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A reverse vaccinology approach to swine dysentery vaccine development

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Short title: Reverse vaccinology for swine dysentery

Abstract

Swine dysentery (SD) is a mucohaemorrhagic colitis of pigs resulting from infection of the large intestine with the anaerobic intestinal spirochaete *Brachyspira hyodysenteriae.* Whole-cell bacterin vaccines are available to help control SD, but their performance has been inconsistent. This study aimed to use a reverse vaccinology approach to identify *B. hyodysenteriae* proteins for use as recombinant vaccine components. Nineteen open reading frames (ORFs) predicted to encode potential vaccine candidate molecules were identified from *in silico* analysis of partial genomic sequence data. The distribution of these ORFs amongst strains of *B. hyodysenteriae* was investigated by PCR, and widely distributed ORFs were cloned. The products were screened with a panel of immune pig sera, and from these a subset of conserved, immunogenic proteins was selected. Mice immunized intramuscularly with these recombinant proteins developed specific systemic antibody responses to them, and their sera agglutinated *B. hyodysenteriae* cells *in vitro*. In a pilot experiment, eight pigs were vaccinated twice intramuscularly with a combination of four of the proteins. The pigs developed antibodies to the proteins, and following experimental challenge only one developed SD compared to five of nine non-vaccinated control pigs. Although these differences in incidence were not significant, they indicated a trend towards protection using the recombinant proteins as immunogens. This study demonstrates that the reverse vaccinology approach has considerable potential for use in developing novel recombinant vaccines for SD.

Keywords: Brachyspira hyodysenteriae; Spirochaete; Swine dysentery; Reverse vaccinology; Recombinant protein

1. Introduction

The anaerobic intestinal spirochaete *Brachyspira hyodysenteriae* is an important enteric pathogen. Infection of growing and fattening pigs results in swine dysentery (SD), a severe mucohaemorrhagic colitis that causes significantly disruption to production (Hampson et al., 2006). Although the incidence and severity of SD can be reduced in infected herds by using antimicrobials, strains of *B. hyodysenteriae* that are resistant to various key antimicrobial agents are increasingly being encountered (Duinhof et al., 2008). The availability of an effective vaccine for SD would be an important alternative or supplement to the use of antimicrobials for control of the disease.

To date vaccines for SD have largely been based on inactivated whole cells of *B. hyodysenteriae*, but these often fail to confer a satisfactory degree of protection against infection, and do not provide adequate cross-protective immunity against strains of different serogroups (Hampson et al., 2006). Efforts have been made to identify *B. hyodysenteriae* proteins for use in subunit vaccines, but vaccination with a recombinant 38 kDa flagellar protein failed to prevent colonization in experimentally infected pigs (Gabe et al., 1995). On the other hand, vaccination with a recombinant 29.7 kDa outer membrane lipoprotein (Bhlp29.7) resulted in partial protection, with fewer animals developing disease than occurred in the control groups (La et al., 2004). This result provided evidence for the potential usefulness of recombinant proteins as subunits for SD vaccines, and encouraged the current investigation.

"Reverse vaccinology" uses a combination of bioinformatics analysis of whole genomic sequence data and laboratory screening to identify novel vaccine candidates for bacterial pathogens (Rappuoli, 2001). This approach was first successfully applied to *Neisseria meningitidis* serogroup B, for which conventional strategies have failed to provide an efficacious vaccine (Pizza et al., 2000).

The aim of the present study was to investigate the use of the reverse vaccinology approach to select potential vaccine candidates for *B. hyodysenteriae,* and to test a small number of these for efficacy in pigs.

2. Materials and methods

2.1. Spirochaete strains and growth

A total of 27 well-characterized *B. hyodysenteriae* strains representing the major serogroups were obtained from the collection held at the Australian Reference Centre for Intestine Spirochaetes (ARCIS), School of Veterinary and Biomedical Sciences, Murdoch University. These comprised 19 strains from Australia, four from the USA, three from Canada, and one from the Netherlands. The strains were thawed and grown at 38°C in Kunkle's pre-reduced anaerobic broth containing 2% (v/v) foetal bovine serum and 1% (v/v) ethanolic cholesterol solution (Kunkle et al., 1986).

2.2. Permissions

The experimental work with animals 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.

2.3. Genomic sequencing and in silico analysis

The genome of Australian *B. hyodysenteriae* strain WA1 (ATCC 49526) was sequenced using a shotgun sequencing approach at the Australian Genome Research Facility, University of Queensland, Australia, with an approximate six times coverage. The program Phred was used for fragment assembly (Ewing et al., 1998), with subsequent viewing using Consed (Gordon et al., 1998). Identification of open reading frames (ORFs)

was carried out using Glimmer and GeneMark (Lukashin and Borodovsky, 1998; Salzberg et al., 1999). The identified ORFs were given a temporary designation starting with ORF-, with the encoded protein identified with the same number but starting with P-. For selected ORFs, searches for functional assignments were conducted using BlastP (ftp.ncbi.nlm.nih.gov) performed with the non-redundant protein database (Altschul et al., 1997). Protein domains were assigned by searching against Pfam (Bateman et al., 2002) and the Conserved Domain Database (CDD) (Wheeler et al., 2001). The cut-off E-value was set to 10^{-7} as a default, and proteins with lower E-values were considered genuine homologies. Cellular localization predictions for each ORF were carried out as follows: prediction of the presence and location of signal peptides in the N-terminal 70 amino acids of an ORF, using SignalP (Version 3.0, http:// www.Cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004); prediction of protein localization sites in Gram-negative bacteria using PSORTb (version 2.0, http://www.psort.org/psortb/) (Nakai, 2000; Gardy et al., 2005); identification of lipoprotein signatures by the Lipop program of the PSORT package (Nakai, 2000), and SpLip (Setubal et al., 2006) for specifically predicting spirochaetal lipoproteins (obtained from Setubal and Haake, Virginia Bioinformatics Institute, USA); and recognition of membrane-spanning regions, using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) (Hofmann et al., 1999). Theoretical molecular weights and isoelectric points were calculated using the Pepstats program (Emboss).

2.4. PCR amplification and sequencing

Pairs of primers that annealed to internal regions of each of 19 selected coding sequences were designed (supplementary table S1), and were used for PCR amplification

from the *B. hyodysenteriae* strains. If a gene could be amplified from more than 90% of the strains examined, it was selected for cloning.

DNA sequencing was conducted using a pair of flanking primers (Supplementary table 2) that annealed to regions external to the coding sequences of the putative genes. For ORF-N17, which was greater than 1600 base pairs (bp), internal primers were used to cover the missing regions. For sequencing, whole ORFs were amplified from the genomes of 6-7 *B. hyodysenteriae* strains. The PCR products were purified using the UltraClean up Kit (Mo Bio Laboratories, Solana Beach, CA, USA), according to the manufacturer's instructions. Sequencing of the PCR products was performed using the ABI PRISM™ Dye Terminator cycles Sequencing Ready Reaction Mix (PE Applied Biosystems, Foster City, CA, USA). Sequence results were edited and compiled using SeqEd v1.0.3 (PE Applied Biosystems), then translated into amino acid sequence using the BioEdit Sequence Alignment Editor (North Carolina State University). The nucleotide and translated protein sequences for all the genes were compared to produce a pair-wise identity matrix using Clustal W (Thompson et al., 1994).

2.5. Cloning and expression of recombinant protein

ORFs were amplified from *B. hyodysenteriae* strain WA1 using primers encoding restriction endonuclease recognition sites for cloning into the *E. coli* expression vector pTrcHisA (Invitrogen, Carlsbad, CA, USA). If an N-terminal signal peptide for secretion was predicted, the corresponding nucleotide sequence was excluded. Among the selected coding sequences, ORF-H17, encoding a large protein with a predicted size of 111.05 kDa was amplified and cloned in two smaller sub-fragments (ORF-H17 N-terminus and ORF-H17 C-terminus) to facilitate subsequent expression and purification. Constructs were confirmed by sequencing using vector (pTrcHisA)-specific primer (FP: 5'-

GAGGTATATATTAATGTATCG-3' and RP: 5'- TCTTCTCTCATCCGCCAAAAC-3'). Recombinant proteins were expressed as inclusion bodies in *E. coli* JM109 in Luria-Bertani broth supplemented with 100 mg/l ampicillin and 1mM isopropyl-B-thiogalactopyranoside. Most of the proteins were purified under denaturing conditions using nickel nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen GmbH), according to the manufacturer's instructions. Two proteins (P-H8 and P-H12) were soluble in the cytoplasm and were purified under native conditions. Protein concentrations were determined using the Bradford Protein assay (Biorad, Madison, WI, USA). The recombinant protein elutions were dialyzed, resuspended in phosphate buffer saline (PBS) and emulsified 1:1 (vol:vol) with Freunds Incomplete Adjuvant (Difco Laboratories) for mouse immunizations.

2.6. Serological assays

For each of the relevant recombinant proteins, five female C3A/HeJ mice of 5-6 weeks of age, housed in one cage, were immunized intramuscularly with 100 μg of the protein, twice at a two-week interval. Two weeks later the mice were individually euthanazed by gassing with methoxyfluorane followed by cervical dislocation. Serum was obtained by heart puncture at necropsy.

For Western blot analysis, 10 μg of recombinant protein was separated by SDS-PAGE and electro-transferred to a 0.2 μm nitrocellulose membrane. Nineteen porcine serum samples were obtained from the collection at ARCIS, and were used to detect the expression and antigenicity of recombinant proteins. The sera included: N1-N3 from healthy grower pigs; M1-M3 from pigs immunized with whole cell bacterins of *B. hyodysenteriae*, *B. pilosicoli* and *B. innocens* respectively; S1-S5 from individual pigs experimentally infected with *B. hyodysenteriae* that developed clinical SD, and had

166 lesions of SD at post-mortem; S6-S9 from individual infected pigs with a serological
167 conversion to *B. hyodysenteriae* using a whole cell ELISA; S10-S13 from four pigs
168 recovered from SD. The sera were used at a 1:100 dilution. Bound antibody was detected
169 with an alkaline phosphatase (AP)-conjugated anti-swine lgG (Sigma; diluted 1:5000).
170 Pre- and post-immunization sera (diluted 1:100) from experimental mice or pigs also were
171 examined by Western blotting using 10 μg recombinant protein and 15 μg of protein from
172 a *B. hyodysenteriae* whole cell preparation. The secondary antibody was anti-mouse lgG-
173 AP for mouse sera (Sigma; diluted 1: 5000).
174 The mouse sera raised against each antigen also were examined for their capacities
175 to agglutinate *B. hyodysenteriae*. Normal mouse serum and serum from a pig
176 hyperimmunized with *B. hyodysenteriae* $B78^T$ bacterin were used as the negative and
177 positive controls respectively. The agglutination test was performed in 96 well U-bottom
178 plates with 5×10^9 cells of *B. hyodysenteriae* strain WA1 resuspended in 50 μl PBS,
179 carried out essentially as previously described (Diarra et al., 1994). Antiserum was serially
180 diluted in 50 μl PBS (1:100 to 1: 6800) and added to each well containing spirochaetes.
181 The last column was left without serum as a control. The plates were incubated overnight
182 at 37°C. The antibody titer was expressed as the reciprocal of the highest dilution of serum
183 showing a definite positive pattern (flat sediment) as compared with the pattern of the
184 negative control (smooth dot) in the center of the well.
185
186 *2.7. Immunization and challenge of pigs*
187 Eighteen female pigs weaned at 3 weeks of age were purchased from a commercial
188 SD-free herd in Western Australia, and transported to an isolation animal house at
189 Murdoch University. The animals were weighed, ear-tagged and randomly assigned to
190 two groups of 9: group 1, an unvaccinated control group; group 2, the experimental

vaccine group. One pig from group 2 subsequently became lame and was removed. The pigs were fed *ad libitum* on a commercial pelleted weaner diet that did not contain any antimicrobial agents. On arrival, and prior to infection, rectal faeces were collected and subjected to selective anaerobic culture for *Brachyspira* species. The pig challenge studies were performed essentially as described by La et al. (2004), starting two weeks after the pigs were purchased. The pigs in the vaccinated group were injected intramuscularly with 2 ml of a vaccine containing 0.5 mg of each of four recombinant proteins emulsified in a commercial water-in-oil adjuvant (Emulsigen; MUP Laboratories Inc., Ralston, NE, USA). Four weeks later they received a second immunization using the same dose and route. Three weeks later, all pigs were inoculated via stomach tube with approximately 10^{10} active viable cells of *B. hyodysenteriae* strain BW1 in 100 ml of Kunkle's broth. Experimental challenge was repeated daily over five days.

The pigs were evaluated daily for the presence of diarrhoea, mucus and/or blood in the faeces, depression and/or anorexia. The consistency of the faeces also was assessed during rectal swabbing for bacteriological examination. Pigs were weighed prior to the first vaccination ("pre-bleed"), just prior to the second vaccination ("pre-boost"), prior to the first day of challenge ("pre-infection") and weekly thereafter. The animals were removed from the experiment if they developed clinical signs, and consequently thereafter their weights were not recorded. Serum samples were taken from the anterior vena cava pre-vaccination, pre-boost, pre-infection and at post-mortem.

Necropsy was performed as previously described (La et al., 2004). For ethical reasons, dysenteric pigs were killed within 24 hours of the first appearance of diarrhoea containing blood and mucus. The remaining pigs were killed 28 days after the last day of experimental infection. At post-mortem, the large intestine was opened and examined for gross pathological changes. Swabs were taken from the wall of the caecum and mid-colon

and plated on selective agar. Fresh tissue from these two areas was placed into 10% (v/v) formalin, and subsequently 4 μm sections were cut and stained with haematoxylin and eosin and with Steiner silver stain.

2.8. Detection of B. hyodysenteriae

Bacteriology swabs were used to collect rectal faeces from each pig every 2-3 days, and to swab the mucosal surface of the colonic and caecal walls at post-mortem. The swabs were used to inoculate selective agar plates and incubated as previously described (La et al., 2004). The presence of low flat spreading growth of spirochaetes on the plate, and any haemolysis around the growth was recorded. Suspected areas of spirochaete growth were resuspended in PBS, and examined under a phase contrast microscope at 400 × magnification. Spirochaetes were confirmed as *B. hyodysenteriae* on the basis of strong β-haemolysis on the plates, microscopic morphology and results of a NADH oxidase (*nox*) PCR of cell growth from the plates. The primers and PCR conditions used have been described previously (La et al., 2003).

2.9. ELISA for serum antibodies

Enzyme-linked immunoabsorbent assays (ELISA) were used to determine lgG and lgM responses in the pig sera, as previously described (La et al., 2004). Briefly, microtitre plates (Sarstedt Technologies, SA, Australia) were coated with the respective purified recombinant proteins (0.5 μg/ml) or a whole-cell (WC) preparation of *B. hyodysenteriae* strain WA1 (1 μg/ml) in 0.1 M carbonate buffer (pH 9.6). Pig sera were diluted 200-fold in 100 μl of PBS-T containing 0.01% skim-milk powder. The conjugates used were goat anti-swine lgG or lgM conjugated with horseradish peroxidase (HRP: Southern Biotechnology, Birmingham, AL, USA) and the substrate was K-Blue TMB substrate

(ELISA Systems, Brisbane, Australia). The colour reaction was stopped after 10 min by adding 1 M sulphuric acid. The optical density (OD) was measured at 450 nm on a microplate reader (Biorad Model 3550-UV).

2.10. ELISA for colonic lgA and lgG

Extraction and assaying of colonic lgA and lgG used a method adapted from Rees et al. (1989). The colonic epithelium was rinsed to remove digesta, stripped off with a scalpel blade and resuspended in 4 ml of PBS containing 1% (w/v) bovine serum albumen fraction V, 2 mM phenylmethylsulphonyl fluoride, 1 mM ethylenediaminetetracetic acid and 0.2% (w/v) sodium azide. The suspensions were vortexed for 1 min at 4°C to pellet the debris. The supernatant was diluted 1:2 with PBS-T, and 100 µl was used for the ELISAs. These were performed as for the serum ELISAs, except a goat anti-swine lgA-HRP (1: 5000 dilution, Southern Biotechnology) was used to detect mucosal lgA.

2.11. Disease scoring

Pigs were scored positive for clinical signs of SD if they had diarrhoea with fresh blood and/or mucus in their faeces. Intestinal lesions were considered typical of SD if haemorrhage, excess mucus, fibrin, and/or necrosis were observed in the caecum and/or colon. Lesion severity was classified as: Mild, patchy reddening in ≤1/3 colon; Severe, lesions in > 1/3 colon with blood, oedema and excess mucus. SD was defined as the presence of both positive clinical signs and typical colonic lesions. Pigs that had mild localized lesions in the colon at post-mortem, but did not have clinical signs, were scored as being “healthy”. Similarly, pigs with mild diarrhoea but no colonic lesions were recorded as “healthy”.

2.12. Statistic analysis

Microsoft Excel and SPSS for windows 14.0 were used for statistic analysis. Body weights and ELISA values were expressed as the arithmetic mean and standard deviation, and statistical differences between the groups were assessed using one-way ANOVA. Faecal excretion of *B. hyodysenteriae*, incidence of SD, and the association between onset of disease and the antibody levels were evaluated using Fisher's exact test. The number of pig sampling days in which cultures were positive or negative for *B. hyodysenteriae* also was compared for the groups. Linear regression was used to compare body weights and antibody levels, and the Pearson correlation coefficient was calculated to determine the association of ELISA reactivity between recombinant proteins and the whole-cell preparation. Significance was accepted at the 5% level.

3. Results

3.1 Identification of vaccine candidates from in silico analysis

Initially 19 ORFs encoding predicted proteins with various functions were selected for further evaluation. These were grouped as predicted lipoproteins, proteases and toxins, flagellar-associated proteins and membrane proteins. Details of the ORFs and their vaccine candidate group are given in Supplementary table 3, together with their GenBank accession numbers.

3.2. Conservation of the candidate genes among B. hyodysenteriae strains

Using gene-specific PCR assays, 17 of the 19 genes were found in >90% of the *B. hyodysenteriae* strains tested. ORF-H28 and ORF-H29 demonstrated 34.9% and 73.9% distribution respectively, and were discarded.

3.3. Cloning and expression of recombinant proteins

Of the 19 molecules that were cloned (including the remaining 17 full ORFs, and both fragments of ORF-H17), 14 (74%) were successfully expressed as determined by Western blot detection with an anti-6x His tag antibody. ORF-H14, ORF-H16, ORF-H20, ORF-H26 and ORF-H27 failed to express even after optimization of the time-course, alteration of isopropyl β-D-thiogalactopyranoside concentration and expression temperature, and using another expression system (pET19b and pQE30) (data not shown). Highly expressing colonies were used for protein purification.

3.4. Serological reactivity of the recombinant proteins

Of the 14 purified proteins screened for reactivity by immunoblotting with a range of porcine sera, seven (P-H7, P-H8, P-H12, P-H17, P-H17 C-terminal, P-H34 and P-H42), derived from six genes, were found to be immunoreactive. Examples are shown in Fig. 1. These protein products also reacted with the hyperimmune pig anti-*B. pilosicoli* and *B. innocens* sera, and showed cross-reactivity with normal pig serum. All seven molecules produced antibody responses in mice, and the sera were specific in that they recognized whole-protein extracts of *B. hyodysenteriae* preparations with native proteins of the correct size (data not shown). Sera from the non-immunized mice did not recognize any of the components tested.

Figure 1 about here

The mouse antisera produced following vaccination with the candidate proteins all agglutinated the *B. hyodysenteriae* cells in a dose-dependent manner. Sera raised against P-H7 and P-H12 agglutinated the cells to a dilution of 400, serum against P-H8 and P-H42 agglutinated to 800, and serum against P-H34, P-H17 and the C-terminal of P-H17

agglutinated to a dilution of 1600. The positive hyperiummune serum agglutinated to a 3200 dilution, while the negative control serum did not agglutinate the spirochaetes.

3.5. Nucleotide sequence conservation

The full ORFs of the six genes from which the seven immunoreactive molecules originated all had 98-100% nucleotide sequence identity and 99-100% predicted amino acid identity amongst the set of *B. hyodysenteriae* strains from which they were amplified.

3.6. Final selection of vaccine proteins

On the basis of ease of expression and purification, products from four of the six ORFs were selected for use in the prototype vaccine. These were P-H7, P-H17 (C-terminal), P-H34 and P-H42. The product from the C-terminal of ORF-H17 was used rather than the whole protein, as it was equally as immunoreactive.

3.7. Body weights of experimental pigs

The body weights of the pigs showed no significant differences between the groups over the course of the experiment.

3.6. Systemic antibody responses

Systemic IgG antibody levels in the pigs against the different antigens are summarized in Figure 2. The overall patterns of response with time were similar whether recombinant antigens or whole cell extracts were used as ELISA plate coating antigens. The non-vaccinated pigs showed a clear trend to have increased circulating IgG antibodies against the recombinant proteins or whole cell proteins through to pre-infection, with further larger increases following infection. Increases in IgM levels occurred in the same

manner (data not shown). For the vaccinated pigs serum lgG and lgM levels to all antigens and the whole cell preparation showed increases after the first vaccination, but a significant response was not detected after the second vaccination, and in most cases infection with live *B. hyodysenteriae* did not further increase the antibody responses. Indeed, in some of the pigs, there was a trend for antibody levels to decline after the second vaccination, but the change was not statistically significant. Mean antibody levels to P-H7 were lower in the vaccinated pigs than in the infected control pigs. Western blot analysis of swine serum from the vaccinated group confirmed that the systemic antibody response was primary directed against the candidate proteins of *B. hyodysenteriae* used in the study. The serum antibody levels of individual pigs did not significantly correlate with whether or not they developed disease.

Figure 2 about here

3.7. Colonic antibody response

The local antibody responses in the colon of the pigs at slaughter are summarized in Table 1. In the vaccinated pigs the local IgA and IgG responses to the candidate proteins, except to IgG in the case of P-H7, were all significantly higher than in the control group. IgA but not IgG responses were also higher with the whole cell preparation. The local antibody levels did not significantly correlate with presence or absence of disease at post-mortem.

Table 1 about here

3.8. Faecal excretion of B. hyodysenteriae

All rectal swabs were negative for *B. hyodysenteriae* and other *Brachyspira* species on arrival and prior to experimental infection. For the control pigs, *B. hyodysenteriae* excretion was first detected in two pigs (C1 and C8) 10 days after the end of experimental infection, and these two developed SD within a few days. Six of the other

seven pigs in the group shed *B. hyodysenteriae* in their faeces, and two developed SD. The other pig was culture positive at post-mortem. For the vaccinated pigs, the first faecal samples positive for *B. hyodysenteriae* occurred at 10 days in two pigs (V1 and V4), however they subsequently did not re-excrete nor develop SD. Five of the other six pigs shed *B. hyodysenteriae* at some time, but only one developed SD. Overall, the numbers of positive faecal samples in the two groups were 26/76 and 22/78, and these differences were not significant ($P = 0.6$).

3.9. Development of clinical signs, and findings at post-mortem

The post-mortem findings are outlined in Table 2. The appearance of clinical signs of SD always coincided with the prior occurrence of positive faecal cultures, although in some pigs positive cultures were not followed by disease development. Four of the nine control pigs developed clinical signs of SD, and severe mucohaemorrhagic colitis was found at post-mortem examination. Another pig (C2) in the control group had diarrhoea for two days, and at necropsy there were mild colonic lesions. This also was scored positive, making the number of positive animals 5/9 (56%) for the control group. The median onset of clinical signs in the control group was 14 (9, 10, 14, 17 21) days after challenge. For the vaccinated group, one pig developed diarrhoea 22 day after challenge, and had mild localized mucohaemorhagic colitis in the upper third of the colon at post-mortem. Another pig had mild lesions in the middle half of the colon at post-mortem, but had not shown clinical signs. The other six pigs remained healthy, and had no colonic lesion at post-mortem, although five of them had *B. hyodysenteriae* in their large intestines. The incidences of disease in the two groups were recorded as 5/9 and 1/8, respectively, and this difference was not significant ($P = 0.13$).

Table 2 about here

4. Discussion

This study explored the use of a reverse vaccinology approach for identifying potential candidates for use in a recombinant *B. hyodysenteriae* vaccine. Reverse vaccinology typically utilizes a complete genome sequence, and usually favours putative secreted toxins and surface exposed membrane proteins or lipoproteins as vaccine targets (Mohavedi and Hampson, 2008). At the time the current pilot study commenced only a partial genome sequence was available, and the choice of potential vaccine candidates was relatively limited. As a result, only a small number (19) of proteins that were either putative lipoproteins, toxin-like proteins, flagellar-associated proteins or membrane-associated proteins (both inner and outer membrane) were selected for further screening.

Analysis of distribution and sequence conservation amongst these vaccine candidates was important as the heterogeneity of some proteins among bacterial isolates can cause problems with cross-protection, which limits their usefulness as vaccines (Tappero et al., 1999). Most of the putative genes were present in all the *B. hyodysenteriae* strains examined, and for the six genes that survived the full screening process, their predicted amino acid sequences in a subset of strains were ≥99% identical to the sequence of the corresponding protein from strain WA1. This suggested that if the selected vaccine candidates were shown to confer protection against one *B. hyodysenteriae* strain they potentially would provide cross-protection against other strains.

All the selected recombinant proteins reacted strongly with the panel of sera from naturally and experimentally infected pigs, suggesting that the corresponding antigens are expressed *in vivo*. Some reactivity with sera from healthy pigs was present, and this was consistent with previous findings that sera from normal pigs are reactive with *B. hyodysenteriae* surface antigens (Wannemuehler et al., 1988). The antigens also reacted with hyperimmune pig sera against *B. pilosicoli* and *B. innocens*. This was not

415 unexpected, as structural proteins in related organisms are likely to share common
416 epitopes that would produce cross-reacting antibodies when used as immunogens.
417 The final experimental vaccine was formulated to contain four candidate proteins
418 for which there was good immunogenicity data, and which were easy to produce. They
419 were combined in the vaccine to reduce the work required to evaluate each protein
420 separately, as well as to potentially improve the protection that would be obtained.
421 Vaccination of mice with the individual proteins generated specific antibodies to the
422 recombinant proteins, and sera from the mice agglutinated *B. hyodysenteriae* cells. The
423 latter activity provided supporting evidence that the vaccine would induce antibodies with
424 relevant activities against the whole spirochaete.
425 Unexpectedly, in the pig trial the non-vaccinated pigs developed increasing
426 systemic lgG and lgM levels to all antigens as their body weights increased, although the
427 highest levels were achieved after bacterial challenge. The reason for these increases was
428 unclear as the experimental pigs came from a herd with no reported history of SD or
429 isolation of *B. hyodysenteriae*, and *Brachyspira* species were not isolated from the animals
430 before the experiment started. It seems possible that the presence of such cross-reacting
431 antibodies could help to explain the relatively small number of control pigs that developed
432 SD (5/9).
433 The vaccinated pigs developed a primary antibody response, but significant
434 secondary responses were not produced, and in most cases experimental challenge did not
435 increase the antibody levels further. This unusual pattern of serological response might be
436 attributed to the use of several proteins in combination, or could be due to the high dose
437 rates used in the vaccine. It did not appear to be a prozone effect, as further serial dilutions
438 of a selection of these sera did not change the relative results (data not shown). At the
439 level of the colon, local lgA and lgG levels to the corresponding recombinant proteins, and

to the whole cell preparation in the case of IgA, were higher in the vaccinated pigs than in the control group at post-mortem. The colon and/or caecum of the majority of pigs in both groups were colonized at post-mortem, but the number of spirochaetes present was not evaluated. In future studies it would be useful to quantify the spirochaetes to determine whether such local antibodies reduced the extent of colonization, potentially to levels where disease does not develop.

Following experimental challenge, some animals began to shed *B. hyodysenteriae* in their faeces around one week after inoculation. The onset and total amount of shedding in the two groups over the experimental period were not significantly different, but this may have been influenced by the fact that four shedding pigs were removed from the control group before the end of the experimental period, and so did not contribute further to the shedding score. Removal of these animals also meant that they could not contribute to a cumulative clinical score. In the control group shedding was more likely to be followed by disease, and again it is possible that the use of quantitative methods might have revealed that the vaccinated pigs were shedding fewer spirochaetes than the control animals that developed disease.

The fact that only one (13%) pig receiving the experimental vaccine developed clinical signs of SD suggested that the vaccine was conferring some protection, even though statistical significance was not achieved. It would be useful to repeat this trial with more pigs to try to achieve more clear-cut differences between the groups. Other evidence suggesting the potential usefulness of the vaccine was the fact that dysenteric pigs in the control group had severe and extensive lesions in their large intestines at post-mortem, whilst the vaccinated pig with clinical signs showed localized lesions, and another pig only had mild colonic lesions and no clinical signs. If these differences were translated to

the farm situation, such a vaccine would be helpful in reducing SD-associated production losses.

In conclusion, this study provided evidence that reverse vaccinology is a useful approach to antigen selection for use in recombinant vaccines for SD. In future work additional *B. hyodysenteriae* proteins should be examined, and it would be helpful to evaluate different protein concentrations, adjuvants and vaccine delivery methods. The supporting vaccine trials should use larger groups of animals and utilize a more quantitative approach to assess the relative level of protection achieved. For reasons of animal ethics, pigs need to be removed when they become ill, so it may not be possible to develop a clinical scoring method. However, quantitation of spirochaetal load should provide useful additional information about vaccine efficacy.

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Figure legends

Fig. 1. Western blot analysis of the recombinant proteins, performed using sera from pigs naturally or experimentally infected with SD, or immunized with *Brachyspira* spp. bacterins. Lane M, molecular mass markers (kDa); Lanes 1-3, normal pig sera. Lanes 4-6, sera from pigs hyperimmunized with *B. hyodysenteriae, B. pilosicoli* and *B. innocens*, respectively. Lanes 7-11, serum from individual experimentally infected SD pigs. Lanes 12-15, sera from pigs showing seroconversion to *B. hyodysenteriae* in a whole cell–ELISA. Lanes 16-19, individual sera from pigs in the convalescent phase of SD. Note the lack of reactivity with the P-H17 N-terminal, and the cross-reactivity of normal pig sera with some of the proteins.

Fig. 2. Comparison of temporal changes in serum antibody concentrations in ELISA to the four recombinant *B. hyodysenteriae* proteins and a whole cell preparation in control and vaccinated pigs.

Fig. 1.

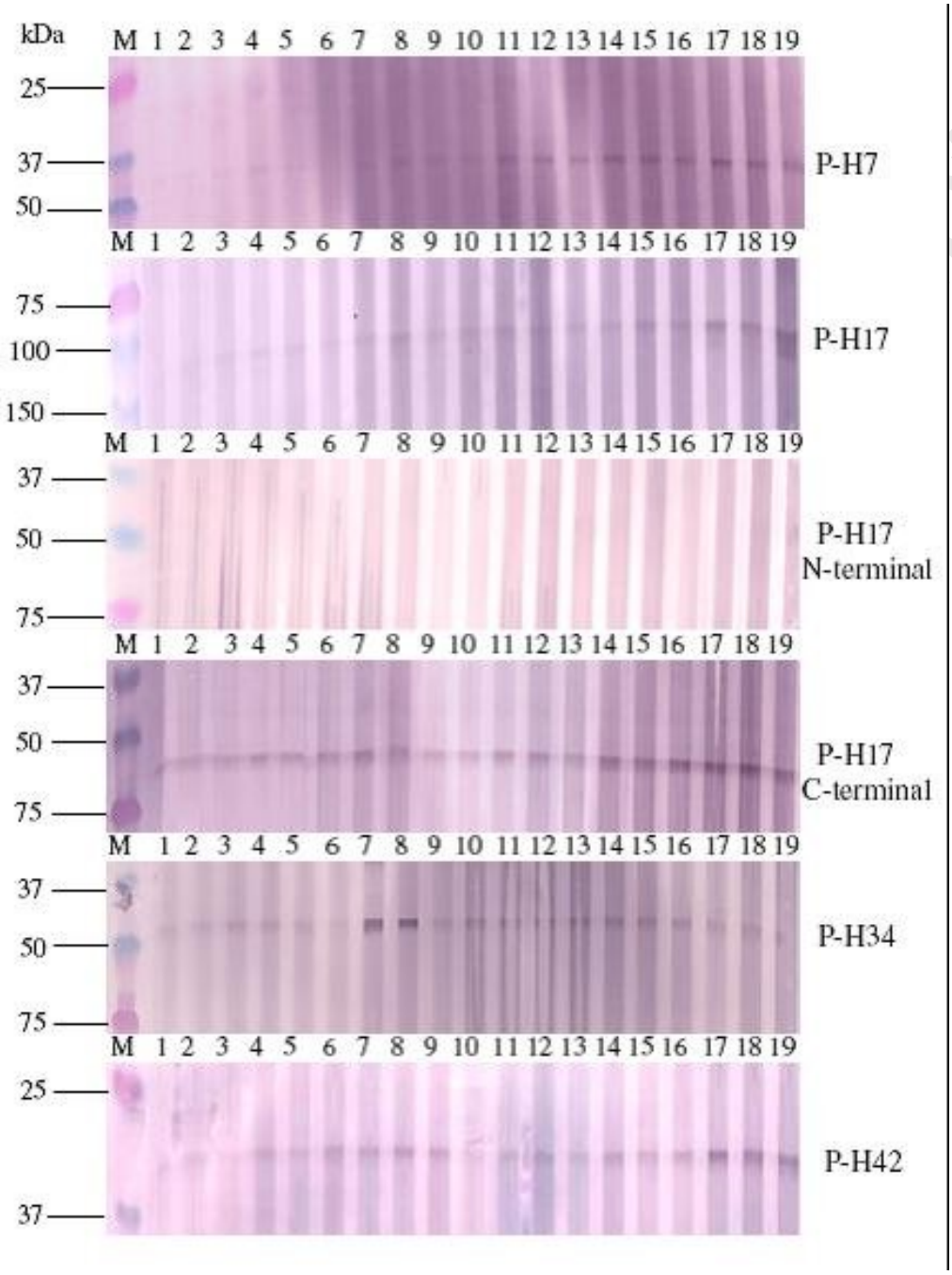

Fig. 2

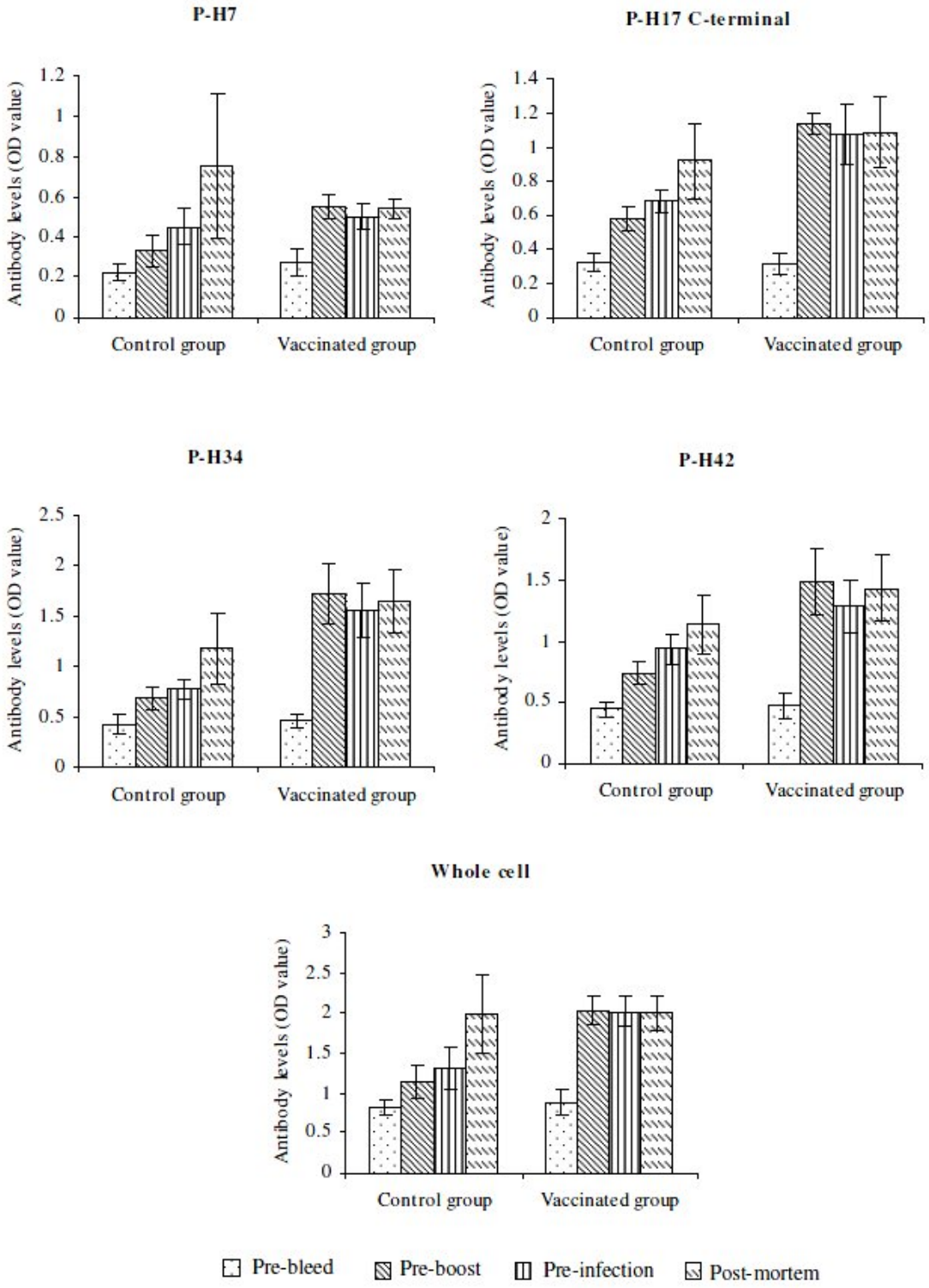

Table 1. Mean and standard deviation of group colonic antibody levels (OD values) to different ELISA antigens at post-mortem.

ELISA antigen	Antibody class	Group[a]	OD value	P value in t test
P-H7	lgA	1	0.50 ± 0.27	0.077
		2	0.75 ± 0.28	
	IgG	1	0.48 ± 0.24	0.125
		2	0.66 ± 0.25	
P-H17, N-terminal	lgA	1	0.32 ± 0.14	0.007
		2	0.69 ± 0.29	
	IgG	1	0.32 ± 0.13	0.018
		2	0.56 ± 0.21	
P-H34	IgA	1	0.42 ± 0.13	0.006
		2	0.72 ± 0.22	
	IgG	1	0.45 ± 0.14	0.038
		2	0.61 ± 0.15	
P-H42	IgA	1	0.50 ± 0.18	0.003
		2	0.84 ± 0.20	
	IgG	1	0.45 ± 0.14	0.023
		2	0.67 ± 0.20	
Whole-cell	IgA	1	0.38 ± 0.14	0.016
		2	0.74 ± 0.21	
	IgG	1	0.56 ± 0.36	0.305
		2	0.71 ± 0.24	

[a] Group 1, non-vaccinated control; group 2, vaccinated.

Table 2.

Clinical signs and post-mortem *B. hyodysenteriae* culture results and colonic lesions in experimental pigs, including the cumulative clinical + lesion score.

		Post-mortem culture[c]			
Pig[a]	Clinical signs[b]	Faeces	Caecum	Colon	Lesion severity
C1	Dys	+	+	+	Severe
C2	Dys	-	-	-	Mild
C3	EOE	+	+	+	Normal
C4	Dys	+	+	+	Severe
C5	Dys	+	+	+	Severe
C6	EOE	-	-	-	Normal
C7	EOE	-	+	+	Normal
C8	Dys	+	+	+	Severe
C9	EOE	+	-	-	Normal
V1	EOE	-	-	-	Normal
V2	EOE	-	+	+	Normal
V3	Dys	+	+	+	Mild
V4	EOE	-	+	-	Normal
V5	EOE	-	+	-	Normal
V6	EOE	+	+	+	Normal
V7	EOE	+	+	+	Mild
V8	EOE	+	+	+	Normal

[a]C1-9: non-vaccinated pigs; V1-8: vaccinated pigs.

[b]Dys; dysentery observed and the pig killed; EOE; end of experiment (no dysentery observed).

[c]+ indicates *B. hyodysenteriae* culture positive, - indicates *B. hyodysenteriae* culture negative.