# Optimisation of the PCR-*invA* primers for the detection of *Salmonella* in drinking and surface waters following a pre-cultivation step

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## Abstract

A polymerase chain reaction (PCR)-based method for the detection of *Salmonella* species in water samples was optimised and evaluated for speed, specificity and sensitivity. Optimisation of $Mg^{2+}$ and primer concentrations and cycling parameters increased the sensitivity and limit of detection of PCR to 2.6 x $10^4$ cfu/mℓ. A 6h non-selective pre-enrichment step further increased the limit of detection to 26 cfu/mℓ. Out of 14 different *Salmonella* strains tested, only two, *Salmonella arizonae* and *Salmonella pullorum*, did not give positive amplification results with primers homologous to a conserved region of the *invA* gene. When environmental and drinking waters were assessed, a non-selective pre-enrichment step was included to increase the detection efficiency of PCR. The PCR method demonstrated specificity in the presence of other competing micro-organisms as confirmed by the conventional culture method. No false positives or negatives were observed when household and environmental water samples were tested by *invA*-PCR analysis parallel to the culture method.

**Keywords**: water quality, *Salmonella*, PCR, invA primers

## Introduction

The availability of safe water remains an urgent human need in many countries and has barely kept pace with population growth. Globally, water is abundant but it is not always available in potable form to many people. One third of the population in the developing countries suffers serious diseases and fatalities due to unhealthy environment and Africa is most vulnerable to waterborne diseases and death due to unsafe drinking water. South Africans who lack access to potable water account for 17% of the population (*The Water Wheel,* 2006), and this water scarcity leads to usage of water sources such as dams, pools, rivers and unprotected springs that pose a health risk to the human population (Obi et al., 2002).

Water sources are often polluted with sewage runoff and become the main causes of diseases such as typhoid and cholera as a result of lack of sanitation facilities. A study funded by Water Research Commission (WRC) revealed the predominance of *Salmonella* in the environment which could possibly be due to faecal contamination from human and animal excreta (Lehloesa and Muyima, 2000 and *The Water Wheel*, 2006). An outbreak of typhoid fever that occurred between August and October 2006 in Mpumalanga, RSA, has resulted in four deaths and 600 people infected. Typhoid fever has become endemic in RSA though at a low frequency (*The Water Wheel*, 2005). In 1997 there were 17 deaths out of 451 cases reported; 93.8% of these were Africans who were 15 years and older and again 25% of these cases were reported in the less populated Limpopo Province (Department of Health, 1998). In 1996, 643 cases and 11 deaths were reported in RSA due to typhoid fever. In 2001, 117 147 cases of cholera with 265 fatalities were reported in the RSA (Nevondo and Cloete, 2002; *The Water Wheel*, 2005), whereas 35 cases were reported in 2004 (Water Services: National Information System, 2006). The high level of microbial contamination that South African water resources receives due to lack of proper sanitary facilities makes routine detection of *Salmonella* spp. in environmental samples a necessary direct component of any public health strategy.

A number of methods for detecting *Salmonella* have been published. Immunoassays and PCR-based methods are currently the dominant methods used for the detection of bacterial pathogens. Immunoassays generally have a problem of cross-reactivity which produce false positives (Mansfield and Forsythe, 2000; Caruso et al., 2000, Liu et al., 2001; Walker et al., 2001 and Fratamico, 2003). Furthermore, immunoassays inherently have a high detection limit even when pre-enrichment of between 18 to 30h is included in the assay (Jaradat et al., 2004; Liu et al., 2001; Chen and Durst, 2006 and De Medici et al., 1998) and this would more often produce false negatives if the level of *Salmonella* is low.

PCR offers a more rapid and reliable method for the detection of *Salmonella* in natural environments. The assay combines simplicity with specificity and sensitivity in detecting organisms. PCR assay has been used successfully to diagnose the presence of bacterial pathogens in aquatic environments, food products and clinical samples (Chen and Griffiths, 2001; Ellingson et al., 2004; Guo et al., 2000; Myint et al., 2006; Patel et al., 2006; Sandery et al., 1996; Schrank et al., 2001; Soumet et al., 1999(a); Soumet et al., 1999(b)). The PCR assay can detect minute amount of the target DNA sequence, however this sensitivity can be influenced by physical dilution in aquatic environments and food products and may result in *Salmonella* escaping

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detection. Pre-enrichment of samples is thus necessary to lower the detection limit and dilute any inhibitory substances present in the samples. Most PCR assays carried out currently include a minimum of 6 to 8 h pre-cultivation step (Ellingson et al., 2004; Fratamico, 2003; Guo et al., 2000; Myint et al., 2006; Oliveira et al., 2002; Oliveira et al., 2003; Patel et al., 2006; Wang and Yeh, 2002) that can increase the sensitivity to one digit number of CFU, which is much better than the immunoassays that pre-enrich the samples for 24 to 30 h but still obtain minimum detection limits of $10^3$ CFU (Soumet et al., 1999(a); Liu et al., 2001; Chen and Durst, 2006).

Rahn et al. (1992) reported the *invA* primer set that was able to discriminate between *Salmonella* and non-*Salmonella* species. They achieved a low detection limit (300 cfu/mℓ) but did not evaluate the method on environmental samples. This current study extends the work done by Rahn et al. (1992) by assessing the efficacy of these *invA* primers in detecting *Salmonella* in natural water systems following optimisation of the PCR conditions. A cultivation step prior to PCR was added to circumvent the problem of detecting dead cells.

## Materials and methods

*Salmonella enteritidis* ATCC 13076 was used as a positive control. Bacterial strains used to determine the specificity of PCR primers are listed in Table 1. Cultures were maintained on nutrient agar (Oxoid) plates and slants.

**Primers and probe**. A 284 bp region of the *Salmonella invA* gene (Galan et al., 1992) was amplified by the primers Sal 1 (5′-GTGAAATTATCGCCACGTTCGGGCAA) and Sal 2 (5′-TCATCGCACCGTCAAAGGAACC) designed by Rahn et al. (1992). The identity of the amplicons was confirmed by hybridisation to an internal probe Sal 3 (5′-GCCCGGTAAACAGATGAGTATTGA) designed by Gericke and Kfir (1995). The primers and probe were purchased from University of Cape Town.

### Template preparation

**Boiling method**: *S. enteritidis* ATCC 13076 was incubated at 37ºC overnight on nutrient agar plates. Thereafter, a colony from the plate was suspended in 1 mℓ of sterilised distilled water in a 2 mℓ micro-centrifuge tube and boiled for 10 min. The lysate was chilled on ice and then spun for 5min in a micro-centrifuge at high speed to pellet the debris. Five µℓ of the supernatant was used as template in the PCR reaction.

**Single colony method**: A colony from an overnight culture of *S. enteritidis* ATCC 13076 was suspended in 1 mℓ of sterile distilled water, and not boiled. Tenfold serial dilutions were prepared and 5µℓ thereof was added directly to the PCR reaction mix. Another 5µℓ was plated on nutrient agar for a bacterial count.

**Total DNA isolation method**: The procedure followed was a combination with modification of the methods of Marmur (1961) and Wallace (1987). Briefly, *S. enteritidis* was grown overnight in 500 mℓ nutrient broth at 37ºC. The cells were pelleted in 50 mℓ tubes and resuspended in 20 mℓ proteinase K reaction buffer [10 mM Tris, pH 7.8; 5mM EDTA and 0.5% sodium dodecyl sulphate (SDS)]. An additional SDS (1.5%) was added and the suspension was incubated at 37ºC for 1 h. RNase A (50 µg/mℓ) and proteinase K (100 µg/mℓ) were added successively and the lysate was incubated at 37ºC for 20 min and 1 h, respectively. The mixture was sequentially extracted with equal volumes of phenol: chloroform: isoamyl alcohol (24:24:1), chloroform: isoamyl alcohol (24:1) and water-saturated diethyl ether. The upper phase was removed in between extractions and transferred to a new tube. DNA was precipitated with 2 vols ethanol and 0.2M NaCl (pH 8.0). The DNA pellet was dissolved in sterile distilled water and quantified by UV spectrophotometry at 260 and 280 nm. Tenfold serial dilutions with sterile distilled water were performed and 5 µℓ from each dilution was added to the PCR reaction mixture.

### PCR optimisation

The PCR reaction mixture for the reference PCR protocol consisted of PCR buffer (50 mM KCl, 10 mM Tris-Cl (pH 9), 0.1% Triton® X-100), 1.5U *Taq* DNA polymerase (Promega), 1.5 mM $MgCl_2$, 200 ng of each primer and 100 µM each of dNTPs. The PCR cycling conditions were: denaturation at 94ºC for 1 min, primer annealing at 55ºC for 1.5 min and extension at 72ºC for 1.5 min for a total of 30 cycles, followed by a 7 min extension period. Amplification was performed in a GeneAmp PCR 2400 version 2.11 thermocycler (Perkin Elmer). Optimisation was performed in duplicate. Parameters that were varied were $MgCl_2$ concentration (0 to 4 mM, Δ=0.5 mM), primer concentration (0 to 200 ng, Δ=25 ng), annealing temperature (55 to 65ºC, Δ=1ºC) and incubation period (30 to 180 s, Δ= 30s) and lastly extension period (0 to 300 s, Δ= 60 s). Each component, in the listed order, was optimised while others were kept constant and the optimised parameter was thereafter used in subsequent experiments.

### Limit of detection

**Cell dilution**: Tenfold serial cell dilutions of *S. enteritidis* ATCC 13076 from $10^0$ to $10^5$ were prepared by the single colony method. Five µℓ from dilutions that gave negative amplification results were added to nutrient broth and incubated for 6h at 37°C.

**DNA dilution**: A 5 µℓ aliquot of each dilution (30 pg to 3 µg/mℓ) of the total DNA of *S. enteritidis* was added to the PCR mixture and the optimised PCR protocol was followed.

### Specificity

*S. enteritidis* ATCC 13076 served as a positive control and a no-template negative control was included to monitor contamination. DNA of all the *Salmonella* and non-*Salmonella* strains (Table 1) was extracted by the boiling method. PCR products were verified by Southern hybridisation with Sal 3 probe. The probe was Dig-11-dUTP labelled (Roche-Diagnostics). Southern transfer of amplicons onto a nylon membrane was carried out as outlined in Sambrook et al. (1989). Hybridisation of the probe to the amplicons was detected with CDP-Star™ according to manufacturer's guidelines (*Dig System User's Guide for Filter Hybridisation*, 1995).

### Field sampling

A total of 39 water samples (2 x 50 mℓ each) were collected from household and communal taps (17), storage tanks (10) and rivers (12) from Kgapane, Mamabolo and Venda areas in the Limpopo Province, RSA. Tap water was allowed to run for 10 s before collection into Sterilin bottles. Water from the storage tanks and rivers (both still and running water) was collected 200 mm below the surface. All the water samples were kept on ice. The


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**TABLE 1**
**Bacterial strains analysed with the PCR *invA* - primer technique**

| Culture | Selection criteria | Culture | Selection criteria |
|---|---|---|---|
| *Salmonella enteritidis* ATCC 13076 | Positive control | *Escherichia coli O:112*<br>*E. coli O:29*<br>*E. coli O:143*<br>*E. hermanii* | *45% DNA relatedness to *Salmonella* |
| | | *Klebsiella pneumoniae* | *40% relatedness to *Salmonella* |
| | | *Citrobacter freundii* | *50% relatedness to *Salmonella* |
| *S. paratyphi* CCRC 12949<br>*S. typhimurium*<br>*S. typhi*<br>*S. saintpaul*<br>*S. Dublin*<br>*S. oxford*<br>*S. bloemfontein*<br>*S. derby*<br>*S. pullorum*<br>*S. arizonae*<br>*S. agona* | Determination of conservation of the *invA* gene within *Salmonella* species | *Shigella dysentery*<br>*S. sonnei*<br>*S. flexneri*<br>*S. boydii* | *45% DNA relatedness to *Salmonella* |
| | | *Pseudomonas aeruginosa* | **reported to produce non-specific product with *invA* primers |
| | | *Bacillus cereus*<br>*B. cereus var mycoides* | **reported to give non-specific product with *invA* primers |

Source: All the micro-organisms were obtained from Environmentek, CSIR.
*: *Bergey's Manual of Systematic Bacteriology* (1984 - 1989).
**: Rahn et al. (1992)

samples were assessed in duplicates, using the PCR technique and the conventional culture method SANS 6340 (2004). Most probable number (MPN) was determined from each sample. For PCR analysis, water samples (50 mℓ) were centrifuged and the pellets were suspended in 1 mℓ sterile distilled water. A 5 µℓ aliquot from each suspension was added directly to the PCR reaction mixture.

Multiple tube fermentation was performed with buffered peptone water (BPW-Biolab) and Rappaport Vassiliadis broth (RV-Merck) as non-selective and selective enrichment media, respectively. Three series of three BPW tubes containing inverted Durham tubes were inoculated with 10, 1 and 0.1 mℓ of each sample and were incubated at 36ºC for 6 h. RV broth was inoculated with 1:10 or 1:100 of BPW culture if the culture has little growth or is turbid, respectively. The cultures were incubated at 43ºC for 24 h. MPN of each sample in three replicates was determined and PCR was run from one tube of the three series of BPW tubes, i.e., one PCR reaction/sample, and also from the RV broth. For confirmation of presence of *Salmonella*, xylose lysine deoxycholate (XLD-oxoid) and BGA (modified brilliant green agar-oxoid) were inoculated with a loopful of RV broth culture and the plates were incubated at 35ºC for 24 h. Red colonies with or without black centres (typical *Salmonella* colonial appearance) were further tested with API 20E and PCR. Samples that were confirmed biochemically by API 20E and produced a 284 bp fragment with PCR were regarded as positive for *Salmonella*. The MPN value was obtained from the probability table (SANS 8199, 2006).

PCR products were verified by probing with Sal 3. The 284 bp bands were cut out from the low melting agarose gel and suspended in 2 vols of sterile distilled water. The gels were incubated at 65°C to melt the gel, and then spun for 5 min. An aliquot of 10 µℓ of heat-denatured PCR products of each sample was dotted on a positively charged nylon membrane and hybridised to the Dig end-labelled Sal 3 probe.

## Results

**Optimisation of PCR**. The concentration of $MgCl_2$ was varied from 0 to 4.0 mM with increments of 0.5 mM. No amplification occurred at 0.0 mM $MgCl_2$. The amplification yield decreased with an increase in magnesium ion concentration beyond 2 mM with the optimum concentration ranging between 1.0 and 2.0 mM. The concentration of 1.5 mM was used in the optimisation of primer concentration, which then produced the concentration of 125 ng as the minimum, efficient concentration of each primer.

Optimisation of cycling conditions was performed with 1.5 mM $MgCl_2$ and 125 ng primers each. Amplification occurred at all annealing temperatures from 55 to 65ºC indicative of high homology of the primers to the target region of the *invA* gene of *Salmonella*. The annealing conditions that produced the highest yield were 60˚C and 30 s and were therefore adopted as the optimum primer annealing conditions.

In this study, amplification occurred efficiently without the extension phase when different extension periods (0 to 300 s) were tested. However, an extension phase may be helpful in early cycles of PCR when the template concentration is low and at late cycles when the product concentration exceeds the enzyme concentration. Thus, a 1min incubation period, which also resulted in a higher PCR yield, was adopted as optimal. The optimised PCR protocol is as follows: PCR buffer, 1.5U Taq DNA polymerase, 125 ng primer each and 100µM of each dNTPs. The cycling conditions were 94°C, 1 min; 60°C, 30 s and 72°C, 1 min followed by 7 min for the last cycle.

**Limit of detection**. The detection limit of *Salmonella* following optimisation of the PCR assay was 2.6 x $10^4$ cfu/mℓ (Fig. 1, Lane 4) without any pre-enrichment step. The sensitivity was further increased to 26 cfu/mℓ (Fig. 1, Lane 7') following a 6 h pre-enrichment step prior to PCR.

**Total DNA dilution**. Serial dilutions of total DNA from *S. enteritidis* gave 300 pg/mℓ (1.5 pg/reaction) as the minimal amount of template DNA required for positive amplification.

**Specificity**. The pair of primers used in this study showed to be specific to all salmonellae tested except the strain *S. pullorum* (Fig. 2a, Lane 12) and *S. arizonae* (Fig. 2a, Lane 13). Non-

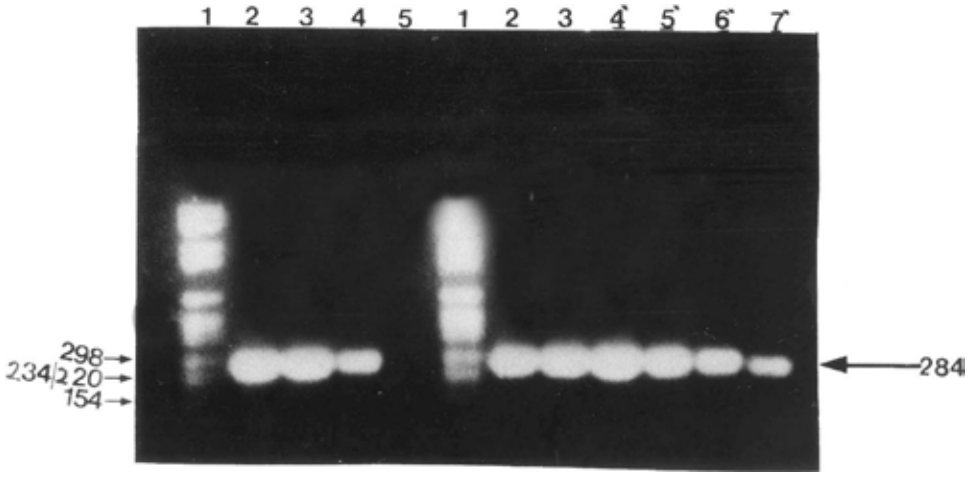


***Figure 1***
*The detection limit of Salmonella cells. Amplification products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. The values on the left are the molecular weight standards and on the right is the size of the target fragment in bp. Lanes without/ with a cap are amplification products obtained prior/after pre-enrichment, respectively. Lanes 1 and 1', 154bp DNA ladder; 2 and 2'- 2.6 x $10^6$ cfu/mℓ; 3 and 3'- 2.6 x $10^5$ cfu/mℓ; 4 and 4'- 2.6 x $10^4$ cfu/mℓ; 5 and 5'- 2.6 x $10^3$ cfu/mℓ; 6'- 2.6 x $10^2$ cfu/mℓ and 7'- 26 cfu/mℓ.*

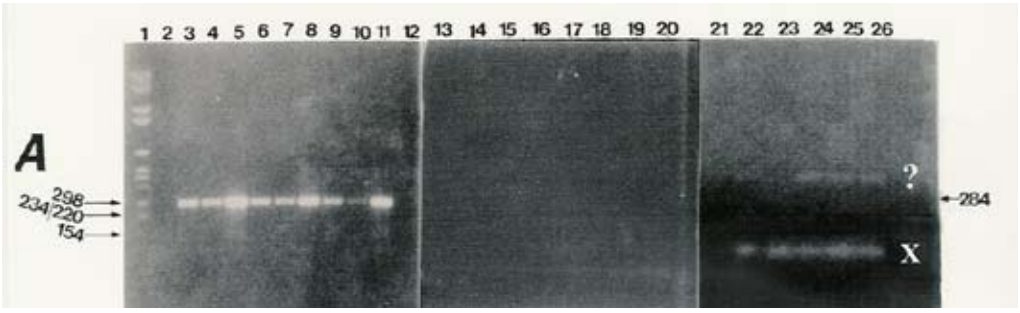


***Figure 2A***
*The specificity of the invA primers tested by PCR on different related bacteria. Amplification products were electrophoresed in a 1.5% agarose gel containing ethidium bromide. The values on the left of the picture are the molecular weight standards and on the right is the size of the target fragment in bp, the question mark (?) shows the non-specific amplification bands and X indicates PCR artifacts. Lanes 1, 154 bp DNA ladder; 2- negative control; 3- S. enteritidis; 4- S. saintpaul; 5- S. dublin; 6- S. typhi; 7- S. paratyphi; 8- S. typhimurium; 9- S. oxford; 10- S. bloemfontein; 11- S. derby; 12- S. pullorum; 13- S. arizonae; 14- E. coli O:112; 15- E. coli O:143; 16- E. hermanii; 17- E. aerogenes; 18- B. cereus; 19- B. cereus var. mycoides; 20- S. dysentry; 21- S. sonnei; 22- S. flexneri; 23- S. boydii; 24- C. freundii; 25- K. pneumoniae; and 26- P. aeruginosa.*


***Figure 2B***
*Southern blot of a selected number of PCR products in A (gel not shown). Amplification products were hybridised to the internal Sal 3 probe. Lane 1- S. typhi; 2- C. freundii; 3- S. enteritidis; 4- K. pneumoniae; 5- S. typhimurium; 6- P. aeruginosa and 7- S. paratyphi.*

specific amplification was occasionally observed with *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Citrobacter freundii* (Fig. 2a, Lanes 24-26) but the amplicon was slightly higher in molecular weight and showed weak amplification. Furthermore, the internal probe did not hybridise to the non-specific products (Fig. 2b). PCR artefacts (X) were occasionally observed.

**Field samples**. PCR detection efficiency of *Salmonella* was evaluated in parallel to the conventional culture method by testing waters collected from houses, communal taps, storage tanks and rivers. PCR was performed along each step of the culture method and *Salmonella* was detected in the presence of other bacteria in all the steps except prior to pre-enrichment. The selective/ differential media RV broth, XLD and BGA, did not inhibit the growth of non-*Salmonella* bacteria but the characteristic colonies of *Salmonella* on XLD and BGA were distinguishable from other non-*Salmonella* bacterial colonies.

The three areas, i.e., Kgapane, Mamabolo and Venda receive their water from different sources that are treated independently of each other. The water treatment was effective in reducing the amount of bacterial contamination and eliminating *Salmonella* from the water as evidenced by both the MPN values and PCR results of the tap waters collected in these


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**TABLE 2**
**Water quality of selected water samples as determined by the PCR method and the culture method**

| Water source | Culture method | PCR technique | MPN value (95% confidence limit |
|---|---|---|---|
| **Kgapane** | | | |
| 1. Communal tap | - | - | 150 (30- 440) |
| 2. Tin tank at home | + | + | 1 100 (150-4800) |
| 3. Stored spring water | + | + | 150 (30- 440) |
| 4. Cement tank at home | - | - | 15 (3- 44) |
| **Mamabolo area** | | | |
| 5. Tin tank at home | - | - | 1 100 (150- 4800) |
| 6. Cement tank at home | - | - | 460 (71- 2400) |
| 7. Cement tank at a school | - | - | 210 (35- 470) |
| **Venda area** | | | |
| 8. Water treatment plant (Phiphidi) | | | |
| i) Raw water | + | + | 1 100 (150- 4800) |
| ii) Coagulation with $AlSO_4$ and lime | + | + | 460 (71-2400) |
| iii) Rapid gravity sand filtration | - | - | 210 (35- 470) |
| iv) Chlorinated water | - | - | 4 (<1-20) |
| 9. Makwarela | | | |
| (i) Tap | - | - | 3 (<1- 13) |
| (ii) Tin tank at home | - | - | 93 (15 380) |
| 10. Ngovhela | | | |
| (i) Tap | - | - | 4 (<1- 20) |
| (ii) Storage tin tank | - | - | 21 (4- 47) |
| 11. Tshinane River | + | + | >1100 |

-: no *Salmonella* was detected
+: presence of *Salmonella*

areas. Samples from the rivers exhibited high bacterial contamination as revealed by both PCR and the culture methods (Table 2).

*Salmonella* was detected by PCR in samples high in competing bacteria as determined by their MPN values. A high amount of indicator bacteria (coliform MPN index >5) did not necessarily coincide with the presence of *Salmonella* (Table 2). No false positives or negatives for *Salmonella* were observed with PCR when assaying field samples as confirmed by the culture method.

## Discussion

The major objective of this study was to develop a rapid and reliable method for the detection of *Salmonella* in water from various sources. The PCR technique was improved by adjusting the concentrations of magnesium ions and primers as well as other cycling parameters to their optimal levels. *Taq* polymerase requires a divalent ion such as $Mg^{2+}$ for activity, i.e.; $Mg^{2+}$ serves as a cofactor and forms soluble complexes with dNTPs to produce the actual substrate that the polymerase recognises (*PCR Applications Manual*, 1999). Hence, no amplification occurred when $MgCl_2$ was not added to the PCR mixture. The concentration of primers and the annealing conditions also affect the specificity of PCR. High primer concentrations and low annealing temperature allow mis-priming, the products of which will actively compete with the target sequence for primers. In the present study amplification occurred at an annealing temperature of as high as 65°C using Sal 1 and Sal 2 primers with Tms of 78°C and 68°C, respectively. Adjusting the PCR components and the cycling parameters resulted in a progressive increase in amplification yield indicating that the concentrations of magnesium ions, primers and the cycling parameters all affected the fidelity of *Taq* polymerase and PCR yield. It is conceivable that further optimisation may still be possible by testing a high number of combinations of PCR parameters.

The limit of detection of the optimised PCR method was $2.6 \times 10^4$ cfu/mℓ. Incorporation of a 6 h non-selective pre-enrichment step further increased the detection limit to 26 cfu/mℓ. Similar results have been reported previously by other authors (Bej et al., 1994; Ellingson et al., 2004; Fratamico, 2003; Guo et al., 2000; Myint et al., 2006; Oliveira et al., 2002; Patel et al., 2006; Pan and Liu, 2002; Soumet et al., 1994) who observed that at least $10^3$to $10^5$ cells/mℓ must be present to give positive results by PCR without a pre-enrichment step and that 1 to 10 cells/mℓ could be detected after a pre-enrichment step.

The target *invA* sequence is conserved among the tested species of *Salmonella* except for *S. pullorum* and *S. arizonae* that gave no amplification results. The absence of PCR products upon *invA* amplification of *S. pullorum* is in contradiction to a report by Galan et al. (1992) that this species tested positive for the presence of *invA* operon. *S. pullorum* is a host-specific avian pathogen and is often avirulent in mammals. *S. pullorum* lacked the ability to invade cultured mammalian epithelial cells when tested for invasiveness (Henderson et al., 1999). Thus, *S. pullorum* poses no possible danger to humans. Galan and Curtiss III (1991) clearly showed that the strains of *S. arizonae* may have significant alterations in the *inv* locus including the absence of some of the genes because they lack the ability to enter cultured epithelial cells (Galan et al., 1992). This deficiency was partially corrected upon addition of a plasmid containing the *inv* locus of *S. typhimurium* (Rahn et al., 1992). Thus, this strain also poses no danger to human beings. Non-specific amplification was occasionally observed with *K. pneumoniae, P. aeruginosa* and *C. freundii*, but the amplicons were found to be slightly higher in molecular weight than the targeted 284 bp fragment and did not hybridise to the *invA* internal probe when tested (Fig. 2b). Rahn et al. (1992) observed similar non-specific amplicons when

*K. pneumoniae*, *P. aeruginosa* were amplified.

No *Salmonella* was detected from direct PCR when environmental waters were assessed. *Salmonella* was detectable by PCR only after enrichment. Myint et al. (2006) observed similar results without pre-enrichment. All the samples tested that contained *Salmonella* according to the culture method showed positive amplification with PCR. No correlation was observed between the occurrence of *Salmonella* and MPN values of the environmental samples (Table 2). The major obstacles of using the PCR technique for direct detection of pathogens in environmental samples are the presence of PCR-inhibitory substances (Feder et al., 2001; Olsen et al., 1995; Picard et al., 1992; Straub et al., 1995; Tebbe and Vahjen, 1993; Tsai and Olson, 1992), the possibility of detecting dead cells (Olsen et al., 1995), and the large amount of start-up material that is required to obtain enough template for PCR (Knight et al., 1990, Sandery et al., 1996). The inclusion of a cultivation step prior to PCR that involved a ten-fold dilution of the sample with medium eliminates the effect of PCR-inhibiting substances on amplification while increasing the number of detectable culturable *Salmonella*. In the past, the presence of various forms of waterborne pathogens was established by using methods that are reliable in detecting bacterial indicator organisms such as *E. coli* in water. McCambridge and McMeekin (1981) clearly showed that *E. coli* is more susceptible to light-induced decay and predation than *S. typhimurium* and other enteric bacteria, which may be a contributing factor to the poor correlation observed in this study. Although it was not observed in water samples tested in this study, pathogens have been detected in waters that have been considered safe on the basis of coliform bacteria (Morinigo et al., 1990).

Waterborne salmonellosis has significantly affected many rural people in South Africa during the past years (Department of Health, 1998). The unavailability of potable water leads to poor hygienic practices as people tend to minimise water usage and use dirty water, flowing and stagnant stream waters for drinking, bathing and laundry. Of the RSA population of 48.61 million, 7% has no infrastructure for provision of potable water and 10% has access below RDP level (Water Services: National Information System, 2006). In the Limpopo Province 7.5% of the population lacks infrastructure to safe water while 21% has access below RDP level (Water Services: National Information System, 2006). This shortage of safe piped water leads to storage of water in open tanks and usage of surface water for domestic purposes (Central Statistical Service Report, 1997). The danger of the shortage of running tap water is supported by the report that communities in the Bochum area of Limpopo Province that used water from the Crocodile River suffered from repeated bilharzia and typhoid fever outbreaks (Chauke, 1996). Fincham and Dhansay (2006) reported bilharzia infections in 80% of children in Limpopo in 2005. The Sekhukhune district of Limpopo Province has reported 93 typhoid fever cases in 2004 (National Institute for Communicable Diseases, 2004), and Mpumalanga encountered an outbreak in 2006 (*The Water Wheel*, 2005). This trend shows than an urgent intervention to thoroughly monitor bacterial contamination of water systems is required to circumvent any possible human infections.

This study showed high prevalence of bacterial contamination including *Salmonella* in surface and stored waters while running tap water exhibited low coliform count and absence of *Salmonella* (Table 2). This observation infers enterobacterial contamination of water during storage either due to aerosols from dust that contains soil and dried animal excreta due to lack of ground cover and sanitary facilities or from persons that dip their contaminated hands into the storage containers to collect water. A similar observation was made in the dry Bochum area of RSA where *Salmonella* was mostly absent from the treated tap water while the stored water had high coliform counts (Chauke, 1996). Nonetheless, the quality of the drinking water tested in these three areas of the Limpopo Province, on average, does not conform to the South African Drinking Water Standard that allows 1 faecal coliform per 100 mℓ of sample (SANS 241, 2005). The implementation and expansion of water and sanitation infrastructure and management thus remain urgent for most rural households in RSA.

PCR offers a great diagnostic tool in comparison to the culture method based on the amount of time required to confirm the presence/absence of *Salmonella*. When optimised, PCR affords high specificity and sensitivity even in the presence of high levels of competing micro-organisms as evident from the MPN values of the samples (Table 2). However, in order to meet the standard detection limits for water quality (SANS 241, 2005), a pre-cultivation step is still required to increase sensitivity and to remove inhibitory substances when monitoring environmental samples. Some authors reported the existence of viable but non-culturable (VBNC) bacteria in environmental samples (Kjelleberg et al., 1993; Knight et al., 1990; Pommepuy et al., 1996 and Roszak and Colwell, 1987), which would not be enriched in a pre-cultivation step and thus may escape detection. Medema et al. (1992), Caro et al. (1999) and Smith et al. (2000) reported a loss of pathogenicity associated with non-culturability of *S. typhimurium* when subjected to seawater stress, solar and ultraviolet-C irradiation. Although the cultivation step excludes the detection of VBNC *Salmonella*, such bacteria most probably pose no significant threat to humans because of the minimum infectious dose of *Salmonella* ($10^5$/ $10^9$ for *S. typhi*/ *S. typhimurium* respectively) required to initiate a disease (Le Minor, 1981). Thus, the 12h cultivation-PCR assay offers a good diagnostic tool for the routine monitoring of *Salmonella* contamination in water used for domestic purposes in contrast to the 102h culture method.

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