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Author: Yong Song, Tom La, Nyree D. Phillips, David J. Hampson

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Development of a serological ELISA using a recombinant protein to identify pig herds infected with *Brachyspira hyodysenteriae*

Yong Song [1], Tom La, Nyree D. Phillips, David J. Hampson*

School of Veterinary and Life Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia.

[1] Current address: School of Anatomy, Physiology & Human Biology, the University of Western Australia, Crawley, Western Australia 6009, Australia

* Corresponding author. *E-mail address*: d.hampson@murdoch.edu.au (David Hampson)

Highlights

- A new ELISA was developed for detecting pig herds infected with *Brachyspira hyodysenteriae*
- Under optimized conditions the test had 100% specificity and 91.7% sensitivity
- The new test should be useful for screening herds for evidence of swine dysentery

Abstract

Brachyspira hyodysenteriae is an anaerobic spirochaete that can induce swine dysentery (SD), a severe mucohaemorrhagic colitis in grower and fattener pigs. The aim of this study was to develop a serological ELISA for use as a screening method to detect evidence of herd infection. Bioinformatic analysis of the complete genome sequence of strain WA1 was used to identify genes predicted to encode outer membrane proteins. Twenty candidate genes were expressed in an *Escherichia coli* mediated system, and purified as histidine-tagged recombinant proteins. Selection of optimal antigens under different conditions was conducted using Western blot and ELISA with a range of pig sera from infected and uninfected pigs. From this analysis, three recombinant proteins were selected as being most suitable for use as antigens. These antigens then were tested under optimized conditions in an indirect ELISA detecting IgG2 using 1551 sera from healthy pigs at slaughter, comprising 896 from 18 herds considered to be free from SD and 655 from 12 infected herds.

Using a cut-off value for positivity of the mean plus five standard deviations of the mean for the negative sera, the best overall results were obtained with the ELISA using antigen H114, which was 100% specific and 91.7% sensitive at detecting the reported status of the herds. This new ELISA should be a useful adjunct for detecting and monitoring the status of herds with respect to the presence of *B. hyodysenteriae*, and should prove useful for understanding the dynamics of infection in herds where the spirochaete is present.

Keywords: *Brachyspira hyodysenteriae*; Diagnosis; Enzyme-linked immunosorbent assay; Spirochaete; Swine dysentery.

Introduction

Swine dysentery (SD) is a severe mucohaemorrhagic diarrhoea that mainly affects pigs in the grower/finisher phase and causes economic losses through reduced growth rates, poorer food conversion, costs of medication, and mortality (Hampson, 2012). SD results from infection of the colon with the anaerobic intestinal spirochaete *Brachyspira hyodysenteriae.* Diagnosis in individual pigs mainly relies on observation of clinical signs and detecting *B. hyodysenteriae* in the faeces or colonic lesions by culture or PCR (La et al., 2003; Song and Hampson, 2009). Unfortunately, these methods are relatively expensive and time-consuming when applied for routine screening to detect herds that are infected. This is particularly so in herds with sub-clinical infections, where large numbers of samples need to be tested to improve the chance of detecting a colonized animal, and so provide evidence that the herd is infected.

Enzyme-linked immunosorbent assays (ELISAs) have the potential to be used as rapid, sensitive and reproducible procedures for quantifying serum antibodies to *B. hyodysenteriae*, and providing indirect evidence of the current or past disease status of an individual pig, and/or of the herd of origin. ELISAs using sonicated whole cells or lipooligosaccharide (LOS) from *B. hyodysenteriae* as plate-coating antigens have been reported to be sensitive enough to allow detection of SD at a herd level, if sufficient pigs are tested (Joens et al., 1982; Wright et al., 1989; Smith et al., 1991; Song et al., 2012). Unfortunately, false-positive reactions hampered further development of these assays (La and Hampson, 2001). Additionally, LOS-based ELISAs require an understanding of the LOS-serogroups of *B. hyodysenteriae* that are present in the region where testing is to be undertaken (Mhoma et al., 1992).

The use of conserved surface proteins of *B. hyodysenteriae* as ELISA antigens should improve sensitivity and specificity, and previous work showed that the use of recombinant

Bhlp29.7 in a whole molecule Ig ELISA could be used to identify infected herds (La et al., 2009). Consequently, the purpose of this study was to identify and test recombinant *B. hyodysenteriae* surface proteins for use as class-specific reagents in ELISAs for the serodiagnosis of SD.

Materials and methods

Permissions

This study was conducted with the approval of the Murdoch University Animal Ethics Committee, permit number R2292/09.

Antigen selection from in silico analysis

Bioinformatic analysis was conducted on the genome of Australian *B. hyodysenteriae* strain WA1 to select surface oriented proteins, as described previously (Song et al., 2009).

Brachyspira hyodysenteriae strains

Twenty-three *B. hyodysenteriae* strains from the USA, Canada and different Australian States, representing a number of different LOS-serogroups, were obtained from the collection held at the Australian Reference Centre for Intestine Spirochaetes, Murdoch University (Table 1).

Distribution study by PCR

Pairs of primers were designed that annealed to internal regions of each of the selected coding sequences and were used for PCR amplification of these from the 23 *B. hyodysenteriae* strains.

Antigen preparation

Selected open reading frames (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). If an N-terminal signal peptide for secretion was predicted, the corresponding nucleotide sequence was excluded. 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 *Escherichia coli* JM109 in Luria-Bertani broth supplemented with 100 mg/L ampicillin and 1 mM isopropyl-B-thiogalactopyranoside. Except for protein H8, the proteins were purified under denaturing conditions using nickel nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen), according to the manufacturer's instructions. Protein concentrations were determined using the Bradford Protein assay (BioRad).

Serum samples

Sera from healthy swine or swine that had either undergone natural infections with SD or had been vaccinated or experimentally challenged with *B. hyodysenteriae* or related *Brachyspira* species were obtained from the Reference Centre for Intestinal Spirochaetes at Murdoch University, and were used to detect the expression and antigenicity of recombinant proteins by Western immunoblotting. These included sera from healthy grower pigs; sera from pigs immunized with whole cell bacterins of *B. hyodysenteriae*, *B. pilosicoli* or *B. innocens*, respectively; sera from individual pigs experimentally infected with *B. hyodysenteriae* that developed clinical SD, and had lesions of SD at post-mortem; pig serum against *B. hyodysenteriae* that had been absorbed with cells of the related intestinal spirochaetes *B. pilosicoli*, *B. intermedia* and *B. innocens*; and sera from individual pigs confirmed to be colonized with *B. pilosicoli* or *B. murdochii* and *B. innocens*, but not *B. hyodysenteriae* by

using culture, PCR testing and DNA sequencing of the products, as described by Osorio et al. (2013).

For final evaluation of the ELISA, a further 1551 swine sera samples comprising 896 from 18 farms considered free from SD and 655 from 12 infected farms were used to evaluate their reactivities with the final three selected antigens. The serum samples were obtained from healthy finisher pigs at slaughter in different states of Australia, and were collected by veterinarians responsible for the health status of the farms. Numbers of animals sampled per farm varied from 35 to 97, with 50 samples being available from most of the farms.

Immunoblotting

For Western blot analysis, 10 μg the recombinant protein was separated by SDS-PAGE and electro-transferred to a 0.2 μm nitrocellulose membrane. The proteins were reacted with a pool of eight sera (diluted 1:100) from naturally infected pigs to detect the expression and antigenicity of the recombinant proteins. Bound antibody was detected with an alkaline phosphatase-conjugated anti-swine IgG (Sigma; diluted 1:5000). For final antigen selection, pig serum against *B. hyodysenteriae* that had been absorbed with cells of *B. pilosicoli*, *B. intermedia* and *B. innocens* was used, and those proteins that still showed reactivity were selected for testing in ELISA.

ELISA procedure

Initially, the standard ELISA assay was performed to screen the selected 20 molecules by determining the signal/noise (S/N) ratio (calculated by average antibody levels of positive sera/average antibody levels of negative sera) using sera from pigs pre- and post-infection with *B. hyodysenteriae* and sera from healthy swine or swine that had undergone natural infections. This was undertaken in ELISA systems detecting IgG, IgM and a combination of IgG and IgM.

Further optimization of the ELISA systems was undertaken using three recombinant antigens in a checkerboard titration at six different concentrations (from 0.63 μg/mL to 20 μg/mL) and with different serum concentrations (from 1:50 to 1:6400); titrating conjugate concentrations; comparing different microplates for efficiency of coating the antigens; comparing skim milk, bovine serum albumen and Tween-20 as blocking agents to decrease non-specific binding; and comparing secondary antibodies and substrates available from different suppliers.

To determine whether the S/N ratio could be improved using different isotypes of IgG, antigens H7 and H114 were tested in IgG, IgG1 and IgG2 ELISAs with sera from four groups: (1) uninfected pigs (*n* =20); (2) pigs infected with *B. hyodysenteriae* (*n*=10); (3) pigs infected with *B. pilosicoli* (*n*=5); (4) pigs colonized with *B. innocens* or *B. murdochii* (*n*=20).

In the finalized optimized protocol the wells in a 96-well microtitre plate (Immulon 4HBX, Dynex Technologies) were coated with recombinant protein antigens at 5 μg/mL in 100 μL 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. The wells were blocked with 3% skim-milk powder in phosphate buffered saline (PBS: pH 7.2) for 1 h, then washing three times with PBS containing 0.05% Tween 20 (PBST). The plate was incubated with diluted serum samples (1:300 for IgG and IgM; 1:600 for IgG1 and IgG2) for 2 h at room temperature (RT). After multiple washing, appropriate horseradish peroxidase (HRP) conjugates were added to each well. The conjugates were Goat anti-swine IgG (1:10,000, KPL) and IgM (1:30,000, SeroTec). Due to lack of commercial anti-swine IG1 and IgG2 conjugated with horseradish peroxidase (HRP), Mouse anti-swine IgG1 (1: 1000 dilution, Serotec) and IgG2 (1:500 dilution, Serotec) antibody solutions were used respectively, followed by adding Goat anti-mouse IgG conjugated with HRP (1:50,000 dilution, Serotec) in an indirect ELISA. The wells were washed three times with 300 μL PBST; 100 μL of 3,3',5,5'-tetramethyl-benzidine liquid substrate (Sigma) were added to each well, and the plate was incubated in the dark at RT. The

colour reaction was stopped after 20 min by adding 0.5 M sulphuric acid. The optical density (OD) was measured at 450 nm on a microplate reader (BioRad Model 3550-UV).

For across-plate standardization, a positive control was added in triplicate to each plate consisting of serum collected from a pig that previously had been experimentally challenged with *B. hyodysenteriae*, and that had recovered after developing SD.

Calibrated optical densities (COD) were calculated according to the following formula: (OD value of test sample - OD value of blank control)/average of (OD value of control sample - OD value of blank control).

Determination of cut-off values for herd positivity

The final three selected antigens were tested in an IgG2 ELISA with the 1,551 sera from 18 herds that were considered not to have SD and 12 herds that had a previous history of SD. The cut-off values for the ELISA were established from the COD of the 896 healthy pigs examined, using the mean plus multiple standard deviations as the cut-off. In this way, if one serum exceeded the cut-off value the herd was regarded as being infected with *B. hyodysenteriae.*

Results

Identification of antigen candidates from the genomic study

Twenty-three predicted outer membrane proteins or lipoproteins from *B. hyodysenteriae* were initially selected based on bioinformatics analysis of the complete genome sequence of *B. hyodysenteriae* strain WA1.

Distribution studies using PCR

Based on the distribution of the selected genes across the 23 *B. hyodysenteriae* strains, 20 genes with wide distribution were selected for cloning (Table 2).

Reactivities of antigens

Following initial ELISA testing, nine antigens (H1, H7, H17, H34, H77, H114, H116, H122 and H173) were selected based on their S/N ratio. Further, the nine antigens demonstrated strong and specific binding reactivities with pools of sera from naturally infected pigs in Western blot, and four antigens (H1, H7, H34 and H114) were still reactive with serum absorbed with other *Brachyspira* species (Fig. 1).

Comparison of antibody classes and antigen selection

Comparison of antibody classes showed that IgM predominated in the serum of pigs recently experimentally infected with *B. hyodysenteriae*, while IgG became predominant with time. Comparison of IgG and the two IgG isotypes showed that IgG2 had the best S/N ratio (Fig. 2). When comparing the nine antigens, the highest S/N ratio for IgG (3.43) occurred with protein H114 whilst in the IgG2 ELISA the highest S/N ratios were with H122 (11.86), H114 (10.81) and H7 (10.46).

ELISA testing with the final three antigens

The three antigens H122, H114 and H7 were tested in IgG2 ELISAs against the 1,551 sera from infected and non-infected herds, and their sensitivities and specificities using different cut off values are shown in Table 3. H114 gave the best performance, having 100% specificity and 91.7% sensitivity when the cut off was set at five standard deviations above the mean of the uninfected herds. Results for H114 are shown in Fig. 3.

Discussion

The ELISA we developed was designed to detect herds infected with *B. hyodysenteriae*. Whether or not they develop disease, colonized pigs from such herds may intermittently shed *B. hyodysenteriae* in their faeces for weeks or months unless they are effectively treated, and they are an important source of infection for other susceptible pigs. Consequently early diagnosis and treatment are important for preventing the spread of the pathogen both within and between herds.

Serological testing of pigs conducted at abattoirs has proved very useful for detecting and monitoring herd-level infection with a number of porcine pathogens, such as with *Salmonella enterica* serovars, and consequently the aim of the current study was to develop a novel ELISA system for detecting herds colonized with *B. hyodysenteriae* that could be used in this context. It was decided to investigate recombinant surface proteins of *B. hyodysenteriae* as the ELISA antigen, as amongst these there could be ones that are both specific for this species and recognized by the immune system during natural infection. Previously recombinant Bhlp29.7 has been shown to be useful for this purpose (La et al., 2009), although it was limited by potential cross-reactivity with strains of the non-pathogenic *Brachyspira innocens* (La et al., 2005), whilst only 58% of German strains of *B. hyodysenteriae* that were tested were found to have the *Bhlp29.7* gene (Barth et al., 2012).

Using our previous reverse vaccinology approach (Song et al., 2009), a set of predicted putative outer-membrane associated proteins was selected from in silico analysis. Molecules encoded by 20 genes that were widely distributed amongst *B. hyodysenteriae* strains were subsequently purified as recombinant proteins and screened for immunogenicity and specificity. The ELISA system was optimized through checkerboard titrations. Specific IgG levels were higher than IgM levels in pigs at slaughter age, and moreover the S/N ratio could be increased by detecting the IgG2 isotype response. This potentially should improve the

specificity of the test. The predominance of the IgG2 isotype may indicate that host immune response is directed into the Th1 pathway following exposure to the spirochaete, which is in agreement with proposed hypothesis that over-expression of the Th1 response is associated with SD development (Hontecillas et al., 2005).

Finally, three molecules with the highest signal to noise ratios were tested in the IgG2 isotype ELISA with sera from herds known to have had *B. hyodysenteriae* infection, or considered by their consulting veterinarians not to be infected. The H114 antigen was the only one to give complete specificity whilst retaining high sensitivity.

Specificity was considered to be more important than sensitivity for this test due to potentially commercial ramifications if an uninfected herd was identified as infected. Neither of the other two antigens gave 100% specificity, and their sensitivity also was inferior to that achieved with H114. The fact that the 100% specificity could only be obtained by adding five standard deviations from the mean was concerning, but reflected the fact that some sera in individual herds had antibody levels that were well above the mean (see herds N2, N3, N10, N12, N16 and N18; Fig. 3). This finding raises some concerns about the true status of some of these herds that were reported as being uninfected. The authors relied on the clinical experience of the submitting veterinarians and their knowledge of the disease history of the herds for which they were responsible. These herds had regular health checks, including abattoir surveillance, and faeces from grower pigs with diarrhoea were routinely checked for *B. hyodysenteriae.* Consequently it is highly unlikely that typical SD was missed in these herds. On the other hand, pigs in some herds may carry *B. hyodysenteriae* strains that have reduced colonization capacity and hence virulence potential, so that few if any clinical signs are observed (La et al., 2015; Hampson et al., 2015). Culture and/or PCR on faeces collected from apparently healthy pigs is unlikely to pick up such strains, due to the limits of detection of

these tests and relatively low numbers of spirochaetes excreted in the absence of diarrhoea. If any of the herds having some pigs with elevated antibody levels but submitted as 'uninfected' actually were sub-clinically infected, then a lower cut off value could be calculated. Doing this would have the added advantage that infected herd P12, which was negative using five standard deviations above the mean of the 'uninfected' herds, would be likely to be detected as being positive. This would increase the sensitivity of the test to 100%.

The status of the infected herds is more certain than the status of the herds reported to be uninfected. The IgG2 ELISA with H114 only failed to detect 1/12 infected herds (P12). In this case it is possible that this negative result arose from the limitations of the sampling regimen. In a previous serological survey using an LPS-based ELISA, the within-herd prevalence of *B. hyodysenteriae* infection was estimated at 10% (Mhoma et al., 1992). Thus it was suggested that a sample size of at least 40 sera should be chosen for herd diagnosis to achieve an appropriate confidence of 95% of detecting an individual infected pig. This level of confidence assumes that the infection is uniformly distributed in the herd, and that the test used is highly sensitive. In our study we aimed to test at least 50 serum samples from each herd to increase the likelihood of detecting animals with elevated specific antibody levels, although this was not always achieved because of practical difficulties. Furthermore the sampled animals were healthy, otherwise they would not have been sent for commercial slaughter, and the test was relying on an immunological memory of a pathological process that may have occurred weeks or months before sampling. As a result of such inherent difficulties it is not surprising that 100% sensitivity was not achieved, especially as the test was configured to achieve optimal sensitivity. Specificity could be improved by testing more individual samples, preferably obtained at a different time so that any pig batch effects were accounted for.

The current optimized ELISA requires additional testing in the field. One complicating factor is the recent discovery in North America and Europe of a new species infecting pigs that is proposed as '*Brachyspira hampsonii*' (Chander et al., 2012). This spirochaete causes a disease that is indistinguishable from SD (Burrough et al., 2012; Rubin et al., 2013). In our study we did not have access to sera from herds infected with this spirochaete, and it remains unsure whether or not the sera would cross-react in the current optimized test. Examination of available genome sequences suggests that strains of *'B. hampsonii'* have a homologue of H114 but these only have around 75% amino acid similarity to H114 in *B. hyodysenteriae.*

Conclusions

This study represented a first systemic investigation of recombinant antigens for use in a serological assay to detect evidence of infection with *B. hyodysenteriae.* The assay that was developed has substantial potential as a test for detecting infected herds.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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389 **Figure legends**

390

391 **Figure 1.** Western immunoblot showing reactivities of nine candidate antigens with a pool of
392 sera from pigs that were naturally infected with *B. hyodysenteriae* (panel A) and with the same
393 sera that had been absorbed with cells of *B. pilosicoli, B. innocens* and *B. murdochii* (panel B).

394

395 **Figure 2.** Comparison of serum IgG, IgG1 and IgG2 against recombinant antigens H7 and
396 H114 in ELISA. The groups of sera include a negative group from uninfected pigs (*n* =20), a
397 positive group from pigs infected with *B. hyodysenteriae* (*n*=10), a group of sera from pigs
398 infected with *B. pilosicoli* (*n*=5), and a group of pigs colonized with *B. innocens/B. murdochii*
399 (*n*=20). S/N, mean antibody level of positive group/mean antibody level of negative group.

400

401 **Figure 3.** Distribution of individual serum IgG2 levels in pigs from 18 herds considered
402 uninfected with *B. hyodysenteriae* and 12 herds with a history of SD. The horizontal line
403 shows the threshold calibrated optical density, calculated as five standard deviations above the
404 mean of 896 negative sera from finisher pigs (cut-off: 0.538). N1-N18: individual negative
405 herd; P1-P12: individual positive herds. Results for herd P12 were below the cut-off value for
406 herd positivity.

407

408

409

410

411 **Table 1**

412 Names, origins and serogroup of 23 *B. hyodysenteriae* strains used in the study.

Working name	Strain name	Origin [1]	Serogroup

HD1	D90.8506	USA	A
HD3	N91.1589.2	NSW	Not determined
HD4	V3821	VIC	F
HD6	D94.00498	USA	Not determined
HD7	Q4662.4	QLD	D
HD8	V2809/87	VIC	H
HD9	155.9	WA	B
HD10	ACK 300/8	USA	B
HD11	N5503/92	NSW	Not determined
HD12	B8044	USA	B
HD13	Q9374	QLD	D
HD14	155.18	WA	B
HD26	N884	NSW	B
HD36	FM88-90	Canada	J
HD37	B234	USA	B
HD44	3391.90B	VIC	B
HD46	155.11	WA	E
HD49	155.8	WA	E
HD51	FMV89-3323	Canada	K
HD52	SA2206	SA	A
HD54	SA43.3	SA	D
HD55	Q95.0715.1	QLD	B/I
HD80	B204	USA	B

[1] Australian States: NSW, New South Wales; VIC, Victoria; QLD, Queensland; WA, Western Australia

416

417

418 **Table 2**

419 Identity of twenty candidate genes encoding potential diagnostic antigens selected from bioinformatics analysis.

Gene name	Gene size (bp)	Protein size (aa)	Predicted molecular weight (Da)	Apparent molecular weight of recombinant protein (kDa)	Predicted identity
H77	702	234	27,291	26.6	Hypothetical protein
H114	996	332	39,199	55.4	Unknown protein
H116	810	270	29,654	32.1	Bifunctional autolysin precursor
H122	639	213	24,082	26.8	Plasma membrane protein
H147	1239	413	45,175	57.9	Unknown protein
H173	639	213	23,789	26.8	Hypothetical protein
H1	1008	336	37.429	41.4	Hypothetical protein
H22	1308	436	47,014	50.7	Unknown protein
H24	729	243	28,009	31.8	OmpA/MotB domain protein
H3	783	261	29,535	34	Extracellular solute-binding protein
H30	969	323	35,982	39.4	Surface antigen BspA

H32	564	188	22,505	26.9	Haemolytic protein HlpA
H41	1068	356	39,872	43.8	Unknown protein
H62	1002	334	37,227	41.1	Unknown protein
H64	1011	337	36,469	40.2	ABC-type nitrate/sulfonate/bicarbonate transport
H7	1038	346	37,360	41.4	Solute-binding protein of ABC transport
H8	1452	484	54,500	60.0	Outer membrane efflux protein
H17	2940	980	110,50	115.05	Pre-protein translocase SecA subunit
H34	1149	383	43,060	47.06	Putative lipoprotein
H42	708	236	27,050	31.05	Flagellar filament outer layer protein

Table 3

Comparison of IgG2 indirect ELISA performance using sera from 18 uninfected herds and 12 infected herds with the final three candidate antigens H122, H114 and H7

	Recombinant proteins					
Cut-off	H122		H114		H7	
	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity
Mean + 3 SD	7/18	12/12	10/18	12/12	8/18	11/12
Mean + 4 SD	11/18	11/12	13/18	11/12	13/18	9/12
Mean + 5 SD	16/18	10/12	18/18	11/12	16/18	9/12

SD, standard deviation.