

Rapid and specific detection of *Salmonella* in water samples using real-time PCR and High Resolution Melt (HRM) curve analysis

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ABSTRACT

A real-time PCR assay combined with a pre-enrichment step for the specific and rapid detection of *Salmonella* in water samples is described. Following amplification of the *invA* gene target, High Resolution Melt (HRM) curve analysis was used to discriminate between products formed and to positively identify *invA* amplification. The real-time PCR assay was evaluated for specificity and sensitivity. The assay displayed 100% specificity for *Salmonella* and combined with a 16–18 h non-selective pre-enrichment step, the assay proved to be highly sensitive with a detection limit of 1.0 CFU/ml for surface water samples. The detection assay also demonstrated a high intra-run and inter-run repeatability with very little variation in *invA* amplicon melting temperature. When applied to water samples received routinely by the laboratory, the assay showed the presence of *Salmonella* in particularly surface water and treated effluent samples. Using the HRM based assay, the time required for *Salmonella* detection was drastically shortened to less than 24 h compared to several days when using standard culturing methods. This assay provides a useful tool for routine water quality monitoring as well as for quick screening during disease outbreaks.

Key words | High Resolution Melt (HRM), real-time PCR, *Salmonella*

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INTRODUCTION

Salmonellosis continues to be one of the most common infectious diseases in the world (Touren *et al.* 2005; Skov *et al.* 2007; Calvo *et al.* 2008). Following the faecal-oral route of infection, *Salmonella* are easily spread by contaminated food and water sources (Chiu *et al.* 2004; Uyanik *et al.* 2008), often leading to disease outbreaks in both humans and animals. Although on track to meet the Millennium Development Goal (MDG), South Africa is prone to *Salmonella* outbreaks as tens of thousands of individuals living in rural and informal communities lack proper sanitation facilities and access to clean, safe water (Bredenhann & Braune 2000; Moganedi *et al.* 2007; WHO/UNICEF 2010). These individuals are directly dependent on often highly polluted surface waters for their daily household and sanitation practices, exposing them to waterborne pathogens and putting them at risk of gastrointestinal diseases (Obi *et al.* 2002; Momba *et al.* 2006; Bessong *et al.* 2009).

Conventional culture based methods for the detection and identification of *Salmonella* species require several days to complete and usually include a number of enrichment steps followed by biochemical and serological identification of presumptive positive isolates (APHA/AWWA/WEF 1998; APHA 2001; ISO 6579:2001). Apart from being laborious and time consuming, culture based methods often lack sensitivity and selectivity (Chiu & Ou 1996; Waage *et al.* 1999; Löfström *et al.* 2004) and require experienced laboratory technicians to perform correctly and accurately.

Several alternative methods for the rapid detection of *Salmonella* species have been developed of which the polymerase chain reaction (PCR) has been shown to be promising (Way *et al.* 1993; Cohen *et al.* 1996; Touren *et al.* 2005; Saroj *et al.* 2008). Further development of fluorescent dyes and instrumentation capable of detecting and measuring fluorescence in real-time during the PCR reaction was a significant advance. Real-time PCR applications can

be completed rapidly since no post-amplification modifications such as agarose gel electrophoresis are required. The analysis of amplified products by means of probes or melt curve analysis is furthermore highly accurate compared to analysis on agarose gels (Berry & Sarre 2007).

As an alternative to gel electrophoresis, melt curve analysis (MCA) is a well known method for the differentiation and identification of amplicons generated by real-time PCR (Berry & Sarre 2007). It is based on the melting temperature (T_m) of the amplification products which strongly relates to the heat facilitated dissociation pattern and subsequently to the nucleotide sequence thereof. High Resolution Melt (HRM) curve analysis of amplification products saturated with double stranded DNA binding dye, compare fluorescence as a function of temperature and allows for the detection of single nucleotide polymorphisms (SNPs) and small deletions in amplicons (Wittwer *et al.* 2003; Smith *et al.* 2010).

While classical MCA can only distinguish gross differences between PCR products, HRM curve analysis allows for the detection of subtle sequence variations between products and provides a much more accurate comparison between amplicons. Important to note is that this feature is mainly due to the binding characteristics of third generation dyes such as EvaGreen. When binding to the double-stranded amplicon, EvaGreen saturates the molecule preventing dye relocation during the melting process as is the case when performing classical MCA which commonly uses non-saturating dyes such as SYBR Green I. Relocation of the dye to non-saturated parts of the amplicon result in less definitive T_m peaks as opposed to HRM curve analysis.

Although widely used in genotypic studies, the ability of HRM curve analysis to discriminate between amplification products based on the nucleotide sequence presents it as a powerful tool for the identification of bacterial species. As different amplicons produce distinct melting curves, these can easily be compared to a reference melting curve to determine the identity of the amplicon.

There is a growing demand for the fast detection of *Salmonella* in water samples, especially during outbreak scenarios when human lives are at stake and the socio-economic impacts are high. We describe here a real-time PCR assay with HRM curve analysis for the rapid, specific and sensitive detection of *Salmonella* in water samples. The assay, combined with a pre-enrichment step is based on the partial amplification and identification of the invasion A (*invA*) gene associated with virulence in *Salmonella* pathogenic to humans (Malorny *et al.* 2003; Gallegos-Robles *et al.* 2009; Jordan *et al.* 2009).

METHODS

Bacterial strains

Salmonella Typhimurium ATCC 14028 was used as a positive control in the detection assay and in all experimental procedures performed. Other bacterial genera as well as environmental strains of *Salmonella*, *Shigella* and entero-pathogenic *E. coli* were used to demonstrate the specificity of the detection assay (Table 1). Environmental strains were supplied by Vermaak and Partners Pathologists (VP) and Aspirata Laboratory Services (ALS), while some reference strains were supplied by the National Health Laboratory Services (NHLS) and the Council for Scientific and Industrial Research (CSIR). All bacterial cultures were maintained on Nutrient Agar plates (Oxoid, UK).

Sample pre-enrichment and genomic DNA extraction

Prior to genomic DNA extraction, 100 ml of water samples examined were filtered through a 0.45 μm nitrocellulose membrane filter using a Sartorius stainless steel vacuum manifold system (Hach, USA). The membrane filter was aseptically transferred to 100 ml sterile Buffered Peptone Water (BPW, Oxoid, UK) followed by incubation at $35 \pm 1.0^\circ\text{C}$ for 16–18 h. For turbid water samples, 20 ml of sample was added directly to 80 ml of sterile BPW followed by incubation at the stated conditions. Genomic DNA was extracted from 1 ml of enriched BPW using the InstaGene Matrix commercial kit (Bio Rad, US). The manufacturer's instructions for DNA extraction were followed precisely and the resulting supernatant contained PCR amplifiable DNA of which 5 μl was used in subsequent real-time PCR reactions.

Primers, real-time PCR and HRM curve analysis

Primer sequences were obtained from a previously published study (Malorny *et al.* 2003) and targets the invasion A (*invA*) gene facilitating invasion of epithelial cells and specific to *Salmonella* pathogenic to humans. All primers used were synthesised by Integrated DNA Technologies (IDT, USA) and primer sequences are listed in Table 2.

Real-time PCR amplification of the *invA* target was performed in either 0.2 ml or 0.1 ml thin walled PCR tubes (Qiagen, Germany) in a total reaction volume of 25 μl .

Table 1 | Bacterial species used in the *Salmonella* real-time PCR detection assay specificity evaluations

Bacterial species	Number of strains used	Source of strain	Real-time PCR with HRM result
<i>Escherichia coli</i>	1	ATCC 25922	–
<i>Shigella flexneri</i>	1	ATCC 12022	–
<i>Shigella boydii</i>	1	ATCC 9207	–
<i>Shigella sonnei</i>	1	ATCC 9290	–
<i>Enterococcus faecium</i>	1	ATCC 6569	–
<i>Enterobacter aerogenes</i>	1	ATCC 13048	–
<i>Klebsiella pneumoniae</i>	1	VP	–
<i>Pseudomonas aeruginosa</i>	1	ATCC 27853	–
<i>Staphylococcus aureus</i>	1	ATCC 25923	–
<i>Proteus mirabilis</i>	1	VP	–
<i>Vibrio cholerae</i> O1 (Ogawa)	1	NHLS	–
<i>Shigella</i> species (environmental strains)	4	VP, ALS	–
Enteropathogenic <i>E. coli</i> (environmental strains)	2	VP	–
<i>Salmonella</i> Typhimurium	1	ATCC 14028	+
<i>Salmonella</i> Enteritidis	1	ATCC 13076	+
<i>Salmonella</i> Typhi	1	NHLS	+
<i>Salmonella</i> Choleraesuis	1	ATCC 10708	+
<i>Salmonella</i> Oxfordii	1	CSIR	+
<i>Salmonella</i> Heidelberg	1	CSIR	+
<i>Salmonella enterica</i> (environmental strains)	7	VP, ALS	+

Table 2 | Nucleotide sequences for primers used in this study

Target	Primer sequence (5'-3')	Amplicon size (bp)	Amplicon T _m (°C)	Reference
<i>invA</i> gene	CACCAAGAAGGTGACTTTATTGTG	284	83.64 ± 0.28	Malorny <i>et al.</i> (2003)
<i>invA</i> gene	GAACTATAACCACCCGCG			

Each reaction consisted of 1x SensiMix HRM (Bioline, UK) reaction buffer containing dNTPs, MgCl₂, a heat activated DNA polymerase and EvaGreen dye, to which 0.2 μM of each primer, nuclease free water (Applied Biosystems, USA) and 5 μl genomic DNA serving as template was added.

Amplification was performed in a RotorGene 6000 2-plex rotary thermal cycler with High Resolution Melt (HRM) capability (Qiagen, Germany) programmed for 45 cycles starting with a single heat activation step of the DNA polymerase at 95 °C for 10 min. This was followed by a DNA denaturation step at 95 °C for 30 s, annealing at 64 °C for 30 s and extension at 72 °C for 30 s. A final extension step was performed at 72 °C for 5 min after cycling.

To differentiate and identify amplification products formed, HRM curve analysis was performed by lowering the temperature to 60 °C for 5 min, followed by an increase

in temperature from 70 to 90 °C at increments of 0.1 °C per second. Fluorescence was measured continuously and T_m peaks were calculated based on the initial fluorescence curve (F/T) by plotting the negative derivative of fluorescence over temperature versus temperature ($-dF/dT$ versus T). The mean T_m value for the *invA* product was calculated using the values from 50 independent PCR analyses of *Salmonella* Typhimurium ATCC 14028 and 37 environmental strains detected in water samples received by the laboratory.

Specificity of the detection assay

To evaluate the specificity of the detection assay, several reference and environmental strains (Table 1) were cultured and maintained on Nutrient Agar (Oxoid) plates. A single

colony of pure culture was re-suspended in 1 ml sterile, deionised water after which the genomic DNA was extracted as described. Extracted genomic DNA was used as template in subsequent real-time PCR reactions.

Size and sequence confirmation of amplicon identity

In addition to HRM curve analysis, agarose gel electrophoresis was used to confirm that amplicons were of the expected size for the *invA* target (284 bp). Five microlitres of real-time PCR reaction were loaded onto a 2% agarose gel stained with ethidium bromide (EtBr) and subjected to electrophoresis at 80 V for 45 min. Following electrophoresis the gel was visualised and images captured using a UV Transluminator 2000 and Digi Doc System (Bio Rad, USA). The identity of the amplification products were further confirmed by nucleotide sequence analysis (Inqaba Biotec, SA) and alignment of the obtained sequences to an existing database (GenBank, NCBI, BLASTn option).

Detection limit of the assay

An overnight culture of *Salmonella* Typhimurium ATCC 14028 was serially diluted 10-fold and used to artificially contaminate surface water samples (250 ml). The theoretical final concentration of *Salmonella* Typhimurium in each contaminated water sample was determined using plating techniques. This was achieved by plating 100 µl of each serial dilution of *Salmonella* Typhimurium used on a non-selective media (Nutrient Agar, Oxoid) in triplicate. Following overnight incubation at 35 ± 1.0 °C the average count was determined for each dilution and the theoretical concentration of *Salmonella* Typhimurium per water sample stated in CFU/ml.

A reagent blank sample was also prepared by contaminating a surface water sample with sterile diluents (Bacteriological Peptone, Oxoid) used to prepare the serial dilutions of *Salmonella* Typhimurium. This was to ensure that all reagents as well as the surface water samples used were free of any *Salmonella*. After artificial contamination the water samples were subjected to the pre-enrichment step and real-time PCR detection assay as described.

Repeatability of the detection assay

The intra-run repeatability of the detection assay was determined by analysing 10 replicates of a single *Salmonella* positive water sample within the same real-time PCR run. The inter-run reproducibility of the detection assay was

determined by analysing a single *Salmonella* positive water sample in triplicate over a period of three consecutive days.

Application to environmental water samples

Once optimised and implemented in the laboratory, the detection assay was applied to a variety of water samples received by the laboratory on a routine basis. A total of 122 water samples were analysed for *Salmonella* using the detection assay. Sources included potable water, surface water, storm water and treated sewage effluents.

RESULTS AND DISCUSSION

Specificity of the detection assay and confirmation of amplicon identity

Though thoroughly tested and demonstrated by the authors (Malorny *et al.* 2003), the specificity of the *invA* primers was further evaluated using 15 reference and 15 environmental bacterial strains. Only strains of *Salmonella* and none of the other genera used in the evaluation produced a positive signal (Table 1). Agarose gel electrophoresis indicated that positive amplification products correlated to a size of 284 bp as expected (Figure 1) and nucleotide sequence analyses of the amplicons confirmed homology to the *invA* gene sequence (BLASTn option, GenBank). The assay therefore appears to be highly specific for *Salmonella* and is not influenced by other bacterial species.

Differentiation and identification of amplicons using HRM curve analysis

Constant HRM profiles with distinct T_m peaks were persistently obtained for reference and environmental strains analysed and some characteristic profiles are displayed in Figure 1. Statistical evaluation of 50 independent PCR analyses using *Salmonella* Typhimurium ATCC 14028 and 37 environmental strains of *Salmonella* presented an *invA* amplification product with an average T_m of 83.64 °C and a SD of 0.28 °C (Table 2). The *invA* amplicon appeared to be stable as little run to run variation in T_m was observed. The variation in amplicon T_m was normally distributed with an inter-quartile range of 83.45–83.85 °C. The non-outlier range was 82.97–84.40 °C and unknown water samples generating amplicons with a T_m within this range were considered positive for *Salmonella*.

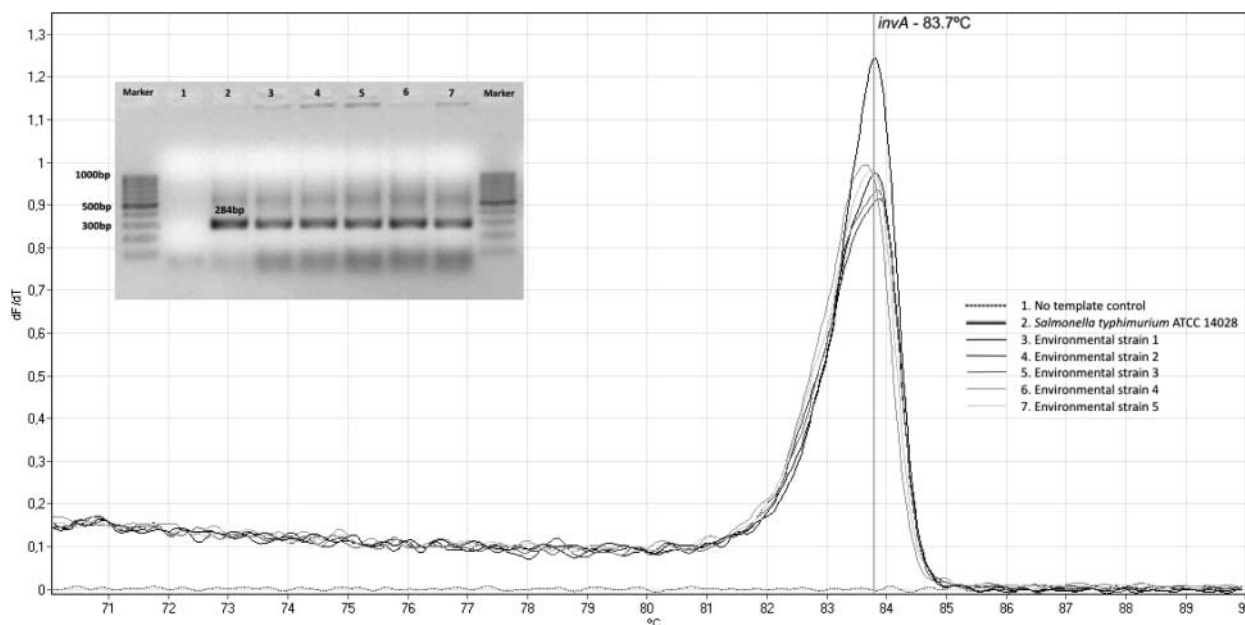


Figure 1 | Some characteristic *Salmonella* positive HRM profiles obtained and size confirmation of amplicons.

Variation in amplicon T_m may be caused by several factors such as poor template DNA purity and quality, salt or metal ion contamination and target sequence variation. However, for this assay variation in T_m due to nucleotide sequence variation seems less likely as accurate SNP detection using HRM is suggested using small sized amplicons (Kristensen & Dobrovic 2008). Variation in T_m is thus rather attributed mainly to the purity of DNA used as template and possible ion contamination when analysing complex matrices such as treated effluents. The variation caused by these factors when analysing complex matrices should not necessarily be viewed as a disadvantage as the T_m variation observed were still well within the non-outlier range. Non-specific amplification was easily identified and resulted in products with melting temperatures either much higher or lower than the control T_m .

Detection limit and repeatability of the assay

The detection limit of the assay was determined using artificially contaminated surface water samples. This was done to specifically evaluate how the natural microbial population present in the surface water samples will affect the sensitivity of the assay. The assay appeared to be highly sensitive and could successfully detect *Salmonella* in seeded surface water samples with a theoretical concentration of 1.0 CFU/ml. The high sensitivity of the assay is mainly attributed to the pre-enrichment step preceding the

PCR detection. The reagent blank showed no presence of *Salmonella*.

The detection assay also displayed a high degree of repeatability. Intra-run analysis successfully detected the presence of *Salmonella* in all 10 replicates with very little variation in amplicon T_m . Successful detection of *Salmonella* in the same water sample in triplicate over a period of three consecutive days also indicated high inter-run reproducibility.

Application to environmental water samples

The real-time PCR detection assay was employed in a routine laboratory set up, using the assay to analyse water samples originating from a variety of sources for the presence of *Salmonella*. Water samples were largely classified as potable water, surface water, storm water and treated

Table 3 | Summarised results of water samples analysed using the detection assay

Sample type	Number of samples analysed	Number of samples positive for <i>Salmonella</i> (%)
Potable water	39	1 (2.6%)
Surface water	25	12 (48.0%)
Stormwater	45	13 (28.9%)
Treated sewage effluent	13	12 (92.3%)
Total	122	38 (31.1%)

sewage effluents. Of the 122 water samples analysed, a total of 38 tested positive for *Salmonella*. Proportionally, the highest incidence of *Salmonella* was detected in the treated sewage effluent followed by the surface water samples. Only one of the potable water samples analysed showed the presence of *Salmonella* using the detection assay. Table 3 provides a relative breakdown of the water samples analysed and the results obtained.

CONCLUSIONS

In developing countries such as South Africa there is a need for the rapid detection and identification of waterborne pathogens such as *Salmonella*, especially during disease outbreaks. Opposed to culturing methods, the assay we described here could successfully detect *Salmonella* in water samples within 24 hours. High Resolution Melt (HRM) curve analysis demonstrated to be a functional tool for the positive identification of *Salmonella*. Amplicon melting peaks could clearly be discriminated from one another and positive amplification of the *invA* target easily identified by comparing it to the positive control peak.

Compared to probe based detection assays, HRM curve based assays have several advantages. The assays are relatively easy to design and the set up thereof do not require complicated probe design procedures. While non-specific binding by probes may be confused with a positive result, non-specific binding is easily identified using HRM. Assays are also cost effective compared to probe based assays.

Successful employment of this detection assay in a routine laboratory environment also demonstrated it as a useful tool for monitoring water quality by water authorities. Though the presence of *Salmonella* in water sources is not specifically regulated by South African law, the assay may greatly aid outbreak investigations and serve as a means of screening water sources for the presence of *Salmonella*.

REFERENCES

- APHA/AWWA/WEF 1998 *Standard Methods for the Examination of Water and Wastewater*, 20th edition. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.
- APHA 2001 *Compendium of Methods for the Microbiological Examination of Foods*, 4th edition. American Public Health Association, Washington, DC, USA.
- Berry, O. & Sarre, S. D. 2007 [Gel-free species identification using melt-curve analysis](#). *Molecular Ecology Notes* **7** (1), 1–4.
- Bessong, P. O., Odiyo, J. O., Musekene, J. N. & Tessema, A. 2009 [Spatial distribution of diarrhoea and microbial quality of domestic water during an outbreak of diarrhoea in the Tshikwi community in Venda, South Africa](#). *Journal of Health, Population and Nutrition* **27** (5), 652–659.
- Bredenhann, L. & Braune, E. 2000 *Policy and Strategy for Groundwater Quality Management in South Africa*. Report W.1.0, Department of Water Affairs and Forestry, Pretoria, South Africa.
- Calvó, L., Martínez-Planells, A., Pardos-Bosch, J. & Garcia-Gil, L. J. 2008 [A new real-time PCR assay for the specific detection of *Salmonella* spp. targeting the *bipA* gene](#). *Food Analytical Methods* **1** (4), 236–242.
- Chiu, C. & Ou, J. T. 1996 Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR combination assay. *Journal of Clinical Microbiology* **34** (10), 2619–2622.
- Chiu, C., Su, L. & Chu, C. 2004 *Salmonella enterica* serotype Choleraesuis: epidemiology, pathogenesis, clinical disease and treatment. *Clinical Microbiology Reviews* **17** (2), 311–322.
- Cohen, H. J., Mechanda, S. M. & Lin, W. 1996 PCR amplification of the *fimA* gene sequence of *Salmonella* Typhimurium, a specific method for detection of *Salmonella* spp. *Applied and Environmental Microbiology* **62** (12), 4303–4308.
- Gallegos-Robles, M. A., Morales-Loredo, A., Álvarez-Ojeda, G., Osuna-García, J. A., Martínez, I. O., Morales-Ramos, L. H. & Fratamico, P. 2009 [PCR detection and microbiological isolation of *Salmonella* spp. from fresh beef and Cantaloupes](#). *Journal of Food Science* **74** (1), 37–40.
- Jordan, R., Van Heerden, E., Hugo, C. J. & Piater, L. A. 2009 Using current molecular techniques for rapid differentiation of *Salmonella* Typhi and *Salmonella* Typhimurium. *African Journal of Biotechnology* **8** (9), 1815–1818.
- Kristensen, L. S. & Dobrovic, A. 2008 [Direct genotyping of single nucleotide polymorphisms in methyl metabolism genes using probe-free high resolution melting analysis](#). *Cancer Epidemiology, Biomarkers and Prevention* **17** (5), 1240–1247.
- Löfström, C., Knutsson, R., Axelsson, C. E. & Rådström, P. 2004 [Rapid and specific detection of *Salmonella* spp. in animal feed samples by PCR after culture enrichment](#). *Applied and Environmental Microbiology* **70** (1), 69–75.
- Malorny, B., Hoorfar, J., Bunge, C. & Helnuth, R. 2003 [Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard](#). *Applied and Environmental Microbiology* **69** (1), 290–296.
- Mogani, K. L. M., Goyvaerts, E. M. A., Venter, S. N. & Sibara, M. M. 2007 [Optimisation of the PCR-*invA* primers for the detection of *Salmonella* in drinking and surface waters following a pre-cultivation step](#). *Water SA* **33** (2), 195–202.
- Momba, M. N. B., Tyafa, Z., Makala, N., Brouckaert, B. M. & Obi, C. L. 2006 [Safe drinking water still a dream in rural areas of South Africa. Case study: The Eastern Cape province](#). *Water SA* **32** (5), 715–720.

- Obi, C. L., Potgieter, N., Bessong, P. O. & Matsaung, G. 2002 Assessment of microbial quality of river water sources in rural Venda communities in South Africa. *Water SA* **23** (3), 287–292.
- Saroj, S. D., Shashidhar, R., Karani, M. & Bandekar, J. R. 2008 Rapid, sensitive, and validated method for the detection of *Salmonella* in food by an enrichment broth culture – nested PCR combination assay. *Molecular and Cellular Probes* **22** (3), 201–206.
- Skov, M. N., Anderson, J. S., Aabo, S., Ethelberg, S., Aarestrup, F. M., Sørensen, A. H., Sørensen, G., Pedersen, K., Nordentoft, S., Olsen, K. E. P., Gerner-Smidt, P. & Baggesen, D. L. 2007 Antimicrobial drug resistance of *Salmonella* isolates from meat and humans, Denmark. *Emerging Infectious Diseases* **13** (4), 638–641.
- Smith, B. L., Lu, C. P. & Bremer, R. A. 2010 High-resolution melting analysis (HRMA): a highly sensitive inexpensive genotyping alternative for population studies. *Molecular Ecology Resources* **10** (1), 193–196.
- Touron, A., Berthe, T., Pawlak, B. & Petit, F. 2005 Detection of *Salmonella* in environmental water and sediment by a nested-multiplex polymerase chain reaction assay. *Research in Microbiology* **156**, 541–553.
- Uyanik, M. H., Yazgi, H. & Ayyildiz, A. 2008 Survival of *Salmonella* Typhi and *Shigella flexneri* in different water samples & at different temperatures. *Turkish Journal of Medical Science* **38** (4), 307–310.
- Waage, A. S., Vardund, T., Lund, V. & Kapperud, G. 1999 Detection of low numbers of *Salmonella* in environmental water, sewage and food samples by a nested polymerase chain reaction assay. *Journal of Applied Microbiology* **87** (3), 418–428.
- Way, J. S., Josephson, K. L., Pillai, S. D., Abbaszadegan, M., Gerba, C. P. & Pepper, I. L. 1993 Specific detection of *Salmonella* spp. by multiplex polymerase chain reaction. *Applied and Environmental Microbiology* **59** (5), 1473–1479.
- WHO/UNICEF 2010 *Progress on Sanitation and Drinking Water: 2010 update*. Water Sanitation and Health Programme, WHO/UNICEF, Geneva, Switzerland.
- Wittwer, C. T., Reed, G. H., Gundry, C. N., Vandersteen, J. G. & Pryor, R. J. 2003 High-resolution genotyping by amplicon melting analysis using LC Green. *Molecular Diagnostics and Genetics* **49** (6), 853–860.

First received 7 July 2011; accepted in revised form 23 August 2011

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Manuscript: WST-WSTWS-EM11802R1

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