Evaluation of recombinant Bhlp29.7 as an ELISA antigen for detecting pig herds with swine dysentery

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Abstract

Swine dysentery (SD) results from infection of the porcine large intestine with the anaerobic intestinal spirochaete Brachyspira hyodysenteriae. Diagnosis of SD traditionally has relied on detecting the spirochaete in the faeces of acutely affected pigs. To date simple and reliable serological assays that can be applied as a diagnostic tool at the herd level have not been available. In the current study a recombinant histidine tagged 29.7 kDa lipoprotein of B. hyodysenteriae (His6-Bhlp29.7) was used as an ELISA plate-coating antigen. Sera (n = 1121) from slaughter-aged pigs on 19 farms were tested in this ELISA. Following optimization of the ELISA conditions using hyperimmune control sera, a set of 464 sera from slaughter-aged pigs from five herds where SD did not occur was tested. From these results a suitable cut-off value for herd negativity was defined as the mean optical density reading plus three standard deviations. Testing of 337 pig sera from six farms with SD then showed that the sensitivity of the test at the herd level was 100%, with all six farms having one or more serum samples exceeding the cut-off value for negativity. Finally, 320 sera from eight herds suspected of having SD were examined. Four of these herds were shown to have pigs with titres consistent with SD. The true health status of the other four herds that were serologically negative could not be confirmed. In conclusion, when used on sets of 40 sera from slaughter-aged pigs the His6-Bhlp29.7 ELISA as established proved to be a useful adjunct to the diagnosis of SD at the herd level.

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1. Introduction

Swine dysentery (SD) is a mucohaemorrhagic colitis of mainly grower/finisher pigs resulting from colonization of the large intestine with the anaerobic intestinal spirochaete Brachyspira hyodysenteriae (Hampson et al., 2006a). Diagnosis of SD primarily relies on the herd history and observation of typical clinical signs of diarrhoea containing fresh blood and/or mucus. A definitive diagnosis of SD traditionally has required the isolation and identification of the spirochaete, although the use of PCR-based assays on faeces or on isolated spirochaetes now allows more...
rapid and reliable diagnosis (Atyeo et al., 1998; Fellström et al., 2001; La et al., 2003). Nevertheless, culture and/or PCR-based testing is time consuming, relatively costly, and may not identify pigs that intermittently excrete low numbers of spirochaetes. Colonization by B. hyodysenteriae elicits a strong systemic immune response against the spirochaete that can last up to 17 weeks (Fisher and Olander, 1981; Fernie et al., 1983; Joens et al., 1979), and hence measuring circulating antibody titres can provide indirect evidence of exposure to B. hyodysenteriae. A number of tests have been developed that detect antibodies to B. hyodysenteriae (reviewed by La and Hampson, 2001). Amongst these, enzyme-linked immunosorbent assays (ELISA) using B. hyodysenteriae lipooligosaccharide (LOS) or sonicated cells as plate-coating antigens have shown the most potential (Joens et al., 1982; Wright et al., 1989), but they have tended to be serogroup-specific (in the case of LOS antigens) and/or lacking in specificity or sensitivity. Consequently there is a need for the use of a more species-specific plate-coating antigen that is applicable to all B. hyodysenteriae serogroups. Previously, it has been suggested that a 29.7 kDa outer membrane lipoprotein of B. hyodysenteriae might be a useful ELISA antigen as it is surface exposed, immunogenic and present in all strains of B. hyodysenteriae (La and Hampson, 2001). This molecule was originally called BmpB (Lee et al., 2000), then BlpA (Cullen et al., 2003), but is now designated as Bhlp29.7 (Hampson et al., 2006b).

The aim of this study was to develop and evaluate an ELISA for the serological detection of SD using recombinant His6-Bhlp29.7 as the discriminatory antigen. The test was targeted at detection of SD at the herd level, and used sera collected from commercial pigs slaughtered in abattoirs since this is a convenient source of sera for screening herd health status.

2. Materials and methods

2.1. Permissions

The work was conducted with the approval of the Murdoch University Animals Ethics Committee. All procedures were carried out under Australian National Health and Medical Research Council guidelines.

2.2. Preparation of ELISA antigen

Recombinant Bhlp29.7 was expressed as an N-terminal histidine-tagged fusion protein (His6-Bhlp29.7), as described previously (La et al., 2004; La et al., 2005). The purified lyophilised antigen was re-hydrated and diluted to a working concentration of 200 μg/ml using sterile phosphate buffered saline (PBS).

2.3. Preparation of reference sera

Reference sera were prepared from pigs that had been hyper-immunized with bacterin preparations of B. hyodysenteriae strain B78T and Brachyspira pilosicoli strain 1648. Spirochaete cells were grown at 37 °C in 500 ml of modified pre-reduced Kunkle’s broth (Kunkle et al., 1986). The mid-log phase cultures were harvested at 10,000 × g at 4 °C and washing twice in PBS. The washed cells were resuspended in 0.3% formaldehyde in PBS and inactivated for 24 h with constant stirring at room temperature. The bacterin suspension was adjusted to a cell density of approximately 10⁹ cells/ml and stored in aliquots at −20 °C.

Two Large White pigs of approximately five weeks of age were obtained from a high health status herd where SD had never been diagnosed clinically or by regular testing by culture and PCR. The pigs were injected intramuscularly in the neck with 3 ml of a 1:1 emulsion of Freund’s Incomplete Adjuvant (FIA; Sigma Chemical Company) and thawed bacterin. After two weeks, the intramuscular injection was repeated weekly for four weeks using the same vaccine. Finally, 1.5 ml of bacterin without adjuvant was given intravenously twice at weekly intervals. Blood was collected by jugular puncture one week after the final immunization and the serum was separated and stored at −20 °C until required.

2.4. ELISA conditions

Assay conditions involved overnight antigen coating in 100 μl carbonate buffer at 4 °C onto 96-well microtitre plates (Immulon 4HBX, Dynex Technologies) followed by blocking of unbound sites with 150 μl PBS containing 1% (w/v) bovine albumin, Fraction V (Sigma Chemical Company, Cat. No.
The wells were washed three times with 150 μl of PBST (137 mM sodium chloride, 2.68 mM potassium chloride, 10 mM disodium hydrogen phosphate, 1.76 mM monobasic potassium phosphate, 0.05% (v/v) Tween 20, pH 7.4), before diluted pig serum (200-fold) was added and the plate incubated for 2 h. The wells were washed as above and the bound serum antibodies were detected with goat anti-swine IgG-HRP (Southern Biotechnology) diluted 5000-fold. All antibodies were diluted in 100 μl of PBST containing 0.1% (w/v) bovine albumin, and incubations occurred at room temperature (RT) with mixing for 1 h. Colour development occurred after the addition of 100 μl of the K-Blue Tetramethylbenzidine (TMB) substrate (ELISA Systems) and was allowed to intensify for 15 min at RT. The colour reaction was stopped by adding 50 μl of 1 M sulphuric acid and the absorbance of the coloured substrate reaction was recorded at 450 nm using a microtitre plate reader (Bio-Rad Model 3550-UV).

2.5. Optimization of the immunoassay

A checkerboard titration involving serial dilutions of plate-coating antigen and primary antibody (reference sera) was performed under the ELISA conditions described above. The titration was performed with a constant 5000-fold dilution of the HRP-conjugated goat anti-pig IgG antibody. The procedure was optimized to give the greatest distinction between the positive signal (absorbance) and the background noise. Immunoassay plates were coated with serial 2-fold dilutions of antigen from 750 to 6000 ng/ml. Reference sera were serially diluted in 2-fold increments from 50-fold to 800-fold. The optimal concentration for antigen coating was chosen as the concentration that gave a signal greater than 1.0 and allowed the highest signal-to-noise ratio. The optimal-coating concentration of His6-Bhlp29.7 was used for all subsequent ELISA tests.

2.6. Across plate standardization

Microtitre plates were coated with the optimal concentration of His6-Bhlp29.7 and assayed in triplicate, with positive and negative control sera used in all tests. To attain cross-plate standardization and normalization a signal to positive ratio was used. The raw absorbance values measured at 450 nm (AX) were manipulated by the equation $X = 100[(A^X - A^N)/(A^P - A^N)]$, where $A^N$ was the absorbance of the negative control serum (pig serum raised against $B. pilosicoli$ strain 1648) and $A^P$ was the absorbance of the positive control serum (pig serum raised against $B. hyodysenteriae$ strain B78T).

2.7. Collection of pig serum

Sera from 1121 finisher pigs originating from 19 piggeries with differing health status from around Australia were collected at slaughter. These samples were provided by consultant veterinarians who were familiar with the health status of the herds. The herds included five high health status herds in which SD had never been observed (group A, n = 464), six in which SD previously had been confirmed to be present by culture and PCR for $B. hyodysenteriae$ (group B, n = 337), and eight herds with a history of diarrhoea where it was thought that SD might be present (group C, n = 320), but where $B. hyodysenteriae$ had not been demonstrated by culture or PCR. In all cases the prior health status of the individual pigs from which the serum was obtained was not known, but none were observed to be diseased at the time of their slaughter for human consumption.

2.8. Strategy used for the development and evaluation of the ELISA

Standardization of the ELISA consisted of three steps. In the first, the 464 serum samples from the five herds in which SD had never been observed (group A) were used to set a suitable cut-off value. Threshold values of two or three standard deviations above the mean of all individuals in this population were assessed, and tested to determine the resultant specificity (i.e. the proportion of negative herds that were correctly identified).

In the second step, the ELISA test was applied to 337 serum samples obtained from six herds in which SD had been confirmed (group B), to check the sensitivity of the selected cut-off value. The sensitivity was defined as the proportion of diseased herds that were correctly identified. Herds were considered infected where one or more individual...
serum samples returned an ELISA value greater than the set cut-off.

The third step involved the application of the assay to 320 serum samples from pigs in eight herds in which diarrhoea had been observed but SD had not been confirmed (group C). For the analysis of these herds, a sample size of 40 sera (where available) was chosen. This sample size was selected in accordance with the results from the herds in group B, and from the results of a similar study which evaluated the use of a LOS-based ELISA test for the diagnosis of SD (Mhoma et al., 1992). Testing 40 serum samples should detect a within-herd prevalence of about 7% with a 95% confidence level of detecting a positive animal when using a perfect test (Cannon and Roe, 1982).

3. Results

3.1. Immunoassay development

Optimized results were obtained with a 200-fold dilution of positive serum and 3000 ng/ml of antigen (Table 1). The reactivity of the serum from the pig immunized with *B. hyodysenteriae* B78T gave an ELISA value of 94.1, which was approximately 2.6-fold greater than the serum raised against *B. pilosicoli* 1648 (ELISA value of 36.3).

Table 1

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<tr>
<th>Antigen concentration (ng/ml)</th>
<th>Reciprocal of serum dilution</th>
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<tr>
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<td>50</td>
</tr>
<tr>
<td>750</td>
<td>5.8</td>
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<td>3000</td>
<td>5.9</td>
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<tr>
<td>6000</td>
<td>4.6</td>
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Optimum values with the best signal-to-noise ratio are highlighted.

3.2. Determination of the cut-off value for the ELISA

The mean and standard deviation of the normalized ELISA absorbance value of sera from pigs in group A (SD not observed) were 16.9 and 9.4, respectively. The mean + three standard deviations cut-off value for the assay was 45.0, and the mean + two standard deviations value was 35.7.

3.3. Specificity and sensitivity of the ELISA

Diagrammatic representations of the ELISA results and cut-off values for the serum from the five healthy herds (group A) and six infected herds (group B) are shown in Figs. 1 and 2, respectively. Using the criterion that a herd was infected if one or more serum sample exceeded the cut-off value, and using the two standard deviations cut-off, the test had zero specificity (all five negative herds scored positive) and a sensitivity of 100% (detecting all six positive herds correctly; group B). Using a cut-off value of three standard deviations above the mean, the ELISA had a specificity of 100% (correctly identifying all five negative herds; group A), and the sensitivity remained unchanged at 100% (detecting all six positive herds, group B). At this cut-off value between 2.5% (1 out of 40 samples) to 10.5% (4 out of 38 samples) of the animals tested were seropositive.

3.4. Application of the Bhlp29.7-ELISA to field samples

Four of the eight (50%) herds where SD was suspected (group C) were determined to be diseased according to the test using three standard deviations as the cut-off value. Prevalence within these four herds ranged from 2.5% (1 of 40 serum samples positive) to 15% (6 of 40 samples), with a mean of 8.75%. The remaining four herds in this group did not contain any tested individuals with ELISA values above the three standard deviation cut-off value.

4. Discussion

An indirect ELISA was established and standardized for the detection of antibodies to Bhlp29.7, an
outer membrane lipoprotein of *B. hyodysenteriae*. As the disease history of individual animals tested was not recorded, it was not possible to investigate the potential usefulness of the ELISA at identifying individual infected pigs.

To determine the disease status of individual herds, a cut-off value was established using the mean and distribution of the serum reactivity of groups of pigs from non-infected herds (group A). Titres varied considerably in these uninfected herds, and it was necessary to apply a cut-off value of three standard deviations above the mean to obtain 100% specificity at the herd level. Relatively large numbers of sera were used in this step (464), which increased confidence in the cut-off value that was eventually selected. Even with this stringent cut-off value the ELISA was still able to correctly identify all six diseased herds in group B, with a within-herd sero-prevalence of...
between 2.5% and 10.5%. When applied to the eight herds in group C, the within-herd prevalence in the four herds identified as positive varied between 2.5% and 15%. In all these 10 positive herds the apparent sero-prevalence was quite low, and in future studies it would be informative to try to establish the actual prevalence of infection in the pigs by taking repeat faecal samples for culture and PCR throughout the growing period – before their serum was collected at slaughter. The low overall sero-prevalence also suggests that it may be necessary to increase the number of pigs tested per herd, to improve the likelihood of detecting herds that have a very low sero-prevalence in this test.

The ELISA tests previously developed for SD that used crude whole cell preparations of *B. hyodysenteriae* were hampered by false-positive readings associated with the cross-reactivity of antibodies produced against intestinal spirochaetes other than *B. hyodysenteriae*. The use of an affinity purified recombinant protein as the coating antigen in the current ELISA reduced the possibility of cross-reactivity, although the wide distribution of ELISA values seen for the healthy herds (group A), suggests that cross-reactivities probably still did occur. These then elevated the cut-off values, and made it more difficult to identify true positive sera from infected herds. Bhlp29.7 is thought to belong to the family of methionine substrate-binding proteins (MetQ), which are found in a wide range of Gram-negative bacteria (La et al., 2005). As such, there are possibly epitopes on Bhlp29.7 that would be similar to those of MetQ proteins in other bacteria, and exposure of pigs to these could induce antibody responses that would result in cross-reactivity with His6-Bhlp29.7. Another possible source of cross-reactivity could be exposure of the pigs to certain strains of *Brachyspira innocens*, a common commensal species colonizing the pig colon. A previous investigation identified the Bhlp29.7 gene in one of 12 *B. innocens* strains tested (La et al., 2005), although it is not known whether the gene is expressed in vivo by *B. innocens*, and if it induces an immune response.

A major constraint in the study was the practical problems associated with obtaining a reliable well-defined set of sera from pigs/herds with confirmed health status. The sera were collected from slaughter-aged animals at the abattoir, and the disease history of the individual animals was not known. Although this method of collection does not provide a truly random representation of the pigs in a herd, and misidentification of herds or individuals is possible, it was far simpler and easier than collecting blood directly from the pigs on farm. Furthermore, it is likely that this is the way that such a test would be used in the field.

Overall, the ELISA that was developed appears useful as an indirect test for determining exposure of herds to *B. hyodysenteriae*. As used, the test was 100% specific and sensitive at the herd level – although it was recognized that relatively few herds were tested, and that additional studies are required to evaluate its usefulness in the field. Consideration also should be given to determining the most appropriate sample size for the current test conditions.

**Acknowledgement**

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**References**


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