Experimental infection of layer hens with a human isolate of *Brachyspira pilosicoli*

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The anaerobic intestinal spirochaete *Brachyspira pilosicoli* commonly colonizes the large intestine of a number of species, including chickens and human beings. The purpose of the current study was to determine whether an isolate of *B. pilosicoli* recovered from an HIV-infected patient with diarrhoea could infect and cause disease in adult chickens. Over a 4-week period following experimental infection, a group of eight inoculated chickens showed a persistent and significant increase in faecal water content (~6–7%). The faeces of three of the eight birds became culture-positive, and remained so. At post-mortem examination, no specific pathological changes were found, and no spirochaetal attachment to the caecal epithelium was observed. These findings confirm that *B. pilosicoli* strains can infect across species barriers and cause chronic mild diarrhoea in intact adult chickens.

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INTRODUCTION

The anaerobic intestinal spirochaete *Brachyspira pilosicoli* has been isolated from the large intestine or faeces of many animal species, including pigs (Trott *et al.*, 1996b), chickens (Stephens & Hampson, 2001), dogs (Duhamel *et al.*, 1998) and water birds (Oxberry *et al.*, 1998). A pathognomonic feature of the colonization, seen in only some infected individuals, is the presence of a 'false brush border' made up of large numbers of spirochaetes attached by one end to the epithelium of the caecum, colon and/or rectum (Thomson *et al.*, 1997). In pigs, *B. pilosicoli* causes porcine intestinal spirochaetosis, a common and widespread colonic infection of weaner and grower pigs, associated with loose faeces and poor growth rates (Trott *et al.*, 1996b; Thomson *et al.*, 1997). In poultry, infection with *B. pilosicoli* is common in adult layer and broiler breeder flocks (Stephens & Hampson, 1999), and has been associated with delayed and reduced egg production and increased faecal moisture content (Stephens & Hampson, 2002a). *B. pilosicoli* also colonizes humans: in developing countries, including Oman (Barrett, 1990) and Papua New Guinea (Trott *et al.*, 1997a), *B. pilosicoli* has been shown to colonize around 30% of individuals, whilst in developed countries it occurs at around this prevalence in rural Aboriginal people in Australia (Lee & Hampson, 1992), in male homosexuals (Trivett-Moore *et al.*, 1998) and in patients infected with human immunodeficiency virus (HIV) (Käsbohrer *et al.*, 1990). In comparison, the spirochaete is rarely isolated from the faeces of the

general population in developed countries (Tompkins *et al.*, 1986; Lee & Hampson, 1992; Brooke *et al.*, 2001). In humans, the colonization has been linked to a number of symptoms including chronic diarrhoea, rectal bleeding, pseudo-appendicitis and lower abdominal discomfort (Gad *et al.*, 1977; Douglas & Cruciani, 1981; Rodgers *et al.*, 1986; Heine *et al.*, 2001). More severe invasive cases have also been described in both immunocompromised and immunocompetent individuals, with intestinal spirochaetes being observed in enterocytes, goblet cells, macrophages and Schwann cells, and associated in some cases with epithelial ulceration and necrosis, and crypt abscessation (Antonakopoulos *et al.*, 1982; Guccion *et al.*, 1995; Padmanabhan *et al.*, 1996). Intestinal spirochaetes resembling *B. pilosicoli* have been observed invading the colon and liver of a homosexual man with HIV, who was suffering from cholestatic hepatitis (Kostman *et al.*, 1995). Isolates of *B. pilosicoli* have also been recovered from the bloodstream of immunocompromised individuals in Europe and the USA, some of whom have had bloody diarrhoea (Lambert & Goursot, 1982; Fournié-Amazouz *et al.*, 1995; Trott *et al.*, 1997b; Kanavaki *et al.*, 2002).

Evidence for the zoonotic spread of *B. pilosicoli* remains equivocal. There are a few examples where the same or very similar strains of the spirochaete have been found in humans and dogs living in the same environment (Koopman *et al.*, 1993; Rayment *et al.*, 1997; Trott *et al.*, 1998). There is experimental evidence that *B. pilosicoli* can be transmitted between animal species, and human strains of *B. pilosicoli* have been used to experimentally infect pigs (Trott *et al.*, 1996a), mice (Sacco *et al.*, 1997) and day-old chicks (Dwars *et al.*, 1992; Trott *et al.*, 1995; Muniappa *et al.*, 1996). Recently,

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Abbreviation: HIV, human immunodeficiency virus.

we reported the infection of adult layer and breeder chickens with strains of *B. pilosicoli* isolated from chickens (Jamshidi & Hampson, 2002; Stephens & Hampson, 2002a). In the current study, we report the successful colonization of layer hens with a strain of *B. pilosicoli* isolated from an HIV-infected patient with diarrhoea (Mikosza *et al.*, 2001).

METHODS

Ethics. This experiment was conducted with the approval of the Murdoch University Animal Ethics Committee.

Experimental birds. Sixteen ISA Brown laying pullets were purchased from a commercial breeder at 18 weeks of age, and were housed in individual cages with mesh floors located in an environmentally controlled (25 °C) facility. The birds were subjected to 12 h artificial light each day. They fed *ad lib* on a commercial wheat and vegetable-protein diet (Wesfeeds). At 37 weeks of age they were allocated randomly to two groups of eight, with each group housed in their individual cages in separate rooms.

Experimental infection. *B. pilosicoli* human strain HIV3AB2 was obtained as a frozen stock culture from the collection held by the Reference Centre for Intestinal Spirochaetes at Murdoch University. The strain was originally isolated from an Australian HIV-infected patient with histological intestinal spirochaetosis and chronic diarrhoea (Mikosza *et al.*, 2001). The strain was thawed and grown in Kunkle's anaerobic broth medium (Kunkle *et al.*, 1986) at 37 °C on a rocking platform until early exponential-phase growth was achieved, when the spirochaetes were actively motile. Growth and absence of contamination were monitored by examining aliquots taken at daily intervals under a phase-contrast microscope. At 38 weeks of age, eight birds in one group were inoculated orally via a crop tube with 2 ml actively growing culture, on three consecutive days. The broth contained approximately 10^8 bacterial cells ml⁻¹. The eight uninfected control birds in the other room were sham-inoculated with sterile broth.

Experimental monitoring. The chickens were weighed on entry to the experiment at 38 weeks of age and at weekly intervals thereafter. Eggs were collected daily and weighed. At weekly intervals, starting immediately before the experimental infection, aluminium foil was placed under the cage of each bird and individual faecal samples were collected after 1 h. Portions (approx. 1 g) were weighed, then dried to constant weight to determine the faecal moisture content. At weekly intervals, starting immediately before the experimental infection, cloacal swabs were taken from each chicken at the time of weighing. A proportion of the faeces was resuspended in PBS and examined under a phase-contrast microscope. The swabs were inoculated onto Trypticase soy agar (BBL) supplemented with 5 % defibrinated ovine blood, 400 µg spectinomycin ml⁻¹ and 25 µg ml⁻¹ each of colistin and vancomycin (Jenkinson & Wingar, 1981). Plates were incubated in an anaerobic environment (94 % N₂/6 % CO₂) generated by Gaspak Plus sachets (BBL), and growth was examined by phase-contrast microscopy after 5 and 10 days. The presence of spirochaetes was initially identified by the appearance of a zone of weak β-haemolysis surrounding a low flat haze of bacterial growth. Spirochaetal growth was subcultured, and isolated cells were then subjected to a PCR protocol which specifically amplifies a 439 bp segment of the 16S rRNA gene of *B. pilosicoli* (Atyeo *et al.*, 1998; Mikosza *et al.*, 1999, 2001). The PCR products were subjected to electrophoresis in 1.5 % agarose gel, stained by immersion for 30 min in ethidium bromide solution, and viewed over UV light.

Post-mortem examination. Five weeks after infection, the birds were killed by cervical dislocation and subjected to post-mortem examination. The caeca and colon were opened to look for gross changes, a

section of one caecum was placed in Bouin's fixative for subsequent histological examination, and a swab was taken from the wall of the other caecum for spirochaete culture. These were processed as for faecal samples. After 4 h fixation, the caecal tissue was washed three times in 50 % ethanol and transferred to 70 % ethanol. The tissue was processed through to paraffin blocks, sectioned at 4 µm and stained with haematoxylin and eosin.

Analysis. The two-tailed *t*-test was used to determine the significance of differences between the infected and control groups in terms of weekly group bird weights, faecal moisture content and egg production.

RESULTS

Before experimental infection, the faeces of both groups of birds were of similar consistency and moisture content (Table 1). One week following infection, the faeces of the infected birds had become softer and less well-formed, and they remained so throughout the experiment. Each week after infection until the end of the experiment, the faecal moisture content of the infected group of birds was significantly greater than that of the control birds, being on average around 6–7 % wetter (Table 1). Over the experimental period, there were no significant differences in bird weights, weekly egg numbers or egg weights between the two experimental groups.

At post-mortem, the caecal size and contents varied between birds, with some caeca being small and empty, and others being large and full of contents of varying consistency. There were no consistent group effects. The mucosal surface of all the caeca examined were grossly normal, and histological examination failed to show any pathological changes. No end-on attachment of spirochaetes to the luminal surface of the caecal enterocytes was observed in any of the birds.

No spirochaetes were detected in the faeces of any of the birds before experimental infection, or in the uninfected birds at any time. Spirochaetes, which were all subsequently identified as *B. pilosicoli* by PCR amplification, were isolated from the faeces of three of the eight infected birds 1 week after infection, and in the same birds at weekly intervals to the end of the experiment. *B. pilosicoli* was also isolated from the caecal wall of these three birds at post-mortem. Occasional

Table 1. Mean ± standard deviation of faecal moisture content (%) in chickens infected with a human strain of *B. pilosicoli* and in uninfected control birds

Weeks post-infection	Infected	Control	P-value*
0	78.93 ± 7.47	78.87 ± 5.91	0.987
1	83.08 ± 5.32	77.50 ± 4.32	0.037
2	87.34 ± 4.83	81.02 ± 3.72	0.011
3	84.84 ± 4.07	77.74 ± 5.32	0.010
4	84.98 ± 4.78	78.05 ± 7.69	0.001

*Comparisons using a two-tailed *t*-test.

spirochaetes were observed in the faeces of the other experimentally infected birds, but these were not isolated.

DISCUSSION

In this study, a strain of *B. pilosicoli* isolated from an HIV-infected patient with histological intestinal spirochaetosis and chronic diarrhoea, was shown to colonize the caeca of three of eight experimentally inoculated adult chickens. This colonization was persistent, extending over a 4-week period. Prolonged colonization, averaging 4 months, is a feature of natural *B. pilosicoli* infection in humans (Trott *et al.*, 1998). Some colonization probably occurred in the other five infected birds, because spirochaetes were observed in their faeces, and the whole infected group showed an increase in faecal moisture content following experimental infection. The sensitivity of detection of *B. pilosicoli* by faecal culture is not particularly high [$>5 \times 10^4$ cells (g faeces) $^{-1}$] (Atyeo *et al.*, 1998), and it is possible that the other birds would have been recorded as colonized if PCR techniques had been applied to the primary growth on the isolation plates, or even directly to the faeces (Atyeo *et al.*, 1998; Mikosza *et al.*, 2001). The relatively low colonization rate as assessed by culture (38%) is consistent with what has been reported in adult chickens that have been experimentally inoculated with chicken strains of *B. pilosicoli* (Jamshidi & Hampson, 2002; Stephens & Hampson, 2002a). Layer hens can be made considerably more susceptible to colonization with *B. pilosicoli* if they receive 50 p.p.m. zinc bacitracin in their diets (Jamshidi & Hampson, 2002). It is assumed that this antimicrobial agent disrupts the normal caecal microflora, reducing colonization resistance and hence enhancing spirochaetal colonization. In the current experiment, this manipulation was not undertaken as one of the aims of the experiment was to judge the susceptibility to infection of adult birds with intact intestinal microflora. It was previously known that day-old chicks could be colonized with human strains of *B. pilosicoli* (Dwars *et al.*, 1992; Trott *et al.*, 1995; Muniappa *et al.*, 1996), but this colonization may be facilitated by the presence of a poorly developed large intestinal microflora in young birds. Colonization of human beings with *B. pilosicoli* could also potentially be enhanced by disruptions to the large intestinal microflora through oral antimicrobial treatments, or following other enteric infections or dietary changes (Hopwood *et al.*, 2002).

The successful colonization of adult birds with a human isolate of *B. pilosicoli* supports previous experimental findings that this spirochaete can infect across species boundaries. Previously, a human isolate of *B. pilosicoli* has been used to experimentally infect a human being (Oxberry *et al.*, 1998), but no attempts have been made to infect humans with animal isolates of *B. pilosicoli*, to determine whether this spirochaete has zoonotic potential.

Infection with *B. pilosicoli* did not alter egg production or cause weight loss. It is possible that had infection occurred at an earlier age, or if colonization had been more prolonged, such changes may have been seen. No pathological changes

were observed in the caeca of colonized birds, but this is not a unique situation as it has been recorded in chickens infected with a chicken strain of *B. pilosicoli* (Jamshidi & Hampson, 2002; Stephens & Hampson, 2002a), and in mice infected with a human strain of *B. pilosicoli* (Sacco *et al.*, 1997). Furthermore, only one caecal site from three culture-positive birds was examined at post-mortem. Traditionally, diagnosis of intestinal spirochaetosis in humans has been made by histological examination of colorectal biopsies. In the absence of spirochaetal attachment and histological changes, diagnosis is therefore reliant on specialized microbiological examination, and this is only available in some veterinary laboratories. As a consequence of these diagnostic difficulties, infection with *B. pilosicoli* may be much more widespread than has been appreciated.

The group of birds that was experimentally infected developed a chronic change in faecal consistency. The 6–7% mean increase in faecal moisture content resulted in the faeces becoming more sloppy and poorly formed, although watery diarrhoea was not observed. Wet faeces are a major problem to the egg-production industry, resulting in faecal staining of egg shells, problems with mechanical cleaning of sheds, increased odour and attraction of flies (Stephens & Hampson, 2001). The mechanisms involved in producing such diarrhoea are unknown, especially as in this case there was no obvious spirochaetal attachment to the epithelium or disruption of the microvilli. The small but significant changes in faecal water content are consistent with the situation described in pigs and chickens naturally infected with *B. pilosicoli*, as well as in humans with intestinal spirochaetosis (Lee & Hampson, 1992; Hopwood *et al.*, 2002; Stephens & Hampson, 2002b).

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