Evidence for systemic spread of the potentially zoonotic intestinal spirochaete *Brachyspira pilosicoli* in experimentally challenged laying chickens

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*Brachyspira pilosicoli* is a potentially zoonotic anaerobic intestinal spirochaete that is one of several species causing avian intestinal spirochaetosis. The aim of this study was to develop a reproducible model of infection in point-of-lay chickens and compare the virulence of two strains of *B. pilosicoli* in a model using experimentally challenged laying chickens. Seventeen-week-old commercial laying chickens were experimentally challenged by oral gavage with either *B. pilosicoli* strain B2904 or CPSp1, following an oral dose of 10 % sodium bicarbonate to neutralize acidity in the crop. Approximately 80 % of the chickens became colonized and exhibited increased faecal moisture content, reduced weight gain and delayed onset of lay. Tissues sampled at post-mortem examination were analysed to produce a quantitative output on the number of spirochaetes present and hence, the extent of colonization. The liver and spleen were colonized, and novel histopathology was observed in these tissues. The infection model we report here has potential use in studies to improve our understanding of the mechanisms by which *Brachyspira* elicit disease in poultry and in testing novel intervention strategies.

INTRODUCTION

Avian intestinal spirochaetosis (AIS) has been recognized as a disease in poultry since 1985 (Davelaar et al., 1985), and the condition arises from the colonization of the caeca and colon of the birds by anaerobic spirochaetes of the genus *Brachyspira* (Swayne & McLaren, 1997; Stephens & Hampson, 2001). Currently, three species of this genus are considered pathogenic in poultry and have been shown to induce AIS in experimentally challenged chickens: *Brachyspira alvinipulli* (Swayne et al., 1995; Stanton et al., 1998), *Brachyspira intermedia* (Hampson & McLaren, 1999) and *Brachyspira pilosicoli* (Stephens & Hampson, 2002a).

*B. pilosicoli* has a wide host range, also causing intestinal spirochaetosis in pigs (PIS) (Trott et al., 1996) and humans (HIS) (Tsganou & Gebbers, 2010), with a potential for zoonosis (Hampson et al., 2006). *B. pilosicoli* have been reported worldwide in chicken flocks with symptoms of AIS such as delayed onset of lay, reduced egg weights, diarrhoea, faecal staining of eggshells, reduced growth rates, increased feed consumption and non-productive ovaries (Davelaar et al., 1986; Griffiths et al., 1987; Swayne et al., 1992). In previous experimental challenge studies, *B. pilosicoli* CPSp1 was associated with reduced egg production, faecal staining of eggs and diarrhoea. However, results from these studies have been variable (Jamshidi & Hampson, 2002; Stephens & Hampson, 2002a, b). A characteristic of *B. pilosicoli* colonization is its ability to form end-on attachments to the intestinal epithelial surface and invade (Jensen et al., 2000, 2001). In poultry, *B. pilosicoli* organisms can form a dense fringe, penetrating between enterocytes inducing mild inflammatory responses such as crypt hyperplasia and increased numbers of goblet cells (Feveree et al., 2008). The aim of the current work was to develop a reproducible model for AIS and compare...
the virulence of Australian strain CPSp1 with a recent isolate of \textit{B. pilosicoli} from the UK, B2904, in an experimental challenge model in point-of-lay chickens.

**METHODS**

**Experimental chickens.** Thirty ISA Warren laying chickens were purchased from a commercial breeder at approximately 16 weeks of age. Upon arrival (day 1), the chickens were randomly divided into three groups of ten chickens each and housed freely in separate, negative pressure rooms of a biosecure containment facility. The chickens were wing-tagged for identification of each chicken. Chickens were housed with 12 h of artificial light per 24 h. Water and a commercial un-medicated wheat, soya bean and alfalfa-based pelleted feed were provided ad libitum.

**Bacteriology.** \textit{B. pilosicoli} strains CPSp1, isolated from a chicken in Australia and used in infection studies (Stephens & Hampson, 2002b), and B2904, isolated from a chicken with clinical signs of AIS in the UK (Mappley et al., 2011), were cultured from frozen stock cultures. The \textit{Brachyspira} were cultured on fastidious anaerobe blood agar (FABA) and in \textit{Brachyspira} enrichment broth (BEB) (Råsbäck et al., 2005) in an anaerobic cabinet (10% H$_2$ and 10% CO$_2$ in N$_2$) (Don Whitley Scientific). Broth cultures were incubated statically at 37°C until early exponential phase growth (approx. 4 days) was achieved, determined from normalized growth curves produced using the FLUOstar Optima instrument (unpublished data) with enumeration of \textit{B. pilosicoli} by Helber cell counting chamber. Bacterial cells were harvested by centrifugation (2447 g, 10 min) and the pellet was resuspended in 0.1 M PBS to yield $5 \times 10^8$ c.f.u. ml$^{-1}$; this was performed rapidly under aerobic conditions to minimize exposure to oxygen.

**Experimental challenge.** After 6 days acclimatization, each of the ten chickens in the three groups was inoculated by oral gavage on three alternate days (days 6, 8 and 10). The chickens were first dosed with 2 ml 10% sodium bicarbonate solution by oral gavage to neutralize the acidity in the crop, as previously described (Carroll et al., 2004; Randall et al., 2006). After 20 min, all chickens in one group were challenged by oral gavage with 1 ml \textit{B. pilosicoli} B2904 suspension ($5 \times 10^8$ c.f.u. ml$^{-1}$), those in another group with \textit{B. pilosicoli} CPSp1 suspension and those in a negative control group were sham-inoculated with 1 ml sterile 0.1 M PBS.

**Experimental monitoring.** The chickens were weighed and cloacally swabbed upon arrival and throughout the study (on the days indicated in Fig. 1a). Cloacal swabs were plated onto \textit{Brachyspira} selective agar (Råsbäck et al., 2005) and incubated anaeroebically at 37°C for 8 days to determine whether the chickens were positive or negative for colonization by \textit{B. pilosicoli}. The plates were examined at 24 h intervals for the presence of growth. When growth was detected visually, cells were picked from the plate using a sterile loop and subjected to PCR to verify genus (Phillips et al., 2005) and species (Mikosza et al., 2001) and detect \textit{B. pilosicoli} in tissues (see below).

Eggs were collected daily and weighed. Three random pools of representative samples of fresh faeces were collected from the floor of each of the rooms for the duration of the study (on the days indicated in Fig. 1b), and portions (1 g) from each pool were weighed and dried to constant weight to determine faecal moisture content.

**Post-mortem examinations.** At 5 and 18 days post final \textit{Brachyspira} challenge, three chickens per group were euthanized by cervical dislocation and subjected to post-mortem examination. Prior to post-mortem examination all birds were sprayed with 70% ethanol, and then the abdominal and thoracic cavity were aseptically opened. Tissue sections (approx. 1 g) of each of the duodenum, jejunum, ileum, caeca, colon, liver and spleen were aseptically sampled using separate sterile instruments for each tissue, and then placed in 0.1 M PBS for bacterial quantification and 10% neutral buffered formalin for histopathological examination. In the first post-mortem examination, at day 5 after the final dose, the oviduct was removed aseptically and fixed in buffered formalin for histopathological examination, whereas in the post-mortem examination at day 18 after the final dose, sections (approx. 1 g) from the ovary, infundibulum, magnum, isthmus and the uterus/vagina region were sampled for bacteriology and histopathology as described above. For bacteriology, the sections were homogenized in 9 ml 0.1 M PBS, diluted serially ($10^{-6}$ to $10^{-7}$), and 20 µl samples plated onto \textit{Brachyspira} selective agar and incubated anaerobically for 3–5 days at 37°C. This process was performed swiftly to minimize exposure to oxygen. \textit{B. pilosicoli} colonies were subsequently counted to provide a quantitative output on colonization by \textit{B. pilosicoli} and identity was confirmed by PCR as described above. Similarly, PCR tests were performed on the tissue homogenates to confirm the presence/absence of \textit{Brachyspira} in tissues that were negative by culture. For histopathology, after a minimum of 24 h fixation, trimmed tissues were processed in paraffin wax and 4 µm sections were stained with haematoxylin and eosin. Post-mortem examinations were performed aseptically and deep tissue samples were taken where possible to exclude the possibility of surface contamination. All animal studies were approved by the Animal Health and Veterinary Laboratories Agency ethics committee and performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

**Statistical analysis.** All results were presented as the mean and ±s of the means. A one-way analysis of variance (ANOVA) was performed with commercially available software (GraphPad Prism), using the Bonferroni test with a 95% confidence interval.

**RESULTS AND DISCUSSION**

All chickens were free from \textit{Brachyspira} prior to challenge as tested by culture and PCR. However, following challenge, cloacal swabs from 80.0 to 85.7% of chickens were positive for \textit{B. pilosicoli} at one or more samplings by culture with species confirmation by PCR. Weight gain was reduced in the challenged groups with the final mean weight significantly lower than the negative control in both the B2904- ($P<0.05$) and CPSp1-challenged ($P<0.01$) groups (Fig. 1a). CPSp1 had little effect on chicken weight in previous experimental infection studies (Stephens & Hampson, 2002a, b; Jamshidi & Hampson, 2003). This suggests that neutralizing the crop prior to infection challenge may improve the survival of \textit{B. pilosicoli} traversing the crop acid barrier facilitating enhanced colonization of the distal intestinal tract and causing disease. Furthermore, housing the chickens freely within the rooms may have improved transmission, and hence increased colonization rates compared to individual caging; also a diet containing wheat was used, which may have had an effect since wheat-based diets have been shown to enhance colonization by \textit{B. intermedia} and may impact on colonization by \textit{B. pilosicoli} (Phillips et al., 2004). Following challenge with CPSp1, faecal moisture content increased by 0.5–5.3%, but at the end of the study there was no significant difference compared to the control group (Fig. 1b). Challenge with B2904 increased faecal moisture by
4.1–7.6% compared to the control group, a significant difference that remained until the end of the study ($P<0.01$).

Control chickens came into lay at approximately 18 weeks of age (day 15) whereas a delay was noted in both challenged groups (onset of lay day 22/23). CPSp1-challenged chickens laid considerably fewer eggs ($n=7$) compared with B2904-challenged chickens ($n=25$) that were productively comparable to the control chickens ($n=26$). However, egg weight for the control group ($54.05\pm 5.92$ g) was significantly greater than the B2904- ($48.55\pm 3.49$ g, $P<0.001$) and CPSp1-challenged ($46.65\pm 3.12$ g, $P<0.01$) groups. Since egg weights are generally lower at the onset of lay, the egg weights of the first 7 eggs were compared and were still significantly greater in the control group ($P<0.05$). Faecal staining was recorded on the eggs of the challenged groups only.

At post-mortem 5 days after the final challenge, *B. pilosicoli* were isolated at approximately 10-fold higher numbers from the caeca of the B2904- than CPSp1-challenged chickens (Fig. 1c). Pathology was consistent with lymphoid hyperplasia, described in chickens colonized by *B. pilosicoli* (Feberwee *et al.*, 2008), and also included an increased frequency of crypt abscesses containing *B. pilosicoli*. Caecal weight of the B2904-challenged ($13.25\pm 1.48$ g, $P<0.05$) but not the CPSp1-challenged ($14.22\pm 0.80$ g) chickens was significantly lower than the control group ($16.00\pm 0.24$ g). As a percentage of mean body weight, the mean caecal weight of the control group (0.99%) was greater than that of the B2904-challenged (0.86%) or CPSp1-challenged (0.93%) groups. The presence of lymphoplasmacytic cells in the caeca and colon was variable in the control and challenged chickens without evidence of

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**Fig. 1.** Clinical outcomes of the chickens experimentally infected with *B. pilosicoli*. Chicken weight (a) and faecal moisture content (b) were monitored; the mean was based on ten chickens until day 15 post final challenge and then seven chickens until the study end, in each of the negative control (closed circle, solid line), B2904-challenged (open square, dashed line) and CPSp1-challenged (closed triangle, dotted line) groups. On the x-axis, arrows indicate the days of challenge. Colonization levels of *B. pilosicoli* for different tissues sampled at post-mortem are shown, both 5 days post final challenge (day 16) (c) and 18 days post final challenge (day 28) (d); the mean was taken from three chickens examined at the two time points, from chickens of the negative control, B2904-challenged (open bar) and CPSp1-challenged (hatched bar) groups. Significance is shown in cases where the final data points of the challenged groups and the negative control group (a and b) or colonization of the two *B. pilosicoli* strains (c and d) differed significantly; *$P<0.05$; **$P<0.01$.**
typhlitis. Other than the caeca and colon, *B. pilosicoli* were recovered in low numbers from the ileum, liver and spleen of two B2904-challenged chickens. Pathology in the ileum included lymphoid hyperplasia, crypt abscesses and intraepithelial trafficking, mainly lymphocytic in villi tips and crypts. In humans, *B. pilosicoli*-like spirochaetes have been observed in extra-intestinal tissues, including the liver (Kostman et al., 1995) and the bloodstream (Trott et al., 1997). In flocks of duck, *Brachyspira* infection was associated with hepatic and splenic amyloidosis (Glavits et al., 2011). Our findings report what is to the best of our knowledge the first isolation of *B. pilosicoli* from the liver and spleen of chickens. Due to the aseptic handling of tissues and use of deep tissues, the possibility of cross-contamination was eliminated and the histopathological findings support the presence of the spirochaetes; however, immunohistochemistry may be an important consideration in the future to confirm the presence of spirochaetes. In the spleen, lymphoid hyperplasia with proliferation of ellipsoid macrophages and germinal centres were recorded with increased pyknotic and karyorrhectic debris. *B. pilosicoli* was isolated from the liver of two B2904-challenged chickens, with moderate to severe hepatic lipidosis noted in all three chickens of this group. Petechiae were detected in the duodenum of CPSp1-challenged chickens, but no spirochaetes were isolated from this tissue.

Despite an improvement of clinical symptoms by the final post-mortem examination, colonization by *B. pilosicoli* persisted in both groups. The number of CPSp1 associated with caecal tissue was significantly greater than the number of B2904 by the end of the study (P<0.05) (Fig. 1d). B2904 was not isolated from the ileum, liver and spleen whereas CPSp1 was. B2904 may have produced pathology in the chickens at an early stage of infection, relating to the normalization of egg production and chicken weight by the end of the study (Figs 1b and 2). Non-specific histological changes were noted, such as haemorrhages in lymphoid tissue, distended crypts and secondary follicle proliferation, in the caeca of B2904-challenged chickens (Fig. 2a). By the end of the study, prominent changes were recorded in tissues of CPSp1-challenged chickens with larger, more

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**Fig. 2.** Histopathological findings in haematoxylin and eosin stained caecal tonsil (a and b), liver (c) and spleen (d) tissues of chickens experimentally challenged with *B. pilosicoli* B2904 (a) and CPSp1 (b–d). Pathology observed included haemorrhages in lymphoid tissue (i), and secondary follicle proliferation in the caecal tonsils and spleen (ii). Furthermore, in the caecal tonsils distended crypts with an accumulation of a mixture of hyaline material, sloughed epithelial cells and inflammatory cells (crypt abscess) (iii) with epithelial attenuation and heterophilic infiltration (iv) were observed. In addition, acute hepatitis was noted with heterophilic infiltration in periportal regions and sinusoids (v). Prominent sheathed capillaries (vi) were observed in the spleen.
numerous crypt abscesses and dilated crypts containing cellular debris and inflammatory cells in the caecal tonsil (Fig. 2b), indicating bacterial colonization. The epithelium surrounding crypts displayed attenuation, degradation and necrosis along crypt hyperplasia, which has been reported to be associated with Brachyspira infection (Feberwee et al., 2008). Caecal haemorrhages were common in CPSp1-challenged chickens and they had more lymphocytic aggregates in the liver (Fig. 2c) with moderate granulocytic hepatitis in one chicken, perhaps associated to the isolation of B. pilosicoli from the liver. Prominent sheathed capillaries and secondary follicle proliferation were also noted in the spleen (Fig. 2d), correlating with the isolation of B. pilosicoli from these tissues. The ileum of two CPSp1-challenged chickens was distended with no spirochaetes isolated from this tissue. Regions of the oviduct were sampled at this point and both B. pilosicoli strains were isolated from one chicken of each group (Fig. 1d). Aside from a focal area of lymphoplasmacytic accumulation in the magnum of a B2904-challenged chicken, no significant histopathological changes were noted. The lack of pathology and absence of colonization of the upper oviduct does not provide an explanation for the delayed and reduced egg production. It is possible that B. pilosicoli produced pathology in this region at an earlier stage of infection and this may have been demonstrated by examining tissues at 5 days after the final challenge; however, the reproductive tract is not well developed at this stage.

These studies have revealed more severe caecal pathological changes, in addition to histopathological changes in the liver and spleen consistent with systemic spread of the spirochaete, which is previously unreported for Brachyspira infection in chickens. In future studies it may be desirable to perform anaerobic blood culture following experimental challenge with Brachyspira. Direct visualization of the aetiological agent will be necessary to determine the specificity of the changes. The differences in the clinical symptoms and pathology between the B2904- and CPSp1-challenged chickens may arise from differences in the pathogenicity of the two strains, and requires further investigation that may be aided by the increasing availability of Brachyspira genome sequence, although they may arise from other factors such as dose. The experiment was controlled and thus, we believe the deep tissue colonization to be a true phenomenon and not the result of an extraneous factor, such as co-infection. Overall, our model of Brachyspira infection has improved rates of colonization and induced hitherto unreported pathology. Furthermore, subsequent and ongoing studies using the B2904-challenge model to evaluate AIS intervention strategies show findings consistent with this study.

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