respectively [34,35], and with excellent correlation coefficients (Table 4). The two sets of $C_T$ values were combined to build a single regression curve, giving an $R^2$ value of 0.997. These results indicate that the duplex RTi-PCR assay developed provides a highly accurate method for the quantification of any *Listeria* species or mixture of them.

To assess the suitability of this method for detecting *L. monocytogenes* among large amounts of other *Listeria* species we performed duplex reactions using as template 100%, 10%, 1%, 0.1% or 0.01% of *L. monocytogenes* DNA in the presence of 10 ng of DNA from *L. welshimeri*. All reactions yielded positive fluorescence signals corresponding to 23S rDNA but also to *hly*, indicating that the system has a detection limit of 0.01% *L. monocytogenes* with around 1 ng of starting DNA. Reliable quantification was only possible down to 10% *L. monocytogenes* DNA, as is the case with other duplex assays (e.g., [36]).

Uniplex RTi-PCR assays have been reported to quantify *Listeria* spp. in cell suspensions accurately [24,25] without the need for any pre-PCR treatment. For our duplex RTi-PCR assay, linear regression analyses of $C_T$ vs. log initial cell numbers yielded $R^2$ and slope values similar to those obtained with purified genomic DNA (data not shown), indicating that the assay is suitable for direct use on cell suspensions.

### 3.5. Limit of quantification of the duplex RTi-PCR assay

The initial amounts of target DNA were linearly correlated with the $C_T$ values obtained in 23S rDNA and *hly* duplex reactions over a 5-log range (Table 4). The correlation coefficients proved that this reaction was highly accurate and the slopes showed that the efficiency was close to 100% [34,35]. Both 23S rDNA (Table 4) and *hly* [25] duplex reactions showed the same results as in the uniplex format, indicating that multiplexing did not negatively influence the RTi-PCR assay to a detectable extent. Thus, 23S rDNA and *hly* standard curves could be constructed in duplex format, decreasing both the amount of reagents required and the number of PCR positions devoted to control reactions. In addition, we statistically quantified the theoretical error associated with serial dilutions of DNA in the experimental design (Table 4). In the higher dilution ranges (i.e., below 30 genomic DNA molecules) both the theoretical and experimental 95%

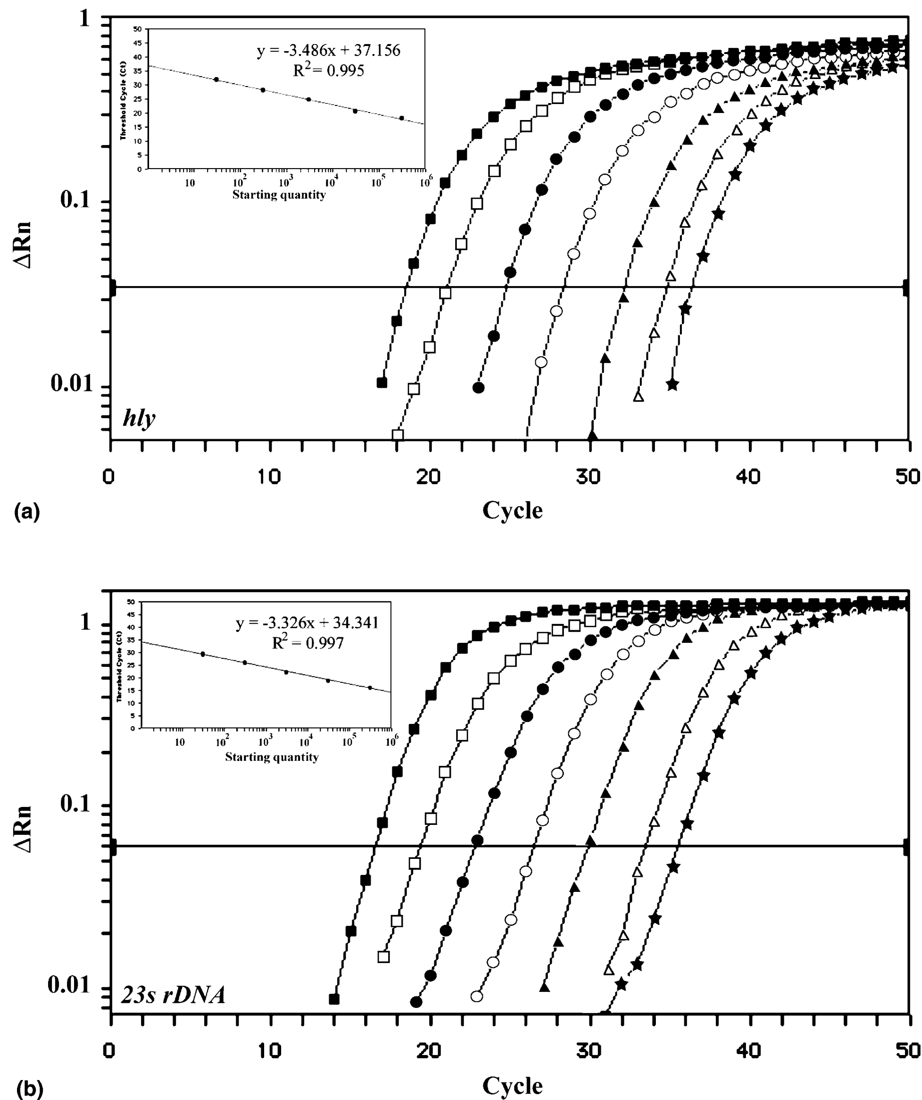


Fig. 1. Use of RTi-PCR for the simultaneous detection and amplification of the (a) *hly* and (b) *23S rDNA* sequences. Representative amplification plots are shown. Serial dilutions of *Listeria monocytogenes* genomic DNA, equivalent to $3 \times 10^5$ (closed squares), $3 \times 10^4$ (open squares), $3 \times 10^3$ (closed circles), $3 \times 10^2$ (open circles), 30 (closed triangles), 3 (open triangles) and 1 (closed stars) target molecules per reaction, were used. Inset, representative standard curve generated from the amplification data.

confidence intervals overlapped for neighbouring dilutions, making reliable quantification impossible. Therefore, the limit of quantification (LOQ) was determined to be 30 genome copies.

### 3.6. Relative accuracy of the 23S rDNA and hly duplex RTi-PCR assay

A high degree of correspondence between the results was observed, with differences always below 15% (Table 5). Moreover, both 23S rDNA and *hly* analyses produced highly similar quantification values with *L. monocytogenes* samples. In addition, when the different species of *Listeria* were analysed individually, the concordance between the values obtained by PicoGreen® and RTi-PCR was 100.45%. Thus, our duplex RTi-PCR assay can accurately quantify *Listeria* spp.

In conclusion, we report, for the first time, a highly specific, sensitive and reliable duplex RTi-PCR assay for the simultaneous quantitative detection of *Listeria spp* and *L. monocytogenes*. This duplex RTi-PCR assay attains an accurate quantification ($R^2$ values higher than 0.99) along 5 log units dynamic range with a PCR efficiency above 99%. Thus, it is a promising alternative to classical methods used in routine analysis in microbiology laboratories. In addition, this assay can be satisfactorily used to evaluate the incidence of *Listeria* spp. and *L. monocytogenes* in food processing environments, and in raw and processed food products.

Table 5
Accuracy of the 23S rDNA and *hly* duplex real-time PCR assay

| | *Listeria monocytogenes* | *Listeria innocua* | *Listeria ivanovii* | *Listeria seeligeri* | *Listeria welshimeri* | *Listeria grayi* | *Listeria* spp. |
|---|---|---|---|---|---|---|---|
| PicoGreen (fg/μl) | $1.00 \times 10^6$ | $1.00 \times 10^6$ | $1.00 \times 10^6$ | $1.00 \times 10^6$ | $1.00 \times 10^6$ | $1.00 \times 10^6$ | $1.00 \times 10^6$ |
| 23S RTi-PCR (fg/μl) | $9.68 \times 10^5$ (96.75) | $9.39 \times 10^5$ (93.87) | $1.03 \times 10^6$ (103.07) | $1.15 \times 10^6$ (114.94) | $9.86 \times 10^5$ (98.58) | $9.69 \times 10^5$ (96.86) | $1.00 \times 10^6$ (100.45) |
| *hly* RTi-PCR (fg/μl) | $9.55 \times 10^5$ (95.55) | – | – | – | – | – | – |

DNA extracted from overnight cultures of *Listeria monocytogenes* ATCC 5577, *Listeria innocua DMSZ* 20649, *Listeria ivanovii* CECT 913, *Listeria seeligeri* CECT 917, *Listeria welshimeri* CECT 919 and *Listeria grayi* CECT 931 was quantified by our assay and by fluorescent dye Picogreen®. $C_T$ values were extrapolated in a regression curve built with *Listeria monocytogenes* DNA dilutions to obtain the number of fg per reaction. Relative accuracy is expressed as the percentage of fg obtained with real-time PCR versus with the fluorescent dye Picogreen®-based method. *Listeria* spp. values were obtain by averaging across the different species.

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