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Recombinant plasmid-based quantitative Real-Time PCR analysis of *Salmonella enterica* serotypes and its application to milk samples

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ABSTRACT

The aim of the current study was to develop, a new, rapid, sensitive and quantitative *Salmonella* detection method using a Real-Time PCR technique based on an inexpensive, easy to produce, convenient and standardized recombinant plasmid positive control. To achieve this, two recombinant plasmids were constructed as reference molecules by cloning the two most commonly used *Salmonella*-specific target gene regions, *invA* and *ttrRSBC*. The more rapid detection enabled by the developed method (21 h) compared to the traditional culture method (90 h) allows the quantitative evaluation of *Salmonella* (quantification limits of 10^1 CFU/ml and 10^0 CFU/ml for the *invA* target and the *ttrRSBC* target, respectively), as illustrated using milk samples. Three advantages illustrated by the current study demonstrate the potential of the newly developed method to be used in routine analyses in the medical, veterinary, food and water/environmental sectors: I – The method provides fast analyses including the simultaneous detection and determination of correct pathogen counts; II – The method is applicable to challenging samples, such as milk; III – The method's positive controls (recombinant plasmids) are reproducible in large quantities without the need to construct new calibration curves.

1. Introduction

The successful detection of *Salmonella* is key to the prevention and identification of problems related to health (medical and veterinary) and safety (food and water/environmental) (Lazcka et al., 2007). Although traditional microbiological methods (ISO 6579, 2004) are accepted standards for *Salmonella* detection, they are labor intensive and time consuming. Therefore, it is important to develop sensitive and rapid methods to detect *Salmonella* (Fey et al., 2004; Hagren et al., 2008; Lazcka et al., 2007; McCabe et al., 2011; Miller et al., 2011; Patel et al., 2006; Pusterla et al., 2010; Sánchez-Jiménez and Cardona-Castro, 2004; Singh et al., 2013; Zhou and Pollard, 2012).

Due to the superior properties (speed and reliability) of Real-Time PCR, the detection of *Salmonella* using Real-Time PCR with either a SYBR green probe (Arrach et al., 2008; Chen et al., 2011; Donhauser et al., 2011; Fukushima et al., 2007) or a TaqMan probe (Gonzalez-Escalona et al., 2009; Hyeon et al., 2010; Josefsen et al., 2007; Lofstrom et al., 2009; Malorny et al., 2004; O'Regan et al., 2008; Woods et al., 2008) has been investigated. In addition, various targets characteristics of *Salmonella* have been used for the detection of this pathogen: *oriC* (replication origin encoding gene) (Woods et al., 2008), *ompC* (major outer membrane protein gene) (Amavisit et al., 2001), *invA* (*Salmonella* invasion protein gene) (Chen et al., 2011; Gonzalez-Escalona et al., 2009); *stn* (enterotoxin gene) (Moore and Feist, 2007), *hilA* (type III secretion system regulation gene) (Donhauser et al., 2011; McCabe et al., 2011); *iroB* (iron-responsive gene) (Murphy et al., 2007); *aceK* (isocitrate dehydrogenase kinase/phosphatase gene) (O'Regan et al., 2008) and the *ttrRSBC* locus (on which the *ttrA*, *ttrB* and *ttrC* tetrathionate reductase structural genes are located) (Hyeon et al., 2010; Josefsen et al., 2007; Lofstrom et al., 2009; Malorny et al., 2004).

Although some of the aforementioned studies have reported promising results using different primers and probes, there is still no internationally accepted standardized protocol for Real-Time PCR-based *Salmonella* detection. More importantly, the Real-Time PCR technique is still used as a presence/absence test or for relative quantification.

In the Real-Time PCR technique, the precise copy number of a specific nucleic acid sequence can be quantified using a calibration curve created with known concentrations of DNA (Lin et al., 2011). Regarding *Salmonella*, known concentrations of DNA have been

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determined relatively, except in a few studies (Fey et al., 2004; Gonzalez-Escalona et al., 2012; Zhang et al., 2011). However, there is a great disadvantage of the standards belonging to these studies for routine analyses; the standards are constructed with genomic DNA to provide accurate quantification results. Among various DNA standards (plasmid DNA, PCR amplicon, synthesized oligonucleotide, genomic DNA, or cDNA), plasmid DNA is the most attractive because it is relatively easy to construct and handle, is relatively stable when frozen, and can be produced in large quantities; hence, plasmids are perfect candidates for standards to produce a calibration curve (Burns et al., 2006; Lin et al., 2011; Ustek et al., 2008). Burns et al. (2006) reported that plasmid calibration standards had equal or better performance characteristics, in terms of precision and closeness to the expected value, than their genomic equivalents for the quantification of the genetically modified (GM) content of food in experiments performed at three different laboratories in Europe. Thus, plasmid calibration standards have gained popularity, and to date, more than 20 plasmid calibration standards have been successfully constructed and used for the detection and quantification of several GM foods (Meng et al., 2012). Although plasmid calibration standards are becoming essential for the practical quantification of genetically modified organisms (GMOs) (Meng et al., 2012), they have limited use in pathogen detection and quantification, one of the most important issues in the medical, veterinary, food and water/environmental sectors.

In light of the promising results of studies on GMOs (Burns et al., 2006; Lin et al., 2011; Meng et al., 2012), the prospective use of plasmids as inexpensive, easy to produce, convenient and standardized positive controls will contribute to the reliability of the Real-Time PCR-based detection and quantification of *Salmonella* for routine analyses. In this study, two recombinant plasmids were constructed as references by cloning the two most commonly used *Salmonella*-specific target gene regions, *invA* and *ttrRSBC*, for the detection and accurate quantification of *Salmonella*.

Milk is an ideal medium for heterotrophic microorganisms due to its nutritious chemical composition; milk is rich in protein, fat, carbohydrates, Ca^{2+} and Fe^{2+} ions and has a relatively neutral pH (6.6) (Jay et al., 2005). *Salmonella* species are perhaps the most frequent pathogens associated with milk and dairy products (Fuquay et al., 2011). Milk contains PCR inhibitors such as fats, proteins and calcium, which cause DNA amplification problems; the extraction of DNA from milk in sufficient concentrations and the purity of the extracted DNA are crucial for successful PCR (Pirondini et al., 2010; Quigley et al., 2012). However, previous studies (Omiccioli et al., 2009; Quigley et al., 2012; Riyaz-Ul-Hassan et al., 2013) have demonstrated the applicability of Real-Time PCR techniques to the detection of various foodborne pathogens. Therefore, milk was chosen in this study as a challenging matrix to test the applicability of the developed method.

To the best of our knowledge, this is the first study in which cloned *Salmonella*-specific gene regions are reported as standard positive controls to assess the efficiency of Real-Time PCR and are used as standard positive markers for accurate quantification, as well as to detect the pathogen in both artificially and naturally contaminated milk samples. Another important contribution of the current study concerns the cost effective and easy quantification of *Salmonella*; the developed approach is thus invaluable for culture-independent routine analyses in the medical, veterinary, food and water/environmental sectors.

2. Materials and methods

2.1. Bacterial reference strains

To test the applicability of recombinant plasmids containing the *invA* or *ttrRSBC* genes as positive controls, *Salmonella* Typhimurium ATCC 14028 was used as the reference strain.

To detect and enumerate *Salmonella* in milk samples using the developed recombinant plasmid-based Real-Time PCR method, 15 *Salmonella enterica* serotypes (*S.* Agona, *S.* Anatum, *S.* Bispebjerg, *S.* Coravallis, *S.* Enteritidis, *S.* Infantis, *S.* Kentucky, *S.* Montevideo, *S.* Nchanga, *S.* Salford, *S.* Telaviv, *S.* Senftenberg, *S.* Thompson, *S.* Typhimurium and *S.* Virchow) were used as positive controls. The ATCC strains of 8 different pathogens other than *Salmonella* were used as negative controls: *Citrobacter freundii* ATCC 6879, *Escherichia coli* O157:H7 ATCC 35150, *Enterococcus faecalis* ATCC 33186, *Listeria innocua* ATCC 33090, *Proteus vulgaris* ATCC 8427, *Shigella sonnei* ATCC 29930, *Staphylococcus auereus* ATCC 13565, and *Yersinia enterocolitica* ATCC 29913.

The *S.* Typhimurium ATCC 14028 and negative controls were purchased from Istanbul Hifzissihha Institute (Istanbul, Turkey). *Salmonella* serotypes used as positive controls were previously isolated from foods at Ankara University, Department of Biology Laboratories (Ankara, Turkey) and identified in the Federal Institute of Risk Assessment, *Salmonella* Reference Laboratories (Germany) (Avsaroglu, 2007). All bacterial strains were kept as frozen stocks at −80 °C and were grown in Brain Heart Infusion (BHI) or Tryptic Soy Broth (TSB) at 37 °C.

2.2. Primers and probes

All primers and probes used (Table 1) were purchased from Fermentas (Thermo Fisher Scientific, USA).

2.3. DNA isolation

Aliquots (1 ml) of bacterial cultures were centrifuged at 6000 *g* for 10 min and DNA samples were isolated from the cell pellets using the Fermentas Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

2.4. PCR

Isolated DNA samples were amplified using Techne TC-512 Thermo Cycler PCR (Bibby Scientific Limited, UK) and PCR kits were supplied by Fermentas (Thermo Fisher Scientific, USA). A 2 μl DNA sample was added to 23 μl of PCR reaction mixture (50 μM dNTPs, 1.5 mM $MgCl_2$, 10 pmol primers, 0.1 U Taq polymerase). The PCR was performed as follows: one cycle at 98 °C for 5 min; 35 cycles of 94 °C for 30 s, 59 °C for 1 min, and 72 °C for 30 s; and one final cycle at 72 °C for 5 min. The resulting PCR products were analyzed on agarose gels to determine the target regions and PCR quality (Ustek et al., 2008).

2.5. Gel electrophoresis

Horizontal agarose (2%) gels (5 × 60 × 3 mm) were prepared with TAE (40 mM Tris-acetate, pH 8.3, 1 mM EDTA) buffer. Electrophoresis was performed at a constant voltage of 130 V. The DNA ladder (1 kb; Life Technologies Corporation, USA) and PCR samples (10 μl each)

Table 1
Primers and probes used.

invA[a]
139 (Forward) (5′-GTGAAATAATCGCCACGTCGGGCAA-3′)
141 (Reverse) (5′-TCATCGCACCGTCAAAGGAACC-3′)
invA-1 probe (5′-FAM-TTATTGGCGATAGCCTGGCGGTGGGTTTTGTTG-TAMRA-3′
ttrRSBC[b]
ttr-6 (Forward) (5′-CTCACCAGGAGATTACAACATGG-3′)
ttr-4 (Reverse) (5′-AGCTCAGACCAAAAGTGACCATC-3′)
Target probe (ttr-5) (5′-FAM-CACCGACGGCGAGACCGACTTT-Dark Quencher-3′)
IAC probe (5′-Yakima Yellow-CACACGGCGACGCGAACGCTTT-Dark Quencher-3′)

[a] Hein et al., 2006; Malorny et al., 2003.
[b] Malorny et al., 2004.

were diluted with sample buffer (1% SDS, 50% Glycerol, 0.05% Bromophenol Blue, pH 8.3) and loaded on agarose gels. The PCR fragments of interest recovered from the agarose gel in slices by elution using a gel extraction kit (Qiagen, Germany).

2.6. Recombinant plasmid construction and sequencing

The Fermentas K1214 cloning kit (Thermo Fisher Scientific, USA) was used; the kit uses a single 3′ thymidine overhang for PCR fragment cloning. All amplified fragments were cloned into the pTZ57R/T cloning vector according to the manufacturer's manual. Briefly, 50 ng of vector DNA and 1 μl of PCR product were incubated (4 °C overnight) with 2X T4 DNA ligase buffer containing 1 μl (3 u/μl) T4 DNA ligase. 2 μl of ligation reaction and 100 μl of DH5 alpha competent cells (Agilent Technologies, USA) were heat-shock transformed and spread on agar plates containing 100 μg/ml ampicillin. Plates were incubated overnight at 37 °C. The plasmids were isolated from bacteria using the Roche High Pure Plasmid Isolation Kit (Roche Applied Science, Germany) according to the manufacturer's manual. To determine whether the *invA* or *ttrRSBC* genes were incorporated into isolated plasmids, the genes were amplified using PCR as described above, and their sequences were determined (Iontek Co, Turkey).

2.7. Calculation of copy numbers of recombinant plasmids containing the invA or the ttrRSBC gene

The concentrations of the isolated recombinant plasmids were measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and the much more sensitive device The Qubit 2.0 Fluorometer (Life Technologies Corporation, USA).

Copy numbers of the recombinant plasmids containing the *invA* gene (P-invA $= 1.75 \times 10^{10}$) and of the recombinant plasmids containing the *ttrRSBC* gene (P-ttrRSBC $= 2.32 \times 10^{10}$) were calculated using a dsDNA copy number calculator (Staroscik, 2004).

2.8. Real-time PCR

To confirm the precision and reproducibility of Real-Time PCR, standard curves were constructed using P-invA and P-ttrRSBC by diluting these standards (10^6 to 10^0) in six different runs on different days with two replicates each. To construct the standard curves for P-invA and P-ttrRSBC, crossing point (Cp) mean values of 12 replicates were plotted against the Log calculated copy numbers. From the slopes of the standard curves, PCR efficiencies and amplification efficiencies for P-invA and P-ttrRSBC were calculated using the following equations, respectively: $E = (10^{-1/slope}) - 1$ and $E_{amp} = 10^{-1/slope}$ (Gallup and Ackermann, 2006).

Detection and quantification limits of the developed technique were determined after three independent experiments using diluted isolated DNA. *Salmonella* Typhimurium ATCC 14028 culture was grown in TSB (Oxoid, Basingstoke, UK) to an optical density (OD_{600} nm) of 0.250, corresponding to 10^8 CFU/ml, and a 10^5 CFU/ml level concentration was obtained through serial dilutions in the same medium. The DNA extracted from the *Salmonella* Typhimurium ATCC 14028 culture at 10^5 CFU/ml was serially diluted to 10^0 CFU/ml using nuclease-free water.

A 25-μl reaction mixture contained 12.5 μl of Maxima Probe qPCR Master Mix (2X) (Maxima Probe qPCR Buffer containing KCl, $(NH_4)_2SO_4$, Maxima® Hot Start *Taq* DNA polymerase, dNTPs, dUTP), 0.5 μL (10 pmol) of each primer (139F and 141R for *invA*; ttr-6F and ttr-4R for *ttrRSBC*), 0.5 μL (10 pmol) of each probe (*invA*-1 probe for *invA*; ttr-5 and IAC probes for *ttrRSBC*), a 5 μL aliquot of DNA, and 150 copies of IAC DNA (purified 303-bp PCR product as described previously (Malorny et al., 2004)) for *ttrRSBC*. Controls with no template DNA, containing 5 μL of TE buffer instead of DNA, were included in each run to detect any contamination. All Real-Time PCR solutions were purchased from Fermentas (Thermo Fisher Scientific, USA).

Real-Time PCR experiments and data analyses were performed using a Roche Light Cycler 480 (Roche Diagnostics, Germany). The thermal cycling conditions were: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 65 °C for 30 s, and 72 °C for 20 s.

2.9. Application of the developed method to milk samples

Raw milk samples were heated to 90 °C, kept at 90 °C for 10 min, and then cooled to room temperature (to make the samples sterile prior to artificial inoculation allowing only analyses of artificially inoculated *Salmonella* serotypes that have known concentrations). Then, 25 ml milk samples were inoculated individually with each of the 15 *Salmonella* serotypes at 10^5 to 10^0 CFU/ml level concentrations, 1 ml of each concentration was added to the 25 ml milk samples, homogeneous mixtures were obtained with gentle vortex mixing, and the samples were diluted tenfold in Buffered Peptone Water (BPW; 225 ml). These mixtures were incubated at 37 °C for 18 h after homogenization. Following pre-enrichment in BPW, the traditional culture method (ISO 6579, 2004) containing a selectively enriched 0.1 ml culture in Rappaport Vasilliadis Soya Broth (RVS) for 24 h at 41.5 °C, and a 1 ml culture in Muller-Kauffmann Tetrathionate-Novobiocin (MKTTn) Broth for 24 h at 37 °C were simultaneously inoculated onto XLD and BGA agars and incubated for 24 h at 37 °C; cultures were also inoculated onto Tryptone Soy Agar (TSA) and incubated for 24 h at 37 °C. Following pre-enrichment in BPW, the newly developed recombinant plasmid-based Real-Time PCR method includes DNA extraction prior to Real-Time PCR analysis. Fig. 1 illustrates the general steps of the traditional culture method and the Real-Time PCR assay.

The newly developed method was applied to naturally contaminated bulk tank milk samples collected from ten milk vendors throughout Istanbul, Turkey. The methodology used was the same as that used to evaluate artificially contaminated milk samples, except for the heating step.

Three independent experiments were performed in triplicate on artificially contaminated and naturally contaminated milk samples. All culture media were purchased from Oxoid (Basingstoke, UK).

2.10. Statistical analysis

A t-test was performed to test the significance of the differences between groups. $p < 0.05$ was considered significant.

3. Results

3.1. Cloning of invA and ttrRSBC and the construction of standard curves

The two most commonly used *Salmonella*-specific target gene regions (*invA* and *ttrRSBC*) were successfully cloned into a pTZ57R/T vector (2886 bp). Negative controls, including genomic DNA from *E. coli* O157:H7, did not yield any PCR product (Fig. 2).

To confirm the precision and reproducibility of Real-Time PCR, the averages of the 12 replicate Cp values presented in Table 2 were used to construct standard curves for P-invA and P-ttrRSBC (Fig. 3).

3.2. Detection and quantification limits of the developed method

The limits of the newly developed method were determined using diluted isolated DNA.

Using the Cp values for the *invA* and *ttrRSBC* targets, concentrations were calculated with constructed standard curves for *invA* and *ttrRSBC* targets (Fig. 4).

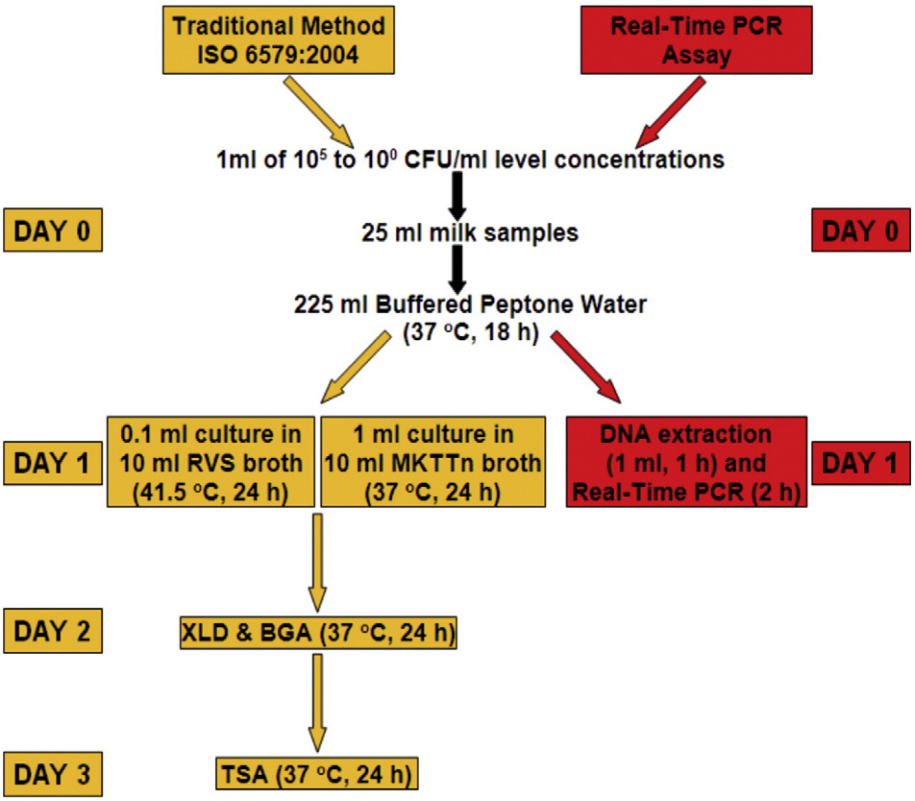

Fig. 1. Flow diagram of the traditional culture method and the Real-Time PCR assay for *Salmonella* analyses in milk samples. Adapted from O'Regan et al. (2008).

3.3. Application of the newly developed method to milk samples

The method developed using *S.* Typhimurium ATCC 14028 was tested to detect and precisely quantify 15 *Salmonella* serotypes inoculated into milk.

Using the Cp values for the *invA* and *ttrRSBC* targets (please see Table S1 and S2), precise concentrations of *Salmonella* cultures, 10^5 to 10^0 CFU/ml, were calculated with the constructed standard curves for the *invA* and *ttrRSBC* targets (Fig. 5).

In addition to artificially contaminated milk samples, the newly developed recombinant plasmid-based Real-Time PCR method was applied to naturally contaminated bulk tank milk samples collected from ten milk vendors throughout Istanbul, Turkey. Detailed analyses revealed that positive samples (the 5th and 8th samples) were detected using both the traditional culture method and the newly developed method. *Salmonella* concentrations were calculated as 10^1 CFU/ml level using the newly developed method (Fig. 6).

4. Discussion

4.1. Cloning and Real-Time PCR efficiency

Statistically significant differences were not found among the Cp values ($p > 0.05$) or among the copy number values ($p > 0.05$) belonging to the *invA* incorporated recombinant plasmids and the *ttrRSBC* incorporated recombinant plasmids. The statistical insignificance of the Cp values illustrates the high reproducibility among Real-Time PCR runs. The statistical insignificance of the copy number values illustrates the consistency of the cloning protocol.

Furthermore, the linear R^2 relationships of both the P-invA and P-ttrRSBC standard curves were 0.999 (Fig. 3), indicating the high reproducibility of Real-Time PCR (Zou et al., 2006) for recombinant plasmids incorporating *invA* and *ttrRSBC*. From the slopes of the constructed standard curves, PCR efficiency values were calculated using the equation $E = (10^{-1/\text{slope}}) - 1$ and were found to be 99%

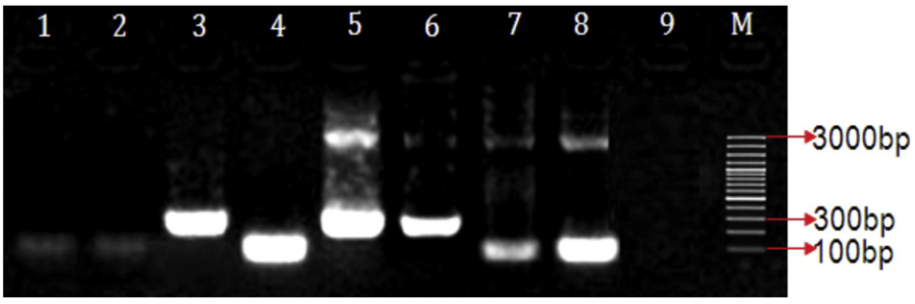

Fig. 2. Ethidium bromide stained agarose gel showing PCR products representing the cloned *invA* and *ttrRSBC* regions. 1: *invA* negative (*invA* forward primer 139F + *invA* reverse primer 141R) 2: *ttrRSBC* negative (*ttrRSBC* forward primer ttr6F + *ttrRSBC* reverse primer ttr4R) 3: PCR product of *invA* Primers + Isolated DNA from *S.* Typhimurium ATCC 14028 culture 4: PCR product of *ttrRSBC* Primers + Isolated DNA from *S.* Typhimurium ATCC 14028 culture 5,6: PCR product of *invA* Primers + P-invA 7,8: PCR product of *ttrRSBC* Primers + P-ttrRSBC 9: *E. coli* O157:H7 as a negative control (PCR product of *invA* Primers and *ttrRSBC* Primers + Isolated DNA from *E. coli* O157:H7) M: Molecular weight marker.

Table 2
Reproducibility of the Real-Time PCR.

Copy number level	P-invA	P-ttrRSBC
	Cp ± SD	Cp ± SD
10^6	12.32 ± 0.08	11.98 ± 0.06
10^4	19.35 ± 0.19	19.23 ± 0.13
10^2	26.18 ± 0.22	25.94 ± 0.18
10^0	32.30 ± 0.66	32.30 ± 0.65

Cp: Crossing point; SD: Standard deviation.

and 98% for P-invA and P-ttrRSBC, respectively. The amplification efficiencies ($E_{amp} = 10^{-1/slope}$) were 1.99 and 1.98 for P-invA and P-ttrRSBC, respectively, and thus a nearly perfect doubling of the template was obtained after each cycle ($E_{amp} = 2$ indicates the perfect doubling of the template every cycle) (Gallup and Ackermann, 2006).

4.2. Detection and quantification limits of the developed method

Our recombinant plasmid-based *Salmonella* detection method has a detection limit of 10^0 CFU/ml for both targets, and the method has quantification limits of 10^1 CFU/ml and 10^0 CFU/ml for the *invA* target and the *ttrRSBC* target, respectively (Fig. 4). The *ttrRSBC* target was easier to detect at lower concentrations. Highly similar Cp and concentration values were obtained for 10^2–10^0 dilutions of the *invA* target. Concentration values of the *ttrRSBC* target were more clearly discriminated at these dilutions.

Although the *invA* gene (essential for invading mammalian cells) is the most commonly used target for the detection of *Salmonella* in PCR assays (O'Regan et al., 2008), instability and natural deletions within *Salmonella* pathogenicity island1, encompassing the *inv*, *spa*, and *hil* loci, has been shown (Ginocchio et al., 1997; Malorny et al., 2004). Malorny et al. (2004) therefore suggested an alternative target, the *ttrRSBC* gene (responsible for tetrathionate respiration), which is genetically stable (Malorny et al., 2004), contrary to the genetic instability of *Salmonella* pathogenicity island1. Genetic instability and natural deletions within *Salmonella* pathogenicity island1 (Ginocchio et al., 1997; Malorny et al., 2004) can explain the lower sensitivity obtained when using the *invA* target at lower concentrations.

4.3. Application to milk samples

According to a scientific/technical report submitted to the European Food Safety Authority (EFSA), 392,485 *Salmonella* cases were reported in the European Union between 2007 and 2009, and *S.* Enteritidis

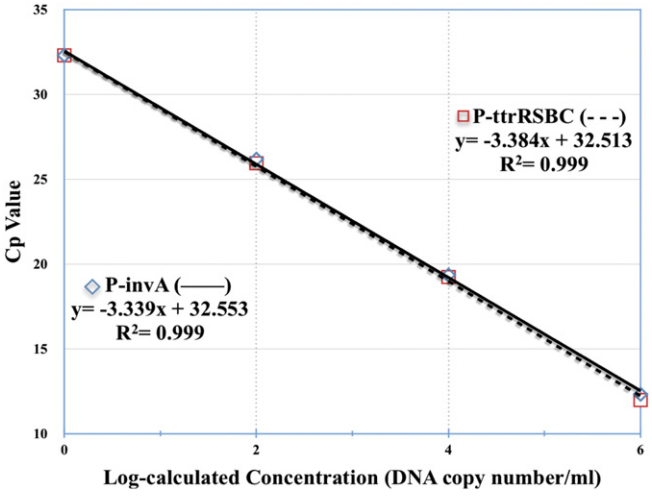

Fig. 3. Standard curves of P-invA: Recombinant plasmids containing *invA* and P-ttrRSBC: Recombinant plasmids containing *ttrRSBC*.

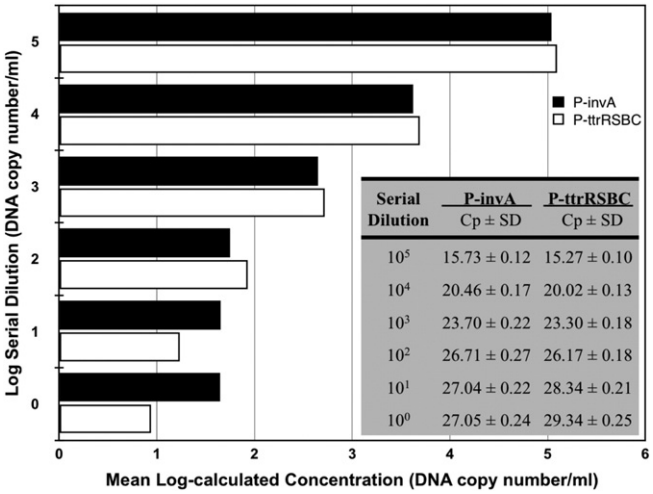

Fig. 4. Cp values and calculated concentrations of diluted DNA samples using constructed standard curves.

was the most widely and frequently reported serovar, followed by *S.* Typhimurium and *S.* Virchow (The total percentages of the three serovars in reported *Salmonella* cases were: 76.3% in 2009, 80.6% in 2008, 81.8% in 2007) (Pires et al., 2011). Thus, 15 *Salmonella* serotypes were used as positive controls, including *S.* Enteritidis, *S.* Typhimurium and *S.* Virchow, and these serotypes are good representatives of the genus *Salmonella* in foods.

Despite the PCR inhibitors present in milk (Pirondini et al., 2010; Quigley et al., 2012), 10 to 100-fold higher bacterial counts were found with the newly developed recombinant plasmid-based Real-Time PCR method than with the traditional culture method (ISO 6579, 2004). The two lowest concentration levels quantified using the newly developed method (Fig. 5E and F) could not be detected using the traditional culture method. These results are likely due to the higher sensitivity of the developed method: (i) the presence of intact DNA from dead cells could not be quantified by plate counts, (ii) the presence of viable but non-culturable forms could not be quantified by plate counts, and (iii) one CFU on an agar plate can be generated by more than one cell (Postollec et al., 2011). Above all, the newly developed recombinant plasmid-based Real-Time PCR method is much faster than the traditional culture method (21 h vs. 90 h), in addition to its higher sensitivity and lower labor intensity.

The newly developed method has a detection limit of 1 CFU/25 ml for both targets and quantification limits of 10^1 CFU/ml and 10^0 CFU/ml for the *invA* target and the *ttrRSBC* target, respectively (quantification limits were determined as the most common minimum calculated concentration levels in Fig. 5). In accordance with the detection and quantification limits of the developed method, plasmids that incorporated *ttrRSBC* were easier to detect, and thus it was more efficient to determine bacterial counts at low concentrations using these plasmids (Fig. 5D,E and F), which may be the result of genetic instability and natural deletions within *Salmonella* pathogenicity island1, encompassing the *inv* loci, as previously mentioned (Ginocchio et al., 1997; Malorny et al., 2004).

Quantitative PCR can be used to quantify nucleic acids by two common methods: relative quantification and absolute quantification. While relative quantification is based on internal reference genes to determine fold differences in expression of the target gene, absolute quantification gives the accurate and exact number of target nucleic acid molecules by comparison with nucleic acid standards using a calibration curve (Bustin, 2000; Hochstart et al., 2015; Li et al., 2009; Popp and Bauer, 2015). In the current study: I – The concentrations of the recombinant plasmids were measured using extremely sensitive device (The Qubit 2.0 Fluorometer, Life Technologies Corporation, USA) and the copy numbers of the recombinant plasmids were calculated

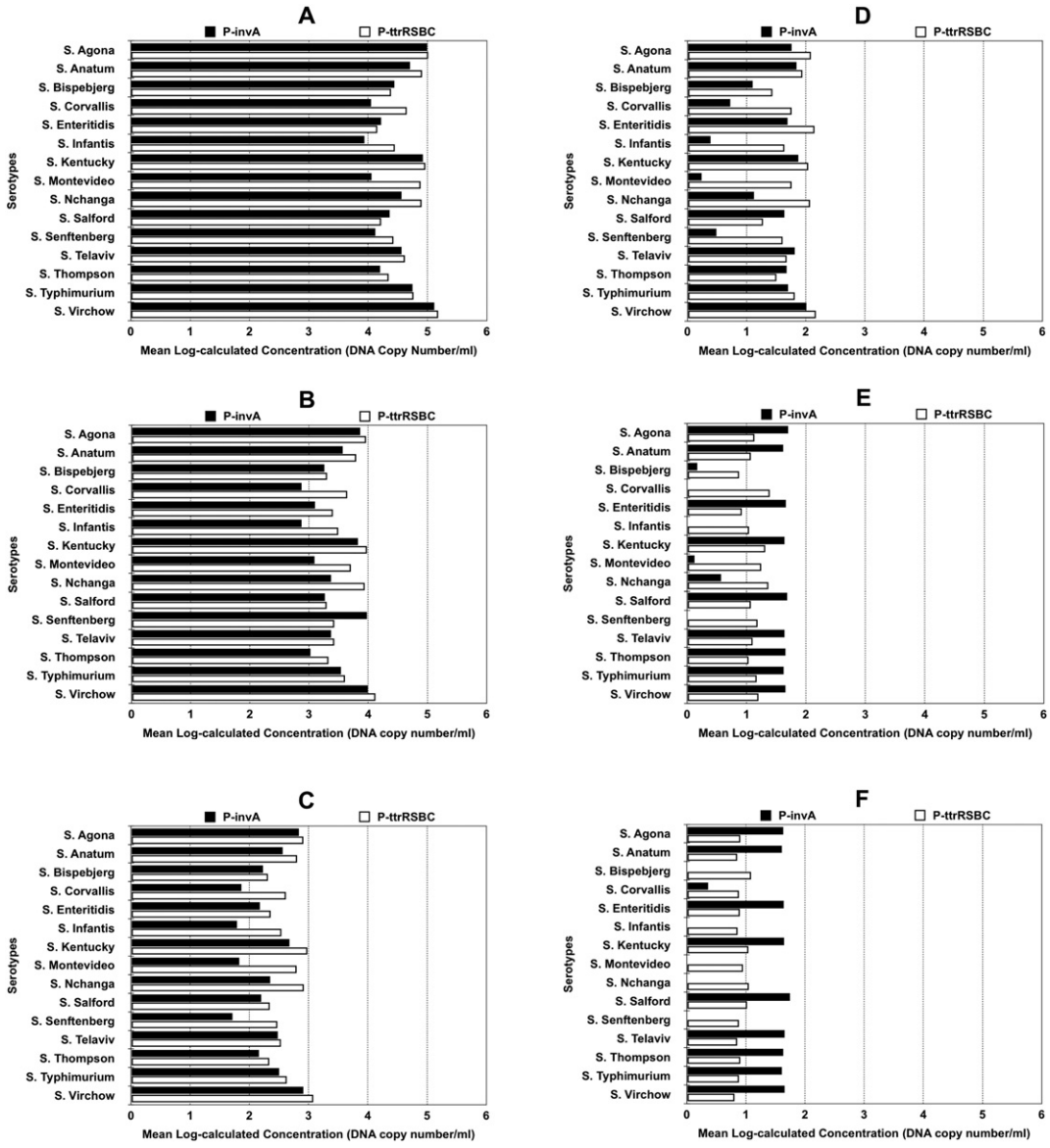

Fig. 5. Mean log10 concentrations of 15 *Salmonella* serotypes. A) Exact concentrations of the serotypes that were measured at 10^3 CFU/ml by the traditional culture method. B) Exact concentrations of the serotypes that were measured at 10^2 CFU/ml by the traditional culture method. C) Exact concentrations of the serotypes that were measured at 10^1 CFU/ml by the traditional culture method. D) Exact concentrations of the serotypes that were measured at 10^0 CFU/ml by the traditional culture method. E) and F) Quantification of the cultures obtained respectively by 10-fold and 100-fold dilution of D. (The concentration levels in E and F could not be detected using the traditional culture method). The negative mean log-calculated concentrations for the *invA* target were omitted.

using a dsDNA copy number calculator (Staroscik, 2004); II – Standard curves were constructed using the results of the repeated experiments (six different runs on different days with two replicates each) carried out by diluting the recombinant plasmids having the precisely measured and calculated values; III – Prior to the application of the developed method to real situations (naturally contaminated milk samples), these precisely constructed standard curves were tested using artificially inoculated *Salmonella* serotypes that have known concentrations (Fig. 5); IV – In accordance with the final purpose of the study, naturally contaminated milk samples were successfully detected, and *Salmonella* counts in these samples were accurately determined by the newly developed method (Fig. 6) thanks to the meticulous application of the absolute quantification principles throughout the study.

The proposed approach showed 100% concordance with the results of the traditional culture method (ISO 6579, 2004) but was much faster (21 h vs. 90 h), much more sensitive, and much less labor intensive than the traditional method for *Salmonella* detection. The developed method was also able to identify exact pathogen counts due to the meticulous application of the absolute quantification principles explained in detail above. These superior properties of the developed approach were demonstrated using both artificially contaminated and naturally contaminated milk samples, which can be challenging for PCR studies.

The presence of a statistically significant difference between *Salmonella* detection using plasmids that incorporated *invA* or *ttrRSBC* was investigated using a t-test. The statistical analyses revealed a statistically insignificant difference ($p > 0.05$) for Cp and concentration parameters, and thus a statistically insignificant difference ($p > 0.05$) between the plasmids that incorporated *invA* and *ttrRSBC* in terms of *Salmonella* detection and quantification ability for both artificially contaminated and naturally contaminated milk samples.

Due to their speed and sensitivity, culture-independent DNA-based technologies are being increasingly used to provide an accurate assessment of the composition of bacteria, including *Salmonella*, in milk (Quigley et al., 2012). Although Real-Time PCR is very fast (the time required in our study was 1 h for DNA extraction and 2 h for Real-Time PCR), DNA-based technologies still require time consuming

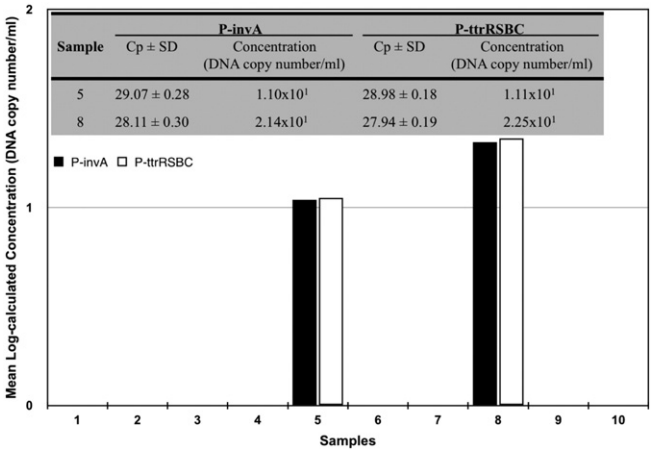

Fig. 6. *Salmonella* analyses of ten raw milk samples with the newly developed recombinant plasmid-based Real-Time PCR method.

pre-enrichment procedures (18 h in our study). Without pre-enrichment, Omiccioli et al. (2009) could not detect contamination levels below 10^4 CFU/ml using a Real-Time PCR method, while the introduction of an enrichment step greatly enhanced sensitivity to a detection limit of as little as one bacterium in a 25 ml milk sample. The DNA-based technologies as well as other technologies, such as electrochemical magneto-immunosensing, suffer from pre-enrichment issues. Using an electrochemical magneto-immunosensing approach, Liebana et al. (2009) detected 5×10^3 and 7.5×10^3 CFU/ml in LB and in milk, respectively, without any pretreatment, but were able to detect 1.4 CFU/ml and 0.108 CFU/ml (2.7 CFU/25 g of milk) in skim milk after 6 h and 8 h of pre-enrichment, respectively. The necessity of a pre-enrichment step is not limited to milk samples; to be able to detect very low levels of *Salmonella* in food and feed samples by molecular methods, the sample preparation step must include a significant amount of time for pre-enrichment (Malorny et al., 2008). However, there is a drawback to including a pre-enrichment step. The step makes it impossible to quantify the initial amount of contamination (Postollec et al., 2011). Although most probable number (MPN) method can allow the determination of initial amount of contamination in enriched samples, it has several limitations for enumeration of pathogens in food and feed samples: I – It is a time consuming (4 to 5 days required for conformation of results), labor intensive, media-intensive and expensive process, when performed appropriately, which does not make it amenable to high throughput processes and routine analyses (Brichta-Harhay et al., 2007; Corry et al., 2012; Malorny et al., 2008); II – The precision is poor unless the number of replicate tubes per dilution is very large (Corry et al., 2012; Motarjemi et al., 2014); III – Inconsistent results of the method were reported (Barkocy-Gallagher et al., 2003; Brichta-Harhay et al., 2007). Miniaturized MPN approach, mini-MSRV MPN technique (miniaturization of the dilution, pre-enrichment, and selective enrichment on modified semi-solid Rappaport-Vassiliadis medium in 12-well microwell plates), was presented to reduce the material and labor cost in conventional MPN method (Fravalo et al., 2003). In spite of the progress related to the material and labor cost, it is possible that the mini-MSRV MPN method is less appropriate to enumerate *Salmonella* ser. Typhi and *Salmonella* ser. Paratyphi; and the method is less sensitive compared to the conventional MPN method (ISO/TS 6579-2, 2012).

The pre-enrichment step must be long enough to achieve the required sensitivity, but not so long that the growth curve reaches the plateau phase (Kramer et al., 2011; Malorny et al., 2008). In the current study, the step was optimized according to these requirements to decrease its effects on quantification as much as possible. The pre-enrichment step is not a drawback of the newly developed method; it is a challenging problem for all food safety analyses, as mentioned above. The newly developed method is able to determine exact counts of *Salmonella* in food samples whether the analysis contains a pre-enrichment step. In the future, instead of a pre-enrichment step the development of a method that does not change the initial bacterial count or instead of MPN/mini-MSRV MPN methods the development of a method that practically and precisely determines the effect of pre-enrichment step on the initial amount of contamination would allow the determination of the initial *Salmonella* count in a food sample by our recombinant plasmid positive controls using Real-Time PCR. Therefore, the elimination or significant reduction of the pre-enrichment step (or steps) or the practical and precise determination of the effect of pre-enrichment step on the initial amount of contamination typically required for the sensitive detection of food borne pathogens is an absolute necessity for the progression of the field of food safety.

4.4. Significance and impact of the study

Quantitative and cost effective methods that can enumerate low concentrations of *Salmonella* are essential to the identification of critical contamination points and to the assessment of microbiological risks in the processing chain (Malorny et al., 2008; Postollec et al., 2011). The estimation of the level of illness that a pathogen can cause in a population is still hampered by a lack of quantitative data (Forsythe, 2002; Malorny et al., 2008; Oscar, 2004). Mainly due to the consumption of contaminated foods (Das et al., 2006; Icgen et al., 2002; Isiker et al., 2003; Malorny et al., 2008; McCabe et al., 2011), nearly 22 million cases of typhoid occur each year with 200,000 deaths globally (Parry, 2005). This striking statistic illustrates the urgent need for quantitative methods and better treatment strategies. The newly developed method is able to determine the exact copy number of *Salmonella* in a sample. This method will provide not only much faster *Salmonella* detection but also better risk evaluation (for food and water/environmental safety) and better treatment strategies (in medicine and veterinary medicine).

Moreover, as analyzed in detail above, the newly developed quantitative recombinant plasmid-based Real-Time PCR method for *Salmonella* spp. is applicable to challenging samples, such as milk.

Another important contribution of the current study concerns the cost effectiveness. Only 1 ml of recombinant plasmid solution yields 200 Real-Time PCR analyses. In each analysis, which contains duplicate recombinant plasmids as positive controls, 72 samples can be analyzed to detect and quantify *Salmonella*. In addition, recombinant plasmids can be produced repeatedly in competent cells in large quantities without the need for new calibration curves (the constructed standard curve is valid for the next generation of recombinant plasmids). On the other hand, the use of genomic DNA as a positive control poses a challenge: When the supply of genomic DNA is exhausted, the entire experimental procedure must be performed anew. Thus, the developed approach using recombinant plasmids as positive controls is invaluable for culture-independent routine analyses in the medical, veterinary, food and water/environmental sectors.

Conflict of Interest

No conflict of interest is declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2016.01.008.

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