Evaluation of day-old SPF chicks for pathogenicity testing of intestinal spirochete species

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Abstract:
One-day-old SPF chicks have previously been used to demonstrate the pathogenicity of *Serpulina hyodysenteriae* and *Serpulina pilosicoli*. *Serpulina innocens* failed to colonize when inoculated into chicks. Recently several new species of intestinal spirochete have been proposed, *Serpulina intermedia* is considered to be non-pathogenic, although it has been associated with colitis in pigs and poor production in commercial poultry, and *Serpulina murdochii* is a non-pathogenic commensal of pigs and rodents. *Brachyspira aalborgi*, a reportedly non-pathogenic human intestinal spirochete has never been tested in an animal model. The aim of this study was to evaluate the day-old SPF chick model for discriminating between potentially pathogenic and non-pathogenic species of intestinal spirochetes. Both virulent (WA 15) and avirulent (SA3) strains of *S. hyodysenteriae* and a strain of *S. intermedia* (889) colonized chicks and caused reduced weight gain and histologic changes including cecal atrophy, epithelial and goblet cell hyperplasia and crypt elongation. Changes were milder with SA3 and 889. Strains of *S. murdochii* and *B. aalborgi* failed to colonise or cause histologic changes. All four strains of *S. pilosicoli* (Kar, Rosie and GAP 401 isolated from humans and 3295 isolated from a pig) colonized and attached in large numbers by one cell end to the cecal epithelium, resulting in microvillus effacement. Strains of *S. hyodysenteriae*, *S. intermedia* and *S. pilosicoli* have pathogenic potential in chick models. Further work is required to assess the pathogenicity of these spirochetes in their natural host species.

Introduction:
Six distinct species of intestinal spirochetes are now recognized, these being *Serpulina hyodysenteriae* [11, 12], *Serpulina intermedia* [13], *Serpulina innocens* [11, 12], *Serpulina murdochii* [13], *Serpulina pilosicoli* [22] and *Brachyspira aalborgi* [4]. *S. hyodysenteriae*, the agent of swine dysentery, is the only member of the genus which is strongly hemolytic. The remaining five species of intestinal spirochetes are all weakly hemolytic and cannot be distinguished on the basis of their cultural characteristics alone [3]. *S. pilosicoli*, the agent of intestinal spirochetosis (IS), is the only weakly hemolytic species that is clearly recognized as a pathogen [18]. In PIS the spirochetes characteristically are found attached in large numbers to the colonic epithelium by one cell end: an associated mild colitis with crypt abscessation can also occur [17, 20]. Strains of *S. pilosicoli* isolated from pigs, humans, dogs and avian species have been shown to cause IS when inoculated into day-old chicks [2, 10, 15, 21] and newly weaned pigs [20]. In humans, confusion has surrounded the etiology and pathogenic significance of IS. The isolation of *B. aalborgi*, an intestinal spirochete believed to be non-pathogenic, from a single healthy individual added to this confusion [4]. The pathogenicity of the remaining species of weakly hemolytic intestinal spirochete is questionable. *S. intermedia* is thought to be a non-pathogenic commensal of the pig [13], however *S. intermedia* is commonly isolated from poultry, where it has been associated with production problems such as wet litter and dirty eggshells [9]. *S. murdochii* is recognized as a non-pathogenic species colonizing pigs and rats [13, 19].

Day-old chicks have been used in a number of previous studies to test the pathogenicity of intestinal spirochetes, including *S. hyodysenteriae* [1, 14], *S. innocens* [21], strains of *S. pilosicoli* from various hosts [2, 10, 15, 21] and a number of uncharacterized isolates that were associated with wet litter and poor production in commercial poultry [16]. Each of the intestinal spirochetes tested has been shown to colonize the cecum and cause mild to moderate lesions, apart from *S. innocens*, which failed to colonize the chicks. The pathogenicity of *S. hyodysenteriae* in its natural host species is unequivocal, however questions still remain regarding the pathogenicity of *S. pilosicoli* in other host species apart from pigs, and of the newly recognized species within the genus *Serpulina*. Furthermore it has yet to be determined whether *B. aalborgi* can colonize an animal model and attach by one cell end to the intestinal...
Materials and Methods: 

**Bacterial strains.** All strains were revived from frozen low subculture (<10) stocks held at the Australian Reference Laboratory for Intestinal Spirochetes, Murdoch University, Perth Western Australia. All strains apart from *B. aalborgi* were of Australian origin. Two porcine strains of *S. hyodysenteriae* (WA15, and SA3), one porcine *S. intermedia* strain (889), one porcine *S. murdochii* strain (155-20), three human *S. pilosicoli* strains (Kar, Gap 401, Rosie-2299), one porcine *S. pilosicoli* strain (3295), and *B. aalborgi* strain 513AT were used.

Each strain was propagated in Kunkle’s medium [6], at 37°C on a rocking platform until early log phase growth was achieved (48-72 hours). Samples were examined each day for contamination, using a phase contrast microscope.

**Pathogenicity testing in day-old SPF chicks.** The birds were specific pathogen free (SPF) day old CSIRO hybrid white leghorn chicks which were obtained from the Western Australian Animal Resource Centre. Experiment 2 involved the use of 38 birds whilst the remaining experiments each used 28 birds. In each experiment, the chicks were weighed and then divided randomly into two or three groups of ten, and one group of eight uninoculated control birds. The remaining groups of birds were inoculated with a strain of intestinal spirochete. A total inoculum of 10^8 spirochetes in early log phase culture was given by crop tube to each chick over three successive days. Each control group was treated simultaneously with an equivalent volume of sterile culture medium. Cloacal swabs were taken weekly and cultured for the presence of spirochetes. A final weight was obtained 21 days post inoculation (dpi).

**Sample preparation for histology.** At post mortem 21 dpi tissue for histological examination was placed in Bouin's fixative. Sections (3 µm) were cut from paraffin embedded tissue and stained with hematoxylin and eosin. A Warthin-Starry stain also was performed to help visualize spirochetes. Crypt length measurements were made using an Optimas image analysis system (Bothell, Washington). Measurements were taken from the edge of the mucosal layer to the neck of the crypt. For statistical analysis, a total of 15 measurements were made from each transverse section taken from the middle cecum.

**Isolation and identification of spirochetes.** Cloacal swabs taken weekly and cecal swabs taken at post-mortem were directly inoculated onto TSVA plates [5] After streaking out, the plates were incubated at 37°C in anaerobic jars in an atmosphere of 94% N2 and 6% CO2 for 5-21 days and then examined for the presence of spirochetes. The identity of single isolates obtained at post-mortem from an infected chick in each treatment group was confirmed by multilocus enzyme electrophoresis, as previously described [7, 8].

**Statistical analysis.** The one-tailed t-test was used to determine significance of differences between groups at day 1 and again at day 21 of the experiments. The 0.05 level of significance was used for the weight data, and the 0.01 level of significance for the crypt length data. One or two chickens in each of the groups failed to thrive and died during the first week of the experiment. Weight data from these chicks was not included in the analysis.

Results: 

**Uninoculated controls.** Intestinal spirochetes were not isolated from any of the control chicks throughout the 21 day test period. Gross post mortem and histologic examination revealed no abnormalities. (Figure 1).

**Serpulina murdochii and Brachyspira aalborgi infection studies.** Chicks that were challenged with either *S. murdochii* or *B. aalborgi* failed to become colonized during the 21 day test period. No gross post-mortem or histologic abnormalities were present in any of the chicks.

**Serpulina hyodysenteriae infection studies.** Intestinal spirochetes were isolated from seven of ten chicks challenged with WA15 and only three of ten chicks challenged with SA3 at 21 days after inoculation (pi). These spirochetes were
all strongly hemolytic, and matched the MEE profiles of the strains originally used to inoculate the chicks. The mean weight of the chicks inoculated with strain WA15 (93.0±19.4) was significantly less than that of the uninoculated controls (127.4±16.1; t=4.0, p<0.005).

At post-mortem the ceca of the colonized chicks were atrophic, thickened, elastic and occasionally hyperemic. Histologic examination showed evidence of epithelial and goblet cell hyperplasia (Figure 2). The crypts were significantly elongated and were characterized by increased numbers of goblet cells and mitotic cells interspersed amongst cuboidal, immature epithelium within intestinal crypts. Sloughed, necrotic epithelial cells were present in the lumen directly over the villus tips, overlying areas of epithelial cell ulceration. The lamina propria was edematous, and contained a heterophilic infiltrate and numerous areas of hemorrhage. Occasional necrotic cells were present in the subepithelium immediately below the villus tips. Changes were more severe in the WA15 treated group than in the SA3 group, and included a greater degree of hemorrhage, edema, and cellularity of the lamina propria, increased numbers of goblet cells, and increased crypt length. Milder changes also were present in uninfected chicks in the WA15 treatment group, suggesting that they may have become colonized but subsequently recovered. Histologic sections from uninfected chicks in the SA3 treatment group could not be distinguished from the control tissue. Warthin Starry silver staining of heavily infected tissue sections showed large numbers of spirochetes deep within intestinal crypts and occasionally invading the lamina propria between epithelial cells or through the extracellular matrix below superficial mucosal erosions.

**Serpulina intermedia infection studies.** *S. intermedia* strain 889 was only isolated from three of nine chicks challenged with the original strain. Diarrhea and wet litter were observed in the filtered box in the final two weeks of the experiment. Whilst mean weight gain was reduced, the value was not significantly different from that of the controls. At post-mortem the ceca of infected birds were grossly indistinguishable from controls. Histologic examination showed similar although significantly milder changes than those recorded for chicks infected with *S. hyodysenteriae* strains, with a hyperplastic epithelium and increased mitotic rate (Figure 3). The mean crypt length was significantly greater than the mean crypt length of the controls and the birds that did not become colonized. The Warthin Starry silver stain also showed that reduced numbers of spirochetes were present in *S. intermedia* tissue sections when compared to tissue sections from birds infected with *S. hyodysenteriae* strains. The spirochetes were confined to the crypts, but occasionally individual cells were noted within the lamina propria.

**Serpulina pilosicoli infection studies.** By 21 dpi, intestinal spirochetes matching the MEE profiles of the challenge strains had been cultured from most birds in each of the four treatment groups; the exceptions were a single bird challenged with strain 3295, and five of the birds challenged with Rosie-2299. Watery diarrhea and wet litter was recorded in the final seven days of the experiment in each of the treatment groups except for the group infected with Rosie-2299. At post mortem, the ceca of infected birds were indistinguishable from those of the controls, except for several birds infected with strains Kar and 3295, in which the cecal wall appeared thickened.

Histologic examination showed marked differences in lesions for each of the *S. pilosicoli* strains as well as differences between individual chicks infected with the same strain. Only patchy attachment of spirochetes to the epithelial cells of the middle or distal cecum was observed in the four chicks that became colonized with Rosie-2299 (Figure 4). Significant crypt elongation of infected tissues was a notable feature. Warthin Starry staining of sections from birds infected with Rosie-2299 confirmed the presence of spirochetes on the surface of the epithelium, however spirochetes were also common within the intestinal crypts. Invasion of the lamina propria was not apparent.

Lesions were much more severe in chicks infected with GAP 401 and Kar. In six of the chicks infected with GAP 401 and five of the chicks infected with Kar, attachment of spirochetes by one cell end to the epithelium was focal in numerous patches. In the remaining chicks in each group, massive numbers of end-on attached spirochetes were diffusely distributed in all sections taken from the cecum (Figure 5). Focal cap-like elevations were common, as were the presence of necrotic sloughed cells on the luminal surface surrounded by intestinal spirochetes. The lamina propria was mildly edematous, and was sometimes characterized by the presence of lymphangectasia in the subepithelial lymph vessels, and occasional heterophilic infiltration. The most striking lesions were observed in the apical enterocytes immediately underlying the spirochetes. These were characterized by vacuolation, the presence of circular eosinophilic droplets in the apical cytoplasm, and irregular spacing of the nuclei. Significant crypt elongation was observed in chicks inoculated with Kar, but not GAP 401. Warthin Starry showed that spirochetal colonization was limited to the superficial enterocytes for Kar sections, however a mild degree of invasion beyond the mucosa and into the lamina propria was observed in GAP 401 sections.

Attachment of strain 3295 to the cecal epithelium was present in all of the colonized chicks, and ranged from focal to
multifocal. Lesions in the underlying epithelial cells and lamina propria were not as severe: eosinophilic droplets but not vacuolation were observed in the apical cytoplasm of underlying cells. Warthin Starry showed that spirochetal attachment was limited to the superficial epithelium. Significant increases in crypt length were observed in tissue sections from chicks infected with Rosie-2299, Kar, and 3295.

Discussion and Conclusions:
This study has demonstrated major differences in the ability of different spirochete species to colonize and cause disease in day-old SPF chicks. Inability to colonize the chicks was associated with two species (*B. aalborgi* and *S. murdochii*) that have previously been suggested to be non-pathogenic. Both pathogenic and non-pathogenic strains of *S. hyodysenteriae* and a porcine strain of *S. intermedia* caused disease but differed in their ability to colonize the cecum. Variation in the degree of colonization and lesion development observed in chicks infected with *S. pilosicoli* reflects the variety in clinical presentation of IS in different host species including humans. Given that the ability of a strain to colonize the epithelium may be a significant virulence determinant, the day-old chick appears to be a useful animal model for pathogenicity testing of intestinal spirochete species. Additionally, the strains of *S. pilosicoli* that are shown to vary in their ability to colonize, attach and cause disease when inoculated into day-old chicks may provide insights into the virulence determinants of this newly described organism.

References:


20. Trott, D. J., C. R. Huxtable, and D. J. Hampson, Infection and Immunity, 64, 4648-4654, 1996


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Address questions and comments about this abstract to Darren Trott (dtrott@numbat.murdoch.edu.au).
Questions and Answers

Q. Your results point to differences among the human strains of S. pilosicoli in the chick model i.e. the Kar strain associated with the most obvious histological changes and the Rosie-2299, with the least. Were there any detectable differences among the original human hosts of these strains, especially regarding intestinal disease?

A. Strains Kar and Rosie 2299 were both isolated from Aboriginal patients with diarrhea, strain Kar from a child with chronic diarrhea (who also had giardiasis) who was sampled as part of a prevalence study and Rosie 2299 from a 50 year old women with chronic diarrhea of unknown aetiology. Unfortunately the woman left the health clinic she was attending and we weren't able to get any followup samples or further epidemiological information. Because the clinicians could not identify any other pathogens from this woman, a sample was sent to our laboratory and we grew S. pilosicoli in very large numbers. Because no other pathogens were isolated from this woman we were very interested to examine the strain in our pathogenicity model.

Q. Are there chicken strains of S. pilosicoli or the other Serpulina species? Have you examined any chicken strains of S. pilosicoli or of other Serpulina species in your model?

A. S. pilosicoli has been cultured from commercial chicks (Trampel et al, 1994 Avian Dis, 38: 895-898; McLaren et al, 1997 J Clin Micro 35: 412-417), from ducks and rheas in the USA (Trott et al, 1996 Lett App Micro 23: 431-436), and from wild ducks in Australia (unpublished data). Avian Sp strains from ducks have been used to infect day-old chicks (Swayne 1994 Proc Ann Meet AM Col Vet Path 45: 224-238) where they caused patchy attachment. Unpublished results of infection studies in our laboratory by A. McLaren showed that S. pilosicoli isolated from commercial chickens attached in a similar way, but in much larger numbers.

Commercial chickens may be colonised by a number of different genomospecies of intestinal spirochete (See McLaren et al 1997, Trott et al, 1996 above). Infection studies with these other groups of intestinal spirochete are not yet complete, however dutch strain 1380, and Hb60 which reside in the Serpulina intermedia MEE group has been shown to be pathogenic in day-old chicks and young laying hens.