Development of a PCR assay for the identification of *Salmonella enterica* serovar Brandenburg

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Currently, *Salmonella enterica* serovar Brandenburg is identified serologically on the basis of two surface antigens, somatic (O) polysaccharide and flagellar (H) proteins. This procedure is time-consuming and requires expensive typing reagents. To overcome these problems, a PCR method was developed and validated for the identification of *S. Brandenburg*. Portions of the *invA*, *rfbJ*(B), *fliC* and *fljB* genes were targeted for amplification using four pairs of oligonucleotide primers. To validate the assay, genomic DNA from an array of 72 *Salmonella* strains representing 28 serotypes and 5 non-*Salmonella* strains from 4 different genera was subjected to PCR.

The four targeted genes were correctly amplified only from *S. Brandenburg*. These results indicate that this PCR assay is a simple, rapid, reliable and reproducible method for the identification of *S. Brandenburg* that will aid in surveillance, prevention and control of this pathogen.

INTRODUCTION

Since 1996, *Salmonella enterica* serovar Brandenburg has been a major cause of ovine abortions and mortality, leading to significant economic impact to farmers in New Zealand (Clark, 2000; Clark *et al.*, 2004; Roe, 1999; Smart, 2000). Sheep that have recovered from clinical disease may become carriers and excrete organisms in faeces. *S. Brandenburg* has also been isolated from humans and other animals, including horses, cattle, goats, deer, pigs and dogs. In New Zealand, human cases occurred through contact with infected animals and not through consumption of animal products (Clark *et al.*, 2004). Therefore, rapid detection of *S. Brandenburg* would aid in controlling the spread of this pathogen to both animals and humans.

The number of *Salmonella* serotypes reported by 2002 was 2541 (CDC, 2007). The somatic O-antigen, together with phase-1 (H1) and phase-2 (H2) flagellar antigens, forms the basis for *Salmonella* serotyping. Each *Salmonella* serotype has a unique combination of O, H1 and H2 antigens. Laboratory methods for the identification of *S. Brandenburg* include serotyping, which is performed by the identification of O, H1 and H2 antigens according to the Kauffmann–White scheme (Popoff & Le Minor, 2001). This procedure is time-consuming, laborious and costly. Rapid and inexpensive PCR assays for the detection of *Salmonella* at the genus level have been developed (Rahn *et al.*, 1992), but the number of assays to determine the *Salmonella* serotype is limited and includes those to detect serotypes Enteritidis (Lampel *et al.*, 1996), Gallinarum (Shah *et al.*, 2005), Typhi (Farrell *et al.*, 2005; Kumar *et al.*,

2006) and Typhimurium (Leon-Velarde *et al.*, 2004). To date, there is no serotype-specific PCR assay for the detection of *S. Brandenburg*.

Somatic O-antigens are polymers of the O-subunit, and the *rfb* gene clusters are responsible for much of its variation. Variability of O-antigens among serogroups is due to differences in the type and arrangement of sugars in the O-subunit (Fitzgerald *et al.*, 2003; Gillespie *et al.*, 2003). The filament of flagella is a polymer of flagellin monomers that are composed of conserved terminal regions and a central variable region (Joys, 1985; Wei & Joys, 1985). The latter is responsible for flagellar antigenic specificity among serotypes (Newton *et al.*, 1991). *S. Brandenburg* carries serogroup B-specific O:4 antigen, H1 antigens l, v, and H2 antigens e, n, z15. These are encoded by *rfbJ*(B), the l and v alleles of *fliC* and the e, n and z15 alleles of *fljB* genes, respectively. Presence of *invA* is specific to *Salmonella*, and the combination of *rfbJ*(B), *fliC* (l, v) and *fljB* (e, n, z15) is specific to *S. Brandenburg*. Therefore, the amplification of *invA* will differentiate *Salmonella* from non-*Salmonella* isolates, and amplification of *rfbJ*(B), *fliC* (l, v) and *fljB* (e, n, z15) will differentiate *S. Brandenburg* from other *Salmonella* serotypes. We describe here the development of a PCR method targeting *invA*, *rfbJ*(B), *fliC* and *fljB* genes for the detection of *S. Brandenburg* from suspected *Salmonella* colonies.

METHODS

Bacterial strains. A total of 72 *Salmonella* strains belonging to 28 different serotypes (Table 1) and 5 non-*Salmonella* strains belonging to 4 different genera (Table 2) were used in this study. *Salmonella* serotypes carrying different O- and H-antigens were used. *Salmonella*

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Table 1. Properties and PCR results of *Salmonella* strains used in this study

<i>Salmonella</i> serotype	Serogroup	Antigenic formula O: H1: H2 antigenic factors	Number tested	Number of strains positive for target gene			
				<i>invA</i>	<i>rfbJ</i> (B)	<i>fliC</i>	<i>fljB</i>
Brandenburg	B	4, 12 : l, v : e, n, z15	25	25	25	25	25
Typhimurium	B	4, [5], 12 : i : 1, 2	4	4	4	0	0
Derby	B	4, [5], 12 : f, g : [1, 2]	4	4	4	0	0
Agona	B	[1], 4, 12 : f, g, s : –	4	4	4	0	0
Saintpaul	B	1, 4, [5], 12 : e, h : 1, 2	1	1	1	0	0
Liverpool	E4	1, 3, 19 : d : e, n, z15	1	1	0	0	1
Potsdam	C1	6, 7 : l, v : e, n, z15	1	1	0	1	1
Livingstone	C1	6, 7 : d : l, w	1	1	0	0	0
Infantis	C1	6, 7 : r : 1, 5	1	1	0	0	0
Singapore	C1	6, 7 : k : e, n, x	1	1	0	0	0
Newport	C2	6, 8 : e, h : 1, 2	4	4	0	0	0
Hindmarsh	C3	8 : r : 1, 5	4	4	0	0	0
Gallinarum	D1	9, 12 : – : –	1	1	0	0	0
Enteritidis	D1	9, 12 : g, m : [1, 7]	3	3	0	0	0
Azteca	B	4, 5, 12 : l, v : 1, 5	1	1	1	1	0
Bredeney	B	1, 4, 12, 27 : l, v : 1, 7	1	1	1	1	0
Jos	B	1, 4, 12, 27 : y : e, n, z15	1	1	1	0	1
Budapest	B	1, 4, 12, 27 : g, t : –	1	1	1	0	0
Abortusovis	B	4, 12 : c : 1, 6	1	1	1	0	0
Wien	B	1, 4, 12, 27 : b : l, w	1	1	1	0	0
Ball	B	1, 4, 12, 27 : y : e, n, x	1	1	1	0	0
Gloucester	B	1, 4, 12, [27] : i : l, w	1	1	1	0	0
Mono	B	4, 12 : l, w : 1, 5	1	1	1	0	0
Togo	B	4, 12 : l, w : 1, 6	1	1	1	0	0
Havana	G2	[1], 13, 23 : f, g, [s] : –	1	1	0	0	0
Cerro	K	18 : z4, z23 : [1, 5]	4	4	0	0	0
Anatum	E1	3, 10 : e, h : 1, 6	1	1	0	0	0
Newington	E2	3, 15 : e, h : 1, 6	1	1	0	0	0

serotypes Potsdam and Livingstone were obtained from the New Zealand Reference Culture Collection, Medical Section, Institute of Environmental Science & Research, Porirua, Wellington, New Zealand. Professor Peter R. Reeves (Department of Microbiology, University of Sydney, Australia) kindly provided genomic DNA of *Salmonella* strains Abortusovis, Azteca, Ball, Bredeney, Budapest, Jos, Gloucester, Mono, Togo and Wien. All other *Salmonella* and non-*Salmonella* strains were obtained from the Institutes of Veterinary,

Animal & Biomedical Sciences (IVABS) and Molecular Biosciences (IMBS) of Massey University, Palmerston North, New Zealand.

DNA extraction. Genomic DNA was extracted from cell suspensions of bacteria grown overnight on xylose-lysine-deoxycholate (XLD) agar at 37 °C. A single colony was resuspended in 30 µl distilled water and boiled at 100 °C for 10 min. The sample was immediately cooled on ice for 5 min, and centrifuged at 13 000 g at 4 °C for 10 min. The supernatant, containing DNA, was used as the template for PCR amplification.

PCR primers. The target genes selected in this study were *invA* (*Salmonella*-specific), *rfbJ*(B) (serogroup B), *fliC* (l, v) and *fljB* (e, n, z15). Oligonucleotide primers (Table 3) used for the amplification of *invA*, *rfbJ*(B) and *fljB* genes were from Rahn *et al.* (1992), Luk *et al.* (1993) and Echeita *et al.* (2002), respectively; the pair used for amplification of *fliC* was designed using the sequence of region IV of the variable domain of *S. Brandenburg fliC* gene (GenBank accession number AY935580). All primers were synthesized by Life Technologies in the desalted form.

DNA amplification and detection. The *invA*, *fliC* and *fljB* genes were amplified in a multiplex PCR and the *rfbJ*(B) gene was amplified in a separate reaction. Multiplex PCR amplification was performed in 0.2 ml thin-walled PCR tubes in a reaction volume of 50 µl, consisting 1 µl genomic DNA, 200 nM each primer *invAF*, *invAR*,

Table 2. PCR results of non-*Salmonella* strains used in this study

Non- <i>Salmonella</i> strain	Number tested	Number of strains positive for target gene			
		<i>invA</i>	<i>rfbJ</i> (B)	<i>fliC</i>	<i>fljB</i>
<i>Escherichia coli</i>	1	0	0	0	0
<i>Klebsiella aerogenes</i>	1	0	0	0	0
<i>Klebsiella edwardsii</i>	1	0	0	0	0
<i>Proteus mirabilis</i>	1	0	0	0	0
<i>Shigella flexneri</i>	1	0	0	0	0

Table 3. PCR primers (F, forward and R, reverse) used in this study

Target gene	Primer	Primer sequence (5'–3')	Position	Size (bp)
<i>invA</i>	<i>invAF</i>	GTGAAATTATCGCCACGTTCTGGGCAA	371–396	285
	<i>invAR</i>	TCATCGCACCGTCAAAGGAACC	655–634	
<i>rfbJ</i> (B)	<i>rfbJ</i> (B)F	AGAATATGTAATTGTCAG	15–32	883
	<i>rfbJ</i> (B)R	TAACCGTTTCAGTAGTTC	897–880	
<i>fliC</i>	<i>fliCF</i>	CACTGGTCTTAATGATGCAGCTC	588–610	222
	<i>fliCR</i>	CCTGTCACTTTCGTGGTTAT	809–790	
<i>fljB</i>	<i>fljBF</i>	GGCAACCCGACAGTAACTGGCGATC	631–656	135
	<i>fljBR</i>	ATCAACGGTAACTTCATATTTG	765–744	

fliCF, *fliCR*, *fljBF* and *fljBR*, 200 µM each of dCTP, dGTP, dATP and dTTP, 2 mM MgCl₂, 1× PCR buffer [20 mM Tris/HCl (pH 8.4), 50 mM KCl], 1.25 U of Platinum *Taq* DNA Polymerase (Life Technologies) and sterile distilled water. The reactions were subjected to a single cycle of denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation (95 °C for 1 min), annealing (60 °C for 30 s), elongation (72 °C for 30 s) and a final elongation (72 °C for 7 min) in a GeneAmp 9600 thermocycler (Perkin Elmer). The *rfbJ*(B) gene was amplified separately in a 50 µl reaction volume containing the same PCR reagents as the multiplex reaction, replacing the primers with *rfbJ*(B)F and *rfbJ*(B)R. The thermocycler parameters were as above, except that annealing was done at 55 °C for 1 min and elongation was at 72 °C for 1 min. A negative control containing sterile distilled water in place of genomic DNA was included in each PCR assay. Reproducibility of the assay was confirmed by testing each strain in duplicate three times. The completed reactions were held at 4 °C for direct use or stored at –20 °C.

Multiplex PCR-amplified sample (10 µl) was mixed with 10 µl of the single reaction and electrophoresed on 2.5 % (w/v) agarose gels in 1× TAE buffer. One-kb plus DNA ladder marker (Life Technologies) was used as a size reference. Following electrophoresis, gels were stained with 0.5 µg ethidium bromide ml^{–1} and visualized on a UV transilluminator. The four PCR products obtained from *S. Brandenburg* amplification were excised from the agarose gel and purified using the QIAquick gel extraction kit (Qiagen). Products were sequenced by the Sequencing Facility of Massey University with

the ABI Prism BigDye Terminator Sequencing Reaction kit on a Capillary ABI3730 DNA analyser (Applied Biosystems). DNA sequences were edited using Chromas software and analysed with the BLASTN network server at <http://www.ncbi.nlm.nih.gov/blast/>.

Validation. Genomic DNA extracted from a panel of *Salmonella* strains representing 28 different serotypes and non-*Salmonella* strains from 4 genera were PCR-amplified to evaluate the validity of the PCR assay.

RESULTS AND DISCUSSION

Preliminary experiments to amplify *invA*, *fliC*, *fljB* and *rfbJ*(B) in a multiplex PCR did not yield consistent results with the *rfbJ*(B) gene. Therefore, this segment was amplified in a separate reaction. Fig. 1 shows the amplification products from a representative number of *Salmonella* strains used in the study. The *invA* gene was amplified in all *Salmonella* isolates but the *rfbJ*(B) gene was only amplified in serogroup B isolates. While *fliC* primers amplified only the *Salmonella* isolates carrying l, v alleles, *fljB* primers amplified only those carrying e, n, z15 alleles (Table 1). The non-*Salmonella* strains did not produce amplification products under these conditions (Table 2). Only *S. Brandenburg* strains gave all four amplification

Fig. 1. PCR amplification of *invA*, *rfbJ*(B), *fliC* (l, v) and *fljB* (e, n, z15) genes. PCR products were electrophoresed on a 2.5 % (w/v) agarose gel, stained with ethidium bromide and photographed under UV light. Lanes: M, One-kb plus DNA ladder marker; 1–5, *S. Brandenburg*; 6, *S. Azteca*; 7, *S. Bredeney*; 8, *S. Jos*; 9, *S. Budapest*; 10, *S. Abortusovis*; 11, *S. Wien*; 12, *S. Ball*; 13, *S. Gloucester*; 14, *S. Mono*; 15, *S. Togo*; 16, *S. Havana*; 17, *S. Newington*; 18, *Klebsiella edwardsii*.

products of the expected sizes of 883 bp (*rfbJ*B), 285 bp (*invA*), 222 bp (*fliC*) and 135 bp (*fljB*). The primer pair sets did not show any cross-reactivity with *Salmonella* strains harbouring other somatic and flagellin gene groups. Sequencing and BLASTN analysis of the amplicons of *S. Brandenburg* confirmed that only the correct gene fragments had been amplified. All 25 *S. Brandenburg* isolates used in this study amplified all 4 genes. Repeated PCRs gave identical results.

The PCR assay developed in this study was based on the amplification of *invA*, *rfbJ*(B), *fliC* and *fljB* genes directly from a single *Salmonella* colony. *invA* was targeted for the diagnosis of *Salmonella* organisms at the genus level, and the combination of three polymorphic genes, *rfbJ*(B), *fliC* and *fljB*, was targeted for the identification of *S. Brandenburg* at the serotype level. *invA*, located on the pathogenicity island 1 of *Salmonella* species, is essential for invasion of epithelial cells (Collazo & Galán, 1997). It is present in all invasive strains of *Salmonella* (Galán, 1996) and absent from closely related genera such as *Escherichia* (Bäumler *et al.*, 1998). The *invA* gene was amplified from all 72 *Salmonella* strains, while it was not amplified from non-*Salmonella* strains belonging to the 4 genera. These results agree with Rahn *et al.* (1992), who designed and established the specificity of this widely used pair of primers. They showed that 630 *Salmonella* strains representing over 100 serotypes yielded the target band of 285 bp, whereas two strains from each of the serotypes Litchfield and Senftenberg were negative. The strains that were shown to lack *invA* sequences were from environmental samples and were not specifically associated with disease (Ginocchio *et al.*, 1997). Furthermore, the *invA* primer pair was also used in a European research project in the validation and standardization of PCR for the detection of *Salmonella* species in food (Hoorfar, 1999). Malorny *et al.* (2003) showed that one *S. Saintpaul* strain did not give the target band. It is speculated that *invA* is absent in these strains, which are not invasive, or that they might be using other invasive mechanisms. However, absence of *invA* in *Salmonella* seems to be rare (Malorny *et al.*, 2003).

The lipopolysaccharide O-antigen together with H1 and H2 flagellar antigens forms the basis for *Salmonella* serotyping (Popoff & Le Minor, 2001). The O-antigen specificity is based on the sugar composition and arrangement in the O-subunit (Fitzgerald *et al.*, 2003). The O-subunit of serogroup B salmonellae is an oligosaccharide of four sugar residues. Of these four sugars, three form a mannosyl-rhamnosyl-galactose backbone common to some other serogroups such as A and D. The fourth sugar is a dideoxyhexose substituted on the mannosyl residue. While the dideoxyhexose sugar present in the O-subunit of serogroups A and D is paratose and tyvelose, respectively, that of both serogroups B and C2 is abequose. Abequose confers on serogroup B strains their specific O:4 antigen (Wyk & Reeves, 1989). The *rfb* gene cluster encodes the enzymes for biosynthesis of O-antigens. The *rfbJ* gene encodes abequose synthase, which is responsible for the final

specific step in the synthesis of abequose. There is a 44% difference between the nucleotide sequence of *rfbJ* gene of serogroup B [*rfbJ*(B)] and that of serogroup C2 [*rfbJ*(C2)] (Luk *et al.*, 1993; Wyk & Reeves, 1989). Luk *et al.* (1993) designed a pair of primers targeting the serogroup B specific *rfbJ* gene that was used in this study and as expected, it amplified only the *rfbJ*(B) gene. In *S. Brandenburg*, H1 and H2 flagellins belong to the L- (l, v) and E-complex (e, n, z15), respectively. Region IV of *Salmonella* flagellin is the most variable, and is believed to carry the major serotype-specific epitopes of the flagellar antigens (Newton *et al.*, 1991; Wei & Joys, 1985). Some of the flagellar antigens are composed of a single antigenic factor (b, c, d, i, r) while others are composed of multiple antigenic factors (l, v; l, w; e, h; e, n, x; e, n, z15; 1, 2). Flagellar antigens are grouped into complexes depending on the antigenic factors that they share. Examples are the E-complex, which contains antigenic factor e (e, h; e, n, x; e, n, z15) and the L-complex, which contains antigenic factor l (l, v; l, w; l, z13, l, z28) (Popoff & Le Minor, 2001). The two primer pairs used to amplify *fliC* and *fljB* (Echeita *et al.*, 2002) were from region IV of the variable region and did not amplify other E- and L-complex genes of the *Salmonella* strains used in this study. This shows the specificity of these two pairs of primers for l, v and e, n, z15 alleles of *fliC* and *fljB* genes, respectively.

All of the *S. Brandenburg* strains in this study were obtained from clinical samples (uterine swabs, faeces, intestinal contents and meat) collected from sheep, cattle and pigs, and sheep yard dust. Pure cultures of *S. Brandenburg* were used for the initial evaluation of the assay because PCR assays are prone to inhibition by substances in the samples (Iijima *et al.*, 2004). All 25 *S. Brandenburg* cultures that were confirmed by serotyping were positive in the PCR, with no false-negative reactions. The PCR described here detected the combination of three genes [*rfbJ*(B), *fliC* (l, v) and *fljB* (e, n, z15)] that is unique to *S. Brandenburg*. A combination of enrichment, subcultures, biochemical tests and serotyping is currently used for the identification of *S. Brandenburg* and the whole process normally takes approximately 7 days. With the PCR assay described in this study, identification of pure cultures of *Salmonella* to the serotype *Brandenburg* could be done in approximately 8 h. In total, the time taken from receipt of the clinical sample to confirming the identity of the organism by this PCR assay will be approximately 4 days. Since this assay requires only genomic DNA, it does not depend on the expression of antigens, thus making the need for time-consuming phase reversal of flagellin antigens unnecessary.

The PCR assay described here will be a useful complement to traditional culture and serotyping techniques for the identification of *S. Brandenburg* in clinical samples.

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