PREVALENCE, PATHOGENICITY and CONTROL OF AVIAN INTESTINAL SPIROCHAETOSIS IN AUSTRALIA

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This thesis is presented for the degree of Doctor of Philosophy of Murdoch University 2008
I declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any tertiary education institution.
Abstract

Avian intestinal spirochaetosis (AIS) is a relatively recently recognized disease of commercial layer and meat breeder chickens resulting from colonization of the gastrointestinal tract by anaerobic spirochaetal bacteria of the genus *Brachyspira*. AIS is characterised by delayed and/or reduced egg production and chronic diarrhoea. This thesis describes an investigation into the prevalence, pathogenicity and control of species of avian intestinal spirochaete in Australia. Faeces samples from chickens in 22 flocks of laying hens, 19 broiler flocks and 28 breeder flocks in Queensland, New South Wales, Victoria, Tasmania and South Australia were subjected to selective anaerobic culture for *Brachyspira* species. Spirochaete isolates then were speciated using phenotypic characteristics and specific polymerase chain reaction amplifications. A highly significant association was found between colonisation with *Brachyspira* species and the occurrence of wet litter and/or reduced production in both broiler breeder and layer flocks in eastern Australia. Multilocus enzyme electrophoresis (MLEE) then was used to help confirm the identity of the spirochaetes, and to examine their genetic relationships and disease associations. MLEE divided the isolates into five known *Brachyspira* species groups: *Brachyspira murdochii*, *B. intermedia*, *B. pilosicoli*, *B. innocens*, and “*B. pulli*”. Three new MLEE groups each containing single isolates were also identified. All farms with production
problems or wet litter were colonised with the pathogenic species, *B. intermedia* and/or *B. pilosicoli*. The pathogenic potential of single isolates of *B. pilosicoli* and *B. innocens* then were experimentally evaluated in adult broiler breeders, confirming that infection with *B. pilosicoli* can result in serious egg production losses, whilst *B. innocens* is non-pathogenic. Antimicrobial susceptibility testing showed that the pathogenic spirochaetes *B. pilosicoli* and *B. intermedia* were both susceptible to tiamulin, lincomycin, metronidazole and tetracycline, while a lack of susceptibility to tylosin was found in some isolates of *B. intermedia* and *B. pilosicoli*. Some isolates of *B. pilosicoli* were not susceptible to ampicillin. Additional studies showed that zinc bacitracin, a common feed additive, can increase susceptibility to colonisation with *B. pilosicoli*. Both tiamulin and lincomycin were shown to be effective in treating infection with *B. pilosicoli* in adult birds.
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Hampson DJ, Stephens CP and Oxberry SL 2006. Antimicrobial susceptibility testing of *Brachyspira intermedia* and *Brachyspira*
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The research upon which this thesis is based was carried out between 1997 and 2002, in a series of studies aimed at elucidating the role played by intestinal spirochaetes in commercial poultry operations in Australia. In 2002, as the final study approached completion, I was given a vaccination for Q fever, a compulsory requirement of my employment. Unfortunately, I experienced a severe adverse reaction to this vaccination, which resulted in progressively worsening physical and mental incapacity over the ensuing 18 months. This was followed by several years of recuperation. As a result, while each of the studies had been published as they were completed, it was not until this year that I have been able to put together this thesis.

I would like to express my appreciation of the understanding shown by my supervisor and by staff of the Graduate Centre of Murdoch University during this time.
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The following literature review is based on the paper:

LITERATURE REVIEW

This thesis is concerned with an investigation into the prevalence, pathogenicity and control of species of avian intestinal spirochaetes in Australia. Accordingly, the literature review has been written to provide an overview of available knowledge about these infections at the time the work described in this thesis commenced in 1997. More recent findings have been included where appropriate, although reference has not been made to the author’s own published work since this time, as this is presented in the main body of this thesis. The literature review concludes with a summary of the aims and objectives of the study.

1.1 INTRODUCTION

Avian intestinal spirochaetosis (AIS) is a relatively recently recognized disease of commercial layer and meat breeder chickens resulting from colonization of the caeca and colon by anaerobic spirochaetal bacteria of the genus *Brachyspira*. Although the spirochaetes involved can also colonise broiler chicks, this does not appear to occur frequently in flocks under field conditions. AIS is characterised by delayed and/or reduced egg production and chronic diarrhoea. The diarrhoea leads to faecal staining of eggs and the resultant wet litter presents problems with cage cleaning, odour emission and attraction of flies.
In the past, the presence of spirochaetes in commercial poultry flocks may have gone unnoticed and their significance unappreciated, because of their poor staining characteristics in histological sections and the fact that they can only be isolated using specialized media and techniques. They do not all induce characteristic histological lesions. Several distinct species of these bacteria colonise poultry, not all of which are considered to be pathogenic. Other uncharacterized species may also be present.

Relatively few studies have been carried out on these organisms or on the pathogenesis of the disease. Moreover, the influences of other factors - such as management practices, diet, strains of bird and climatic conditions - have largely not been investigated.

1.2 THE NATURE OF INTESTINAL SPIROCHAETES

Spirochaetes are members of the order of bacteria known as Spirochaetales. These are helical shaped, motile bacteria (Figure 1.1). The cell is contained within a multi-layered membrane referred to as the outer sheath. This sheath completely surrounds the protoplasmic cylinder, which consists of the cytoplasmic and nuclear regions enclosed by the cytoplasmic membrane-cell wall complex. Periplasmic flagella wind around the helical protoplasmic cylinder, contained between the outer sheath and the protoplasmic cylinder (Canale-Parola, 1984). The order Spirochaetales contains three families, Spirochaetaceae, Leptospiraceae and Brachyspiraceae (Paster and Dewhirst, 2001).
The intestinal spirochaetes described in this thesis are members of the family Brachyspiraceae, genus *Brachyspira* (formerly *Serpulina*) (Ochiai *et al.*, 1997; Hampson and La, 2006). Although they may persist for a short time in the external environment, particularly in moist conditions, their primary habitat is the large intestine of various species of birds and animals. Some *Brachyspira* species are restricted to only one or a few host species, whilst others are less constrained and are more widely distributed. They are all fastidious anaerobes and include species that are considered to be pathogenic and others that are essentially commensal.

There are currently seven officially named species of *Brachyspira* and their morphological characteristics, common host species and disease associations are summarized in Table 1.1. Other unofficial species include “*B. canis*” (Duhamel *et al.*, 1998), “*B. pullii*” (McLaren *et al.*, 1997) and “*B. suanatina*” (Råsbäck *et al.*, 2007a). The best known and most studied of the species is *B. hyodysenteriae*, the agent of swine dysentery. To date the three main species that have been associated with AIS are *B. intermedia*, *B. pilosicoli* and *B. alvinipulli*. These will be discussed in more detail in later sections.
Figure 1.1 Wet mount of spirochaetes (*Brachyspira* spp.) viewed under phase contrast (x1000).

Figure 1.2. Electron micrograph of *B. pilosicoli* showing four periplasmic flagella inserted at one cell end.
<table>
<thead>
<tr>
<th>Species</th>
<th>Length (µm)</th>
<th>Diameter (µm)</th>
<th>Flagella per cell</th>
<th>Cell end</th>
<th>Major host</th>
<th>Disease association</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. hyodysenteriae</em></td>
<td>6 - 13</td>
<td>0.3 - 0.4</td>
<td>22 - 28</td>
<td>Blunt</td>
<td>Pigs</td>
<td>Swine dysentery</td>
</tr>
<tr>
<td><em>B. intermedia</em></td>
<td>7.5 - 10</td>
<td>0.36 – 0.45</td>
<td>24 - 28</td>
<td>Blunt</td>
<td>Pigs, birds</td>
<td>Spirochaetal colitis, AIS</td>
</tr>
<tr>
<td><em>B. innocens</em></td>
<td>5.3 – 14.1</td>
<td>0.25 – 0.40</td>
<td>8 - 12</td>
<td>Blunt</td>
<td>Pigs, birds</td>
<td>Commensal</td>
</tr>
<tr>
<td><em>B. alvinipulli</em></td>
<td>8 - 11</td>
<td>0.22 – 0.34</td>
<td>22 - 30</td>
<td>Blunt</td>
<td>Birds</td>
<td>AIS</td>
</tr>
<tr>
<td><em>B. murdochii</em></td>
<td>5 - 8</td>
<td>0.3</td>
<td>22 - 36</td>
<td>Blunt</td>
<td>Pigs, birds</td>
<td>Commensal</td>
</tr>
<tr>
<td><em>B. pilosicoli</em></td>
<td>4 - 12</td>
<td>0.2 – 0.4</td>
<td>8 - 12</td>
<td>At least one end pointed</td>
<td>Pigs, birds, humans, rodents</td>
<td>Intestinal spirochaetosis in pigs, humans and birds (AIS)</td>
</tr>
<tr>
<td><em>B. aalborgi</em></td>
<td>1.7 – 6.0</td>
<td>0.2</td>
<td>8</td>
<td>Tapered</td>
<td>Humans</td>
<td>Intestinal spirochaetosis</td>
</tr>
</tbody>
</table>
1.3 AVIAN INTESTINAL SPIROCHAETES (*BRACHYSPIRA SPP.*)

1.3.1 Morphology

*Brachyspira* spp. all possess a Gram-negative cell wall structure and the same characteristic cell morphology as other spirochaetes. They are helical with an outer sheath and a central protoplasmic cylinder enclosed by a cytoplasmic membrane. There are two sets of periplasmic flagella, each set originating from opposite ends of the protoplasmic cylinder (Figure 1.2). These run along the length of the cell, between the protoplasmic cylinder and the outer sheath, each set overlapping with the other midway along the cell. This unique arrangement accounts for the variation in the number of flagella reported for the different species of spirochaete. If flagellar numbers are reported as a ratio between end:middle:end numbers, then the most commonly reported ratios for spirochaetes obtained from avian samples are either 8:16:8 or 5:10:5.

The characteristic sinuous movement of spirochaete cells is brought about by the rotation of the periplasmic flagella between the outer sheath and the inner protoplasmic cylinder. This type of motility allows spirochaetes to readily move through viscous fluids, such as the mucus layer overlying the epithelium in the large intestine.

Intestinal spirochaetes stain poorly with routine stains, such as haematoxylin-eosin and may easily be missed in sections stained by such
means. However, they stain well with silver-impregnation staining techniques, such as Warthin-Starry or Steiner, appearing deep brown to black in colour.

Intestinal spirochaetes may also be observed by dark-field or phase contrast microscopy in wet mounts. These may be prepared from caecal contents or caecal droppings.

1.3.2 Growth requirements and colony morphology

*Brachyspira* spp. are anaerobic, but can tolerate exposure to oxygen. They have been isolated from birds using the same or similar media and conditions as used for intestinal spirochaetes from pigs and other species. Media used have included Trypticase Soy agar with 5% defibrinated blood and 400 μg per mL spectinomycin (*Davelaar et al.*, 1986), or the same medium with the addition of 25 μg per mL each of colistin or vancomycin (*McLaren et al.*, 1996). Stoutenburg and Swayne (1992) added rifampicin. The addition of mucin appears to improve growth, while the use of citrated rather than defibrinated blood may enhance haemolytic variation. BJ medium which contains the above four antibiotics as well as spiramycin is a highly selective medium for the isolation of *B. hyodysenteriae*, the causative agent of swine dysentery (Kunkle and Kinyon, 1988). However the growth of some avian intestinal spirochaetes may be inhibited on this medium.
Where large numbers of *Brachyspira* cells are required, as in the case of the preparation of inoculum for experimental infections, the spirochaetes can be grown in broth media, for example in pre-reduced Trypticase Soy broth with the addition of 2% foetal calf serum and a 1% cholesterol solution (Kunkle *et al.*, 1986).

The usual culture conditions for intestinal spirochaetes are an atmosphere of 94% N₂/6% CO₂ at a temperature of 37°C (Lee *et al.*, 1993; Lee and Hampson, 1994). They can be cultured successfully using commercial anaerobic atmosphere generation systems. Some researchers have used an incubation temperature of 38°C (Stanton and Lebo, 1988), while others have used a temperature of 42°C (Songer *et al.*, 1976). Optimal temperatures for the culture of avian intestinal spirochaetes remain to be determined.

On solid media, *Brachyspira* spp. usually do not form discrete colonies. Instead, they spread over the agar surface, forming a thin haze-like film after three or more days of incubation. Growth is indicated in some strains by varying levels of haemolysis of the blood agar. However non-haemolytic or weakly-growing strains may only be visible as a dull sheen on the surface of the agar. Colony formation can be enhanced by using fastidious anaerobe agar.
Figure 1.3 Culture of *B. pilosicoli* on agar, showing weak haemolysis.

The presence of spirochaetes should always be confirmed by microscopy. Viewing a wet mount using dark-field or phase microscopy not only allows the confirmation of any growth present as that of spirochaetes, but also enables the characteristic motility to be seen. Experienced workers will thus be able to assess the viability of the culture.
1.4 HISTORICAL BACKGROUND TO AIS

The first apparent observation of what would now be considered to be avian intestinal spirochaetes was made by Fantham in the early 1900s, who described helical bacteria that he named "*Spirochaeta lovati*" in grouse in Great Britain (Fantham, 1910). The bacteria were observed in the caeca and recti of both young and mature birds but did not appear to be associated with disease.

Twenty years later Harris reported observing spirochaetal bacteria in the caecal droppings of one-quarter of chickens (*Gallus gallus domesticus*) he randomly selected from the Baltimore live poultry markets (Harris, 1930). The chickens included both clinically normal and sick adult birds. The caecal droppings of many of the birds were yellowish-brown, semi-solid to pasty and had a strong odour. Harris did not observe spirochaetes in immature or baby birds, a finding that has been consistently repeated to the present day. Harris reported three morphologically distinct forms of spirochaetal organisms. No lesions were produced in chickens orally inoculated with faeces containing these three types of spirochaetal bacteria.

In 1955 Mathey and Zander reported finding large caseous nodules associated with spirochaetal bacteria in the caecal walls of turkeys (*Meleagridis galloparvo*), chickens, and pheasants (*Phasianus colchicus*) in the United States (Mathey and Zander, 1955). When these organisms
were orally inoculated into turkeys, caecal nodules were produced. A spirochaetal bacterium was isolated from these nodules and cultured in chicken embryos. Swayne and McLaren (1997) have suggested that the nodules may have been dilated intestinal crypts with associated granulomatous inflammation. When the organisms were intravenously inoculated into day-old chicks no lesions or infection resulted.

None of the organisms from these early reports are available for examination. However, the descriptions of their shape, size and mode of motility are consistent with them being spirochaetes. All subsequent observations of intestinal spirochaetes in poultry have been made since 1986 and are discussed below.

1.5 OCCURRENCE AND PREVALENCE OF AIS IN EUROPE, THE UNITED STATES AND AUSTRALIA

1.5.1 Studies in Europe

In what constituted the first genuine report of AIS and so named by them, Davelaar and colleagues (1986) demonstrated spirochaetes in the caecal mucosa of laying hens with diarrhoea in the Netherlands. They used these isolates to induce inflammation of the caeca in ten-week-old hens, in association with an increase in faecal moisture content and growth retardation. In the United Kingdom, Griffiths et al (1987) described a
similar syndrome in pullets involving reduced growth rate, delayed onset of egg production and poor shell quality.

In 1989, Dwars and colleagues in the Netherlands described the results of diagnostic testing for the presence of intestinal spirochaetes in poultry flocks over an eight-month period. Using immunofluorescence staining with an anti-spirochaete serum raised against *B. hyodysenteriae*, the agent of swine dysentery, they demonstrated spirochaetes in the caeca of birds from 37 of 134 (27.6%) flocks with enteritis but in the caeca of birds from only two of 45 (4.4%) flocks with no intestinal disease. These findings demonstrated that infection was relatively common in the Netherlands, and that there was a significant correlation between intestinal disorder and the presence of spirochaetes.

Although AIS is not considered to be a problem in broiler flocks, Dwars and colleagues (1992b) used one of their isolates in a series of experimental infections of day-old chicks. They demonstrated that the spirochaetes caused reduced growth rates, decreased serum concentrations of protein, bilirubin and lipid carotenoids and an increased faecal fat content.

When laying hens were experimentally infected with spirochaetes from the above survey, they developed mild persistent gastrointestinal dysfunction (Dwars et al., 1990). At nine months post-inoculation spirochaetes were still present in the caecal faeces, indicating that
Colonization and disease in adult birds tend to be chronic. An increase in faecal lipids was also found in laying hens experimentally infected with the spirochaetes (Dwars et al., 1992a).

Experimental infection of broiler parent hens with the same spirochaete caused persistent infection, with wet droppings and reduced egg production (Dwars et al., 1993). The broilers that hatched from the eggs produced by these birds had reduced growth rates, wet droppings, low plasma carotenoid concentrations and elevated alkaline phosphatase activity. They were not colonised by spirochaetes. These findings emphasise the important effects of AIS on broiler performance following infection of the breeding flock. In a more recent study in the Netherlands that extended this work, Smit and colleagues (1998) recorded the effect of AIS on the performance of broiler progeny in eight infected broiler breeder flocks. Correlation of data from 136 broiler flocks derived from these breeder flocks showed that broiler flocks from infected breeders, with clinical signs of AIS, had poorer feed conversion, of approximately 90 g per kg growth, an increased number of weak chicks, slower growth and poorer feed digestion than the offspring of flocks where spirochaetes were not present. Breeder flocks with clinical signs of AIS produced 7.5% fewer eggs than apparently healthy flocks. Consistent with earlier findings, the broilers themselves were not colonised by spirochaetes.

Dwars and colleagues (1992a) suggested that the clinical signs seen in the field, including wet droppings, pale egg yolks, production loss and
poor shell quality could be explained by an impaired resorption of dietary fat, carotenoids and vitamin D3. As spirochaetes colonise the caeca and the small intestine is the major site for absorption of lipids, carotenoids or bilirubin, then this effect on resorptive capacity would appear to be indirect. The clinical signs could also be due in part to malabsorption of nutrients, while the poor shell quality often seen in infected birds could have its origins in poor calcium absorption. More experimental work is required to clarify this area.

Other studies that have been reported from Europe since the current study commenced included the description of colonization with various *Brachyspira* spp. in feral mallards in Sweden (Jansson *et al.*, 2001), infection with *B. pilosicoli* in three laying flocks in the UK (Burch *et al.*, 2006) and a case of severe typhlocolitis associated with *B. alvinipulli* in two large commercial flocks of geese in Hungary (Nemes *et al.*, 2006). Most recently, a study of laying hen farms in northeastern Italy found that faeces from 14 sheds (31%) on 10 farms (34.5%) contained pathogenic *B. intermedia* and/or *B. pilosicoli* and disease consistent with AIS was observed in nine of these sheds on seven farms. There was a significant association between the pathogenic species and reduced egg production (Bano *et al.*, 2008). A study of laying hen flocks on 25 farms in the Netherlands with symptoms consistent with AIS found that the most commonly detected spirochaetes were the pathogenic species *B. intermedia* and *B. pilosicoli*. Pathogenic *B. alvinipulli* was found in two flocks and *B. hyodysenteriae* was identified in three flocks (Feberwee *et
In Sweden, comparative phylogenetic analysis in combination with PCR and phenotyping of 30 spirochaetes obtained from commercial laying hens identified seven isolates as *B. intermedia*, eight as *B. innocens*, five as *B. murdochii* and three isolates each as *B. alvinipulli* and “*B. pulli*”. The remaining four isolates could not be identified as any currently recognised species, while no *B. pilosicoli* was isolated (Jansson *et al*., 2008).

### 1.5.2 Studies in the USA

To date there have been only two published reports of AIS in chicken flocks in the USA. In the first report, spirochaetes were observed in the caecal lumina and crypts of hens from two commercial layer flocks that exhibited faeces-stained eggshells, diarrhoea and typhlitis (Swayne *et al*., 1992). In pathogenicity tests, this spirochaete produced pale-yellow, watery caecal contents and mild lymphocytic typhlitis in day-old chicks and in 14-month-old chickens (Swayne *et al*., 1995). This spirochaete was subsequently named *Serpulina alvinipulli* (Stanton *et al*., 1998). In the second report, Trampel and co-workers (1994) described overgrowth of the caecal mucosa by spirochaetes in a flock of 100,000 chickens, of 30 weeks of age, experiencing diarrhoea and a 5% drop in egg production. Spirochaetes were seen attached by one cell end to the caecal mucosa, and these were later identified as *B. pilosicoli* (McLaren *et al*., 1997).
Intestinal spirochaetes have also been observed in wild waterfowl in the US (Swayne, 1997) and in captive juvenile ring-necked pheasants (Webb et al., 1997). Recently *B. pilosicoli* was isolated from turkeys in California (Shivaprasad and Duhamel, 2005). The intestinal spirochaete *Brachyspira hyodysenteriae*, the agent of swine dysentery, has also been isolated in the US from captive rheas (*Rhea americana*) with typhlocolitis (Sagartz et al., 1992; Buckles et al., 1997) and this seems to be a fairly widespread problem. This spirochaete can cause typhlitis in experimentally infected chicks (Sueyoshi and Adachi, 1990) and has recently been described in feral mallards in Sweden (Jansson et al., 2004) and in laying hens in the Netherlands (Feberwee et al., 2008).

### 1.5.3 Studies in Australia

In 1996, a study was published from Western Australia (WA), where selective culture techniques were used to isolate intestinal spirochaetes from faecal samples from birds in 30 broiler breeder and 37 layer flocks. Spirochaetes were isolated from 64% of flocks with signs of reduced egg production and/or wet litter, compared with 28% of clinically normal flocks (McLaren et al., 1996). The higher apparent overall prevalence of infection in WA compared to the Netherlands (Dwars et al., 1989) may have been due to the greater sensitivity of detection by faecal culture compared to the use of immunofluorescence, rather than being due to true differences in prevalence.
As in the USA, studies in Australia also have revealed a high rate of faecal carriage of intestinal spirochaetes (\textit{B. pilosicoli}) in wild waterbirds (Oxberry \textit{et al.}, 1998) and these birds have been suggested as a possible reservoir of infection for chickens.

1.6 Classification and \textit{Brachyspira} species prevalence in AIS

Historically, the classification of spirochaetes was based on their morphology, motility, host species and site of colonization. However, modern techniques that are able to analyse genetic and phenotypic characteristics, such as analysis of RNA and DNA sequences, protein profiles and the distribution of isoenzymes have led to a reassessment of this classification (Swayne, 1997). Although the majority of intestinal spirochaetes probably remain unclassified, degrees of relatedness between avian and mammalian intestinal spirochaetes have been determined for many strains. This information will form the basis for a future comprehensive taxonomic scheme for these bacteria. The development of a multilocus sequence typing (MLST) scheme for the genus \textit{Brachyspira} has recently been reported and its application may help to clarify the relationships between \textit{Brachyspira} spp. from birds and other species (Råsbäck \textit{et al.}, 2007b).

The spirochaetes originally isolated from the studies in adult poultry in Europe and the US were not identified to the species level and this created difficulties of interpretation of both the field studies and
experimental infections conducted there. An important study using multilocus enzyme electrophoresis (MLEE) analysed a collection of organisms from Europe, the US and WA and identified at least six species (McLaren et al., 1997). Three of these species are currently considered to be capable of causing disease in chickens: *B. intermedia*, *B. pilosicoli* and *B. alvinipulli*. The MLEE study showed that the pathogenic species workers in Europe appeared to have been most concerned with and which they had used in their experimental infections of day-old chicks and adult birds, was *B. intermedia* (Griffiths et al., 1987; Dwars et al., 1992a, b, 1993), although *B. pilosicoli* was also identified from the field surveys. In contrast the reports from the US had involved either *B. pilosicoli* (Trampel et al., 1994) or *B. alvinipulli* (Swayne et al., 1992, 1995; Stanton et al., 1998). Interestingly both *B. intermedia* and *B. pilosicoli* are also considered to be potential pathogens of pigs (Hampson and Trott, 1995) and *B. pilosicoli* also infects dogs (Duhamel et al., 1998) and other animal species including human beings (Trott et al., 1997; Trivett-Moore et al., 1998). As such, *B. pilosicoli* may be considered a potentially zoonotic infection, although this has not been investigated in detail in relation to the poultry industries.

A subset of the isolates obtained in the study in WA was analysed in the MLEE study and 41% were identified as *B. intermedia*, 56.4% as belonging to a previously undescribed and unnamed group of uncertain pathogenicity ( provisionally designated as “*Brachyspira pulli*”) and the rest as *B. innocens*, a non-pathogenic species known to infect pigs.
No isolates of the pathogenic species *B. pilosicoli* or *B. alvinipulli* were identified. Strains of *B. pilosicoli* have however been recovered in WA from wild waterbirds (Oxberry et al., 1998) and more recently from birds in layer flocks (Phillips et al., 2005).

Generally, the presence of the pathogenic species *B. intermedia* and *B. pilosicoli* in chicken flocks in Australia appears to be more similar to the situation in Europe than to the findings from the USA. More studies are required to investigate the prevalence and distribution of the various spirochaete species in chickens in the US and in other poultry-producing countries, before firm comparisons can be made.

### 1.7 EXPERIMENTAL INFECTION STUDIES IN AUSTRALIA

To clarify the pathogenic potential of Australian isolates, an isolate of *B. intermedia* from WA and a *B. pilosicoli* isolate from Queensland, both obtained from chickens, were inoculated orally into day-old chicks and/or chickens approaching lay. In the chicks, infection with *B. intermedia* and *B. pilosicoli* resulted in diarrhoea within seven to nine days, whilst chicks infected with *B. innocens* remained healthy (Hampson and McLaren, 1997). *B. pilosicoli* was found attached by one cell end to the caecal mucosa – a feature also seen with this spirochaete in other animal species (Trott et al., 1996; Trivett-Moore et al., 1998). Infection of 14-week-old layer hens with an isolate of *B. intermedia*, obtained from a WA layer with diarrhoea, resulted in reduced growth rates, significantly
increased faecal moisture content (wet droppings) and a significant reduction in egg production over a six week period following commencement of lay (Hampson and McLaren, 1999). These results demonstrated the pathogenic potential of a WA isolate of *B. intermedia* in chicks and layers and a Queensland isolate of *B. pilosicoli* in day-old chicks.

### 1.8 CLINICAL SIGNS OF AIS

The clinical signs of AIS are not diagnostic. Rather they are indicative of infection with a non-specific intestinal pathogen. Layer chickens with AIS can have wet droppings, higher lipid content in faeces, pasty vents, chronic diarrhoea, reduced growth rates, delayed onset of egg laying, eggshells stained with faeces, reduced average egg weights and reduced egg carotenoid content (Davelaar et al., 1986; Griffiths et al., 1987; Swayne et al., 1992; Dwars et al., 1992 a and b, 1993)

### 1.9 DIAGNOSIS

At post-mortem affected birds may have pale-coloured, foul-smelling, foamy caecal contents. There are usually no gross lesions, so initial diagnosis is mainly made on the basis of microbiological findings. As not all intestinal spirochaetes are pathogenic, clinical signs indicative of AIS should also be present to enable a presumptive diagnosis.
Figure 1.4 Eggs from birds experimentally infected with *B. pilosicoli* showing faecal staining and soiling.

Figure 1.5 Thin shelled eggs from birds experimentally infected with *B. pilosicoli*. 
Histological examination of caeca from infected birds also may not reveal any abnormalities or there may be mild lymphocytic inflammation (Swayne and McLaren, 1997). In infection with *B. pilosicoli* the spirochaetes may be seen attached by one cell end to the caecal mucosa (Trampel *et al.*, 1994), but penetration of the caecal mucosa is rarely seen (Swayne *et al.*, 1992, 1995; Trampel *et al.*, 1994) (Figure 1.8). In contrast, penetration of spirochaetes between and below caecal epithelial cells and erosion of these cells or necrosis of the mucosa are often associated with infection with *B. intermedia* (Davelaar *et al.*, 1986; Dwars *et al.*, 1990, 1992a).
Fig. 1.7 Section of caecum showing presence of spirochaetes (Warthin-Starry x 400). Spirochaetes stained black.

Confirmation that the organisms are spirochaetes may be obtained by demonstration of spirochaete-specific antigens through direct or indirect fluorescent antibody tests (Hunter and Clark, 1975), by visualization of characteristic periplasmic flagella by electron microscopy, or by isolation and identification of the spirochaetes (see below). The latter allows discrimination between species and provides a clear diagnosis of AIS where pathogenic species are identified. It also allows determination of antimicrobial sensitivity of the isolates. Fresh faeces or caecal droppings are the samples of choice, or caecal contents obtained at necropsy. Samples should not be frozen but should be kept at 4°C in transit to the laboratory and cultured as soon as possible following receipt. The sensitivity of culture is dependent on the number of organisms present and the condition of the sample. Culture may be unsuccessful from birds.
that have received antimicrobial therapy, despite spirochaetes being observed in the samples. Generally it can take 3-10 days before spirochaetes are recovered in pure culture.

Fig. 1.8 End-on attachment of *B. pilosicoli* to caecal enterocytes producing brush border effect (Warthin-Starry x 400). Spirochaetes stained black.

Speciation of avian intestinal spirochaetes is somewhat difficult, as not all the cultured species have been fully defined. Some information can be obtained by biochemical profiling using commercial kits (Hunter and Wood, 1979), or determination of carbohydrate fermentation patterns (Phillips, 1976). For example, weakly haemolytic indole positive isolates are likely to be *B. intermedia* (Lee *et al.*, 1993), whilst hippurate positive
isolates are likely to be *B. pilosicoli* (Fellström and Gunnarsson, 1995) or *B. alvinipulli* (Stanton *et al.*, 1998). Analysis of 16S rRNA gene restriction patterns also may be a useful pointer to the species involved (Jensen *et al.*, 1992). To date, however, the most definitive methods of speciation have been MLEE (Lymbery *et al.*, 1990; Lee *et al.*, 1993; McLaren *et al.*, 1997), or polymerase chain reaction tests amplifying 16S rRNA, 23S rRNA or the gene for NADH oxidase (*nox*) (Park *et al.*, 1995; Leser *et al.*, 1997; Atyeo *et al.*, 1998, 1999; Suriyaarachahi *et al.*, 2000). The 16S rDNA gene appears to be the target of choice for identifying *B. pilosicoli* (Park *et al.*, 1995), whilst the 23S rDNA gene is better for *B. intermedia* (Leser *et al.*, 1997; Suriyaarachahi *et al.*, 2000). These PCRs can be used to amplify the initial growth from the primary isolation plate (Atyeo *et al.*, 1998). No PCRs have been developed to identify or detect *B. alvinipulli*. Recently these PCR assays have been adapted for direct detection of the spirochaetes in chicken faeces. The presence of inhibitors in the faeces is removed by washing them and then the DNA is extracted using a commercial spin column. A two-step nested duplex PCR assay subsequently has then been used for the rapid detection of *B. pilosicoli* (16S rRNA gene) and *B. intermedia* (*nox*) in the faeces (Phillips *et al.*, 2006). In the future, studies on AIS may be further facilitated by the use of PCR on DNA extracted from intestinal biopsy material, as used for human intestinal spirochaetosis (Mikosza *et al.*, 1999), by the use of *in situ* hybridization, as developed for porcine spirochaetes (Boye *et al.*, 1998), or by quantitative PCR to help determine the spirochaetal load in infected chickens.
Subspecific differentiation of avian isolates of *B. pilosicoli* and *B. intermedia* has been achieved using MLEE (McLaren *et al.*, 1997) and by pulsed field gel electrophoresis (PFGE) (Atyeo *et al.*, 1996; Suriyaarachahi *et al.*, 2000; Phillips *et al.*, 2005). PFGE in particular seems to be a useful technique for studying the molecular epidemiology of AIS, although to date few studies have been undertaken in this area. New multilocus sequence typing methods also show promise for examining relationships between *Brachyspira* isolates (Råsbäck *et al.*, 2007b).

1.10 **ANTIMICROBIAL TREATMENT**

There is little published information on the treatment of AIS in commercial poultry. Swayne (1997) has suggested that compounds used for the treatment of swine dysentery would be useful in chickens, and he also reported the successful use of neomycin to prevent clinical signs of AIS. Antimicrobial sensitivity testing of two US chicken isolates of *B. pilosicoli* and two of *B. alvinipulli* in agar dilution showed that they had high susceptibility to lincomycin, carbadox and tiamulin (drugs used for swine dysentery), but gave highly variable results for a range of other antimicrobials (Trampel *et al.*, 1999). These authors suggested that lincomycin was probably the drug of choice for AIS in the US since it was registered for use in broilers, whilst carbadox and tiamulin had not been cleared by the Food and Drug Administration’s Center for Veterinary Medicine for use in poultry. Smit and colleagues (1998) in the
Netherlands described the treatment of infected hens in the field with a 5-nitroimidazole compound, at a concentration of 120 ppm in the drinking water, for six days. They found that treatment of hens before the onset of lay prevented the negative effects on egg production, but treatment administered later was less effective and resulted in only a temporary effect. They postulated that this was due to either reinfection of the birds from the litter or to effective treatment of only part of the flock.

A recent study in the UK described the use of tiamulin to treat three laying hen flocks with AIS caused by *B. pilosicoli* (Burch et al., 2006). Treatment resulted in improved egg production, although it was not confirmed that the spirochaetes had been removed from the treated flocks. Tiamulin is now licensed for use in laying chickens in the UK.

1.11 CONTROL

As with most infectious diseases, it is more economic to prevent AIS than to treat the condition. Careful attention to biosecurity to prevent the introduction of spirochaetes on contaminated machinery, boots or clothing and effective rodent, insect and wild bird control, with consistently high levels of shed hygiene should reduce the chances of spirochaete infection. In infected sheds measures should be taken to minimize the birds’ contact with infected faeces and care also should be exercised to ensure that the birds are not subjected to undue stress (Kouwenhoven, 1993).
Studies have shown that avian intestinal spirochaetes are susceptible to commonly used disinfectants and do not survive for long in the environment of an infected shed (Phillips et al., 2003). Hence it should be relatively easy to remove the cycle of infection by disinfecting and leaving sheds empty before a new batch of chickens is introduced.

Recent studies have shown that diets based on wheat may increase susceptibility to colonization of laying hens with \emph{B. intermedia} compared to diets based on other cereal grains (Phillips et al., 2004a). Furthermore, some wheat varieties may be more problematic than others (Phillips et al., 2004b). The reason for this is not clear, but it may reflect the presence of specific non-starch polysaccharide components in the wheat. Where AIS is a problem, consideration should be given to examining and perhaps modifying the diet that is being used.

It is clear that further work is required to improve understanding of the epidemiology of the various species of intestinal spirochaetes involved in AIS and their relative contribution to disease. This knowledge should assist in the development of appropriate control programmes for AIS.

\subsection*{1.11 AIMS AND OBJECTIVES}

It will be clear from this review that the literature on AIS is sparse and in particular, very little work has been carried out on this syndrome in
Australia. In consequence, the work described in this thesis aimed to improve understanding of the distribution, disease association and control of AIS in Australia. To achieve this, the following objectives were identified:

a) to carry out a survey of commercial layer and meat breeder flocks in Australia to determine the occurrence and disease association of AIS;

b) to gather a representative collection of intestinal spirochaete isolates;

c) to attempt to identify and type the isolates in the collection and determine their antimicrobial susceptibilities;

d) to carry out infection studies using selected isolates; and

e) to examine possible methods of prevention and control of AIS
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Paper I


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In the late 1980s, there were reports of intestinal spirochaetes being isolated from commercial poultry flocks, particularly in the Netherlands. Although these isolates were not identified, they were reported as being associated with enteric disease.

In the study described in the following paper, the situation in commercial poultry flocks in eastern Australia was examined. The study aimed to determine whether intestinal spirochaetes were present, if so to what extent, what species were present and whether they were associated with disease.

Note that at the time this paper was written the spirochaetes were still called *Serpulina* spp. The paper is set out in the style of Avian Pathology.
Prevalence and disease association of intestinal spirochaetes in
chickens in eastern Australia

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SUMMARY

Faecal samples (n=1786) from chickens in broiler breeder (n=28), layer (n=22) or broiler (n=19) flocks in the eastern states of Australia were cultured for intestinal spirochaetes. Overall, birds in 42.9% of broiler breeder and 68.2% of layer flocks were colonised with spirochaetes, but no birds in broiler flocks were infected. Colonisation rates in infected flocks ranged from 10 to 100% of birds sampled. Faeces from colonised flocks were on average 14% wetter than those from non-colonised flocks. There was a highly significant association between colonisation with spirochaetes and the occurrence of wet litter and/or reduced production. A subset of 57 spirochaete isolates from birds in 16 flocks were identified to the species level using a panel of polymerase chain reaction tests. Isolates from nine (56%) of these flocks were spirochaetes that are known to be pathogens of poultry: thus *Serpulina pilosicoli* was isolated from birds from five flocks, birds from two flocks were infected with *Serpulina intermedia*, and in two other flocks both species were identified. Isolates from the other seven flocks belonged to other *Serpulina* species which are currently of unknown pathogenicity. This study indicates that infections with intestinal spirochaetes are a common but currently under-diagnosed cause of wet litter and/or reduced egg production in broiler breeder and layer flocks in Australia.
Introduction

Intestinal spirochaetes of the genus *Serpulina* are anaerobic, spiral-shaped bacteria which colonise the large intestine and can cause enteric disease in a range of animal species (Hampson and Stanton, 1997). These organisms have fastidious growth requirements, and hence can only be isolated using specialised media and anaerobic growth conditions. Over the past decade colonisation with intestinal spirochaetes has been recognised as a reason for previously unexplained production losses and/or diarrhoea in layers and broiler breeders in Europe, the United States and Western Australia (Davelaar *et al*., 1986; Griffiths *et al*., 1987; Dwars *et al*., 1989; Swayne *et al*., 1992, 1995; Trampel *et al*., 1994; McLaren *et al*., 1996).

Colonisation of adult birds with intestinal spirochaetes has been associated with a number of clinical signs and production problems, including diarrhoea, increased fat content of the faeces, faecal staining of eggshells, delayed onset of egg laying, reduced egg weights, reduced growth rates, increased feed consumption and poor digestion of feed (Davelaar *et al*., 1986; Griffiths *et al*., 1987; Swayne *et al*., 1992; Dwars *et al*., 1990, 1992a, 1993; Trampel *et al*., 1994). Moreover, in a recent comprehensive study carried out in the Netherlands (Smit *et al*., 1998), broiler flocks derived from breeder flocks infected with spirochaetes were found to have poorer feed conversion, an increased number of weak chicks, slower growth and a poorer feed digestion than the offspring of
flocks where spirochaetes were not present. The broilers themselves were not colonised by the spirochaetes. Colonisation and disease in adult birds tend to be chronic (Dwars et al., 1990). In experimentally-infected broilers retarded growth is the main indicator of infection (Dwars et al., 1992b), but natural colonisation of broilers in the field has not been described.

The spirochaetes originally isolated from adult poultry in Europe and the United States were not all identified to the species level, but a recent analysis of a subset of these organisms has demonstrated that a number of species are involved, not all of which are necessarily capable of causing disease (McLaren et al., 1997). The pathogenic species workers in Europe appear to have been concerned with was Serpulina intermedia (Dwars et al., 1992a,b, 1993), whilst reports from the US involved Serpulina pilosicoli (Trampel et al., 1994) or Serpulina alvinipulli (Swayne et al., 1992, 1995; Stanton et al., 1998). Interestingly both S. intermedia and S. pilosicoli are also considered to be pathogens of pigs (Hampson and Trott, 1995), and S. pilosicoli also infects dogs, other animal species and human beings (Trott et al., 1997a).

In the Netherlands, Dwars and colleagues (1990) used immunofluorescence staining to demonstrate spirochaetes in the faeces of birds in 28% of flocks with diarrhoea, compared to their presence in 4% of flocks with no disease signs. Similarly in a more recent study in Western Australia (WA), intestinal spirochaetes were isolated from birds
in 64% of flocks with signs of intestinal disease, compared with 28% of clinically normal flocks (McLaren et al., 1996). The lower apparent overall prevalence of infection in Europe than WA may reflect the different methodologies used to detect the organisms, rather than true differences in prevalence.

Using multilocus enzyme electrophoresis (MLEE), 41% of the WA isolates analysed were identified as *S. intermedia*, 56.4% as belonging to a previously undescribed and unnamed group of uncertain pathogenicity (provisionally designated as “*Serpulina pulli*”), and the rest as *Serpulina innocens*, a non-pathogenic species known to infect pigs (McLaren et al, 1997). No isolates of *S. pilosicoli* nor *S. alvinipulli* were recovered in WA. An isolate of *S. intermedia* from a WA layer was subsequently used to experimentally infect layer hens, causing increased faecal water content and reduced egg production, and thus confirming its pathogenic potential (Hampson and McLaren, 1999).

The current survey was designed to determine whether the prevalence of intestinal spirochaete infections in flocks in the eastern states of Australia was as high as already demonstrated in WA, and hence whether these organisms represent a national problem to the industries. Other aims were to determine what species of spirochaetes are involved, and to gain more information as to what extent these infections are associated with intestinal disease and/or reduced production in Australia.
Materials and methods

Collection of faecal samples

Faecal samples (n=1786) were collected from chickens on 69 farms, comprising 19 broiler, 28 broiler breeder and 22 layer farms located in the Australian states of Queensland, New South Wales, Victoria, Tasmania and South Australia (Table 1). Wherever possible, a minimum of 20 freshly-dropped caecal faeces samples of 5-20 g were collected from individual birds on each farm, and a bulk pooled sample was collected for faecal moisture determination. Samples were collected into sealed containers, held on ice and transported by overnight courier to the Toowoomba Veterinary Laboratory (TVL). At the time of collection, management at the farms was asked to supply information on the age and type of bird, housing, whether adult birds were caged or not, litter type and litter management practices (eg litter completely cleaned out and replaced between batches of birds, or new litter only provided in brooding areas), disinfectants used, and whether or not there were any perceived problems with growth rates, egg production, egg quality or wet litter.

Antimicrobial treatment of an infected flock

A broiler breeder farm reported unexplained diarrhoea in 20% of the flock with reduced production that had been ongoing for several months.
Problems were particularly pronounced in two sheds, each housing 8000 broiler breeders at 40 weeks of age. Initially, as part of the survey for spirochaetes, 10 samples from one shed and 15 from the second shed were cultured. Subsequently two birds from the first shed were subjected to postmortem examination, and 10 faecal samples from each of the other three sheds on the farm were also cultured for spirochaetes.

The birds in the first two sheds were then treated with antimicrobials in the water. The first shed was treated with lincospectin for seven days at a rate of 50 mg per bird per day, and the second shed was treated with tiamulin at 25 mg/kg for five days. Samples from both sheds were cultured on the last day of treatment and at weekly intervals thereafter. After one month sampling was carried out fortnightly and monthly after three months. Nineteen weeks after the original treatment, both sheds were water-medicated with oxytetracycline at 60 mg/kg for four days. Samples were collected from both sheds two days after treatment and again four weeks later.

Sample culture

Each faecal sample was plated on selective Trypticase Soy Agar (Micro Diagnostics, Brisbane, Australia), supplemented with 5% defibrinated bovine blood, 0.1% porcine mucin (Sigma, Castle Hill, Australia), 200 μg per ml spectinomycin and 6.25 μg per ml each of colistin and vancomycin. Plates were incubated in an anaerobic atmosphere
generated by Anaerogen sachets (Oxoid, Basingstoke, United Kingdom). Plates were checked after five and 10 days incubation for spirochaetal growth using dark field microscopy. Isolates were repeatedly subcultured until pure.

*Faecal moisture content*

Bulk faecal samples were weighed and then dried to constant weight. The percentage faecal moisture was determined using the formula: % faecal moisture = wet weight – dry weight X 100/wet weight.

*Identification of isolates by Polymerase Chain Reaction (PCR)*

A total of 57 isolates from 16 farms were subjected to further identification. Twenty eight isolates were from one broiler breeder farm and the others were from three other broiler breeder farms and 12 layer flocks, with between 1-7 isolates tested per flock. Polymerase chain reaction (PCR) tests were used to identify pure cultures of the isolates. Cells picked from the plate with a sterile toothpick were added directly to the PCR mix. The methodology has been described previously (Park *et al.*, 1995; Atyeo *et al.*, 1998). Amplification targets used were the 16S rRNA gene for *Serpulina pilosicoli* (Park *et al.*, 1995; Atyeo *et al.*, 1998), the 23S rRNA gene for *S. intermedia* (Leser *et al.*, 1997), and the nox gene for both the non-pathogenic species *S. innocens* and *S. murdochii* (Atyeo *et al.*, 1999).
Analysis of results

Results for the survey were analysed to determine prevalence rates of infection overall, prevalence for each of the three types of flock, and association between infection and signs of intestinal disease and/or reduced production. Flocks were divided into those reporting wet litter and/or production problems and those reporting normal health and production, and the significance of the difference was determined by chi-squared analysis. Faecal moisture content was also compared for infected and non-infected flocks, using Student’s t-test. The results were also examined to determine if there was any apparent correlation between infection and other factors recorded. Where isolates were identified, results were analysed in relation to the disease history of the relevant farm, where this was available.

Results

Prevalence of infection

Results are summarised in Table 1. Weakly beta-haemolytic intestinal spirochaetes were isolated from birds on 27 of 69 (39.1%) farms sampled, with 287 of 1786 (16.1%) individual faecal samples being positive. Spirochaetes were not isolated from any of the 19 broiler units. In contrast 12 of the 28 (42.9%) broiler breeder farms were colonised,
with spirochaetes detected in 29 of the 112 (25.9 \%) sheds on these farms, and in 126 of 812 (15.5 \%) individual samples. Spirochaetes were detected in 15 (68.2\%) of the 22 commercial layer farms, being isolated from 37 of the 68 (54.4 \%) sheds tested and 161 of the 589 (27.3 \%) faecal samples.

**Table 1.** Proportion of farms, sheds and faecal samples from which intestinal spirochaetes were detected

<table>
<thead>
<tr>
<th></th>
<th>Broilers</th>
<th>Broiler breeders</th>
<th>Layers</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farms</td>
<td>0/19</td>
<td>12/28</td>
<td>15/22</td>
<td>27/69</td>
</tr>
<tr>
<td>Sheds</td>
<td>0/45</td>
<td>29/112</td>
<td>37/68</td>
<td>66/225</td>
</tr>
<tr>
<td>Samples</td>
<td>0/385</td>
<td>126/812</td>
<td>161/589</td>
<td>287/1786</td>
</tr>
</tbody>
</table>

Comparison of prevalence of infection in birds of different ages

Faecal samples from birds of ages from one day to 100 weeks were cultured. Spirochaetes were not isolated from samples from birds under 10 weeks of age. Above this age, rates of infection (\% of positive samples) in sheds that were positive for spirochaetes gradually increased, reaching the highest prevalence in the oldest birds. This analysis was based on 68 infected sheds for which age data were available. In sheds containing birds aged from 10 to 39 weeks (n=23) the average prevalence of infection was 40.5\%; in sheds containing
birds aged from 40 to 69 weeks (n=36) the average prevalence of colonisation was 44.9%; in sheds containing birds aged from 70 to 100 weeks (n=7) the average prevalence was 81.1%. The trend for colonisation rates to increase with the age of the birds just failed to reach significance.

**Distribution of colonisation with respect to sheds**

Samples were obtained from birds in multiple sheds on 52 farms, with the maximum number of sheds sampled on any one farm being 12. Birds on 21 of these farms were positive for spirochaetes and in 13 cases (61.9%) all sheds on the individual farm had colonised birds.

**Within-flock prevalence of colonisation**

The within-flock prevalence of colonisation in the positive broiler breeder and layer flocks varied from 10% to 100%. The mean prevalence over all flocks was 46.7% (Standard Error 5.4), with 37.3% (SE 9.8) for the broiler breeders and 52.6% (SE 6.0) for the layers.

**Significance of management factors**

No consistent association was identified between colonisation with intestinal spirochaetes and the management factors recorded or the breed of bird involved.
Association with wet litter and/or reduced production

Information on the occurrence of wet litter or production problems was only available for 54 of the 69 flocks tested. Of these, 34 reported no disease signs and spirochaetes were isolated from five (14.7%). Twenty flocks reported disease signs, including wet litter, production drop or both. Fourteen of these flocks (70%) were colonised with spirochaetes (Table 2). These differences were highly statistically significant in the chi-square test with Yates adjustment (P < 0.0001). Spirochaetes were not isolated from six flocks that reported problems of wet litter. Four of these were broiler flocks from one company, located in the same area, and the wet litter was suspected to be associated with the poor quality of their water supply. The other two flocks with diarrhoea but no spirochaetes were unrelated broiler breeder flocks.

Spirochaetes were obtained from 11 layer flocks for which adequate disease information was available. Of these, nine (81.8%) reported wet litter and/or production drop, while two flocks were healthy. Adequate disease information was only available for seven of the eight broiler breeder flocks that were positive for spirochaetes. Of these seven, five (71.4%) reported problems, with three having wet litter and two wet litter associated with reduced production. The other two flocks reported no disease signs.
**Table 2.** Association between the isolation of intestinal spirochaetes and signs of disease

<table>
<thead>
<tr>
<th>Intestinal disease status of flock</th>
<th>Flocks tested</th>
<th>Flocks infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>- wet litter</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>- production drop</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>- both the above</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>No disease signs</td>
<td>34</td>
<td>5</td>
</tr>
</tbody>
</table>

**Association with increased faecal moisture**

Average percent faecal moisture was determined for 49 of the 69 flocks sampled (71.0%) and the results are summarised in Table 3. The faecal moisture contents of colonised broiler breeder flocks and layer flocks were not significantly different. The average moisture contents in uncolonised flocks of the three flock types also did not differ significantly. Average faecal moisture however was significantly higher for both broiler breeder and layer flocks which were colonised with intestinal spirochaetes than for non-colonised flocks. Overall, broiler breeder and layer flocks that were colonised with intestinal spirochaetes had significantly wetter faeces than uncolonised flocks (by approximately 14%; p<0.001).
Identification of isolates and association with disease

Overall 18 of the 57 (31.6%) isolates from birds on 16 farms that were examined by PCR were identified as *Serpulina pilosicoli*, seven (12.3%) as *S. intermedia*, 18 (31.6%) were positive in the *S. innocens/S. murdochii* PCR, and 14 (24.5%) did not give a positive result with any of the tests (Table 4). Of the 18 isolates identified as *S. innocens/murdochii* 17 were from one farm where multiple isolates were examined. *S. pilosicoli* was isolated from birds on six of the 16 farms from which spirochaetes were tested, and *S. intermedia* was recovered from birds on four farms (two of which were also colonised with *S. pilosicoli*).

Spirochaetes of unknown species were identified as the sole isolates on five farms, and were isolated with other species on three farms. Isolates identified as *S. innocens/S. murdochii* were found on two farms, one of which was also colonised with *S. pilosicoli*.

Only eight of these 16 farms reported the health status of their birds, and all eight considered they had clinical problems (Table 4). These included diarrhoea, lethargy, egg production drop and poor growth rates. Isolates of *S. pilosicoli*, *S. intermedia* or both species were recovered from five of these farms, but the spirochaetes recovered from the other three farms could not be speciated using the PCRs.
Table 3. Association between faecal moisture content in flocks and detection of intestinal spirochaetes.

<table>
<thead>
<tr>
<th></th>
<th>Average faecal moisture (%) ± Standard deviation (n= number of flocks)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Broilers</td>
<td>Broiler breeders</td>
</tr>
<tr>
<td>Infected flocks</td>
<td>NA</td>
<td>69.0 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>(n=6)</td>
<td></td>
</tr>
<tr>
<td>Uninfected flocks</td>
<td>57.4 ± 5.3</td>
<td>58.9 ± 11.9</td>
</tr>
<tr>
<td></td>
<td>(n=18)</td>
<td></td>
</tr>
<tr>
<td>Significance</td>
<td>NA</td>
<td>p&gt;0.005</td>
</tr>
</tbody>
</table>

NA, Not applicable; NS, Not significant
Table 4. Identification of intestinal spirochaete isolates from 16 farms using PCR tests, and clinical history on the farms.

<table>
<thead>
<tr>
<th>Farm</th>
<th>Isolates</th>
<th>Identification</th>
<th>Clinical History</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler breeder 1</td>
<td>17 isolates</td>
<td><em>S. innocens/murdochii</em></td>
<td>Diarrhoea in 5-20% of flock, production drop.</td>
</tr>
<tr>
<td></td>
<td>6 isolates</td>
<td><em>S. pilosicoli</em></td>
<td></td>
</tr>
<tr>
<td>Broiler breeder 2</td>
<td>1 isolate</td>
<td>Unknown</td>
<td>Diarrhoea, production drop, lethargic, anorexic</td>
</tr>
<tr>
<td></td>
<td>2 isolates</td>
<td><em>S. pilosicoli</em></td>
<td></td>
</tr>
<tr>
<td>Broiler breeder 3</td>
<td>3 isolates</td>
<td><em>S. pilosicoli</em></td>
<td>N/A Abattoir sample</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>S. pilosicoli</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 isolates</td>
<td><em>S. intermedia</em></td>
<td></td>
</tr>
<tr>
<td>Layer 2</td>
<td>1 isolate</td>
<td><em>S. pilosicoli</em></td>
<td>Lethargic, depressed, off lay.</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>S. intermedia</em></td>
<td></td>
</tr>
<tr>
<td>Layer 3</td>
<td>1 isolate</td>
<td>Unknown</td>
<td>Severe diarrhoea, production drop.</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>S. pilosicoli</em></td>
<td></td>
</tr>
<tr>
<td>Layer 4</td>
<td>1 isolate</td>
<td><em>S. pilosicoli</em></td>
<td>Obvious illness, diarrhoea, egg drop</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>S. intermedia</em></td>
<td></td>
</tr>
<tr>
<td>Layer 5</td>
<td>1 isolate</td>
<td><em>S. intermedia</em></td>
<td>Poor growth rate, stunting, mortalities</td>
</tr>
<tr>
<td>Farm</td>
<td>Isolates</td>
<td>Identification</td>
<td>Clinical History</td>
</tr>
<tr>
<td>------------</td>
<td>----------</td>
<td>-------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Layer 6</td>
<td>2 isolates</td>
<td><em>S. intermedia</em></td>
<td>Weakness, lethargy, some mortalities.</td>
</tr>
<tr>
<td></td>
<td>1 isolate</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Layer 7</td>
<td>1 isolate</td>
<td><em>S. intermedia</em></td>
<td>N/A</td>
</tr>
<tr>
<td>Layer 8</td>
<td>1 isolate</td>
<td>Unknown</td>
<td>Wet litter</td>
</tr>
<tr>
<td>Layer 9</td>
<td>5 isolates</td>
<td><em>S. pilosicoli</em></td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>2 isolates</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Layer 10</td>
<td>1 isolate</td>
<td>Unknown</td>
<td>N/A</td>
</tr>
<tr>
<td>Layer 11</td>
<td>1 isolate</td>
<td><em>S. innocens/murdochii</em></td>
<td>N/A</td>
</tr>
<tr>
<td>Layer 12</td>
<td>3 isolates</td>
<td>Unknown</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A: not available. Unknown: could not be identified by PCR methods.

*Effect of treatment with antimicrobials*

Spirochaetes were isolated from 10/10 and 11/15 samples respectively from the first two sheds sampled on the farm in question, and from 8/10, 6/10 and 4/10 samples from the other three sheds. Histological examination of sections of caeca from two birds revealed large numbers of spirochaetes in crypts, with occasional penetration of the mucosa.
Culture of caecal contents yielded very heavy growth of spirochaetes from both birds. Two isolates from these birds were identified as *S. innocens/murdochii*. An isolate obtained from a faecal sample from the second shed was identified as *S. pilosicoli*. Three further isolates obtained from faecal samples from two other sheds on this farm were identified as *S. innocens/murdochii*

Following antimicrobial treatment the condition of the birds was reported as improving slowly, although production figures were not made available. Treatment with lincospectin resulted in the occurrence of slimy faeces, which lasted for two to three weeks. After three weeks, spirochaetes were isolated from the shed that had been treated with tiamulin, although at a reduced prevalence (30% positive). This prevalence continued in this shed for almost three months after which it increased to 80%, slightly greater than the level prior to treatment. The shed treated with lincospectin remained negative for almost three months, when 30% of samples cultured were positive. This level of colonisation continued for almost four months. Following oxytetracycline medication samples from the first shed were negative, while the prevalence of colonisation in the second shed was reduced from 80% to 60%. Four weeks later, samples from the first shed were still negative, while the prevalence in shed two had increased to 70%.
**Discussion**

The survey detected intestinal spirochaetes in birds from 40.6% of the 69 commercial poultry farms tested. This overall result is very similar to that of the previous WA survey, which detected spirochaetes in 43.3% of 67 flocks tested (McLaren *et al*., 1996). These results confirm that significant levels of colonisation with intestinal spirochaetes are present in flocks throughout Australia.

An important finding was that all 19 broiler farms tested were negative for spirochaetes, although it should be noted that only four of these reported problems with wet litter. This lack of colonisation is consistent with reports from the Netherlands. Whilst broiler chicks are highly susceptible to experimental infection with intestinal spirochaetes, it is assumed that the all-in all-out management systems used to raise these birds commercially, or the specific litter management practices employed on broiler farms, may prevent access to and/or build up of infection over the brief life of the birds.

In contrast to broilers, 42.9% of the 28 broiler breeder flocks sampled were colonised with spirochaetes. This is very similar to the corresponding result from the WA study, which found 53.3% of 30 broiler breeder flocks to be positive. Together these studies indicate a significant level of colonisation of broiler breeder flocks in Australia. This result is particularly relevant given the recent work of Smit *et al* (1998),
which showed that broiler flocks derived from breeder flocks colonised by spirochaetes have poorer feed conversion rates, an increased number of weak chicks and slower growth rates than the progeny of flocks that were not colonised.

An extremely high level of colonisation was found in layer flocks, with 15 of the 22 (68.2%) farms sampled being positive. This was almost twice the prevalence found amongst the 37 layer flocks previously sampled in WA (35.1%). The high level of colonisation might have been contributed to by the disease status of the farms that elected to participate in the survey, since a majority reported health problems. Three layer farms volunteered to participate in the survey specifically because of persistent problems with wet litter and diarrhoea, whilst the same was true for two breeder flocks which had production problems of unknown aetiology.

A positive trend was observed between within-flock prevalence of colonisation and increasing age of bird. No spirochaetes were isolated from birds below 10 weeks of age but above that age prevalences of colonisation in positive flocks gradually increased to reach 81.1% in birds aged from 70 to 100 weeks. The high rates seen in some layer flocks may be a consequence of the older average age of this type of flock, that is, colonisation is a function of length of exposure. This is an area that requires more detailed investigation.
Some information was obtained on the distribution of colonisation on individual farms. Twenty one of 52 farms with multiple sheds, the majority with five to eight sheds, were positive for spirochaetes, and all sheds on the farm were positive in 13 (61.9%) cases. In addition, the colonisation seen in the different sheds frequently correlated with the increasing ages of the birds. This suggests that infection gradually builds up with time, but it does not identify the original source of the organisms. Colonised birds may be brought onto the farm, or the organisms may already exist there and the birds are gradually colonised as they become exposed. Within-shed prevalence varied from 10% to 100% of samples (overall mean of 46.7% of samples). Future work should investigate aspects of strain dissemination within and between flocks by the use of molecular strain typing techniques.

Overall the results revealed a significant association between colonisation and disease. Spirochaetes were isolated from 14 of 20 (70.0%) flocks with either signs of intestinal disease such as diarrhoea or wet litter, or egg production drop, or both, but from only five of 34 (14.7%) flocks with no reported evidence of disease. This difference was highly statistically significant, and again was very similar to the previous findings in WA, and to the situation reported in Europe. Colonised flocks had faeces which were on average approximately 14% wetter than those from non-colonised flocks. Accurate measurement of the water content of bulked faecal samples is difficult, and is influenced by selection of samples and storage, but nevertheless the differences
recorded between the flocks that were colonised or uncolonised were substantial. Such clear increases in faecal water content are likely to cause problems with mechanical cleaning, build up of faeces on cages, faecal staining of eggs, and problems of odour and flies. Six flocks comprising four broiler and two broiler breeder flocks were identified which had wet litter, but from which no spirochaetes could be isolated. Thus, not unexpectedly, it is apparent that intestinal spirochaetes are not the only cause of wet litter problems. Similarly the presence of intestinal spirochaetes in some flocks which did not report signs of disease suggest that certain of these organisms may be apathogenic or require other predisposing factors before they can induce disease.

Of the 57 isolates examined by PCR 31.6% were \textit{S. pilosicoli} and 12.3% were \textit{S. intermedia}. Both species are known to be pathogenic to poultry and both are also pathogens of swine, a fact that may have important implications for farm management. Furthermore \textit{S. pilosicoli} infects human beings (Trott \textit{et al.}, 1997a), so the possibility of zoonotic spread of these organisms from chickens to humans must now also be considered. Besides causing intestinal disease in humans, \textit{S. pilosicoli} has been isolated from the bloodstream of a series of debilitated patients (Trott \textit{et al.}, 1997b). It is unclear whether the same extraintestinal spread may occur in naturally infected chickens, and whether it may contribute to systemic disease.
Interestingly, no *S. pilosicoli* isolates were identified in the earlier WA study, although infection with *S. intermedia* strains was common. Both these species have been shown to induce diarrhoea and reduced growth rates in experimentally-infected day old broiler chicks (Hampson and McLaren, 1997), whilst *S. intermedia* has caused increased faecal water and reduced egg production in experimentally-infected layer hens (Dwars *et al.*, 1992; Hampson and McLaren, 1999). To our knowledge, although natural infection with *S. pilosicoli* has been associated with a 5% reduction in flock egg production (Trampel *et al.*, 1994), layer hens have not been experimentally infected with these organisms to confirm their pathogenicity in adult birds.

Of the other 32 isolates, 18 (31.6% overall) reacted with the *S. innocens/S. murdochii* PCR, and 14 (24.5% overall) gave no product with any of the three PCRs. In pigs, the *S. innocens/S. murdochii* group of spirochaetes are considered to be apathogenic, but the situation in poultry is less clear. In broiler breeder farm 1, where 17 of these isolates originated, the birds had diarrhoea and production drop. Interpretation of this finding however is clouded by the fact that *S. pilosicoli* was also isolated from birds on the farm. The identity of the spirochaete isolates that did not react with any of the PCRs remains uncertain. Chickens are known to be infected with two other *Serpulina* spp. which so far have not been recorded in other species - the pathogenic *S. alvinipulli* (currently only detected in chickens in the USA), and a large group of organisms provisionally designated “*S. pulli*” detected in the WA study (McLaren *et al.* 1997).
The latter group was mildly pathogenic in experimentally-infected broilers (Hampson and McLaren, 1997). All the spirochaetes recovered in this study now should be fully identified to the species level, and the pathogenic potential of selected isolates tested in experimentally-infected birds.

The spirochaetes that were speciated all originated from 16 farms. Amongst these there was no clear pattern of association between the clinical data and the specific species isolated. Only eight of these farms reported clinical data, and all these had various forms of production problems including wet litter and reduced egg production. Five farms were infected with *S. pilosicoli* or *S. intermedia* or both, and this could account for the disease seen. On the other three farms single isolates of spirochaetes of unknown species were recovered. Testing of single isolates from these farms complicates interpretation, since on seven farms other where multiple isolates were examined five were found to be colonised by spirochaetes of more than one species. Even where spirochaetes of the same species were isolated from a farm, no subspecific differentiation was undertaken to determine whether these were the same or different strains of the species.

In the therapeutic trial, water medication with both lincospectin and tiamulin appeared to be effective in removing colonisation in the short term. Unfortunately in the birds treated with tiamulin spirochaetes were again detected after three weeks, although this did not occur for 10
weeks in the shed treated with lincospectin. It is not known whether the same strains or even species of spirochaetes re-emerged after treatment, and this requires analysis. Treatment with lincospectin apparently caused a transient slimy diarrhoea, suggesting that although effective at removing spirochaetes temporarily, this drug may not be particularly appropriate for use in chickens at the dose rates utilised. Subsequent treatment of both sheds with oxytetracycline appeared to eliminate spirochaetes from the birds in the shed treated with lincospectin, but not from the shed treated with tiamulin. This failure may have been the result of the presence of a higher initial infectious load, as tiamulin had failed to keep the infection under control for some weeks before oxytetracycline was administered.

The only other report describing the use of antimicrobials to control intestinal spirochaetal infections in chickens was recently published by Smit et al. (1998). Treatment of breeder hens before the onset of lay with a 5-nitroimidazole compound (120 ppm Ridzol) in the drinking water for approximately six days helped maintain egg production in colonised birds, although in some cases only temporarily. Treatment after the onset of lay was less effective. Although antimicrobial treatment of breeders is possible, their use in layer flocks is problematic because of potential drug residues in eggs produced for human consumption. Clearly much more work is required to investigate means to control intestinal spirochaetes in chickens.
In summary this study has demonstrated a high prevalence of colonisation with intestinal spirochaetes in broiler breeder and layer flocks in Australia. Several species of organisms are involved, including pathogenic *S. intermedia* and *S. pilosicoli*. Colonisation is significantly associated with production problems and wet litter. Colonisation tends to be chronic, and to date antimicrobial treatment has met with limited success at long term control.

**Acknowledgements**

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Experimental infection of broiler breeder hens with the intestinal spirochaete *Brachyspira (Serpulina) pilosicoli* causes reduced egg production.

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Following the prevalence study described above, the next aim was to examine, in a controlled environment, the pathogenicity of two species of intestinal spirochaete, both of which species had been prevalent in the isolations obtained during the course of the aforementioned study. In order to make the study as relevant as possible to industry, commercial meat breeder birds were inoculated with strains previously isolated from commercial birds in eastern Australia.
Experimental infection of broiler breeder hens with the intestinal spirochaete *Brachyspira* (Serpulina) *pilosicoli* causes reduced egg production

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SUMMARY

The pathogenic potential of the anaerobic intestinal spirochaetes *Brachyspira* (Serpulina) *pilosicoli* and *Brachyspira innocens* was evaluated in adult chickens. Thirty 17-week-old Cobb broiler breeder hens were individually caged in three equal groups of 10 birds. Control birds were sham inoculated with sterile broth medium. Birds in the other two groups were inoculated respectively with an isolate of *B. innocens* or of *B. pilosicoli*. Birds were monitored daily, and killed at 41 weeks of age. Infection had no consistent effect on body weight gain, but inoculation with *B. pilosicoli* resulted in a brief increase in faecal water content. *B. innocens* had no effect on egg production, but *B. pilosicoli* infection caused a delayed onset of laying, and a highly significant reduction in egg production over the first 11 weeks of lay. This study confirms that *B. pilosicoli* can cause serious egg production losses in adult chickens, whilst *B. innocens* is non-pathogenic.
Introduction

Spirochaetes belonging to the genus *Brachyspira* (formerly *Serpulina*) are anaerobic, spiral-shaped bacteria which colonise the large intestine and can cause enteric disease in a number of animal species (Hampson & Stanton, 1997). These intestinal spirochaetes can only be isolated after a minimum of 3-5 days incubation using specialised selective media and anaerobic growth conditions.

To date, colonisation of layer and broiler breeder birds with intestinal spirochaetes has only been recorded in continental Europe, the United Kingdom, the United States and Australia (Davelaar et al., 1986; Griffiths et al., 1987; Dwars et al., 1989; Swayne et al., 1992, 1995; Trampel et al., 1994; McLaren et al., 1996; Stephens & Hampson, 1999). Colonisation tends to be chronic in adult birds in infected flocks (Dwars et al., 1990), and has been associated with a variety of symptoms including diarrhoea, increased faecal fat content, faecal staining of eggshells, delayed onset of egg laying, reduced egg weights, reduced growth rates, increased feed consumption and poor digestion of feed (Davelaar et al., 1986; Griffiths et al., 1987; Swayne et al., 1992; Dwars et al., 1990, 1992a, 1993; Trampel et al., 1994). Broiler flocks derived from the offspring of breeder flocks infected with spirochaetes have been shown to have poorer feed conversion, an increased number of weak chicks, slower growth and a poorer feed digestion than the offspring of flocks where spirochaetes are not present (Smit et al., 1998). Experimentally-infected broiler chicks
show retarded growth (Dwars et al., 1992b), but, despite several investigations, natural colonisation of broilers with spirochaetes has not been detected in the field (Stephens & Hampson, 2001).

Several different species of intestinal spirochaetes naturally colonise chickens, and not all are necessarily capable of causing disease (McLaren et al., 1997). The pathogenic species that workers in Europe and the UK appear to have been most concerned with is *Brachyspira intermedia* (Griffiths et al., 1987; Dwars et al., 1992a,b, 1993), although strains of *B. pilosicoli* also have been identified (McLaren et al., 1997). Reports from the US have involved either *B. pilosicoli* (Trampel et al., 1994) or *Brachyspira alvinipulli* (Swayne et al., 1992, 1995; Stanton et al., 1998). In Australia both *B. intermedia* and *B. pilosicoli* have been isolated from layer and broiler breeder flocks with production problems (Stephens & Hampson, 1999). Other intestinal spirochaete species, including *B. innocens*, also have been isolated from Australian chickens. *Brachyspira innocens* is generally considered to be a non-pathogenic spirochaete in conventional pigs (Kinyon & Harris, 1979), although porcine strains of this species have caused mucoid faeces and typhlocolitis in gnotobiotic pigs (Neef et al., 1994).

In the Netherlands, experimental infection of adult birds with spirochaete strain 1380, later identified as *B. intermedia* (McLaren et al., 1997), resulted in increased faecal water content, reduced egg production and poor performance in broilers hatched from the infected birds (Dwars et
al., 1989; 1992a; 1993). In Australia, experimentally infection of layer hens with an Australian chicken isolate of *B. intermedia* resulted in prolonged caecal colonisation, increased faecal moisture content and reduced egg production (Hampson & McLaren, 1999). In contrast to the situation with *B. intermedia*, there have been no reports of studies using either *B. pilosicoli* or *B. innocens* to experimentally infect adult birds. *B. pilosicoli* is of particular comparative interest because it colonises many bird and animal species, including human beings (Trott *et al*., 1997a, 1997b; Oxberry *et al*., 1998; Trivett-Moore *et al*., 1998; Brooke *et al*., 2001). The existence of cross-species transmission of *B. pilosicoli* has important implications for control of the infection in chicken flocks, whilst the possibility of zoonotic transfer increases the need for further study of this spirochaete.

The overall aim of the current study was to investigate the pathogenic potential of Australian chicken strains of *B. pilosicoli* and *B. innocens* in meat breeder hens, by inoculating them into birds from the same flock from which the strains were recovered.

**Materials and Methods**

*Experimental birds*

Thirty Cobb 500 broiler breeder females were obtained from a commercial producer at 13 weeks of age. The birds were placed in
individual cages with mesh floors and egg roll-out trays. The cages were specially constructed to be large enough to accommodate breeder females. Each cage was provided with a waste tray for the collection of faeces. Clear plastic sheet was hung between cages to minimise the risk of transmission of infection between cages. The birds were kept in an air-conditioned room with temperatures varying between 17-23 °C. The day-length was set at 8 hours until 19 weeks of age, then gradually increased to 15 hours until 23 weeks of age and thereafter maintained at 16 hours. The birds were fed commercial diets supplied by the farm of origin, and these contained 50 ppm zinc bacitracin. They received a pullet developer diet until 19 weeks of age, then a pre-breeder ration. When egg production in the control group reached approximately 15%, all the birds were given a breeder production mix. Feed intake was restricted, with the birds being given 62 grams daily at 13 weeks of age, and this being gradually increased to a maximum of 165 grams per day by 27 weeks of age. Water was provided ad libitum by means of individual water bottles with nipple drinkers.

*Spirochaete strains used for experimental infection*

*Brachyspira pilosicoli* strain CPSp1 and *B. innocens* strain CPSi1, which were used to infect the birds, were isolated from breeders on the same farm from which the test birds originated. The strains had been isolated two years before the current study, during the course of a disease investigation, and their species identity had been confirmed through
biochemical testing and polymerase chain reaction amplification of portions of their 16S rRNA and NADH oxidase genes (Stephens & Hampson, 1999). For experimental infection, the spirochaetes were grown to mid-log phase in Kunkle's broth (Kunkle et al., 1986). The inocula used to infect the birds contained approximately $10^8$ bacterial cells per mL.

**Experimental infection and monitoring of birds**

The birds were acclimatised for four weeks. Over this period, individual faecal samples from each bird were taken weekly. These were cultured for spirochaetes on selective Trypticase Soy Agar (Micro Diagnostics, Brsibane, Australia), supplemented with 5% defibrinated bovine blood, 0.1% porcine mucin (Sigma, Castle Hill, Australia), 200 µg/ml spectinomycin and 6.25% µg/ml each of colistin and vancomycin. Plates were incubated in an anaerobic environment generated by Anaerogen sachets (Oxoid, Basingstoke, United Kingdom), and growth examined by dark field microscopy after 5 and 10 days. The species identity of isolates obtained during the course of the study was determined using a species-specific PCR protocol, as previously described (Stephens & Hampson, 1999).

After four weeks the birds were weighed and randomly assigned on the basis of body weight to one of three groups, each of ten birds. Birds in Group A (control group) were inoculated orally with one mL of sterile
broth. Birds in Groups B and C were inoculated with one mL of a broth culture of either *B. innocens* or *B. pilosicoli* respectively.

Eggs from each bird were collected, counted and weighed every day. Once a week the birds were weighed, and individual faecal samples collected and cultured for intestinal spirochaetes. The faecal samples were weighed, dried to constant weight in a hot air oven, and the percentage faecal moisture calculated.

*Post mortem examination*

At 41 weeks of age the birds were killed by cervical dislocation, and subjected to post mortem examination. The caeca, ovaries and oviducts were examined grossly and sections fixed in 10% buffered formalin for subsequent histological examination. Caecal contents were cultured for intestinal spirochaetes.

*Analysis*

Weekly group bird weights and faecal moisture content were compared using one-way analysis of variance. Means were compared using Fisher’s protected least significant difference method, and significance was accepted at the 0.5% level. Group egg production per week was compared using Chi square tests, except where values per cell were less than five, when Fisher’s exact test was used in two by two
contingency tables. Full egg production was assumed to be one egg/bird/day. The weights of the eggs produced were analysed by calculating a mean weight of eggs produced from each bird per week (ie total weight/number of eggs), then calculating group means of these bird means each week. These group means were compared by one-way analysis of variance.

Results

Body weights

There were few significant group differences in the body weights of the birds (Table 1). On week 26 the birds in group C were significantly heavier than those in the other two groups, and these birds were also significantly heavier than those in group B, but not in group A, on weeks 28, 29 and 32. Generally the birds gained weight as expected for the type and age of bird, and remained comparable with birds of the same batch in commercial production.

Colonisation

None of the birds were colonised with intestinal spirochaetes prior to the start of the experiment, and no control birds were colonised at any time. On week 18, one week following inoculation, *B. innocens* was isolated from one bird in group B. On week 19 an additional two birds in this
group were positive for *B. innocens*, and all three remained positive for a further week. On week 21, four weeks after inoculation, only one of these three birds was positive. Thereafter and for the duration of the trial, none of the birds in group B were culture positive.

On week 18, one week following inoculation, *B. pilosicoli* was isolated from faecal samples from three birds in group C. These birds were positive the following week, but only one was positive the next week. All birds were culture negative by week 21, and remained so throughout the rest of the experiment.

*Faecal water content*

In weeks 19 and 20 the faecal moisture content of birds in group C was significantly higher than that of birds in group B, but not that of birds in group A (Table 2). At this time the faeces of birds in group C were 2-7% wetter than those in the other two groups. Thereafter there was no significant difference in the faecal moisture content between the groups, and there was no overall group difference in faecal moisture content.
Table 1. Group mean (± standard error) body weight of chickens (g) in the three experimental groups. Means within a row having different superscripts differ at the 5% level of significance.

<table>
<thead>
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<th>Week</th>
<th>Group A (control)</th>
<th>Group B (B. innocens)</th>
<th>Group C (B. pilosicoli)</th>
<th>P-value</th>
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<td>1695 (44)</td>
<td>1635 (61)</td>
<td>0.7627</td>
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<tr>
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<td>1755 (35)</td>
<td>1805 (45)</td>
<td>1765 (62)</td>
<td>0.3359</td>
</tr>
<tr>
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<td>1845 (46)</td>
<td>1927 (37)</td>
<td>1855 (59)</td>
<td>0.5005</td>
</tr>
<tr>
<td>21</td>
<td>2010 (57)</td>
<td>2146 (42.62)</td>
<td>2055 (63)</td>
<td>0.2223</td>
</tr>
<tr>
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<td>2345 (75)</td>
<td>2525 (52)</td>
<td>2355 (82)</td>
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<td>2655 (20)</td>
<td>2550 (95)</td>
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<tr>
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<td>2790 (30)</td>
<td>2745 (108)</td>
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<tr>
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<td>2980 (120)</td>
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<tr>
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<tr>
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<td>3265 (70)</td>
<td>3439 (47)</td>
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<tr>
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<td>3745 (75)</td>
<td>3575 (96)</td>
<td>3669 (3)</td>
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<td>3710 (96)</td>
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<td>3790 (97)</td>
<td>3887 (64)</td>
<td>0.7780</td>
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</table>
Table 2. Group mean (± standard error) percent faecal water content of chickens in the three experimental groups. Means within a row having different superscripts differ at the 5% level of significance.

<table>
<thead>
<tr>
<th>Week</th>
<th>Group A (control)</th>
<th>Group B (B. innocens)</th>
<th>Group C (B. pilosicoli)</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>18</td>
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<td>53.29 (2.33)</td>
<td>58.52 (1.15)</td>
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</tr>
<tr>
<td>19</td>
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<td>50.41 (2.36)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57.56 (1.28)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0143</td>
</tr>
<tr>
<td>20</td>
<td>54.0 (1.55)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.7 (1.29)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55.4 (0.96)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0024</td>
</tr>
<tr>
<td>21</td>
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<td>53.8 (1.12)</td>
<td>53.7 (1.47)</td>
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<td>57.2 (0.99)</td>
<td>56.8 (1.23)</td>
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<tr>
<td>24</td>
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<tr>
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<tr>
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<tr>
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<td>57.4 (1.30)</td>
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<tr>
<td>29</td>
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<td>57.1 (1.19)</td>
<td>0.5688</td>
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<tr>
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<tr>
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<td>56.0 (1.22)</td>
<td>58.3 (0.79)</td>
<td>56.4 (1.06)</td>
<td>0.2630</td>
</tr>
</tbody>
</table>
Egg production

Birds in groups A and B both commenced laying at 23 weeks of age, however the onset of egg production in birds in group C was delayed for 2 weeks (Table 3). Ninety per cent of birds in groups A and B had commenced laying by 27 and 26 weeks of age respectively, but Group C did not reach this figure until the birds were 30 weeks of age.

Egg production by birds in group C was significantly less than by birds in the other two groups from weeks 23 to 33, except in week 31. Thereafter no significant differences occurred. Overall birds in group C produced highly significantly ($P < 0.0000$) fewer eggs than birds in the other two groups.

Egg weights

Comparisons of the mean weights of eggs layed revealed no significant group effects either on a weekly basis or overall. Mean egg weights increased gradually from around 45 grams at start of lay to approximately 67 grams at week 41.
Bird health

Birds in group C had frothy brown faeces when sampled in the first two weeks following experimental inoculation, but not thereafter. The faeces of the birds in the other groups remained normal throughout.

Two birds in group C were euthanased, both one week after they were noticed to be depressed and off their feed. The first, killed in week 24, was diagnosed with tibial dyschondroplasia, and the second, killed in week 34, with hepatoma. No other abnormalities were found in these birds, and no spirochaetes were isolated from their caecae.
Table 3. Total number of eggs produced per group of 10 chickens per week (maximum possible 70/week). Means within rows having different superscripts differ at the 5% level of significance.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Group A (control)</th>
<th>Group B (B. innocens)</th>
<th>Group C (B. pilosicoli)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
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<td>5\textsuperscript{a}</td>
<td>0\textsuperscript{b}</td>
<td>0.0272</td>
</tr>
<tr>
<td>24</td>
<td>4\textsuperscript{a}</td>
<td>14\textsuperscript{b}</td>
<td>0\textsuperscript{c}</td>
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</tr>
<tr>
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<td>22\textsuperscript{a}</td>
<td>5\textsuperscript{b}</td>
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</tr>
<tr>
<td>26</td>
<td>22\textsuperscript{a}</td>
<td>40\textsuperscript{b}</td>
<td>5\textsuperscript{c}</td>
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</tr>
<tr>
<td>27</td>
<td>31\textsuperscript{a}</td>
<td>52\textsuperscript{b}</td>
<td>17\textsuperscript{c}</td>
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</tr>
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<td>50\textsuperscript{a}</td>
<td>26\textsuperscript{b}</td>
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<td>49\textsuperscript{a}</td>
<td>33\textsuperscript{b}</td>
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<td>37\textsuperscript{b}</td>
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</tr>
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<td>49</td>
<td>35</td>
<td>0.6473</td>
</tr>
<tr>
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<td>45</td>
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<td>50</td>
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<td>0.2222</td>
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<td>48</td>
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<tr>
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<td>48</td>
<td>48</td>
<td>34</td>
<td>0.6519</td>
</tr>
<tr>
<td>40</td>
<td>44</td>
<td>35</td>
<td>34</td>
<td>0.2631</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>787\textsuperscript{a}</strong></td>
<td><strong>823\textsuperscript{a}</strong></td>
<td><strong>532\textsuperscript{b}</strong></td>
<td><strong>0.0000</strong></td>
</tr>
</tbody>
</table>
Post-mortem findings

At necropsy, the caeca of the birds from group C contained more gas and their contents were more frothy, fluid and considerably paler than those of the birds in the other two groups. No gross or histological lesions were found in the caeca, ovaries or oviducts of any of the birds. There was no evidence of end-on attachment of spirochaetes to the caecal epithelium, and no spirochaetes were isolated from any of the birds.

Discussion

In Australia, natural infection with both *B. pilosicoli* and *B. innocens* has been reported in layer and broiler breeder flocks (Stephens & Hampson, 1999). The current study is the first report of experimental infection of commercial adult birds with these organisms.

Overall, the study showed that *B. innocens* is unlikely to have much pathogenic significance in adult birds. The presence of non-pathogenic spirochaetes in certain flocks however does complicate the diagnosis of avian intestinal spirochaetosis, and it requires the availability of reliable species-specific techniques, such as PCR, to differentiate pathogenic from non-pathogenic spirochaete isolates from chickens.
Following experimental inoculation of the birds in groups B and C with spirochaetes, only a small number of birds became culture positive, and this colonisation only persisted for a maximum of four weeks. The birds were given only a single challenge with a relatively small number of spirochaetes, and this may help account for the low proportion of birds which became culture positive. Interestingly, there was no evidence of cross-transmission of spirochaetes between or within groups of birds, but again this may have failed to arise due to the careful hygiene that was practised and the low level of colonisation overall. The low colonisation rate and relatively short duration of colonisation in individual birds may have been influenced by the inclusion of 50 ppm of zinc bacitracin in the commercial diets used. This antimicrobial was added to mimic the situation in the commercial flock from which the birds and the spirochaetes originated. It would be interesting to determine the extent of both colonisation and production losses that would result if the experiment were repeated without the inclusion of any growth promotants. Similarly, the good husbandry conditions practiced in the experiment may have minimised potential production problems that would occur in a more stressful commercial situation.

Faeces were only cultured weekly, and it is possible that transient colonisation of individual birds was missed. The culture detection method used also may not have been sufficiently sensitive to detect a low level of colonisation in some birds. Atyeo et al. (1998) have shown that the use of PCR on growth from primary plates can substantially increase detection
rates for *B. pilosicoli* in pig faeces, achieving detection limits of around $10^4$ cells/g faeces. More recently, *B. pilosicoli* has been detected in human faeces using PCR on DNA extracted directly from the faeces (Mikosza *et al.*, 2001). Future studies of *B. pilosicoli* in chickens should include use of such PCR protocols to improve the sensitivity of detection.

The experimental challenge with *B. pilosicoli* did not cause a reduction in body weight, and, on several occasions in the earlier part of the experiment, birds in group C were actually heavier than the other birds. This probably reflects the fact that the birds in group C were producing fewer eggs, and putting more energy into carcase growth at this time.

The birds infected with *B. pilosicoli* showed a transient increase in faecal moisture in the first few weeks after infection, as well as having brown frothy faeces, but this effect did not persist. Had colonisation with the spirochaetes continued, faecal moisture content may have remained elevated. Although the increase in moisture content was only of a few percentage points, commercially this could be sufficient to cause problems with mechanical cleaning of manure, faecal staining of eggs, increased odour and attraction of flies.

The most striking and significant finding in the study was the delay in both onset of egg production and in reduced total egg production in the birds inoculated with *B. pilosicoli*. When these birds did produce eggs, however, these were not significantly lighter than those produced by birds
in the other two groups. The major losses in production occurred in the first 11 weeks of lay, where in 10 of these weeks total egg production was significantly reduced in birds of group C. Over this 11 week period average egg productions per bird in groups A, B and C were 40.7, 45.0 and 26.8 respectively. Hence, over this initial period, birds in group C on average produced 14 less eggs than the birds in the control group, or had only two thirds of their level of production. In a commercial situation this loss would have an extremely serious economic impact. That such losses can occur was seen in Iowa, where a 100,000 bird layer flock infected with a spirochaete, which was later identified as \textit{B. pilosicoli} (McLaren \textit{et al.}, 1997), was shown to have an overall 5% reduction in egg production (Trampel \textit{et al.}, 1994).

No gross nor histological abnormalities were found in the caeca of any of the birds at post mortem examination. The lack of pathological changes at necropsy is not altogether surprising, because the birds were not colonised at this time, and they had had many weeks for any lesions to resolve. In future studies it would be useful to kill birds at the time they were culture positive. A practical outcome of the lack of gross and histological changes in the caecae is that it may not necessarily always be possible to diagnose infection with intestinal spirochaetes purely on pathological grounds. Diagnosis may have to be based on microbiological culture of appropriate samples from birds showing clinical signs. Previous studies have demonstrated that in flocks infected with spirochaetes and experiencing production problems, not all birds are necessarily positive
on culture (Stephens & Hampson, 1999). Thus samples from a number of birds might have to be examined to enable a diagnosis to be made. Moreover, the anaerobic nature and fastidious growth requirements of intestinal spirochaetes make microbiological culture relatively difficult.

There was no obvious explanation for the delay and subsequent persistent reduction in egg production in the group of birds inoculated with *B. pilosicoli*, particularly as only three birds were confirmed to be colonised. It is possible that the other birds were colonised, but were not detected because samples were only tested once a week. Colonisation had ceased by the time egg production had started, and it could be speculated that greater losses may have occurred had colonisation persisted. At post mortem no evidence was found for abnormalities in the ovaries or oviducts, although again by the time the birds were killed they were laying normally. Possibly their intestinal function was temporarily impaired, resulting in reduced nutrient uptake, although this was not reflected in reduced body weight gain. The birds did develop transient frothy brown faeces with slightly increased faecal water content, but did not have obvious diarrhoea. It was of interest that the birds in this group had altered caecal contents at post mortem, and this may reflect persistent subtle changes in the caecal microflora or in caecal function.

This investigation has confirmed that infection of commercial meat breeders with *B. pilosicoli* can significantly reduce egg production.
Previously we have shown that infection with this organism is widespread in commercial layer and meat breeder flocks in Australia (Hampson & Stephens, 1999). Thus it would appear almost certain that the infection is causing important economic losses. Further experimental studies are needed to find reliable means to establish experimental colonisation, to examine the pathological basis of production losses, and to improve diagnosis and control of *B. pilosicoli* infections.

**Acknowledgments**

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This work was carried out with the approval of the Animal Ethics Committee, Department of Primary Industries and Fisheries, Queensland.
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Serpulina pilosicoli isolates from the blood of critically-ill patients.

*Journal of Clinical Microbiology* **35**:482-485.
Antimicrobial susceptibility testing of *Brachyspira intermedia* and *Brachyspira pilosicoli* isolates from Australian chickens

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Having shown that certain species of intestinal spirochaete were capable of causing enteric disease and production loss, the next aim was to examine the antibiotic susceptibility of these species. Although carried out in vitro, the results of this study provided producers with some guidance with respect to the control of intestinal spirochaete infection in poultry flocks. The author assisted with this part of the final study.
Antimicrobial susceptibility testing of \textit{Brachyspira intermedia} and \textit{Brachyspira pilosicoli} isolates from Australian chickens

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$^2$ Toowoomba Veterinary Laboratory, Biosecurity, Department of Primary Industries and Fisheries, Toowoomba, Queensland 4350, Australia
Summary

The susceptibilities of predominantly Australian isolates of the pathogenic intestinal spirochaetes Brachyspira intermedia (n=25) and Brachyspira pilosicoli (n=17) from chickens were tested in agar dilution against four concentrations each of the antimicrobials tiamulin, lincomycin, tylosin, metronidazole, tetracycline and ampicillin. Based on available minimum inhibitory concentration (MIC) breakpoint values for Brachyspira hyodysenteriae or other Gram-negative enteric veterinary pathogens, isolates of both species generally were susceptible to tiamulin, lincomycin, metronidazole and tetracycline. The B. intermedia isolates tended to be less susceptible to tiamulin and more susceptible to lincomycin, tylosin and ampicillin than the B. pilosicoli isolates. Although not classed as resistant, four isolates of B. intermedia had an elevated MIC range for tiamulin (1-4 mg/l), 11 isolates of B. intermedia and five of B. pilosicoli had an elevated MIC range for lincomycin (10-50 mg/l), one isolate of B. pilosicoli had an elevated MIC range for tetracycline (10-20 mg/l), and one isolate of B. intermedia and five of B. pilosicoli had an elevated MIC range for ampicillin (10-50 mg/l). A clear lack of susceptibility to tylosin (MIC >4 mg/l) was seen in 11 isolates each of B. intermedia and B. pilosicoli, and to ampicillin (MIC >32 mg/l) in two isolates of B. pilosicoli.
Introduction

Anaerobic intestinal spirochaetes of the genus *Brachyspira* commonly colonise the caeca and colon/rectum of various species of birds (Stephens & Hampson, 2001). *Brachyspira* species identified in birds include the potentially pathogenic *B. intermedia, B. pilosicoli, B. alvinipulli,* and *B. hyodysenteriae,* as well as the presumed commensal “non-pathogenic” species *B. innocens, B. murdochii* and “*B. pulli*” (Jensen et al., 1996; McLaren et al., 1997; Stanton et al., 1998; Stephens & Hampson, 2001; Jansson et al., 2004).

Intestinal spirochaete infections have been reported to cause problems in flocks of laying hens and broiler breeder hens, where the associated clinical condition has been called “avian intestinal spirochaetosis” (AIS) (Swayne & McLaren, 1997). AIS has been linked to reduced egg production, delayed onset of laying, reduced growth rates, increased feed consumption and increased faecal moisture content that leads to wet litter (Davelaar et al., 1986; Griffiths et al., 1987; Dwars et al., 1989, 1992; Swayne et al., 1992; Trampel et al., 1994; Smit et al., 1998). The two pathogenic species most commonly involved in AIS are *B. intermedia* and *B. pilosicoli* (Stephens & Hampson, 2001; Stephens et al., 2005). To date, *B. alvinipulli* has only been reported from diseased chickens in one flock in the USA (Swayne et al., 1992; McLaren et al., 1997), and infection with *B. hyodysenteriae* (the agent of swine dysentery) has not been reported as a natural cause of disease in commercial poultry.
In comparison to the situation with *B. hyodysenteriae* and *B. pilosicoli* infections in pigs, where a large body of literature is available, relatively little has been published on means to treat AIS (Swayne, 2003). Treatment of infected laying hens with antimicrobials is complicated by a lack of registered products, and potential problems of antimicrobial residues in eggs for human consumption. Antimicrobial agents that have been reported to temporarily reduce clinical signs of AIS in chickens include 5-nitroimidazole in the drinking water of breeder birds (Smit *et al*., 1998), and tiamulin, lincospectin or oxytetracycline in the drinking water of laying hens (Stephens & Hampson, 1999).

It is known that isolates of *B. hyodysenteriae* and *B. pilosicoli* that infect pigs may develop resistance if regularly exposed to antimicrobials (Karlsson *et al*., 2002, 2004), and the same is likely to be true for *Brachyspira* spp. isolates from chickens. Consequently, prior to medicating for pathogenic *Brachyspira* spp. in chickens it is important to determine the likely effectiveness of available antimicrobial agents. To date there has been only one published report on the *in-vitro* antimicrobial susceptibilities of intestinal spirochaetes from chickens, and this involved small numbers of US isolates (Trampel *et al*., 1999). The two isolates of *B. pilosicoli* and the two of *B. alvinipulli* tested were susceptible to tiamulin, lincomycin and carbadox, resistant to streptomycin, and gave strain dependant results for chlortetracycline, oxytetracycline, tylosin, bacitracin, erythromycin, neomycin, and penicillin.
The aim of the current study was to gain more information about the likely antimicrobial susceptibilities of intestinal spirochaetes from chickens. The antimicrobials tested were mainly those used to treat *B. hyodysenteriae* and *B. pilosicoli* infections in pigs, because it was presumed that these also would be effective for treating *Brachyspira* spp. in chickens.

The type strain of *B. hyodysenteriae* (*B78^T*) was used as a positive control strain throughout the study, as MICs for this strain have been established in our laboratory (Karlsson et al., 2002), and no chicken reference strains of *B. intermedia* and *B. pilosicoli* of known antimicrobial susceptibility profiles are available. Since there have been no widely accepted MIC breakpoints for *Brachyspira* spp., other than for *B. hyodysenteriae*, breakpoints for *B. hyodysenteriae* were used where available.

**Materials and Methods**

**Spirochaete isolates**

Isolates of *B. intermedia* (n=25) and *B. pilosicoli* (n=17) from chickens were obtained as frozen stock from the culture collection held at the Reference Centre for Intestinal Spirochaetes at Murdoch University. Before being stored the isolates had been subcultured at least three times to ensure their purity. All isolates had been identified and typed using species-specific polymerase chain reactions (PCR) and multilocus
The enzyme electrophoresis (MLEE) (Stephens & Hampson, 1999; Stephens et al., 2005) was used to help assist with interpretation of the results of the current study. Twenty seven of the isolates of both species originated from five laying hen flocks and three broiler breeder flocks in Queensland (Stephens & Hampson, 1999), 10 isolates of *B. intermedia* were from nine assorted laying hen and broiler breeder flocks in Western Australia (WA) (McLaren et al., 1996, 1997), three isolates of *B. pilosicoli* and one of *B. intermedia* were from chickens in the Netherlands, and one *B. pilosicoli* isolate was from a chicken in the USA (McLaren et al., 1997). The type strain of *B. hyodysenteriae*, B78T, was included as a control strain for standardisation of antimicrobial susceptibility testing.

**Antimicrobial susceptibility testing**

Stock solutions were made by mixing the following antimicrobial powders with sterile distilled water: tiamulin hydrogen fumerate (99.5% activity); lincomycin hydrochloride (808 units/mg); tylosin tartrate (906 mg/g); metronidazole (100%); tetracycline hydrochloride (950 mg/g); and ampicillin (100%). Tiamulin hydrogen fumarate was obtained from Novartis Animal Health (Sydney, Australia) and the other antimicrobials were from Sigma (Castle Hill, Australia). Stock solutions were stored at 4°C for less than 24 hours before use.
Antimicrobial susceptibility plates were made using Trypticase Soy Agar (TSA), supplemented with 5% defibrinated ovine blood. Four concentrations of each antimicrobial were tested and the appropriate amount of each stock solution was added to the agar immediately before pouring the plates (Table 1). The antimicrobial sensitivity plates and control plates containing TSA with 5% ovine blood but no antimicrobials were dried for 15 min at 37°C before inoculation with the measured amount of test organisms. The inoculum was allowed to dry before incubating in an atmosphere of 94% H₂ and 6% CO₂ at 37°C.

Table 1. Antimicrobial concentrations used in the agar dilution antimicrobial sensitivity test

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Concentrations tested (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tiamulin</td>
<td>0.1, 1, 4^a, 10</td>
</tr>
<tr>
<td>lincomycin</td>
<td>1, 10, 50, 100</td>
</tr>
<tr>
<td>tylosin</td>
<td>4^a, 20, 50, 100</td>
</tr>
<tr>
<td>metronidazole</td>
<td>0.1, 1, 10, 20</td>
</tr>
<tr>
<td>tetracycline</td>
<td>1, 5, 10, 20</td>
</tr>
<tr>
<td>ampicillin</td>
<td>1, 10, 50, 100</td>
</tr>
</tbody>
</table>

^a A concentration of 5 mg/l was tested initially

For preparation of the inoculum, isolates were plated onto TSA with 5% defibrinated ovine blood and incubated in an atmosphere of 94% H₂, 6%
O₂ at 37°C for 5 days. The cells were gently resuspended in 1 ml of sterile phosphate buffered saline and then counted using a haemocytometer and phase-contrast microscope. A total of 10⁵ cells were drop-inoculated onto the control and sensitivity plates. Each isolate was tested at least in duplicate, and *B. hyodysenteriae* control strain B78ᵀ was included in each batch of tests.

Growth of the strains on the control and sensitivity plates was checked visually after five days incubation. Zones of weak haemolysis were present around growth on the control plates, and isolates were recorded as being susceptible to the antimicrobial concentration in the test plates if no such zones were observed. Any surface growth was scraped off the plate and examined under a phase contrast microscope to confirm purity and the endpoint. As only four dilutions of each antimicrobial were used, and in some cases these were quite widely separated, the minimum inhibitory concentration (MIC) values were recorded as being in the range between the highest sensitive concentration and the lowest resistant concentration. The MIC ranges of the antimicrobials at which 50% and 90% of the isolates were inhibited (MIC₅₀ and MIC₉₀, respectively) also were calculated for each species. MIC breakpoints used to assist interpretation of the results are shown in Table 2. For tiamulin, lincomycin, tylosin and metronidazole, values identified for *B. hyodysenteriae* by Rønne and Szancer (1990) were applied, whilst for tetracycline and ampicillin MIC breakpoints for Gram negative enteric
veterinary pathogens were used (National Committee for Clinical Laboratory Standards, 1999).

**Results**

The MIC ranges obtained for the antimicrobials tested against *B. hyodysenteriae* strain B78\(^T\) were consistent between batches, and the values agreed with previous results obtained in our laboratory for this strain (Karlsson *et al.*, 2002). The MIC ranges for the chicken *B. intermedia* and *B. pilosicoli* isolates are presented in Table 3. Generally, isolates of both species were susceptible to most of the antimicrobials tested, although sometimes the MIC breakpoints fell within the MIC range, making interpretation difficult. For example, 11 isolates of *B. intermedia* and five of *B. pilosicoli* had an elevated MIC range for lincomycin (10-50 mg/l), spanning the value of 36 mg/l considered to represent resistance in *B. hyodysenteriae* (Rønne & Szancer, 1990). Four isolates of *B. intermedia* from three farms had an elevated MIC range for tiamulin (1-4 mg/l), one isolate of *B. pilosicoli* had an elevated MIC range for tetracycline (10-20 mg/l), and one isolate of *B. intermedia* and five of *B. pilosicoli* from three farms had an elevated MIC range for ampicillin (10-50 mg/l). In comparison, a clear lack of susceptibility to tylosin (MIC >4 mg/l) was seen for 11 isolates of *B. intermedia* (44%) and 11 isolates of *B. pilosicoli* (65%). Similarly, two isolates of *B. pilosicoli* (12%), both from farm Q-BB 4, were clearly not susceptible to ampicillin *in vitro* (MIC >32 mg/l). The MIC\(_{50}\) and MIC\(_{90}\) values for the two species are shown in
Table 4. These values reflect a tendency for the collection of \textit{B. intermedia} isolates to be less susceptible to tiamulin than the \textit{B. pilosicoli} collection, with the latter tending to be less susceptible to lincomycin and tylosin, and clearly less susceptible to ampicillin than the \textit{B. intermedia} collection.

\textbf{Table 2}. MIC breakpoints (mg/l) for in-vitro antimicrobial susceptibility tests as used to assist in interpretation in the study

\begin{tabular}{lccc}
  \hline
  Antimicrobial & Sensitive$^a$ & Intermediate$^a$ & Resistant$^a$ \\
  \hline
  Tiamulin & $\leq 1$ & $> 1 \leq 4$ & $> 4$ \\
  Lincomycin & $\leq 4$ & $> 4 \leq 36$ & $> 36$ \\
  Tylosin & $\leq 1$ & $> 1 \leq 4$ & $> 4$ \\
  Metronidazole & $\leq 4$ & $> 4 \leq 16$ & $> 16$ \\
  Tetracycline & $\leq 4$ & 8 & $\geq 16$ \\
  Ampicillin & $\leq 8$ & 16 & $\geq 32$ \\
  \hline
\end{tabular}

$^a$ Based on MICs determined for \textit{B. hyodysenteriae} against tiamulin, lincomycin, tylosin and metronidazole and for Gram-negative enteric veterinary pathogens against tetracycline and ampicillin

Three farms had multiple isolates that belonged to the same \textit{Brachyspira} species and had the same electrophoretic type (ET) in MLEE. Farm Q-L 2 had three isolates of \textit{B. intermedia} in ET 60, of which isolate QAW3 had lower MIC ranges for tiamulin, tylosin and tetracycline than isolates
Table 3. Susceptibilities to six antimicrobials of 25 isolates of *Brachyspira intermedia* and 17 isolates of *Brachyspira pilosicoli* from hens in layer and broiler breeder flocks in Queensland, Western Australia, the Netherlands and the USA.

<table>
<thead>
<tr>
<th>ETa</th>
<th>Isolate</th>
<th>Flock&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tiamulin</th>
<th>Lincomycin</th>
<th>Tylosin</th>
<th>Metronidazole</th>
<th>Tetracycline</th>
<th>Ampicillin</th>
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<td>42</td>
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<td>4-20</td>
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<td>&lt;1</td>
<td>&lt;1</td>
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<td>43</td>
<td>QAW32</td>
<td>Q-L 3</td>
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<td>4-20</td>
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<tr>
<td>44</td>
<td>histo 6</td>
<td>WA 1</td>
<td>0.1-1</td>
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<td>&lt;4</td>
<td>&lt;0.1</td>
<td>&lt;1</td>
<td>1-10</td>
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<td>0.1-1</td>
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<td>&lt;1</td>
</tr>
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<td>46</td>
<td>E2</td>
<td>WA 4</td>
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</table>

<sup>b</sup> Location: QAW, Queensland; WA, Western Australia; ABB, Netherlands; E2, USA.
<table>
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<tr>
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<th>Isolate</th>
<th>Flock&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tiamulin</th>
<th>Lincomycin</th>
<th>Tylosin</th>
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<td>&lt;1</td>
<td>&lt;1</td>
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*B. intermedia*
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<th>Lincomycin</th>
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*B. intermedia*
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<td>Tiamulin</td>
<td>Lincomycin</td>
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<sup>a</sup>ET: Enterotype
<sup>b</sup>Flock: Flock type

**B. pilosicoli**
<table>
<thead>
<tr>
<th>ET&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Isolate</th>
<th>Flock&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tiamulin</th>
<th>Lincomycin</th>
<th>Tylosin</th>
<th>Metronidazole</th>
<th>Tetracycline</th>
<th>Ampicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>81</td>
<td>42167</td>
<td>USA</td>
<td>0.1-1</td>
<td>10-50</td>
<td>&gt;100</td>
<td>&lt;0.1</td>
<td>10-20</td>
<td>1-10</td>
</tr>
<tr>
<td>82</td>
<td>13316</td>
<td>The Netherlands</td>
<td>&lt;0.1</td>
<td>10-50</td>
<td>&gt;100</td>
<td>0.1-1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

<sup>a</sup> ET, electrophoretic type in multilocus enzyme electrophoresis: from Stephens et al. (2005)

<sup>b</sup> Q, Queensland. WA, Western Australia. BB, broiler breeder flock. L, laying hen flock.
<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC\textsubscript{50} (B. intermedia)</th>
<th>MIC\textsubscript{90} (B. intermedia)</th>
<th>MIC\textsubscript{50} (B. pilosicoli)</th>
<th>MIC\textsubscript{90} (B. pilosicoli)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiamulin</td>
<td>0.1-1</td>
<td>1-4</td>
<td>&lt;0.1</td>
<td>0.1-1</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>&lt;1</td>
<td>10-50</td>
<td>1-10</td>
<td>10-50</td>
</tr>
<tr>
<td>Tylosin</td>
<td>&lt;4</td>
<td>&gt;100</td>
<td>4-20</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>0.1-1</td>
<td>0.1-1</td>
<td>0.1-1</td>
<td>0.1-1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>&lt;1</td>
<td>1-5</td>
<td>&lt;1</td>
<td>1-5</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>1-10</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Table 4. MIC\textsubscript{50} and MIC\textsubscript{90} ranges (mg/l) for the six antimicrobials tested with the *B. intermedia* and *B. pilosicoli* isolates.
QAW1 and QAW2. Farm Q-BB 4 had five isolates of *B. pilosicoli* in ET 73, and again there were differences between some isolates in their MIC ranges for the antimicrobials tested, except for tiamulin. Farm Q-BB 1 had five isolates of *B. pilosicoli* in ET 76, and differences were observed between some of them in MIC ranges for lincomycin, tyllosin, and ampicillin. Other farms had multiple isolates belonging to closely related ETs, and again there was a tendency for some of these isolates to vary in their MIC ranges for some of the antimicrobials (eg isolates QAW15, QAW17 and QAW12 in related ETs 57, 58 and 59).

There were no obvious or consistent differences in the antimicrobial susceptibilities of isolates from laying hen flocks compared to those from broiler breeder flocks. The 10 WA isolates of *B. intermedia* were susceptible to tyllosin (MICs of <4 mg/l), whilst 11 of 14 (79%) Queensland isolates of *B. intermedia* were not susceptible. There were no other consistent differences in susceptibility between the isolates from the two Australian States.

**Discussion**

This study was the largest to date examining antimicrobial susceptibilities in *Brachyspira* isolates from chickens. The isolates tested were mainly from Australia, as currently there are few other isolates available worldwide. The agar dilution technique has been widely used for antimicrobial susceptibility testing of porcine *Brachyspira* isolates
(Hommez et al., 1998), usually using doubling dilutions of antimicrobials throughout the MIC range to obtain accurate MIC values. Due to constraints on resources, in the current study only four dilutions of each antimicrobial were tested for each isolate - so results were expressed as MIC ranges. Despite these limitations of geographic origin of isolates and the use of broad MIC ranges, the current data set increase our understanding of what antimicrobial agents might prove useful to treat AIS caused by one or other of the main two pathogenic Brachyspira species infecting chickens.

The MIC ranges obtained for most isolates of both Brachyspira species for tiamulin, lincomycin, metronidazole and tetracycline suggested that these antimicrobials generally are likely to be effective for treatment. This conclusion is supported from the results of other studies where these drugs have been used to control AIS in naturally infected flocks (Smit et al., 1998; Stephens & Hampson, 1999), and for tiamulin and lincomycin in hens experimentally infected with B. intermedia and B. pilosicoli (Hampson et al., 2002; Stephens & Hampson, 2002). Whether or not these antimicrobials can be used to treat AIS in infected flocks, particularly in hens laying eggs intended for human consumption, will also depend on local regulations.

The two Brachyspira species had identical MIC$_{50}$ and MIC$_{90}$ ranges for metronidazole and tetracycline, but there were trends for some species-related differences in susceptibility to the other four antimicrobials.
Reduced susceptibility to tiamulin occurred more commonly amongst the 
*B. intermedia* isolates than amongst the *B. pilosicoli* isolates, whilst the 
reverse was true for ampicillin. The MIC<sub>50</sub> figures also suggested a trend 
for reduced susceptibility to lincomycin and tylosin amongst *B. pilosicoli* 
isolates compared to *B. intermedia* isolates. These trends emphasize the 
need to identify the spirochaete species involved in individual cases of 
AIS, and preferably to conduct antimicrobial susceptibility testing before 
selecting an antimicrobial for treatment.

In some cases, one-dilution differences in susceptibility ranges were 
detected amongst different isolates from the same farm that grouped into 
a single ET, and hence were presumed to represent a single strain or 
closely related clonal group of strains. These differences occurred for 
both *B. pilosicoli* and *B. intermedia* isolates, and may represent the 
beginning of a loss of susceptibility to particular agents by members of a 
given clonal group on a farm. In future work it would be useful to confirm 
these trends by monitoring susceptibility of strains over a longer period, 
testing with more antimicrobial dilutions across the appropriate 
concentration ranges.

Resistance to tylosin was relatively common, being recorded in seven of 
the 13 (54%) *B. pilosicoli* isolates from Queensland, all four non-
Australian *B. pilosicoli* isolates, and in 11 of the 14 (79%) isolates of *B.
intermedia* from Queensland flocks. In comparison, all 10 isolates of *B.
intermedia* from WA flocks were susceptible to tylosin (MIC <4 mg/l).
Again, these differences reinforce the need to undertake MIC testing before selecting an antimicrobial to treat AIS. Tylosin is sometimes used to treat mycoplasma infections in poultry, and it is possible that such exposure in the Queensland and non-Australian flocks had encouraged the development of resistance. The generally low levels of susceptibility to tylosin amongst the chicken isolates, and an associated tendency for these also to have elevated MIC ranges to lincomycin, mirrors the situation seen amongst porcine isolates of *B. hyodysenteriae* and *B. pilosicoli* (Karlsson *et al.*, 2002, 2004). Currently it is not known whether this resistance in the chicken isolates was caused by the common point mutations in the 23S rRNA described as being responsible for macrolide and lincosamide resistance amongst porcine isolates of *B. hyodysenteriae* and *B. pilosicoli* (Karlsson *et al.*, 1999, 2004).

Two isolates of *B. pilosicoli* clearly showed resistance to ampicillin, whilst five other *B. pilosicoli* and one *B. intermedia* isolate had elevated MIC ranges. Previously, certain human and porcine isolates of *B. pilosicoli* have been shown to be resistant to amoxicillin (Brooke *et al.*, 2003). In that study resistance was associated with beta-lactamase enzyme activity, and it was suggested that this could have been acquired by horizontal transfer of beta-lactamase genes from other *Brachyspira* strains or bacterial species. The genetic basis of the ampicillin resistance observed in the chicken isolates, particularly in *B. pilosicoli*, deserves further investigation.
Acknowledgment

This study was supported in part by a grant from the Australian Chicken Meat Council and the former Australian Egg Industry Council, administered through the Rural Industries Research and Development Corporation. We thank the Queensland Department of Primary Industries and Fisheries and Murdoch University for provision of facilities.


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Karlsson M, Oxberry SL and Hampson DJ 2002. Antimicrobial susceptibility testing of Australian isolates of *Brachyspira"
hyodysenteriae using a new broth dilution method. *Veterinary Microbiology* **84**:123-133.


National Committee for Clinical Laboratory Standards. 1999. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard M31-A. National Committee for Clinical Laboratory Standards Wayne PA.


Evaluation of tiamulin and lincomycin for the treatment of broiler breeders experimentally infected with the intestinal spirochaete *Brachyspira pilosicoli*
Having examined the efficacy of a range of antimicrobials against pathogenic species of intestinal spirochaete \textit{in vitro}, the following study evaluated the effectiveness of two of these antimicrobials in treating birds experimentally infected with a pathogenic intestinal spirochaete.
Evaluation of tiamulin and lincomycin for the treatment of broiler breeders experimentally infected with the intestinal spirochaete

*Brachyspira pilosicoli*

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²Division of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, WA 6150, Australia
SUMMARY

*Brachyspira pilosicoli* strain CPSp1 isolated from a chicken in a broiler breeder flock in Queensland was used to experimentally infect 30 individually caged 22-week-old Cobb 500 broiler breeder hens. Another 10 birds were sham inoculated with sterile broth. All birds failed to become colonised. At 29 weeks of age all birds were transferred to a diet containing 50 ppm zinc bacitracin (ZnB) and were rechallenged with the same *B. pilosicoli* strain at 32 weeks of age, weekly for five weeks. The majority of the inoculated birds then became colonised, confirming previous findings that ZnB can increase susceptibility to colonisation with *B. pilosicoli*. The control group remained uninfected. Infected groups tended to have an increased faecal water content and faecal staining of eggshells. Ten birds were then treated by crop tube with 25 mg/kg body weight tiamulin for five days, and 10 with 20 mg/kg body weight lincomycin for five days. Both treatments removed the infection, whilst untreated birds remained infected. The results support previous observations that ZnB at 50 ppm in the diet increases the susceptibility of birds to *B. pilosicoli* infection, and demonstrated the usefulness of both tiamulin and lincomycin for treatment of infection with *B. pilosicoli* in adult birds.
Avian intestinal spirochaetosis (AIS) is a condition resulting from colonisation of the caeca and colon of birds by anaerobic intestinal spirochaetes belonging to the genus *Brachyspira* (Swayne, 1997; Stephens & Hampson, 2001). Infection with intestinal spirochaetes is widespread in layer and broiler breeder flocks. Several *Brachyspira* species infect chickens, of which three are known to cause AIS (McLaren *et al*., 1997). The pathogenic species are *B. pilosicoli* (Trampel *et al*., 1994; Stephens & Hampson, 1999), *B. intermedia* (Griffiths *et al*., 1987; Dwars *et al*., 1992, 1993; Stanton *et al*., 1997; Hampson & McLaren, 1999), and *B. alvinipulli* (Swayne *et al*., 1992, 1995; Stanton *et al*., 1998).

AIS is believed to cause a variety of production problems, including diarrhoea, wet litter, faecal staining of eggshells, 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 weak chicks, with slower growth and a poorer feed digestion (Davelaar *et al*., 1986; Griffiths *et al*., 1987; Swayne *et al*., 1992; Dwars *et al*., 1990, 1992, 1993; Trampel *et al*., 1994; Smit *et al*., 1998).

Little has been published about how to control AIS, although antimicrobials are regularly used to prevent and treat intestinal spirochaete infections in pigs. Recently we showed that the antimicrobials
tiamulin and lincomycin, which are commonly used to treat pigs, were both effective in treating sheds of birds on a broiler breeder farm where AIS had been identified as a cause of production problems (Stephens & Hampson, 1999). Tiamulin was also effective when used to treat layer hens experimentally infected with *B. intermedia* (Hampson *et al.*, 2002). To date there have been no reports of the use of either of these antimicrobials to treat adult birds experimentally infected with *B. pilosicoli*.

Recently we developed a model in which we were able to induce production losses in adult broiler breeder hens experimentally infected with *B. pilosicoli* (Stephens & Hampson, 2002). The current experiment was designed to test the efficacy of tiamulin and lincomycin for the treatment of AIS in broiler breeder hens experimentally infected with *B. pilosicoli*.

**Materials and Methods**

This study was conducted with the approval of the Animal Ethics Review Committee, Southeast Region, Department of Primary Industries, Queensland.

*Experimental birds*

Forty Cobb 500 broiler breeder females were obtained from a commercial producer at 20 weeks of age. The birds were placed in individual cages with mesh floors and egg rollout trays. The cages were constructed to be
large enough to accommodate breeder females. Each cage was provided with a waste tray for the collection of faeces. The birds were placed in four adjoining rooms. The birds were maintained under conditions of a common, controlled airflow with temperatures varying from an average minimum of 20.2°C to an average maximum of 23.1°C. The day-length was initially set at 8 hours, then gradually increased to 15 hours until 25 weeks of age and thereafter maintained at 16 hours.

**Diets**

The birds were initially fed a commercial breeder production diet which did not contain any growth promoters. From 29 weeks of age the birds’ diet was changed to include 50 ppm of zinc bacitracin (ZnB). Feed intake was restricted, with the birds initially being given 100 grams daily at 20 weeks of age. This amount was increased to 110 g per day at 21 weeks of age, 115 g per day at 22 weeks of age and 120 g daily at 23 weeks of age. This feed rate was maintained until the birds reached 33 weeks of age, when the feed rate was reduced to 115 g per day. Water was provided *ad libitum* by means of water pipes running past the rear of the cages in each room, and accessed individually by each bird in that room.

*Spirochaete strain used for experimental infection*

*Brachyspira pilosicoli* strain CPSp1, which was used to infect the birds, was isolated from a breeder on the same farm from which the test birds
originated. The strain had been isolated four years before the current study, during the course of a disease investigation, and its species identity had been confirmed through biochemical testing and polymerase chain reaction amplification of portions of their 16S rRNA and NADH oxidase genes (Stephens & Hampson, 1999). This strain previously had been used to induce production losses in experimentally infected broiler breeder hens (Stephens & Hampson, 2002). For experimental infection, the spirochaetes were grown to mid-log phase in Kunkle’s broth (Kunkle et al., 1986). The inocula used to infect the birds contained approximately $10^8$ actively motile bacterial cells per mL, as determined by direct counting of spirochaetes in a counting chamber placed under a dark field microscope.

*Experimental infection*

The birds were acclimatised for two weeks. Over this period, individual faecal samples from each bird were taken three times. These were cultured for spirochaetes on selective Trypticase Soy Agar (Micro Diagnostics, Brisbane, Australia), supplemented with 5% defibrinated ovine blood, 0.1% porcine mucin (Sigma, Castle Hill, Australia), 200 µg/mL spectinomycin and 6.25% µg/mL each of colistin and vancomycin. Plates were incubated in an anaerobic environment generated by Anaerogen sachets (Basingstoke, United Kingdom) and growth examined by dark field microscopy after 5 and 10 days.
After two weeks, when the birds were 22 weeks of age, they were weighed and randomly assigned on the basis of body weight to one of four groups, each of ten birds. Each group was placed in one of the four rooms. The 30 birds in Groups B, C and D were orally inoculated via a crop tube with two mL of culture of *B. pilosicoli* on three occasions, each 48 hours apart. This inoculation procedure was repeated a further three times at weekly intervals. The 10 control birds in group A were inoculated with sterile broth following the same routine used for the infected groups.

After another six weeks, when the birds were 32 weeks of age, they were again inoculated as above, and the inoculation procedure was repeated five times at weekly intervals.

**Treatment**

When the birds were 39 weeks of age, the birds in group B were orally dosed daily on five consecutive days by crop tube with 2 mL of a solution of tiamulin (Jurox Pty Ltd, Rutherford, Australia) dissolved in sterile distilled water, at a rate of 25 mg tiamulin per kg bodyweight per day. This was given approximately seven hours post feeding. Birds in Group C were orally dosed daily on five consecutive days by crop tube with 2 mL of a solution of lincomycin (Pharmacia and Upjohn, Brisbane, Australia), at a rate of 20 mg lincomycin per kg bodyweight per day.
Monitoring of infection, faecal moisture, egg production and faecal staining of eggshells

Eggs from each bird were collected, counted and weighed every day. Eggs were also scored for degree of faecal eggshell staining. Eggs were scored from zero for a clean shell to five for a heavily stained shell. Once a week the birds were weighed and individual faecal samples were collected. Faecal samples were cultured for intestinal spirochaetes as previously described. The faecal samples were then weighed, dried to constant weight in a hot air oven, and the percentage faecal moisture calculated.

Post mortem examination

At 43 weeks of age the birds were killed by cervical dislocation, and subjected to post mortem examination. The caeca, ovaries and oviducts were examined grossly and sections fixed in 10% buffered formalin for subsequent histological examination. Caecal contents were cultured for intestinal spirochaetes.

Statistical analysis

Weekly group bird weights, faecal moisture content and faecal staining of eggshells were compared using one-way analysis of variance. Means were compared using Fisher’s protected least significant difference
method, and significance was accepted at the 5% level. Group egg production per week was compared using Chi square tests, except where values per cell were less than five, when Fisher’s exact test was used in two by two contingency tables. Full egg production was assumed to be one egg/bird/day. The weights of the eggs produced were analysed by calculating a mean weight of eggs produced from each bird per week (i.e. total weight/number of eggs), then calculating group means of these bird means each week. These group means were compared by one-way analysis of variance.

Results

Body weights

There were few significant group differences in the body weights of the birds, and those that were identified are shown in Table 1. At 24 weeks, two weeks after initial inoculation, the average weight of birds in the control group (Group A) was significantly greater than that of the birds in Groups B and C. At 25, 35, 36 and 38 weeks of age the birds in group A were significantly heavier than those of the birds in Group B only. At 40, 41 and 43 weeks of age the average weight of birds in Group A was significantly greater than that of birds in both Groups B and C.
Table 1. Group mean bodyweight of chickens (g) in the four experimental groups in weeks where significant differences were found

<table>
<thead>
<tr>
<th>Age (wks)</th>
<th>Group A (control)</th>
<th>Group B (infected/tiamulin)</th>
<th>Group C (infected/lincomycin)</th>
<th>Group D (infected)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>350^C</td>
<td>210^A</td>
<td>260^{AB}</td>
<td>295^{BC}</td>
<td>0.010</td>
</tr>
<tr>
<td>25</td>
<td>470^B</td>
<td>365^A</td>
<td>435^{AB}</td>
<td>455^B</td>
<td>0.049</td>
</tr>
<tr>
<td>35</td>
<td>3478^B</td>
<td>3255^A</td>
<td>3400^{AB}</td>
<td>3525^B</td>
<td>0.013</td>
</tr>
<tr>
<td>36</td>
<td>3528^B</td>
<td>3285^A</td>
<td>3430^{AB}</td>
<td>3530^B</td>
<td>0.038</td>
</tr>
<tr>
<td>38</td>
<td>3606^B</td>
<td>3305^A</td>
<td>3405^{AB}</td>
<td>3555^B</td>
<td>0.045</td>
</tr>
<tr>
<td>40</td>
<td>3650^B</td>
<td>3335^A</td>
<td>3415^A</td>
<td>3495^{AB}</td>
<td>0.007</td>
</tr>
<tr>
<td>41</td>
<td>3661^B</td>
<td>3405^A</td>
<td>3435^A</td>
<td>3615^{AB}</td>
<td>0.044</td>
</tr>
</tbody>
</table>

Within each row, values with different superscripts differ at the 5% level of significance

Colonisation

None of the birds were colonised with intestinal spirochaetes prior to the start of the experiment and no control birds were colonised at any time throughout the experiment. Over the first six weeks following the original inoculations, no birds were found to be culture positive. Each week from 33 weeks between 3-8 birds in each infected group were positive by faecal culture, until treatment of groups B and C at 39 weeks of age.
Results for the experimentally infected birds from 33 weeks of age onwards are shown in Table 2.

**Table 2.** Number of experimentally infected birds in groups B, C and D with faecal cultures positive for *B. pilosicoli*

<table>
<thead>
<tr>
<th>Age in weeks&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Group B&lt;sup&gt;b&lt;/sup&gt; (tiamulin)</th>
<th>Group C (lincomycin)</th>
<th>Group D (no treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>7</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>34</td>
<td>7</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>35</td>
<td>8</td>
<td>6</td>
<td>5</td>
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<td>6</td>
<td>5</td>
</tr>
<tr>
<td>37</td>
<td>6</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>38</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>39</td>
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<td>0</td>
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</tr>
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<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>41</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>42</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>43</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reinfected weekly from weeks 32 to 37, treated in week 39;  
<sup>b</sup> Number of birds positive, of 10 in each group
One day after the completion of treatment all birds in both Groups B and C were negative on culture, and remained so over the four week period to the end of the experiment. During this time three untreated birds in Group D remained positive for three weeks, and two birds were positive in the last week of the trial.

*Faecal moisture content*

At 34 weeks of age, two weeks after the commencement of the second set of inoculations, the mean faecal moisture content of birds in Groups B and D was significantly greater than that of Groups A and C (Table 3). At 37 weeks, mean faecal moisture content of birds in Group D was significantly greater than that of Groups A and B, although not significantly greater than that of Group C. At 40 weeks of age, mean faecal moisture content of birds in Group B was significantly higher than that of Groups A and D. This was again the case in the final week of the trial.

*Egg production*

There were no significant differences in egg numbers produced between the four groups of birds until 38 weeks of age. At 38 and 39 weeks, two and three weeks respectively following the last inoculation, the mean weight of eggs produced by Group B was significantly less than that produced by either Group A or Group D, although it was not significantly
less than Group C (Table 4). At 42 weeks of age, the mean weight of eggs produced by Group A was significantly greater than that produced by either Group B or Group C.

**Table 3.** Group mean faecal moisture of chickens (%) in the four experimental groups

<table>
<thead>
<tr>
<th>Age (wks)</th>
<th>Group A control</th>
<th>Group B infected tiamulin</th>
<th>Group C infected lincomycin</th>
<th>Group D infected</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>58.3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>63.2&lt;sup&gt;B&lt;/sup&gt;</td>
<td>61.3&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>62.6&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.012</td>
</tr>
<tr>
<td>37</td>
<td>55.0&lt;sup&gt;A&lt;/sup&gt;</td>
<td>54.8&lt;sup&gt;A&lt;/sup&gt;</td>
<td>57.7&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>61.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.034</td>
</tr>
<tr>
<td>40</td>
<td>59.3&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>63.5&lt;sup&gt;C&lt;/sup&gt;</td>
<td>62.8&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>56.5&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td>43</td>
<td>59.9&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>60.6&lt;sup&gt;C&lt;/sup&gt;</td>
<td>56.6&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>54.4&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Within each row, values with different superscripts differ at the 5% level of significance

*Faecal staining of eggs*

There was significantly less faecal staining of the eggshells amongst the uninfected birds of Group A compared with the other three groups at 31 weeks of age, six weeks after the completion of the initial inoculations (Table 5). Over the following week, the first week of the second set of
inoculations, the mean degree of faecal staining of eggs of Group A was significantly less than that of either of Groups B and D. During the third and fourth weeks of the second set of inoculations, faecal staining of eggshells of Group A was significantly less than that of all three infected groups. This was also the case at 37 and 39 weeks.

**Table 4.** Group mean egg weights (g) in the four experimental groups

<table>
<thead>
<tr>
<th>Age wks</th>
<th>Group A control</th>
<th>Group B Infected</th>
<th>Group C Infected</th>
<th>Group D infected</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>67.4^B</td>
<td>62.0^A</td>
<td>63.6^{AB}</td>
<td>67.1^B</td>
<td>0.043</td>
</tr>
<tr>
<td>39</td>
<td>68.1^C</td>
<td>62.5^A</td>
<td>64.1^{AB}</td>
<td>66.2^{BC}</td>
<td>0.025</td>
</tr>
<tr>
<td>42</td>
<td>70.8^{A}</td>
<td>64.9^{B}</td>
<td>65.6^{B}</td>
<td>67.6^{AB}</td>
<td>0.030</td>
</tr>
</tbody>
</table>

Within each row, values with different superscripts differ at the 5% level of significance

*Bird health*

One bird in group A was euthanased at 32 weeks of age, prior to the commencement of the second set of inoculations. The bird had been depressed and not standing, although still eating and drinking, for a period of one week. It was diagnosed with fatty liver haemorrhagic syndrome. Silver impregnation stains of histological sections of the
caecum failed to show any spirochaetes and caecal contents were
negative on culture.

Table 5. Group mean faecal staining of eggshells in the four
experimental groups

<table>
<thead>
<tr>
<th>Age wks</th>
<th>Group A control</th>
<th>Group B Infected tiamulin</th>
<th>Group C Infected lincomycin</th>
<th>Group D infected</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>0.47&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.86&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.81&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.78&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.012</td>
</tr>
<tr>
<td>32</td>
<td>0.41&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.92&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.80&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.03&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.032</td>
</tr>
<tr>
<td>34</td>
<td>0.51&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.95&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.05&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.01&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.021</td>
</tr>
<tr>
<td>35</td>
<td>0.50&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.90&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>1.22&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.94&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.002</td>
</tr>
<tr>
<td>37</td>
<td>0.57&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.04&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.15&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.24&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.007</td>
</tr>
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<td>38</td>
<td>0.50&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.90&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>1.25&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.87&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.013</td>
</tr>
<tr>
<td>39</td>
<td>0.48&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.28&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.35&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.15&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.001</td>
</tr>
<tr>
<td>40</td>
<td>0.49&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.97&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>1.23&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.76&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.020</td>
</tr>
<tr>
<td>41</td>
<td>0.56&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.33&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.23&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.87&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.008</td>
</tr>
<tr>
<td>42</td>
<td>0.54&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.78&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.29&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.87&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Within each row, values with different superscripts differ at the 5% level of significance
Post mortem examination

At necropsy, no gross nor histological lesions were found in the caecae, ovaries or oviducts of any of the birds. Silver impregnation stains revealed spirochaetes in the caeca of the two birds from Group D that had remained culture positive throughout. There was no evidence of end-on attachment of spirochaetes to the caecal epithelium. Caecal contents of all birds were negative on culture for spirochaetes with the exception of those of the two birds from Group D, both of which yielded heavy growth of spirochaetes which were subsequently identified as *B. pilosicoli* on the basis of their phenotypic properties.

Discussion

This study was originally designed as a simple experiment to test the efficacy of tiamulin and lincomycin for the control of *B. pilosicoli* infection in broiler breeders, in a model system that we previously had developed (Stephens and Hampson, 2002). Initially, the birds failed to become colonised following repeated experimental challenge. In the original model, the birds received 100 ppm ZnB in their diet, but none was initially included in the diet in the current experiment. In a previous experimental study in layers infected with *B. pilosicoli*, we found that 50 ppm ZnB significantly increased colonisation rates (Jamshidi & Hampson, 2002). In the current experiment, the birds were readily colonised following the addition of ZnB to the diet, and this observation helps to confirm that ZnB
can increase the susceptibility of adult chickens to *B. pilosicoli* infections. Clearly, further work is required to investigate the basis for this change in susceptibility, but presumably it is related to changes in the microflora brought about by the antimicrobial activities of ZnB. From a practical perspective, the use of ZnB in flocks with AIS caused by *B. pilosicoli* needs to be approached with caution. On the other hand, ZnB has been shown to be effective at reducing colonisation with *B. intermedia* (Hampson *et al.*, 2002), and this difference emphasises the need for appropriate diagnostic tests to differentiate between the two spirochaete species in flocks with AIS.

In the first part of the experiment, in weeks 24 and 25, there was a tendency for birds in the experimentally infected groups to be lighter, even though colonisation was not detected by faecal culture. Subsequently, even though the birds became colonised with *B. pilosicoli* after they received ZnB, no differences in weight or egg production were found. In part this could be due to other beneficial effects of the ZnB masking potential production problems caused by the *B. pilosicoli*. Faecal water content did increase in colonised birds however, and this was associated with increased faecal staining of eggshells. These results are consistent with descriptions of diarrhoea and wet litter problems in flocks with AIS.

Once the birds became colonised with the *B. pilosicoli* strain, it became reasonably easy to demonstrate that both tiamulin and lincomycin were
effective in removing the infection. In this study the treated birds did not become reinfected, as previously had been found in treated experimentally-infected layers (Hampson *et al*., 2002), and in birds in the field (Smit *et al*., 1998; Stephens & Hampson, 1999). This may have been because in the current experiment the birds were unable to physically contact each other, and treated birds were housed in isolation from untreated birds. This observation suggests that reinfection on a farm is most likely to occur from other incompletely treated birds, rather than from an endogenous residual infection, or from the immediate environment in the case of caged birds.

In the current experiment no attempts were made to optimise the dose rates for tiamulin and lincomycin, and this needs to be examined. Potential issues about drug residues in eggs also need to be considered, and until this is clarified these antimicrobials will be more practical for treating AIS in breeders than in layers.

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Industries, Queensland and Murdoch University. The support of the Darwalla Milling Company also is gratefully acknowledged.

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Paper V

The use of multilocus enzyme electrophoresis to characterise intestinal spirochaetes (*Brachyspira* spp.) colonising hens in commercial flocks

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Foreword

In the following study, multilocus enzyme electrophoresis (MLEE) was used to identify, examine genetic relationships and study the disease associations of a collection of intestinal spirochaetes, all of which had been isolated during the course of the initial prevalence and disease association study. The paper is written in the style of Veterinary Microbiology.
The use of multilocus enzyme electrophoresis to characterise intestinal spirochaetes (*Brachyspira* spp.) colonising hens in commercial flocks

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Abstract

Multilocus enzyme electrophoresis (MLEE) was used to identify, examine genetic relationships and study the disease associations of a collection of 53 intestinal spirochaete isolates previously recovered from the faeces of adult hens on 14 farms in Queensland, Australia. The MLEE results were compared with those previously obtained using species-specific PCR amplifications. The isolates were divided into five *Brachyspira* species groups by MLEE: *Brachyspira murdochii* (n=17), *B. intermedia* (n=15), *B. pilosicoli* (n=14), *B. innocens* (n=2), and “B. pulli” (n=1). Three new MLEE groups each containing single isolates also were identified. The results of the PCR assay for *B. pilosicoli* were concordant with the MLEE results, but the 23S rDNA-based PCR for *B. intermedia* had failed to detect eight of the 15 isolates. The *B. innocens/B. murdochii* nox-based PCR had correctly identified all the isolates of *B. murdochii*, but did not identify either of the two *B. innocens* isolates. Using MLEE, isolates from two farms (14%) were identified as *B. murdochii*, whilst the pathogenic species *B. intermedia* and *B. pilosicoli* were present in hens from eight (57%) and five (36%) farms respectively, and were identified together in four (29%) farms. All seven of the farms with production problems or wet litter were colonised with *B. intermedia* and/or *B. pilosicoli*. Six farms had multiple spirochaete isolates available for examination. Two broiler breeder farms both had five isolates of *B. pilosicoli* that shared the same MLEE electrophoretic type (ET), whilst one laying hen farm had three isolates of *B. intermedia* that all belonged to the same ET. Hence on each
of these farms a predominant strain of a pathogenic species was present. On the other farms isolates of the same species were more diverse, and belonged to different ETs. These results show that the epidemiology of intestinal spirochaetal infections in broiler breeder and laying hen flocks can vary considerably between farms, although the reasons for these differences were not established.
Introduction

Anaerobic intestinal spirochaetes commonly colonise the caeca and colon/rectum of birds (Stephens and Hampson, 2001). These bacteria were originally placed in the genus *Serpulina*, but now have been transferred to the genus *Brachyspira* (Ochiai et al., 1997). *Brachyspira* species found colonising birds include the potentially pathogenic *B. pilosicoli*, *B. intermedia*, *B. alvinipulli*, and *B. hyodysenteriae*, as well as the presumed commensal “non-pathogenic” species *B. innocens*, *B. murdochii* and “*B. pulli*” (Jensen et al., 1996; McLaren et al., 1997; Stanton et al., 1998; Stephens and Hampson, 2001; Jansson et al., 2004). The name “*B. pulli*” has not been officially proposed, but it corresponds to the multilocus enzyme electrophoresis (MLEE) “group d” spirochaetes of McLaren et al. (1997), which were called “*B. pulli*” by Stephens and Hampson (1999, 2001).

Phenotypic differences between the various avian intestinal spirochaete species are inconsistent, and this makes their routine identification difficult. PCR assays have been used to identify chicken strains of *B. pilosicoli*, *B. intermedia*, *B. innocens* and *B. murdochii* (Atyeo et al., 1999; Suriyaarachichi et al., 2000), and *B. hyodysenteriae* isolates from mallards (Jansson et al., 2004). MLEE has been shown to be a powerful alternative method for identifying isolates of various bacterial species, including *Brachyspira* species (Lee et al., 1993), and is most effective means currently available to identify the recognised *Brachyspira* species.
that colonise birds (McLaren et al., 1997). Using this technique, the latter authors divided 56 intestinal spirochaete isolates from commercial poultry flocks in Australia, the USA and Europe into six distinct groups, each equating to the above mentioned *Brachyspira* species. Porcine isolates of *B. hyodysenteriae* made up a seventh species group in the study, but no *B. hyodysenteriae* isolates were present amongst the chicken isolates examined (McLaren et al., 1997).

In the current study, MLEE was used to more fully characterise a collection of Queensland poultry isolates from the 14 flocks, that were originally examined in a study by Stephens and Hampson (1999). The intention was to examine genetic relationships within and between the isolates from these flocks. This information would increase understanding of the distribution, diversity and disease associations of *Brachyspira* species in commercial flocks of adult hens.

**Materials and methods**

*Spirochaete isolates*

Intestinal spirochaete isolates tested by PCR in the study of Stephens and Hampson (1999) were obtained as frozen stock from the culture collection held at the Reference Centre for Intestinal Spirochaetes at Murdoch University. Before being stored the isolates had been subcultured at least three times to ensure their purity. A total of 53 of
these isolates, which originated from 11 laying hen flocks and three broiler breeder flocks in Queensland, were successfully revived. These included 14 isolates that had previously been identified as *B. pilosicoli*, seven identified as *B. intermedia*, 18 identified as *B. innocens/B. murdochii*, and 14 that had not reacted in the PCRs, and therefore were not allocated to a species (Stephens and Hampson, 1999).

**Phenotypic characterisation of spirochaetes**

All isolates were plated to Trypticase Soy agar containing 5% (v/v) defibrinated ovine blood, and were incubated for 5 d at 37°C in an anaerobic jar in an atmosphere of 94% H₂ and 6% CO₂. The cells were subcultured to the same media. Spirochaetal isolates picked from the plates were examined using a phase contrast microscope to ensure their purity, and then part of a colony was transferred to 10ml Kunkle’s pre-reduced anaerobic broth (Kunkle et al., 1986). The broth was incubated on a rocking platform at 37°C for 3-5 d before being subcultured. Growth and purity were confirmed by monitoring samples using a phase contrast microscope. To examine indole production, two ml quantities of broth containing actively growing spirochaetes were extracted with xylol, and then 2 drops of Kovács’ reagent were added. The development of a red colour in the upper layer was indicative of indole production by the spirochaete isolate.
To prepare cells for MLEE analysis, these were harvested in mid-log phase from 300 ml of Kunkle’s broth by centrifugation at 12,000 × g for 20 min at 4°C. The cells were resuspended in 100 ml of sterile phosphate buffered saline and centrifuged again. This was repeated, then the cells were resuspended in 1 ml of sterile distilled water before being stored at -20°C for later enzyme extraction.

*Characterisation of intestinal spirochaetes by MLEE*

The MLEE methodology used was as described by Lee et al. (1993) and McLaren et al. (1997). Washed spirochaete cells were thawed at 37°C, placed on ice and lysed by three 30-s cycles of sonication (Sonicator XL2015 ultrasonic liquid processor, Heat Systems, New York, USA). The suspension was centrifuged at 15,000 × g for 10 min at 4°C, and the supernatant stored at -80°C.

The electrophoretic mobilities of 15 constitutive enzymes were determined by electrophoresis of the thawed cell suspensions in 11.4% horizontal starch gels. The enzymes examined were: acid phosphatase (ACP), alcohol dehydrogenase (ADH), alkaline phosphatase (ALP), arginine phosphokinase (APK), esterase (EST), fructose 1,6 diphosphate (FDP), glutamate dehydrogenase (GDH), guanine deaminase (GDA), hexokinase (HEX), l-leucyl-glycyl-glycine peptidase (LGG), mannose phosphate isomerase (MPI), nucleoside phosphorylase (NP), phosphoglucomutase (PGM), phosphoglycose isomerase (PGI) and
superoxide dismutase (SOD). One of four different buffer systems was used for each enzyme: ACP, ADH, HEX and NP were assayed using a Tris malate (pH 7.4) buffer system; ALP, PGI, GDA and MPI were run in a phosphate (pH 7.0) buffer system; EST, FDP, LGG, PGM and SOD were assayed in a discontinuous lithium hydroxide buffer system; APK and GDH were assayed in a discontinuous Poulik buffer system. Enzymes were localised by the addition of suitable specific substrates, as detailed previously (Lee et al., 1993).

**Interpretation of MLEE data**

Variations in the electrophoretic mobility of an enzyme were interpreted as representing products of different alleles at the specific locus coding for that enzyme. Distinctive mobility variants were numbered in order of decreasing rate of anodal migration. Isolates that did not show activity for some of the enzymes were assigned a null allele (zero). For pair-wise comparison, the null alleles were not included in the analysis and were assigned the value -1. Gel runs were repeated up to four times to ensure correct allele designation.

Isolates with identical enzymatic profiles at all 15 loci were grouped into an electrophoretic type (ET). ETs obtained for the 53 new isolates were directly compared with those obtained in a previous MLEE study of 56 intestinal spirochaetes from chickens (McLaren et al., 1997). The latter isolates comprised 50 from Australia, four from the USA and two from the
Netherlands, including type strains and well-characterised isolates for the main species.

Genetic distance between ETs was calculated by pair-wise comparison as the proportions of loci at which dissimilar alleles occurred. The computer program PHYLIP version 3.51c (Phylogeny Inference Package, Department of Genetics, University of Washington, USA) was used to analyse the data and to generate a phenogram illustrating genetic relationships between newly identified ETs, using the unweighted paired group method of arithmetic averages clustering fusion strategy. A genetic distance of 5.1 was arbitrarily used to delineate the species, as it gave relatively clear separation between the groups.

Results

Phenotypic characteristics

All 53 isolates were weakly beta-haemolytic. Nine produced indole, all of which were identified as *B. intermedia* on the basis of MLEE grouping (Table 1, Fig. 1). Six other isolates in the *B. intermedia* grouping did not produce indole, although three of these were from the same farm and shared the same ET.
MLEE results

On the basis of their allelic profiles, the 53 isolates from this study plus
the 56 from the study of McLaren et al. (1997) were divided into 82 ETs in
10 MLEE groupings (a to j), as defined at a genetic distance of 5.1 (Fig.
1). The isolates from the current study were placed into 41 of these ETs,
whilst the isolates from the study of McLaren et al. (1997) were located in
the other 41 ETs. Some ETs contained multiple isolates, but in no case
did ETs contain isolates from both studies. The names of the 53 isolates
in the current study, their MLEE grouping and their ET affiliations are
listed in Table 1.

The MLEE phenogram clustered the isolates into six groups
corresponding to known Brachyspira species, as defined in the study of
McLaren et al. (1997). Fifty (94%) of the current 53 Queensland isolates
were present in five of these six named groups (B. murdochii, B.
innocens, “B. pulli”, B. intermedia and B. pilosicoli), whilst no new isolates
of the sixth named group, B. alvinipulli, were identified. The other four
MLEE groups marked on the phenogram (a, g, h, and i) were new, in that
they were not defined in the MLEE study of McLaren et al. (1997). New
group h contained four isolates, PHB-9, 2A-15, A5 and A6, all from the
MLEE study of McLaren et al. (1997), that had belonged to the “B. pulli”
group (ETs A30–A32) in that study. The other three new MLEE groups
each contained single isolates from the current study.
Table 1. Isolate name, multilocus enzyme electrophoresis (MLEE) grouping, electrophoretic type (ET) in Fig. 1, indole production, flock identity, PCR results and age of bird sampled for 53 isolates of weakly haemolytic *Brachyspira* spp. from 14 Queensland poultry flocks

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Flock(^b)</th>
<th>MLEE(^b) group</th>
<th>ET(^c)</th>
<th>Indole</th>
<th>PCR(^d)</th>
<th>Age of bird (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QAW14</td>
<td>Broiler breeder 1</td>
<td>a</td>
<td>1</td>
<td>-ve</td>
<td><em>B. innocens/murdochii</em></td>
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<td>Broiler breeder 1</td>
<td>b</td>
<td>2</td>
<td>-ve</td>
<td><em>B. innocens/murdochii</em></td>
<td>N/A</td>
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<td>QAW23</td>
<td>Broiler breeder 1</td>
<td>b</td>
<td>3</td>
<td>-ve</td>
<td><em>B. innocens/murdochii</em></td>
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<td>Broiler breeder 1</td>
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<td>4</td>
<td>-ve</td>
<td><em>B. innocens/murdochii</em></td>
<td>N/A</td>
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<td>-ve</td>
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<td>-ve</td>
<td><em>B. innocens/murdochii</em></td>
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<td>Broiler breeder 1</td>
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<td>5</td>
<td>-ve</td>
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<td>Broiler breeder 1</td>
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<td>6</td>
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<td><em>B. innocens/murdochii</em></td>
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<td>7</td>
<td>-ve</td>
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<td>Broiler breeder 1</td>
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<td>8</td>
<td>-ve</td>
<td><em>B. innocens/murdochii</em></td>
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<tr>
<td>Isolate</td>
<td>Flock$^b$</td>
<td>MLEE$^b$ group</td>
<td>ET$^c$</td>
<td>Indole</td>
<td>PCR$^d$</td>
<td>Age of bird (weeks)</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
<td>----------------</td>
<td>-------</td>
<td>--------</td>
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<td>QAW11</td>
<td>Broiler breeder 1</td>
<td>b</td>
<td>9</td>
<td>-ve</td>
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<td>Broiler breeder 1</td>
<td>b</td>
<td>10</td>
<td>-ve</td>
<td>B. innocens/murdochii</td>
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</tr>
<tr>
<td>QAW25</td>
<td>Broiler breeder 1</td>
<td>b</td>
<td>11</td>
<td>-ve</td>
<td>B. innocens/murdochii</td>
<td>40</td>
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<td>12</td>
<td>-ve</td>
<td>B. innocens/murdochii</td>
<td>N/A</td>
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<tr>
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<td>Broiler breeder 1</td>
<td>b</td>
<td>14</td>
<td>-ve</td>
<td>B. innocens/murdochii</td>
<td>N/A</td>
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<tr>
<td>QAW19</td>
<td>Broiler breeder 1</td>
<td>b</td>
<td>15</td>
<td>-ve</td>
<td>B. innocens/murdochii</td>
<td>25</td>
</tr>
<tr>
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<td>Broiler breeder 1</td>
<td>b</td>
<td>16</td>
<td>-ve</td>
<td>B. innocens/murdochii</td>
<td>N/A</td>
</tr>
<tr>
<td>QAW5</td>
<td>Layer 11</td>
<td>b</td>
<td>17</td>
<td>-ve</td>
<td>B. innocens/murdochii</td>
<td>N/A</td>
</tr>
<tr>
<td>QAW28</td>
<td>Layer 12</td>
<td>c</td>
<td>19</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
</tr>
<tr>
<td>QAW33</td>
<td>Layer 1</td>
<td>c</td>
<td>20</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
</tr>
<tr>
<td>QAW34</td>
<td>Layer 10</td>
<td>d</td>
<td>23</td>
<td>-ve</td>
<td>-ve</td>
<td>74</td>
</tr>
<tr>
<td>QAW38</td>
<td>Layer 8</td>
<td>d</td>
<td>24</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
</tr>
<tr>
<td>Isolate</td>
<td>Flock(^b)</td>
<td>MLEE(^b) group</td>
<td>ET(^c)</td>
<td>Indole</td>
<td>PCR(^d)</td>
<td>Age of bird (weeks)</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
<td>-----------------</td>
<td>--------</td>
<td>--------</td>
<td>----------</td>
<td>-------------------</td>
</tr>
<tr>
<td>QAW40</td>
<td>Layer 2</td>
<td>e</td>
<td>42</td>
<td>+ve</td>
<td>-ve</td>
<td>15</td>
</tr>
<tr>
<td>QAW32</td>
<td>Layer 3</td>
<td>e</td>
<td>43</td>
<td>+ve</td>
<td>-ve</td>
<td>N/A</td>
</tr>
<tr>
<td>QAW20</td>
<td>Layer 7</td>
<td>e</td>
<td>52</td>
<td>+ve</td>
<td>B. intermedia</td>
<td>41</td>
</tr>
<tr>
<td>QAW4</td>
<td>Layer 5</td>
<td>e</td>
<td>54</td>
<td>+ve</td>
<td>B. intermedia</td>
<td>21</td>
</tr>
<tr>
<td>QAW16</td>
<td>Layer 6</td>
<td>e</td>
<td>56</td>
<td>+ve</td>
<td>-ve</td>
<td>N/A</td>
</tr>
<tr>
<td>QAW15</td>
<td>Layer 6</td>
<td>e</td>
<td>57</td>
<td>+ve</td>
<td>B. intermedia</td>
<td>32</td>
</tr>
<tr>
<td>QAW17</td>
<td>Layer 6</td>
<td>e</td>
<td>58</td>
<td>+ve</td>
<td>-ve</td>
<td>32</td>
</tr>
<tr>
<td>QAW12</td>
<td>Layer 6</td>
<td>e</td>
<td>59</td>
<td>-ve</td>
<td>B. intermedia</td>
<td>32</td>
</tr>
<tr>
<td>QAW1</td>
<td>Layer 2</td>
<td>e</td>
<td>60</td>
<td>-ve</td>
<td>B. intermedia</td>
<td>62</td>
</tr>
<tr>
<td>QAW2</td>
<td>Layer 2</td>
<td>e</td>
<td>60</td>
<td>-ve</td>
<td>B. intermedia</td>
<td>62</td>
</tr>
<tr>
<td>QAW3</td>
<td>Layer 2</td>
<td>e</td>
<td>60</td>
<td>-ve</td>
<td>B. intermedia</td>
<td>62</td>
</tr>
<tr>
<td>QAW18</td>
<td>Broiler breeder 4</td>
<td>e</td>
<td>61</td>
<td>+ve</td>
<td>-ve</td>
<td>N/A</td>
</tr>
<tr>
<td>Isolate</td>
<td>Flock&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MLEE&lt;sup&gt;b&lt;/sup&gt; group</td>
<td>ET&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Indole</td>
<td>PCR&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Age of bird (weeks)</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------</td>
<td>-------------------------</td>
<td>---------------</td>
<td>--------</td>
<td>-----------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>QAW21</td>
<td>Broiler breeder 4</td>
<td>e</td>
<td>62</td>
<td>+ve</td>
<td>-ve</td>
<td>N/A</td>
</tr>
<tr>
<td>QAW7</td>
<td>Broiler breeder 1</td>
<td>e</td>
<td>63</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
</tr>
<tr>
<td>QAW36</td>
<td>Broiler breeder 2</td>
<td>e</td>
<td>64</td>
<td>-ve</td>
<td>-ve</td>
<td>43</td>
</tr>
<tr>
<td>QAW37</td>
<td>Layer 12</td>
<td>g</td>
<td>66</td>
<td>-ve</td>
<td>-ve</td>
<td>N/A</td>
</tr>
<tr>
<td>QAW41</td>
<td>Layer 12</td>
<td>i</td>
<td>70</td>
<td>-ve</td>
<td>-ve</td>
<td>36</td>
</tr>
<tr>
<td>QAP3</td>
<td>Layer 6</td>
<td>j</td>
<td>71</td>
<td>-ve</td>
<td></td>
<td>B. pilosicoli</td>
</tr>
<tr>
<td>QAP1</td>
<td>Broiler breeder 4</td>
<td>j</td>
<td>73</td>
<td>-ve</td>
<td></td>
<td>B. pilosicoli</td>
</tr>
<tr>
<td>QAP5</td>
<td>Broiler breeder 4</td>
<td>j</td>
<td>73</td>
<td>-ve</td>
<td></td>
<td>B. pilosicoli</td>
</tr>
<tr>
<td>QAP6</td>
<td>Broiler breeder 4</td>
<td>j</td>
<td>73</td>
<td>-ve</td>
<td></td>
<td>B. pilosicoli</td>
</tr>
<tr>
<td>QAP7</td>
<td>Broiler breeder 4</td>
<td>j</td>
<td>73</td>
<td>-ve</td>
<td></td>
<td>B. pilosicoli</td>
</tr>
<tr>
<td>QAP10</td>
<td>Broiler breeder 4</td>
<td>j</td>
<td>73</td>
<td>-ve</td>
<td></td>
<td>B. pilosicoli</td>
</tr>
<tr>
<td>QAP4</td>
<td>Layer 2</td>
<td>j</td>
<td>74</td>
<td>-ve</td>
<td></td>
<td>B. pilosicoli</td>
</tr>
<tr>
<td>Isolate</td>
<td>Flock&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MLEE&lt;sup&gt;b&lt;/sup&gt; group</td>
<td>ET&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Indole</td>
<td>PCR&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Age of bird (weeks)</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------</td>
<td>------------------------</td>
<td>--------------</td>
<td>--------</td>
<td>---------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>QAP9</td>
<td>Layer 9</td>
<td>j</td>
<td>75</td>
<td>-ve</td>
<td>B. pilosicoli</td>
<td>21</td>
</tr>
<tr>
<td>QAP8</td>
<td>Broiler breeder 1</td>
<td>j</td>
<td>76</td>
<td>-ve</td>
<td>B. pilosicoli</td>
<td>N/A</td>
</tr>
<tr>
<td>QAP12</td>
<td>Broiler breeder 1</td>
<td>j</td>
<td>76</td>
<td>-ve</td>
<td>B. pilosicoli</td>
<td>N/A</td>
</tr>
<tr>
<td>QAP14</td>
<td>Broiler breeder 1</td>
<td>j</td>
<td>76</td>
<td>-ve</td>
<td>B. pilosicoli</td>
<td>N/A</td>
</tr>
<tr>
<td>QAP15</td>
<td>Broiler breeder 1</td>
<td>j</td>
<td>76</td>
<td>-ve</td>
<td>B. pilosicoli</td>
<td>N/A</td>
</tr>
<tr>
<td>QAP16</td>
<td>Broiler breeder 1</td>
<td>j</td>
<td>76</td>
<td>-ve</td>
<td>B. pilosicoli</td>
<td>N/A</td>
</tr>
<tr>
<td>QAP11</td>
<td>Broiler breeder 4</td>
<td>j</td>
<td>77</td>
<td>-ve</td>
<td>B. pilosicoli</td>
<td>N/A</td>
</tr>
</tbody>
</table>

<sup>a</sup> Flock identity is from Stephens and Hampson (1999).

<sup>b</sup> MLEE groups are as shown in Fig 1. Groups a, g, h and i are “unknowns”. Group b = B. murdochii; c = B. innocens; d = “B. pulli”; e = B. intermedia; f = B. alvinipulli; j = B. pilosicoli.

<sup>c</sup> Chicken isolates from McLaren et al. (1997) are located in the 41 ETs unlisted above.

<sup>d</sup> PCR results reported in the previous study of Stephens and Hampson (1999)

<sup>e</sup> N/A, data not available.
Fig 1. Multilocus enzyme electrophoresis (MLEE) phenogram showing genetic distance among 82 electrophoretic types (ETs) containing 110 intestinal spirochaetes isolated from chickens. Fifty-three isolates are from the current study and 57 from the study of McLaren et al. (1997). To assist in orientation, the names of 11 isolates from the latter study are written in the ET column on the phenogram. Ten MLEE groups are shown, of which groups a, g, h and i are newly identified unnamed groups not previously described in the study of McLaren et al. (1997). Group h was formed using allelic profiles of four isolates from the study of McLaren et al. (1997) that had belonged to the “B. pulli” group (ETs A30-A32) in that study. The other three new MLEE groups each contained single isolates from the current study.
The identities of the 14 flocks, the number of isolates in these flocks, their group by MLEE, and the disease status of the flocks are presented in Table 2. These data are listed in the same format as in the publication by Stephens and Hampson (1999), except no isolates from Broiler breeder flock 3 or Layer flock 4 were available for the current study. Overall, 29 of the 53 (55%) isolates were identified by MLEE as belonging to the two known pathogenic species *B. intermedia* (n=15) and *B. pilosicoli* (n=14). *B. intermedia* was isolated from eight (57%) flocks, including five laying hen flocks and all three broiler breeder flocks. Seven (88%) of these flocks were classified as being diseased. *B. pilosicoli* was isolated from hens from five flocks (36%), including three of the 11 laying hen flocks, and two of the three broiler breeder flocks. Three (60.0%) of these five flocks were diseased, whilst the health status of the other two was not known. The health status of the two flocks from which isolates of *B. innocens* were recovered was unknown. One of the two flocks colonised with *B. murdochii* had wet litter (although *B. pilosicoli* and *B. intermedia* were also present in this flock, Broiler breeder 1), while the other colonised flock was of unknown health status. “*B. pulli*” was isolated from one flock of unknown health status. The single isolate of MLEE group a came from a flock with wet litter (Broiler breeder 1), whilst isolates from groups g and i both came from the same flock of unknown health status (Layer 12).
Spirochaete species in flocks colonised with multiple isolates

Multiple isolates were available for six of the flocks, and five of these had isolates of more than one species present. Broiler breeder 1 was concurrently colonised with *B. murdochii*, *B. intermedia*, *B. pilosicoli* and an MLEE group a spirochaete. Flocks Broiler breeder 4, Layer 2 and Layer 6 were colonised with both *B. intermedia* and *B. pilosicoli*. Flock Layer 12 was colonised with *B. innocens* and the two isolates of *Brachyspira* MLEE groups g and i.

*ET distribution of isolates within and between flocks*

No isolates from different farms belonged to the same ET, although occasionally such isolates were closely related (eg. the *B. pilosicoli* isolate in ET 74 from Layer 2 was closely related to the *B. pilosicoli* isolates in ET 73 on Broiler breeder 4) (Fig. 1).
Table 2. Distribution of *Brachyspira* spp. isolates with respect to flocks of origin and disease status of flock

<table>
<thead>
<tr>
<th>Flock</th>
<th>No. of isolates</th>
<th>Identity by MLEE (no. of isolates)</th>
<th>Disease status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler breeder 1</td>
<td>23</td>
<td><em>B. murdochii</em> (16); <em>B. pilosicoli</em> (5); <em>B. intermedia</em> (1); a (1)</td>
<td>Wet litter in 20% flock</td>
</tr>
<tr>
<td>Broiler breeder 2</td>
<td>1</td>
<td><em>B. intermedia</em> (1)</td>
<td>Diarrhoea/production loss</td>
</tr>
<tr>
<td>Broiler breeder 4</td>
<td>8</td>
<td><em>B. pilosicoli</em> (6); <em>B. intermedia</em> (2)</td>
<td>N/A</td>
</tr>
<tr>
<td>Layer 1</td>
<td>1</td>
<td><em>B. innocens</em> (1)</td>
<td>N/A</td>
</tr>
<tr>
<td>Layer 2</td>
<td>4</td>
<td><em>B. pilosicoli</em> (1); <em>B. intermedia</em> (3)</td>
<td>Depressed/lethargic/off lay</td>
</tr>
<tr>
<td>Layer 3</td>
<td>2</td>
<td><em>B. intermedia</em> (2)</td>
<td>Diarrhoea/production loss</td>
</tr>
<tr>
<td>Layer 5</td>
<td>1</td>
<td><em>B. intermedia</em> (1)</td>
<td>Poor growth rate/death</td>
</tr>
<tr>
<td>Flock&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No. of isolates</td>
<td>Identity by MLEE (no. of isolates)</td>
<td>Disease status&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>-------------------</td>
<td>----------------</td>
<td>----------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Layer 6</td>
<td>5</td>
<td><em>B. pilosicoli</em> (1); <em>B. intermedia</em> (4)</td>
<td>Weak/lethargic/deaths</td>
</tr>
<tr>
<td>Layer 8</td>
<td>1</td>
<td><em>B. intermedia</em> (1)</td>
<td>Wet litter</td>
</tr>
<tr>
<td>Layer 7</td>
<td>1</td>
<td><em>B. innocens</em> (1)</td>
<td>N/A</td>
</tr>
<tr>
<td>Layer 9</td>
<td>1</td>
<td>“<em>B. pulli</em>” (1)</td>
<td>N/A</td>
</tr>
<tr>
<td>Layer 10</td>
<td>1</td>
<td><em>B. murdochii</em> (1)</td>
<td>N/A</td>
</tr>
<tr>
<td>Layer 11</td>
<td>1</td>
<td><em>B. innocens</em> (1); g (1); i (1)</td>
<td>N/A</td>
</tr>
<tr>
<td>Layer 12</td>
<td>3</td>
<td><em>B. innocens</em> (1); g (1); i (1)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

<sup>a</sup> Flock identity is from Stephens and Hampson (1999).

<sup>b</sup> N/A, not available.
More than one isolate of the same species was recovered on five farms. On Broiler breeder 1, the 16 isolates of *B. murdochii* were present in 14 different ETS, widely distributed across the species group. In contrast, the five isolates of *B. pilosicoli* from this farm all belonged to the same ET (76). On broiler breeder 4, five of the six *B. pilosicoli* isolates belonged to the same ET (73), whilst the sixth was more distantly related (ET 77). The two *B. intermedia* isolates from this farm were in adjacent ETs that were clearly distinct from each other (ETs 61 and 62). On Layer 2, the three *B. intermedia* isolates all belonged to the same ET (60). On Layer 6, the four *B. intermedia* isolates were different, belonging to four adjacent ETs (56-59), but in this case they were more closely related, and may have belonged to the same clonal group.

**Discussion**

The results of this study confirm that MLEE is a useful technique for identifying intestinal spirochaete species isolated from hens. All but three of the 53 isolates examined were clustered into five of six named *Brachyspira* spp. groups, together with other previously-identified intestinal spirochaetes from hens, as described in the study of McLaren et al. (1997). Four new minor groups, each containing small numbers of isolates, were also identified. Whether these may represent new *Brachyspira* species, or are affiliated with existing species remains unclear. In the case of the isolate in MLEE group a, despite the comparatively large genetic distance from its nearest neighbour (MLEE
group b, *B. murdochii*), it may actually belonged to this neighbouring group. This is supported by the fact that the original *nox*-based PCR identified the isolate in MLEE group a as *B. innocens/murdochii*, and it originated from farm Broiler breeder 1, where many other diverse isolates of *B. murdochii* were identified. In the case of the isolate in new MLEE group g, it was related to *B. alvinipulli*, but its true identity could not be determined. New MLEE group h was formed from four isolates all previously identified as “*B. pulli*” in the MLEE study of McLaren et al. (1997). In the current study, their relocation to a distinct group was related to the fact that the pair-wise comparison of allelic profiles was over a much larger number of isolates than it was in the earlier study. The final new MLEE group i contained a single isolate that was most closely related to *B. pilosicoli*. It is unlikely to belong to this species however as it failed to amplify in the *B. pilosicoli* PCR.

The MLEE results helped to validate the results of the original PCRs used by Stephens and Hampson (1999). The *B. innocens/B. murdochii* PCR had ~98% specificity and ~90% specificity. It amplified all 17 isolates identified as *B. murdochii* (as well as the isolate in MLEE group a), but failed to amplify both isolates of *B. innocens* (MLEE group c). The PCR used to identify *B. intermedia* had 100% specificity but only ~47% sensitivity, as it was positive for only seven of the 15 isolates in MLEE group e. When this same PCR was used in another study on 34 strains of *B. intermedia* from pigs and chickens and 195 strains of other *Brachyspira* species, it was found to be 100% sensitive but only ~94%
specific (Suriyaarachchi et al., 1999). These contrasting results emphasise the need to optimise conditions for diagnostic PCRs, and to include a sufficient number of positive and negative controls.

The finding that not all *B. intermedia* isolates were indole positive was unexpected, as porcine isolates of *B. intermedia* are typically indole positive (Lee et al., 1993). Nevertheless, McLaren et al. (1997) also found one of 15 chicken isolates of *B. intermedia* to be indole negative, so it appears that indole production cannot be fully relied upon for identifying avian isolates of *B. intermedia*.

The spirochaetal isolates represented a range of *Brachyspira* species, the most common being *B. murdochii* (32%), *B. intermedia* (28%) and *B. pilosicoli* (26%). On a farm basis, *B. intermedia* was the most common species, being identified on eight farms (57%), compared to five (36%) for *B. pilosicoli* and two (14%) for *B. murdochii*. The wide distribution of *B. intermedia* amongst these Queensland farms was not reported in the study of Stephens and Hampson (1999), due to the poor sensitivity of the *B. intermedia* PCR used in that study. The current results are more consistent with those previously found in Western Australia, where 41% of isolates from flocks were identified as *B. intermedia* (McLaren et al., 1996, 1997). Hence, Australia-wide, *B. intermedia* is probably more commonly present in hens than is *B. pilosicoli*. All seven of the flocks having a history of disease (reduced production/wet litter) were colonised with *B. intermedia* and/or *B. pilosicoli*, and both species were present
together on four farms. These results help to confirm the likely pathogenic potential of these two *Brachyspira* species. *B. alvinipulli*, the other confirmed pathogen of hens, was not identified in this study, although a related isolate was identified in MLEE group g. To date *B. alvinipulli* has only been isolated from one flock in the USA, and therefore it seems to have a restricted geographical range.

The non-pathogenic species were not well represented amongst the isolates in the current study, except for *B. murdochii* which predominated on farm Broiler breeder 1. The identification of only one “B. pulli” isolate was unexpected, as in the study of McLaren et al. (1996, 1997) these “MLEE group d” isolates formed the most common single group (39% of isolates). Hens on Broiler breeder 1 were colonised with strains of many different ETs of *B. murdochii* (16 isolates in 14 ETs), and the origin and significance of these diverse strains was unclear. The farm had a wet litter problem, but some hens also were colonised with *B. pilosicoli* or *B. intermedia*. Further work is required to determine whether *B. murdochii* has the potential to cause wet litter problems alone, or whether it simply proliferates more readily in situations where there are underlying gastrointestinal disturbances.

Overall, the other *Brachyspira* species also showed considerable strain diversity, but this was generally observed between farms rather than within farms. On farms Broiler breeder 1 and Broiler breeder 4 a predominant strain (ET) of *B. pilosicoli* was present (on each farm, five
isolates of \textit{B. pilosicoli} in an ET), whilst on Layer 2 the three isolates of \textit{B. intermedia} all belonged to one ET. On farms Layer 2 and Layer 6 the \textit{B. pilosicoli} isolates belonged to different ETs. The results suggest that the epidemiology of avian intestinal spirochaetosis is complex and variable, with certain farms colonised by a predominant strain of either \textit{B. pilosicoli} or \textit{B. intermedia}, and others with mixed strains of these and other species. Further work is required to determine the clinical significance of these different patterns of infection, and to investigate the factors responsible for the different species and strain distributions.

\textit{Acknowledgments}

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\textbf{References}


McLaren AJ, Trott DJ, Swayne DE, Oxberry SL and Hampson DJ 1997. Genetic and phenotypic characterisation of intestinal spirochetes


Appendix

Questionnaire supplied to meat breeder and meat chicken farms at the time of sample collection during the study described in Paper I.
Poultry Spirochaete Prevalence Survey
Questionnaire for Meat Breeder/Meat Chicken Farms

Toowoomba Veterinary Laboratory
203 Tor Street Toowoomba Qld 4350
Phone 07 4688 1351  Fax 07 4688 1195

Aim:

To attempt to answer whether spirochaetal infection is:

1. present in meat chickens ?
2. carried over from batch to batch ?

If present is this related to:

1. age of birds ?
2. stocking density ?
3. litter management ?

Information requested:

Company Name: ___________________     Farm Name: ___________________

Farm location: __________________________ Shed Name or No. _____________

Strain of bird:__________________________ Age of bird: _____wks ? or days ?

Diarrhoea in birds during growing period?   Yes           No

1. Seasonal or age influence
Date placed:   ____/_____/199___  or

Date lifted:   ____/_____/199___

Age at turn off:   ____ days

2.  Stocking density

Number of birds:   ______ thousand

Shed dimensions:   ______ x ______ metres   or   _____ x ______ feet

3.  Litter conditions

Litter material:   ________________

Litter treatment prior to this batch:

  e.g. Complete clean out and all new litter,

  or new  litter brooding area, composted rest of shed

Disinfectant used:   Yes  [ ]  No  [ ]

Type/Name of disinfectant:   __________________________

Thank you for your assistance.     Carol Stephens
The survey that formed the basis for this work demonstrated that intestinal spirochaetes of the genus *Brachyspira* are prevalent in Australian commercial poultry flocks, with intestinal spirochaetes being detected in birds from more than 40% of the 69 commercial poultry farms tested. The survey also demonstrated that infection with intestinal spirochaetes is associated with intestinal disease, resulting in wet litter and reduced production.

A very high level of colonisation was found in layer flocks, with over 68% of farms sampled being positive. A majority of the layer farms that elected to participate in the survey reported health problems, which may have contributed to the high rate of colonisation found. Further work needs to be done in order to confirm levels of colonisation in egg layers. This would require comparing colonisation rates in layers of varying health status.

The survey found that almost 43% of broiler breeder flocks sampled were colonised with spirochaetes, indicating a significant level of colonisation of broiler breeder flocks in Australia. Further investigation is needed to determine whether in Australia such colonisation affects the production of broiler flocks derived from these infected breeder birds.
Comparison of mortality, feed conversion and growth rates of broilers derived from infected and uninfected broiler breeder flocks is required.

The lack of colonisation in all broiler farms tested was a significant finding. While consistent with overseas research, the reason for this lack of colonisation has not been satisfactorily explained. It would seem likely that the all-in all-out management practices employed on broiler farms, combined with complete litter removal between batches, prevents infection. The widespread use of ionophores as coccidiostats may also inhibit infection with \textit{Brachyspira}. However it may also be that the relative immaturity of the broiler chicken intestinal flora does not provide the spirochaetes with an environment conducive to colonisation. The fact that spirochaetes were not detected in any birds under the age of 10 weeks, irrespective of husbandry practices, would tend to support this theory. This is an area requiring further investigation, particularly as it may provide insights into ways of preventing the onset of colonisation.

The results of the survey showed that levels of colonisation frequently correlated with increased bird age, suggesting that infection gradually builds up over time. However, the original source of the infection was not determined. It is possible that infection originates from birds that are already colonised being brought onto farms. However this is unlikely to be the case with meat breeders, which are usually moved into the growing sheds at around 12 weeks of age. The survey results showed that they are unlikely to be colonised at this age. It is possible that the
organisms may already exist on the farm and the birds are gradually colonised as they become exposed. External sources of infection may exist, such as pigs and wild birds. Future work should investigate aspects of strain dissemination within and between flocks by the use of molecular strain typing techniques.

Overall the survey results revealed a significant association between colonisation and disease, including wet litter, diarrhoea and egg production drop. However, the presence of intestinal spirochaetes in some flocks which did not report signs of disease suggested that certain strains of these organisms were apathogenic.

**Paper II**

The subsequent experimental infection of birds with *B. innocens* and *B. pilosicoli* demonstrated that some strains of *Brachyspira* are non-pathogenic. Overall, the study showed that *B. innocens* is unlikely to have pathogenic significance in commercial adult birds. The presence of non-pathogenic spirochaetes complicates the diagnosis of avian intestinal spirochaetosis and the availability of reliable species-specific techniques, such as PCR and RFLP, are required to differentiate pathogenic from non-pathogenic spirochaete isolates.

The significant finding in the study was the delay in both onset of egg production and in reduced total egg production in the birds inoculated
with *B. pilosicoli*. The major losses in production occurred in the first 11 weeks of lay, during which birds experimentally infected with *B. pilosicoli* produced only two thirds of their expected number of eggs. In a commercial situation this loss would have an extremely serious economic impact.

No gross or histological abnormalities were found in the caeca of any of the birds at post mortem examination. The lack of pathological changes at necropsy was not completely unexpected, as the birds were not colonised at this time and they had had many weeks for any lesions to resolve. However the failure to detect any lesions meant that no information was obtained which might have assisted in elucidating the pathological mechanisms involved. In future experimental infection studies it would be useful to necropsy birds at the time they were culture positive. A practical outcome of the lack of gross and histological changes in the caeca is that it may not always be possible to diagnose infection with intestinal spirochaetes purely on histological grounds. Diagnosis may have to be based on microbiological culture of appropriate samples from birds showing clinical signs. Given that the anaerobic nature and fastidious growth requirements of intestinal spirochaetes make microbiological culture relatively difficult and that mixed infections may occur, it would be desirable to culture samples from a number of birds in order to make an accurate diagnosis. Further experimental studies are needed to find a reliable means of establishing experimental colonisation and to subsequently examine the pathological basis of production losses.
Having confirmed the pathogenic potential of *B. pilosicoli* in commercial
meat breeders, the next experiment examined antimicrobial
susceptibilities in *Brachyspira* isolates from chickens. The main two
pathogenic *Brachyspira* species infecting chickens, *B. pilosicoli* and *B.
intermedia* were tested against tiamulin, lincomycin, metronidazole,
tetracycline, ampicillin and tylosin. This study increased understanding of
what antimicrobial agents might prove useful in treating AIS caused by
one or other of the main two pathogenic *Brachyspira* species infecting
chickens.

The minimum inhibitory concentration (MIC) ranges obtained for most
isolates of both *Brachyspira* species for tiamulin, lincomycin,
metronidazole and tetracycline suggested that these antimicrobials are in
general likely to be effective in treating AIS. Whether or not these
antimicrobials can be used in infected flocks, particularly in hens laying
eggs intended for human consumption, will depend on local regulations.

The two *Brachyspira* species had identical MIC$_{50}$ and MIC$_{90}$ ranges for
metronidazole and tetracycline, but there were trends for some species-
related differences in susceptibility to the other four antimicrobials.
Reduced susceptibility to tiamulin occurred more commonly amongst the
*B. intermedia* isolates than amongst the *B. pilosicoli* isolates, whilst the
reverse was true for ampicillin. The MIC$_{50}$ figures also suggested a trend
for reduced susceptibility to lincomycin and tylosin amongst \textit{B. pilosicoli} isolates compared to \textit{B. intermedia} isolates. These trends emphasize the need to identify the spirochaete species involved in individual cases of AIS and preferably to conduct antimicrobial susceptibility testing before selecting an antimicrobial for treatment. In future work it would be useful to confirm these trends by monitoring susceptibility of a greater number of strains over a longer period and testing with more antimicrobial dilutions across the appropriate concentration ranges.

**Paper IV**

Following the determination of MIC ranges, two antibiotics were selected for use in treating broiler breeder hens experimentally infected with \textit{B. pilosicoli}. Attempts to infect the birds with spirochaetes initially failed, the birds only becoming infected following the addition of zinc bacitracin at a rate of 50 ppm to their diet. Subsequent treatment with tiamulin and with lincomycin both removed the infection, whilst untreated birds remained infected, thus demonstrating the usefulness of both tiamulin and lincomycin for treatment of infection with \textit{B. pilosicoli} in adult birds. The mechanism by which zinc bacitracin, a feed additive commonly used in the commercial poultry industry, appears to promote colonisation is unknown and certainly requires further investigation. Clarification could shed further light on the mechanism by which spirochaetes establish intestinal infection.
Multilocus enzyme electrophoresis (MLEE) was used to identify, examine genetic relationships and look at disease associations of a collection of intestinal spirochaete isolates previously recovered from commercial poultry farms in Queensland. The results showed that the epidemiology of intestinal spirochaetal infections in broiler breeder and laying hen flocks is complex and can vary considerably between farms.

The isolates were divided into five Brachyspira species groups by MLEE: Brachyspira murdochii (n=17), B. intermedia (n=15), B. pilosicoli (n=14), B. innocens (n=2), and “B. pulli” (n=1). Three new MLEE groups each containing single isolates also were identified. Using MLEE, isolates from two farms (14%) were identified as B. murdochii, whilst the pathogenic species B. intermedia and B. pilosicoli were present in hens from eight (57%) and five (36%) farms respectively and were identified together in four (29%) farms. All seven of the farms with production problems or wet litter were colonised with B. intermedia and/or B. pilosicoli. Six farms had multiple spirochaete isolates available for examination. Two broiler breeder farms both had five isolates of B. pilosicoli that shared the same MLEE electrophoretic type (ET), whilst one laying hen farm had three isolates of B. intermedia that all belonged to the same ET. Hence on each of these farms a predominant strain of a pathogenic species was present. On the other farms isolates of the same species were more diverse and belonged to different ETs. Further investigation is required to elucidate
the basis for this variation. Longitudinal studies, involving characterisation of isolates from individual farms over time are desirable. Detailed comparisons of strains and husbandry practices on different farms are also required. This study once again confirmed the desirability of accurately identifying the individual strains involved in order to make an accurate diagnosis of AIS.

Conclusions

This work has shown that intestinal spirochaetes of the genus *Brachyspira* are prevalent in Australian commercial poultry flocks and are associated with enteric disease. All farms with production problems were found to be colonised with either *B. pilosicoli* or *B. intermedia*. Infection studies confirmed that the two species *B. pilosicoli* and *B. intermedia* are pathogenic and are capable of causing chronic diarrhoea, production loss and wet litter. Antimicrobial susceptibility testing showed that lincomycin and tiamulin can be effective in the control of avian intestinal spirochaetes. Examination of genetic relationships using MLEE demonstrated the complex epidemiology of spirochaetal infections. This research has provided new insights into the role of intestinal spirochaetes in enteric disease and production loss in commercial poultry production in Australia.