# Comparison between two standardized cultural methods and 24 hour Duplex SYBR Green Real-Time PCR assay for *Salmonella* detection in meat samples

**Elisabetta Delibato[1], Alfonsina Fiore[1], Fabrizio Anniballi[1], Bruna Auricchio[1], Emma Filetici[2], Leucio Orefice[1], Marina Nadia Losio[3], Dario De Medici[1]**

*[1]Department of Food Safety and Veterinary Public Health, Istituto Superiore di Sanità, Rome, Italy;*
*[2]Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy;*
*[3]Istituto Zooprofilattico della Lombardia ed Emilia Romagna, Brescia, Italy*

**SUMMARY**

Food-borne diseases caused by *Salmonella* represent a worldwide public health problem. *Salmonella* must be absent in an established amount depending on the kind of the product and usually cultural methods have to be applied to evaluate the compliance of the products. ISO 6579:2002 in Europe and FSIS MLG 4.04.:2008 in the USA have usually been employed to detect *Salmonella* in meat, poultry and egg products. A Real Time PCR method using probes has recently been validated against the NMKL (Nordic Committee on Food Analysis) standard method. This method has been modified using the less expensive Sybr Green Real Time PCR approach and applied directly in the 18 hours pre-enrichment broth for the purpose of detecting *Salmonella* in meat products in less than 24 hours. The purpose of this study was to:
- compare the effectiveness of ISO and FSIS cultural methods;
- develop a new 24 hour duplex Sybr Green Real Time PCR-melting curve analysis;
- evaluate the performance of Salmonella, Standard Method, Rapid Method, SYBR Green Real Time PCR.
The equivalence between ISO and FSIS methods was demonstrated and the use of SYBR Green Real Time PCR as a screening tool for negative results seems appealing especially to evaluate compliance with the HACCP systems.

*KEY WORDS:* Salmonella, Rapid Method, Real Time PCR, Sybr Green

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## INTRODUCTION

Food-borne diseases caused by *Salmonella* represent a worldwide public health problem. (Gomez *et al*., 1997). In Europe, Salmonellosis is still the second most commonly recorded zoonosis accounting for 151,995 confirmed human cases. *Salmonella* is most often found in fresh poultry and pig meat where proportions of positive samples, on average 5.5% and 1.1%, were detected respectively (EFSA, 2009).
According to European criteria (Commission Regulation No 2073/2005, updated by Commission Regulation 1441/2007) *Salmonella* must be absent in an established amount depending on the kind of the product, and a standardized ISO cultural method (ISO, 2002) has to be applied to evaluate product compliance. A similar standardized culture method, FSIS method (FSIS, 2008), has been used in USA for *Salmonella* detection in meat, poultry and egg products. Both standard culture methods (SCMs) for detecting *Salmonella* require up to 5 days to produce positive results. These methods include stages of pre-enrichment, selective enrichment,

*Corresponding author*
Delibato Elisabetta Ph D
Department of Food Safety and Veterinary Public Health
Istituto Superiore di Sanità
Viale Regina Elena, 299 - 00161 Rome, Italy
E-mail: elisabetta.delibato@iss.it

isolation in selective agars, biochemical characterization of suspected colonies and final serological confirmation. Cultural microbiological methods are powerful but laborious, costly and time-consuming and often ineffective because they are not compatible with the speed at which the products are manufactured and the short shelf-life of products (Bhunia, 2008).
Thus, there is a need to have novel methods able to detect in a short time, preferably in about 24 hours, a small quantity of *Salmonella* within a given amount of food. Rapid methods have to be almost as efficient as the standardized cultural method for their use to evaluate product compliance (Piknova *et al*., 2005). Real Time PCR has become the most promising technique for rapid detection of pathogens in food. The main advantages of Real Time PCR are high sensitivity, high specificity, excellent efficiency, and no post-PCR steps that reduce risks of cross-contamination (Rodriguez-Lazaro *et al.,* 2003). Sybr Green Real Time PCR is a useful tool when a large number of samples have to be analysed because it is less expensive that probe Real-Time PCR assays (De Medici *et al*., 2003), (Elizaquivel and Aznar, 2008), (Fenicia *et al*., 2007), (Fricker *et al*., 2007), (Fukushima and Tsunomori, 2005), (Yang *et al*., 2004). The aims of this study were to:
- compare the effectiveness of ISO and FSIS cultural methods;
- develop a new 24 hour duplex Sybr Green Real Time PCR including a competitive chimerical internal amplification control (IAC);
- evaluate the performance of Sybr Green Real Time PCR with both cultural methods.

## MATERIALS AND METHODS

### Bacterial strains

All the strains (Table 1) were cultured in Triptone Soya Broth (Oxoid, Basingstoke, Hampshire, United Kingdom) at 37°C for 24 h. The broth cultures were then washed three times by means of centrifugation (8000 x *g* for 10 min) in a solution of 0.8% NaCl. *Salmonella* enterica serovar Typhimurium ATCC 14028 was used for the evaluation of the limit of detection (LOD) of the three methods. The microbial suspension of *S*. Typhimurium standardized by count on Triptone Soy Agar (Oxoid) yielded $10^8$ log CFU/ml.

### Real and spiked meat samples

Eighty samples of the various edible parts of pork, chicken, beef and turkey were purchased in Italy from various local food stores.
In addition thirty samples of 25 g of minced pork samples were spiked with $10^0$-$10^1$ CFU of three different strains of *Salmonella* (10 samples were spiked with *S.* Enteritidis, 10 samples with *S.* Infantis and 10 samples with *S.* Derby).

### SYBR Green Real Time PCR method

*DNA extraction*

One milliliter of the pre-enrichment broth was transferred to a micro-centrifuge tube with a capacity of 1.5 ml. The broth was centrifuged for 10 min at 14,000 x *g* at 4°C and the supernatant was discarded carefully. The pellet was re-suspended in 200 µl of 6% Chelex 100 (Bio-Rad Life Science Research) by vortexing. The micro-centrifuge tube was incubated for 20 min at 56°C and then for 8 min at 100°C. The suspension was immediately chilled on ice and centrifuged for 5 min at 14,000 x *g* at 4°C. Five microliters of supernatant was used as the template DNA in the PCR (Malorny *et al*., 2003).

*IAC construction and optimization*

In order to identify false-negative results, a competitive IAC was synthesized. The IAC was constructed according to the procedure of Abdulmawjood *et al.* (Abdulmawjood et al., 2002). The primers used to amplify the IAC, designed using Primer Express 1.5 software, generate a 170 bp PCR product. One microliter of pUC 19 (Gene Bank accession number L09137) was used as template in a 50 µl of reaction mixture containing: 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCL (pH 8.3), 200 µM of each deoxynucleoside triphosphate, 1 µM of each primer ttr6-pUC19 F (CTCACCAGGAGATTACAACATGGTGACGAGCATCACAAAAATCG) and ttr4-pUC19 R (AGCTACGACCAAAAGTGACCATCGAAGGGAGAAAGGCGGACAG) (M-Medical, Florence, Italy), and 2.5 U of *Taq* polymerase (Applied BioSystems, Roche Molecular Systems). PCR was carried out using the following conditions: 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 60°C, extension for 1 min at 72°C. After the 35$^{th}$ cycle, the extension reaction was continued for another 7 min at 72°C. Optimization of the copy number of the IAC in-

TABLE 1 - *Strains used for selectivity study and results obtained using ISO, FSIS and SYBR Green Real Time PCR.*

| *Strain* | *No. of tested strains* | *Real-Time PCR* | *Strain* | *No. of tested strains* | *Real-Time PCR* |
|---|---|---|---|---|---|
| S. Blockley | 4 | + | *Aeromonas hydrophila* | 1 | - |
| S. Blukwa | 1 | + | *Bacillus subtilis* | 1 | - |
| S. Bovismorbificans | 2 | + | *Campylobacter jejuni* | 1 | - |
| S. Brandenburg | 3 | + | *Campylobacter coli* | 1 | - |
| S. Bredeney | 2 | + | *Enterobacter aerogenes* | 1 | - |
| S. Brooklyn | 1 | + | *Enterobacter cloacae* | 1 | - |
| S. Cerro | 1 | + | *Escherichia coli* | 2 | - |
| S. Choleraesuis | 3 | + | *Listeria innocua* | 1 | - |
| S. Derby | 2 | + | *Listeria monocytogenes* | 1 | - |
| S. Enteritidis | 7 | + | *Proteus hauseri* | 1 | - |
| S. Gallinarum | 1 | + | *Pseudomonas aeruginosa* | 1- | |
| S. Give | 3 | + | *Shigella boydii* | 1 | - |
| S. Goldcoast | 1 | + | *Shigella flexneri* | 1 | - |
| S. Hadar | 5 | + | *Shigella sonnei* | 1 | - |
| S. Heidelberg | 2 | + | *Staphylococcus aureus* | 3 | - |
| S. Kibusi | 1 | + | *Staphylococcus epidermidis* | 1 | - |
| S. Kottbus | 4 | + | *Staphylococcus xylosus* | 1 | - |
| S. Liverpool | 1 | + | *Yersinia enterocolitica* | 6 | - |
| S. London | 2 | + | *Yersinia intermedia* | 1 | - |
| S. Mbandaka | 1 | + | *Yersinia kristensenii* | 2 | - |
| S. Napoli | 4 | + | *Yersinia pseudotubercolosis* | 1 | - |
| S. Newport | 4 | + | | | |
| S. Othmarschen | 1 | + | | | |
| S. Parathyphi | 2 | + | | | |
| S. Pomona | 2 | + | | | |
| S. Saintpaul | 2 | + | | | |
| S. Thompson | 4 | + | | | |
| S. Tomegbe | 1 | + | | | |
| S. Typhimurium | 4 | + | | | |
| S. Umbilo | 1 | + | | | |
| S. Urbana | 1 | + | | | |
| S. Veneziana | 1 | + | | | |
| S. Virchow | 2 | + | | | |
| S. Worthington | 1 | + | | | |
| S.IIIb38:lv:z35 | 1 | + | | | |

cluded in each PCR reaction was performed as reported elsewhere (Fenicia *et al*., 2007; Fricker *et al*., 2007).

*SYBR Green Real Time PCR and melting curve analysis*

Real Time PCR and data analysis were performed using 96-microwell plates and a Stratagene Mx3005p sequence detector (Agilent, Santa Clara, CA, USA). Five microliters of purified DNA, 25 µl of SYBR Green Master Mix (Applied BioSystems), 200 nM of primers ttr6 (CTCACCAGGAGATTACAACATGG) and ttr4 (AGCTCAGACCAAAAGTGACCATC) (Malorny *et al*., 2004a), and 150 copies of IAC were added to each micro-well to reach a total volume of 50 µl per well, DNase-RNase-free distilled water (Sigma) was added. The reaction was run at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s and melting curve analysis.

To evaluate the efficiency of the amplification, a standard curve was constructed using the threshold cycle ($C_T$) versus 10-fold dilution of *S.* Typhimurium ($1x10^0$ to $1x10^7$ copies of DNA). The amplification efficiency ($E$) was calculated using the following equation: $E = (10^{-1/slope}) - 1$ (Klein *et al*., 1999).

The specificity of the reaction is given by the detection of the $T_m$ of the amplification products

immediately after the last reaction cycle. The melting curve was visualized with the software MxPro (Mx3005P v 4.00 - Agilent).
The inclusivity and exclusivity were performed using a panel of 75 *Salmonella* strains and 38 non-*Salmonella* strains (Table 1).

**Standard cultural methods (SMCs)**
The detection of *Salmonella* was performed according to ISO and FSIS methods respectively. The presumptive *Salmonella* colonies were serologically typed using commercial sera (Statens Serum Institut, Copenhagen, Denmark).

**Limit of detection of the SCMs and SYBR Green Real Time PCR method**
Forty samples each of 25 g were put into separate stomacher bags. Ten samples were spiked each with $10^0$-$10^1$ CFU, 10 samples with $10^1$-$10^2$ CFU, 10 samples with $10^2$-$10^3$ CFU of *S. Typhimurium* ATCC 14028, and the last 10 samples were un-inoculated. Two hundred twenty-five ml of buffered peptone water (BPW - Oxoid) were added to each stomacher bag prior to homogenization and incubated at 37°C±2 for 18 h. After incubation, 1 ml of pre-enrichment broth was subjected to the DNA extraction and the presence of *Salmonella* was determinated using SYBR Green Real Time PCR method.. The leftover pre-enrichment broth was used for the detection of *Salmonella* using the two SCMs.

**Statistical analysis**
The means and standard deviations of the $C_T$ and of the $T_m$ were calculated for each target strain and non-target strain, respectively. The statistical significance of the difference in the means of the $T_m$ value obtained by analyzing bacterial strains or meat samples was determined by Student's *t* test. A $P < 0.05$ value was chosen as significant.

## RESULTS

The optimal number of IAC copies was established when the melting peak was always present in the *Salmonella* negative broth cultures/samples and absent in the *Salmonella* positive broth cultures/samples (Fenicia *et al*., 2007).
SYBR Green Real Time PCR showed that, when using a concentration of $1x10^3$ genome copies/well of *Salmonella*, a concentration of 150 copies/well of IAC is optimal. In fact the addition of 150,000 copies of IAC produced complete competition of the target *ttr* gene, while the addition of 15,000 and 1,500 copies of IAC produced a melting curve in which the presence of both amplicons is clear. When 150 and 15 copies were added, only the peak resulting from the *Salmonella* amplicon was found (Figure 1).
SYBR Green Ral Time PCR shows that all *Salmonella* serotypes gave a specific amplicon with a reproducible $T_m$ at 81.29±0.20°C; instead negative control and non-*Salmonella* strains showed an IAC reproducible $T_m$ at 84.89±0.18°C (Figure 2). The mean peak obtained analyzing real and spiked samples, showed 66 positive curves for *Salmonella* at $T_m$ of 81.20±0.25°C, and 154 curves specific for the IAC at 84.88±0.20°C. Student's *t* test showed no significant difference ($P>0.05$) either between the results obtained from the mean peaks obtained analyzing *Salmonella* strains and positive food samples and the IAC mean peaks obtained from non-target and from negative samples.
A standard curve obtained by plotting the mean $C_T$ (n=3) versus logarithmic concentrations of *S.* Typhimurium ($1x10^0$ to $1x10^7$ copies of DNA) displayed a good linearity of response ($R^2=0.997$). Standard regression analyses of the linear part of the slope gave a coefficient of -3.3373, and the PCR efficiency was 0.9936.
The selectivity of the methods tested against a panel of 75 *Salmonella* strains and 38 non-*Salmonella* strains showed 100% of inclusivity and 100% of exclusivity (Table 1).
The LOD of all three methods was detected using spiked samples and the presence *S.* Typhimurium was confirmed for SCMs by serological characterization of characteristic colonies, and for Real Time PCR using the $T_m$ of the PCR products. Spiked samples inoculated with all three inoculation levels of *S.* Typhimurium produced characteristic colonies on selective agar media. No characteristic colonies were detected in un-inoculated samples. The presence of *S.* Typhimurium was also confirmed by Real Time PCR at all inoculation levels after 18 h of pre-enrichment. The LOD of the three methods results $10^0$-$10^1$ CFU/25 g of meat.
Three of the 80 real samples analysed resulted positive for *Salmonella* with the two SCMs and

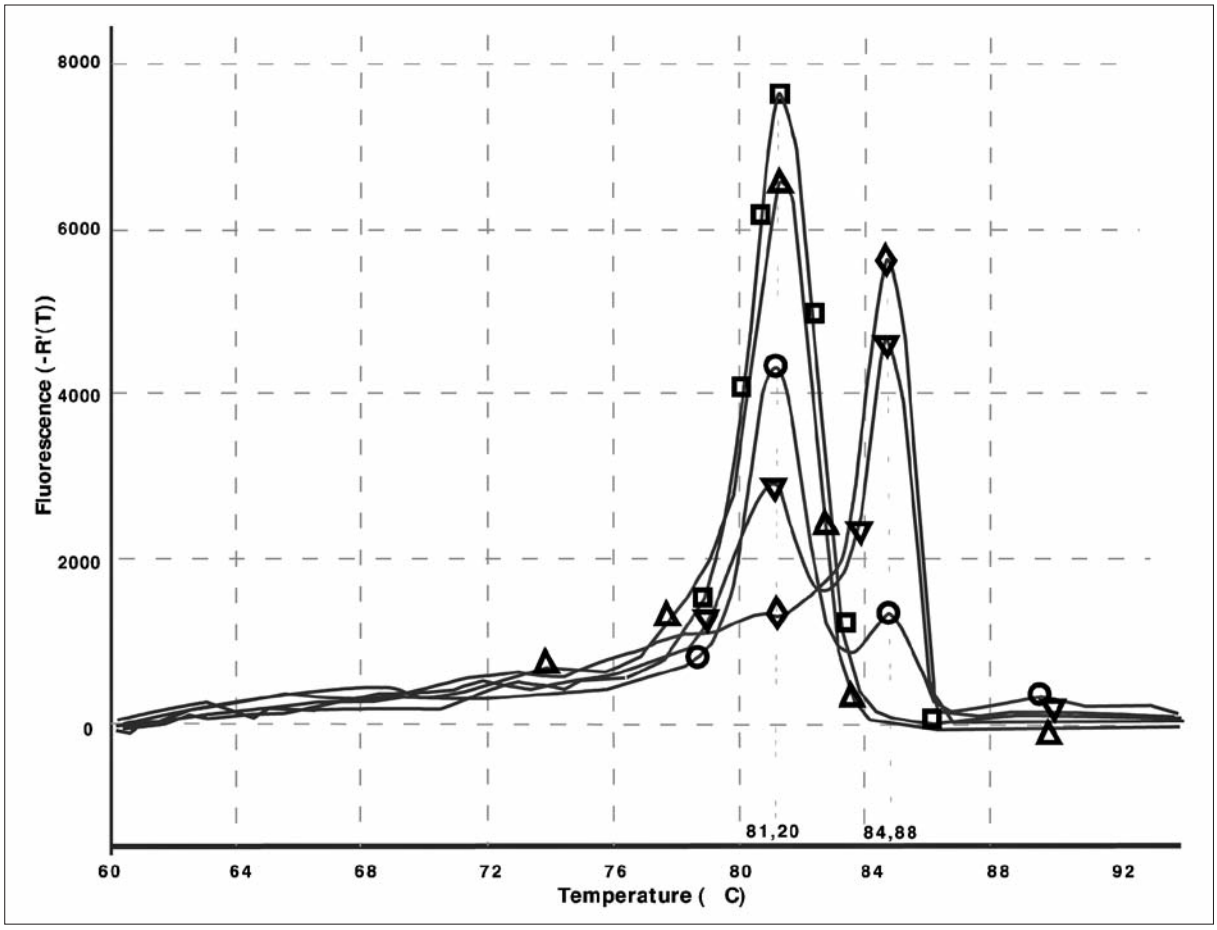


FIGURE 1 - *Melting curves obtained after coamplification of 1x10$^3$ genome copies of Salmonella* Enteritidis *ATCC 13076 and 15 copies(□), 150 copies (△), 1,500 copies (▽), 15,000 copies (○) , 150,000 copies (◇) of IAC.*

the SYBR Green Real Time PCR. In these positive samples *S.* Typhimurium, *S.* Infantis, *S.* London were identified by slide agglutination. In addition, all the thirty spiked samples were correctly identified through the two SCMs and the SYBR Green Real Time PCR methods.

## DISCUSSION

The present study was undertaken to compare the effectiveness of the two main SCMs used for *Salmonella* detection in Europe (ISO method) and in the USA (FSIS method). The results demonstrated that these two SMCs can be considered indistinguishable because both are able to detect the same positive and negative samples in naturally and spike contaminated meat samples.

However, both of the compared SCMs take over five days to confirm results.

A real improvement for an acceptable alternative system would be a method able to detect the target in not more than 24 hours. A Real Time PCR using hydrolysis probes was developed by Malorny *et al.* (Malorny *et al.*, 2004b) and recently validated against the NMKL (Nordic Committee on Food Analysis) standard method (Lofstrom *et al.*, 2009). This method has been modified using the less expensive SYBR Green Real Time PCR approach and evaluated in comparison with both the SCMs. In the absence of the probe, the specificity of the SYBR Green Real Time PCR was determined by the definition of the melting temperature of the PCR product obtained (De Medici *et al.*, 2003). Since IAC is mandatory in PCR-based methods for the detec-

tion of foodborne pathogens ((ISO 22174), to avoid false negative results due to the lack of PCR amplification, a competitive IAC was included in the PCR reaction as suggested by other authors (Fenicia *et al*., 2007; Hoorfar *et al*., 2004).
The selectivity of the SYBR Green Real Time PCR method was demonstrated using 75 *Salmonella* strains and 38 non-*Salmonella* strains where an inclusivity and exclusivity of 100% was found.
The robustness of the three methods (ISO, FSIS, and SYBR Green Real Time PCR) was also verified using real and spiked samples. However, the results obtained using spiked samples have less value than naturally contaminated samples, but spiked samples were needed to improve the validation process, since the prevalence of naturally *Salmonella*-contaminated samples is very low. To obtained data in conditions similar to those obtained from naturally contaminated samples, a very low level of contamination was chosen (Piknova *et al*., 2002). A LOD of $10^0$-$10^1$ CFU/25 g was confirmed in all samples by all the three methods.
The isolation of *S.* Typhimurium, *S.* Infantis and *S.* London in naturally contaminated samples demonstrates the effectiveness of the SYBR Green Real Time PCR as well as in SCMs in revealing different *Salmonella* serotypes in naturally contaminated meat samples. The determination of the melting temperature, which was consistently specific for each amplicon obtained, confirmed the specificity of both, *Salmonella* and IAC amplicons (De Medici *et al*., 2003). In fact the melting temperature of the target and IAC amplicons did not give statistically significant differences between the melting temperatures determined using isolated strains or naturally and spiked meat samples. No statistically significant difference was detected comparing the $T_m$ obtained from different levels of inoculation (DNA target). This demonstrated that the melting curve analysis was not influenced by any substance present in meat samples or by different target concentrations.
This SYBR Green Real Time PCR method seems to be very appealing as a screening method, although the isolation and serotyping of the *Salmonella* in food samples remain important for analytical and epidemiological purposes. To this end the remaining pre-enrichment broth found positive to SYBR Green Real TIme PCR should be submitted to one of the SCMs at the aim to isolate the strain. This strategy should be particularly important when widescale monitoring is applied to evaluate the presence of *Salmonella* in food with low prevalence of contamination.

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## REFERENCES

Abdulmawjood A., Roth S., Bulte M. (2002). Two methods for construction of internal amplification controls for the detection of Escherichia coli O157 by polymerase chain reaction. *Mol. Cell. Probes*. **16**, 335-339.

Bhunia A.K. (2008). Biosensors and bio-based methods for the separation and detection of foodborne pathogens. *Adv. Food Nutr. Res*. **54**, 1-44.

De Medici D., Croci L., Delibato E., Di Pasquale S., Filetici E., Toti L. (2003). Evaluation of DNA extraction methods for use in combination with SYBR green I real-time PCR to detect *Salmonella* enterica serotype enteritidis in poultry. *Appl. Environ. Microbiol*. **69**, 3456-3461.

EFSA. (2009). Trends and Sources of Zoonoses and Zoonotic Agents in the European Union in 2007. *The EFSA Journal.* 223.

Elizaquivel P., Aznar R. (2008). A multiplex RTi-PCR reaction for simultaneous detection of Escherichia coli O157:H7, *Salmonella* spp. and Staphylococcus aureus on fresh, minimally processed vegetables. *Food Microbiol.* **25**, 705-713.

Fenicia L., Anniballi F., De Medici D., Delibato E., Aureli P. (2007). SYBR green real-time PCR method to detect Clostridium botulinum type A. *Appl. Environ. Microbiol*. **73**, 2891-2896.

Fricker M., Messelhausser U., Busch U., Scherer S., Ehling-Schulz M. (2007). Diagnostic real-time PCR assays for the detection of emetic Bacillus cereus strains in foods and recent food-borne outbreaks. *Appl. Environ. Microbiol.* **73**, 1892-1898.

FSIS, Ed. (2008). Isolation and Identification of *Salmonella* from Meat, Poultry, and Egg Products (MLG 4.04). Laboratory Guidebook. Edited by U.S.D. o. A.-F.S. a. I. Service. Athens, GA, USA: United States Department of Agriculture - Food Safety and Inspection Service.

Fukushima H., and Tsunomori Y. (2005). [Study of real-time PCR assays for rapid detection of food-borne pathogens]. *Kansenshogaku Zasshi*. **79**, 644-655.

Gomez T.M., Motarjemi Y., Miyagawa S., Kaferstein F.K., Stohr K. (1997). Foodborne salmonellosis. *World Health Stat Q.* **50**, 81-89.

HOORFAR J., COOK N., MALORNY B., WAGNER M., DE MEDICI D., ABDULMAWJOOD A., FACH P. (2004). Diagnostic PCR: making internal amplification control mandatory. *J. Appl. Microbiol*. **96**, 221-222.

ISO, Ed. (2002). International Standard ISO/FDIS 6579. Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. Edited by I.O.f. Standardization. Geneva. Switzerland.

KLEIN D., JANDA P., STEINBORN R., MULLER M., SALMONS B., GUNZBURG W.H. (1999). Proviral load determination of different feline immunodeficiency virus isolates using real-time polymerase chain reaction: influence of mismatches on quantification. *Electrophoresis*. **20**, 291-299.

LOFSTROM C., KRAUSE M., JOSEFSEN M.H., HANSEN F., HOORFAR J. (2009). Validation of a same-day real-time PCR method for screening of meat and carcass swabs for *Salmonella*. *BMC Microbiol.* **9**, 85.

MALORNY B., COOK N., D'AGOSTINO M., DE MEDICI D., CROCI L., ABDULMAWJOOD A., FACH P., KARPISKOVA R., AYMERICH T., KWAITEK K. AND HOORFAR J. (2004a). Multicenter validation of PCR-based method for detection of *Salmonella* in chicken and pig samples. *J. AOAC Int.* **87**, 861-866.

MALORNY B., HOORFAR J., HUGAS M., HEUVELINK A., FACH P., ELLERBROEK L., BUNGE C., DORN C., HELMUTH R. (2003). Interlaboratory diagnostic accuracy of a *Salmonella* specific PCR-based method. *Int. J. Food Microbiol*. **89**, 241-249.

MALORNY B., PACCASSONI E., FACH P., BUNGE C., MARTIN A., HELMUTH R. (2004b). Diagnostic real-time PCR for detection of *Salmonella* in food. *Appl. Environ. Microbiol*. **70**, 7046-7052.

PIKNOVA L., KACLIKOVA E., PANGALLO D., POLEK B., KUCHTA T. (2005). Quantification of *Salmonella* by 5'-nuclease real-time polymerase chain reaction targeted to fimC gene. *Curr. Microbiol.* **50**, 38-42.

PIKNOVA L., STEFANOVICOVA A., DRAHOVSKA H., SASIK M., KUCHTA, T. (2002). Detection of *Salmonella* in food, equivalent to ISO 6579, by a three-days polimerase chain reaction-based method. *Food Control.* **13**, 191-194.

RODRIGUEZ-LAZARO D., HERNANDEZ M., ESTEVE T., HOORFAR J., PLA M. (2003). A rapid and direct real time PCR-based method for identification of *Salmonella* spp. *J. Microbiol. Methods.* **54**, 381-390.

YANG C., JIANG Y., HUANG K., ZHU C., YIN Y., GONG J.H., YU H. (2004). A real-time PCR assay for the detection and quantitation of Campylobacter jejuni using SYBR Green I and the LightCycler. *Yale J. Biol. Med*. **77**, 125-132.