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Combination of most-probable-number method with light cycler real-time PCR assay (MPN-real-time PCR) for rapid quantification of *Salmonella* in artificially and naturally contaminated bovine fecal samples at slaughter house

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Abstract

As a part of our effort in quantitative risk analysis of food-borne diseases, the objective of this study was to develop rapid and reliable protocols for detection and quantification of *Salmonella* in bovine fecal samples at slaughter house. First, for the detection of *Salmonella* in artificially and naturally contaminated fecal samples, SYBR Green I real-time PCR assay was used, where quantification of *Salmonella* was achieved by combining this assay with most-probable-number (MPN) method (MPN-real-time PCR). To develop the protocol for detecting and enumerating of *Salmonella* in artificially contaminated fecal samples, a *Salmonella enterica* serotype Typhimurium DT104 strain was inoculated into fecal samples at different levels of contamination. Data on artificially contaminated fecal samples indicated that both detection and quantification protocols were able to detect and enumerate as few as 1 CFU/mL of faeces after 8-h of a single non-selective pre-enrichment step in buffered peptone water. All MPN estimates corresponded well to inoculum levels. The protocol was then applied to naturally contaminate fecal samples. A total of 296 fecal and 26 environmental samples were aseptically collected from slaughterhouse located in Meaux, France weekly in February and March 2006. 9.1% (27/296), and 34.6% (9/26) fecal and environmental samples, respectively, were found *Salmonella*-positive, with estimated MPN values of *Salmonella* ranging from <1.8 - 1609 MPN/g of faeces. The mean of the log10 concentration of *Salmonella* is 0.62 MPN/g with standard deviations of 2.7 by using the censored regression approach. Counts were generally low, with the exception of 6 animals (>1400 MPN/g), while all the other 21 *Salmonella* positive animals had faeces with less than 80 MPN/g. The prevalence of *Salmonella* showed no significant difference (p=1) between French (8.63%, 17/197) and Belgian cattle (10%, 10/99). Furthermore, neither the animals' area of origin (p=0.75), age (p=0.18), race (p=0.94), breed (p=0.23), or movement of the animal (p=0.89) had any impact on the prevalence of *Salmonella*. The results of this study demonstrate that the combination of real-time PCR assay and MPN method constitutes an effective, rapid and easy-to-perform method for quantifying low levels of *Salmonella* in bovine fecal samples.

Keywords: *Salomonella* spp., MPN-real-time PCR, Quantification, Faeces.
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الربط بين طريقة العدد الأكثر احتمالا مع قياس الوقت الحقيقي لتفاعل البلمرة المتسلسل للتقدير الكمي السريع للسالمونيلا في عينات براز الأبقار الملوثة طبيعيا وتجريبيا في المسالخ

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الخلاصة

كجزء من المحاولات في التحليل الكمي لخطورة الأمراض التي تنتقل عن طريق الغذاء، كان الهدف من هذه الدراسة لتطوير خطة أو طريقة سريعة لتحديد وتقدير كمية جراثيم السالمونيلا المتواجدة لنماذج براز الأبقار في المسلخ، أولا لتحديد السالمونيلا في عينات

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البراز الطبيعي والملوث بالسالمونيلا استخدمت طريقة الوقت الحقيقي لتفاعل البلمرة المتسلسل وقد تم انجاز تقدير كمية السالمونيلا من خلال دمج هذه الطريقة مع طريقة العد الأكثر احتمالا. ولتطوير طريقة لتحديد وعد جرثومة السالمونيلا في عينات البراز الملوث تجريبيا استخدمت جرثومة السالمونيلا من نوع انتيريكا نمط تايفيميوريوم DT104 حيث تم حقنها في عينات البراز وبتراكيز تلويث مختلفة. أظهرت المعلومات حول عينات البراز الملوثة تجريبيا إمكانية تحديد وتقدير كمية السالمونيلا وتحديد العدد إلى حد جرثومة واحدة / مل في البراز بعد 8 ساعة في الخطوة الأولى من مرحلة الزرع غير الانتخابية للجرثومة على مرق الببتون، بعد ذلك تم تطبيق الطريقة على نماذج البراز الملوثة طبيعيا بجرثومة السالمونيلا. تم جمع 296 عينة براز و 26 عينة من محيط المجزرة في مدينة ميوكس الفرنسية للفترة من شباط إلى آذار 2006 فقد أظهرت الدراسة أن 9.1 % و 34.6 % كانت موجبة لوجود السالمونيلا في عينات البراز ومحيط المجزرة على التوالي ومع حساب قيمة العدد الأكثر احتمالا للسالمونيلا تراوح العدد بين اقل من 1.8 – 1609 جرثومة / غم من البراز. أن معدل لوغارتم 10 للتلوث بالسالمونيلا هو 0.62 جرثومة / غم مع انحراف معياري 2.7. بصورة عامة فان أعداد جراثيم السالمونيلا قليلة ما عدا ستة حيوانات كانت أعدادها 1400 جرثومة / غم براز بينما كانت أعدادها في العينات الموجبة الباقية اقل من 80 جرثومة / غم. كما وان الدراسة لم تظهر أية فروقات معنوية بين الأبقار الفرنسية والبلجيكية ولا هناك فرق في موطنها الأصلي والعمر والعرق والسلالة أو خلال حركة الحيوانات من منطقة إلى أخرى. وأوضحت نتائج الدراسة أن دمج طريقة الوقت الحقيقي لتفاعل البلمرة المتسلسل مع طريقة العدد الأكثر احتمالا أنها كانت أكثر فاعلية وسريعة وسهلة كطريقة تستخدم لتقدير التراكيز والأعداد القليلة من جرثومة السالمونيلا في عينات براز الأبقار.

Introduction

Salmonella is one of the most common foodborne pathogens transmitted to humans (1). Foodborne salmonellosis has been the major cause of all foodborne disease caused by salmonella species ((2). Over 2500 different serotypes of *Salmonella* have been described to date, the most prevalent of which are being Enteritidis, Typhimurium and Heidelberg (3). This enterobacterial pathogen is responsible for a foodborne illness called salmonellosis. Fever, nausea, sometimes vomiting, abdominal cramps and diarrhoea characterize human salmonellosis. Meat and meat products from beef have been associated with severe food poisoning outbreaks caused by *Salmonella enterica* serotypes around the world (4). *Salmonella* can be carried by healthy cattle at slaughter (5) and therefore may serve as a reservoir and source of contamination of carcasses during processing. Therefore, for food safety, rapid, sensitive, and specific detection and quantification techniques of foodborne pathogens in food products and fecal samples that might contaminate foodstuffs are needed. Conventional cultural methods for the detection of *Salmonella* in fecal samples are time consuming and usually require 4 days to presumptively identify *Salmonella* in a test sample. For this reason, methods based on polymerase chain reaction (PCR) have been increasingly used for the detection of *Salmonella* cells in various foods and different matrices (6) However, these assays usually rely on visualizing the amplification product by ethidium bromide staining after agarose gel electrophoresis which is labor and time-intensive. To reduce the time required for detection of *Salmonella* spp. in foods, the time-consuming conventional PCR assays are gradually being replaced by more convenient real-time PCR assays, which represent a significant progress to PCR-based methods for a broad range of applications. A number of real-time PCR-based assays for the detection of *Salmonella* in foods have already been described (7). This technology which combines amplification and detection in a one step closed-tube reaction, presents many advantages such as high sensitivity, high specificity, and lower risks of cross-contamination (8).

Several studies have identified the lack of quantitative data on levels of contamination in contaminated foods as a key data gap for the development of risk assessment for pathogens (9). *Salmonella* cells can be enumerated by applying the classical microbiological quantification techniques, such as the plate counting methods and the most-probable-number (MPN) method. Some of these techniques require up to 6 days for detection and quantification, thus once again pose the problem of being labor-intense and time-consuming. Recently, the use of real-time PCR assays for quantification of initial target DNA has overcome this disadvantage of the time factor. Unfortunately, amplification efficiencies of these quantification assays can be difficult to ensure and their suitability for exact quantification of initial amount of target DNA has therefore been questioned (10).

In this study, an alternative approach is presented. PCR products can be quantified by combining the principles of the Most-Probable-Number (MPN) statistics and LightCycler real-time PCR. Through the use of this approach, we sought to develop a rapid and simple MPN-real-time PCR protocol (MPN-real-time PCR) based on the double-stranded DNA (dsDNA) binding dye SYBR Green I for the detection and quantification of *Salmonella* spp. in contaminated fecal samples. To our knowledge, this is the

first report in which a LightCycler real-time PCR detection method is combined with the MPN method to enumerate *Salmonella* spp. in fecal samples. However, methods based on MPN-conventional PCR (MPN-PCR) have previously been described for the detection and enumeration of different micro-organisms (11). The first objective of the present work was to develop MPN-real-time PCR assay for the quantification of *Salmonella* in experimentally contaminated fecal samples. The second objective was to apply this developed assay to enumerate *Salmonella* in naturally contaminated fecal samples obtained from a slaughter house located in Meaux, France.

Materials and methods

Bacterial strains

To determine the specificity of the LightCycler real-time PCR assay, frozen stock cultures of 3 different serotypes of *Salmonella enterica* and 7 strains of non-*Salmonella* species, including strains in the family of *Enterobacteriaceae* closely related to *Salmonella*, such as *E. coli*, *C. freundii*, *K. pneumoniae* and *Shigella* spp. (Table 1). The bacteria were transferred into BHI broth and incubated overnight at 37°C. These overnight bacterial cultures were subsequently subjected to DNA extraction and real-time PCR assay. The bacterial strains were obtained from the collection of the LERQAP (Laboratoire d'étude et de recherche sur la qualité des aliments et des procédés agroalimentaires) of the French Food Safety Agency (Agence Française de Sécurité Sanitaire des Aliments, AFSSA), located in Maisons-Alfort, France.

Sensitivity of the real-time PCR assay with pure cultures

The sensitivity of the real time-PCR assay was evaluated using pure cultures of three strains of *Salmonella enterica* belonging to different serotypes (Table 1). Cells were grown overnight at 37°C in BHI broth. Ten-fold serial dilutions of each pure culture were prepared in Buffered Peptone Water (BPW; Difco, Becton Dickinson). To determine cell numbers, appropriately diluted cultures were spread-plated on Xylose-Lysine-Tergitol-4 agar (XLT-4; Difco) in ten replicate plates. Plates were incubated overnight at 37°C. All dilutions were then incubated at 37°C for 6, 8 and 16 h of nonselective enrichment. After each pre-enrichment period, 1.5 mL-aliquot was collected from each dilution into microcentrifuge tubes and subjected to DNA extraction and real-time PCR assay. Reproducibility of SYBR Green real-time PCR was assessed by running samples independently on different days.

Fecal samples

For the development of detection and quantification protocols, beef fecal samples were initially simultaneously analysed by cultural methods and real-time PCR to determine that they were negative for *Salmonella*. Then, negative fecal samples were artificially inoculated with *S. enterica* serotype Typhimurium DT104 at different levels of contamination.

The application of developed protocols was performed on naturally contaminated beef fecal samples; a total of 296 fecal and 26 environmental samples were aseptically collected from slaughterhouse located in Meaux, France. The collection of samples was done weekly in February and March 2006. An average number of 40 samples per visit were collected. For each animal, approximately 75 g of fecal was collected after evisceration from the lower intestine and placed into a cool box with ice packs and transported to the laboratory at the end of the morning. In addition, 26 environmental samples were carried out. For each fecal sample, the information collected related to breed, sex, age, animal weight, area of origin, place of birth and its movement.

Real-time PCR detection protocol of fecal samples

Development of the detection assay on artificially contaminated fecal samples

For the artificial inoculation procedures, the exact numbers of *Salmonella* cells were determined by plating 0.1-mL aliquots of suitable 10-fold dilutions onto XLT-4 agar plates in ten replicates and incubating them overnight at 37°C. The dilutions were then kept refrigerated at 4°C for 24 h. When the *Salmonella* cells were added to the feces after being confirmed *Salmonella*-negative by both culture methods and real-time PCR, the estimated cell concentration of the inoculum was determined for a second time. 25 g of fecal samples were inoculated with the following estimated levels of contamination: 1 to 5, 10 to 20, and 100 CFU/g before being homogenized in 225 mL of BPW by mixing. The homogenates were then pre-enriched for 6, 8, and 16 h at 37°C in order to determine the shortest enrichment time needed to detect the lowest level of contamination. At each time point, aliquots were withdrawn and appropriate 10-fold serial dilutions of each spiked pre-enrichment broth were spread-plated on XLT-4 agar plates in triplicates and incubated overnight at 37°C before counting colonies and calculating CFU. In addition, other aliquots of each spiked pre-enrichment broth were collected and subjected to DNA extraction for the real-time PCR assay.

Application of the detection assay on naturally contaminated fecal samples

For fecal samples, 5 g of each fecal sample were added to 45 mL BPW. After being thoroughly mixed, the mixtures were pre-enriched for 18 h at 37°C. 1.5-mL aliquots of each

pre-enrichment broth were collected and subjected to DNA extraction for the real-time PCR assay as described below. The remaining quantities of fecal samples were stored at 4°C to be used for quantification protocol in case of *Salmonella*-positive results.

The MPN- real-time PCR quantification protocol of fecal samples

Development of the method on artificially contaminated fecal samples

MPN assays (ten-tube method) for *S. enterica* serotype Typhimurium DT104 was performed according to the procedures described in FDA' Bacteriological Analytical Manual (BAM) (12). The tubes were then incubated for 6, 8 and 16 h at 37°C in order to optimize the incubation period for the MPN-real-time PCR method. After each incubation period, 1.5 mL of each enriched tube was drawn and processed for DNA extraction. Immediately after DNA extraction, the MPN-real-time PCR runs were carried out on DNA templates by following the procedures described in the SYBR Green real-time PCR assay section. From the amplification results the number of positive and negative capillary tubes was scored and the MPN calculations were made with a computer-assisted spreadsheet (13). The spreadsheet can be found on the website of the Bacteriological Analytical Manual (BAM) of the U.S Food and Drug Administration (BAM/FDA). The repeatability of the MPN-real-time PCR assay was determined by repeated measurements of the same sample. The repeatability was estimated by computing the Coefficient of Variation of log MPN (CV%). The CV was calculated as the mean divided the standard deviation. If the CV values were less than 20%, the repeatability considered to be acceptable.

Application of the method on naturally contaminated fecal samples

Fecal samples that tested *Salmonella*-positive with the real-time PCR detection assay were subjected to enumeration assay with MPN-real-time PCR and to isolation of presumptive *Salmonella* colonies using conventional culture methods. 25 g of each *Salmonella*-positive fecal sample was homogenized in 225 mL of BPW by mixing. MPN assays (five-tube method) were performed according to the procedures described in BAM (12). The tubes were then incubated at 37°C for 8 h (optimal incubation time determined by MPN real-time PCR assay of artificially contaminated fecal samples). After incubation period, 1.5 mL of each enriched tube was drawn and processed for DNA extraction. The MPN-real-time PCR runs were carried out on DNA templates by following the procedures described in SYBR Green real-time PCR conditions section. From the amplification results the number of positive and negative capillary tubes was scored and the MPN calculations were made with a computer-assisted spreadsheet (13).

DNA extraction procedures

DNA was extracted from pure cultured strains and from pre-enriched cultures of artificially and naturally contaminated fecal samples. Aliquot of enriched sample was transferred to 2-mL microcentrifuge tube. The cell suspension was centrifuged for 10 min at 12,000 rpm. The supernatant was discarded carefully. The pellet was resuspended in 100 µL of sterile distilled water by vortexing. The tube was centrifuged again at 12,000 rpm for 10 min, and the supernatant was discarded carefully. The pellet was resuspended once again in 100 µL of sterile distilled water by vortexing and boiled in a water bath for 10 min. After heat treatment the tube was immediately centrifuged for 10 min at 12,000 rpm. The supernatant was carefully transferred to a new microcentrifuge tube and stored at –20 °C until the real-time PCR assay was performed. An aliquot of 2 µL of the supernatant was used as the template DNA in the real-time PCR assay.

SYBR Green real- time PCR assay

The *Salmonella* specific primers ST11 (5'-AGCCAACCATTGCTAAATTGGCGCA-3') and ST15 (5'-GGTAGAAATTCCCAGCGGGTACTG-3'), originally designed by Aabo et al.,(14) 1993), and previously shown to be highly specific for *Salmonella* (12,14,15) were purchased from Proligo (Paris, France) and used to amplify a 429-bp.

Real-time PCR reactions were performed with the LightCycler PCR instrument (Roche Diagnostics) using the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Diagnostics, Meyla, France). The reaction mixture contained the following concentrations of reactants: 2 µL of LightCycler-Faststart DNA Master SYBR Green I (1 X concentration), 4 mM $MgCl_2$, 0.4 µM of each primer, 2 µL of template DNA, and sterile PCR grade water to a total volume of 20 µL per capillary. Each LightCycler run contained one negative control consisting of H_2O without any template DNA to monitor for possible contamination and one positive control (*S. enterica* serotype Typhimurium DT104; AFSSA 13887.03). Mixing of the reagents for the PCR was accomplished under laminar flow in a clean room separate from the one where DNA templates were prepared. Master mixture and extracted DNA were placed into glass capillaries, sealed with a plastic cap, centrifuged, (3000 rpm for 15 sec.) and placed into the LightCycler™ carousel (Roche Diagnostics).

The thermal cycling program for the LightCycler™ has four phases: denaturation, amplification, melting and cooling. In the initial denaturation phase the capillary is heated to 95°C for 10 min, followed by 40 to 45 cycles of

amplification phase of 10 s at 95°C, annealing for 10 s at 66°C, and extension for 20 s at 72°C. Signal detection was performed at the end of the extension step with a single fluorescence acquisition for each capillary. The melting curve analysis phase began with 95°C for 0 s, then cooled to 73°C for 30 s before the temperature was raised to 95°C at a rate of 0.1 °C/s. Fluorescence acquisition was performed continuously during this phase. Finally, the cooling phase lasted for one minute at 40°C. Melting temperature (*T*m) peaks were calculated based on initial fluorescence curves (*F*/*T*) by plotting negative derivative of fluorescence over temperature versus temperature (-d(*F*)/ d*T* versus *T*).

Isolation of positive colonies from fecal samples

For isolation of presumptive *Salmonella* colonies from PCR-positive fecal samples, 0.1 mL of non-selective pre-enrichment mixture was transferred to 10 mL of Rappaport-Vassiliadis (RV) medium and another 0.1 mL to 10 mL of Mueller-Kauffman tetrathionate broth (AES Laboratoire, France). Rappaport-Vassiliadis (RV) medium selective enrichment was carried out for 22-24 h at 42°C, and Muller-Kauffmann tetrathionate broth was incubated for 22-24 h at 37°C. Both selective enrichment broths were streaked onto xylose lysine tergitol-4 (XLT-4) agar plates (Difco) and xylose lysine deoxycholate (XLD) agar plates. Plates were then incubated for 22-24 h at 37°C. If growth was slight or if no typical colonies were found, the plates were reincubated for a further 24 h at 37°C.

Antimicrobial susceptibility testing

The antimicrobial susceptibility tests were performed by the disk diffusion method on Mueller-Hinton agar (BioRad, Marne la Coquette, France). The panel of antibiotics tested (load, breakpoints (mm)) was recommended by the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM): ampicillin (10 µg, 19-14), amoxicillin + clavulanic acid (20 µg, 21-14), cephalothin (30 µg, 21-15), streptomycin (10 IU, 15-13), gentamicin (10 IU, 16-14), kanamycin (30 IU, 17-15), chloramphenicol (30µg, 23-19), tetracycline (30 IU, 19-17), sulfamethoxazole-trimethoprim (23.75 + 1.25µg, 16-10), sulphonamides (200 µg, 17-12), nalidixic acid (30 µg, 20-15), ofloxacin (5µg, 22-16), enrofloxacin (5 µg, 22-17) and colistin (50 µg, 15). Zone diameters were read using the automated scanner Osiris (BioRad). In this study, if an isolate resistant to, at least, two antimicrobials within two different antimicrobial families were considered multidrug resistant.

Data management and statistical procedure

The collected data and the laboratory results were stored in a Microsoft excel file and coherence tests were applied to these data. The SAS software v.9.1 (SAS Institute, Inc.) (16) was used for statistical analysis. The Chi-square or Fisher testes were used to identify a significant difference ($p < 0.05$) between breed, sex, age, area of origin, place of birth and its movement. And also the FREQ procedure was used for each level of data. The maximum likelihood estimation (MLE) with proc lifereg on SAS (17), assuming an underlying normal distribution for the log10 concentraction, was used to calculate an estimate for the mean and standard deviation of the concentration of the *Salmonella* in bovine feces.

Results

Sample description

A total of 296 fecal and 26 environmental samples were collected from slaughterhouse located in Meaux, France. The animals were 32% blanc-bleu (n=95), 27.4% charolaise (n=80), 12.5% blonde-d'Aquitaine (n=37) and the other 13 breeds accounted for 28% (Table 2). Cows represented 72.9% (n=216) of the animals, bulls 18.6% (n=55) and castrated animals 7.7% (n=23), while two animals were not identified (missing data). Table 3 shows the proportion of each age group with its percentage. Most of the animals came from Basse-Normandie (27%, n=81), 11.8% (n=35) from Haute-Normandie and 33.4% (n=99) from different regions of Belgium as showed in table 4. The obtained data from the slaughter house showed that 58% of the animals (n=171) had been reared in their region of birth (no movement), while the remaining ones (n=123) had been reared in a region different from their birth region (movement). Most of the animals (n=245, 82.8%) were beef cattle, while 14.9% were dairy cattle (n=44) and the remainder (n=7, 2.3%) belonged to mixed breeds as shown in Table 5.

Specificity of real-time PCR

The real-time PCR assay correctly classified all the serotypes of *Salmonella* tested as *Salmonella* and the other the non-*Salmonella* species, including strains in the family of *Enterobacteriaceae* closely related to *Salmonella*, such as *E. coli*, *C. freundii*, *K. pneumoniae* and *Shigella* spp. as non-*Salmonella*. Table 1 shows the C_T (Threshold PCR cycle) and T_m (Melting temperature) values from the SYBR Green I real-time PCR analysis. Specificity was assessed by the T_ms of the amplification products immediately after the reaction cycle. This amplification resulted in product with a T_m of 87.2 ± 0.5°C. Negative controls and samples confirmed negative did not show peaks in T_m that corresponded to 87.2 ± 0.5°C. Figure 1 shows the melting peak analysis of the amplified products in real-time PCR for positive (*S.* Enteritidis, *S.*Hadar and *S. entrica* serotype Typhimurium DT104 and representative negative (*Escherichia coli*, *Enterobacter cloacae*, *Citrobacter freundii* and *Klebsiella pneumoniae).*

Table 1 : Strains used in this study.

Species	Source	Strain no.	SYBR Green I real-time PCR	
			C_T[1]	T_m= 87.2± 0.5°C[2]
Other strains				
Escherichia coli	Hospital	49	33.82	_
Klebsiella pneumoniae	Hospital	84	> 36	_
Enterobacter cloacae	Hospital	59	> 36	_
Shigella sonnei	Hospital	65	> 36	_
Yersinia spp.	Hospital	81	32.06	_
Citrobacter freundii no[1]	Hospital	55	31.07	_
Citrobacter freundii no[2]	Bovine/kidney	4525.04	31.77	_
S. enterica serotype				
S. Hadar	Steak/Gorden-bleu [3]	TQA 042	11.72	+
S. Enteritidis	Bovine/feces	9211.02	12.24	+
S.Typhimurium DT104	Avian	13887.03	11.94	+

[1]C_T= Threshold PCR cycle is defined as the cycle at which a significant increase in the fluorescence is first recorded. [2]The presence of PCR product (+) indicates amplification of specific product. [3]Cordon-bleu = specific turkey product.

Table 2: Breeds and types of the studied animals.

Race	Type of the animal	Number of animals (Percentage %)	Real-time-PCR Positive (Percentage %)
Armoricaine	Mixed breed	3 (1.01%)	0 (0%)
Bazadaise	Beef cattle	1 (0.33%)	0 (0%)
Blanc-Bleu	Beef cattle	95 (32.09%)	9 (9.47%)
Blonde-d'Aquitaine	Beef cattle	37 (12.5%)	5 (13.51%)
Casta-(Autre-et-ST-Girons)	Beef cattle	2 (0.67%)	0 (0%)
Charolaise	Beef cattle	80 (27.03%)	7 (8.75%)
Croisé	Dairy cattle	29 (9.79%)	1 (3.45%)
Dairy-Shorthorn	Dairy cattle	1 (0.33%)	0 (0.00%)
Limousine	Beef cattle	13 (4.39%)	2 (15.38%)
Montbeliarde	Mixed breed	3 (1.01%)	0 (0.00%)
Normande	Mixed breed	1 (0.33%)	0 (0.00%)
Parthenaise	Beef cattle	2 (0.68%)	0 (0.00%)
Prim'Holstein	Dairy cattle	14 (4.73%)	0 (0.00%)
Rouge-des-Prés	Beef cattle	10 (3.38%)	1 (10.00%)
Salers	Beef cattle	3 (1.01%)	1 (33.33%)
Unknown		2 (0.68%)	1 (50.00%)
Total		296 (100.00%)	27 (9.12%)

Table 3: Age groups and average weights of sampled animals.

	Number of animals (Percentage %)	Average of the Weight (sd)	Real time PCR Positive (Percentage %)
Male calf	12 (4.05%)	154,84 (26,99)	1 (8.33%)
Young bull	19 (6.42%)	349,29 (52,30)	0 (0%)
Bull	24 (8.11%)	404,14 (102,31)	1 (4.17%)
Castrated animal	23 (7.77%)	526,42 (53,49)	1 (4.35%)
Female Calf	3 (1.01%)	150,27 (26,45)	0 (0%)
Heifer	31 (10.47%)	433,01 (56,96)	7 (22.58%)
Cow	182 (61.49%)	459,02 (66,60)	16 (8.79%)
Unknown	2 (0.68%)		1 (50%)
Total	296		27 (9.12%)

Table 4: Geographical origin of sampled animals.

Region or country	Number of animals (Percentage %)	Real time PCR Positive (Percentage %)
Aquitaine	2 (0.68%)	0 (0%)
Basse-Normandie	81 (27.36%)	9 (11.11%)
Bourgogne	6 (2.03%)	0 (0%)
Champagne-Ardenne	7 (2.36%)	0 (0%)
Haute-Normandie	35 (11.82%)	2 (5.71%)
Ile-de-France	21 (7.09%)	3 (14.29)
Nord-pas-de-Calais	14 (4.73%)	0 (0%)
Pays de Loire	2 (0.68 %)	0 (0%)
Picardie	27 (9.12%)	2 (7.41%)
Belgium	99 (33.4%)	10 (10%)
Unknown	2 (0.68%)	1 (50%)
Total	296	27 (9.12%)

Table 5: Animal breed with its percentage, prevalence.

Breed	Number of animals (Percentage %)	Real time PCR Positive (Percentage %)
Beef cattle	245 (82.77%)	26 (8.78%)
Dairy cattle	44 (14.86%)	1 (0.34%)
Mixed	7 (2.36%)	0 (0%)
Total	296	27 (9.12%)

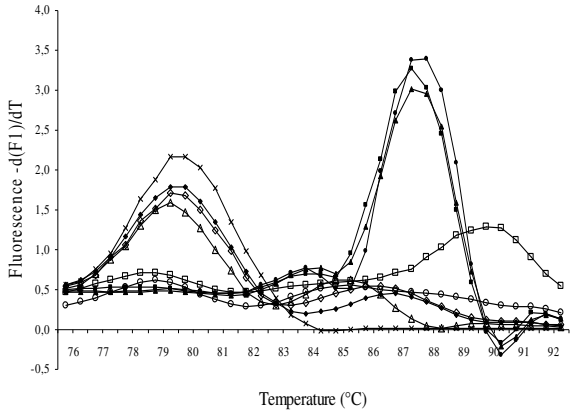

Figure 1: Melting curve analysis of amplified PCR products using ST11 and ST15 primers for Salmonella enterica serotypes: S. typhimurium DT104 (●); S. Hadar (■); and S. enteritidis (▲); and non-Salmonella strains: Escherichia coli (Δ); Enterobacter cloacae(○); Klebsiella pneumoniae(□); Citrobacter freundii no1 (◊);Citrobacter freundii no2 (♦); and water(negative control (x).

Detection limits in pure cultures

The study was performed on the three *Salmonella* strains. Using our described real-time PCR assay with an 8 h pre-enrichment step in BPW, it was possible to detect as few as 1 CFU/g of pure cultures from each of the three strains. Experiments were carried out three times and good reproducibility was observed (data not shown).

Detection of Salmonella from artificially contaminated fecal samples

Fecal samples inoculated with *S. enterica* serotype Typhimurium DT104 at the estimated levels of contamination (1-5, 10-20, and 100 CFU/g) gave negative results without enrichment, whereas the real-time PCR assay detected the bacteria in fecal samples even at a low level of contamination after enrichment. When spiked fecal samples were enriched in BPW for 6 h, inoculum levels of 10 and 100 CFU/g were detected. When the inoculation levels were 1 CFU/g, 8 or 16 h of enrichment were necessary to detect them, thus leading to the definition of the optimal enrichment time as 8 h. The relative detection limits of the real-time PCR assay for *S. enterica* serotype Typhimurium DT104 in artificially contaminated fecal samples were consistent with DNA purified from serial dilutions of broth cultures of the three *Salmonella* strains and with inoculated fecal samples.

Detection of Salmonella from naturally contaminated fecal samples

The collection of samples was done weekly in February and March 2006 (an average of 40 samples per visit). After 18 h of enrichment, 9.12% (27/296), 34.62% (9/26) fecal and environmental samples respectively were found *Salmonella* positive. The prevalence of *Salmonella* positive

samples didn't show any significant difference (p=1) between French (8.63%, 17/197) and Belgian cattle (10%, 10/99). Furthermore, neither the animals' area of origin (p=0.75), age (p=0.18), race (p=0.94), breed (p=0.23), or movement of the animal (p=0.89) had any impact on the prevalence of *Salmonella*. The real-time PCR positive samples were further analyzed with the traditional cultural methods while the negative ones were discarded.

Enumeration of Salmonella in artificial and naturally contaminated fecal samples

For fecal samples inoculated with *S. enterica* serotype Typhimurium DT104, a non-selective pre-enrichment time of 8 h in buffered peptone water was found optimal to obtain MPN-real-time PCR estimates close to the contamination levels (Table 6). With the developed MPN-real-time PCR assay it was possible to enumerate approximately 1 CFU *Salmonella* per g of feces within 11-12 h, which included an 8 h enrichment and 3-4 h period to carry out the sample preparation and real-time PCR assay. The MPN-real-time PCR estimates correspond well to the estimated level of contamination inoculated into the samples. Clear positive peaks were observed in most positive tubes originating from fecal samples inoculated with 100 and 10-20 cells per g of faeces (Figure 2 A, B), while in the case of the samples inoculated with low levels 1-5 cells per g of faeces, weak fluorescence peaks were observed (Figure 2 C). Fares (2007) has demonstrated that the MPN-real-time PCR assay (by using contaminated milk samples) has acceptable repeatability with a coefficient of variation (CV%) of less than 20% for inoculum levels of 100 and 10-20 CFU/mL; but larger variation were observed in samples with inoculum level of 1-5 CFU/mL. Arguably, this was due to the low level of inoculum.

All fecal samples that tested positive with the LightCycler real-time PCR detection assay were subjected to enumeration assay with MPN-real-time PCR for enumeration of *Salmonella* per gram faeces.. The mean of the log10 concentration of *Salmonella* in positive bovine fecal samples are 0.6189 MPN/g with standard deviations of 2.7112 (Table 7). Counts were generally low, with the exception of 6 animals (>1400 MPN/g), while all the other 21 animals were less than 80 MPN/g (from them 13 animals <1.8 MPN/g) as show in Table (8).

Confirmation of the specificity of real-time PCR products by DNA melting temperature analysis

In the SYBR Green I real-time PCR, the amplification of the DNA target is expressed as a threshold cycle (C_T). The C_T represents the number of reaction cycle at which the reporter fluorescence raises above a set baseline threshold, and indicates that the DNA amplicon is replicating exponentially. Immediately following amplification, the products were melted, and the release of fluorescence dye

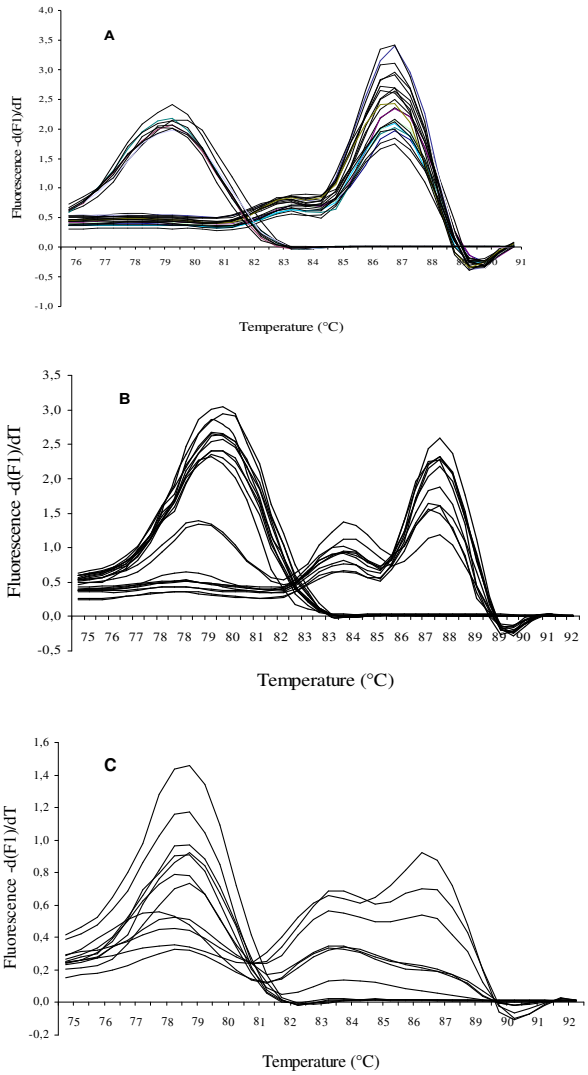

Figure 2: MPN-real-time-PCR analysis of feces inoculated with *Salmonella enterica* serotype Typhimurium DT104. DNA extracted from fecal samples after 8h non-selective enrichment in BPW: Inoculum level of 100 CFU/g (A); Inoculum level of 10-20 CFU/g (B); and Inoculum level of 1-5 CFU/g (C). Some positive and negative peaks were omitted from these graphs for clarity.

measured to generate melting curves from which T_m was calculated. T_m is dependent upon the length of the amplified DNA, as well as the G/C content of the sequence (18). As the T_m is reached, the DNA denatures and releases SYBR Green I, causing a sharp decline in fluorescence.

This decrease in fluorescence is plotted as the negative derivative of fluorescence over temperature versus temperature ($-d(F)/dT$ versus T) giving a melting peak and

T_m for each PCR product. In artificially contaminated milk assay, the average real-time PCR T_m value (mean plus standard deviation from a range of 8 to10 assays) of the specific products was 87.6°C (±0.4). These results were consistent with naturally contaminated fecal samples in which the mean real-time PCR T_m of twenty seven positive fecal samples was 87.2°C (±0.4). Other real-time assays (19) have reported similar results. However, variations of more than 1°C in the minimum and maximum T_ms have been reported from other studies (20). The average T_m of the negative controls was 79.7°C (±0.2). (18) reported that primer-dimers which are typically shorter in length usually melt at a much lower T_m than the intended product and are therefore easy to distinguish whereas secondary or non-specific products can be of varying lengths and sequences and therefore have a large range of possible melting temperatures.

Classic PCR and isolation of Salmonella

All fecal samples that tested positive with the LightCycler real-time PCR detection assay were subjected to classic PCR and to isolation of presumptive *Salmonella* colonies using conventional culture methods. 66.7% (18/27) of samples that tested positive by the LightCycler real-time PCR detection assay were positive with conventional PCR. The majority of the conventional negative samples (78%, 7/9) was <1.8 MPN/g, while the other two were 27 MNP/g and 53.9 MPN/g as show in Table 8.

Salmonella Typhimurium was isolated in this study from the fecal samples with multidrug resistance (penta « ampicillin, chloramphenicol, streptomycin, sulphonamide and tetracycline », fluoroquinolone and rifampin) and *Salmonella* Derby from environmental samples with multidrug resistance (streptomycin, sulphonamide and tetracycline).

Table 6: Enumeration of *Salmonella enterica* serotype Typhimurium DT104 in artificially contaminated fecal samples using MPN-real-time PCR method after 8 h pre-enrichment in BPW.

Sample no.	Contamination level (CFU/mL)	Plate counting (CFU/mL)	MPN estimate (MPN/mL)	MPN confidence limits (low/high)
1	1-5	2	4.3	1.9 - 9.5
2	1-5	1	2.2	0.6 - 8.9
3	1-5	2	3.6	1.1 - 11.1
4	10-20	8	9.5	4.9 - 18.3
5	10-20	15	28..7	13.1 - 63.0
6	10-20	12	19.6	10.2 - 37.9
7	100-150	105	120.3	55.4- 160.5
8	100-150	111	138.1	133.3 - 464.1
9	100-150	95	111.6	105.2 – 360

Table 7: Parameter estimates for the log10 concentration of *Salmonella* in gram bovine fecal by using maximum likelihood estimation (MLE) with proc lifereg on SAS.

	Estimation	Standrdard errour	95 confidence limits	
			low	high
Mean	0.6189	0.6117	-0.5800	1.8178
Standrdard deviations	2.7112	0.7296	1.5999	4.5945

Discussion

Dairy cattle and its environment harbor pathogens that pose a potential human health hazard. Meat and meat products serve as vehicles for the transmission of pathogenic bacteria including *Salmonella* spp. from cattle to humans. Outbreak investigations and volunteer studies have shown that very low doses of certain *Salmonella* strains can cause disease in a significant proportion of the consumers (21. It has, therefore, become increasingly important to develop rapid and sensitive methods not only for the detection but also for the quantification of low numbers of S*almonella* cells in foods and fecal samples. In risk assessment investigation, enumeration rather than presence/absence is important to estimate the rate of human exposure.

The aim of the present study was first to develop a sensitive, simple, and rapid MPN-real-time PCR based method for the quantification of *Salmonella* in artificially contaminated fecal samples. The method has been successfully used for detection and quantification of *Salmonella* in artificially contaminated fecal samples.

Therefore, we evaluated the utility of this developed method to enumerate *Salmonella* spp. in naturally contaminated fecal samples collected from slaughterhouse located in Meaux, France, and to be used afterwards for the development of quantitative risk assessment of human salmonellosis linked to the consumption of ground meat. This is, to our knowledge, the first report on quantification of *Salmonella* in artificially and naturally contaminated fecal samples by the MPN method combined with LightCycler real-time PCR based on the double-stranded DNA (dsDNA) binding dye SYBR Green I. The major advantage of the LightCycler real-time-PCR assay is that it is easy to perform and has been shown to save time and effort. In this study, we have shown that with a little optimisation step, the simple and less expensive option of SYBR Green I can be used as an effective alternative.

Table 8: Quantification estimates of *Salmonella* in fecal samples obtained with MPN-real time PCR assay after 8 h pre-enrichment in BPW and result of the classic PCR of all fecal samples that tested positive with the LightCycler real-time PCR detection assay.

Week	Sample order	Weight (Kg)	Type of animals	Real time PCR	MPN-PCR (g)	Classic PCR
1	4	517	Cow	+	53.9	-
1	5	522	Cow	+	<1.8	-
1	16	493	Heifer	+	5.56	+
1	20	296	Cow	+	20.5	+
2	5	543	Cow	+	>1600	+
2	20	464	Cow	+	>1600	+
2	32	473	Cow	+	27.1	-
3	20	ND	ND	+	<1.8	+
4	4	604	Cow	+	9.3	+
4	18	597	Castrated animal	+	1.98	+
4	43	308	Bull	+	<1.8	-
6	1	196	Cow	+	1609	+
6	11	471	Cow	+	>1600	+
6	16	408	Cow	+	42.6	+
6	18	389	Male Calf	+	>1600	+
6	35	344	Cow	+	>1600	+
7	1	454	Cow	+	<1.8	+
7	15	473	Heifer	+	<1.8	-
7	21	466	Cow	+	<1.8	-
7	22	498	Cow	+	74.8	+
7	23	466	Cow	+	<1.8	-
8	6	368	Heifer	+	18	+
8	10	402	Heifer	+	<1.8	-
8	12	413	Heifer	+	<1.8	+
8	15	370	Cow	+	<1.8	-
8	21	436	Cow	+	<1.8	+
8	29	431	Heifer	+	<1.8	+

In our study, *Salmonella* could not be detected in inoculated fecal samples when DNA was extracted directly and without enrichment (Table 2). Previously reported studies (22) suggested the use of preenrichment step prior to DNA extraction methods to improve the detection of low numbers of *Salmonella* in foods and to overcome the problems of certain inhibitors present in food. Therefore, we carried out one step enrichment in BPW prior to DNA extraction, while the DNA extraction procedure based on a simple boiling method gave optimal results and no inhibition was seen in inoculated and naturally contaminated fecal samples.

In general, as a simple, but widely used method, boiling method provides a fast and efficient way of extracting DNA that can be used in PCR assays in less well equipped laboratories. When we compare the boiling method with the commercial kit for extracting DNA from artificially contaminated fecal samples, clear positive peaks were

observed in more positive tubes originating artificially contaminated fecal samples extracted by the commercial kit than by the boiling method. Even though the boiling method gave the same result in term of the positive and negative tube comparing with the extracted kit; extraction procedures have to be improved to isolate DNA from naturally contaminated fecal samples.

The detection of low numbers of cells is particularly important for *Salmonella* spp., since epidemiological evidence suggests that the infectious dose for some strains could be in the range of 10-100 cells (23). In the present study, experiments carried out on artificially contaminated fecal samples showed that real-time PCR could detect 1 CFU/g of *Salmonella* contaminated fecal samples after 8 h of incubation in the non-selective pre-enrichment medium. Published papers describing real-time PCR-based detection of *Salmonella* from either spiked or naturally contaminated foods have claimed detection limits ranging from 1 to less than 10^3 CFU/g or mL after enrichment at different times ranging from 6 h to overnight incubation (20, 23). Real-time PCR assays developed with SYBR green were able to detect 1 to 2 cfu of *Salmonella* in poultry and meat products (24), 4 cfu in raw pork sausage (25), 1 to 2.5 cfu in pasteurized milk, ground beef, and alfalfa sprouts (Mercanoğlu and Griffiths, 2005), 2.5 cfu in milk and water and 6 cfu in chicken intestinal samples (20).

The potential of MPN-real-time PCR method for the quantification of *Salmonella* spp. from artificially contaminated faeces was investigated and resulted in MPN counts that corresponded well to the estimated level of contamination inoculated into the samples. Generally, the MPN-real-time PCR tended to give higher estimates than the inoculum level. However, the inoculum level estimates fell well within the 95% confidence limits of the MPN estimates while remaining lower than MPN-real-time PCR results. These results are supported by the findings of (26). In their results, they found that MPN-PCR tended to give higher estimates than plate counting; which was probably due to DNA from dead and stressed cells, which were not able to form colonies. As the post-PCR melting curve analysis of the amplified product was performed, it was very important to establish whether the level of contamination has an influence on the position of the melting peak at 87.6°C (± 0.4°C). As can be seen in Figure 2 A, B, and C, the significant peak at 87.6 (± 0.4°C) remains unaltered at the different levels of contamination even though a variety of lesser peaks are evident at lower levels of contamination, presumably as a result of the amount of accumulated product. (27) indicated also that the height of the peaks varies relatively to the amount of accumulated product. Generally, using the protocol described here, satisfactory peak heights were produced using a 40-cycle real-time-PCR.

When the real-time PCR assay was used to examine of 296 fecal samples collected from slaughterhouse located in Meaux, France, it indicated that 9.12% (27/296) were contaminated by *Salmonella*, which is within the range of prevalence rates reported in the literature. This level of contamination is in agreement with another French study (28), which showed the prevalence of *Salmonella* in fecal samples to be 9.5% (29) reported prevalence herd with salmonella (8.1%). However, previously reported surveys of cattle fecal samples in Europe, Australia, United States and Canada have shown large variations in the prevalence of *Salmonella* in feces ranging from 2% to 50% (30). The large variations in levels of fecal *Salmonella* contamination observed in these studies have been attributed to several factors such as variations in sampling and detection techniques, seasonal differences, herd size, geographic area, hygiene, and farm management practices. These reported findings clearly suggest *Salmonella* can be carried by healthy cattle at slaughter (31) and can therefore serve as a reservoir and source of contamination of carcases during processing and may pose a health hazard.

Although the traditional culture method remains the accepted procedure for confirming the presence of *Salmonella* in food because this method allows isolation and culturing of Salmonellae from samples, the real-time PCR assay is substantially faster and usually more sensitive than the standard culture procedure. In addition, this real-time PCR assay can be combined with subculture of enrichment broths from PCR-positive samples for the isolation of the pathogen, which is of great benefit to the food industry and to regulatory or public health authorities engaged in food safety and the management of salmonellosis.

MPN-real-time PCR assay of fecal samples conducted in this study suggested that *Salmonella* count in feces were generally low, with the exception of 6 animals (>1400 MPN/g), while all the other 21 animals were less than 80 MPN/g (from them 13 animals <1.8 MPN/g) as show in table (8). Since 63% of the *Salmonella* concentration in positive fecal samples fall below or above the detection limit of MPN-real-time PCR assay (censored observations), the maximum likelihood estimation (MLE) with proc lifereg on SAS (censored or Tobit regression approach) was used to calculate an estimate for the mean and standard deviation of the log10 concentration of the *Salmonella* in gram bovine faeces.

(32) has reported that counts of *Salmonella* in positive fecal samples varied from <3 MPN/g of feces to 2.8 X 10^3 MPN/g and 71% of positive samples had count <10 MPN/g, and the same author (2005) found that *Salmonella* in positive fecal samples varied from <3 MPN/g of fecal to 93 MPN/g and 64% of positive samples had count <10 MPN/g. In our study the *Salmonella* in positive fecal samples varied from <1.8 MPN/g and 1609 MPN/g, and

63% of positive samples <20 MPN/g. 66.7% (18/27) of samples that tested positive by the LightCycler real-time PCR detection assay were positive with conventional PCR. The majority of the conventional negative samples (78%, 7/9) was <1.8 MPN/g, while the other two were 27 MNP/g and 53.9 MPN/g as show in Table 8.

Identification of isolates is of particular importance for epidemiological data and public health authorities. PCR procedure, on the other hand, can be used only as a screening tool because it indicates only presence or absence of the pathogen. In our study, *Salmonella* Typhimurium was the only serotype isolated from the fecal samles with multidrug resistant (penta, FQ and R) and *Salmonella* Derby the only serotype isolated from environmental samples with multidrug resistant (Sm, Te and Su) by cultural procedures from the twenty seven fecal and nine environmental real-time PCR *Salmonella*-positive samples. This finding might not be surprising because for a variety of reasons including the relatively low number of real-time PCR positive samples in this study. Moreover, in all cases the number of *Salmonella* in the fecal samples was very low. Conventional cultural procedures will not always detect small numbers of *Salmonella* cells in certain food or fecal samples. (15) reported some factors that can influence recovery rates including sensitivity of the methods, the susceptibility of *Salmonella* strains to inhibitors in the food or media, and overgrowth by competitors during incubation. (33) mentioned many reasons why *Salmonella* were not isolated from PCR positive raw milk samples; bulk tank milk can contain many other organisms that may compete with *Salmonella* in the enrichment broth; the presence of other organisms on the XLT4 selective agar plates may interfere with the production of H_2S by *Salmonella*; H_2S production is required for the formation of the black colour in *Salmonella* colonies. (34) showed that RV broth has a low selectivity for Enterobacteriaceae present in pig faeces and that XLT4 agar has a low discriminatory power and the study emphasise the need for new and more selective enrichment and different media to be developed. found that 22% (10/45) of real-time PCR *Salmonella* positive from artificially contaminated bovine fecal samples was negative with culture method, the author explained that due to low number of cell after enrichment may be there are false-positives obtained by real-time PCR method; however, supplementary analyses showed that *Salmonella* could be cultured from these negative samples when additional enrichment and IMS used. This would indicate either a very low number of *Salmonella* that the culture method described above could not detect, or that there was a high degree of background flora, and additional measures were needed to increase the number of *Salmonella* to detectable levels and minimize background microflora.

A data gap that is routinely identified in risk assessment is the lack of quantitative data on the level of contamination in the contaminated foods with pathogens (9). The application of MPN method combined with LightCycler real-time PCR to quantify *Salmonella* spp. in feces proved to be rapid and highly sensitive and small numbers of *Salmonella* could be found in fecal samples. This assay yields significant labor and time savings since the quantification of *Salmonella* spp. can completed within 12 h which included an 8-h non-selective enrichment step and 4 h to carry out the sample preparation and real-time PCR assay as opposed to the classical methods, which require at least 5 days of work.

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