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Full Length Research Paper

Using current molecular techniques for rapid differentiation of *Salmonella* Typhi and *Salmonella* Typhimurium

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Typhoid fever is responsible for the deaths of many people annually. However, conventional and time-consuming detection methods for *Salmonella* Typhi still dominate. By using a molecular based approach, it was possible to identify *Salmonella* Typhi by amplifying two specific genes (*viaB* and *tyv*) and by using RFLP analysis on the 16S rRNA gene as a first step for identification. We were also able to identify *Salmonella* Typhi using multiple gene targets in a single multiplex PCR reaction. Here we show that, as opposed to conventional methods, molecular based approaches are more rapid and should thus be used for any initial detection of *Salmonella* Typhi.

Key words: Detection, API, BIOLOG, RFLP, PCR, 16S rRNA *Salmonella* Typhi, *Salmonella* Typhimurium.

INTRODUCTION

Typhoid fever is a major health problem in many parts of the world, especially in the rural communities. Between 17 and 33 million cases are reported annually with 600,000 associated deaths (Parry et al., 2002). *Salmonella* Typhi, the causative agent, is a serious human pathogen that is usually transmitted *via* the faecal-oral route. This bacterium is generally excluded from the group of food-borne salmonellas because it is not a pre-harvest food safety issue (Kumar et al., 2006). For this reason, rapid detection methods thereof in environmental samples have become a great necessity to start effective treatment (Kumar et al., 2006).

Salmonella Typhi is gram-negative, rod-shaped and non-spore forming, and is a member of the family *Enterobacteriaceae* (Everest et al., 2001). This family is classified into serovars (serotypes) based on the lipopoly-saccharide (O), flagellar protein (H) and sometimes the capsular (Vi) antigens. There are currently 2463 known serovars and within a serovar, there may be strains that differ in virulence. The problem with conventional detection methods is that they are laborious and time-consuming, and cannot distinguish between serovars (Kumar et al., 2006). This can become a serious issue since serovars may differ in the level of pathogenicity (Brenner et al., 2000). Pertaining hereto, *Salmonella* Typhi causes typhoid fever which could result in death; however on the other hand *Salmonella* Typhimurium merely causes gastroenteritis, which can easily be treated.

PCR has proven an invaluable tool for detection of pathogens in water and food samples (Hashimoto et al., 1995). Some PCR assays have previously been described for the detection of *Salmonella* Typhi and other *Salmonella* serovars (Hashimoto et al., 1995; Zhu et al., 1996; Hirose et al., 2002), however these mostly rely on the amplifi-cation of a single target gene.

Here we evaluate molecular techniques for the rapid identification of *Salmonella* Typhi as well as to differentiate this dangerous pathogen from the other less pathogenic *Salmonella* Typhimurium serovar using specific restriction enzymes and multiplex PCR in comparison to the conventional 2 - 5 days for speciation.

EXPERIMENTAL

Conventional methods

The following conventional methods were evaluated [enrichment, selective plating and biochemical tests according to the ISO 6579: 1993 method as described by Harrigan (1998), API (bioMérieux)

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and BIOLOG (BIOLOG, Inc.) for the timescale for detection of as well as the ability to discriminate between serovars. No serological tests were performed. All the media were prepared according to the Difco Manual (Becton and Dickinson, 1998).

Molecular identification techniques

All the PCR reactions were carried out using genomic DNA (gDNA) as template. For *Salmonella* Typhimurium, gDNA was extracted from liquid cultures with the ChargeSwitch gDNA Mini Bacteria kit (Invitrogen) according to the manufacturer's instructions, while a positive control gDNA sample from *Salmonella* Typhi was received from the National Health Laboratory Service, South Africa.

16S rRNA analysis

The 16S rRNA sequence was PCR amplified using the universal bacterial primers (27F and 1492R, both to a final concentration of 0.2 mM) (Weisburg et al., 1991), SuperTherm reagents (Southern Cross Biotechnology), and dNTP's (0.2 mM final concentration; Fermentas). The standard cycling conditions were as follows: i.) an initial denaturation at 94°C for 5 min. ii.) 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 45 s and elongation at 72°C for 45 s. iii.) A final 10 min extension period at 72°C.

Following visualization by electrophoresis on a 1% (w/v) agarose gel, PCR fragments were purified using the GFX PCR DNA purification kit (Amersham Biosciences). Ligation with T_4 DNA ligase (Fermentas) followed using the pGEM®-T Easy vector system (Promega), transformation into Top 10 *Escherichia coli* competent cells and small scale plasmid isolation using the GeneJET™ Plasmid Miniprep Kit (Fermentas) according to the manufacturer's instructions, followed by RFLP analysis. Selected clones, identified as carrying the plasmid-borne insert of interest, were sequenced using the SP6 and T7 primers with the ABI 377 Genetic Analyser (Applied Biosystems) to verify strain identity.

RFLP analysis was further used as a first step in the molecular based approach for distinction between different organisms. In this technique, the 16S rRNA sequence of each strain was digested using *Avr*II (Fermentas) according to the manufacturer's instructions and assessed on a 1% (w/v) agarose gel.

Gene-specific PCR for distinction between Salmonella species

A specific LPS (*tyv*) gene (329 bp), found only in *Salmonella* Typhi (Hirose et al., 2002), was PCR amplified to distinguish the *Salmonella* Typhi strain from *Salmonella* Typhimurium, which was used as a negative control. The forward (STFR: GCG CGA ATT CAG CTT TTA ATT ACC GGT GGA TGT GGC TTC C) and reverse primers (STRR: GCG CGA ATT CGC CGT ACT GCC TCA AGT AAA TTT AAA GTT C) were used with the standard cycling PCR conditions except for the annealing temperature which was set at 65°C. Also, the V_i antigen (*viaB*) gene (599 bp) found only in *Salmonella* Typhi (Hashimoto et al., 1995) was PCR amplified using the forward (STF: GTT ATT TCA GCA TAA GGA G) and reverse primers (STR: ACT TGT CCG TGT TTT ACT C) at an annealing temperature of 52°C.

In addition, the *invA* gene was also targeted since it is found in both *Salmonella* Typhi and *Salmonella* Typhimurium, and was used for a multiplex PCR using the forward (S 139: GTG AAA TTA TCG CCA CGT TCG GGC AA) and reverse (S 141: TCA TCG CAC CGT CAA AGG AAC) primers (Salehi et al., 2005) in conjunction with the LPS gene primers at an annealing temperature of 65°C.

RESULTS AND DISCUSSION

Conventional methods

Following evaluation of all the conventional methods, it was concluded that these could only be used as general detection techniques for *Salmonella* and are not able to discriminate between different *Salmonella* serovars, a critical aspect since these have very different levels of pathogenicity. Furthermore, all the conventional techniques required at least 48 h in order to obtain results (data not shown). Another drawback of these techniques is that pure cultures are required, thus further increasing the time required for detection and speciation.

Molecular identification techniques

16S rRNA analysis

A PCR was carried out using the universal bacterial 16S rRNA primers and visualization by UV illumination showed the expected bands of about 1500 bp (data not shown). A restriction digest was performed on the purified insert-containing plasmid DNA using the restriction endonuclease, *Eco*RI, to determine which clones contained the insert of interest. Three bands (3015 867 and 667 bp) were expected since *Eco*RI cuts the insert from the pGEM®-T Easy backbone and once in the insert. These results were obtained in all clones analyzed. Following RFLP analysis, selected clones were sequenced and identified using the BLASTn algorithm at NCBI (National Center for Biotechnology Information) (data not shown).

Further RFLP analysis of the 16S rRNA sequence was done using *Avr*II and, as a result of the variable regions, a unique pattern was expected and obtained (Figure 1) for both *Salmonella* Typhimurium (X80681) and *Salmonella* Typhi (U88545).

Gene-specific PCR for distinction between Salmonella species

The LPS (*tyv*) gene encodes a CDP-tyvelose epimerase, which converts CDP-paratose to CDP-tyvelose, and plays an important role in the formation of the outer membrane proteins that render severe resistance against high temperatures and denaturing reagents like SDS and urea (Hirose et al., 2002). The V_i antigen (*viaB*) gene encodes an exopolysaccharide known as the Vi antigen and is produced from a homopolymer of high molecular mass (Virlogeux et al., 1996) to form a capsular structure protecting *Salmonella* Typhi against complement-mediated lysis as well as phagocytosis. The two above-mentioned genes were targeted in order to specifically detect the presence of *Salmonella* Typhi as these genes are only found in this serovar. Furthermore, the *invA* gene, which aids the bacterium in the penetration of the small intestinal wall (Salehi et al., 2005), was selected to detect the presence of *Salmonella* in the samples as this gene is found in all *Salmonella* species.

The LPS (*tyv*) gene was PCR amplified using the gene-specific primers and the expected size band of ~329 bp

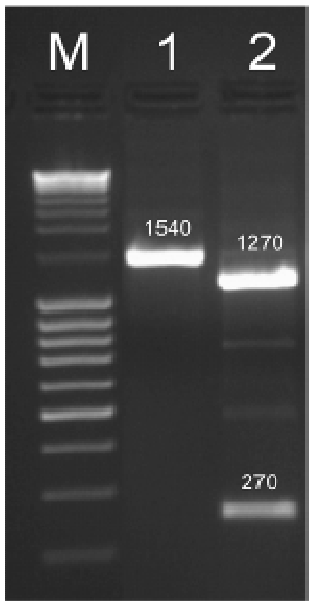

Figure 1. RFLP analysis of the 16S rRNA fragments with *Avr* II. Molecular marker MassRuler™ (Fermentas); lane 1, *Salmonella* Typhimurium (X80681); lane 2, *Salmonella* Typhi (U88545).

obtained (data not shown). The negative control, namely *Salmonella* Typhimurium, does not contain the LPS gene and hence no amplicon was obtained. This gene can thus be used to distinguish between *Salmonella* Typhimurium and *Salmonella* Typhi.

Subsequently, the V_i antigen (*viaB*) gene was also amplified by PCR, using the primers specific for this gene. The expected size band of ~599 bp was obtained (data not shown). Again, *Salmonella* Typhimurium which does not contain the V_i antigen gene yielded the expected negative results. This also proved that it was possible to distinguish between *Salmonella* Typhimurium and *Salmonella* Typhi making use of the amplification of the *viaB* gene.

The *invA* gene is found in all *Salmonella* species, and hence can be utilized to detect the presence of *Salmonella* in any sample. The expected size band of ~284 bp was obtained for both *Salmonella* Typhi and *Salmonella* Typhimurium (data not shown). Thus amplification of this gene can be used to merely detect the presence of *Salmonella* in a sample. Salehi and co-workers (2005) showed that it was possible to identify 30 *Salmonella* isolates using these primers. They were also able to show that *Citobacter freundii*, *Shigella boydii*, *Shigella sonnei*, *E. coli* O2 K12, and *Proteous mirabilis* did not show any PCR amplified fragment.

Multiplex PCR

A multiplex PCR was performed to amplify both the *invA* and *tyv* genes simultaneously. As was expected, *Salmonella* Typhi, which contains both these genes, produced two bands of the respective sizes on the agarose gel, while *Salmonella* Typhimurium, which only has the *invA* gene, produced only one band on the gel (Figure 2).

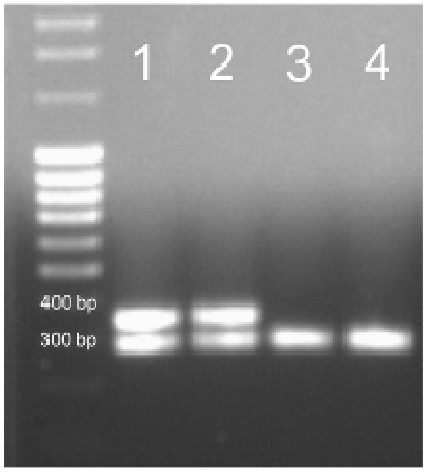

Figure 2. Multiplex PCR amplification of the *invA* and the LPS gene, respectively. Molecular marker MassRuler™ (Fermentas); lanes 1 and 2, *Salmonella* Typhi (U88545), PCR done in separate reactions; lanes 3 and 4, *Salmonella* Typhimurium (X80681), PCR done in separate reactions.

Conclusion

The rapid detection of microbial pathogens is critical since people's lives may depend on it. Thus, there is a need for more reliable and faster methods. PCR has proven an invaluable tool for this detection and it should be implemented to obtain a rapid yes/no answer on-site. It is possible, using molecular methods, to identify and distinguish between different *Salmonella* serovars within 4 h if a whole cell PCR is performed or 7 h if genomic DNA is to be extracted first. With gene specific PCR, it is also possible to specifically detect a pathogenic organism from a mixed bacterial culture.

It was also proven that RFLP analysis of 16S rRNA PCR amplicons could be used as a first step fingerprint in the molecular based approach for distinction between different *Salmonella* serovars. With Multiplex PCR, multiple gene products can be amplified in a single PCR reaction and in this study it is clearly shown as a rapid method to distinguish between *Salmonella* Typhimurium and *Salmonella* Typhi, taking 4 h to make that distinction.

From this study it was shown that the minimum time needed for conventional methods to preliminarily identify *Salmonella* is 48 h and these results still do not discriminate between serovars (e.g. *Salmonella* Typhi and *Salmonella* Typhimurium) which differ greatly in their levels of pathogenicity, while these techniques also require pure, cultured bacteria. In comparison, the molecular approaches were rapid and specific for each

serovar. Such early detection will help to control pathogenic bacteria and, in turn, this will prevent the spread of disease.

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REFERENCES

Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B (2000). *Salmonella* nomenclature. J. Clin. Microbiol. 38: 2465-2467.

Difco Manual (1998). 11th Edition. Difco Laboratories., Division of Becton Dickinson and Company, Sparks, Maryland, USA.

Everest P, Wain J, Roberts M, Rook G, Dougan G (2001). The molecular mechanisms of severe typhoid fever. Trends Microbiol. 9: 316-320.

Harrigan WF (1998). Laboratory Methods in Food Microbiology. Academic Press: London. pp. 180-184.

Hashimoto Y, Itho Y, Fujinaga Y, Khan AQ, Sultana F, Miyake M, Hirose K, Yamamoto H, Ezaki T (1995). Development of nested PCR based on the *viaB* sequence to detect *Salmonella* Typhi. J. Clin. Microbiol. 33: 775-777.

Hirose K, Itoh KI, Nakajima H, Kurazono T, Yamaguchi M, Moriya K, Ezaki T, Kawamura Y, Tamura K, Watanabe H (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.

Kumar S, Balakrishna K, Batra HV (2006). Detection of *Salmonella* enterica serovar Typhi (*Salmonella* Typhi) by selective amplification of *invA, viaB, fliC-d* and *prt* genes by polymerase chain reaction in multiplex format. Appl. Microbiol. 42: 149-154.

Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ (2002). Typhoid fever. N. Engl. J. Med. 347: 1770-1782.

Salehi TZ, Mahzounieh M, Saeedzadeh A (2005). Detection of *invA* gene in isolated *Salmonella* from broilers by PCR method. Int. J. Poult. Sci. 4: 557-559.

Virlogeux I, Waxin H, Ecobichon C, Lee JO, Popoff MY (1996). Characterization of the *rcsA* and *rcsB* genes from *Salmonella* Typhi: *rcsB* through *tviA* is involved in regulation of Vi antigen synthesis. J. Bacteriol. 178: 1690-1698.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991). 16S Ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697-703.

Zhu Q, Lim CK, Chan YN (1996). Detection of *Salmonella* Typhi by polymerase chain reaction. J. Appl. Bacteriol. 80: 244-251.