
Vaccination with an autogenous bacterin fails to prevent colonization by
Brachyspira intermedia in experimentally infected laying chickens

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Abstract

Avian intestinal spirochaetosis (AIS) is a disease complex affecting adult laying and breeding chickens associated with infection by anaerobic intestinal spirochaetes of the genus Brachyspira. Options for control of AIS are limited, as few effective antimicrobial agents are registered for use in laying chickens. One of the two most commonly encountered pathogenic species in AIS is B. intermedia, and the aim of the current study was to determine whether a B. intermedia bacterin vaccine would help control AIS caused by this species. An autogenous bacterin was prepared from B. intermedia strain HB60 and given twice intramuscularly at a 3-week interval to 12 laying chickens housed in individual cages. Twelve non-vaccinated control chickens were placed in adjacent cages in the same room. Two weeks after the second vaccination all the chickens were experimentally challenged with B. intermedia HB60 by crop tube. Subsequently faeces were cultured for spirochaetes every 2–3 days, faecal water content and chicken weight were measured weekly, and egg numbers and weights were recorded daily. Serum was taken prior to both vaccinations, at the time of challenge
and at euthanasia. The chickens were killed 6 weeks post-challenge. The vaccinated chickens showed seroconversion to the vaccine, but antibody levels declined significantly post-infection. In comparison, the non-vaccinated chickens showed seroconversion post-infection. The reason for the reduction in the antibody levels in the vaccinated chickens after infection was not explained. At some point all the chickens excreted spirochaetes in their faeces, and the duration of excretion was not different between vaccinated and non-vaccinated chickens. There were no differences in faecal water content, chicken weights, egg production, or gross and microscopic caecal lesions between vaccinated and non-vaccinated chickens. In conclusion, an autogenous bacterin vaccine did not prevent infection with *B. intermedia* in laying chickens.

**Keywords:** *Brachyspira intermedia*; Vaccine; Chickens; Avian intestinal spirochaetosis

**Introduction**

Avian intestinal spirochaetosis (AIS) is a disease complex associated with colonization of adult chickens or other poultry species by pathogenic intestinal spirochaetes of the genus *Brachyspira* (formerly *Serpulina*) (Swayne and McLaren, 1997, Stephens and Hampson, 2001 and Hampson and Swayne, 2008). AIS most commonly has been reported in flocks of laying hens and broiler breeders where it has been associated with a variety of non-specific clinical signs and production problems. Reported examples of problems include pasty vents, wet litter, faecal staining of eggshells, increased fat content of faeces, delayed onset of egg laying, reduced egg production, increased mortalities, and a reduced feed conversion ratio (Devalaar et al., 1986, Griffiths et al., 1987, Dwars et al., 1989, Swayne et al., 1992, Swayne et al., 1995, Trampel et al., 1994, Hampson and McLaren, 1999, Stephens and Hampson, 2001, Burch et al., 2006 and Bano et al., 2008). Broiler flocks derived from infected breeders may show poor feed conversion ratios, a high number of weak chicks, slow growth, and poor feed digestion (Dwars et al., 1993 and Smith et al., 1998).
Infections with the pathogenic species *B. intermedia*, *B. pilosicoli*, *B. alvinipulli*, and *B. hyodysenteriae* have all been reported in chickens, with *B. intermedia* usually being most consistently and commonly encountered in epidemiological surveys of flocks with AIS (McLaren et al., 1997, Stephens and Hampson, 1999, Stephens et al., 2005, Bano et al., 2008, Feberwee et al., 2008 and Jansson et al., 2008).

Infections with *Brachyspira* species in pigs are routinely controlled using antimicrobial agents, but there are few antimicrobials available that are registered for use in laying chickens, and this makes control of AIS difficult. Implementing good shed hygiene and strict biosecurity measures can be used to try to prevent infection being introduced into flocks (Phillips et al., 2003), but this strategy is not always successful. To date there are no commercial vaccines available to help prevent or control AIS.

The aim of the current work was to undertake a pilot study to determine whether vaccination with an autogenous bacterin could confer protection against AIS caused by *B. intermedia* in laying chickens.

**Materials and methods**

**Permissions**

The experimental work was conducted with the approval of the Murdoch University Animals Ethics Committee.

**Spirochaete growth**

*B. intermedia* strain HB60, originally isolated from a chicken in a Western Australian layer flock with a wet litter problem, was obtained as frozen stock from the culture collection held at the Australian
Reference Centre for Intestine Spirochaetes, School of Veterinary and Biomedical Sciences, Murdoch University. The cells were thawed, and grown at 37 °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). The spirochaetes were harvested in early log-phase, when they were actively motile. Spirochaete growth and absence of contamination were monitored by examining aliquots under a phase contrast microscope.

**Bacterin vaccine**

The spirochaete cells were harvested by centrifugation, washed three times in sterile phosphate buffered saline (PBS), pH 7.2, resuspended in PBS and inactivated by addition of 0.4% formalin followed by stirring overnight at 4 °C. The spirochaetes were counted in a haemocytometer chamber under a phase contrast microscope, adjusted to $10^{10}$ cells/ml, aliquoted and stored at −20 °C. Immediately before administration the bacterin was thawed and sonicated in a commercial water-in-oil adjuvant (Emulsigen; MUP Laboratories Inc., Ralston, USA) to produce an emulsion.

**Serum samples**

The chickens were bled from the marginal wing vein four times during the course of the experiment, commencing immediately prior to the first vaccination (pre-vaccination), immediately prior to the second vaccination (pre-boost), immediately prior to the experimental challenge (pre-infection) and at post-mortem. The blood was held at 4 °C overnight and the serum was removed, divided into aliquots and stored at −20 °C. Immediately prior to use the sera were thawed on ice and briefly vortexed.
ELISA

Whole cells of *B. intermedia* HB60 (10^8 ml^-1 in PBS) were sonicated on ice using three cycles of 30 s with 2 min between cycles. The sonicate was centrifuged at 10,000 × g for 10 min and the supernatant used as plate coating antigen stock. Total protein content was determined using a commercial dye-binding assay kit (Bio-Rad Laboratories, Hercules, CA, USA), then the sonicate was diluted in bicarbonate/carbonate coating buffer (pH 9.6) to a working dilution of 2 μg total protein/ml. 100 μl of the working dilution was added to each well of a 96-well microtitre plate (Becton Dickinson, Lincoln Park, NJ, USA) and the plate was left at 4 °C overnight. The coating solution was poured off and each well was blocked for 1 h at 25 °C with 150 μl of PBS containing 1% (w/v) bovine albumin, fraction V (Sigma, St Louis, MO, USA; Cat. No. A3803). The blocking solution was poured off and the plate was washed three times in PBS–0.05% (v/v) Tween 20 (PBST). A single serum dilution ELISA was used, as dilutions in the range 100–500 give a linear response (T. La and D.J. Hampson, unpublished data). Each serum sample was diluted 1:100 in PBST containing 0.1% (w/v) bovine albumin, fraction V, and 100 μl was added to each well. Plates were incubated at 25 °C for 2 h with gentle shaking, then washed three times with PBST. 100 μl of diluted (1:20,000) goat anti-chicken IgG (whole molecule) horseradish peroxidase conjugate (Sigma–Aldrich, Castle Hill, Australia) was added to each well and the plates incubated at 25 °C for 1 h with gentle shaking. The plates were washed three times with PBST and 100 μl of 1-Step™ Turbo TMB-ELISA (Thermo Scientific, Rockford, IL, USA) substrate solution was added to each well and incubated for 10 min at 25 °C. Colour development was stopped with 100 μl of 1 M H_2SO_4 before reading the absorbance in an ELISA plate reader at 450 nm. Mean absorbance values were plotted for the chickens in each group.

Spirochaete isolation and PCR

Bacteriology swabs were used to collect discrete samples of fresh faeces from the cage floor of each chicken, and also to swab the mucosal surface of the caecal walls and rectum at post-mortem. The swabs were used to inoculate selective agar plates (Jenkinson and Wingar, 1981), consisting of
Trypticase Soy agar (BBL, Cockeysville, USA) containing 5% (v/v) defibrinated sheep blood, 400 μg/ml spectinomycin and 25 μg/ml each of colistin and vancomycin (Sigma–Aldrich). The plates were incubated for 5–7 days at 37 °C in a jar with an anaerobic environment generated using a GasPak Plus™ disposable hydrogen plus carbon dioxide generator envelope with a palladium catalyst (Becton Dickinson Microbiology Systems). The presence of low flat spreading growth of spirochaetes on the plate was recorded. The suspected areas of spirochaete growth were resuspended in PBS, and examined under a phase contrast microscope at 400× magnification. Spirochaetal growth was collected from the surface of the plate using a wooden toothpick, resuspended in ultra-pure water and used as a template for *B. intermedia* PCR amplification, as previously described (Phillips et al., 2005).

**Experimental protocol**

Twenty-four ISA-Brown laying chickens of 20 weeks of age were purchased from a commercial breeder and housed in individual cages with mesh floors located in a room in an environmentally controlled (22–25 °C) facility. The chickens were subjected to 12 h artificial light each day. They were fed *ad libitum* on a commercial mash formulated to meet the nutritional requirements of laying chickens, and water was available to them *ad libitum*. Twelve of the chickens were vaccinated with the bacterin and the other 12 chickens were left unvaccinated. The cages were arranged so that each alternate cage contained a vaccinated chicken.

One week after arrival, at 21 weeks of age, the chickens were vaccinated with 1 ml of the bacterin vaccine by injection into the pectoral muscles. They were vaccinated again 3 weeks later, at 24 weeks of age.

The chickens were weighed on entry to the experiment and at weekly intervals thereafter. Eggs were collected daily and weighed. At weekly intervals aluminum foil was placed under the cage of each
chicken, and after 1-h individual caecal faeces samples were collected. Approximately 2 g of the samples were weighed, and then dried to constant weight in a hot air oven to determine their moisture content.

Faeces were collected from all chickens prior to experimental infection, and then every 2–3 days until the end of the experiment. For each subgroup a total of 180 excreta samples were obtained at 15 sampling times over the 5-week post-infection period.

**Experimental infection**

Experimental infection commenced 2 weeks after the second vaccination, when the chickens were 26 weeks of age, and all were laying eggs. The chickens were orally inoculated via a crop tube with 4 ml of the appropriate actively growing culture, on 4 consecutive days. The broths contained approximately $10^8$ to $10^9$ motile bacterial cells/ml.

**Post-mortem examination**

Six weeks after the experimental challenge (at 32 weeks of age) the chickens were killed by cervical dislocation, and subjected to post-mortem examination. The caecum and rectum were opened to look for evidence of gross changes. A section of one caecum was placed in 10% neutral buffered formalin as a fixative for subsequent histological examination, and swabs were taken from the wall of the other caecum and the rectum for spirochaete culture. These were processed as for faecal samples. The fixed tissue was processed through to paraffin blocks, sectioned at 4 μm and stained with haematoxylin and eosin and with the Warthin–Starry silver stain.
Analysis of data

Comparisons were made between the two groups of chickens using InStat version 3 (Graphpad Software, San Diego, CA, USA). The ratio of the total number of faecal samples collected that were positive and negative for *B. intermedia* were compared between the vaccinated and non-vaccinated groups using Chi-squared tests. Weekly weights, faecal moisture content, egg numbers, and egg weights were compared using one-way analysis of variance. Means were compared using the Tukey–Kramer multiple comparisons test, with significance accepted at the 5% level.

Results

Antibody levels

Vaccinated chickens showed a significant (*P* < 0.05) primary antibody response following the first vaccination (Fig. 1). The mean antibody level increased following the second vaccination, but this increase just failed to reach statistical significance. The antibody level then showed a significant (*P* < 0.01) reduction at post-mortem, with 10 of the 12 vaccinated chickens showing reductions in antibody levels. In comparison, the non-vaccinated chickens only showed a significant (*P* < 0.001) increase in antibody levels following experimental infection.

Colonization

None of the chickens were positive for intestinal spirochaetes prior to experimental infection. Following experimental challenge, all chickens excreted spirochaetes in their faeces at some point (range 1–13 samplings positive). Of the 180 faecal samples that were collected over the post-infection period, 82 (45.6%) were *B. intermedia* positive for the vaccinated chickens and 80 (44.4%) were positive for the unvaccinated chickens. These differences were not statistically significant. At post-
mortem *B. intermedia* was isolated from the caecum and/or rectum of 8 vaccinated chickens and from 11 of the non-vaccinated chickens. Again these differences were not significant.

**Faecal water content**

Within each group the faecal water content of the chickens did not change significantly with time (Table 1), and pair-wise comparisons between the vaccinated and non-vaccinated chickens were not significantly different at any sampling time.

**Chicken weights, egg numbers, and egg weights**

No significant differences were found between the vaccinated and non-vaccinated chickens in weekly body weights, weekly egg weights, or weekly egg numbers (data not shown).

**Post-mortem findings**

At post-mortem chickens from both groups had enlarged ballooning caeca with frothy and mucoid contents. No gross pathological lesions were observed. Histologically all sections showed variable oedema and congestion of the lamina propria, and some sections showed localized areas of separation of the epithelium from the underlying lamina. Bacteria with the appearance of spirochaetes were seen in the crypts and adjacent to the luminal epithelium in all sections, with spirochaete-like forms seen in the lamina propria just under the epithelium in occasional sections. There were no consistent differences between the two groups of chickens in the appearances of the sections or in the number or disposition of the spirochaete-like bacteria that were observed.
Discussion

*B. intermedia* strain HB60 previously has been used to experimentally infect laying chickens (Hampson and McLaren, 1999, Hampson et al., 2002 and Phillips et al., 2004). In the current study this strain colonized the chickens, and the control chickens developed antibodies to whole cell extracts of the *B. intermedia* strain following experimental challenge. Unlike previous studies, the colonization was not associated with any obvious clinical signs such as reduced egg production and increased faecal water content, although there were abnormalities of the caecal contents and microscopic changes in the caeca. Whilst vaccination did result in elevated serum antibody levels to HB60, there was no evidence that this influenced colonization or reduced the mild pathological changes found in the caeca following experimental infection. Hence it appears that bacterin vaccines are unlikely to be particularly useful in preventing AIS caused by *B. intermedia*.

Previously, bacterin vaccines against *B. hyodysenteriae* have been reported to offer variable and generally incomplete protection against swine dysentery in pigs (Fernie et al., 1983, Hampson et al., 1993, Diego et al., 1995 and Waters et al., 1999), whilst a bacterin against *B. pilosicoli* did not prevent colonization with this spirochaete in experimentally infected pigs (Hampson et al., 2000). Hence the failure of the current bacterin to offer protection in laying chickens is not altogether surprising. More recently the use of a swine dysentery vaccine based on a recombinant 29.7 kDa outer membrane lipoprotein (Bhlp29.7) of *B. hyodysenteriae* did provide a degree of protection to experimentally infected pigs (La et al., 2004). Hence it may be worth investigating similar outer membrane proteins and lipoproteins in *B. intermedia* to see if they could be used as the basis for a vaccine against *B. intermedia* in chickens. A mucosal route of delivery might also improve protection against colonization and lesion development in the caeca.
An unexpected observation in the current experiment was the significant reduction in antibody levels in the vaccinated chickens following experimental infection. It appeared that the serum antibody generated by vaccination was removed from the circulation during the period following infection. Furthermore, despite the spirochaete colonizing the large intestine, there was no evidence of this boosting circulating antibody levels in the vaccinated chickens. These findings suggest the possibility that some form of immunological tolerance to *B. intermedia* developed as a result of vaccination. Although the control chickens did not receive the vaccine adjuvant, it seems unlikely that it would have been responsible for these results. Overall the current observations need further investigation, and should be taken into consideration when developing vaccines against *B. intermedia* in chickens.

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Fig. 1. Mean and S.D. of serum ELISA absorbance values against whole cell preparations of *B. intermedia* strain HB60 in vaccinated and non-vaccinated laying chickens at four time points during the experiment.
Table 1. Mean (and S.D.) percent faecal water content in vaccinated and non-vaccinated chickens pre-challenge and in weeks 1–5 post-challenge with *B. intermedia* strain HB60

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<th>Pre-infection</th>
<th>Weeks post-infection</th>
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<td>1</td>
<td>2</td>
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<td>5</td>
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<tr>
<td>Vaccinated</td>
<td>77.3 (9.0)</td>
<td>81.0 (1.8)</td>
<td>80.3 (3.2)</td>
<td>78.0 (7.0)</td>
<td>78.3 (2.3)</td>
<td>78.3 (6.4)</td>
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<td>Non-vaccinated</td>
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<td>76.4 (4.1)</td>
<td>77.1 (7.9)</td>
<td>73.9 (16.9)</td>
<td>77.6 (4.4)</td>
<td>75.4 (6.4)</td>
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