Development of an indirect ELISA for the detection of serum IgG antibodies against region IV of phase 1 flagellin of *Salmonella enterica* serovar Brandenburg in sheep

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Region IV of phase 1 flagellin (FliC) of *Salmonella enterica* serovar Brandenburg (S. Brandenburg) was expressed in *Escherichia coli* and purified by nickel chelate affinity chromatography. The purified recombinant protein was evaluated for its suitability as an antigen in an indirect ELISA for the detection of antibodies in sheep sera. A cut-off value of 0.1 was calculated using 80 serum samples collected from sheep with no previous history of S. Brandenburg. In the present study we show the results of ELISA with field sera collected from 81 sheep naturally infected with S. Brandenburg. The assay was able to detect antibodies belonging to the IgG class with a sensitivity of 93.8%. Thus, indirect ELISA might be a suitable screening tool for serological monitoring of sheep flocks infected with S. Brandenburg.

INTRODUCTION

Salmonella enterica serovar Brandenburg (S. Brandenburg) has been a major cause of ovine abortions and mortality in New Zealand since 1996 (Clark, 2000; Clark *et al.*, 2004; Roe, 1999; Smart, 2000). In addition, this pathogen infects humans through contact with infected animals and contaminated farming environments (Clark *et al.*, 2004). Currently, diagnosis is carried out by standard bacteriological methods. When dealing with a large number of samples, a serological test such as ELISA is faster and cheaper than bacteriology (Funk *et al.*, 2005; van Zijderveld *et al.*, 1992). A number of ELISAs for detecting *Salmonella* antibodies have been described based on LPS (House *et al.*, 2005; Veling *et al.*, 2000) and flagellar (Dalby *et al.*, 2005; Gast *et al.*, 2002; Veling *et al.*, 2000) antigens. These antigens result in a lack of specificity from cross-reactions among serotypes due to a sharing of somatic antigens of LPS and conserved terminal regions of flagellin (Cooper *et al.*, 1989; Nicholas & Cullen, 1991; Timoney *et al.*, 1990).

Flagellins are highly immunogenic proteins and phase 1 flagellin is encoded by the *fliC* gene (Iino *et al.*, 1988). Monomers are composed of conserved amino-terminal (regions I, II) and carboxy-terminal regions (region VIII), and a central variable region (regions III, IV, V, VI and VII) (de Vries *et al.*, 1998; Wei & Joys, 1985). Wei & Joys

(1985) showed that the predicted amino acid sequence of region IV showed less than 32% homology for any pair-wise comparison of four different flagellins used in their study. Studies have shown that major serotype-specific flagellin antigenic determinants are located in region IV (de Vries *et al.*, 1998; Newton *et al.*, 1991).

Currently, recombinant proteins are used for the detection of serum antibodies in many bacterial diseases (Kwang & Littledike, 1995). ELISAs using recombinant proteins containing phase 1 flagellin fragments for the detection of antibodies in poultry against *S. enterica* serotype Enteritidis have been reported (Mizumoto *et al.*, 2004; Yap *et al.*, 2001). To date, ELISAs based on recombinant flagellar antigens for the serodiagnosis of S. Brandenburg infection in sheep are not available. In this study, we first reacted sheep sera against recombinant region IV of phase 1 flagellin in Western blots to confirm the specificity of the binding, and then used the same antigen in an ELISA. This paper shows the results of an indirect ELISA for the detection of serum IgG antibodies against region IV of phase 1 flagellin in sheep naturally infected with S. Brandenburg.

METHODS

DNA extraction. S. Brandenburg field isolate S59 was obtained from the Microbiology Laboratory of the Institute of Veterinary, Animal & Biomedical Sciences of Massey University, Palmerston North, New Zealand. Genomic DNA was extracted using a standard phenol/chloroform method.

PCR and cloning. Genomic DNA was used as the PCR template. Two oligonucleotide primers containing restriction site *NdeI*

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Abbreviation: AP, alkaline phosphatase.

(forward 5'-GGGAATTCATATGAAGAGCGAAGCAGTTAAAGCGG-3' and reverse 5'-GGGAATTCATATGTAAAGCAATA-CCACTGTCGG-3') were designed to amplify a 369 bp fragment of region IV (nucleotides 544–912 bp) of the *fliC* gene (GenBank accession no. AY935580). The forward primer was designed to be in-frame with the N-terminal histidine tag of the pET14b expression vector (Novagen). The amplified 400 bp PCR product was digested with *NdeI* and cloned into pET14b using standard cloning techniques. Following transformation of *Escherichia coli* One Shot BL21 (DE3) competent cells (Life Technologies), plasmids were sequenced by the Sequencing Facility of Massey University to verify the reading frame and the sequence of the insert.

Expression and purification of recombinant protein. For protein expression, recombinant *E. coli* cells were grown on LB agar containing 50 µg carbenicillin ml⁻¹. A 5 ml overnight LB culture originating from a single colony from the agar was inoculated into 500 ml LB and incubated at 37 °C until mid-exponential phase was reached. IPTG was added to a final concentration of 1 mM, and the culture was incubated at 37 °C for a further 3 h. At the end of the 3 h incubation, the culture was pelleted by centrifugation.

The histidine-tagged fusion protein was found to be soluble, and purification was performed under native conditions using BD Talon metal affinity resin (Clontech Laboratories). Cell lysates of recombinant *E. coli* cultures were centrifuged at 10 000 g for 30 min at 4 °C, and the supernatant was used for protein purification according to manufacturer's instructions. The recombinant protein was eluted and collected in fractions of 1 ml with elution buffer pH 7.0 (50 mM sodium phosphate, 500 mM sodium chloride, 150 mM imidazole). Fractions were analysed by SDS-PAGE, and those containing the purified protein were pooled and dialysed for 24 h against three changes of 4 l PBS pH 7.4. Protein concentration was estimated using a Bio-Rad Protein assay kit (Bradford, 1976).

SDS-PAGE and Western immunoblotting. A modified Laemmli (1970) procedure was used to separate proteins on a discontinuous polyacrylamide gel consisting of a 4% stacking gel and a 12% separating gel. The proteins were transferred onto a PVDF membrane (Gelman Sciences), and the non-specific sites were blocked with 5% (w/v) skimmed milk powder in Tris-buffered saline pH 7.4 supplemented with 0.1% (v/v) Tween 20 overnight at 4 °C. A preliminary Western blot was carried out to confirm the presence of histidine-tagged proteins post-purification. Subsequently, the membrane containing recombinant protein was cut into strips, and each strip was incubated with 10 ml diluted sheep sera and with 10 ml diluted alkaline phosphatase (AP)-labelled donkey anti-sheep whole IgG (Sigma) antibody. Each step was for 1 h at room temperature followed by a washing step. The strips were then incubated with 10 ml phosphatase substrate, 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (Sigma), for colorimetric detection. Sera from a positive and a negative control sheep were included in each blot for comparison.

Serum samples

Samples were collected from three groups.

Group 1: sheep not exposed to *S. Brandenburg*. Sera were collected from 80 ewes in farms without a history of *S. Brandenburg*. These samples were used to determine the cut-off value in the ELISA. Faecal samples collected from these sheep were subjected to routine bacteriological procedures, and were negative for *S. Brandenburg*.

Group 2: sheep naturally infected with *S. Brandenburg*. Sera collected approximately 3–4 weeks post-abortion from 81 *S. Brandenburg*-confirmed ewes were used in the ELISA. Vaginal

swabs collected from these ewes were subjected to routine bacteriological procedures to confirm *S. Brandenburg* infection.

Group 3: sheep vaccinated with Salvexin and Salvexin + B. Salvexin vaccine (Schering-Plough Animal Health) contains inactivated *S. enterica* serovar Typhimurium (*S. Typhimurium*), *S. enterica* serovar Hindmarsh (*S. Hindmarsh*) and *S. enterica* serovar Bovismorbificans, while Salvexin + B vaccine contains inactivated *S. Brandenburg* in addition to the above three *Salmonella* serotypes. Sera collected from ewes that received Salvexin and Salvexin + B were kindly provided by Dr Stanley Fenwick of Massey University, New Zealand, and used as a preliminary assessment of ELISA specificity. In this trial, 15 sheep were used as controls, 15 were vaccinated with Salvexin, and the other 15 were vaccinated with Salvexin + B. Ten samples collected pre-vaccination, and also at 2 weeks post-booster, from each group were used in the ELISA.

Indirect ELISA. Optimal concentrations of reagents were determined by checkerboard titrations (Crowther, 1995). Each well of 96-well MaxiSorp microtitre plates (Nunc) was coated with 100 µl (2.5 µg ml⁻¹) recombinant protein in 0.1 M bicarbonate buffer pH 9.6 (0.06 M NaHCO₃, 0.04 M Na₂CO₃) for 2 h at 37 °C. The plates were washed three times with PBS pH 7.4 containing 0.05% (v/v) Tween 20, and once with PBS alone to remove unbound antigen. The non-specific sites were blocked by incubation with 200 µl PBS containing 5% (w/v) skimmed milk powder per well for 1 h at 37 °C, and washed. One hundred microlitres of sera diluted (1:100) in PBS containing 5% (w/v) skimmed milk powder was added to each well in duplicate, and incubated for 1 h at 37 °C. After another cycle of washing, 100 µl 1:10 000 diluted AP-labelled donkey anti-sheep IgG was added to each well and incubated for 1 h at 37 °C. After a final series of washes, 100 µl (1 mg ml⁻¹) *p*-nitrophenylphosphate (Sigma) dissolved freshly in 1 M diethanolamine buffer pH 9.8, 0.5 mM MgCl₂, was added to each well, and incubated for 30 min in the dark at room temperature. The resultant yellow colour reaction was stopped by the addition of 50 µl 2 M Na₂CO₃ per well and the absorbance values at 405 nm (*A*₄₀₅) were determined using an ELISA plate reader (Molecular Devices). Negative control serum, positive control serum and dilution buffer were included on each plate. Each sample and control was assayed in duplicate. The cut-off value of the ELISA was determined as the mean absorbance of the 80 negative sera plus 3 SDs.

Statistical analysis. Excel (Microsoft) was used for basic calculations including means, SDs and SEMs. Results were analysed by Student's *t*-test (Excel; Microsoft) and considered significant when *P* values were <0.05.

RESULTS AND DISCUSSION

SDS-PAGE, and the subsequent Western blotting of the purified 15 kDa protein and analysis with anti-histidine mAb are shown in Fig. 1. None of the 80 sera collected from the non-infected group (group 1) contained antibody that recognized the purified protein. However, 81 of those sera collected from naturally infected sheep (group 2) reacted with it (Fig. 2, Table 1). All 10 sera from the Salvexin + B-vaccinated group reacted with the protein, but the 10 sera from the Salvexin group were non-reactive. All the pre-vaccination sera, and those collected from control sheep were negative in the blots (Table 1). The immunogenic characteristic of this recombinant flagellin elucidated by Western blots enabled the protein to be evaluated as a

Fig. 1. (a) Purification of histidine-tagged recombinant protein by affinity chromatography. Sonicated cell lysate of *E. coli* was applied to a cobalt affinity column, and the bound protein was eluted with 150 mM imidazole. A total of 5 μ l eluate was electrophoresed in a 12% SDS-PAGE gel, and stained with Coomassie blue. M, Prestained BenchMark protein ladder (kDa); 1, purified recombinant protein. (b) Western blot detection of purified recombinant protein with anti-histidine mAb. A dilution of 1:1000 anti-histidine mouse IgG mAb, and a 1:300 dilution of peroxidase-labelled sheep anti-mouse IgG were used. M, Precision Plus Protein dual colour standard (kDa); 1, purified recombinant protein. The arrows indicate the position of histidine-tagged recombinant protein.

diagnostic antigen for incorporation into an ELISA for immunodetection of *S. Brandenburg* infection in sheep.

During optimization, chequerboard titrations indicated 250 ng coating antigen, and a 1:100 dilution of sheep sera should be used in the ELISA. The mean background absorbance of the ELISA was 0.08. The absorbance value of each sample was calculated by subtracting the background absorbance from the mean absorbance of the duplicates. All 80 sera collected from sheep not exposed to *S. Brandenburg* had absorbance values ranging from 0.002 to 0.070. This population had a mean of 0.033 and an SD of 0.021. The calculated cut-off value (mean plus 3 SDs of the absorbance values) from these 80 control sera was 0.096, and was set at 0.1 (data not shown).

Sera collected from the 81 sheep naturally infected with *S. Brandenburg* were used to evaluate the sensitivity of the ELISA. Sera with an absorbance value higher than 0.1 were scored as positive. Of the 81 sera, 76 were scored positive (Table 1). For these 81 samples, absorbance values ranged from 0.059 to 2.430 with a mean of 0.71. Most had an absorbance in the range of 0.10–0.50 (Table 2). Five of the samples that had absorbance values less than 0.1 were scored negative. Infection with *Salmonella* leads to increased levels of serum IgG antibodies (Withanage *et al.*, 2005). This preliminary ELISA was carried out with sera

Fig. 2. Western blot detection of serum IgG antibodies against the recombinant protein in sheep naturally infected with *S. Brandenburg*. A 1:100 dilution of sheep sera, and a 1:10 000 dilution of AP-labelled donkey anti-sheep IgG were used. (a) Lanes 1 to 8, sera from sheep not exposed to *S. Brandenburg* (sample numbers N1, N8, N17, N23, N35, N52, N54, N60, respectively); lane 9, negative control; lane 10, positive control; M, Prestained BenchMark protein ladder (kDa). (b) Lanes 1 to 6, sera from sheep naturally infected with *S. Brandenburg* (sample numbers S7, S12, S2, S44, S9 and S11, respectively); lane 7, negative control; lane 8, positive control; lanes 9 and 10, sera from sheep naturally infected with *S. Brandenburg* (sample numbers S29 and S78, respectively); M, Prestained BenchMark protein ladder (kDa). The arrows indicate the position of the recombinant protein.

from recently infected ewes (3–4 weeks post-abortion). Using a positive cut-off absorbance value of 0.1, the ELISA correctly classified 76 out of 81 sheep naturally infected with *S. Brandenburg* as positive giving the assay a sensitivity of 93.8%. The ELISA-negativity of the other five samples may be due to insufficient IgG at the time of sampling. The time interval between infection and blood sampling may have been too short for these five sheep to result in sufficient IgG levels to give an absorbance of 0.1. The data provided indicate the potential usefulness of recombinant region IV protein in an ELISA to detect *S. Brandenburg*-infected sheep flocks in surveillance programs.

The use of sensitive methods for the identification of *Salmonella* is important because information on the true *Salmonella* status in sheep flocks is essential for the control of *S. Brandenburg* infection. Bacteriological culture is the 'gold standard' for *Salmonella* diagnosis, and is the only one that enables identification of the specific serovars. Although bacteriological culture methods are highly specific, there are disadvantages (Funk *et al.*, 2005). Conventional bacteriological isolations from clinical sam-

Table 1. Summary of serum IgG antibody detection of the recombinant protein in sheep by Western blot and indirect ELISA

Source of samples (time of sample collection)	No. of sera tested	No. of sera positive in Western blot	No. of sera positive in ELISA	Mean A_{405} (range)
Sheep naturally infected with <i>S. Brandenburg</i> (3–4 weeks post-abortion)	81	81	76	0.71 (0.059–2.43)
Sheep experimentally vaccinated with:				
Salvexin	10	0	0	0.03 (0–0.06)
Salvexin + B (2 weeks post-booster)	10	10	10	0.72 (0.28–1.82)

ples may not identify all infected animals due to the intermittent shedding of *Salmonella*. The actual shedding of *Salmonella* indicates true infection and transmission, whereas positive serology also indicates silent transmission within the flock (van Winsen *et al.*, 2001). Therefore, serology can be used to identify infected and carrier animals, and to aid surveillance and epidemiological studies. However, the presence of serum antibodies will not discriminate between previous and current exposure, and between vaccination and natural exposure. A paired serum sample testing may be beneficial in this regard. In order to evaluate its suitability for detecting carriers, it will be necessary to determine for how long this ELISA remains able to detect antibodies in previously infected sheep. It is believed that the antibody response against *Salmonella* flagella is long lasting (Frimpong *et al.*, 2000; Willke *et al.*, 2002).

A preliminary assessment of ELISA specificity was carried out with sera collected from sheep vaccinated with Salvexin/Salvexin + B. Sera collected 2 weeks post-booster from ewes that received Salvexin and Salvexin + B vaccines were used in the ELISA. Control sheep and sheep vaccinated with Salvexin had ELISA absorbance values less than 0.1, whereas sheep vaccinated with Salvexin + B had values more than 0.1. For the latter group, values ranged from 0.28 to 1.82 with a mean of 0.72 (Fig. 3, Table 1). Only the sera from sheep vaccinated with Salvexin + B were positive in the ELISA indicating the specificity of the assay.

Table 2. Detection of the serum IgG antibody response of naturally infected sheep against the recombinant protein by indirect ELISA

Sera collected 3–4 weeks post-abortion from 81 sheep naturally infected with *S. Brandenburg* were used at a dilution of 1:100.

A_{405} range	No. of samples
<0.10	5
0.10–0.50	34
0.51–0.90	13
0.91–1.30	17
1.31–1.70	6
1.71–2.10	3
2.11–2.50	3
Total	81

However, studies with sera from sheep infected with other *Salmonella* serotypes and bacterial strains need to be performed to determine the true specificity of this ELISA. Non-availability of sera from sheep infected with other *Salmonella* serotypes at the time of the study was a limitation.

Serotype-specific flagellar antigenic determinants that are located in region IV of the FlhC protein induce the production of serotype-specific antibodies in the infected host. Some of the serotype-specific flagellar antigens are composed of a single antigen (b, c, d, i, r) while others are composed of multiple antigens (l, v; l, w; e, h; e, n, x; e, n, z15; 1, 2). Flagellar antigens are grouped into complexes depending on the antigens they share. Examples are the E complex that contains antigen e (e, h; e, n, x; e, n, z15) and the L complex that contains antigen l (l, v; l, w; l, z13; l, z28). Phase 1 flagellin of *S. Brandenburg* belongs to the L

Fig. 3. Detection of IgG antibody response against the recombinant protein in sera from sheep vaccinated with Salvexin and Salvexin+B by indirect ELISA. Sera collected 2 weeks post-booster from ewes that received Salvexin (sample numbers Sa1, Sa2, Sa3, Sa4, Sa5, Sa6, Sa7, Sa8, Sa9 and Sa10), Salvexin + B (sample numbers SB1, SB2, SB3, SB4, SB5, SB6, SB7, SB8, SB9 and SB10) and from ewes used as controls (C1, C2, C3, C4, C5, C6, C7, C8, C9 and C10) were used. A serum dilution of 1:100 and 1:10 000 dilution of AP-labelled donkey anti-sheep IgG were used. Each absorbance value represents the mean value of two determinations minus background absorbance. The cut-off value was set at 0.1. A significant difference ($P < 0.001$) (Student's *t* test) was found between the Salvexin- and Salvexin+B-vaccinated groups.

complex since it carries l, v epitopes. *Salmonella* serotypes that carry phase 1 (H1) or phase 2 (H2) flagellins belonging to the L complex would react with varying intensities in this ELISA due to shared epitopes among them. Examples of serotypes that possess H1 or H2 belonging to the L complex include Ayton (H1: l, w), Bredeney (H1: l, v), Give (H1: l, v), Glidji (H1: l, w), Gloucester (H2: l, w), Kimberly (H1: l, v), Livingstone (H2: l, w), London (H1: l, v), Ohio (H2: l, w), Potsdam (H1: l, v), Victoria (H1: l, w) and Wien (H2: l, w). These serotypes are not common sheep pathogens and have not been shown to cause ovine abortions. However, it is advisable to complement positive ELISA results with history and bacteriological findings.

In Denmark, until the end of 1994, pigs were tested for salmonellosis exclusively by bacteriological methods. Since 1995, *Salmonella* testing is based on a combination of both bacteriological and serological methods (Skov *et al.*, 2002). Currently, in Denmark, the screening of pigs for monitoring and controlling salmonellosis is by the identification of antibodies in serum and muscle juice (the juice released from small pieces of muscle frozen at -20°C and thawed after 1–3 days) using an indirect ELISA with a mixture of flagellar LPS as coating antigens (Mousing *et al.*, 1997). This program has reduced human cases of pork-related salmonellosis (Korsak *et al.*, 2006). The purpose of this program is to identify common *Salmonellae* including *S. Brandenburg* without differentiating them. Since both *S. Brandenburg* and *S. Typhimurium* belong to serogroup B sharing LPS 'O' antigens 4 and 12, an LPS-based ELISA is not suitable to differentiate them. In New Zealand, *S. Typhimurium* and *S. Hindmarsh* are the most common causes of ovine salmonellosis, leading to diarrhoea and mortality with occasional abortions (Clark *et al.*, 2002). *S. enterica* serovar Oranienburg has also been isolated from aborted fetuses in sporadic outbreaks of abortions in New Zealand (Russell & Tannock, 1964). *S. enterica* serotypes Abortusovis, Dublin and Montevideo are causes of ovine abortions in other countries (Sharp *et al.*, 1983; Wray & Linklater, 2000). All these serotypes do not contain flagellar antigens of the L complex. Therefore, the ELISA described here would be able to differentiate *S. Brandenburg* from the above serotypes and this feature is important for disease monitoring purposes in New Zealand.

This is believed to be the first reported ELISA to detect seroconversion of *S. Brandenburg*-infected sheep using a recombinant *Salmonella* flagellin antigen. Recombinant flagellin proteins are easily produced through heterologous expression systems, and therefore can be obtained in large quantities that are relatively pure. Therefore, inclusion of a recombinant protein with region IV in coating ELISA plates rather than the whole flagellin isolated from cultures has advantages such as purity, low cost and non-infectiousness. Moreover, it increases the specificity of the assay due to the absence of cross-reactions caused by conserved regions of whole flagellin. In conclusion, the use of this ELISA in combination with clinical, bacteriological and/or molecular diagnostic methods would constitute an

improved diagnostic assay for the detection of *S. Brandenburg* infection in sheep flocks.

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