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Detection of *Salmonella* Enteritidis in asymptomatic carrier animals: comparison of quantitative real-time PCR and bacteriological culture methods

B.M. Maciel[1], J.C.T. Dias[1], C.C. Romano[1], N. Sriranganathan[2], M. Brendel[1] and R.P. Rezende[1]

[1]Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Salobrinho, Ilhéus, BA, Brasil
[2]Department of Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Center for Molecular Medicine and Infectious Diseases, Blacksburg, VA, USA

Corresponding author: R.P. Rezende
E-mail: rachel@uesc.br

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ABSTRACT. Quantification of *Salmonella* in asymptomatic carrier animals can be used to assess microbial risk and monitor the level of contamination in domestic animals used for food production. We examined the sensitivity, specificity and accuracy of real-time qPCR, without pre-enrichment or selective enrichment stages, for the quantification of *S. enterica* serovar Enteritidis in resistant mice, as a model of asymptomatic carrier animal. The results were compared with those obtained by traditional bacteriological culture methods, the gold standard test. Two hundred and forty-three samples, including spleen, liver, mesenteric lymph nodes, portions of intestine, intestinal content of the ileocecal portion, and feces, were collected from a group of 27 C57BL/6 mice, that had been intragastrically inoculated with high doses of *S. enterica* serovar Enteritidis. The real-time qPCR assay

presented a consistent linearity of the standard curve ($r^2 = 0.999$), with very low differences between melting temperatures, and low coefficients of variation in intra- (<1%) and interassay (<2%) comparisons. The primers were highly specific; there was no amplification with other *Salmonella* serovars or with DNA from uninfected tissues and feces from mice. The detection limit of the technique was defined as 32 copies of *S. enterica* serovar Enteritidis. A sensitivity of 90%, a specificity of 77% and an accuracy of 79% were obtained. The higher sensitivity of PCR was reflected in a kappa coefficient of 0.41, showing moderate agreement between tests. We conclude that real-time qPCR is a good alternative for diagnostic scanning in asymptomatic carrier animals, due to its high sensitivity and rapidity.

Key words: *Salmonella* Enteritidis detection; Quantitative real-time PCR; Asymptomatic carrier animals; Sensitivity; Specificity; Accuracy

INTRODUCTION

Salmonella enterica serotype Enteritidis (*SE*) is the most common food borne disease worldwide (WHO-GFN, 2009). This serovar is closely associated with consumption of eggs, poultry meat (Center for Diseases Control and Prevention (CDC), 1990, 1992, 2003) and pork (Hald et al., 2004), because it generally causes infection in the herd in absence of a clinical disease (O'Brien, 1990). These asymptomatic carrier animals can become a natural reservoir of *Salmonella* responsible for a silent introduction of the bacteria into the food chain and environment, making the control strategies difficult. Therefore, it is necessary to reduce the prevalence of *Salmonella* serovars of public health significance in food-producing animals. To reduce this risk a number of measures have been recommended by the World Health Organization, including: i) the control of *Salmonella* in the food-producing animal (pre-harvest control), ii) the improvement of hygiene during slaughter and further processing of meat (harvest control), and iii) the consumer, who concerns the application of effective hygienic measures (post-harvest control) (EFSA, 2006; Malorny et al., 2008). However, the quantification of *Salmonella* in infected animals can monitor the level of contamination of a herd and may be of help in assessing microbial risk. In pathogen control measures it is important to evaluate if *Salmonella* infection is eliminated or strongly reduced at the pre-harvest level as this will also reduce the risk of contamination at harvest and post-harvest levels (EFSA, 2006).

Currently, international guidelines and regulations for the detection of *Salmonella* spp are based on traditional culture methods, which are laborious and take at least 3 days for negative samples and 5 days for confirmation of positive results (APHA, 1992). In addition, the use of pre-enrichment and selective enrichment media during the culture methodology prevents a real bacteria count. Thus, quantitative real-time PCR assay (real-time qPCR) for the specific quantification of *Salmonella* has been widely applied due to its sensitivity, specificity and speed of assay (Chen et al., 1997; De Medici et al., 2003; Ellingson et al., 2004; Malorny et al., 2004; Perelle et al., 2004; Nam et al., 2005; Wolffs et al., 2006; Josefsen et al., 2007; Temelli et al., 2010), including the replacement of the traditional serotyping procedure (Hadjinicolaou et al., 2009). This molecular technology has been widely used for clinical diagnosis of both viral and bacterial pathogens (Espy et al., 2006). However, there is concern regarding

the validation of these tests, with respect to sensitivity and specificity of the primers used. It applies, primarily, to the diagnosis of Enterobacteriaceae, in which a low sensitivity can be obtained (Lemmon and Gardner, 2008). This is valid for detection of invasive *Salmonella* serovars in peripheral blood (Nga et al., 2010). In food, real-time PCR has been demonstrated to be a good choice for *Salmonella* spp detection, supporting its use as an international standard method (Malorny et al., 2003, 2007). Nevertheless, because of low levels of *Salmonella* in food from subclinically infected herds, a pre-enrichment stage is still necessary prior to PCR assay (Josefsen et al., 2007).

In this study, we propose the quantification of *SE* in different tissues and feces of asymptomatic carrier mice by real-time qPCR without pre-enrichment or selective enrichment stages. Our experimental study may contribute to further research to test this molecular approach as a useful tool for detection and quantification of *Salmonella* in the early stages of infection in an asymptomatic carrier herd, allowing an early strategic intervention to pre-harvest control of this pathogen. For this, we determined the sensitivity, specificity and accuracy of real-time qPCR performed with the Plexor™ System for the quantification of *SE* in C57BL/6 mice, in comparison with traditional bacteriological culture method as the gold standard test.

MATERIAL AND METHODS

Bacterium sample

Standard curve for the *Salmonella* different fragment (Sdf I) gene specific for serovar Enteritidis (Agron et al., 2001) was constructed using the bacterial sample *Salmonella* Enteritidis phage type 1 (*SE* PT1), kindly provided by Fundação Oswaldo Cruz (Enterobacteria Laboratory, Instituto Gonçalo Muniz, RJ, Brazil). One colony of *SE* PT1 was grown in 1.0 mL Trypticate Soy Broth (Merck) at 37°C for 16-18 h. Then, 500 µL bacterial suspension was used for extraction and purification of DNA using the Wizard® SV DNA Purification System (Promega Corporation, Madison, WI, USA), according to manufacturer recommendations for isolation of genomic DNA from Gram-negative bacteria (http://www.promega.com/enotes/applications/ap0051_tabs.htm#b02).

Amplification of the Sdf I gene

A conventional PCR was performed using the DNA of *SE* PT1 as a template, with primers forward (5'-TGTGTTTTATCTGATGCAAGAGG-3') and reverse (5'-CGTTCTTCTGGTACTTACGATGAC-3') (Deng et al., 2007, 2008) for the Sdf I gene (GenBank® Accession No. AF370707.1, generated by Promega). Amplification was carried out in a total volume of 50 µL, containing 0.4 µL 25 µM of each primer, 0.5 µL 10 mM dNTPs, 2.5 U Tag DNA Polymerase (Cenbiot, Porto Alegre, Brazil), 5 µL 10X PCR buffer, 2.5 µL 25 mM $MgCl_2$, and 3 µL template at 10 ng DNA. The volume was completed to 50 µL with Milli-Q sterilized water. PCR amplification was performed using a Mastercycler Gradient (Eppendorf, Germany) as follows: one cycle (94°C for 5 min), 32 cycles (94°C for 60 s, 58°C for 30 s, 72°C for 60 s) and one cycle (72°C for 10 min). The PCR product was visualized by electrophorese on 1% agarose gel in 1X TAE, stained by SYBR® Safe (Invitrogen, Carlsbad, CA, USA). The size of PCR-produced DNA fragment was 293 bp.

Standard curve for the Sdf I gene

The PCR product was excised from the gel and purified by using the Wizard® SV Gel and PCR Clean-up System (Promega) and measured spectrophotometrically at 260 and 280 nm using GeneQuant™ (Amersham Biosciences, Sweden). It was diluted in a 10-fold series to create the standards for a five-point standard curve (3.2×10^5 to 3.2×10^1 copies of the Sdf I gene) that was run in triplicate. The number of copies per µL was calculated as follows:

- Molecular weight of fragment = 293 bp x 660 Da = 1.93×10^5 g
- One molecule or 1 copy of fragment = $1.93 \times 10^5 / 6.02 \times 10^{23} = 3.21 \times 10^{-19}$ g
- Therefore, 10 ng DNA contains $10 \times 10^{-9} / 3.21 \times 10^{-19}$ copies = 3.2×10^{10} copies.

Real-time qPCR

Real-time qPCR was performed on an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, CA, USA) by using Plexor™ System (Promega). In this system, the fluorescent label effectively quenches the fluorescent signal during PCR product accumulation. The set of FAM labeled primers for an internal region of the Sdf I gene (5'-CGGTTTGATGTGGTTGGTTCGTCA-3' and 5'-AATGGTGAGCAGACAACAGGCTGA-3') was designed by the PrimerQuest Program (IDT SciTools, http://www.idtdna.com/Scitools/Applications/PrimerQuest/). Amplification was carried out in a total volume of 25 µL, containing 0.4 µL 5 µM each primer, 12.5 µL 2X Plexor™ Master Mix and 5 µL templates (standard dilutions at 3.2×10^5 to 3.2×10^1 copies of the Sdf I gene), brought up to a volume of 25 µL with nuclease-free water. Each run consisted of a one cycle (95°C for 2 min), 40 cycles (95°C for 5 s, 60°C for 35 s) and a dissociation stage (95°C for 15 s, 60°C for 60 s and 95°C for 15 s). The size of PCR-produced DNA fragment was 181 bp. For internal control of the reaction, a total volume of 25 µL, containing 12.5 µL 2X Plexor™ Master Mix, 5.0 µL 5X Plexor™ qPCR control and 7.5 µL nuclease free-water, was used. The results were analyzed by the Plexor™ Analysis version 1.1.4 Software.

Sensitivity, specificity and reproducibility of the real-time qPCR primers

For the sensitivity evaluation of the primer set of real-time qPCR, the detection limit of the reaction was determined using the serial dilutions of DNA at the concentrations of 3.2×10^5, 3.2×10^3, 3.2×10^2, 3.2×10^1, and 1.6×10^1 in 45 reaction cycles (five more than the reaction described above).

To confirm the specificity of the primers, the sequence of the Sdf I gene region amplified by the set of FAM-labeled primers was determined using BLASTn Programs from the National Center for Biotechnology Information, to check the similarity of the sequence with *SE*. The primers were used for conventional PCR with *SE* PT1 DNA templates, to confirm a PCR product of 181 bp. Eleven strains of *S. enterica* (Table 1), previously isolated by Maciel et al. (2004, 2010) and DNA of uninfected tissues and feces were also used as a control to certify the absence of any other *Salmonella* or mouse DNA amplification.

To evaluate the intra- and interexperimental reproducibility the five different known concentrations of DNA (3.2×10^5 to 3.2×10^1) were amplified by three different runs performed as described above, including three replicates. For each experiment the average of

amplification threshold, standard deviation and coefficient of variation (CV) for each assay were calculated.

In vivo experiment

All animal procedures were approved by the local Animal Ethics Committee and performed according to the legal requirements of the scientific community. The animals were housed in cages, acclimatized to the vivarium conditions and fed with sterilized standard pellet diet and sterilized water *ad libitum*.

A total of 243 samples including spleen, liver, mesenteric lymph nodes, portions of intestine (duodenum, jejunum, ileum, and cecum), intestinal content of ileocecal portion, and feces were analyzed from a group of 27, 16 to 20 g, 7-week-old C57BL/6 mice, intragastrically inoculated with 5 x 10^8 cfu/mL *SE* PT1. Another group of seven mice was treated with an equal volume of water as a control. At nine different post-inoculation times, three mice from infected and one from the control group were sacrificed. The tissues and feces were immediately processed for DNA extraction using the Wizard® SV DNA Purification System (Promega), according to manufacturer recommendations. For the real-time qPCR assay, the DNA samples were diluted in MOPS/EDTA (Promega) to 20 ng/µL and 5 µL DNA template was used in the reaction, for a total of 100 ng DNA (maximal amount recommended by the manufacturer). The real-time qPCR assay was performed as described above. The tissues and feces were parallel processed for bacteriological culture method for *Salmonella* detection.

Bacteriological culture method for *SE* detection

In order to identify the accuracy of real-time qPCR, all samples of mice were also tested by bacteriological culture methods, i.e., the gold standard test. Approximately 50 mg feces, intestinal content of ileocecal portion and tissue samples were incubated in selective enrichment media Rappaport Vassiliadis Broth (Merck, Germany) at 37°C for 16 h. Then, a sample from the broth was plated onto xylose-tergitol 4 agar (Merck) and *Salmonella-Shigella* agar (Merck) plates. The plates were incubated overnight at 37°C. Presumptive *Salmonella* colonies were tested using the biochemical method according the APHA (1992) and then subjected to PCR for the Sdf I gene detection, as described previously. Amplification products were visualized on 1% agarose gel in 1X TAE buffer, stained with SYBR® Safe (Invitrogen).

Comparing both tests, the sensitivity and specificity of the qPCR test were calculated using the following formulas (Martin, 1984):

- Sensitivity % = [number of true-positive samples / (number of true-positive samples + number of false-negative samples)] × 100

- Specificity % = [number of true-negative samples / (number of true-negative samples + number of false-positive samples)] × 100

RESULTS

Real-time qPCR standard curve for *SE* quantification

The standard curve constructed using the dilutions from 3.2 x 10^5 to 3.2 x 10^1 copies

of the Sdf I gene showed a good linearity with consistent correlation coefficient ($R^2 = 0.999$). Through the formula y = -0.269x + 11.52 (where *y* is the threshold cycle, and *x* is the log of the starting quantity), the number of copies of *SE* for unknown samples could be quantified (Figure 1A). There was no signal in the non-template control. The absolute cycle threshold values (*C*t) were calculated at optimal baseline regions and the default threshold was 10 standard deviation below the mean fluorescence generated during baseline cycles, as shown in amplification curves (Figure 1B). Melt-curve analysis allows distinguishing of amplification products with different sequences and lengths and was used to check for the production of secondary products such as primer dimmers in the assay. The melting temperature of the standard templates varied between 80.3° and 80.7°C (Figure 1C). Another peak in melt curves, varying between 77.5° and 77.9°C, was observed in samples with low DNA concentration or high primer concentration. When the primer concentration was optimized, this second peak tended to disappear.

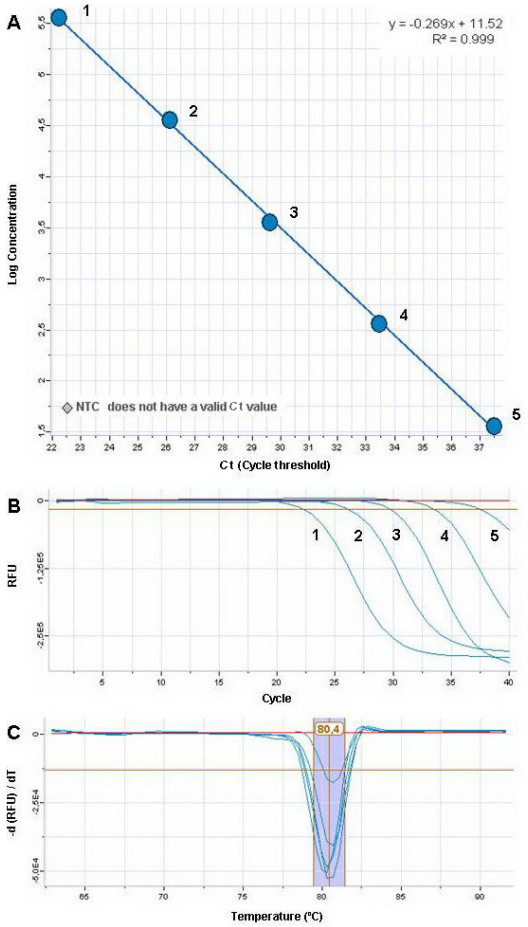

Figure 1. A. Standard curve; **B.** amplification curves, and **C.** melt curves of standard DNA templates with 10-fold serial dilutions for quantification of *Salmonella* Enteritidis. No. of copies of the Sdf I gene in A: 1 = 3.2 x 10^5 copies; 2 = 3.2 x 10^4 copies; 3 = 3.2 x 10^3 copies; 4 = 3.2 x 10^2 copies; 5 = 3.2 x 10^1 copies. NTC = non-template control; RFU = relative fluorescence units.

Sensitivity, specificity and reproducibility of the real-time qPCR assay

A range from 3.2 x 10^5 to 1.2 x 10^1 copies of the Sdf I gene was used to determine the sensitivity limit of this assay, and the limit of detection was defined in 32 copies of *SE*. Even increasing the run for 45 cycles, lower DNA concentration (1.6 x 10^1 copies of the Sdf I gene) could not be detected and primer dimmers were observed by the melt-curve (data not shown).

For determination of specificity the sequence of the region Sdf I gene amplified by the set of FAM-labeled primers was analyzed by BLASTn Programs of the National Center for Biotechnology Information and resulted in 100% homology with DNA of *SE*, GeneBank® Accession No. AF370707.1. Through conventional PCR, the primers specifically amplified *SE* DNA, confirming a PCR product of 181 bp, whereas no amplification was obtained with any of the other *Salmonella* serovars tested (Table 1), or even with any DNA of uninfected tissues and feces from mice. Furthermore, the nucleotide sequences of the Sdf I gene have been previously tested by other authors, showing high specificity for *SE* (Agron et al., 2001; Deng et al., 2007, 2008).

Table 1. Serovars of *Salmonella enterica* subsp *enterica* used to study specificity of PCR primers for *S. enterica* serovar Enteritidis detection.

Serovar	Serogroup	Isolate or type
Salmonella Brandenburg	B	UESC, Tegu No. 22-1
Salmonella Agona	B	UESC, Tegu No. 13-1
Salmonella Saintpaul	B	UESC, Tegu No. 26-1
Salmonella Infantis	C_1	UESC, Tegu No. 40-1
Salmonella Typhi	D	ATCC 6539
Salmonella Panama	D	UESC, Tegu No. 21-3
Salmonella Rubislaw	G	UESC, Dog No. 92
Salmonella Rubislaw	G	UESC, Tegu No. 20-2
Salmonella Carrau	H	UESC, Dog No. 72
Salmonella Carrau	H	UESC, Tegu No. 11-1
Salmonella enterica (rough strain)	-	UESC, Tegu No. 26-4

UESC = Universidade Estadual de Santa Cruz.

To assess the intra-assay reproducibility, the same sample was analyzed three times within the same run. The CV was statistically low, at <1% and the *C*t for each concentration ranged between 0.1-0.2 cycles and was highly reproducible (Table 2). The interassay variation was assessed by three different runs including triplicates of the same sample in each run. The CV was higher than the intra-assay variation but, at <2.2%, still statistically low and the *C*t for each concentration ranged between 0.7-2 cycles (Table 2).

Table 2. Intra- and interassay reproducibility of real-time qPCR.

Standard dilutions		Intra-assay reproducibility[a]		Interassay reproducibility[b]	
Sample	DNA concentration	*C*t	CV (%)	*C*t	CV (%)
1	3.2 x 10^5 copies	23.21	0.51	23.70	0.88
2	3.2 x 10^4 copies	26.68	0.03	26.80	1.21
3	3.2 x 10^3 copies	29.86	0.38	30.46	0.74
4	3.2 x 10^2 copies	33.14	0.28	34.30	0.99
5	3.2 x 10^1 copies	38.45	0.02	38.26	2.01

[a]Average between triplicates; [b]average between experiments; *C*t = cycle threshold value; CV = coefficient of variation.

Validation of the real-time qPCR for *in vivo* test of *SE* quantification

The number of positive results determined by both detection methods is summarized in a two-by-two matrix (Table 3). *SE* was cultured from 30 (12.3%) of the 243 samples analyzed. Twenty-seven culture-positive samples also appeared to be positive by real-time qPCR (90% sensitivity). In addition, 75 samples were positive for *SE* by the real-time qPCR (30.9%) whereas 48 were negative by culture. Of the 213 culture-negative samples, 168 were negative by this PCR assay (77% specificity). Considering the bacteriological culture method for the presence of *SE* as the gold standard test, the accuracy of the real-time qPCR was 79% and the kappa coefficient was 0.41, showing a moderate agreement between tests (Table 3).

Table 3. Correlation between detection of *Salmonella enterica* serovar Enteritidis by real-time qPCR and bacteriological culture method in mice.

Bacteriological culture result	No. of samples with the following real-time qPCR result[a]		Total
	Positive	Negative	
Positive	27	3	30
Negative	48	165	213
Total	75	168	243

[a]Sensitivity = 90%; specificity = 77%; accuracy = 79%; kappa coefficient = 0.41.

Table 4 compares the numbers of positive results in each sample obtained by the culture method and real-time qPCR, showing the minimal number of *SE* cells determined by this PCR assay. In all samples tested, the real-time qPCR was more sensitive, detecting more positive samples than the bacteriological culture method. In mesenteric lymph node, jejunum and ileum, *SE* could be detected only by the real-time qPCR assay. Samples with quantities less than 10^2 bacteria were positive only by PCR assay. This occurred in liver, where the minimal number of the bacteria detectable was 9×10^1 copies of Sdf I gene. Little difference occurred between the detection limit of the tests in the other positive samples, except in ileocecal content, where the culture-positive results were derived from 2.9×10^3 copies of the Sdf I gene, one log more than the real-time qPCR assay, where the limit of detection was 3×10^2 copies of the Sdf I gene (Table 4).

Table 4. Real-time qPCR versus bacteriological culture method: number of positive samples in each assay of the *in vivo* experiment and minimal quantity detected of *Salmonella enterica* serovar Enteritidis in each sample tested.

Sample	Real-time qPCR assay		Bacteriological culture method	
	No. of positive samples (%)	Minimal quantity detected[a]	No. of positive samples (%)	Minimal quantity detected[a]
Spleen	9 (33.3)	2.86×10^2	6 (22.2)	3.07×10^2
Liver	9 (33.3)	8.91×10^1	6 (22.2)	2.60×10^2
Mesenteric lymph node	3 (11.1)	2.30×10^2	Negative	-
Duodenum	Negative	-	Negative	-
Jejunum	3 (11.1)	8.16×10^2	Negative	-
Ileum	9 (33.3)	3.34×10^2	Negative	-
Cecum	15 (55.6)	2.19×10^2	6 (22.2)	2.19×10^2
Ileocecal content	15 (55.6)	3.02×10^2	6 (22.2)	2.85×10^3
Feces	12 (44.4)	1.12×10^3	6 (22.2)	1.12×10^3
Total	75 (30.9)		30 (12.3)	

[a]No. of copies of the Sdf I gene.

DISCUSSION

The real-time qPCR assay described here presented a consistent linearity of the standard curve (Figure 1A), with very low difference between the melting temperatures (Figure 1C) and was highly reproducible (Table 2). The primers demonstrated high specificity as no amplification was obtained either with any of the other *Salmonella* serovars tested (Table 1) or even with any DNA of uninfected tissues and feces from mice. Thus, due to the simplicity, rapidity, reproducibility, specificity, and sensitivity of this technique it could be considered a powerful tool for the rapid detection and quantification of *Salmonella* in animals. In a previous comparative study of four different techniques employed with real-time PCR, the Plexor™ System showed to be the most robust method against inhibitory substances and proved to be practical for routine use; it thus could be the method of choice for qualitative analysis where sensitivity, low cost and simplicity of use prevail (Buh et al., 2008).

In this study, we compared the results of a bacteriological culture method for the detection of *SE* in asymptomatic animals with those of a real-time qPCR performed with Plexor™ System. The real-time qPCR demonstrated to be a sensitive method for *Salmonella* diagnosis, detecting and quantifying low amounts of DNA of *SE* in animals without clinical symptoms, based on the detection limit of 32 copies. Forty-five samples were positive using the PCR assay, which were not detectable by bacteriological culture methods (Tables 3 and 4). The percentage of positive results was 31% by real-time qPCR, whereas only 12% were positive by the culture method. This higher sensitivity of the molecular technique (90%) has been explained by the fact that PCR is based on DNA detection and, consequently, also detects nonviable bacterial cells (Malorny et al., 2008). Nevertheless, in this study we consider that dead cells or free nucleic acids do not interfere with the *Salmonella* quantification, because the samples were immediately analyzed and there were no cooling or freezing stages. As regards *Salmonella*, stressed cells have been reported in temperatures below 8°C (Pintar et al., 2007). It is important to emphasize that in all samples tested, real-time qPCR was more sensitive, including the feces and ileocecal content (Table 4). These samples tend to have many polymerase inhibitors but, even without the selective enrichment stage, *SE* could be detected and quantified by this molecular methodology. This fact is very important to be considered because these two samples can be used to monitor and quantify the bacteria shedding into the environment, which is a serious problem in resistant hosts, resulting in a contamination of an entire herd (Galán and Zhou, 2000). Thus, the quantification of *SE* by real-time qPCR may be an alternative for the diagnosis of *Salmonella* and may improve pathogen control in breeding where the presence of asymptomatic carriers constitutes a risk to public health. The bacteriological culture method for diagnosis of *Salmonella* demonstrated to be less sensitive and thus may result in a false-negative diagnosis with the consequence of non-implementation or omission of control strategies. Moreover, the selective enrichment stage is necessary to detect low levels of *Salmonella* by culture methods, and this prevents the real bacteria count in the samples. Despite these shortcomings, the bacteriological method is still very useful for controlling *Salmonella* in a herd and it must be adopted in cases where the isolation of the strain is necessary: i) for *Salmonella* identification, ii) for information about all serovars of *Salmonella* infections, iii) to test the antimicrobial sensitivity, iv) to determine the current *Salmonella* status of individual animals, v) to investigate a description of the general diversity of infections with different *Salmonella* serovars in a population, and vi) to evaluate *Salmonella*-free status of herds (EFSA, 2006).

The corrected agreement between both tests was measured by kappa coefficient, which obtained a score of 0.41. This moderate agreement can be explained by many factors such as: higher sensitivity of real-time qPCR, insufficient homogenization of the samples, factors affecting the growth of different isolates, or possibly, the presence of antagonistic bacterial species (Boutaga et al., 2003). The choice of each approach for *Salmonella* monitoring or their use in combination will depend on the questions that have to be answered. Real-time qPCR is a good choice when the quantification is necessary to evaluate the level of herd contamination, rate of pathogen dissemination into the environment and for assessment of microbial risk. Bacteriological culture method is mandatory when the pathogen's isolation is necessary. Thus, when used appropriately, each of these approaches is of benefit for specific purposes.

In conclusion, our experimental study showed that real-time qPCR is an effective method to detect and quantify *SE* in asymptomatic mice. Therefore, this technique may be a good choice for the diagnostic scanning in herds where the presence of asymptomatic carriers constitutes a risk to other animals and to public health. PCR offers significant advantages over culture methods with respect to the rapidity, reproducibility, specificity, and sensitivity for the detection and quantification of *SE* in different stages of infection. Moreover, this technique, which allows quantifying the microbial risk assessment, is an important tool to monitor the shedding of pathogenic bacteria, thus assisting in early application of control strategies. Nevertheless, real-time qPCR cannot totally substitute bacteriological methods, and the choice of the technique to be applied will depend on the objective of pathogen control.

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