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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 cm^2 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 food-borne infections and intoxications in Europe, revealed the majority of outbreaks, where causative agents were reported, were caused by *Salmonella* 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/seafood, 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 (Ellingson, 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

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standards are not compulsory, their implementation could encourage the international acceptance of validated alternative methods (Malorny et al., 2003).

Commercially available real-time PCR based kits for the detection of *Salmonella* include the BAX System (Oxoid), the LightCycler *Salmonella* detection kit (Roche) and the TaqMan® *Salmonella* Gold detection and quantitation kit (Applied Biosystems). The Oxoid system is ISO accredited and the Roche and Applied Biosystems kits are AOAC approved and all have been independently evaluated (Cheung, Chan, Wong, Cheung, & Kam, 2004; Cheung, Kwok, & Kam, 2007).

A *Salmonella* real-time PCR assay was developed in this study, targeting the *ssrA* gene, a novel nucleic acid diagnostic target for bacterial detection and identification (O' Grady, Sedano-Balbas, Maher, Smith, & Barry, 2008). The *ssrA* gene codes for transfer messenger RNA (tmRNA) and has been identified in all sequenced bacterial genomes (Keiler, Shapiro, & Williams, 2000; Moore & Sauer, 2007). tmRNA has many diverse functions, including tagging of abnormal proteins for degradation and modulating the activity of DNA binding proteins (Julio, Heithoff, & Mahan, 2000).

The real-time PCR assay was combined with a modified enrichment procedure for the rapid detection of *Salmonella* on fresh meat. This alternative method was validated against the traditional culture method following requirements outlined in ISO 16140 (Anonymous, 2003a), ISO 6579 (Anonymous, 2002) and ISO 22174 (Anonymous, 2005b) standards.

2. Methods

2.1. Bacterial strains and culture conditions

A total of 30 *Salmonella* strains (representing 26 serovars) and 30 non-*Salmonella* species/strains were used (Table 1) for inclusivity and exclusivity testing respectively, as required by ISO 16140 (Anonymous, 2003a). All strains were incubated in tryptone soya broth (TSB; Oxoid, Hampshire, UK) at 30 °C or 37 °C, as appropriate.

2.2. Traditional culture method – ISO 6579:2002

The traditional culture method (reference method) was performed as described in ISO 6579:2002. The following materials were used: Buffered peptone water (BPW; Oxoid), Rappaport Vasilliadis Soya (RVS; Oxoid), Müller Kauffmann Tetrathionate with novobiocin broth (MKTTn; AES Laboratoire, Bron, France), xylose–lysine-desoxycholate (XLD; Oxoid), brilliant green agar (BGA; Oxoid), tryptone soya agar (TSA; Oxoid), API 20E strips (Marcy l'Etoile, France), API James (Biomerieux), API TDA (Biomerieux), API VP1/VP2 (Biomerieux), identification sticks oxidase (Oxoid), hydrogen peroxide 4% (Sigma–Aldrich, Missouri, USA). Positive and negative controls (*Salmonella* Typhimurium ATCC 14028 and *E. coli* ATCC 25922, respectively) were also included.

2.3. Modified culture method

The modified culture method required primary enrichment in BPW (100 ml) for 18 h at 37 °C. Following incubation, 100 μl of pre-enrichment culture was transferred to 10 ml RVS and incubated at 42 °C for 6 h. One millilitre of *Salmonella*/RVS culture was then centrifuged (5000*g* for 10 min) and the cell pellet washed in 1 ml phosphate buffered saline (PBS; Oxoid). DNA isolations were performed as described below and stored at −20 °C until use.

2.4. DNA isolation and quantification

Genomic DNA was prepared from 1 ml of TSB or RVS cultures. 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 $MgCl_2$), 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 H_2O (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-tm F and Entero-tm R, 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 (Sequiserve, 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 *ssrA* 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 $MgCl_2$ 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 H_2O. 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 ssrA* 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

Table 1
Salmonella and non-*Salmonella* serovars/strains used in real-time based assay design and validation.

Salmonella Serovar	Strain	Non-*Salmonella* Organism	Strain
Agona	NCTC 11377[a]	*Acinetobacter calcoaceticus*	ATCC 23055[c]
Anatum	SARB 2[b]	*Aeromonas hydrophila*	ATCC 35654[c]
Braenderup	NCTC 05750[a]	*Arthrobacter globiformis*	ATCC 8010[c]
Bredeney	NCTC 05731[a]	*Bacillus cereus*	NCTC 07464[a]
Derby	SARB 11[b]	*Citrobacter diversus*	CCFRA7119[g]
Dublin	NCTC 09676[a]	*Citrobacter freundii*	NCTC 8090[a]
Enteritidis	ATCC 13076[c]	*Citrobacter freundii*	NCTC 09750[a]
Enteritidis PT4	NCTC 13349[a]	*Citrobacter koseri*	NCTC 10768[a]
Gallinarum	NCTC 423,287/91[a]	*Enterobacter aerogenes*	NCTC 10006[a]
Gallinarum	NCTC 13346[a]	*Enterobacter agglomerans*	NCTC 09381[a]
Goldcoast	NSRL[d]	*Enterobacter cloacae*	NCTC 11933[a]
Hadar	ULjub MI 2[e]	*Enterobacter intermedius*	NDC 427[f]
Heidelberg	NCTC 5717[a]	*Enterobacter sakazaki*	NCTC 11467[a]
Infantis	Uljub VF 35/94[e]	*Enterococcus faecalis*	NCTC 12697[a]
Kentucky	NCTC 05799[a]	*Enterococcus faecium*	ATCC 35667[c]
Livingstone	NCTC 09125[a]	*Escherichia coli*	ATCC 25922[c]
London	NCTC 05777[a]	*Escherichia coli*	NDC 544[f]
Manhattan	NCTC 06245[a]	*Escherichia coli*	NCTC 09001[a]
Newport	SARB 36[b]	*Klebsiella oxytoca*	ATCC 43086[c]
Nottingham	NCTC 07832[a]	*Klebsiella pneumoniae*	ATCC 13883[c]
Panama	SARB 40[b]	*Lactobacillus plantarum*	ATCC 8014[c]
Saint-Paul	Uljub VF S-13/95[e]	*Leuconostoc mesenteroides*	ATCC 8293[c]
Senftenberg	SARB 59[b]	*Proteus mirabilis*	DSM 4479[h]
Stanley	SARB 60[b]	*Pseudomonas aeruginosa*	NCTC 12903[a]
Typhimurium	ATCC 14028[c]	*Pseudomonas fragi*	DSM 3456[h]
Typhimurium LT 2	NCTC 12416[a]	*Pseudomonas putida*	ATCC 49128[c]
Typhimurium DT 104	NCTC 13348[a]	*Staphylococcus epidermidis*	AFRC[i]
Uganda	NCTC 06015[a]	*Staphylococcus haemolyticus*	ATCC 29970[c]
Virchow	NCTC 05742[a]	*Staphylococcus saprophyticus*	ATCC 15305[c]
Gaminara	NCTC 5797[a]	*Streptococcus lactis*	NCDO 2003[j]

[a] NCTC, National collection of type cultures.
[b] SARB, *Salmonella* reference collection B, University College Cork, Ireland.
[c] ATCC, American type culture collection.
[d] NSRL, National Salmonella Reference Laboratory, University College Hospital Galway, Ireland, ULjub University of Ljubljana, Slovenia.
[e] ULjub, University of Ljubljana, Slovenia.
[f] NDC, National Diagnostic Centre, NUI Galway, Ireland.
[g] CCFRA, Campden and Chorleywood Food Research Association.
[h] DSM, German collection of microorganisms and cell cultures.
[i] AFRC, Ashtown Food Research Centre, Teagasc, Dublin, Ireland.
[j] NCDO, National Collection of Dairy Organisms c/o NCIMB Ltd., Aberdeen, Scotland, United Kingdom.

Table 2
Sequences of primers and probes used in design and development of the *ssrA Salmonella* assay.

Name	Sequence	Size (bp)	T_m (°C)
Entero-tm F	5′-GGGGCTGATTCTGGATTCGA-3′	20	63.6
Entero-tm R	5′-TGGTGGAGCTGGCGGGA-3′	17	67.5
FPF	5′-CCTCGTAAAAAGCCGCA-3′	17	59.1
FPR	5′-GAGTTGAACCCGCGTC-3′	16	59.0
SAM 1	FAM-CAAACGACGAAACCTACGCTTTAGC-BBQ	25	66.8
SAM 2	FAM-AGACTAGCCTGATTCGTTTTAACGCT-BBQ	26	66.6
IAC-Entero	ROX-TCAAACCCAAAAGAGATCGCGTGGA-BHQ2	25	68.9

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* (10^3–10^0 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 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 ($4 \times 100\ cm^2$) using pre-moistened sterile sponge swabs (Technical Service Consultants Limited,

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 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 (VRBGA; 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 cm^2). 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.

Table 3
Detection of *Salmonella* in spiked carcass swabs using the alternative method.

	Inoculum level (cfu/100 cm^2)				
	1	10	100	1000	5000
Beef replicate 1	4/5 +	5/5 +	5/5 +	5/5 +	5/5 +
Beef replicate 2	4/5 +	5/5 +	5/5 +	5/5 +	5/5 +
Beef replicate 3	4/5 +	5/5 +	5/5 +	5/5 +	5/5 +
Pork replicate 1	4/5 +	5/5 +	5/5 +	5/5 +	5/5 +
Pork replicate 2	5/5 +	5/5 +	5/5 +	5/5 +	5/5 +
Pork replicate 3	1/5 +	5/5 +	5/5 +	5/5 +	5/5 +
Total	22/30 +	30/30 +	30/30 +	30/30 +	30/30 +

2.10.3. Blind sample study

Twenty-four spiked cotton swab blind samples were prepared in an independent microbiology laboratory (Ashtown Food Research Centre, Teagasc, Dublin, Ireland). All cultures used for inoculation (*S.* Derby SARB 11, *S.* Dublin NCTC 09676, *S.* Livingstone NCTC 09125, *S.* Typhimurium DT 104 NCTC 13348, *S.* Typhimurium ATCC 14028, *E. coli* NCTC 09001, *E. cloacae* NCTC 10005 and *C. freundi* NCTC 09750) were grown in nutrient broth for 24 h at 37 °C. Serial 10-fold dilutions were performed and ~1000–1 CFUs were used to inoculate the swabs. Each sterile cotton swab (Nuova Aptaca, Regione Monforte, Canelli, Italy) was inoculated with different strains at different inoculation levels (Table 4). The dry cotton swabs were placed in the inoculum and allowed to stand for 30 min. The swabs were then immersed in semi-solid nutrient agar, shipped on ice to our laboratory and analysed using the alternative method described above.

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* Anatum 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.* Anatum. 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

Table 4
Blind study samples – inoculation data and combined enrichment/real-time PCR results.

Swab no.	Non-*Salmonella* (CFU/ml)	*Salmonella* (CFU/ml)	Results (Ct) Target/IAC
Swab inoculated with:			
Swab 1	*E. coli* (~10)	Typ. DT104 (~100), Derby (~10)	21.71/14.88
Swab 2	*E. cloacae* (~10)	Typ. (~10), Dublin (~10)	19.72/14.40
Swab 3	*Citro.* (~1000)	Living. (~100), Typ. DT104 (~10)	19.62/13.91
Swab 4	*E. coli* (~100)	N/A	–/24.99
Swab 5	*E. coli* (~10)	Typ. (~10), Typ. DT104 (~1)	20.05/14.28
Swab 6	*Citro.* (~100)	Derby (~10), Typ. (~100)	21.54/14.97
Swab 7	*E. coli* (~10), *E. cloacae* (~10)	Dublin (~10)	23.03/15.46
Swab 8	*E. coli* (~10)	Derby (~10), Living. (~10)	20.21/14.65
Swab 9	Blank	Blank	–/17.39
Swab 10	*E. cloacae* (~10)	Typ. (~10), Typ. DT104 (~100)	19.74/14.46
Swab 11	*Citro.* (~100)	Derby (~10), Living. (~1)	25.27/14.92
Swab 12	*Citro.* (~100)	Typ. DT104 (~10)	19.22/13.44
Swab 13	*Citro.* (~100)	Derby (~10)	24.70/15.59
Swab 14	*E. cloacae* (~100)	N/A	–/31.28
Swab 15	*E. coli* (~10)	Typ. (~10)	20.26/14.96
Swab 16	*Citro.* (~100)	Dublin (~10)	20.22/14.86
Swab 17	*Citro.* (~100)	Living. (~100)	19.37/13.96
Swab 18	*Citro.* (~100), *E. coli* (~10)	Typ. (~10)	20.16/14.59
Swab 19	*E. cloacae* (~10)	Derby (~1), Dublin (~100)	20.82/15.82
Swab 20	*Citro.* (~100)	N/A	–/26.00
Swab 21	N/A	Typ. DT104 (~10), Typ. (~10), Living. (~10)	19.86/14.54
Swab 22	*E. coli* (~10)	Derby (~1), Typ. (~1)	–/31.61
Swab 23	*E. cloacae* (~100)	Typ. DT104 (~10), Living. (~10)	18.95/13.82
Swab 24	*Citro.* (~100)	Derby (~10), Typ. (~1), Living. (~10)	24.03/14.65
Pos (Salm)		Dublin (~10^6 genome equivalents)	20.87/23.88
PCR neg			–/31.40

Derby = *S.* Derby SARB 11; Dublin = *S.* Dublin NCTC 09676; Living. = *S.* Livingstone NCTC 09125; Typ. DT104 = *S.* Typhimurium DT 104 NCTC 13348; Typ. = *S.* Typhimurium ATCC 14028; *E. coli* = *E. coli* NCTC 09001; *E. cloacae* = *Enterobacter cloacae* NCTC 10005; *Citro.* = *Citrobacter freundii* NCTC 09750.

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.

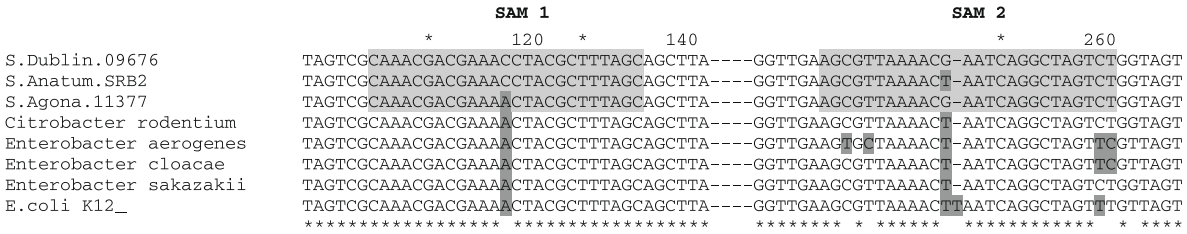

Fig. 1. Sequence alignment of three *Salmonella* serovars and five non-*Salmonella* species at SAM 1 and SAM 2 probe regions. Left: SAM 1 probe region (light grey) highlighting mismatches (dark grey) with one *Salmonella* serovar (*S.* Agona) and closely related species. Right: SAM 2 probe region (light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

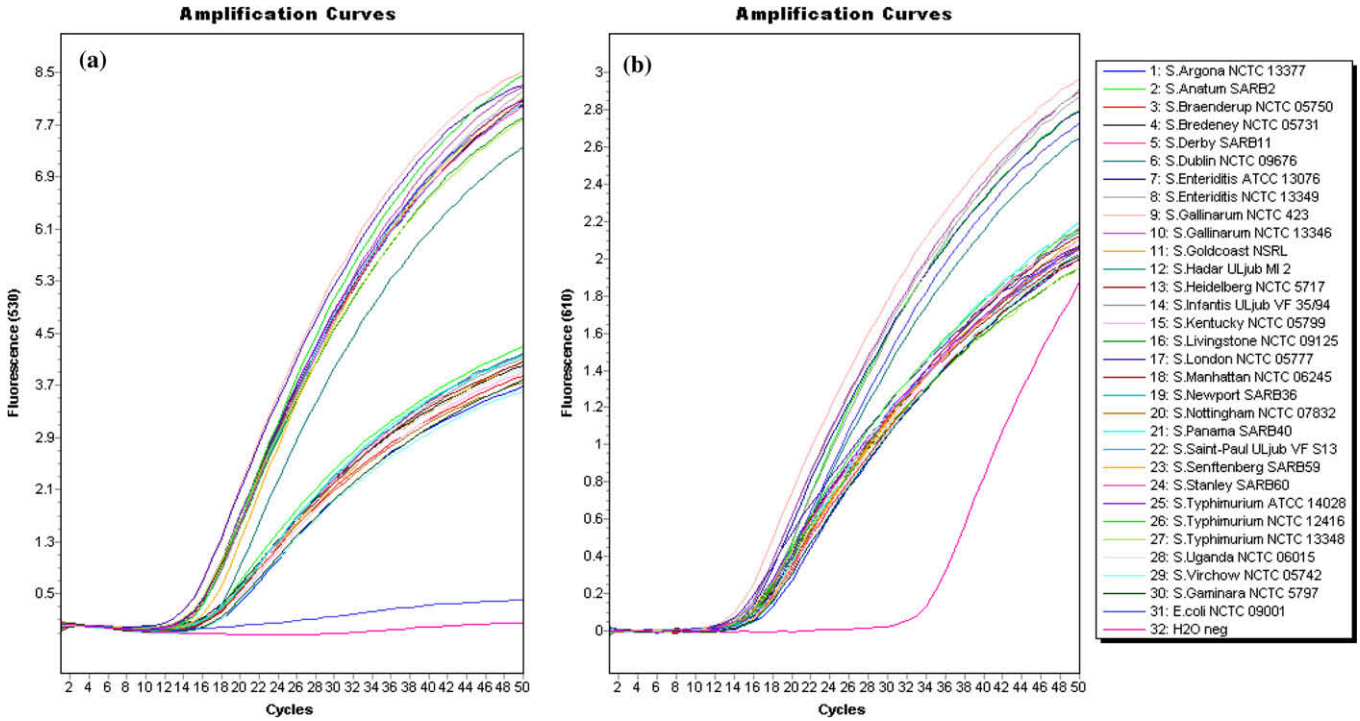

Fig. 2. Amplification curves demonstrating (a) inclusivity of the *Salmonella* real-time PCR assay and (b) IAC detection for all samples in the same experiment.

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 cm^2, and 100% when spiked with $\geqslant$10 CFU/100 cm^2.

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

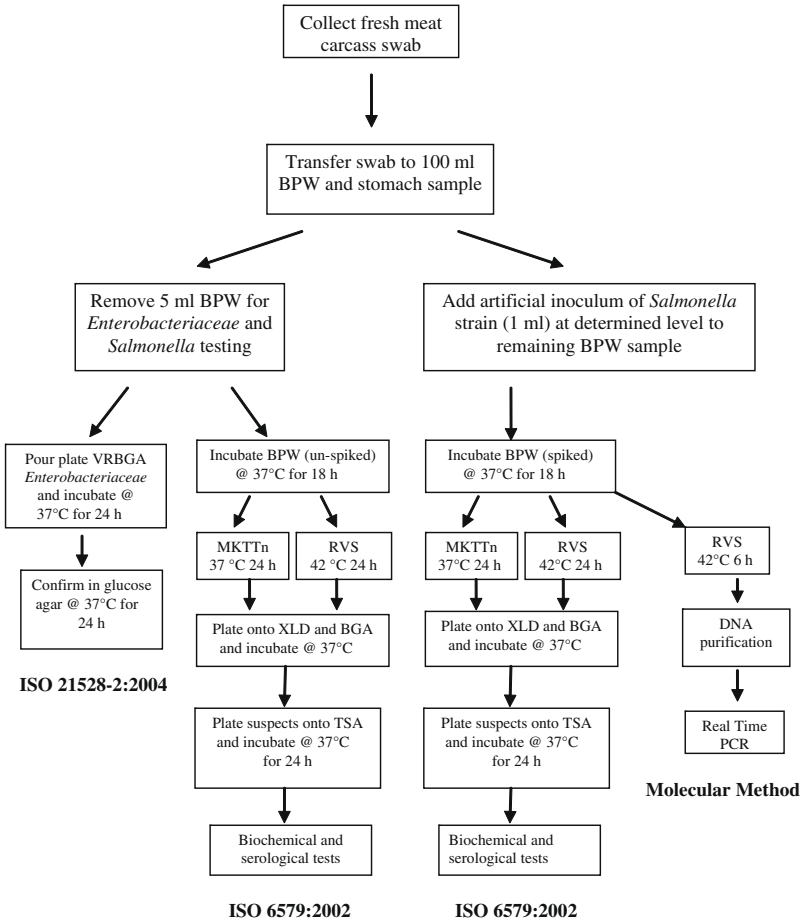

Fig. 3. Flow chart demonstrating steps involved in validation of alternative molecular method to traditional ISO method for spiked carcass swabs (Phase 2).

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

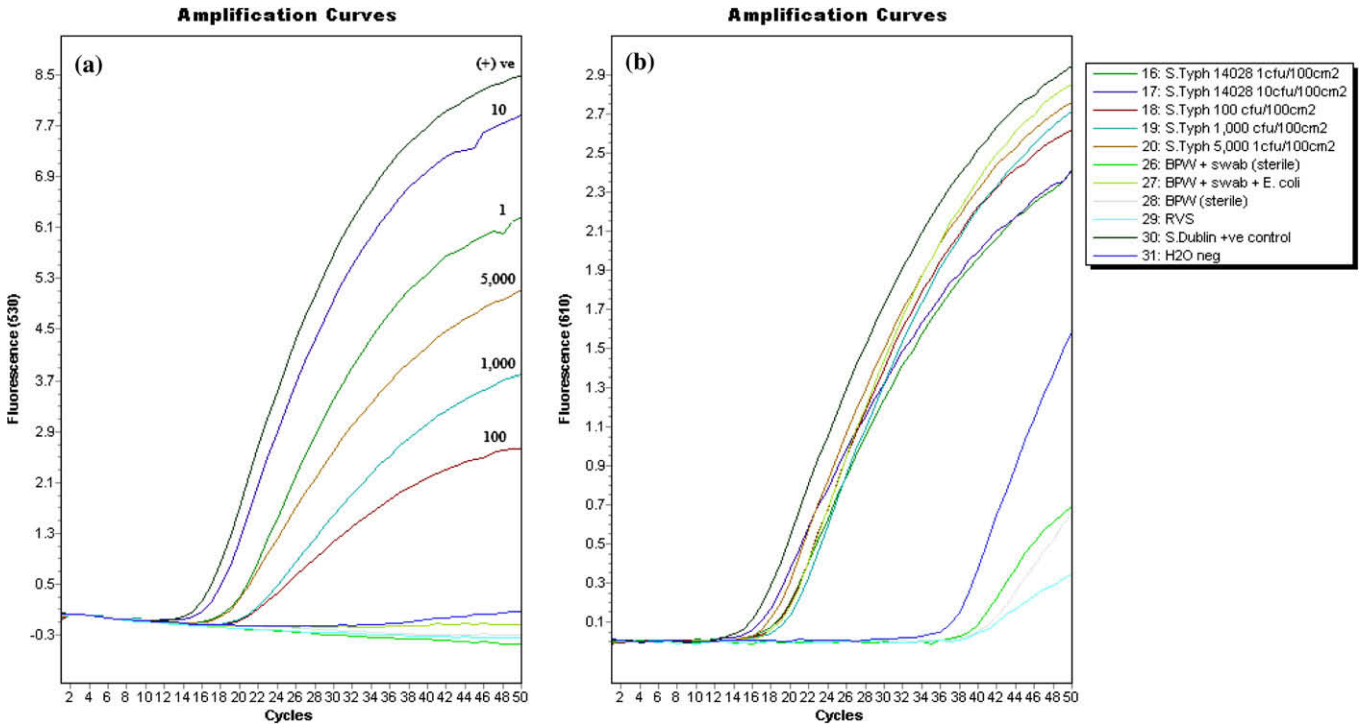

Fig. 4. Phase 2 validation – representative results (third replicate; 1 strain): Amplification curves demonstrating (a) detection of 5/5 pork carcass swabs spiked with *S.* Typhimurium ATCC 14028 ranging from 1–5000 CFU per 100 cm^2 and (b) IAC detection for all samples in same experiment.

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 (C_T) values determined using the *Salmonella* real-time PCR assay e.g. a swab spiked with 1000 CFU/100 cm^2 had a later C_T value than a swab spiked with 10 CFU/100 cm^2 (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 cm^2 while vastly reducing the analysis time would make it a valuable asset to the food testing industry.

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