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

G. N. van Blerk, L. Leibach, A. Mabunda, A. Chapman and D. Louw

**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*

**INTRODUCTION**

Salmonellosis continues to be one of the most common infectious diseases in the world (Touron et al. 2005; Skov et al. 2007; Calvó 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; Touron 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 (Tm) 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 Tm 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. 2005; 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 ± 1.0 °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. 2005) 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.
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 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 Tm peaks were calculated based on the initial fluorescence curve (F/T) by plotting the negative derivative of fluorescence over temperature versus temperature (ΔF/ΔT versus T). The mean Tm 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.

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

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

### Table 2 | Nucleotide sequences for primers used in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence (5’–3’ )</th>
<th>Amplicon size (bp)</th>
<th>Amplicon Tm (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>invA gene</td>
<td>CACCAAGAAGGTGACTTTATTGTG</td>
<td>284</td>
<td>83.64 ± 0.28</td>
<td>Malorny et al. (2003)</td>
</tr>
<tr>
<td>invA gene</td>
<td>GAACGTTATAACCACCCCGCG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A 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. 2005), 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 Tm 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 Tm 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 Tm was observed. The variation in amplicon Tm 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 Tm within this range were considered positive for Salmonella.
Variation in amplicon Tm 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 Tm 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 Tm 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 Tm 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 Tm.

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 Tm. 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 sewage effluent. The results are summarised in Table 3.

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

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Number of samples analysed</th>
<th>Number of samples positive for Salmonella (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potable water</td>
<td>39</td>
<td>1 (2.6%)</td>
</tr>
<tr>
<td>Surface water</td>
<td>25</td>
<td>12 (48.0%)</td>
</tr>
<tr>
<td>Stormwater</td>
<td>45</td>
<td>13 (28.9%)</td>
</tr>
<tr>
<td>Treated sewage effluent</td>
<td>13</td>
<td>12 (92.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>122</td>
<td>38 (31.1%)</td>
</tr>
</tbody>
</table>
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 assays we described here could successfully detect *Salmonella* in water samples within 24 hours. 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**


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