Brachyspira hyodysenteriae isolated from apparently healthy pig herds following an evaluation of a prototype commercial serological ELISA

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Abstract

Swine dysentery (SD) is a disease mainly of grower/finisher pigs characterised by severe mucohaemorragic colitis. The classical aetiological agent is the anaerobic intestinal spirochaete *Brachyspira hyodysenteriae*, although "*Brachyspira hampsonii*" and *Brachyspira suanatina* also cause SD. This study reports on the unexpected isolation of *B. hyodysenteriae* from pigs in apparently healthy herds that gave positive reactions when tested with a prototype commercial serological ELISA for detecting herds infected with *B. hyodysenteriae* (Priocheck® Brachyspira porcine Ab ELISA). The ELISA was tested with sera collected at abattoirs from 1,770 slaughtered pigs from 30 Australian herds, including 12 with a history of SD and18 that were considered by their consulting veterinarians to be healthy. The latter herds had no history of SD and did not routinely use antimicrobials that may have masked the disease. Based on the recommended ELISA cut-off value, 25 herds were recorded as showing evidence of infection, including 11 of 12 herds that were considered infected by the submitters and 14 of the 18 "healthy" herds. When faecal or colonic wall samples from 11 of the 14 "false positive" herds subsequently were culturing 6-24 months after the original ELISA testing was completed, different strains of *B. hyodysenteriae* were isolated from six herds, including a high-health status breeding herd. The existence of apparently healthy herds that are colonised by *B. hyodysenteriae* has major implications for the control of SD. Had the ELISA not been trialled it is unlikely that colonic samples from these herds would have been cultured and the colonisation identified.

Keywords: Swine dysentery; *Brachyspira hyodysenteriae*; diagnosis; ELISA; MLST

1. Introduction

Swine dysentery (SD) is endemic in many regions of the world. The disease typically presents as a severe mucohaemorrhagic colitis mainly in grower and finisher pigs, although clinical signs may be ameliorated or prevented by the use of antimicrobial agents (Hampson 2012). The classical agent of SD is the strongly haemolytic anaerobic intestinal spirochaete *Brachyspira hyodysenteriae*, but latterly the related strongly haemolytic species *Brachyspira suanatina* and "*Brachyspira hampsonii"* also have been shown to be able to cause SD (Råsbäck et al. 2007; Burrough et al. 2012; Chander et al. 2012; Rubin et al. 2013; Mushtaq et al. 2015).

Diagnosis of SD largely rests on observation of clinical signs and pathology, together with demonstration and identification of the aetiological spirochaetes in the colon or faeces. *Brachyspira* spp. grow slowly on specialised selective agar plates under anaerobic conditions, and their subsequent identification traditionally has been difficult and time consuming. The application of PCR technology to identify spirochaetes growing on plates or on DNA extracted from faecal or colonic samples represented an important diagnostic advance (Atyeo et al. 1998; La et al. 2003). Although culture and PCR can detect spirochaetes in the faeces of pigs that have diarrhoea and are excreting them in large numbers, they may not detect low numbers in healthy carrier animals (Fellström et al. 2001). Consequently these methods are not well suited for routine screening of apparently healthy herds to detect animals with low levels of colonisation, mainly because very large numbers of samples need to be tested and this is expensive and time consuming.

Enzyme-linked immunosorbent assays (ELISAs) have the potential to be used for quantifying serum antibodies to pathogens, and hence providing indirect evidence of past exposure to the pathogen. As a consequence they are very useful in routine screening of apparently healthy

animals at the abattoir to assess the likely status of the herd of origin, particularly where the herd may have sub-clinical infection. ELISAs using either sonicated whole cells of *B. hyodysenteriae* or extracted lipooligosaccharide (LOS) as plate-coating antigens have been reported to be sensitive enough to allow detection of SD at a herd level, if sufficient numbers of pigs are tested (Joens et al. 1982; Wright et al. 1989; Smith et al. 1991; Mhoma et al. 1992; Song et al. 2012). Unfortunately as these antigens potentially could be shared by other *Brachyspira* species and generate false-positive cross-reactions this has hampered their further commercial development (La and Hampson 2001). To overcome cross-reactivity, recombinant specific conserved surface proteins of *B. hyodysenteriae* have been trialled as ELISA antigens. One such antigen, Bhlp29.7 (formerly BmpB) has been used in a serological ELISA to identify infected herds (La et al. 2009a). Unfortunately the test also has potential for cross-reactivity with strains of the non-pathogenic *Brachyspira innocens* (La et al. 2005), and more importantly the *Bhlp29.7* gene was found to be absent in a significant number of *B. hyodysenteriae* strains (Barth et al. 2012). More recently an immunogenic surface protein named H114 was shown to be widespread amongst *B. hyodysenteriae* strains and to have potential to be used as an ELISA antigen to detect infected herds (Song et al. 2015). In the current study a prototype test using H114 developed by Prionics AG (Priocheck® Brachyspira porcine Ab ELISA) was tested on sera from pigs in 30 herds with or without a history of having SD. Fourteen of these herds gave apparently false positive results, but subsequent investigation by culture and PCR revealed that at least six were colonised with strains of *B. hyodysenteriae.*

2. Materials and Methods

2.1. Permissions

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

2.2. Herds and sample collection

Serum samples originating from 30 pig herds were collected by members of the Australian Pig Veterinarians (APV), a special interest group of the Australian Veterinary Association. The herds all had >500 sows, and were located in the States of Western Australia (n=10), Victoria (n=9), New South Wales (n=8) and South Australia (n=3). The samples were from 12 herds where clinical SD had been recorded and where it was considered still to be present, even though controlled, and 18 where the consulting specialist veterinarian considered that the infection did not occur. Disease status was assessed based on clinical history, lack of use of routine medication in healthy herds, regular abattoir monitoring for disease signs and diagnostic testing of faecal samples from pigs with diarrhoea, including PCR for *Brachyspira* species. One of these herds was a breeding herd that was considered to be of very high health status. In most cases a minimum of 40 blood samples per herd was collected as a batch from healthy slaughter-aged pigs during exsanguination at the abattoir. Additional batches of sera were collected from seven herds where less than 40 samples were obtained at the initial sampling, or where consulting veterinarians questioned the initial results and sought additional testing. The blood samples were allowed to stand overnight at 4^{o}C and the serum was pipetted off and tested within 48 hours.

2.3. ELISA testing

The testing was conducted blind to the origin of the samples. Serum samples were tested in a prototype commercial serological ELISA (Priocheck® Brachyspira porcine Ab ELISA; Prionics AG), as optimised by the manufacturer and following their detailed instructions. Recombinant antigen H114 was pre-coated on the plates by the manufacturer. Single strong positive, weak positive and negative control sera were supplied and included in each test run. Serum samples were diluted 1:20, added to wells on the plate, incubated at room temperature for 60 minutes, and after washing as recommended were reacted with a peroxidase labelled anti-pig antibody

in a direct ELISA. Colour development using TMB substrate was measured optically at a wavelength of 450nm. Percentage Positivity was calculated as the OD_{450nm} of the sample minus the OD_{450nm} of the negative control, divided by the OD_{450nm} of the positive control minus the OD_{450nm} of the negative control, multiplied by 100. Test samples with values equal to or above the cut-off of 30 Percent Positivity were considered positive in the test. Herds with one or more positive serum sample were considered to show evidence of infection with *B. hyodysenteriae.*

2.4. Culture and PCR

For 22 of the herds, limited numbers of colon samples from pigs at the abattoir or faecal samples on farm subsequently were obtained for culture and PCR for *B. hyodysenteriae* (Table 1). Sampling occurred 6 – 24 months after the original ELISA testing, and was not possible in all cases as some producers did not want to be involved, or there had been changes in management, and one had closed down. The preferred samples were colonic mucosa obtained at the abattoir following slaughter, where minor thickening or irregularities in the otherwise apparently normal colonic wall were identified by external palpation. Between 1 and 20 colonic samples were obtained from 13 of the 22 herds. Faecal samples (between 1 and 50) were obtained from fattening pigs in 16 of the herds, including seven from herds where colon samples also were submitted. Loose faeces were requested, but in a number of cases the faeces received were of normal consistency and were considered unlikely to contain detectable levels of spirochaetes even if the animal was colonised.

Table 1 about here

Samples were plated onto selective Trypticase Soy Agar (BBL) plates containing 5% (vol/vol) defibrinated ovine blood, 400 μg of spectinomycin per ml, and 25 μg each of colistin and

147 vancomycin (Sigma–Aldrich) per ml (Jenkinson and Wingar 1981). The plates were incubated
148 for 5–7 days at 37 °C in an anaerobic environment of 94% H_2 and 6% CO_2 generated with
149 anaerobic Gaspak plus sachets (BBL). The plates were examined for the presence of a low, flat,
150 spreading growth and associated haemolysis. Surface growth was re-suspending in phosphate-
151 buffered saline and examined under a phase-contrast microscope. The harvested growth on
152 plates suspected to have spirochaete growth were subjected to a PCR reaction for *B.*
153 *hyodysenteriae*, as previously described (La et al. 2003).
154
155 *2.5. Multilocus sequence typing (MLST)*
156 Six *B. hyodysenteriae* isolates obtained in pure culture were analysed by multilocus sequence
157 typing (MLST), as previous described (La et al. 2009b). Two isolates came from herds where
158 the veterinarian considered that SD was present and four came from apparently healthy herds.
159
160 **3. Results**
161 *3.1. Culture and PCR*
162 *B. hyodysenteriae* was isolated from faecal or colonic samples from nine of the 22 sampled
163 herds, including three reported as being infected and six which were considered to be
164 uninfected by their veterinarians (Table 1). Between one and 13 *B. hyodysenteriae* isolates
165 were recovered from samples from these six herds, with their identity confirmed by PCR. All
166 isolates were strongly beta-haemolytic.
167
168 *3.2. MLST*
169 The six isolates examined by MLST were confirmed as being *B. hyodysenteriae* on the basis of
170 their clustering with other strains of the species, although they belonged to six different new
171 sequence types (STs): ST140, ST144, ST149, ST158, ST160 and ST161. These STs have been
172 submitted to the PubMLST database (http://pubmlst.org/brachyspira/).

173

174 *3.3. ELISA*

175 A total of 1,770 serum samples were obtained from the 30 herds (Table 1). Multiple sets of
176 samples were submitted from seven herds, with a total of 42 batches of sera received from the
177 30 herds. In the case of the 12 herds that were reported as being infected by the submitting
178 veterinarians, 11 were positive by ELISA (91.7%). The herd that was negative by ELISA (herd
179 12) was only sampled once. Herd 10 was negative with the first batch of sera test, but was
180 positive with two other batches of sera. Faecal or colonic samples were collected from pigs
181 from seven of the 12 herds considered to be infected, and single isolates of *B. hyodysenteriae*
182 were detected by culture in three (including herd 12 that was negative by ELISA).

183

184 Of the other 18 herds that were classified as not being infected by the veterinarians, 14 (herds
185 13 through 26) were positive by ELISA. Three of these herds had more than one batch of sera
186 tested, and batches from two herds also were positive in the subsequent tests (herds 15 and 24).
187 Faecal or colonic samples were obtained from 11 of these 14 herds, and *B. hyodysenteriae* was
188 isolated from samples from six of these (54.5%). The final four herds (herds 27-30) were
189 considered negative by their veterinarians and also gave a negative ELISA results. Faeces or
190 colon samples cultured from three of these four herds were negative. Confirmatory samples
191 were not made available for the fourth herd.

192

193 **4. Discussion**

194 Use of the prototype ELISA was able to correctly identify 11 of 12 herds that had a history of
195 SD. Herd 10 was only correctly identified as infected when a second batch of 40 sera was
196 examined, while herd 12 that was incorrectly recorded as uninfected by ELISA only had 40
197 samples collected at a single time point. In this case the negative result may have arisen from
198 the limitations of the sampling regimen. With a perfect test, and assuming an infected herd has

199 a 10% within-herd prevalence of infection, a sample size of 40 sera should achieve 95%
200 confidence of detecting an individual infected pig, provided that the infection is uniformly
201 distributed in the herd (Mhoma et al. 1992; Song et al. 2015). If additional samples had been
202 tested from this herd, and preferable samples from another batch of pigs, the chances of
203 identifying the herd as infected would have been improved (as it was for herd 10). Indeed, for
204 routine surveillance it is recommended that the ELISA should be used regularly on different
205 batches of serum samples: this regimen would both help detect new infections in disease-free
206 herds, and could be used to monitor changes in the numbers of infected pigs in herds that are
207 known to be colonised.
208
209 Of the 18 herds reported to be non-infected, only four were ELISA negative. Samples from
210 three of the latter were tested by culture and PCR and the negative results helped to confirm
211 that they were likely to be true negative herds. On the basis of the overall findings it appeared
212 that the ELISA had quite a poor specificity as a herd test. Of the other 14 “false positive” herds,
213 however, six that initially were considered to be non-infected by the consulting veterinarians
214 subsequently were shown to be colonised when *B. hyodysenteriae* was isolated from animals
215 from the herds. Sampling occurred 6-24 months after ELISA testing, and it is possible that they
216 were newly infected during this intervening period, but against this possibility was the fact that
217 their clinical status of the herds did not change over this period and they remained healthy. As
218 a result of this finding the apparent specificity of the test improved, and indeed it might have
219 been even greater if more samples from others of the eight “false positive” herds were
220 examined by culture and PCR. This was emphasised by the fact that *B. hyodysenteriae* was
221 only isolated from samples from a minority of the 12 herds that were known to be infected. To
222 obtain a better understanding of the true specificity of the ELISA it would be necessary to
223 examine samples from many more herds that are confirmed to be uninfected based on prior
224 extensive screening of large number of colonic samples by culture and PCR. Serological cross-

225 reactivities resulting from infection with other *Brachyspira* species also remain a possible way
226 that false positive results could be generated. In this case the gene for H114 is present in some
227 strains of "*B. hampsonii*", *B. intermedia*, *B. murdochii* and *B. pilosicoli*, although at the
228 translated amino acid level the similarity to H114 in *B. hyodysenteriae* is only 70-78%,
229 meaning that serological cross-reactivity is unlikely (unpublished data). Such potential cross-
230 reativities also were screened for in the original selection of the ELISA antigen (Song et al.
231 2015).
232
233 The most important finding in this study was the demonstration that *B. hyodysenteriae* is
234 present in a number of Australian pig herds that are not showing signs of disease, including in
235 a high health status breeding herd that does not routinely use antimicrobials. There had been no
236 reason to suspect that these herds were colonised, and they would not have been investigated
237 for SD had they not submitted serum samples as being from "negative control SD-free herds".
238 It is apparent that routine herd screening based simply on observation of clinical signs,
239 checking production records and submitting faecal samples from healthy pigs to look for *B.*
240 *hyodysenteriae* by culture and/or PCR may fail to identify colonisation in apparently healthy
241 herds. A more reliable means of routine screening of healthy herds can be achieved at the
242 abattoir by culturing any thickened areas of the colonic wall in pigs after slaughter.
243
244 Although the sampled herds were not randomly selected, *B. hyodysenteriae* was detected by
245 culture in six of the 18 healthy herds. Although this is a small sample size, it does give a
246 remarkably high 33% prevalence of infection amongst apparently healthy Australian herds.
247 Interestingly, this is the same as in a much earlier study where 35 of 106 randomly selected
248 Western Australian herds were considered to be infected based on testing blood collected at
249 abattoirs in a serological ELISA using extracted *B. hyodysenteriae* LOS as the plate coating
250 antigen (Mhoma et al. 1992). It appears that colonisation of herds may be more common than

is generally appreciated, and that colonisation does not necessarily result in typical disease at the herd level.

A similar situation to that described here was reported in an Australian study published in 1992, where an isolate of *B. hyodysenteriae* was recovered from a pig in a high-health status herd with minimal antimicrobial usage and no disease (Hampson et al. 1992). Significantly, this isolate caused typical SD when it was used to experimentally challenge pigs in a research facility. The reason for lack of disease expression in the herd of origin was unclear, but it is known that dietary ingredients may influence the occurrence and severity of SD by creating conditions within the large intestine that influence colonisation by the spirochaete (Pluske et al. 1996; Siba et al. 1996; Thomsen et al. 2007; Hansen et al. 2010, 2011). In this scenario, should isolates from healthy herds be transmitted to other herds where different conditions exist then disease eventually may develop (Hampson et al. 2015).

A question remains as to the pathogenic potential of the isolates in the current study that were recovered from herds where signs of disease were not observed. Six different sequence types of *B. hyodysenteriae* were detected for six isolates, including four STs for the four isolates tested from herds without disease. This demonstrates that the absence of disease in these colonised herds was not associated with one shared strain of low virulence. Isolates of *B. hyodysenteriae* with low or reduced virulence have been described previously in Europe and North America (Lyson et al. 1982; Jensen and Stanton 1993; Achacha et al. 1996; Thomson et al. 2003), and it will be important to test the current isolates in a standardised experimental infection model to evaluate their potential to colonise and cause disease. If they prove to have reduced capacity to cause disease then the molecular basis of this will need to be investigated further (La et al. 2011; La et al. 2014). Interestingly when the prototype test kit was used in Europe and the USA it was again found to be sensitive but apparently lacking in specificity, but in this case the

277 "false positive" herds were not investigated further (unpublished data). It seems likely that at
278 least some of these herds also were colonised. Use of the ELISA could help alert veterinarians
279 to the possibility that a healthy herd is colonised, so that it can undergo further investigation,
280 and as such the test should be a useful addition to the available diagnostic repertoire.
281
282 **Conflict of interest**
283 The Prionics test kits were produced under licence from Murdoch University and the
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285 interest with the research and/or publication of this study.
286
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295
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406 **Table 1**

407 Comparison of reported health status of the 30 herds, and results in ELISA and by culture of

408 faeces and/or colonic contents where available

Herd	Reported herd SD status	ELISA results[a]	Samples subsequently tested by culture and PCR[b]	Number of positive samples
1	Positive	2/45 positive	NT	NT
2	Positive	5/40 positive	NT	NT
3	Positive	5/60 positive	10 faeces, 2 colon samples	Negative
4	Positive	1/39 positive	2 colon samples	Negative
5	Positive	8/62 positive	NT	NT
6	Positive	1/40 positive 60/60 negative (2^{nd} test)	NT	NT
7	Positive	5/40 positive	1 colon sample	Negative
8	Positive	3/39 positive	12 faeces samples	Negative
9	Positive	5/20 positive 45/45 negative (2^{nd} test)	4 faeces, 2 colon samples	Positive (1)
10	Positive	40/40 negative 2/40 positive (2^{nd} test) 1/60 positive (3^{rd} test)	10 faeces samples	Positive (1)
11	Positive	25/25 negative 2/25 positive (2^{nd} test)	1 faeces sample	Negative
12	Positive	40/40 negative	6 faeces, 7 colon samples	Positive (1)
13	Negative	1/47 positive	8 faeces samples	Positive (1)
14	Negative	8/40 positive	36 faeces, 4 colon samples	Positive (9)

15	Negative	2/40 positive 3/40 positive (2nd test) 57/57 negative (3rd test) 2/30 positive (4th test)	22 faeces, 17 colon samples	Positive (1)
16	Negative	5/63 positive	50 faeces samples	Positive (13)
17	Negative	6/61 positive	6 faeces samples	Positive (4)
18	Negative	2/40 positive	6 colon samples	Positive (1)
19	Negative	1/17 positive 43/43 negative (2nd test) 56/56 negative (3rd test)	42 faeces, 2 colon samples	Negative
20	Negative	5/57 positive	41 faeces samples	Negative
21	Negative	1/50 positive	20 faeces samples	Negative
22	Negative	1/40 positive	2 colon samples	Negative
23	Negative	3/60 positive	20 colon samples	Negative
24	Negative	5/35 positive 9/35 positive (2nd test) 1/16 positive (3rd test)	NT	NT
25	Negative	2/30 positive	NT	NT
26	Negative	2/60 positive	NT	NT
27	Negative	40/40 negative	2 faeces, 2 colon samples	Negative
28	Negative	40/40 negative	NT	NT
29	Negative	20/20 negative	2 faeces samples	Negative
30	Negative	33/33 negative	11 colon samples	Negative

409 NT, not tested

410 [a]Second, third and fourth tests conducted 2-6 months after the first test

411 [b]Samples collected 6-24 months after serological testing