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Evaluation of PCR amplification from fixed caecal tissue to determine the infection of experimentally infected laying hens with *Brachyspira pilosicoli*

Jamshidi, A.[1*] **and Hampson, D. J.**[2]

[1]Department of Food Hygiene and Public Health, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; [2]Division of Veterinary and Biomedical Sciences, University of Murdoch, Murdoch, Western Australia 6150, Australia

[*]**Correspondence:** A. Jamshidi, Department of Food Hygiene and Public Health, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. E-mail: ajamshid@ferdowsi.um.ac.ir

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Summary

Avian intestinal spirochaetosis (AIS) is a condition arising from colonization of the caeca and colon of birds with anaerobic spirochaetes of the genus *Brchyspira*. The purpose of the present study was to evaluate PCR amplification from formalin-fixed, paraffin-embedded tissue method for detecting infected laying hens with *Brachyspira pilosicoli* with the same primers that have been used for detecting the infection in live birds. Over a 4-week period following experimental infection, the faeces of three of the eight inoculated birds became culture-positive. At post-mortem examination, no specific pathological changes were found, and in histological examination of caeca, no forming a "false brush border" was observed, but the spirochaete-form bacteria were present between the intestinal villi. Faecal and swab cultures from caeca of three infected birds became positive and confirmed by PCR amplification of 16S rRNA gene of *B. pilosicoli*. But this gene was not detected by PCR amplification from fixed caecal tissues of infected birds. This result might be due to low number of bacteria in each section or damage and fragmentation of target DNA.

Key words: *Brachyspira pilosicoli*, Laying hens, Fixed tissue, PCR

Introduction

Anaerobic intestinal spirochaetes of the genus *Brachyspira* (formerly *Serpulina*) colonize the large intestine of a variety of animal species (Hmpson and McLaren, 1999). Avian intestinal spirochaetosis (AIS) is a condition arising from colonization of the caeca and colon of birds with these bacteria (Stephens and Hampson, 2001). The condition occurs in both layer hens and broiler breeders, in which it has been associated with a variety of production problems, including diarrhoea, wet litter, faecal staining of eggshell, pasty vents, increased faecal fat content, delayed onset of egg laying, reduced egg weights, reduced growth rates, increased feed consumption, poor digestion of feed and increased number of week chicks, with slower growth and poorer feed digestion (Jamshidi and Hampson, 2002). To date, three species of these organisms have been identified as being potential pathogens of chickens, these being *B. pilosicoli*, *B. intermedia* and *B. alvinipulli* (Hampson and McLaren, 1999). The anaerobic intestinal spirochaete *B. pilosicoli* can infect across species boundaries (Jamshidi and Hampson, 2003). It causes intestinal spirochaetosis in human that is a milder form of colitis and diarrhoea than swine dysentery (Trott *et al*., 1996). It was shown that the human isolate of *B. pilosicoli* can also colonize the intestinal lumen of layer hens (Jamshidi and Hampson, 2003).

B. pilosicoli has been isolated from the large intestine or faeces of many animal species, including pigs (Trott *et al*., 1996a), chickens (Stephens and Hampson, 2001), dogs (Duhamel *et al*., 1998) and water birds (Oxberry *et al*., 1998). The presence of *B. pilosicoli* has been reported in laying hens flocks from Iran (Jamshidi *et al*., 2007).

Traditional laboratory diagnosis of *Brachyspira* spp by culture and biochemical tests are laborious and time-consuming, because their fastidious and slow-growing nature, requiring specialized selective media and anaerobic growth conditions and also limited range of phenotypic differences between them (Jamshidi and Hampson, 2002; La *et al*., 2003). To reduce the time needed for detection of *B. pilosicoli*, and to improve the specificity of the diagnostics, a variety of polymerase chain reaction (PCR) assays have been developed (Park *et al*., 1995; Fellstrom *et al*., 1997; Atyeo *et al*., 1998; Nyree *et al*., 2006). These PCRs target conserved gene such as the 16S rRNA gene (rDNA), the 23S rDNA, the NADH oxidase (*nox*) gene, or the haemolysin (*tly*) gene. The assays typically are conducted on intestinal spirochaete isolates or on spirochaetal growth harvested from primary isolation plates (La *et al*., 2003).

Sometimes diagnosis of infection requires identification of the organism within the tissue. However many pathological specimens are routinely fixed in formalin and embedded in paraffin prior to histological examination. Such samples are easy to store, transport, and section for histological staining. Tissue architecture and proteins are well maintained with this method. PCR-based detection of pathogens in formalin-fixed paraffin-embedded tissues (FFPE) is a routine technique (Levine *et al*., 2002). Although FFPE tissues are one of the commonest sources of diagnostic material, it is also known that nucleic acids including DNA and RNA, extracted from them are of lower quality than those recovered from fresh tissues (Bielawski *et al*., 2001). The yield of DNA that can be obtained from paraffin-embedded tissue and the length of DNA fragments gradually decrease with time (Bielawski *et al*., 2001).

The current study was designed to determine the sensitivity of PCR amplification from FFPE tissue to detect infected laying hens with *B. pilosicoli* with the same primers that have been used for detecting the infection in live birds.

Materials and Methods

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

Experimental birds

Sixteen 18-week-old ISA-Brown layer hens were obtained from a commercial breeder. The birds were housed in a controlled environment (25°C) facility, and randomly allocated to individual cages with mesh floors set in banks of 10 cages. The birds were subjected to 12 hrs artificial light each day. They were fed *ad libitum* on commercial wheat and vegetable-protein based layer diet (Wesfeeds Pty Ltd, Bentley, WA). At 37 weeks of age they were randomly allocated into two groups of eight, and each group housed in their individual cages in different 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 patient with histological IS 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 log-phase growth was achieved, when the spirochaetes were actively motile. Growth and absence of contamination was monitored by examining aliquots taken at daily intervals under a phase contrast microscope. At 38 weeks of age, eight birds in one group were orally inoculated via a crop tube with 2 ml of the actively growing culture, on three consecutive days. The broth contained approximately 10^8 bacterial cells/ml. The eight uninfected control birds in the other room were sham-inoculated with sterile broth.

Monitoring for spirochaetes in faeces

The birds were weighed on entry to the experiment at 38 weeks of age and at weekly intervals thereafter. Cloacal swab were obtained weekly from each bird at the time they were handled for weighing, commencing prior to experimental inoculation with *B. pilosicoli*. The swabs were streaked onto selective Trypticase soy

agar (BBL, Cockeysville, MD, USA) supplemented with 5% defibrinated ovine blood, 400 µg/ml spectinomycin and 25 µg/ml each of colistin and vancomycin (Jenkinson and Wingar, 1981). Plates were incubated in an anaerobic environment (94% N_2 and 6% CO_2) 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 ß-haemolysis surrounding a low flat haze of bacterial growth. Spirochaetal growth was subcultured, and then isolated cells were subjected to a PCR protocol which specifically amplifies a 439 base pair segment of the 16S rRNA gene of *B. pilosicoli* (Atyeo *et al*., 1998; Mikosza *et al*., 1999; Mikosza *et al*., 2001). The PCR products were subjected to electrophoresis in a 1.5% agarose gel, stained by immersion for 30 min in an ethidium bromide solution, and viewed over ultraviolet light. The inoculated bacteria considered as positive control and *B. aalborgi* and *B. intermedia* as negative control.

Post-mortem examination

When the birds were 43 weeks of age, they were killed by cervical dislocation, and subjected to post-mortem examination. The caeca and colon were opened to look for evidence of gross changes, sections of one caecum placed in 10% buffered formalin for subsequent histological examination, and swabs taken from the other caecum for spirochaete culture. For histological examination, the fixed tissue was processed through to paraffin blocks, sectioned at 4 µm and stained with haematoxylin and eosin.

PCR amplification from fixed tissue

DNA from paraffin-embedded tissue (PET) samples was extracted by a modification of a previously described method (Frank *et al*., 1996; Mikosza *et al*., 1999). PET samples were sliced into 15 to 20 µm sections with a microtome blade, which was thoroughly cleaned between samples. Each section was dewaxed by placing it in 200 µl of xylene and then on a rocker for 5 min, at room temperature. The samples were then centrifuged at 10000 × g for 10 min, and the supernatant was discarded. A second xylene incubation was performed, and 200 µl of 100% ethanol was added, incubated, and centrifuged, as for xylene. A second ethanol incubation and centrifugation was performed, the supernatant was discarded, and the samples were dried at 50°C in an oven for 30 min. Twenty micrograms of proteinase K in 100 µl of 50 mM Tris-HCl, pH = 8.3 was added and incubated overnight at 37°C. The samples were then boiled for 8 min, and 1 µl of each resultant extract was used as template DNA for PCR analysis.

Pair of primers which specifically detect the presence of the *B. pilosicoli* 16S rRNA gene (Atyeo *et al*., 1998; Mikosza *et al*., 2001), the thermocycling conditions, and predicted size outlined in Table 1.

Results

The mucosal surface of all the caeca examined and were grossly normal. In histological examination, no end-on attachment of spirochaetes to the lumenal surface of the caecal enterocytes was observed in any of the birds, but the spirochaete form bacteria was present between the intestinal villi of the infected hens.

No spirochaetes were detected in swab culture of the uninfected birds at any time. Spirochaetes, which were identified as *B. pilosicoli* by PCR amplification, were isolated from the swab culture of three of the eight infected birds one week after infection, and in these same birds at weekly intervals

Table 1: Primers and thermocycling condition for *B. pilosicoli*-specific reactions*

Target gene	Primer sequence (name)	Predicted product size (bp)	Thermicycling program
B. pilosicoli 16S rRNA	5′- CCC CTA CAA TAT CCA AGA CT -3′ (R -SERP 16Sr-583) 5′- AGA GGA AAG TTT TTT CGC TTC-3′ (F- A-coli PIL 16S f-187)	439	94ºC for 5 min – (94ºC for 30s-51ºC for 30s-72ºC for 30s)*33 cycles

*All reactions were performed on an Applied Biosystems 2400 thermocycler

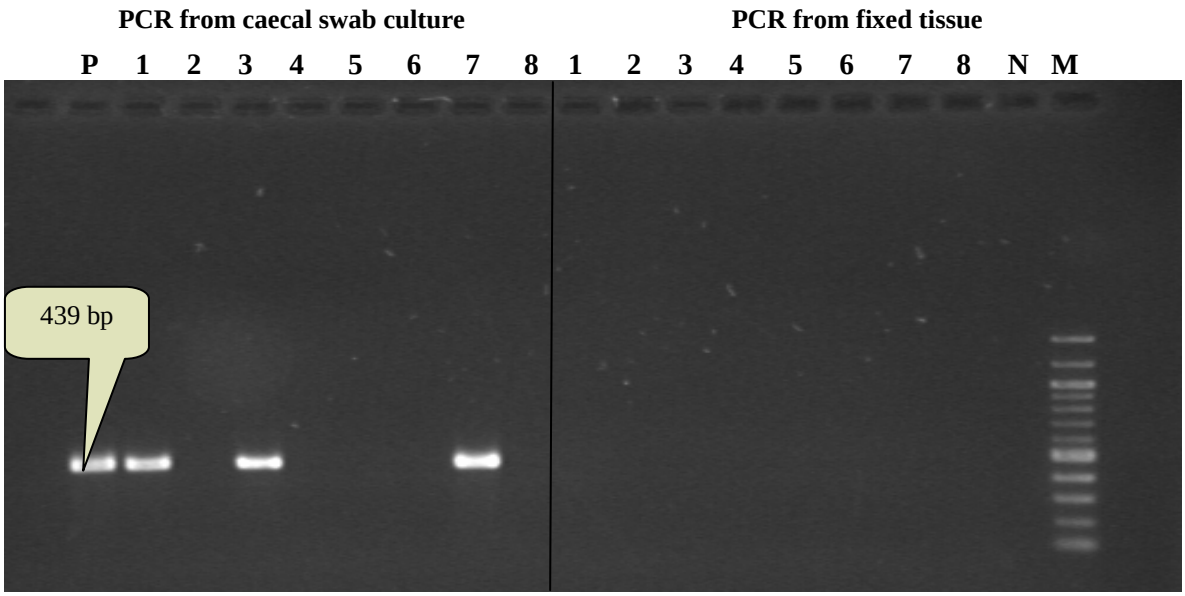

Fig. 1: Results of the PCR assay, amplifying a 439 base pair segment of the 16SrRNA gene of *Brachyspira pilosicoli*. Lane 1, 3, 7 positive samples. P: positive control (HIV3AB2); M:100 bp Marker and N: negative control

to the end of the experiment (Fig. 1). *B. pilosicoli* was also isolated from the caecal wall of these three birds at post-mortem. *B. pilosicoli* were not detected by PCR amplification of FFPE tissues obtained from caeca of infected birds.

Discussion

In this study a strain of *B. pilosicoli* which had been isolated from an HIV patient with histological intestinal spirochaetosis and chronic diarrhoea was shown to colonize the caecae of three of eight experimentally inoculated adult chickens. This colonization was persistent, extending over a four week period. Prolonged colonization, averaging four months, is a feature of natural *B. pilosicoli* infection in human beings (Trott *et al*., 1998). The relatively low colonization rate as assessed by culture (38%) is consistent with what has been reported in adult birds which have been experimentally inoculated with chicken strains of *B. pilosicoli* (Stephens and Hampson, 2001; Jamshidi and Hampson, 2002). It was previously known that day-old chicks could be colonized with human strains of *B. pilosicoli* (Dwars *et al*., 1992; Muniappa *et al*., 1996), but this colonization may be due to the presence of a poorly-developed large intestinal microflora in young birds.

No pathological changes were observed in the caecae 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 and Hampson, 2002; Stephens and Hampson, 2002), and in mice infected with a human strain of *B. pilosicoli* (Sacco *et al*., 1997).

Extraction of nucleic acid from archival tissue allows retrospective analysis and correlation of clinical end points or histological appearance structures with molecular biological markers (Coombs *et al*., 1999). Extraction of nucleic acid from fixed tissues is difficult and often yields only degraded DNA (Coombs *et al*., 1999; Levine *et al*., 2002). The factors that may affect the sensitivity of PCR of FFPE tissues include tissue type, length of the product to be amplified, copy number of the gene to be amplified, concentration of target DNA, concentration of non-target DNA and other inhibitors remaining in the DNA extracts and duration of fixation (Greer *et al*., 1991). Failing to detect 16S rRNA gene of *B. pilosicoli* in PCR amplification from fixed caecal tissues may be due to presence of PCR inhibitory factors, these PCR inhibitory factors which are found in genomic DNA extracted from FFPE tissues, are the high concentration of small DNA fragments that appear to compete with template DNA and denatured protein which cannot be

completely removed by phenolic DNA extraction (Liborio *et al*., 2005). A large number of 3`-end of small DNA fragment that are not long enough to allow efficient amplification, serve as abortive PCR template and compete for the enzyme *taq* polymerase (Liborio *et al*., 2005).

Absence of end-on attachment of spirochaetes to the luminal surface of the caecal enterocytes my be due to the presence of normal microflora in laying hens, because in an experiment, infected one-day-old specific-pathogen-free (SPF) chicks, showed a dense carpet of spirochaetes that were attached end-on the epithelial surface of caeca (Trott *et al*., 1995). Although the spirochaete-form bacteria was present between the intestinal villi, another possible reason for failing to detect 16S rRNA gene of *B. pilosicoli*, was low number of bacteria in each section, because in histological examination no false brush border of spirochaetes attached to the intestinal mucosa was observed. In addition, in the case of low template concentration, the relative primer excess makes more likely taq-related primer extension and primer dimmer formation resulting from 3` primer complementarity. These primer dimmers will compete with template with the result that low or absent amplification can occur in these situations (Liborio *et al*., 2005). Failing to detect the specific gene of *B. pilosicoli* in PCR amplification from fixed caecal tissue is not a unique situation, as it has been recorded that despite clear histological evidence of intestinal spirochaetosis in human, insufficient specific DNA in the piece of tissue that was processed for PCR, failed to produce a positive result, possibly because the target DNA was too damaged and/or fragmented (Mikosza *et al*., 1999).

Because large DNA fragments are more possibly to be damaged in FFPE tissues, for increasing the sensitivity of PCR test, it is pertinent to use primers that amplifies shorter DNA segments, or using nested PCR to determine the presence of the target DNA fragment.

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