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Development and validation of a rapid real-time PCR based method for the specific detection of Salmonella on fresh meat

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Abstract

In this study, a combined enrichment/real-time PCR method for the rapid detection of Salmonella on fresh meat carcasses, was designed, developed and validated in-house following requirements outlined in ISO 16140:2003. The method included an 18 h non-selective enrichment in buffered peptone water (BPW) and a 6 h selective enrichment in Rappaport Vasilliadis Soya (RVS) broth, based on the traditional culture method, ISO 6579:2002. The real-time PCR assay included an internal amplification control (IAC), was 100% specific and was sensitive to one cell equivalent. The alternative method was validated against the traditional culture method and relative accuracy of 94.9%, sensitivity of 94.7% and specificity of 100% were determined using 150 fresh meat carcass swabs. This alternative method had a detection limit of 1–10 CFU/100 cm² for fresh meat carcass swabs and was performed in 26 h. Following further inter-laboratory studies, this alternative method could be suitable for implementation in testing laboratories for the analysis of carcass swabs.

1. Introduction

Salmonella is one of the most prevalent foodborne pathogens and infects over 160,000 individuals in the EU annually, with an incidence rate of 35 cases per 100,000 (Anonymous, 2007a). The annual cost of foodborne Salmonella is believed to reach up to €2.8 billion per year (Anonymous, 2003c). Reports from the World Health Organisation surveillance programme for control of foodborne infections and intoxications in Europe, revealed the majority of outbreaks, where causative agents were reported, were caused by Salmonellae serotypes (Anonymous, 1998, 2000).

Salmonellae are most often associated with any raw food of animal origin which may be subject to faecal contamination, such as raw meat, poultry, fish/seafod, eggs and dairy (Anonymous, 2007c). Salmonella testing in the slaughter environment is important as intestinal pathogens are carried into the abattoir in the bowels and on the skin of the animals (Wray, 2000). Although total viable counts (TVC) and Enterobacteriaceae testing are routinely performed on fresh meat carcasses, there was no requirement to test for Salmonella contamination prior to 2006 (Anonymous, 2005a).

Good hygiene practice (GHP) and a hazard analysis critical control point (HACCP) system must be employed to ensure minimal microbial contamination of meat carcasses during slaughter (Bolton et al., 2002). Microbiological food testing is then used to validate and verify these HACCP based procedures. The traditional culture based method for the detection of Salmonella is labour intensive and time-consuming, taking greater than 5 days to determine a positive result.Alternative analytical methods, in particular, more rapid methods are permissible by regulatory authorities once they have been validated against the reference method (Anonymous, 2003a, 2005a).

A number of real-time PCR based assays for the detection of Salmonella have been developed and published in recent years (Ellington, Anderson, Carlson, & Sharma, 2004; Hoorfar, Ahrens, & Rådström, 2000; Malorny et al., 2004; Moore & Feist, 2007). Earlier assays lacked appropriate controls such as an internal amplification control (IAC), which is now becoming mandatory (Hoorfar et al., 2003, 2004; Moore & Feist, 2007). Others were not validated against traditional culture methods as described in ISO 16140 (Anonymous, 2003a) and/or did not meet diagnostic PCR requirements outlined in ISO 22174 (Anonymous, 2005b). Although these
was then centrifuged (5000 g) and stored at 4°C until use. DNA isolations were performed as described below and stored in 1 ml phosphate buffered saline (PBS; Oxoid). DNA isolations were performed using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) following manufacturer’s instructions. Total genomic DNA was quantified using the TBS-380 mini-fluorometer (Turner Biosystems, California, USA) and PicoGreen dsDNA quantitation kit (Invitrogen Corporation, California, USA).

2.5. Conventional PCR

Conventional PCR was performed using the iCycler iQ thermal cycler (Bio-Rad Laboratories Inc., California, USA). Reactions were performed in 50 μl volumes consisting of: 10X buffer (containing 15 mM MgCl₂), 1 μl Taq DNA polymerase (1 U/μl; Roche Diagnostics, Mannheim, Germany), 1 μl dNTP mix (10 mM; deoxynucleoside triphosphate set – Roche Diagnostics), 1 μl forward and reverse primers (20 μM), 39 μl nuclease free H₂O (Applied Biosystems/Ambion, Texas, USA) and 2 μl PCR template (genomic DNA). The cycling parameters consisted of 30 cycles of denaturation at 94°C (30 s), annealing at 50°C (60 s) and extension at 72°C (30 s) followed by a final extension at 72°C for 10 min.

2.6. Generation of sequence data

Salmonella sequencing primers, Entero-tmF and Entero-tmR, were supplied by MWG Biotech, Ebersberg, Germany (Table 2). PCR products were purified using the high pure PCR product purification kit (Roche Diagnostics Ltd., West Sussex, UK), and sent for sequencing (Sequiseerve, Vaterstetten, Germany). Sequence alignments were performed using Clustal W multiple sequence alignment programme (http://www.ebi.ac.uk/tools/clustalw/index.html).

2.7. Primer and probe design for Salmonella real-time PCR assay

Salmonella assay primers and probes were designed following alignment of ssaR gene sequences of Salmonella serovars and related organisms (Table 2) according to general guidelines and recommendations (Dorak, 2006; Rybicki, 2001). The primers and probes were suspended in nuclease free water to a concentration of 100 μM and stored at −20°C.

2.8. Real-time PCR

Real-time PCR reactions were performed on the LightCycler® 2.0 Instrument (Roche Diagnostics) using the LightCycler® FastStart DNA Master HybProbe kit (Roche Diagnostics). PCR was performed in a final volume of 20 μl including 2 μl DNA templates and 2 μl of iAC template in LightCycler hybridisation buffer with MgCl₂ adjusted to 5 mM concentration. Uracil-DNA glycosylase (Roche Diagnostics; 0.3 μl), PCR primers (0.5 μM final concentration) and probes (0.2 μM final concentration) were added to the reaction mixture. The volume was adjusted to 20 μl with nuclease free H₂O. Real-time PCR cycling conditions consisted of incubation for 10 min at 95°C followed by 50 amplification cycles with denaturation for 10 s at 95°C and an annealing/extension for 30 s at 64°C. Prior to sample analysis, a colour compensation file was generated on the LightCycler following the protocol outlined in Technical Note No. LC 21/2007 (Anonymous, 2007d).

2.9. Internal amplification control

The E. coli ssaR gene was amplified using Salmonella assay primers (Table 2). This PCR product was ligated into a plasmid and cloned into chemically competent E. coli cells using the pCR®2.1-TOPO® TA cloning kit (Invitrogen) according to manufacturer’s instructions. Plasmid purification was carried out using the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer’s
instructions. The optimum number of IAC plasmids to be included in the real-time PCR reaction was determined by performing titrations of serial dilutions of the IAC plasmid (10^4–10^1 copies) with known numbers of Salmonella (10^3–10^6 cell equivalents).

### 2.10. Validation

The combined enrichment/real-time PCR method i.e. the alternative method was validated against the traditional culture method ISO 6579 (Anonymous, 2002), in accordance with ISO 16140 (Anonymous, 2003a).

#### 2.10.1. Phase 1 – validation in pure culture

Phase 1 validation was performed using pure cultures of 30 Salmonella strains and 30 non-Salmonella species/strains (closely related species or common meat microflora). One hundred millilitres of BPW was inoculated with ~1 CFU for Salmonella serovars (final concentration of 0.01 CFU/ml) and ~1000 CFU/ml for non-Salmonella species/strains (final concentration of 10 CFU/ml). Approximate cell density of each test strain was established by plate counts. Following inoculation of the BPW, presumptive inoculation figures were confirmed by spread plating 100 µl of inoculum onto TSA followed by plate counts. Following 18 h enrichment in BPW, reference and alternative methods were performed in parallel.

#### 2.10.2. Phase 2 – validation in spiked carcass swabs

Fresh meat carcass swabs were collected in local abattoirs. Four sites on the carcass were swabbed (4 × 100 cm²) using pre-moistened sterile sponge swabs (Technical Service Consultants Limited, Technical Service Consultants Limited, National Collection of Dairy Organisms c/o NCIMB Ltd., Aberdeen, Scotland, United Kingdom, and the National Collection of Dairy Organisms c/o NCIMB Ltd., Aberdeen, Scotland, United Kingdom).
Heywood, Lancashire, UK) i.e. neck, brisket, flank and rump for beef; jowl, back, belly and ham for pork. Two swabs, front and back, were used to sample each carcass and then placed together in a sterile bag (i.e. one sample). A minimum of 25 carcasses were swabbed on each visit to the abattoir (i.e. one replicate). Six replicates of fresh carcass swabs (three beef and three pork) were collected for this study. Samples were immediately placed on ice. Sample analysis was performed after a maximum of 24 h after sample collection.

Each carcass swab was tested for naturally occurring Salmonella according to ISO 6579 (Anonymous, 2002) and Enterobacteriaceae according to ISO 21528-2 (Anonymous, 2004) to determine the level of background contamination (Fig. 3). Violet red bile green agar (VRBA; Oxoid) and glucose agar (Mast Group Ltd., Merseyside, UK) were used in Enterobacteriaceae enumeration.

In parallel, carcass swabs (homogenised in BPW) were inoculated with five different Salmonella strains (Derby, Dublin, Livingstone, Typhimurium, Typhimurium DT104) at five inoculation levels (1, 10, 100, 1000, 5000 CFU/100 cm²). These cultures were grown for 18 h at 37 °C in BPW followed by incubation for 4 h at 4 °C. The spiked samples were then tested for the presence of Salmonella using the ISO culture based method and the alternative molecular method as previously described.

### 3. Results

#### 3.1. Assay design and development

*In-silico* analysis of Salmonella *ssrA* sequence data revealed that there was no single probe-binding site that would enable detection of all *Salmonella* serovars. One probe region (SAM 2) was suitable for the detection of 29/30 *Salmonella* strains (25/26 serovars), however *Salmonella* Anatium had a single mismatch in the probe region which would lead to a false negative result. A second probe region (SAM 1) was required to detect *S. Anatium*. Fig. 1 illustrates the two probe-binding regions necessary for the detection of all *Salmonella* serovars.

*Salmonella* specific probes were designed to have similar melting temperatures (Table 2) and both probes were labelled with the same fluorophore and quencher molecules. Detection of *Salmonella* can result from three probe combinations i.e. SAM 1 only, SAM 2 only or SAM 1 + SAM 2. The SAM 1 probe detected...
16/30 of the strains used in this study while the SAM 2 probe detected 29/30 strains.

An IAC probe was designed to detect the ssrA gene of *Escherichia coli* and closely related species belonging to the Enterobacteriaceae family including *Salmonella*. The IAC probe region was common to all but one of the Enterobacteriaceae strains (i.e. *Enterobacter aerogenes*) examined. One hundred copies of the IAC plasmid (containing the 286 bp *E. coli* ssrA gene fragment) was determined to be the optimum concentration for use in the real-time PCR assay, such that the IAC would be detected without affecting detection of the primary *Salmonella* target.

3.2. Performance of the ssrA *Salmonella* real-time PCR assay

A detection limit of 1–10 genome equivalents was determined for three *Salmonella* serovars, representing the three probe-binding combinations. Average PCR amplification efficiency from nine sensitivity experiments was ~100%.

Inclusivity of the *Salmonella* real-time PCR assay was confirmed using 100 ng genomic DNA from 30 *Salmonella* strains (Fig. 2). A negative control (*E. coli* ATCC 25922) and a no-template control were included in the assay. Exclusivity of the assay was confirmed using 100 ng genomic DNA from 30 non-*Salmonella* species/strains (previously determined to be PCR amplifiable), a positive control (*Salmonella* Dublin NCTC 09676) and a no-template control. Inclusivity and exclusivity was not affected by the inclusion of 100 copies of IAC plasmid.

3.3. Validation

Validation of the alternative method was performed according to ISO 16140 ([Anonymous, 2003a](#)) in pure culture and in spiked carcass swabs. A blind sample study was also performed using spiked samples prepared by an independent laboratory.

3.3.1. Phase 1 – validation in pure culture

All *Salmonella* serovars grown in pure culture (according to 2.10.1) were detected using the alternative method while the non-*Salmonella* species/strains were not detected. Samples analysed in parallel using the traditional culture method, ISO 6579:2002, yielded identical results.

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**Fig. 1.** Sequence alignment of three *Salmonella* serovars and five non-*Salmonella* species at SAM 1 and SAM 2 probe regions. Left: SAM 1 probe region (light grey) highlighting mismatches (dark grey) with one *Salmonella* serovar (*S. Agona*) and closely related species. Right: SAM 2 probe region (light grey) highlighting mismatches (dark grey) with one *Salmonella* serovar (*S. Anatum*) and closely related species.

**Fig. 2.** Amplification curves demonstrating (a) inclusivity of the *Salmonella* real-time PCR assay and (b) IAC detection for all samples in the same experiment.
3.3.2. Phase 2 – validation in spiked carcass swabs

One hundred and fifty carcass swabs were inoculated with *Salmonella* and tested according to Section 2.10.2. None of the swabs were naturally contaminated with *Salmonella*, the majority contained Enterobacteriaceae at varying levels, and all swabs contained some microbial contamination. In the presence of such natural background flora, the alternative method had a relative accuracy of 94.9%, sensitivity of 94.7% and specificity of 100% when compared to the traditional ISO method. Representative results (S. Typhimurium ATCC 14028 replicate 3) are shown in Fig. 4. Table 3 summarises results from the three beef and three pork carcass swab replicates tested using the alternative method. The *Salmonella* detection probability was 73% when carcass swabs were spiked with 1 CFU/100 cm², and 100% when spiked with ≥10 CFU/100 cm².

3.3.3. Blind sample study

As the resources were not available to perform an inter-laboratory trial to complete the validation of the alternative method (i.e. participation of at least 10 collaborative laboratories as required by ISO 16140:2003), a small blind sample study was performed as described in Section 2.11.3. Cotton swabs (n = 24) were spiked with varying levels of *Salmonella*, with and without artificially introduced background Enterobacteriaceae.

With the exception of one swab, samples were identified correctly using the alternative method (Table 4), i.e. results reflected inoculation data which was received post-analysis. Sample 22 contained ~1 CFU/ml of *Salmonella* Derby and ~1 CFU/ml of *Salmonella* Typhimurium (the lowest inoculum tested), in a background of ~10 CFU/ml of *E. coli* and was not detected by the alternative method.

4. Discussion

Although there has been a fall in the number of human *Salmonella* infections in Europe over the past number of years (Anonymous, 2007a), foodborne infection from *Salmonella* continues to pose a great risk to public health. It is widely believed that pathogen reduction in animals, efficient Quality Control Systems (e.g. HACCP principles and GHP) and Quality Assurance (QA) at all stages of the food chain from “farm to fork” is the most effective way to prevent the spread of infection via food (Anonymous, 2003b). The presence of *Salmonella* on fresh meat carcasses was addressed by the European Union in 2001 and new regulatory microbiological criteria was published in 2005 (Anonymous, 2005a). According to this regulation, from a total of 50 carcasses tested, a maximum of two beef or five pork carcasses can be positive for *Salmonella*.

The aim of this research was to develop a rapid test for the detection of *Salmonella* on fresh meat carcasses. A real-time PCR assay, targeting the *ssrA* gene, combined with a two step enrichment for the detection of *Salmonella* was developed and compared to the traditional culture based method, ISO 6579:2002 (Anonymous, 2002).

*ssrA* is present as a single copy gene in all sequenced bacterial genomes (Keiler et al., 2000; Moore & Sauer, 2007). Conserved regions at the extremities flank divergent sequences, making the

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Fig. 3. Flow chart demonstrating steps involved in validation of alternative molecular method to traditional ISO method for spiked carcass swabs (Phase 2).
gene an ideal target for nucleic acid diagnostics (O’Grady et al., 2008). The ssrA gene has been demonstrated as a suitable diagnostic target for the detection of Listeria monocytogenes in enriched food samples (O’Grady et al., 2008, 2009).

There was limited heterogeneity in the ssrA gene between genera of the Enterobacteriaceae family, making specific assay design for Salmonella challenging. Two Salmonella specific TaqMan probes were required to achieve 100% specificity. As there is only one base difference between Salmonella serovars and related species in the two probe regions, the mismatch was placed in the centre of the sequence to increase probe differentiation ability (1). It has been reported that a C–A mismatch is the most destabilising mismatch, reducing the melting temperature of the probe of the non-target sequence by 8 °C (Lay & Wittwer, 1997). The SAM 1 and SAM 2 probes both contain a central C–A mismatch.

An IAC was included in the assay to control for the presence of inhibitory substances, malfunctions with the thermocycler, poor enzyme activity and incorrect reaction mixture which can lead to false negative results (Rossen, Norskov, Holmstrom, & Rasmussen, 1992). As the Salmonella assay primers used to amplify the target and IAC are not Salmonella specific, the presence of background Enterobacteriaceae on meat carcasses can cause downstream competition for primers between the target sequence, background Enterobacteriaceae, and the IAC. In cases where Salmonella are absent on the carcass swabs, competition arises for primers between Enterobacteriaceae and low copy numbers of the IAC. The IAC probe will bind to the ssrA sequence of the Enterobacteriaceae, thus eliminating the risk of an invalid result.

In the presence of a high concentration of exclusively E. aerogenes DNA an invalid result was obtained. This is because the IAC probe does not bind to the E. aerogenes ssrA gene. This situation is almost certain never to arise however, as it would be extremely unlikely to collect an environmental sample containing a pure culture of E. aerogenes.

To increase the concentration of the target organism and to ensure detection of viable bacteria, samples were homogenised and enriched in non-selective BPW (1:10 dilution) followed by semi-selective enrichment in RVS (1:100 dilution). The significant dilution factor incorporated as a result of the enrichment steps (1:1000 dilution) minimises the detection of any initial non-culturable or dead cells. Possible biological contaminants such as blood and fats present on animal carcasses which can cause PCR inhibition are also diluted. The bacterial cell pellet was washed in PBS prior to DNA isolation to reduce the concentration of enrichment medium which may also cause PCR inhibition (Rodriguez-Lazaro & Hernandez, 2006).

Of the 2500 or more Salmonella serovars, the majority (2300) belong to Salmonella enterica subspecies enterica, from which 30 strains were chosen for validation as required by ISO 16140. Salmonella Enteritidis and Salmonella Typhimurium were the two most common serovars isolated from humans, pigs and cattle in 2006 in the EU. The 10 most frequently isolated serovars from humans in the European Union in 2006 and other serovars commonly isolated from cattle, pigs and feedstuffs were included in the Salmonella panel used in this study (Anonymous, 2007a).

While it was of the highest priority to find naturally contaminated samples for the purpose of validation, the 75 porcine and 75 bovine samples collected in abattoirs for this study tested negative for naturally contaminating Salmonella using the traditional ISO culture method (Anonymous, 2002). This may be explained by the fact that a relatively low number of Salmonellae are isolated from animal sources in Ireland annually (Anonymous, 2007b). There were 653 non-human Salmonella isolates submitted to the National Salmonella Reference Laboratory (NSRL) Ireland in 2007 including 304 swine and 12 bovine isolates, with serovars Typhimurium, Derby, Dublin and Infantis being most frequently isolated.

As naturally contaminated samples were unavailable, carcass swabs containing natural flora were spiked with five Salmonella strains at five inoculum levels and used for validation. The Salmonella cells were cold-shocked by placing the bacterial cultures at 4 °C for 4 h prior to spiking. This stressing of the

Fig. 4. Phase 2 validation – representative results (third replicate; 1 strain): Amplification curves demonstrating (a) detection of 5/5 pork carcass swabs spiked with S. Typhimurium ATCC 14028 ranging from 1–5000 CFU per 100 cm² and (b) IAC detection for all samples in same experiment.
inoculum was performed to mimic the conditions experienced by naturally contaminating cells in the sample environment.

Validation of the alternative method was performed in pure culture and spiked carcass swabs according to ISO 16140 (Anonymous, 2003a). In pure culture, the alternative method and the traditional method yielded identical results. In spiked carcass swabs, the alternative method had a relative sensitivity of 94.7% and specificity of 100% when compared to the traditional method. There does not appear to be a correlation between Salmonella inoculum levels used to spike carcass swabs and the corresponding crossing threshold (Ct) values determined using the Salmonella real-time PCR assay e.g. a swab spiked with 1000 CFU/100 cm² had a later Ct value than a swab spiked with 10 CFU/100 cm² (Fig. 4). This is possibly due to varying levels of background microflora present on the swabs and the resultant competition that would arise during enrichment.

A blind sample study was performed and only one sample was not correctly identified. As the number of Salmonella present in the inoculum used to spike the samples was estimated rather than experimentally determined, it was not possible to determine whether the 1 CFU/ml inocula contained any Salmonella cells. The traditional culture method was not performed in parallel; therefore this result could not be confirmed.

This rapid Salmonella test can be performed in 26 h, a significant reduction in labour and turnaround time compared with the 5 days necessary to perform the traditional culture method. The alternative method could currently be used as a screening method however, following an inter-laboratory trial, this assay has the potential to become a standardised method for routine analysis of carcass swabs for the presence of Salmonella. It is reassuring from the perspective of both the fresh meat producer and the consumer, that throughout this study no Salmonella was found on fresh meat carcasses. However, carcasses from herds which tested positive for Salmonella could be investigated to increase the likelihood of detecting naturally contaminated samples. The ability of the alternative method to specifically detect Salmonella with a sensitivity of 1–10 CFU/100 cm² while vastly reducing the analysis time would make it a valuable asset to the food testing industry.

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