Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Detection of *Salmonella enterica* serovar Typhi (*S.* Typhi) by selective amplification of *invA*, *viaB*, *fliC-d* and *prt* genes by polymerase chain reaction in mutiplex format

S. Kumar, K. Balakrishna and H.V. Batra

Division of Microbiology, Defence R&D Establishment, Gwalior, Madhya Pradesh, India

Keywords
detection, food, PCR, *Salmonella* Typhi, water.

Correspondence
S. Kumar, Division of Microbiology, Defence R&D Establishment, Jhansi Road, Gwalior 474 002, Madhya Pradesh, India.
E-mail: subodh_kumar@email.com

2005/0363: received 7 April 2005, revised 19 July 2005 and accepted 22 July 2005

doi:10.1111/j.1472-765X.2005.01813.x

Abstract

Aims: Development of a PCR assay that can target multiple genes for rapid detection of *Salmonella enterica* serovar Typhi (*S.* Typhi) from water and food samples.

Methods and Results: PCR primers for invasion, O, H and Vi antigen genes, *invA*, *prt*, *fliC-d* and *viaB* were designed and used for the rapid detection of *S.* Typhi by multiplex PCR. Internal amplification control, which coamplified with *prt* primers, was also included in the assay. The results showed that all cultures of *Salmonella* were accurately identified by the assay with no nonspecific amplification in other cultures. The assay had 100% detection probability when a cell suspension of 10^4 CFU ml^{-1} (500 CFU per reaction) was used. *Salmonella* Typhi bacteria were artificially inoculated in the water and food (milk and meat rinse) samples and detected by mPCR after overnight pre-enrichment in buffered peptone water. No *Salmonella* bacteria could be detected from water samples collected from the field by mPCR or standard culture method.

Conclusions: The developed mPCR assay provides specific detection of *S.* Typhi.

Significance and Impact of the Study: Rapid methods for detection of *S.* Typhi from complex environmental matrices are almost nonexistent. The mPCR assay reported in this study can be useful to identify *S.* Typhi bacteria in field environmental samples.

Introduction

Typhoid fever continues to be a major health problem in many parts of the world particularly the developing countries. The incidence of typhoid fever has been estimated to be between 17 and 33 million cases annually with 600 000 associated deaths (Pang 1998). *Salmonella* Typhi, the causative agent of typhoid fever, is an obligate human pathogen. Typhoid fever is typically acquired by ingesting food or water that has been contaminated by the faeces of a typhoid infected individual. However, unlike ubiquitous serovars such as *S.* Typhimurium, *S.* Typhi is generally excluded from the group of foodborne salmonellas because it is not a preharvest food safety issue. Probably, for this reason, the rapid methods for detection of *S.* Typhi from environmental sources and food matrices are almost nonexistent. However, many typhoid fever outbreaks have been reported, which were caused by consumption of either contaminated water (Mermin *et al.* 1999) or food (Cote *et al.* 1995).

The laboratory procedures for detection and identification of *Salmonella* by conventional methods are laborious and time consuming taking 3–5 days. PCR has proven to be an indispensable tool for detection of infectious agents in the laboratory. Many PCR assays have been described for detection of *S.* Typhi and other *Salmonella* serovars (Hashimoto *et al.* 1995; Zhu *et al.* 1996; Chaudhry *et al.* 1997; Agarwal *et al.* 2002; Hirose *et al.* 2002; Nakano

© 2006 The Society for Applied Microbiology,

et al. 2003). Most of these assays are monoplex PCRs, which rely on the amplification of a single target. This is suitable for clinical samples such as blood but for identification of *S.* Typhi from environmental matrices, e.g. water/food, where a range of bacteria can be expected to be present, targeting amplification of multiple genes can obviously be more reliable. Moreover, they lack internal amplification control (IAC), which has now become almost mandatory in diagnostic PCRs particularly when tested on environmental or food samples. We herein report a robust and specific multiplex PCR assay for identification of *S.* Typhi from water and food matrices.

Materials and methods

Bacterial strains, culture media, growth conditions and DNA sample preparations

The bacterial strains used in this study are listed in Table 1. These cultures were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, Christian Medical College (CMC), Vellore, Postgraduate Institute of Medical Education & Research (PGI), Chandigarh and Central Research Institute (CRI), Kasauli (HP). The cultures were grown in LB broth (Difco; Becton Dickinson, Sparks, MD, USA) at 37°C in shaking conditions. *Salmonella* Typhi (strain SKST) bacteria were enumerated on nutrient agar plates by pour plate method. Template DNA from each bacterial strain was purified by boiling method (Theron *et al.* 2001) or by DNA extraction kit (Qiagen, Hilden, Germany). One-millilitre culture was used to prepare DNA by boiling, harvested in 50 μl sterile double distilled water (DDW) and stored at −20°C until used.

Table 1 Bacterial strains used for the evaluation of specificity of PCR primers

Bacterial strain (source, no. of strains tested)	PCR detection of *inv*A	*viaB*	*fliC*-d	*prt*
Salmonella				
Typhi (lab strain, strain SKST)	+	+	+	+
Typhi (PGI, 10)	+	+	+	+
Typhi (CMC, 4)	+	+	+	+
Livingstone (CRI, 1)	+	−	+	−
Strasbourg (CRI, 1)	+	−	+	+
Schwarzengrund (CRI, 1)	+	−	+	−
Paratyphi A (PGI, 1; CMC, 2)	+	−	−	+
Typhimurium MTCC 1251, 1253	+	−	−	−
Enteritidis (CRI, 1)	+	−	−	+
Weltevreden MTCC 1169	+	−	−	−
Senftenberg (PGI, 2)	+	−	−	−
Infantis MTCC 1167	+	−	−	−
Virchow MTCC 1163, 1164	+	−	−	−
Proteus mirabilis MTCC 1429	−	−	−	−
Proteus vulgaris MTCC 744	−	−	−	−
Proteus morganii MTCC 662	−			−
Shigella flexneri MTCC 1457, (CMC, 1)	−	−	−	−
Pseudomonas aeruginosa MTCC 424	−	−	−	−
Ps. aeruginosa (CMC,1)	−	−	−	−
Enterobacter cloacae MTCC 509	−			−
Serratia marcescens MTCC 97	−	−	−	−
Serratia liquifaciens MTCC 1620	−	−	−	−
Klebsiella pneumoniae MTCC 109	−	−	−	−
Kl. pneumoniae MTCC 432	−	−	−	−
Klebsiella rhinoscleromatis MTCC 661	−	−	−	−
Escherichia coli MTCC 730, 732, 739	−	−	−	−
Staphylococcus aureus MTCC 97, (lab strain, 4)	−	−	−	−
Yersinia enterocolitica (lab strain, 2)	−	−	−	−
Yersinia pseudotuberculosis (lab strain,1)	−	−	−	−

Primers and internal amplification control

Four pairs of primers were designed using the GenBank database sequences (Table 2). All primers used in the study were synthesized by Operon Biotechnologies, Cologne, Germany. To check the presence of inhibitors within PCR mixture, IAC was constructed. The primers used in this reaction had 5′ overhanging ends, which were identical to the primers used in mPCR specific for *prt* (prtF and prtR), whereas 3′ ends were complementary to a DNA sequence of pBluescript SK phagemid (Table 2).

The PCR reaction mixture for generation of IAC DNA contained 1·0 μmol l^{-1} of each primer, 0·2 mmol l^{-1} of each dNTP (MBI Fermentas, Vilnius, Lithuania), 0·5 units of *Taq* polymerase, 1·5 mmol l^{-1} $MgCl_2$ in 1×PCR buffer (MBI Fermentas) with 500 pg of template DNA. The reaction procedure consisted of 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min. The DNA was denatured for 4 min in the beginning and finally extended for 5 min at 72°C. PCR product was purified using commercially available kit (Qiagen). The concentration of IAC DNA was determined spectrophotometrically at 260 nm and was stored in DDW at −20°C. The following equation was used to calculate the copy number of the PCR product concentration: weight of PCR fragment (in g μl^{-1}) × $(6{\cdot}023 \times 10^{23})/(660$ g mol^{-1} × number of base pairs of PCR fragment) = the number of genomic copy per microlitre.

PCR amplification and analysis of PCR products

Multiplex PCR was carried out in 25 μl reaction containing 0·3 μmol l^{-1} of prtF and prtR primers, 0·2 μmol l^{-1} of all other primers, 0·2 mmol l^{-1} of each dNTP, 1000 copies of IAC DNA, 1·0 unit of Hot Start *Taq* polymerase, 2·5 mmol l^{-1} $MgCl_2$ in 1×Hot Start PCR Buffer (MBI Fermentas) with 2·5 μl of template DNA. Various

© 2006 The Society for Applied Microbiology,

Table 2 Primers for multiplex PCR amplification of *S.* Typhi

Primer	Primer sequence 5′ to 3′	Target gene	Amplicon size (bp)	Position of primers	Accession No.
InvAF	CGAGCAGCCGCTTAGTATTGAG	*invA*	881	978–999	U43273
InvAR	CCATCAAATTAGCGGAGGCTTC			1858–1837	
ViaBF	CACGCACCATCATTTCACCG	*viaB*	738	165–184	D14156
ViaBR	AACAGGCTGTAGCGATTTAGG			902–882	
DhF	GCTTAATGTCCAAGATGCCTAC	*fliC*-d	587	516–537	L21912
DhR	GAGCAACGCCAGTACCATCTG			1102–1082	
PrtF	CGTTTGGGTTCCTTGGATCACG	*prt*	369	383–404	M29682
PrtR	CTATAATGGCGGCGGCGAGTTC			751–730	
IAC1	CGTTTGGGTTCCTTGGATCACGGT GCCACCTAAATTGTAAGCG	IAC	455	2955–14	X52328
IAC2	CTATAATGGCGGCGGCGAGTTCTG ACCGCTACACTTGCCAGC			404–385	

IAC primers are flanked by prt primers on 5′ ends.

concentrations of IAC DNA were tried before choosing 1000 copies per reaction. PCR was taken through 38 cycles in Bio-Rad iCycler (Thermal cycler) (Bio-Rad Laboratories, Hercules, CA, USA) at 94°C for 30 s (denaturation), 60°C for 90 s (annealing) and 72°C for 2 min (extension). Gradient temperature from 57 to 63°C was initially used before finalizing 60°C as annealing temperature. The DNA was denatured for 4 min in the beginning and finally extended for 5 min at 72°C. PCR products were analysed in 2% agarose.

Determination of detection probability

DNA prepared from *S.* Typhi cell suspension at a concentration of 10^0–10^6 CFU ml^{-1} was used in this experiment. A 2·5-μl aliquot of each dilution was added to five separate PCR tubes in the presence of about 1000 copies of IAC DNA. Negative control reaction mixture contained sterile DDW in place of template DNA. The PCR conditions were same as described above. The experiment was repeated four times. The detection probability was obtained by plotting the relative number of positive PCRs observed against the concentration of cell suspension.

Robustness test

To determine the robustness of the assay eight replicates of 1000 copies of *S.* Typhi DNA were made simultaneously in one run at optimized concentrations and suboptimal concentrations of 10% or 20% less and 10% or 20% more PCR reagent. The IAC template (1000 copies) was added ±10% or 20% in corresponding run. The experiments were performed at annealing temperatures of 58, 60 and 62°C. All other conditions were kept constant as described above.

Determination of specificity of mPCR

The specificity of primers was checked against cultures as shown in Table 1 by taking 5 μl of template DNA (*c.* 10^7 CFU). The procedure for PCR was essentially the same as described above.

Analysis of artificially contaminated food/water samples

In order to validate the mPCR method for detection of *S.* Typhi bacteria, water and food samples were collected and artificially inoculated. Water samples (five) were collected from laboratory and household supply and five sets of 100 ml from each water sample were inoculated with *S.* Typhi bacteria to achieve a concentration of 10^3, 10^2, 10^1, 10^0 and 10^{-1} CFU ml^{-1}. Each 100-ml sample was filtered through 0·45 μm size membrane filter. The filter was inoculated in 50 ml buffered peptone water (BPW) and incubated overnight at 37°C. Samples of milk and meat (three each) were procured from the local market. Meat rinse was prepared by suspending 150 g of meat sample in 150 ml of BPW and rinsing thoroughly with the medium. The rinse was centrifuged at 1500 ***g*** to get rid of the particulate debris and supernatant was stored in aliquots of 10 ml at −20°C for further use. Ten millilitre each of meat rinse or milk sample were inoculated with 100 μl of *S.* Typhi culture to achieve a concentration of 10^3–10^{-1} CFU ml^{-1}. An uninoculated control was prepared by seeding samples with 100 μl of BPW. Each food sample was diluted with BPW at a ratio of 1 : 10, mixed well and incubated overnight (18 h) at 37°C. One millilitre of BPW growth was taken at the end of incubation period from all samples and processed for DNA extraction by boiling. The DNA (2·5 μl) was used as template in PCR assay.

Analysis of field samples

Twenty-five potable water samples (100 ml), which included 10 groundwater (hand pump water) samples and 15 samples from municipal water supply (tap water) were collected from different locations in Gwalior city in sterile containers. The samples were processed for DNA extraction by above mentioned procedure for water (except for artificial contamination) and analysed by mPCR.

Investigation of water and food samples by conventional culture method

In order to confirm the results of mPCR, conventional culture method was also attempted for isolation of *S.* Typhi from field samples. The pre-enriched growth in BPW (as previously described) was used to inoculate (1 : 10) tetrathionate brilliant green bile broth and selenite cystine broth for enrichment. After incubation of 24 h at 37°C selective plating was performed on Xylose Lysine Deoxycholate agar and MacConkey agar. The presumptive colonies were screened by inoculation of triple sugar iron and lysine iron agar slants. Culture showing positive reactions were taken for further biochemical and serological tests according to methods of AOAC (Williams 1984).

Results

Multiplex PCR

mPCR was optimized using the profile as outlined in Materials and methods. The annealing temperature of 60°C was finally selected though bands were visible at all the temperatures. The concentration of prtF and prtR primers was increased to 0·3 μmol l^{-1} because a faint band was seen at 0·2 μmol l^{-1} concentration in the presence of IAC DNA.

Analysis by BLAST search indicated significant specificity of the primers for the desired gene sequences. All *Salmonella* and non-*Salmonella* cultures were identified correctly (Table 1, Fig. 1).

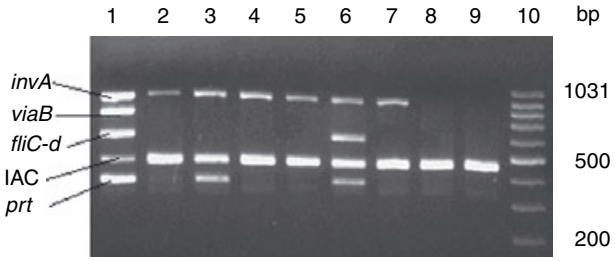

Figure 1 Agrose gel electrophoresis of PCR products. PCR was carried out with DNA obtained from *Salmonella* Typhi (lane 1), *S.* Virchow (lane 2), *S.* Enteritidis (lane 3), *S.* Typhimurium (lane 4), *S.* Senftenberg (lane 5), *S.* Strasbourg (lane 6), *S.* Infantis (lane 7) and *E. coli* (lane 8), lane 9 – negative control. Lane 10 shows the profile of 100 bp marker.

IAC and detection probability

The IAC coamplified with target DNA and had amplicon size of 455 bp. Inclusion of varying concentrations of IAC DNA in mPCR mix did not change the detection limit of the assay (Fig. 2) and 1000 copies were found to be optimum. The detection probability of mPCR was found to be 20% at a concentration of 10^3 CFU ml^{-1} (50 CFU per reaction) and 100% at a concentration of 10^4 CFU ml^{-1} (500 CFU per reaction). To determine the precision of the assay three replicates of 10-fold dilutions of purified DNA of *S.* Typhi containing 10^6–10^0 genome equivalents were also determined simultaneously in a single run. The assay had detection limit of 3 pg.

Robustness

There was no significant loss in the visibility of bands at less (10%, 20%) or more (10%, 20%) concentrations of PCR reagents and IAC DNA. The most prominent change of all concentrations was at 20% less concentration.

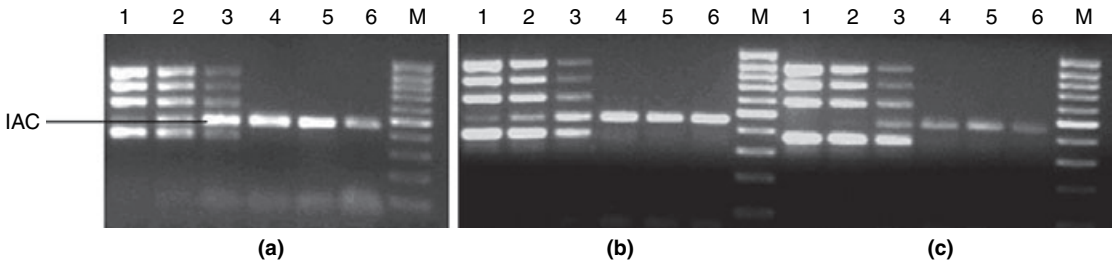

Figure 2 Coamplificaton of IAC (455 bp) and *Salmonella* Typhi genomic DNA. The initial number of *S.* Typhi DNA per reaction was 5×10^4, 10^3, 10^2, 10, 1 CFU (a, b and c: lanes 1–5) and IAC copy number was $3{\cdot}9 \times 10^4$ (a), $3{\cdot}9 \times 10^3$ (b) or $3{\cdot}9 \times 10^2$ (c). Lane 6 (a, b and c) represents the negative control where no *S.* Typhi DNA was taken. Marker 100 bp (MBI Fermentas) was used as molecular weight standard.

© 2006 The Society for Applied Microbiology,

Temperature variation of ±2°C also did not make any change in the PCR profile.

Analysis of artificially contaminated food/water samples

Salmonella Typhi could be detected in all artificially inoculated water and food samples after overnight pre-enrichment in BPW. Detection limit of order of 10^{-1} bacteria (70 CFU/100 ml) was observed in water samples. Detection of bacteria in milk and meat rinse samples showed that mPCR could detect $4{\cdot}8 \times 10^{1}$ CFU ml^{-1} in milk and $2{\cdot}0 \times 10^{1}$ CFU ml^{-1} in meat rinse samples.

Analysis of field samples

None of the sample tested positive for *S.* Typhi by mPCR or by bacteriological isolation.

Discussion

In this study, a multiplex PCR assay was developed for rapid detection of *S.* Typhi from environmental matrices. The assay targeted the amplification of four genes instead of one as in many other studies. The genes *viaB*, *prt* and *fliC-d* encode for the synthesis of Vi (capsule), O (LPS) and H (flagellar) antigens respectively. These antigens form the basis of classification for *Salmonella* by Kauffman–White scheme. The gene *invA*, along with other invasion genes, is responsible for invasion of epithelial cells and has been reported to be present in all salmonellas (Chiu and Ou 1996). The *viaB* gene is present in *Citrobacter freundii* and three serovars of *Salmonella* (*S.* Paratyphi C and *S.* Dublin being the other two). The gene *prt* is part of *rfb* cluster of genes and encodes for CDP paratose synthase which converts CDP-4-keto-3,6-dideoxyglucose to CDP paratose. This gene is present in serovar Typhi, Paratyphi A and few others. The *fliC*-d gene is present in over 100 *Salmonella* serovars. Earlier researchers who had targeted single genes, e.g. *fliC*-d (Chaudhry *et al.* 1997), *ViaB* (Hashimoto *et al.* 1995) and 16S rRNA gene (Zhu *et al.* 1996) for specific detection of *S.* Typhi, understandably, strains of *Salmonella* other than Typhi were also detected in some cases. To specifically identify *S.* Typhi from environmental samples, we utilized amplification of four gene sequences (*invA*, *viaB*, *fliC-d*, *prt*) producing a specific profile.

To examine the possible cross reactions, selected primers were tested both by homology searches using BLAST search and screening of *Salmonella* and non-*Salmonella* strains. No false positives and negatives were recorded. PCR amplicons of different genes also yielded the products of expected sizes after restriction enzyme digestion (results not shown). This shows high specificity of the assay.

In order to make the PCR assay acceptable to the present norms of a diagnostic PCR (Hoorfar *et al.* 2003), IAC was included in the assay. The IAC was pBluescript phagemid DNA flanked by prt primers. Minimum number of copies of IAC DNA that gave good visible band, was selected in order to avoid competition between target DNA and IAC DNA for prt primers particularly at low concentration of target DNA.

Another important criterion for a diagnostic PCR is robustness. The present assay worked in presence of 20% lower to 20% more concentration of PCR reagents and IAC DNA. A temperature variation of ±2°C was also well-tolerated, which shows the robustness of the assay.

The mPCR detected *S.* Typhi cell suspension of 10^{4} CFU ml^{-1} (500 CFU per reaction) at a probability of 100%. The detection limit was 3 pg with pure genomic DNA. The level of *S.* Typhi cells can be obtained by inclusion of a pre-enrichment step even if low levels of bacteria are present. This has been demonstrated in artificially contaminated water, meat rinse and milk samples. Detection limit of 0·7 CFU ml^{-1} was observed in water samples after overnight growth in BPW, however, two log higher concentration of bacteria could be detected in milk and meat rinse samples. This may be because of the presence of large number of competing bacteria in foods that might have restricted the growth of *S.* Typhi. No reports are available for comparison of *S.* Typhi detection in foods but detection of other *Salmonella* serovars by PCR has been reported. The detection limit in these studies varies widely and in many of them it ranges from 1–3 CFU g^{-1} (Agarwal *et al.* 2002) to 10–40 CFU g^{-1} (Nakano *et al.* 2003) of food. Our results are similar to these studies. Although limited numbers of food samples were included for artificial contamination in the present study, which obviously limits the representation of sampling as well as the reliability and accuracy of mPCR yet detection of *S.* Typhi from all the samples prove the utility of the assay.

The likelihood of contamination of the field water by *S.* Typhi is largely expected only in the event of an outbreak of typhoid fever. As only sporadic cases of typhoid fever were reported in Gwalior this season with no major outbreak, we did not process a large number of field samples. No *S.* Typhi bacteria could be detected by mPCR or conventional culture method from the field samples. Although the assay could not be tested on naturally contaminated samples, we still believe that the highly specific and robust mPCR assay developed in this study can be used to detect *S.* Typhi from environmental samples in less than 24 h instead of 4–5 days by conventional culture method.

Acknowledgements

Authors are thankful to Director, DRDE for providing the necessary facilities and encouragement and to Ms Kavita Pol and Ms Bushra Siddiqui for technical help.

References

Agarwal, A., Makker, A. and Goel, S.K. (2002) Application of the PCR technique for a rapid, specific and sensitive detection of *Salmonella* spp. in foods. *Mol Cell Probes* **16**, 243–250.

Chaudhry, R., Laxmi, B.V., Nisar, N., Ray, K. and Kumar, D. (1997) Standardisation of polymerase chain reaction for the detection of *Salmonella typhi* in typhoid fever. *J Clin Pathol* **50**, 437–439.

Chiu, C.-H. and 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. *J Clin Microbiol* **34**, 2619–2622.

Cote, T.R., Convery, H., Robinson, D., Ries, A., Barrett, T., Frank, L., Furlong, W., Horan, J. *et al.* (1995) Typhoid fever in the park: epidemiology of an outbreak at a cultural interface. *J Community Health* **20**, 451–458.

Hashimoto, Y., Itho, Y., Fujinaga, Y., Khan, A.Q., Sultana, F., Miyake, M., Hirose, K., Yamamoto, H. *et al.* (1995) Development of nested PCR based on the *ViaB* sequence to detect *Salmonella typhi*. *J Clin Microbiol* **33**, 775–777.

Hirose, K., Itoh, K.-I., Nakajima, H., Kurazono, T., Yamaguchi, M., Moriya, K., Ezaki, T., Kawamura, Y. *et al.* (2002) Selective amplification of *tyv* (*rfbE*), *prt* (*rfbS*), *viaB*, and *fliC* genes by multiplex PCR for identification of *Salmonella enterica* serovars Typhi and Paratyphi A. *J Clin Microbiol* **40**, 633–636.

Hoorfar, J., Cook, N., Malorny, B., Wagner, M., De Medici, D., Abdul-mawjood, A. and Fach, P. (2003) Making internal amplification control mandatory for diagnostic PCR. *J Clin Microbiol* **41**, 5835.

Mermin, J.H., Villar, R., Carpenter, J., Roberts, L., Samaridden, A., Gasanova, L., Lomakina, S., Bopp, C. *et al.* (1999) A massive epidemic of multidrug-resistant typhoid fever in Tajikistan associated with consumption of municipal water. *J Infect Dis* **179**, 1416–1422.

Nakano, S., Kobayashi, T., Funabiki, K., Matsumura, A., Nagao, Y. and Yamada, T. (2003) Development of a PCR assay for detection of *Enterobacteriaceae* in foods. *J Food Prot* **66**, 1798–1804.

Pang, T. (1998) Genetic dynamics of *Salmonella typhi* – diversity in clonality. *Trends Microbiol* **6**, 339–342.

Theron, J., Morar, D., Preez, M.Du., Brozel, V.S. and Venter, S.N. (2001) A sensitive seminested PCR method for the detection of *Shigella* in spiked environmental water samples. *Water Res* **35**, 869–874.

Williams, S. (1984) *Official Methods of Association of Official Analytical Chemists, AOAC Inc., Virginia, USA*. Richmond, Virginia: The William Byrd Press Inc..

Zhu, Q., Lim, C.K. and Chan, Y.N. (1996) Detection of *Salmonella typhi* by polymerase chain reaction. *J Appl Bacteriol* **80**, 244–251.

 © 2006 The Society for Applied Microbiology,