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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 mucohaemorrhagic 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<sup>®</sup> 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 and 18 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
*B. hyodysenteriae* 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 \textit{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°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
vancomycin (Sigma–Aldrich) per ml (Jenkinson and Wingar 1981). The plates were incubated for 5–7 days at 37 °C in an anaerobic environment of 94% H₂ and 6% CO₂ generated with anaerobic Gaspak plus sachets (BBL). The plates were examined for the presence of a low, flat, spreading growth and associated haemolysis. Surface growth was re-suspending in phosphate-buffered saline and examined under a phase-contrast microscope. The harvested growth on plates suspected to have spirochaete growth were subjected to a PCR reaction for *B. hyodysenteriae*, as previously described (La et al. 2003).

2.5. Multilocus sequence typing (MLST)

Six *B. hyodysenteriae* isolates obtained in pure culture were analysed by multilocus sequence typing (MLST), as previous described (La et al. 2009b). Two isolates came from herds where the veterinarian considered that SD was present and four came from apparently healthy herds.

3. Results

3.1. Culture and PCR

*B. hyodysenteriae* was isolated from faecal or colonic samples from nine of the 22 sampled herds, including three reported as being infected and six which were considered to be uninfected by their veterinarians (Table 1). Between one and 13 *B. hyodysenteriae* isolates were recovered from samples from these six herds, with their identity confirmed by PCR. All isolates were strongly beta-haemolytic.

3.2. MLST

The six isolates examined by MLST were confirmed as being *B. hyodysenteriae* on the basis of their clustering with other strains of the species, although they belonged to six different new sequence types (STs): ST140, ST144, ST149, ST158, ST160 and ST161. These STs have been submitted to the PubMLST database (http://pubmlst.org/brachyspira/).
3.3. ELISA

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

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

4. Discussion

Use of the prototype ELISA was able to correctly identify 11 of 12 herds that had a history of SD. Herd 10 was only correctly identified as infected when a second batch of 40 sera was examined, while herd 12 that was incorrectly recorded as uninfected by ELISA only had 40 samples collected at a single time point. In this case the negative result may have arisen from the limitations of the sampling regimen. With a perfect test, and assuming an infected herd has
a 10% within-herd prevalence of infection, a sample size of 40 sera should achieve 95% confidence of detecting an individual infected pig, provided that the infection is uniformly distributed in the herd (Mhoma et al. 1992; Song et al. 2015). If additional samples had been tested from this herd, and preferable samples from another batch of pigs, the chances of identifying the herd as infected would have been improved (as it was for herd 10). Indeed, for routine surveillance it is recommended that the ELISA should be used regularly on different batches of serum samples: this regimen would both help detect new infections in disease-free herds, and could be used to monitor changes in the numbers of infected pigs in herds that are known to be colonised.

Of the 18 herds reported to be non-infected, only four were ELISA negative. Samples from three of the latter were tested by culture and PCR and the negative results helped to confirm that they were likely to be true negative herds. On the basis of the overall findings it appeared that the ELISA had quite a poor specificity as a herd test. Of the other 14 “false positive” herds, however, six that initially were considered to be non-infected by the consulting veterinarians subsequently were shown to be colonised when *B. hyodysenteriae* was isolated from animals from the herds. Sampling occurred 6-24 months after ELISA testing, and it is possible that they were newly infected during this intervening period, but against this possibility was the fact that their clinical status of the herds did not change over this period and they remained healthy. As a result of this finding the apparent specificity of the test improved, and indeed it might have been even greater if more samples from others of the eight “false positive” herds were examined by culture and PCR. This was emphasised by the fact that *B. hyodysenteriae* was only isolated from samples from a minority of the 12 herds that were known to be infected. To obtain a better understanding of the true specificity of the ELISA it would be necessary to examine samples from many more herds that are confirmed to be uninfected based on prior extensive screening of large number of colonic samples by culture and PCR. Serological cross-
reactivities resulting from infection with other *Brachyspira* species also remain a possible way that false positive results could be generated. In this case the gene for H114 is present in some strains of “*B. hampsonii*, *B. intermedia*, *B. murdochii* and *B. pilosicoli*, although at the translated amino acid level the similarity to H114 in *B. hyodysenteriae* is only 70-78%, meaning that serological cross-reactivity is unlikely (unpublished data). Such potential cross-reativities also were screened for in the original selection of the ELISA antigen (Song et al. 2015).

The most important finding in this study was the demonstration that *B. hyodysenteriae* is present in a number of Australian pig herds that are not showing signs of disease, including in a high health status breeding herd that does not routinely use antimicrobials. There had been no reason to suspect that these herds were colonised, and they would not have been investigated for SD had they not submitted serum samples as being from “negative control SD-free herds”. It is apparent that routine herd screening based simply on observation of clinical signs, checking production records and submitting faecal samples from healthy pigs to look for *B. hyodysenteriae* by culture and/or PCR may fail to identify colonisation in apparently healthy herds. A more reliable means of routine screening of healthy herds can be achieved at the abattoir by culturing any thickened areas of the colonic wall in pigs after slaughter.

Although the sampled herds were not randomly selected, *B. hyodysenteriae* was detected by culture in six of the 18 healthy herds. Although this is a small sample size, it does give a remarkably high 33% prevalence of infection amongst apparently healthy Australian herds. Interestingly, this is the same as in a much earlier study where 35 of 106 randomly selected Western Australian herds were considered to be infected based on testing blood collected at abattoirs in a serological ELISA using extracted *B. hyodysenteriae* LOS as the plate coating 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
“false positive” herds were not investigated further (unpublished data). It seems likely that at least some of these herds also were colonised. Use of the ELISA could help alert veterinarians to the possibility that a healthy herd is colonised, so that it can undergo further investigation, and as such the test should be a useful addition to the available diagnostic repertoire.

Conflict of interest

The Prionics test kits were produced under licence from Murdoch University and the Australian Pork CRC. The authors declare that they have no personal potential conflicts of interest with the research and/or publication of this study.

Acknowledgements

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References


La, T., Phillips, N.D., Thomson, J.R., Hampson, D.J., 2014. Absence of a set of plasmid-


**Table 1**

Comparison of reported health status of the 30 herds, and results in ELISA and by culture of faeces and/or colonic contents where available

<table>
<thead>
<tr>
<th>Herd</th>
<th>Reported herd SD status</th>
<th>ELISA results$^a$</th>
<th>Samples subsequently tested by culture and PCR$^b$</th>
<th>Number of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive</td>
<td>2/45 positive</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
<td>5/40 positive</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
<td>5/60 positive</td>
<td>10 faeces, 2 colon samples</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>Positive</td>
<td>1/39 positive</td>
<td>2 colon samples</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>8/62 positive</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>6</td>
<td>Positive</td>
<td>1/40 positive</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60/60 negative (2$^{nd}$ test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Positive</td>
<td>5/40 positive</td>
<td>1 colon sample</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>Positive</td>
<td>3/39 positive</td>
<td>12 faeces samples</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
<td>Positive</td>
<td>5/20 positive</td>
<td>4 faeces, 2 colon samples</td>
<td>Positive (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45/45 negative (2$^{nd}$ test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Positive</td>
<td>40/40 negative</td>
<td>10 faeces samples</td>
<td>Positive (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2/40 positive (2$^{nd}$ test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1/60 positive (3$^{rd}$ test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Positive</td>
<td>25/25 negative</td>
<td>1 faeces sample</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2/25 positive (2$^{nd}$ test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Positive</td>
<td>40/40 negative</td>
<td>6 faeces, 7 colon samples</td>
<td>Positive (1)</td>
</tr>
<tr>
<td>13</td>
<td>Negative</td>
<td>1/47 positive</td>
<td>8 faeces samples</td>
<td>Positive (1)</td>
</tr>
<tr>
<td>14</td>
<td>Negative</td>
<td>8/40 positive</td>
<td>36 faeces, 4 colon samples</td>
<td>Positive (9)</td>
</tr>
<tr>
<td></td>
<td>Test 1</td>
<td>Test 2</td>
<td>Test 3</td>
<td>Test 4</td>
</tr>
<tr>
<td>---</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>15</td>
<td>Negative</td>
<td>2/40 positive</td>
<td>22 faeces, 17 colon samples</td>
<td>Positive (1)</td>
</tr>
<tr>
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<td>3/40 positive (2nd test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>57/57 negative (3rd test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2/30 positive (4th test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Negative</td>
<td>5/63 positive</td>
<td>50 faeces samples</td>
<td>Positive (13)</td>
</tr>
<tr>
<td>17</td>
<td>Negative</td>
<td>6/61 positive</td>
<td>6 faeces samples</td>
<td>Positive (4)</td>
</tr>
<tr>
<td>18</td>
<td>Negative</td>
<td>2/40 positive</td>
<td>6 colon samples</td>
<td>Positive (1)</td>
</tr>
<tr>
<td>19</td>
<td>Negative</td>
<td>1/17 positive</td>
<td>42 faeces, 2 colon samples</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43/43 negative (2nd test)</td>
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<td></td>
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<tr>
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<td></td>
<td>56/56 negative (3rd test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Negative</td>
<td>5/57 positive</td>
<td>41 faeces samples</td>
<td>Negative</td>
</tr>
<tr>
<td>21</td>
<td>Negative</td>
<td>1/50 positive</td>
<td>20 faeces samples</td>
<td>Negative</td>
</tr>
<tr>
<td>22</td>
<td>Negative</td>
<td>1/40 positive</td>
<td>2 colon samples</td>
<td>Negative</td>
</tr>
<tr>
<td>23</td>
<td>Negative</td>
<td>3/60 positive</td>
<td>20 colon samples</td>
<td>Negative</td>
</tr>
<tr>
<td>24</td>
<td>Negative</td>
<td>5/35 positive</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9/35 positive (2nd test)</td>
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<td>1/16 positive (3rd test)</td>
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<td>Negative</td>
<td>2/30 positive</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>26</td>
<td>Negative</td>
<td>2/60 positive</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>27</td>
<td>Negative</td>
<td>40/40 negative</td>
<td>2 faeces, 2 colon samples</td>
<td>Negative</td>
</tr>
<tr>
<td>28</td>
<td>Negative</td>
<td>40/40 negative</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>29</td>
<td>Negative</td>
<td>20/20 negative</td>
<td>2 faeces samples</td>
<td>Negative</td>
</tr>
<tr>
<td>30</td>
<td>Negative</td>
<td>33/33 negative</td>
<td>11 colon samples</td>
<td>Negative</td>
</tr>
</tbody>
</table>

NT, not tested

aSecond, third and fourth tests conducted 2-6 months after the first test

bSamples collected 6-24 months after serological testing