Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
International Journal of Food Microbiology 89 (2003) 241–249
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

# Interlaboratory diagnostic accuracy of a *Salmonella* specific PCR-based method

Burkhard Malorny[a,*], Jeffrey Hoorfar[b], Marta Hugas[c], Annet Heuvelink[d], Patrick Fach[e], Lüppo Ellerbroek[a], Cornelia Bunge[a], Christina Dorn[a], Reiner Helmuth[a]

[a] *Federal Institute for Risk Assessment, Diedersdorfer Weg 1, D-12277 Berlin, Germany*
[b] *Danish Veterinary Institute, Bülowsvej 27, DK-1790 Copenhagen, Denmark*
[c] *Institute for Food and Agricultural Research and Technology, Granja Camps I Armet S/N, 17121 Monells, Spain*
[d] *Inspectorate for Health Protection and Veterinary Public Health, P.O. Box 202, 7200 AE Zutphen, The Netherlands*
[e] *Agence Française de Sécurité Sanitaire des Aliments, 1 à 5 rue de Belfort, 94700 Maisons-Alfort, France*

Received 30 September 2002; received in revised form 3 March 2003; accepted 15 March 2003

**Abstract**

A collaborative study involving four European laboratories was conducted to investigate the diagnostic accuracy of a *Salmonella* specific PCR-based method, which was evaluated within the European FOOD-PCR project (http://www.pcr.dk). Each laboratory analysed by the PCR a set of independent obtained presumably naturally contaminated samples and compared the results with the microbiological culture method. The PCR-based method comprised a preenrichment step in buffered peptone water followed by a thermal cell lysis using a closed tube resin-based method. Artificially contaminated minced beef and whole broiler carcass-rinse resulted in a detection limit of less than 5 cells per 25 g meat or 100 ml broiler rinse. A total of 435 samples from four countries, including pig carcass swabs ($n=285$), whole broiler carcass-rinse ($n=25$), various raw meat ($n=33$), and environmental samples ($n=92$) were investigated. The interlaboratory diagnostic accuracy, i.e. diagnostic specificity and sensitivity, was shown to be 97.5%. The co-amplification of an internal amplification control indicated possible inhibitory substances derived from the sample. This work can contribute to the quality assurance of PCR-based diagnostic methods and is currently proposed as international standard document.


*Keywords:* Detection; Food; PCR; *Salmonella*; Accuracy; Standard

## 1. Introduction

*Salmonella* continues to be a major foodborne pathogen for animals and humans (Humphrey, 2000); in many countries, it is the leading cause of foodborne outbreaks and infections (BgVV-FAO/WHO Collaborating Centre for Research and Training in Food Hygiene and Zoonoses, 2000; Wallace et al., 2000). Due to the worldwide trade of food and feeding stuffs, the risk of microbial infections for consumers increases. Thus, for food safety, the availability of reliable, rapid and internationally accepted test systems to detect the presence or absence of foodborne pathogens becomes increasingly important for the

* Corresponding author. Bundesinstitut für Risikobewertung, National Salmonella Reference Laboratory, Diedersdorfer Weg 1, D-12277 Berlin, Germany. Tel.: +49-30-8412-2237; fax: +49-30-8412-2953.
*E-mail address:* b.malorny@bfr.bund.de (B. Malorny).

0168-1605/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0168-1605(03)00154-5

agricultural and food industry, as well as the legislative control. Currently, international guidelines and regulations for the detection of *Salmonella* are based on traditional microbiological methods resulting in 4–6 days isolation procedures (Anonymous, 1993). In vitro amplification of DNA by the polymerase chain reaction (PCR) has become the potential of a powerful alternative in microbiological diagnostics due to its rapidity and accuracy.

However, due to lack of international validation and standard protocols, as well as variable quality of reagents and equipment, the methodology has difficulties to move from expert-to end-user laboratories. For example, many PCR-based methods published for the detection of *Salmonella* differ in specificity, detection limit and sample treatment (Aabo et al., 1993; Bäumler et al., 1997; Burkhalter et al., 1995; Cohen et al., 1994; Jones et al., 1993; Kwang et al., 1996). Furthermore, an internal amplification control (IAC), necessary to indicate false-negative results caused by PCR inhibitors, is rarely included in the final diagnostic test. A European research project (Malorny et al., 2003a) is currently working on the validation and standardization of PCR for the detection of five major pathogens, namely thermophilic *Campylobacter* spp., *Escherichia coli* O157, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Salmonella* spp. A *Salmonella* specific PCR assay, which was validated within this project, showed a high selectivity on 242 *Salmonella* strains (inclusivity 99.6%) and 122 non-*Salmonella* strains (exclusivity 100%) (Malorny et al., 2003b). The primer set used for specific amplification of a *Salmonella* genomic DNA fragment has previously been published (Rahn et al., 1992) and amplifies a 284-bp sequence of the *invA* gene. For the indication of possible PCR inhibitors derived from the sample, an internal amplification control (IAC) was included, which co-amplifies with the *invA* target gene. A European collaborative study resulted in an analytical accuracy, i.e. inclusivity and exclusivity, of 98% (Malorny et al., 2003b).

The present study reports an interlaboratory assessment of the diagnostic accuracy of a *Salmonella* PCR-based method. The method comprises a protocol for sample treatment combined with the previously validated PCR assay. Naturally contaminated samples, especially swab samples from pig carcasses, whole broiler carcass-rinse, various raw meat samples, and environmental samples obtained in broiler-houses, were investigated in four European laboratories. The detection limit using artificially contaminated minced beef and carcass-rinse has been determined. The influence of background flora on the sensitivity of the PCR-based method was considered.

## 2. Materials and methods

### 2.1. Sample collection

Table 1 shows the types and numbers of the naturally contaminated samples investigated in each of the four laboratories located in Germany (Berlin, BfR), Spain (Monells, IRTA), The Netherlands (Zutphen, IHP) and France (Maisons-Alfort, AFSSA). Swab samples were taken from pig carcasses (neck, belly, ham) according Kitchell et al. (1973) and were obtained in one local slaughterhouse in Spain (10–15 swabs per week in eight consecutive weeks starting in January 2001), in seven local slaughterhouses in the Netherlands (in the period November 2001 to April 2002) and one local slaughterhouse in Germany (1 day in August 2002). Environmental swabs and socks were taken in five different local broiler houses in Germany within four consecutive weeks in April 2001 (Ellerbroek et al., 2002). Chilled or frozen broilers were obtained in Germany from various local food stores; minced beef and other meat types including neck-skins of broilers were obtained from various locations in France (food stores and producers). Samples from France were preselected in order to have a maximum of naturally positive *Salmonella* samples.

### 2.2. Artificial inoculation of minced beef

One strain each of *Salmonella enteritidis* (AFSSA-SE45, France), *Salmonella typhimurium* (AFSSA-STM3, France), *Salmonella virchow* (AFSSA-SV17, France) and *Salmonella derby* (AFSSA-SD1352, France) were aerobically grown at 37 °C with shaking in tryptone soy broth (TSB) (Biokar, Beauvais, France) to exponential phase. Viable counts were obtained by plating a dilution made in ice-cold buffered peptone water (BWP) (Merck, Darmstadt, Germany) onto plate count agar (PCA) (AES Laboratories, Combourg, France) in duplicate and incubating

Table 1
Results of the interlaboratory study of a PCR-based method compared to the traditional culture method for the detection of *Salmonella* from various samples collected across Europe

| Type of sample | Location of analysis | Total no. of samples | Traditional culture method | | *Salmonella* PCR | | Sensitivity (%) | Specificity (%) | Accuracy (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | Positive | Negative | $False_{neg}$ | $False_{pos}$ | | | |
| Carcass swabs from pig | Spain, Netherlands, Germany | 285 | 66 | 219 | 1[a] | 1 | 98.4 | 99.5 | 99.3 |
| Environmental socks, broiler | Germany | 68 | 53 | 15 | 4[b] | 2 | 92.4 | 86.6 | 91.2 |
| Environmental swabs, broiler | Germany | 24 | 19 | 5 | 1[a] | 0 | 94.7 | 100 | 95.8 |
| Carcass-rinse, broiler | Germany | 25 | 8 | 17 | 0 | 0 | 100 | 100 | 100 |
| Various meat samples[d] | France | 33 | 22 | 11 | 2[c] | 0 | 90.9 | 100 | 93.9 |
| Total | | 435 | 168 | 267 | 8 | 3 | 98.2 | 97.7 | 97.5 |

[a] Positive case if the internal amplification control (IAC) copy number in the PCR reaction was reduced to 30.
[b] Three positives cases when the IAC copy number in the PCR reaction was reduced to 30.
[c] Both false-negatives obtained from neck-skin of broilers. One positive case when no IAC was added to the PCR reaction.
[d] Eighteen samples minced beef, eight samples neck-skin from broilers, three samples meat from turkey, one sample each sausage, pork meat, horse meat and pâté.

the plates for 24 h at 37 °C. The concentration of the culture was estimated by calculating the average number of the colony-forming units. The artificial inoculation procedure of 25 g chilled (4 °C) minced beef is shown in Fig. 1. In order to confirm the absence of *Salmonella*, uninoculated minced beef was tested by the standard culture method (Fig. 1). The experiment was repeated two times.

### 2.3. Artificial inoculation of whole broiler carcass-rinses and enumeration of the natural background flora

*S. enteritidis* phage type PT4 (BgVV 98-425, Germany) was aerobically grown at 37 °C with shaking in BPW to exponential phase. Viable cell counts were obtained by plating a dilution made in 0.9% sodium chloride onto Luria-Bertani agar (Miller, 1972) and incubation of the plates at 37 °C for 24 h. The concentration of the culture was estimated by calculating the average number of the colony-forming units from five agar plates. The preparation of whole broiler carcass-rinse and artificial inoculation procedure is shown in Fig. 1. In order to confirm the absence of *Salmonella*, uninoculated carcass-rinses were tested by the standard culture method (Fig. 1). The experiment was repeated three times on three consecutive days. Enumeration of viable cells derived from the natural background flora of the broiler carcass-rinses was determined by plating 10-fold dilutions of carcass-rinses (in 0.9% sodium chloride) in the range of $10^{-1}$ to $10^{-12}$ on 2× nutrient agar plates (Miller, 1972) in triplicate. After aerobical incubation for 24 h at 37 °C, the total number of viable cells per milliliter carcass-rinse was calculated by counting the colonies.

### 2.4. Traditional enrichment

The traditional enrichment method for the detection of *Salmonella* in artificially and naturally contaminated samples was based on EN 12824:1997 (Anonymous, 1997) with some modifications in respect to the use of the selective enrichment broths and agars. All participating laboratories performed the preenrichment at 37 °C for 18–20 h using BPW (Merck). Briefly, swab samples from pig were placed in 100 ml BPW. Neck-skins, environmental swabs or socks from poultry were placed in 200 ml BPW. Raw meat (25 g) was homogenized in 225 ml BPW using a stomacher (see Section 2.2). Whole broiler carcass-rinses were prepared as recommended in ISO/CD 6887 (Anonymous, 2000) (Fig. 1).

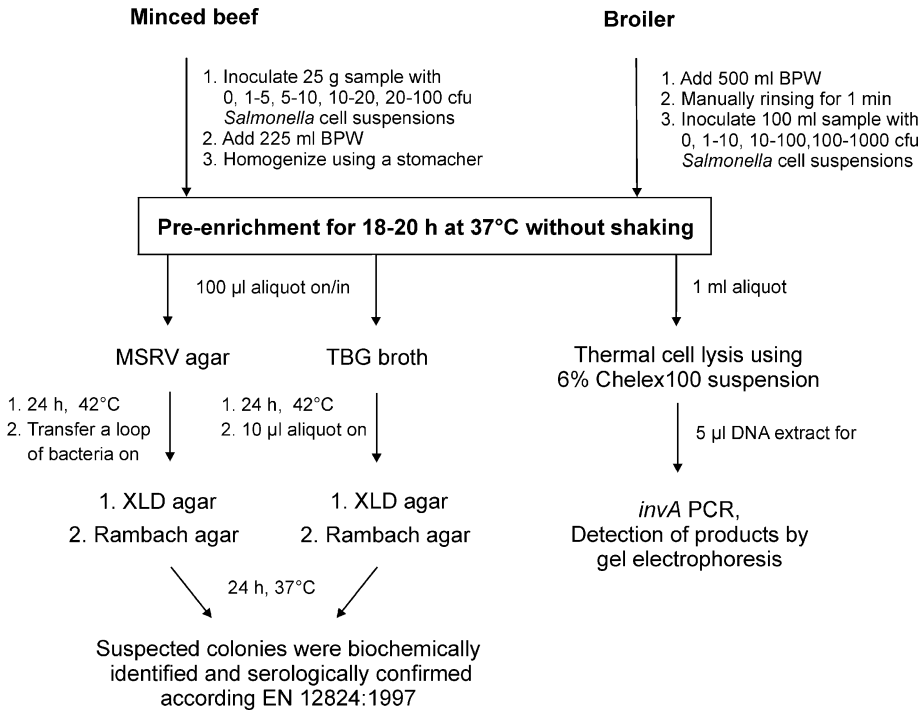


Fig. 1. Schematic flow diagram of the experimental protocol for the artificial inoculation of minced beef or whole broiler carcass-rinse. For inoculation of minced beef, four *Salmonella* serovars (*S. enteritidis*, *S. typhimurium*, *S. virchow* and *S. derby*) were used. Whole carcass-rinse was inoculated with *S. enteritidis*. Abbreviations: BPW, buffered peptone water; MSRV, modified semi-solid Rappaport Vassiliadis; TBG, tetrathionate brilliant green bile broth.

Selective enrichment broths and selective plating agars were used in the participating laboratories according to their in-house method and were as follows. Laboratory in Germany (Berlin, BfR): First enrichment in tetrathionate brilliant green bile broth (TBG) (Merck) and modified semi-solid Rappaport Vassiliadis (MSRV) (Merck); second enrichment on agar plates: xylose lysine desoxycholate (XLD) (Merck), Rambach (Merck) and brilliant green phenol red lactose saccharose (BPLS) (Merck). Laboratory in Spain (Monells, IRTA): First enrichment in selenite cystine (SC) (Merck) and MSRV (Merck); second enrichment on agar plates: XLD (Merck) and brilliant green agar (BGA) (Oxoid, Basingstoke, UK). Laboratory in The Netherlands (Zupthen, IHPV): First enrichment on MSRV (Merck); second enrichment: BGA (Oxoid) and mannitol lysine crystal violet brilliant green (MLCB) (Oxoid). Laboratory in France (Maisons-Alfort, AFSSA): First enrichment in RV (AES Laboratoire, Combourg, France), SC (AES Laboratoire) + MSRV (Merck); second enrichment on agar plates: XLD (AES Laboratoire) and BGA (AES Laboratoire).

Modified semi-solid Rappaport Vassiliadis (MSRV) agar plates were spotted with three drops (approximately 100 µl) of the preenriched culture and incubated 20–22 h at 42 °C. 10 ml TBG or RV broth was inoculated with 100 µl preenrichment, and 10 ml SC broth with 1 ml preenrichment followed by incubation at 42 °C (MSRV, RV) and 37 °C (TBG, SC) for 20–22 h, respectively. If presumptive *Salmonella* strains migrated around the inoculation spot, a loopful from MSRV agar was streaked onto a selective agar plate. Liquid enrichment (10 µl) was streaked onto the selective agar plates. The plates were incubated 24 h at 37 °C. Presumptive colonies were serologically and biochemically confirmed as described in EN 12824: 1997 (Anonymous, 1997).

### 2.5. Sample DNA extraction for PCR

One milliliter preenrichment (see Section 2.4) was centrifuged for 5 min at 10,000 × *g* and 4 °C. The supernatant was carefully discarded, the pellet was resuspended in 300 μl Chelex 100 (cat. no. 142-2832, Bio-Rad, München, Germany) suspension (6%) and incubated at 56 °C for 15–20 min. The tube was briefly mixed and incubated at 100 °C for 8 min. After chilling on ice for 2 min, a centrifugation step followed for 5 min at 14,000 × *g* and 4 °C. A 5-μl aliquot of the supernatant containing DNA was directly used as template in the PCR reaction.

### 2.6. PCR

PCR reaction mix (25 μl) contained 0.4 μM of each primer 139 and 141 (salt-free) (Rahn et al., 1992), 200 μM of each dNTP, 1 × PCR reaction buffer (20 mM Tris–HCl (pH 8.4), 50 mM KCl), 1.5 mM $MgCl_2$, 1 μg $\mu l^{-1}$ BSA Fraction V, 1 U Platinum *Taq* polymerase (Invitrogen, Karlsruhe, Germany), 300 copies of IAC (498-bp purified PCR product) (Malorny et al., 2003b) and 5 μl Chelex-treated sample. The manufacturers of the reagents, except the Platinum *Taq* polymerase and buffer, differed between the laboratories but had the same purity. PCR reactions were carried out in a GenAmp PCR System 9700 thermocycler (Applied Biosystems, Weiterstadt, Germany) in the laboratories located in Germany, Spain and France, and a Mastercycler Gradient (Eppendorf, Hamburg, Germany) in the laboratory located in The Netherlands. The incubation conditions were 95 °C for 1 min, followed by 38 cycles of 95 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s. A final extension of 72 °C for 4 min was applied.

A 10-μl aliquot of a PCR product was loaded on a 1.8% agarose gel and electrophoresed at 6 V $cm^{-1}$ for 90 min. After staining with ethidium bromide, the gel was documented with a video camera. A positive response was defined by the presence of a visible band at the expected size, while a negative response was defined as lack of any band at the expected size.

### 2.7. Detection of Salmonella in the presence of background flora

Three meat-associated species *Pseudomonas aeruginosa* DSM 50071, *Citrobacter freundii* EU-NS26 (isolated in Germany) and *E. coli* ATCC 25922 as well as *S. typhimurium* reference strain 51K61 (Malorny et al., 2001) were cultured in BPW overnight at 37 °C. The number of colony-forming units was determined by 10-fold dilutions in BPW, followed by plating onto Luria-Bertani agar plates in triplicate and incubation at 37 °C for 24 h. An equal number of the three non-*Salmonella* bacterial species was mixed in 10 ml BPW at low (approximately $10^4$ cfu $ml^{-1}$), intermediate (approximately $10^6$ cfu $ml^{-1}$) or high (approximately $10^8$ cfu $ml^{-1}$) level. Various concentrations of *S. typhimurium* strain 51K61 (Malorny et al., 2001) were added, respectively (0, 1–5, 5–10, 10–50, 50–500, 5–10 × $10^2$ and 5–10 × $10^3$ cfu $ml^{-1}$). A DNA extraction of 1 ml of each sample using Chelex 100 (see Section 2.5) was performed before and after incubation at 37 °C for 20 h followed by a PCR analysis. The detection limit of the *S. typhimurium* strain 51K61 was also determined in the absence of the background flora. The experiment was repeated one time.

### 2.8. Statistical analysis and terms used

Relative sensitivity, specificity and diagnostic accuracy was calculated according to MICROVAL protocol (Anonymous, 2002b). The formulas used for the analysis were: relative sensitivity (%) = 100 × (positive agreement between culture and PCR)/(positive agreement between culture and PCR)+(false-negatives by PCR); relative specificity (%) = 100 × (negative agreement between culture and PCR)/(negative agreement between culture and PCR)+(false-positives by PCR); relative accuracy (%) = 100 × (positive and negative agreement between culture and PCR)/total number of samples tested. The diagnostic accuracy takes into account the target and nontarget microorganisms in the presence of a biological matrix (Malorny et al., 2003a) and comprises the terms sensitivity and specificity. The relative sensitivity is the ability of the alternative method (here PCR) to detect the analyte compared to the reference method (here traditional culture method) in the presence of a biological matrix. The relative specificity is the ability of the PCR not to detect the target organism when it is not detected by the reference method. The relative accuracy is the degree of correspondence between the response obtained by

the alternative method and the reference method on identical samples.

## 3. Results

### 3.1. Detection limit and influence of background flora on the detection of Salmonella

The detection limit of the PCR method using thermal cell lysed DNA of *Salmonella* cells (strain 51K61) was $6 \times 10^2$ cfu $ml^{-1}$ (10 cfu in the PCR reaction) in the presence of 300 IAC copy numbers per reaction in two independent determinations. The identical detection limit was obtained in the presence of a background flora mix of the species *P. aeruginosa*, *E. coli* and *C. freundii* at low (approximately $10^4$ cfu $ml^{-1}$), intermediate (approximately $10^6$ cfu $ml^{-1}$) or high level (approximately $10^8$ cfu $ml^{-1}$) (Fig. 2). After enrichment of the artificial culture mixes for 20 h, the detection limit decreased to lower than 3 cfu $ml^{-1}$ at the three background flora levels tested. After competitive amplification, the 157-bp IAC was clearly visible on the agarose gel in the non-inoculated and inoculated samples prior to enrichment. For post enrichment samples, the IAC was not detected or weakly detected in positive PCRs, probably due to the excess of the target *invA* fragments in the PCR reactions.

### 3.2. Artificial inoculated minced beef and whole broiler carcass-rinse

Fig. 1 shows the procedure of the artificial inoculation experiments. The inoculation of 25 g minced beef at five levels (0, 1–5, 5–10, 10–20 and 20–100 cfu per 25 g) using four different *Salmonella* serovars (*S. typhimurium*, *S. enteritidis*, *S. virchow* and *S. derby*) resulted in 100% agreement between the detection of *Salmonella* by traditional culture and PCR-based method in two independent experiments. Non-inoculated minced beef was *Salmonella*-negative, whereas all *Salmonella* contamination levels gave positive results. Similarly, the artificial contamination of 100 ml whole broiler carcass-rinse at four levels (0, 5, 26 and 474 cfu per 100 ml) resulted in 100% agreement between the traditional and the PCR method in four independent experiments. The non-inoculated carcass-rinse was *Salmonella*-negative. The total viable colony counts of aerobically grown bacteria on nutrient agar of the four non-inoculated

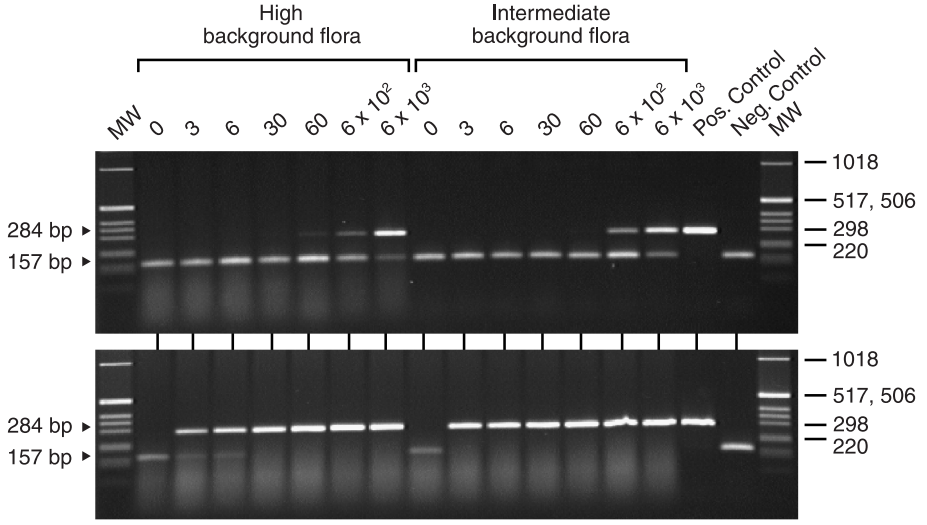


Fig. 2. Detection limit of the PCR-based method for the detection of *Salmonella* in the presence of high (approximately $10^8$ cfu $ml^{-1}$) and intermediate (approximately $10^6$ cfu $ml^{-1}$) levels of background flora before (upper gel) and after (lower gel) enrichment in BPW. On the top of the figure, the inoculation levels of *S. typhimurium* strain 51K61 in cfu $ml^{-1}$ are indicated. The left side of the gels shows the sizes of PCR products (157 bp for internal amplification control and 284 bp for *invA Salmonella* target fragment). The molecular weight standard (MW) marker X (Roche Diagnostics, Mannheim, Germany) is shown at the right side of the gels.

carcass-rinse samples ranged between 2.7 and $7.3 \times 10^5$ cfu $ml^{-1}$ carcass-rinse.

### 3.3. Naturally contaminated samples

A total of 435 samples were analysed by the traditional culture and the PCR-based method for the detection of *Salmonella* (Table 1). Of these, a total of 165 samples were positive and 259 samples were negative by both methods. Eight samples were false-negatives and three false-positives by PCR resulting in a general relative diagnostic accuracy of 97.4%. Emphasis was given on the analysis of swab samples from pig carcasses since their microbiological inspection plays in Europe an important role (Council directive 64/433/EEC of June 1964, http://www.europa.eu.int/comm/food/fs/inspections/index_en.html). Of 285 pig swab samples investigated, 65 were positive and 218 negative by both methods. Only one false-negative and one false-positive were obtained by PCR. However, subsequent selective enrichment of the false-positive swab sample by repeated subculturing onto brilliant green agar (BGA) and mannitol lysine crystal violet brilliant green (MLCB) revealed a positive isolate. It was observed that this *Salmonella* strain did not show typical growth on modified semi-solid Rappaport Vassiliadis (MSRV). The strain was later serotyped as a rough variant. False-negative PCR reactions using the same sample preparation were repeated in the presence of 30 and 0 instead of 300 initial copies of the IAC. Of these, five were positive in the presence of 30 copies IAC, and one in the absence of an IAC. Two could not be detected. After diluting the DNA extract by fivefold, a weak PCR positive result was obtained from one of the two samples. However, all eight samples showed a weak amplification of the IAC indicating inhibitory substances in the PCR reaction or the presence of high concentrations of background microorganisms. In all other negative samples, the IAC was clearly visible. Purified *Salmonella* isolates of the two PCR negative samples could be readily detected by PCR.

## 4. Discussion

In a previous study, a PCR assay for the detection of *Salmonella* was evaluated and validated in an international collaborative study showing an analytical accuracy of 98% (Malorny et al., 2003b). This study is directed to determine the diagnostic accuracy of the PCR-based method for the detection of *Salmonella*, which was assessed in four European laboratories, and will contribute to an international standard. The relative diagnostic accuracy of 435 samples obtained from pig and broiler carcasses, in broiler houses and from raw meat was at least 97.5% and showed the applicability of the PCR-based method as alternative to the traditional culture method. The sample treatment comprised nonselective preenrichment in BPW followed by a thermal cell lysis using Chelex 100, a common chelating resin that has a high affinity for polyvalent metal ions (chelating agent). The procedure is simple, rapid, involves no organic solvents, and can be done in the same tube which decrease the risk of cross-contamination. The Chelex 100 extraction method has been previously described for the successful extraction of amplifiable *Salmonella* DNA from various food samples (Brasher et al., 1998; Cano et al., 1993; Fach et al., 1999; Kimura et al., 1999; Soumet et al., 1999). The combination of preenrichment and DNA extraction overcomes the detection of dead cells in the sample by diluting them in the preenrichment medium, and Chelex 100 removes and neutralizes many PCR inhibitory substances (Walsh et al., 1991).

However, the diagnostic accuracy of the selected naturally contaminated samples varied between the matrix categories. Whereas food associated samples (swabs from meat, whole broiler carcass-rinse, meat) showed an accuracy of 99–100%, the accuracy in environmental samples obtained in broiler houses decreased down to 91%. This indicates that the sample treatment used here for environmental samples works not optimal. Environmental samples from broiler houses often include faeces, which is known as a PCR inhibitor (Widjojoatmodjo et al., 1992). The Chelex 100 extraction method seems not to sufficiently neutralize such inhibitors in all cases. Two out of six *Salmonella* culture-positive neck-skin samples obtained from broilers were negative in PCR. Since only eight neck-skin samples were investigated, it is not possible to conclude that this method does not work for the food category neck-skin. More samples have to be investigated in future.

The addition of an IAC in each PCR tube is becoming mandatory according to draft standards (Anonymous, 2002a), if the PCR is used as diagnostic tool for the indication of PCR inhibitors derived from the sample template. The screening of the samples investigated here showed that the IAC works well in practise. Weak or no amplification of the IAC in 8 of the 435 samples, which were initially *Salmonella*-negative by PCR but positive by traditional culture method, indicated the presence of PCR inhibitors in the PCR reaction.

Background microorganisms occurring in the food matrix can negatively influence the growth of the target pathogen (Beckers et al., 1987; Thisted et al., 1998, 2000). Under the conditions used here, the PCR detection limit was not influenced by background microorganisms as *Salmonella* cells grew well even in the presence of high levels of background flora. However, we are aware that the use of well-grown *Salmonella* and other bacterial cells may not reflect completely the natural situation in food samples. The degree and type of sublethally injured or stressed *Salmonella* cells can influence the recovery and consequently the detection limit (Wuytack et al., 2003).

In conclusion, the PCR-based method, presented here, can be useful as a more rapid screening tool to sort out negative samples at an earlier stage. After preenrichment, the sample preparation and PCR can be immediately performed within 3–4 h. PCR positive cases can be then continued by selective enrichment and further isolation of the *Salmonella* strains. The interlaboratory validation results support the potential of the method as international standard.

## Acknowledgements

The work was supported by the European Commission, proposal no. PL QLRT-1999-00226.

We thank Dr. M. Garriga and Dr. T. Aymerich as experts, and C. van Heerwaarden, A. Zwartkruis-Nahuis, F. Dilasser, J. Grout, M. Haarmann and C. Dittmar-Gabor for excellent technical assistance.

## References

Aabo, S., Rasmussen, O.F., Rossen, L., Sorensen, P.D., Olsen, J.E., 1993. *Salmonella* identification by the polymerase chain reaction. Mol. Cell. Probes 7, 171–178.

Anonymous, 1993. Microbiology of Food and Animal Feeding Stuffs—Horizontal Method for the Detection of *Salmonella* (ISO 6579:1993). International Organization for Standardization, Geneva, Switzerland.

Anonymous, 1997. Microbiology of Food and Animal Feeding Stuffs—Horizontal Method for the Detection of *Salmonella* (EN 12824:1997). European Committee for Standardization, Brussels, Belgium.

Anonymous, 2000. Microbiology of Food and Animal Feeding Stuffs—Preparation of Test Samples, Initial Suspension and Decimal Dilution for Microbiological Examination—Part 2: Specific Rules for the Preparation of the Initial Suspension and Decimal Dilutions of Meat and Meat Products. International Organization for Standardization, Geneva, Switzerland.

Anonymous, 2002a. Microbiology of Food and Animal Feeding Stuffs—Polymerase Chain Reaction (PCR) for the Detection of Food-Borne Pathogens—General Method Specific Requirements (prEN 22174). International Organization for Standardization, Geneva, Switzerland.

Anonymous, 2002b. Microbiology of Food and Animal Feeding Stuffs—Protocol for the Validation of Alternative Methods (EN ISO 16140). European Committee for Standardization, Paris, France.

Bäumler, A.J., Heffron, F., Reissbrodt, R., 1997. Rapid detection of *Salmonella enterica* with primers specific for *iroB*. J. Clin. Microbiol. 35, 1224–1230.

Beckers, H.J., Heide, J.V.D., Fenigsen-Narucka, U., Peters, R., 1987. Fate of salmonellas and competing flora in meat sample enrichments in buffered peptone water and in Müller–Kauffmann's tetrathionate medium. J. Appl. Bacteriol. 62, 97–104.

BgVV-FAO/WHO Collaborating Centre for Research and Training in Food Hygiene and Zoonoses, 2000. WHO surveillance programme for control of foodborne infections and intoxications in Europe: Seventh Report 1993–1998, http://www.bfr.bund.de, Berlin, Germany.

Brasher, C.W., DePaola, A., Jones, D.D., Bej, A.K., 1998. Detection of microbial pathogens in shellfish with multiplex PCR. Curr. Microbiol. 37, 101–107.

Burkhalter, P.W., Müller, C., Luthy, J., Candrian, U., 1995. Detection of *Salmonella* spp. in eggs: DNA analyses, culture techniques, and serology. J. AOAC Int. 78, 1531–1537.

Cano, R.J., Rasmussen, S.R., Sánchez, F.G., Palomares, J.C., 1993. Fluorescent detection-polymerase chain reaction (FD-PCR) assay on microwell plates as a screening test for salmonellas in foods. J. Appl. Bacteriol. 75, 247–253.

Cohen, N.D., Neibergs, H.L., Wallis, D.E., Simpson, R.B., McGruder, E.D., Hargis, B.M., 1994. Genus-specific detection of salmonellae in equine feces by use of the polymerase chain reaction. Am. J. Vet. Res. 55, 1049–1054.

Ellerbroek, L.H., Haarmann, M., Wichman-Schauer, H., Malorny, B., 2002. Studies to optimise the detection of *Salmonella* in fattening poultry flocks. Fleischwirtschaft 3, 47–48.

Fach, P., Dilasser, F., Grout, J., Tache, J., 1999. Evaluation of a polymerase chain reaction-based test for detecting *Salmonella* spp. in food samples: Probelia *Salmonella* spp. J. Food Prot. 62, 1387–1393.

Humphrey, T., 2000. Public-health aspects of *Salmonella* infection.

In: Way, C., Way, A. (Eds.), Salmonella in Domestic Animals. CABI Publishing, Oxon, UK, pp. 245–263.

Jones, D.D., Law, R., Bej, A.K., 1993. Detection of *Salmonella* spp. in oysters using polymerase chain reaction (PCR) and gene probes. J. Food Sci. 58, 1191–1197.

Kimura, B., Kawasaki, S., Fujii, T., Kusunoki, J., Itoh, T., Flood, S.J., 1999. Evaluation of TaqMan PCR assay for detecting *Salmonella* in raw meat and shrimp. J. Food Prot. 62, 329–335.

Kitchell, A.G., Ingram, G.C., Hudson, W.R., 1973. Microbiological sampling in abattoirs. S.A.B. Technical Series, vol. 7. Academic Press, London, UK, pp. 43–61.

Kwang, J., Littledike, E.T., Keen, J.E., 1996. Use of the polymerase chain reaction for *Salmonella* detection. Lett. Appl. Microbiol. 22, 46–51.

Malorny, B., Schroeter, A., Bunge, C., Hoog, B., Steinbeck, A., Helmuth, R., 2001. Evaluation of molecular typing methods for *Salmonella enterica* serovar *typhimurium* DT104 isolated in Germany from healthy pigs. Vet. Res. 32, 119–129.

Malorny, B., Tassios, P.T., Rådström, P., Cook, N., Wagner, M., Hoorfar, J., 2003a. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int. J. Food Microbiol. 83, 39–48.

Malorny, B., Bunge, C., Hoorfar, J., Helmuth, R., 2003b. Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. Appl. Environ. Microbiol. 69, 290–296.

Miller, J.H., 1972. Experiments in molecular genetics. Formulas and Recipes, 3rd ed. Cold Spring Harbor, New York, p. 433.

Rahn, K., De Grandis, S.A., Clarke, R.C., McEwen, S.A., Galán, J.E., Ginocchio, C., Curtiss III., R., Gyles, C.L., 1992. Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. Mol. Cell. Probes 6, 271–279.

Soumet, C., Ermel, G., Rose, N., Rose, V., Drouin, P., Salvat, G., Colin, P., 1999. Evaluation of a multiplex PCR assay for simultaneous identification of *Salmonella* sp., *Salmonella enteritidis* and *Salmonella typhimurium* from environmental swabs of poultry houses. Lett. Appl. Microbiol. 28, 113–117.

Thisted, L.S., Ballagi-Pordány, A., Lindqvist, R., 1998. A mimic as internal standard to monitor PCR analysis of food-borne pathogens. Lett. Appl. Microbiol. 26, 9–11.

Thisted, L.S., Lindqvist, R., Ballagi-Pordány, A., Danielsson-Tham, M.-L., 2000. A combined culture and PCR method for detection of pathogenic *Yersinia enterocolitica* in food. Int. J. Food Microbiol. 57, 63–73.

Wallace, D.J., Van Gilder, T., Shallow, S., Fiorentino, T., Segler, S.D., Smith, K.E., Shiferaw, B., Etzel, R., Garthright, W.E., Angulo, F.J., 2000. Incidence of foodborne illnesses reported by the foodborne diseases active surveillance network (FoodNet)—1997. FoodNet working group. J. Food Prot. 63, 807–809.

Walsh, P.S., Metzger, D.A., Higuchi, R., 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques 10, 506–513.

Widjojoatmodjo, M.N., Fluit, A.C., Torensma, R., Verdonk, G.P., Verhoef, J., 1992. The magnetic immunopolymerase chain reaction assay for direct detection of salmonellae in fecal samples. J. Clin. Microbiol. 30, 3195–3199.

Wuytack, E.Y., Phuong, L.D., Aertsen, A., Reyns, K.M., Marquenie, D., De Ketelaere, B., Masschalck, B., Van Opstal, I., Diels, A.M.J., Michiels, C.W., 2003. Comparison of sublethal injury induced in *Salmonella enterica* serovar *typhimurium* by heat and by different nonthermal treatments. J. Food Prot. 66, 31–37.