THE EPIDEMIOLOGY OF *BRACHYSPIRA* SPECIES IN ONTARIO LAYER CHICKEN FLOCKS

by

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ABSTRACT

THE EPIDEMIOLOGY OF *BRACHYSPIRA* SPECIES IN ONTARIO LAYER CHICKEN FLOCKS

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Avian intestinal spirochetosis is a disease of poultry caused by the spirochete bacterium *Brachyspira*, and is characterized by increased mortality, reduced egg production, weight loss, diarrhoea, and fecal staining of egg shells, which leads to downgrading of eggs. The presence of *Brachyspira* species in Ontario layer chicken flocks and its association with downgrades for dirty eggs was explored. Further, farm interviews were conducted to determine risk factors associated with the presence of *Brachyspira* species. The prevalence of *Brachyspira* species was significantly higher in flocks with a higher proportion of dirty eggs (dirty flocks) compared to those with a lower proportion of dirty eggs (clean flocks). *Brachyspira pilosicoli* was the only pathogenic species detected. Risk factors associated with the presence of *Brachyspira* species were flock age, barn age, multi-age farms, and housing type. Based on these findings, recommendations were suggested to control the occurrence of *Brachyspira* species and the associated dirty egg problem.
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CHAPTER ONE

INTRODUCTION, LITERATURE REVIEW, AND OBJECTIVES

INTRODUCTION

Avian intestinal spirochetosis (AIS) is primarily a disease of layer and broiler breeder chickens exhibited by delayed and/or reduced egg production, increased mortality, weight loss, and chronic diarrhoea that leads to faecal staining of egg shells. Avian intestinal spirochetosis is caused by *Brachyspira* species (Davelaar *et al*., 1986; Trampel *et al*., 1994; Jordan and Hampson, 2008).

*Brachyspira* species are anaerobic gram-negative bacteria that colonize the large intestine of different species of animals and humans (Hirsh, 2004). Not all *Brachyspira* species are pathogenic. Of the seven known species that colonize chickens, four of them, namely *Brachyspira alvinipulli, Brachyspira hyodysenteriae, Brachyspira intermedia,* and *Brachyspira pilosicoli* are pathogenic. Various factors, such as flock age, housing type, manure disposal system, and feed have been associated with the occurrence of *Brachyspira* species in chickens (Stephens and Hampson, 1999; Phillips *et al*., 2005; Bano *et al*., 2008; Myers *et al*., 2009).

Avian intestinal spirochetosis is a concern to the egg layer industry primarily due to its economic significance (Trampel *et al*., 1994; Smith, 2005; Burch *et al*., 2006) as a consequence of faecal staining of egg shells and reduced egg production (Trampel *et al*., 1994). Ontario has the largest table egg industry in Canada (9,836,000 layer chickens), with a revenue of $912,357,000 from 239,247,000 dozen eggs per year (Statistics Canada, 2011). Although there are no published reports of AIS in chickens in Canada, faecal-stained eggs (dirty eggs) have been a problem in the Ontario layer industry for two decades (Dr. Lloyd Weber, 2009, personal
communication), and are the most common reason for down grading of eggs (Scott Houghton, Leanne Cooley, 2009, personal communication). Based on studies in other countries that have reported spirochetes as a cause of AIS (Dwars et al., 1991; McLaren et al., 1996; Stephen and Hampson, 1999), a preliminary study was initiated in 2008 at the Animal Health Laboratory, University of Guelph (unpublished). In that study, four of five farms with a history of a dirty egg problem were positive for *Brachyspira* species using culture and polymerase chain reaction (PCR); the two pathogenic species identified were *B. intermedia* and *B. pilosicoli* (Dr. Durda Slavic, 2009, personal communication).

The literature review that follows examines the relationship between *Brachyspira* species and AIS, presence of pathogenic and non-pathogenic *Brachyspira* species in poultry and other animal species, zoonotic importance of *Brachyspira*, descriptive epidemiology of *Brachyspira* species in chickens and other avian hosts, and risk factors associated with the presence of *Brachyspira* species in chicken and other hosts.

**LITERATURE REVIEW**

*Brachyspira* and avian intestinal spirochetosis

The genus *Brachyspira* (formerly *Treponema* and later *Serpulina*) is a member of the family *Brachyspiraceae* (Ochiai et al., 1997; Olsen et al., 2000), within the class Spirochetes (Paster et al., 2000). Spirochetes are gram-negative, long, thin, helical, and motile bacteria. *Brachyspira* species are obligate anaerobes that attach to the mucin layer of the colon and can cause inflammation in some animal species and humans (Lux et al., 2000; Hirsh, 2004).
There are relatively few studies on *Brachyspira* and AIS, and the pathogenic potential is not established for all species of *Brachyspira* because this bacterium requires specialized techniques and methods to isolate. At present, there are nine species of *Brachyspira* described (isolated) from avian species (Table 1.1). The presence of pathogenic and non-pathogenic species in infected animals makes it difficult to attribute infection to pathogenic or commensal species (Duhamel, 1998). For instance, *B. innocence*, *B. murdochii* and *B. pulli*, the presumed non-pathogenic species in chickens, were isolated from chickens with signs of AIS in conjunction with pathogenic species of *Brachyspira* (Feberwee et al., 2008). Provisionally-proposed species, (*B. corvi* and *B. suanatina*) have also been isolated from avian species; however, their clinical significance is questionable (Stephen and Hampson, 1999; Rasback et al., 2007; Janasson et al., 2008). To-date, *B. corvi*, which has been isolated from corvid birds has not been associated with AIS; *B. pulli*, which has been isolated from chickens and dogs, is presumed to be non-pathogenic (Feberwee et al., 2008). *Brachyspira suanatina* has been recovered from mallard ducks, and caused diarrhoea in experimentally-infected pigs (Rasback et al., 2007).

All of the pathogenic species except *B. alvinipulli* have been experimentally shown to cause AIS (Trott et al., 1995; Hampson and McLaren, 1999; Jamshidi and Hampson, 2003). Avian intestinal spirochetosis is a disease complex of mainly layer chickens (Adachi et al., 1985; Swayne and McLaren, 1997). In chickens, AIS can be manifested by reduced growth rate, delayed onset of egg laying, chronic diarrhoea, faecal staining of egg shells, weight loss, reduced egg production, and increased mortality (Davelaar et al., 1986; Griffiths et al., 1987; Dwars et al., 1992; Swayne et al., 1992; Stephens and Hampson, 1999; Burch et al., 2006; Bano et al., 2008). Of the pathogenic species, only *B. alvinipulli* is exclusively pathogenic to poultry (Swayne et al., 1995). The other pathogenic species colonize other animals as well. For instance,
*B. hyodysenteriae* causes swine dysentery in pigs, whereas *B. pilosicoli* causes porcine intestinal spirochetosis (Trott *et al.*, 1996). *Brachyspira pilosicoli* has a wider host range, and has been isolated from a number of other species of animals, including humans with symptoms of intestinal spirochetosis (Hampson *et al.*, 2006b).

**Pathogenic *Brachyspira* species**

As indicated, pathogenic species of *Brachyspira* cause gastrointestinal symptoms in different animals. A brief review of each pathogenic species follows.

**Brachyspira hyodysenteriae**

Adachi and *et al.*, (1985) observed lesions in the caecal mucosa of chickens that were experimentally-exposed to *B. hyodysenteriae*. However, the first natural infection of chickens with this species was confirmed in 2007 from three commercial layer flocks in the Netherlands that exhibited signs of AIS (Feberwee, *et al.*, 2008). It has subsequently been recovered from layer hens with a history of AIS in Hungary (Ivanics *et al.*, 2009). *Brachyspira hyodysenteriae* has also been isolated from rheas and mallards with typhlocolitis (Jensen *et al.*, 1996; Janasson *et al.*, 2001).

*Brachyspira hyodysenteriae* is highly virulent in pigs (Jensen *et al.*, 1996), in which it causes swine dysentery in growers and finishers. Signs include chronic diarrhoea, weight loss, and high mortality (Hampson *et al.*, 1995; Taylor, 1997). Mortality can reach up to 30%, incurring a significant economic loss (Harris, *et al.*, 1972). Recovered pigs continue to shed the bacteria, serving as a source of infection for susceptible pigs (Songer and Harris, 1978). Pigs recovered from swine dysentery are resistant to re-infection (Hirsh, 2004). *Brachyspira*
*Hyodysenteriae* has been detected from the faeces of dogs, rats, and mice living on swine farms, indicating possible cross-species transmission (Hirsh, 2004).

**Brachyspira pilosicoli**

*Brachyspira pilosicoli* causes severe reduction of egg production (Stephens and Hampson, 2002b), and increased mortality in layers (Burch *et al*., 2006). *Brachyspira pilosicoli* can be isolated from various animal species and humans (Munshi *et al*., 2003; Smith, 2005).

*Brachyspira pilosicoli* isolates from infected humans have resulted in wet faeces when inoculated into chickens (Jamshidi and Hampson, 2003). Typhlocolitis has also been observed in turkeys colonized by this species (Shivaprasad and Duhamel, 2005). Although the association between clinical signs and colonization was not established, this bacterium was also isolated from faecal samples of water birds, mainly from ducks (Oxberry *et al*., 1998). *Brachyspira pilosicoli* has been associated with intestinal spirochetosis in pigs, dogs, cattle, horses, Sika deer, wild mice, rats, and humans (Joens and Kinyon, 1982; Trott *et al*., 1996; Shibahara *et al*., 2000b; Jamshidi and Hampson 2003; Hirsh, 2004; Hampson *et al*., 2006a). It has also been isolated from animals with diarrhoea, including colts (Shibahara *et al*., 2002a), puppies (Oxberry and Hampson, 2003), and pigs (Trott *et al*., 1996). In pigs, it causes porcine intestinal spirochetosis, which is manifested primarily by typhlocolitis and watery diarrhoea (Trott *et al*., 1996). Natural spread among humans and animals was established after ducks, pigs, and dogs living around infected humans were found positive for this species (Trott *et al*., 1996; Trott *et al*., 1997). The zoonotic importance of *B. pilosicoli* will be discussed later in this review.
**Brachyspira intermedia**

*Brachyspira intermedia* was originally recognized as a new species of *Serpulina* in 1997 (Stanton *et al.*, 1997), and was later classified under the genus *Brachyspira* in 2006 (Hampson and La, 2006). *Brachyspira intermedia* affects mainly poultry and pigs (Jordan and Hampson, 2008). It is commonly isolated from older layer flocks with chronic diarrhoea (Myers *et al.*, 2009). The within-flock prevalence in layers older than 40 weeks ranged from 10 to 100%, and it was detected from 80% of the flocks (Myers *et al.*, 2009). This species is responsible for significant economic loss due to reduced egg production and faecal staining of eggs (Hampson and McLaren, 1999). For example, an average of 1.4% weekly decrease in egg production and 1.16 grams lower egg weight has been reported in flocks experimentally-infected with this species (Hampson and McLaren, 1999).

**Brachyspira alvinipulli**

A fourth distinct virulent species of *Brachyspira* in chickens was described in 1995 (Swayne *et al.*, 1995). Thereafter, the name *B. alvinipulli* was proposed (Stanton *et al.*, 1998). Following this first report from the United States (U.S.) (Stanton *et al.*, 1998), *B. alvinipulli* has been reported in layer flocks in Hungary (Nemes *et al.*, 2006) and the Netherlands (Feberewee *et al.*, 2008). *Brachyspira alvinipulli* was the most commonly detected species in geese with signs of typhlocolitis (Nemes *et al.*, 2006; Glavitis *et al.*, 2008).

**Non-pathogenic species of Brachyspira**

The presumably non-pathogenic species of *Brachyspira* can be found in the faeces of animals with or without signs of spirochetosis (Hirsh, 2004). The three known species that
colonize chickens are *B. innocens*, *B. murdochii*, and *B. pulli* (Kinyon and Harris, 1979). One-day-old chicks experimentally-inoculated with *B. innocens* and followed for 21 days did not show any clinical signs, whereas those infected with *B. pilosicoli* and *B. hyodysenteriae* exhibited stunted growth and diarrhoea (Trott *et al*., 1995). Of broiler breeder chickens inoculated with *B. innocens* and *B. pilosicoli* at 17 weeks of age, only those inoculated with *B. pilosicoli* showed decreased egg production and diarrhoea (Stephens and Hampson, 2002b). In contrast, decreased egg production due to *B. innocens* in free range layer flocks has been reported (Burch, 2010). *Brachyspira murdochii*, which is an apparently non-pathogenic species, was found to be slightly pathogenic to pigs when present in high numbers in the intestine (Weissenböck *et al*., 2005; Jensen *et al*., 2010). This observation led to the conclusion that under certain unidentified conditions, all *Brachyspira* species could be potential pathogens (Weissenböck *et al*., 2005).

**Zoonotic importance of *Brachyspira* species**

*Brachyspira pilosicoli* is a potentially zoonotic species that colonizes the large intestine of humans, and is considered to be the agent of human intestinal spirochetosis (HIS) (Trivett *et al*., 1998; Mikosza and Hampson, 2001). Common symptoms of HIS due to *B. pilosicoli* are rectal bleeding, abdominal cramps, and chronic diarrhoea (Trott *et al*., 1996; Smith, 2005). A person who voluntarily ingested *B. pilosicoli* developed nausea, abdominal pain, and headache, in addition to shedding the bacteria until therapy was initiated on day 52 (Oxberry *et al*., 1998). To-date, there is no evidence that *Brachyspira* is transmitted through consumption of eggs from affected hens. It has been suggested that this is because of the anaerobic nature of the bacteria and its inability to survive the dry environment on the egg surface (Jamshidi and Hampson,
Transmission to humans likely occurs by the faecal–oral route from the faeces of animals, such as dogs and birds (Hampson et al., 2006). Wild birds and rodents are recognized as potential sources of transmission by contaminating water sources with faecal material (Hampson, 1991; Oxberry et al., 1998; Jansson et al., 2001).

Reported prevalence of *B. pilosicoli* estimates in humans vary depending on geographic location; estimates range from 2 to 7% in western countries, to as high as 30% in developing countries (Trott et al., 1997). A lower prevalence was reported in children younger than two years of age compared to those who were older than two years of age (Trott et al., 1997).

Prevalence estimates also vary depending on the immune status of individuals. Generally, a higher prevalence was observed among immune-compromised individuals (Fournieamazouz et al., 1995). Prevalence estimates as high as 54% have been reported among HIV-infected patients (Trivett et al., 1998), and spirochetemia has been observed in terminally ill patients (Fournieamazouz et al., 1995; Kanavaki et al., 2002; Smith, 2005). Spirochetemia occurs due to injury to the gut mucosa that facilitates mucosal invasion (Trott et al., 1997; Bait-Merabet et al., 2008). Antimicrobials, such as metronidazole, mebendazole, and erythromycin, were found to be successful in eradicating *B. pilosicoli* from the stool of infected individuals (Ruane et al., 1989; Oxberry et al., 1998; Calderaro et al., 2008).

**Epidemiology of *Brachyspira* in chickens and other avian hosts**

*Brachyspira* species have been identified in chicken flocks in Australia, Sweden, Italy, Poland, the United Kingdom, the Netherlands, Hungary, Iran, and the U.S. (Davelaar et al., 1986; Dwars et al., 1989; Swayne et al., 1992; Jansson et al., 2000; Kizerwetter-Świda et al., 2005).
2005; Burch, 2006; Bano et al., 2008; Ivanics et al., 2009; Jamshidi et al., 2009). Several studies have indicated an association between pathogenic species of \textit{Brachyspira} and the occurrence of delayed egg production, reduced weight gain, chronic diarrhoea, faecal staining of egg shells, and increased mortality in chicken flocks (Davelaar et al., 1986; Griffiths et al., 1987; Swayne et al., 1992, Trampel et al., 1994; Smit et al., 1998; Hampson and McLaren, 1999; Stephens and Hampson, 1999; Hampson et al., 2002a; Burch et al., 2006; Bano et al., 2008; Feberwee et al., 2008).

Although AIS due to \textit{Brachyspira} species is particularly a problem of layer chickens, it can also affect broiler breeders (Stephens and Hampson, 1999). The prevalence of \textit{Brachyspira} species among broiler breeder and layer flocks from eastern Australia was 43\% and 68\%, respectively (Stephens and Hampson, 1999). Globally, the prevalence of \textit{Brachyspira} species among layer flocks has been reported as 32\% in the Netherlands (Dwars et al., 1989), 35\% in Western Australia (McLaren et al., 1996), 50\% in Poland (Kizerwetter-Świda et al., 2005), 71\% in Italy (Bano et al., 2008), and 17\% in Iran (Jamshidi et al., 2009). Higher prevalence has been observed among chicken flocks with AIS compared to those without AIS. For example, 28\% of the positive layer flocks in the Netherlands and 70\% of the positive layer and broiler breeder flocks in eastern Australia had signs of AIS; whereas only 4\% of the positive layer flocks in the Netherlands and 15\% of the positive layer and broiler flocks in eastern Australia did not show signs of AIS (Dwars et al., 1989; Stephens and Hampson, 1999; Kizerwetter-Świda et al., 2005). Reported within-flock prevalence estimates of \textit{Brachyspira} in eastern Australia, Italy, and the U.S. range from 10 to 100\% (Stephens and Hampson, 1999; Bano et al., 2008; Myers, et al., 2009).
Of the colonized flocks in the eastern Australian study (Stephens and Hampson, 1999), 56% were colonized with pathogenic species; 12.6% were colonized with *B. intermedia*, 31% with *B. pilosicoli*, and 12.5% with both species. Bano *et al.* (2008) reported that, of the *Brachyspira*-positive farms in northern Italy, 41.3% were colonized with pathogenic species; *B. intermedia* was the most frequent species (27.5%) followed by *B. pilosicoli* (13.8%). Similarly, a study from the U.S. reported that the two most prevalent pathogenic species were *B. intermedia* and *B. pilosicoli*, affecting 81% and 24% of the flocks, respectively; for several flocks, both species were isolated concurrently (Myers *et al.*, 2009). *Brachyspira intermedia* was reported as the most prevalent species in Western Australia and Poland (Kizerwetter-Świda *et al.*, 2005; Phillips and Hampson, 2005). Even in eastern Australia, where *B. pilosicoli* was observed to have higher occurrence, *B. intermedia* was found to be more prevalent when a gel electrophoresis technique was employed (Stephens and Hampson, 1999).

*Brachyspira* survives for a prolonged period in aquatic environments. *Brachyspira pilosicoli* was recovered for up to 66 days in lake water at 4°C, and for 4 days at 25°C (Oxberry *et al.*, 1998). The experimentally documented survival time for *B. pilosicoli* and *B. intermedia* in porcine faeces mixed with soil at 10°C was up to 210 and 119 days, respectively (Boye *et al.*, 2001). However, a shorter survival time was recorded under natural conditions above 10°C, especially when the bacteria were directly exposed to sunlight (Boye *et al.*, 2001). In chicken faeces, *Brachyspira* remained viable for 2 to 17 hours at 37°C, and up to 3.5 days at 4°C, depending on the concentration of the bacteria (Phillips *et al.*, 2003). The authors indicated that the shorter survival time could be due to the acidic nature of chicken faeces and the small bulk that did not provide a protective habitat.
Risk factors associated with *Brachyspira* species in chickens and other hosts

**Age**

Several studies have shown a significant positive association between age and *Brachyspira* colonization (Phillips *et al*., 2005; Myers *et al*., 2009). In particular, pathogenic *Brachyspira* species have been isolated in a higher proportion among layer flocks older than 40 weeks compared to those younger than 40 weeks (Bano *et al*., 2008; Myers *et al*., 2009). Within-flock prevalence also increased to 100% as flocks became older (Jordan and Hampson, 2008).

Others have also identified a positive association between age and *Brachyspira* prevalence, although the association failed to reach statistical significance. For example, the among flock prevalence in chickens 10 to 39 weeks of age was 41%, increasing to 81% in those 40 to 100 weeks of age, whereas chickens less than 10 weeks of age were negative for *Brachyspira* species (Stephens and Hampson, 1999). Jordan and Hampson (2008) also documented that pullets up to 15 weeks of age were rarely colonized. Experimentally-infected one-day-old chicks did not exhibit diarrhoea until they were 21 days of age, although they started shedding at 7 days of age (Trott *et al*., 1995). This age-associated increase in *Brachyspira* colonization has also been noted in other avian species. For instance, mortality due to *Brachyspira* in ducks and geese increased with age (Nemes *et al*., 2006; Glavitis *et al*., 2008). There is no clear explanation why younger birds are not easily infected; however, it is possible that colonization could be a result of long exposure time to the bacteria (Stephens and Hampson, 1999). Once the bacterium is introduced into a barn, there can be cross-contamination within and between flocks by means of equipment and personnel (Phillips *et al*., 2005). Signs, such as chronic diarrhoea, have been observed approximately four weeks after exposure (Dwars *et al*., 1991).
Housing and manure disposal

Several studies have shown that flocks with outdoor access were at a higher risk of contracting *Brachyspira* species than caged flocks (Griffiths *et al*., 1987; Bano *et al*., 2008; Jansson *et al*., 2008). However, a Dutch study found that the risk of colonization with *Brachyspira* did not differ between flocks housed in floor barns with access to litter and flocks housed in cages (Dwars *et al*., 1989). Flocks from multi-age farms were positively associated with the presence of *Brachyspira* (Burch *et al*., 2006). No significant association was reported between flock size and the presence of *Brachyspira* (Bano *et al*., 2008; Myers *et al*., 2009).

Layer flocks housed in barns that had belts for manure disposal had a significantly lower risk of *Brachyspira* species than flocks housed in barns with deep pits. However, there was no association between the presence of pathogenic *Brachyspira* species and manure disposal system (Bano *et al*., 2008).

Feed and medication

Wheat-based feed has been reported to enhance colonization by *Brachyspira* in layer flocks (Phillips *et al*., 2004). Layers experimentally infected with *B. intermedia* that were fed a wheat diet had a significantly higher risk of colonization than layers that were fed barley, sorghum, or a combination of barley and sorghum (Phillips *et al*., 2004). Higher numbers of dirty eggs were produced from hens that were fed wheat and rye compared to those fed a corn diet (Lazaro *et al*., 2003). Adding an enzyme supplement, which hydrolyses the non-starch polysaccharide in wheat, was shown to significantly decrease the number of faecal-stained eggs (Lazaro *et al*., 2003). Colonization of laboratory mice by *B. pilosicoli* was witnessed only when they were fed a special
diet that increased fermentation in the large intestine (Jamshidian et al., 2004; Sacco et al., 1997). Furthermore, although the feed ingredients did not differ, pelleted feed was associated with increased occurrence of \textit{B. pilosicoli} and \textit{B. innocens} in pigs compared to non-pelleted feed (Stege et al., 2001).

Several studies have examined the effect of antimicrobials on the occurrence of \textit{Brachyspira}. Zinc bacitracin (ZnB) mixed with feed at a dose of 100 ppm decreased colonization in layer hens experimentally-infected with \textit{B. intermedia}; of the hens on this diet, 16.7\% were positive for \textit{B. intermedia}, whereas the number of colonized hens increased to 60\% after removal of ZnB from the feed (Hampson et al., 2002a). Zinc bacitracin at a dose 50 ppm also inhibited colonization with \textit{B. intermedia} (Hampson et al., 2002b). In contrast, 50 ppm ZnB augmented colonization with \textit{B. pilosicoli} (Jamshidi and Hampson, 2002; Stephen and Hampson, 2002). Among layers experimentally-infected with \textit{B. pilosicoli}, 70\% of those that were treated with ZnB remained positive for \textit{B. pilosicoli} compared to 10\% of those that were not treated with ZnB (Jamshidi and Hampson, 2002). Myers et al. (2009) reported that flocks that were treated by producers with tetracycline and bacitracin within 3 months during the study were all (100\%) positive for \textit{B. intermedia} and 14\% of the flocks were positive for \textit{B. pilosicoli}.

Flocks were found negative for \textit{Brachyspira} after being treated with 60 mg/kg oxytetracycline for four consecutive days (Stephen and Hampson, 1999). Treatment with 25mg/kg tiamulin for five consecutive days also eliminated \textit{B. pilosicoli} from an experimentally infected flock; however, 90\% of the chickens were re-infected after five weeks (Hampson et al., 2002a). In another study involving experimentally infected broiler breeder chickens, \textit{B. pilosicoli} appeared to have been permanently eradicated by treating all of the chickens with 25mg/kg
tiamulin and 20mg/kg lincomycin (Stephen and Hampson, 2002). The authors concluded that re-infection might be the result of contamination from untreated birds rather than from internal residual infection.

**Reservoir animals**

Various birds and mammals are considered reservoirs of *Brachyspira* (Buckles *et al*., 1997; Hirsh, 2004). Pigs and rodents are well-established reservoirs of *B. hyodysenteriae* (Joens and Kinyon, 1982). Non-caged pullets that were living near pigs exhibited some signs of AIS (Griffiths *et al*., 1987). It has also been reported that experimentally-infected mice were able to shed *B. hyodysenteriae* for up to 180 days and were able to transmit the pathogen to swine five to seven days after being infected (Joens, 1980). In addition, *B. hyodysenteriae* have been found in the caeca of wild rodents, suggesting that wild rodents can be potential carriers of spirochetes (Joens and Kinyon, 1982).

**Other management factors**

It has been suggested that transmission of *Brachyspira* species in barns can be controlled with proper hygiene and management, such as cleaning and disinfecting the premises, and by effective control of rodents and wild birds (Stephen and Hampson, 2001). Allowing downtime of a few days between flocks in combination with disinfection has been shown to eradicate *Brachyspira* (Phillips *et al*., 2003). The maximum survival time for *B. intermedia* and *B. pilosicoli* when exposed to common disinfectants under in vitro conditions was only a few minutes (Phillips *et al*., 2003). Quaternary ammonium, iodine (active), chlorine, glutaraldehyde,
and hydrogen peroxide at the manufacturer’s recommended concentration have been shown to destroy the bacteria in less than one minute (Phillips et al., 2003).

OBJECTIVES

Because there are only limited published studies on Brachyspira, numerous potentially critical factors still need to be investigated. In particular, the prevalence of Brachyspira in Ontario poultry flocks is unknown; the association between Brachyspira and AIS has not been studied in Ontario or elsewhere in Canada. However, downgrading of eggs as a result of faecal staining of the shells is an important economic problem in the Ontario layer industry. Therefore, there is a need to study the potential association between Brachyspira species and dirty eggs.

The objectives of this thesis are therefore;

1. To investigate the presence of Brachyspira species in Ontario layer chicken flocks;

2. To compare the prevalence of pathogenic and non-pathogenic Brachyspira species in flocks with downgrades for dirty eggs to flocks without downgrades for dirty eggs in Ontario; and

3. To identify risk factors associated with the presence of Brachyspira species in Ontario layer chicken flocks.
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*Brachyspira* species pathogenic to chickens

* Provisionaly proposed *Brachyspira* species
CHAPTER TWO

*Brachyspira* species in Ontario layer chicken flocks and its association with downgrades for dirty eggs

Prepared in the style of *Avian Diseases*

**ABSTRACT**

The prevalence of *Brachyspira* species in faeces of layers were compared between flocks with downgrades for dirty eggs (dirty flocks), and those without (clean flocks). A total of 534 pooled faecal samples from 89 flocks on 58 farms were examined using a real-time polymerase chain reaction. *Brachyspira* species were detected from 23 (39.7%; 95% CI: 26.7 - 52.6%) of the farms, 42 (47.2%; 95% CI: 36.5 - 58.1%) of the flocks, and 123 (23.0%; 95% CI: 19.5 - 26.8%) of the faecal samples. The association between flock status and presence of *Brachyspira* species was determined using logistic regression with a random effect for farm. The odds of *Brachyspira* among dirty flocks were higher (OR= 18.3; 95% CI: 1.4 - 238.3; *P*=0.026) than clean flocks. The amount of variation explained at the farm level was 58.8%. Of the 42 *Brachyspira*-positive flocks, 78.6% were dirty flocks. Samples positive for *Brachyspira* were further processed for species identification of the pathogenic species *B. hyodysenteriae*, *B. intermedia*, and *B. pilosicoli*. *Brachyspira pilosicoli* was detected from 12.4% (95% CI: 6.3 – 21.0%) of all flocks tested. This species was detected from 9/23 (39.1%), 11/42 (26.2%), and 26/123 (21.1%) of *Brachyspira*-positive farms, flocks, and samples, respectively. Results indicated a strong positive association between dirty egg shells and the presence of *Brachyspira* species in Ontario layer
flocks. The relatively high variation in the prevalence of *Brachyspira* species among farms indicates that identification of risk factors and interventions at the farm level should be investigated.

**Keywords:** Avian intestinal spirochetosis; *Brachyspira pilosicoli*; layer chickens; Ontario; prevalence; PCR

**Abbreviations:** AIS= avian intestinal spirochetosis; AHL= Animal Health Laboratory; PCR= polymerase chain reaction

**INTRODUCTION**

Avian intestinal spirochetosis due to pathogenic *Brachyspira* species is an enteric disease in poultry. In chickens, it is characterized by chronic diarrhoea, weight loss, delayed and/or reduced egg production, and increased mortality (6, 7, 8, 25, 26). It is a concern especially in the layer industry due to the economic loss from decreased egg production and downgrading of eggs as a result of faecal staining of the shells (3, 24, 28). The pathogenic species of *Brachyspira* in chickens are *Brachyspira alvinipulli*, *Brachyspira hyodysenteriae*, *Brachyspira intermedia* and *Brachyspira pilosicoli* (5, 7, 14).

World-wide, prevalence estimates of *Brachyspira* species in chicken range from 4.4% to 71.1% (1, 4), although sample sizes, sampling strategies, and diagnostic techniques were not consistent among studies. Individual faecal samples were collected in some studies (15, 25), whereas pooled samples were collected in most observational studies (1, 18, 26). The majority of studies used both bacterial culture and PCR techniques (1, 12, 25), and one study processed the samples further with gel electrophoresis (20). To-date, only one study has employed a PCR method alone, in which DNA was extracted directly from faecal samples (18).
The highest prevalence (71.1%) was reported in layer flocks in Italy using culture and PCR (1). The proportion of *Brachyspira* species was higher in the group affected with AIS compared to the group that did not show any signs of AIS (1, 4, 17). The highest reported prevalence in flocks exhibiting signs of AIS was 64% (1), and the lowest reported prevalence in flocks without signs of AIS was 4.4% (4). Within-flock prevalence estimates range from 10% to 100% (1, 18, 25).

*Brachyspira intermedia* and *B. pilosicoli* are the most prevalent pathogenic species reported in layers (18, 20, 25). *Brachyspira intermedia* have been found in a slightly higher proportion of samples compared to *B. pilosicoli*, although both species have occasionally been isolated concurrently (18, 25). An important aspect of AIS is egg shell staining, whereby stained eggs are downgraded and cannot be sold as unpasteurized, shell-on table eggs. Faecal staining of eggs due to increased wetness of the faeces has been shown in both experimental and observational studies (11, 18). Stephen and Hampson (1999) reported a 14% increase in water content of the faeces from *Brachyspira*-colonized flocks. Jamshidi *et al.* (2003) also reported 6% to 7% increases in faecal water content from adult chickens experimentally-infected with human isolates of *B. pilosicoli*.

The Ontario layer industry has experienced a dirty egg problem for several years (Dr. Lloyd Weber, 2009, Lloyd Weber Consulting Services, personal communication); however, the role of *Brachyspira* has not been investigated until recently. An interest in this bacterium emerged in the winter of 2008 after spirochetes were observed on gram smears of caecal material collected from case submissions (hens with signs of AIS) to the AHL, Guelph, Ontario. Subsequently, a preliminary study was initiated to determine if *Brachyspira* species were present in clinically-affected Ontario layer flocks (unpublished). Culture and gel-based PCR methods were used to
screen 100 faecal samples from five farms with a history of AIS. Of the five farms, three were positive for *Brachyspira* species by culture and four were positive using PCR. *Brachyspira* species isolated by culture were further identified as *B. pilosicoli* and *B. intermedia*. This study found that six of seven flocks (86%) were positive for *Brachyspira* species using PCR. Although the study confirmed the presence of this bacterium in Ontario, the sample size was not large enough to estimate the prevalence of *Brachyspira* species. Moreover, the presence of *Brachyspira* species in flocks without signs of AIS was not investigated. Therefore, the objective of this study was to investigate the presence of pathogenic and non-pathogenic *Brachyspira* species in Ontario layer flocks, and compare the prevalence of *Brachyspira* species in flocks with downgrades for dirty eggs to flocks without downgrades for dirty eggs.

**MATERIAL AND METHODS**

**Sample size**

The prevalence of *Brachyspira* species has been estimated to be between 19% and 56% lower for flocks with clean eggs compared to flocks with dirty eggs (1, 17, 25); therefore, we assumed there would be a difference in prevalence of 50% between groups in our study population. Based on results from the preliminary study conducted at the AHL (86% of seven dirty egg flocks were positive for *Brachyspira* species using PCR), we assumed that the prevalence in the clean egg group would be 43%. Sample size calculations indicated that a total of 88 flocks should be enrolled in the study to detect a difference between groups with a confidence of 95%, power of 90%, average cluster size of two flocks per farm, and intra-class correlation of 0.5.

The sample size determination for demonstrating flock-level freedom from disease using pooled testing was based on the following: a pool size of five, a conservative estimate of pool-
level test sensitivity of 50%, desired flock-sensitivity of 95%, and an expected prevalence as low as 28% in the clean egg group (17). Thus, a minimum of six pooled faecal samples were tested for each flock.

**Flock recruitment**

Lists of egg producers were provided by the major egg grading companies in Ontario. Each company comprises approximately 50% of the market share of Ontario table eggs. These lists were provided after the purpose of the research was explained to the producers by their respective grading station representative. The project was approved by the University of Guelph Research Ethics Board. Producers were contacted by telephone to confirm their willingness to participate in the study. Voluntary participants were scheduled for a face-to-face interview and faecal sample collection from their barns.

Designation of flocks as dirty or clean was based on the percentage of eggs graded ‘B’ each week for four consecutive weeks before the day of sampling, in conjunction with the company’s knowledge of flocks with a long-term history of a dirty egg problem. Accordingly, a flock was designated as ‘a flock with a dirty egg problem’ if ≥ 0.5% of the eggs were graded ‘B’ at least one week during the indicated time period, or the grading company was aware that the flock had a history of a dirty egg problem. Hereafter, flocks with a dirty egg problem will be referred to as ‘dirty flocks’, and flocks without a problem (< 0.5% of the eggs were graded ‘B’ each week during the indicated time period or no history of a dirty egg problem) will be referred to as ‘clean flocks’. If a farm had more than one flock, all flocks on the farm were sampled, with the exception of six farms whose owners were not willing to allow access to more than one barn. Sampling all the flocks on a farm was considered important because flocks on the same farm can be of different ages or breeds, or have a different flock status, housing system, manure disposal,
or feeding practices. Information about different risk factors, such as management, flock characteristics, and farm location, were collected from the questionnaire administered prior to sample collection.

**Sample collection**

Sampling was conducted from the end of August 2010 to the end of February 2011 by visiting each farm once. For caged and free run houses with manure belts, producers were asked to run the belt at the time of sampling so that fresh faeces could be picked from each tier of the belts. For free run houses without manure belts, faecal samples were collected from under the slats. For high-rise houses, samples were collected from different locations of the manure pit. For A-frame caged houses with scrapers, samples were collected from different locations under the scraper and the cages. Random selection of faeces was not possible; however, approximately six to eight grams of fresh (moist) faeces were purposively selected from different sections of the barn or belt. Pooled faecal samples (six pools of five) from each flock were collected in graduated sealed containers, placed in an ice box, and transported to the AHL. Farms and flocks were assigned a coded number to maintain confidentiality.

**Detection of *Brachyspira* species from faecal samples**

Nucleic acid was extracted from faecal samples as follows. Faeces were re-suspended in 30 ml of 1x phosphate buffered saline. Two millilitres of this faecal suspension was used for DNA extraction using the MagMAX™ faeces total nucleic acid purification protocol. All samples were initially tested for the presence of *Brachyspira* species by real-time polymerase chain reaction (RT-PCR). The PCR reaction was carried in a total volume of 20 μl (2 μl of DNA template and 18 μl of master mix) on the Roche LightCycler 480™ under the following thermal cycling conditions: 5 minutes of initial denaturation period at 95°C, then 45 cycles of 30 seconds...
at 95°C, and 30 seconds at 60°C, and finally 10 seconds at 40°C for cooling. These conditions were optimized to detect 440 bp region of the *Brachyspira* species *nox* gene using forward *Brachyspira* spp-F284, [5’- aaagctacagatcctaattgtca -3’] and reverse *Brachyspira* spp-R723 [5’- ccagcaccaactaccataact -3’] primers and a probe 6FAM-TTg CTACTggTTCTT+g+gCCT--BBQ. Detection occurred via excitation – emission of the FAM dye at 465-510 nm. A screening probe was used in combination with the primers: [5’-, 20 mer].

Species identification of pathogenic *Brachyspira* species (*B. hyodysenteriae* and *B. intermedia*) was conducted using multiplex RT-PCR according to AHL’s standard operating protocol. Real-time PCR for the identification of *B. pilosicoli* was run separately because the probe for detection of this species could not be successfully incorporated.

**Data analysis**

Data were entered into and organized using Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, Washington, USA), and then imported into Stata IC 11 (Stata Corp, College Station, Texas, USA) for descriptive and quantitative analyses. A flock was considered positive for *Brachyspira* species if at least one of the pooled samples tested positive on RT-PCR. Between-flock prevalence was calculated as the number of positive flocks divided by the total number of flocks sampled. The prevalence of *Brachyspira* species and *B. pilosicoli* were calculated in dirty flocks and clean flocks. Prevalence was estimated with 95% confidence intervals using Stata’s, binomial exact confidence interval calculator. Univariable logistic regression models with a random effect for farm were used to investigate the association between flock status (dirty vs. clean flocks), and the presence of *Brachyspira* species and *B. pilosicoli* at 

(P< 0.05). The predicted probability of the prevalence of *Brachyspira* species and *B. pilosicoli* in
dirty and clean flocks after controlling for farm-level clustering was estimated from the regression models. Intra class correlation was calculated using the latent variable technique.

RESULTS

Flock and barn description

Each flock was housed in one barn, and all farms with more than one flock were multi-age farms. The number of flocks per farm ranged from 1 to 10 with mean and median of 3.6 and 2, respectively; 37 of 58 farms (63.8%) had only one flock. The age of flocks ranged from 21 to 79 weeks (mean 47, median 46). Flock size ranged from 4,740 to 115,050 layers (mean 31,730, median 24,930). The housing types were stacked traditional cages (70.8%); low rise A-frame cages with manure curtains (7.9%); low rise A-frame cages without manure curtains (5.6%); high- rise barns, in which manure was deposited in a pit under the cages (11.2%); free run houses with slats and access to the outside (1.1%); and free run houses without outdoor access, in which the manure was removed by a belt system (3.4%). Breeds included Lohman white (49.4%), Lohman brown (3.4%), Shaver white (7.9%), Bovan white (24.7%), Bovan brown (1.1%), Hy-line white (3.4%), Hy-line brown (2.2%), Hissex (1.1%), and ISA-brown (6.7%). All barns were ‘all-in-all-out’.

Prevalence and association between flock status and presence of *Brachyspira* species

A total of 534 pooled faecal samples were collected from 89 layer flocks on 58 farms in Ontario. *Brachyspira* species were detected from 23 (39.7%; CI: 27.0 - 53.4%) of the farms, 42 (47.2%; CI: 36.5 - 58.1 %) of the flocks, and 123 (23.0%; CI: 19.5 - 26.8%) of the faecal samples (Table 2.1). Only one of six pooled samples was positive in seven flocks, whereas all six
samples were positive in three flocks. Within-flock, the number of positive pooled samples ranged from 1/6 (16.7%) to 6/6 (100%), with a mean of 1.4/6 (23.3%).

Twenty-one of 58 farms had more than one flock in production; all flocks on the farm were included in the study for 15 of the 21 multi-flock farms. Of those 15 farms, seven farms had 100% of the flocks testing positive for *Brachyspira* species, one had 85.7% of flocks positive, three had 66.7% of flocks positive, one had 50% of flocks positive, and three had 100% of the flocks testing negative. Sixty-seven percent of the 21 multi-flock farms tested positive for *Brachyspira* species.

Of the 123 *Brachyspira*-positive samples, 98 (79.7%) were from dirty flocks. Of the 42 *Brachyspira*-positive flocks, 33 (78.6%) were dirty flocks (Table 2.2). Moreover, all of the flocks in which 100% of the pooled faecal samples were positive for *Brachyspira* species were dirty flocks. The prevalence of *Brachyspira* species among dirty and clean flocks is shown in Table 2.2. The odds of *Brachyspira* species among dirty flocks were higher (OR = 18.3; 95% CI: 1.4 - 238.3; *P* = 0.026) than clean flocks, after controlling for clustering by farm. The amount of variation explained at the farm level was 58.8 (σ²_farm = 4.7). The predicted probability of *Brachyspira* species in dirty and clean flocks was (67.3%; CI: -0.5 – 1.9) and (10.1%; CI: -4.2 to -0.1) respectively, after controlling for clustering by farm.

*Brachyspira pilosicoli* was the only pathogenic species detected. It was detected from 12.4% (CI: 6.3 – 21.0%) of all flocks tested. *Brachyspira pilosicoli* was detected from 9/23 (39.1%), 11/42 (26.2%), and 26/123 (21.1%) of *Brachyspira*-positive farms, flocks, and samples, respectively. The prevalence of *B. pilosicoli* species among dirty and clean flocks is shown in Table 2.2. The odds of *B. pilosicoli* among dirty flocks were not statistically different from clean flocks (OR=1.3; CI: 0.3 - 4.9; *P* = 0.72), after controlling for clustering by farm. The amount of
variation explained for *B. pilosicoli* at the farm level was 5.3% ($\sigma^2_{farm} = 0.2$). Seven of the 11 (63.6%) *B. pilosicoli*-positive flocks were dirty flocks. The predicted probability of *B. pilosicoli* in dirty and clean flocks was (12.6%; CI: -3.6 to -0.3) and (10.1%; CI: -3.9 to -0.5), respectively, after controlling for clustering by farm.

**DISCUSSION**

We found a strong association between the presence of *Brachyspira* species and a dirty egg problem, with a prevalence of 63.5% among dirty flocks. This prevalence is comparable to, or higher than, previously reported prevalence in flocks with AIS. For instance, a prevalence of 64.2% in flocks with AIS was reported in Italy (1), whereas a lower prevalence was reported in the Netherlands (28%) and Western Australia (50%) (4, 17). This difference in prevalence could mainly be due to different detection methods and sampling strategies. It is worth noting that the sensitivity and specificity of the tests used could be different. For example, PCR performed on pure culture is more sensitive than PCR performed directly on faeces because of the potential inhibitory factors present in faeces (22). The study in the Netherlands used a fluorescent antibody test (4), whereas most of the other studies used PCR following pure faecal culture (1, 25). A study in eastern Australia on flocks that had wet faeces reported 20% positive samples, using culture and PCR (20). To-date, only one study used a direct PCR method (18), reporting 81% *B. intermedia*, and 23.5% *B. pilosicoli*-colonized flocks. However, comparison is difficult because the study included only flocks that were 46 weeks and older. The study by Swayne *et al.* (1992) was the only study designed to specifically associate dirty eggs with *Brachyspira* species. However, only two farms were included in that study.

*Brachyspira pilosicoli*, a species with a diverse host range, could have been introduced to flocks by animals, such as wild birds, waterfowl, and/or rodents, through contamination of water
sources with faecal material (7, 12, 19). Although the prevalence of *B. pilosicoli* among dirty flocks was higher compared to clean flocks, the association between *B. pilosicoli* presence and flock status was not statistically significant. A similar finding was reported by a study in Italy, in which there was no significant association between flocks with wet faeces and the presence of the pathogenic species, *B. pilosicoli* and *B. intermedia* (1). Our finding of *B. pilosicoli* in both clean and dirty flocks and also a high number of samples positive for *Brachyspira* species indicates that *B. pilosicoli* might not account for all of the dirty egg problems, and implies the potential role of other *Brachyspira* species in AIS affected flocks. There is a possibility that *B. alvinipulli* (not investigated in the current study due to lack of specific primer) might be present in Ontario layer flocks. Feberwee *et al.* (2008) indicated that the pathogenic species, *B. alvinipulli*, could be an under-diagnosed yet important cause of AIS. It is also possible that presumably non-pathogenic species, such as *B. murdochii* and *B. innocence*, might have contributed to the dirty egg problem; *B. murdochii* has been shown to be pathogenic under some undefined conditions (2, 25). A recent experimental study indicated that pigs infected with high numbers of *B. murdochii* developed catarrhal colitis (13). Further research is necessary to determine what other species of *Brachyspira* might be present in AIS flocks and what their role is in this clinical condition.

Another reason for detecting *B. pilosicoli* in both dirty and clean flocks could be misclassification of flocks into dirty and clean categories. The method used to classify flocks, which depended on the flock’s history, and whether it was graded ‘B’, could have led to false positive or false negative classification of dirty flocks. Eggs can be graded ‘B’ for reasons other than dirty shells, including leakers (eggs with cracked or broken shells), abnormal shells (distorted shell with ridges on the surface), dark yolk, and weak air sac (30). However, in our
study population, dirty shells were the most common cause of ‘B’ downgrades. There is also a possibility that flocks owned by producers who do not routinely send dirty eggs to the grading station were classified as clean when they should have been classified as dirty. We attempted to gather this information in the questionnaire; however, it was not possible for producers to estimate the exact number of dirty eggs that were kept from being shipped to the grading station. Incorrect categorization of flocks might subject the study to non-differential misclassification that biases the odds ratio toward the null.

The lack of detection of *B. intermedia*, one of the most prevalent species identified in previous studies (including the preliminary study by the AHL), was unexpected. The results of previous studies suggest that flocks do not acquire infection from their surroundings provided that the barns were disinfected between batches (14, 20). The maximum survival time for *B. intermedia* and *B. pilosicoli* when exposed to common disinfectants was only a few minutes (21). Therefore, it is possible that *B. intermedia* could be introduced to farms by reservoir animals. *Brachyspira intermedia* has been reported exclusively in pigs and birds, and pigs are the potential reservoirs for this species (14). None of the layer farms in this study raised pigs on the same farm. Further, no statistical association was observed between the presence of *Brachyspira* species and swine farms within 2 km area of the participating layer farms (Chapter 3). All barns were cleaned and disinfected and allowed a minimum of 24 hours downtime between flocks (Chapter 3). Therefore, we consider that there is a small possibility that the flocks were infected from the environment. However, this is an area that needs further investigation.

*Brachyspira pilosicoli* has been reported in humans in developed countries, such as Norway and Australia (16, 29). *Brachyspira pilosicoli* can inhabit the human intestine without visible symptoms of human intestinal spirochetosis (29). Although it has been suggested that
*Brachyspira* might not be transmitted to humans from eggs (10), zoonotic transmission of this species from human food or water contaminated by faeces of infected animals and birds has been indicated (9). *Brachyspira pilosicoli* isolates from infected humans have also resulted in wet faeces when inoculated into chickens (11).

In summary, this study has shown that *Brachyspira* species is significantly associated with the dirty egg problem in Ontario layer flocks. The relatively high variation in the prevalence of *Brachyspira* species among farms indicates that identification of risk factors and interventions at the farm level should be investigated.

**ACKNOWLEDGEMENTS**

This project was supported by the OMAFRA-University of Guelph Agreement through the Animal Health Strategic Investment (AHSI) fund managed by the Animal Health Laboratory of the University of Guelph. The authors would like to thank Dr. David Pearl, Dr. Marina Brash, Dr. Mike Petrik, Dr. Agnes Agunos, Jennifer Zoethout, and Amanda Drexler. We appreciate the cooperation of the Egg Farmers of Ontario and the participating producers. The authors wish to thank the Public Health Agency of Canada for providing supplies for field work.

**REFERENCES**

2. Burch, D. Egg production drops from Brachyspira.  


Table 2.1. Prevalence of *Brachyspira* species in Ontario layer chicken flocks

<table>
<thead>
<tr>
<th>Sampling Level</th>
<th><em>Brachyspira</em> spp.</th>
<th></th>
<th><em>B. pilosicoli</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive (%)</td>
<td></td>
<td>No. positive (%)</td>
<td></td>
</tr>
<tr>
<td>Farms (n = 58)</td>
<td>23 (39.7%)</td>
<td></td>
<td>9 (15.5%)</td>
<td></td>
</tr>
<tr>
<td>Flocks (n = 89)</td>
<td>42 (47.2%)</td>
<td></td>
<td>11 (12.4%)</td>
<td></td>
</tr>
<tr>
<td>Samples (n = 534)</td>
<td>123 (23.0%)</td>
<td></td>
<td>26 (4.9%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. Comparison of the prevalence of *Brachyspira* species and *B. pilosicoli* between dirty and clean flocks in Ontario layer chicken flocks

<table>
<thead>
<tr>
<th>Flock status</th>
<th><em>Brachyspira</em> spp.</th>
<th></th>
<th><em>B. pilosicoli</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive (%)</td>
<td></td>
<td>No. positive (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td></td>
<td>95% CI</td>
<td></td>
</tr>
<tr>
<td>Dirty flocks (n = 52)</td>
<td>33 (63.5%; 49.0 - 76.4%)</td>
<td></td>
<td>7 (13.5%; 5.6 - 25.8%)</td>
<td></td>
</tr>
<tr>
<td>Clean flocks (n = 37)</td>
<td>9 (24.3%; 11.8 - 41.2%)</td>
<td></td>
<td>4 (10.8%; 3.0 - 25.4%)</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER THREE
Risk factors associated with Brachyspira species in Ontario layer chicken flocks

Prepared in the style of Preventive Veterinary Medicine

ABSTRACT

Avian intestinal spirochetosis, caused by pathogenic species of Brachyspira, is a disease of mainly layer and broiler breeder chickens, characterized by chronic diarrhoea, weight loss, low egg production, and faecal-stained eggs. The purpose of this study was to identify risk factors associated with the presence of Brachyspira species in Ontario layer chicken flocks. Pooled faecal samples were collected from 89 flocks from 58 farms between August 2010 and February 2011; 52 flocks were classified as dirty flocks (history of downgrades for dirty eggs) and 37 were classified as clean flocks (no history of downgrades for dirty eggs). A questionnaire related to management and biosecurity practices, and antimicrobial use, was administered prior to sample collection. Using real-time polymerase chain reaction; 63.5% of the dirty flocks and 23.4% of the clean flocks were positive for Brachyspira species.

A logistic regression model with a random effect for farm showed that the odds of Brachyspira species for flocks ≥ 60 weeks of age were higher than for flocks ≤ 34 weeks (OR = 9.3; P = 0.014). The odds of Brachyspira species in flocks housed in A-frame cages with manure curtains (OR = 20.0; P = 0.002) and flocks from multi-age farms (OR = 8.5; P = 0.001) were higher than for flocks in cage-stacked houses and from single-age farms, respectively. The odds of Brachyspira species for flocks housed in barns ≥ 30 years old was lower than for flocks
housed in barns ≤ 14 years old (OR = 0.1; \( P = 0.002 \)). The calculated intra class coefficient was 5.6 x 10^{-14}; the notably low proportion of variation among farms after the fixed effects were included in the model suggests that the farm-level variable (multi-age farm) included in the final model accounted for most of the farm-to-farm variation in \textit{Brachyspira} presence. Therefore, it is recommended that strict biosecurity be followed on farms with multiple flocks of different ages to avoid transmission of the bacteria between flocks.

**Key words:** Downgrading; egg shells; table eggs; housing

1. INTRODUCTION

Avian intestinal spirochetosis (AIS) is a disease of birds caused by pathogenic spirochete bacteria, which inhabit the gastrointestinal tract (Dwars et al., 1992b; Stephens and Hampson, 1999). \textit{Brachyspira} species cause chronic diarrhoea, increased mortality, decreased egg production, wet litter, and pasty vents, which are the signs of AIS (Bano et al., 2008; Burch et al., 2006; Griffiths et al., 1987; Hampson and McLaren, 1999; Hampson et al., 2002; Smit et al., 1998; Stephens and Hampson, 1999; Swayne et al., 1992, Trampel et al., 1994). \textit{Brachyspira} is particularly important in the layer industry because it is implicated in egg shell staining (Swayne et al., 1992) and subsequent downgrading of table eggs.

Factors that have been associated with the occurrence of \textit{Brachyspira} species include flock age, housing type, manure disposal system, direct and indirect contact with reservoir animals, feed, and medications (Bano et al., 2008; Griffiths et al., 1987; Hirsh, 2004; Jansson et al., 2008; Stephens and Hampson, 1999). Previous studies have shown that the presence of \textit{Brachyspira} species increased with age (Myers et al., 2009; Phillips et al, 2005; Stephens and Hampson, 1999). The prevalence of \textit{Brachyspira} species in flocks older than 40 weeks was significantly
higher than in flocks younger than 40 weeks (Bano et al., 2008; Myers et al., 2009). The proportion of flocks positive for *Brachyspira* species was lower for caged flocks than for those in housing types that allowed access to the outside (Bano et al., 2008; Griffiths et al., 1987; Jansson et al., 2008); however, the prevalence of *Brachyspira* species was not different for flocks housed in cages compared to those housed in floor barns with access to litter (Dwars et al., 1989). The prevalence of *Brachyspira* species was also higher for flocks from housing types with manure pits in the barn than for those from housing types with conveyor belts for manure disposal (Bano et al., 2008; Burch et al., 2006). Pigs, rodents, wild birds, and waterfowl can be carriers of *Brachyspira* species (Griffiths et al., 1987; Joens and Kinyon, 1982), especially *B. pilosicoli*. *Brachyspira pilosicoli* affects various species of animals, including humans, and can be transmitted to poultry from reservoir animals and dogs (Hampson et al., 2006). Transmission of *B. pilosicoli* between humans and poultry has been reported (Oxberry et al., 1998). Antimicrobials, such as tiamulin and lincomycin, eliminated *B. pilosicoli* from experimentally-infected layer flocks (Stephens and Hampson, 2002), whereas zinc bacitracin increased its prevalence (Jamshidi and Hampson, 2002). Wheat-based feed has also been shown to enhance colonization of flocks with *Brachyspira* species (Durmic et al., 2002; Phillips et al., 2004).

*Brachyspira* species were recently identified from case submissions to the Animal Health Laboratory (AHL), University of Guelph, in which six of seven Ontario layer flocks with a dirty egg problem (faecal-stained egg shells) were positive on polymerase chain reaction (unpublished). To-date, there have been no observational studies conducted in Canada to identify risk factors for *Brachyspira* species in layers. Thus, the objective of this study was to determine risk factors associated with the presence of *Brachyspira* species in Ontario layer chicken flocks.
2. MATERIAL AND METHODS

2.1. Sample size

It is noted that sample size calculations for this study were not based on determining risk factors for *Brachyspira*. Rather, they were based on an *a priori* interest by the Ontario layer chicken industry (Chapter 2) to detect an association between egg status (dirty vs. clean egg flocks) and *Brachyspira* (although, from a causal standpoint, it was deemed more prudent to investigate risk factors for *Brachyspira* because it is a more proximal outcome than egg status). The prevalence of *Brachyspira* species has been estimated to be 19% to 56% lower for flocks with clean eggs compared to flocks with dirty eggs (Bano et al., 2008; McLaren et al., 1996; Stephens and Hampson, 1999); therefore, we assumed there would be a difference in prevalence of 50% between clean and dirty egg groups in our study population. Based on results from the preliminary study conducted at the AHL (six of seven (86%) flocks with a dirty egg problem were positive), we assumed that the prevalence in the clean egg group would be 43%. Therefore, a total of 88 flocks were enrolled in the study to detect a difference between groups with a confidence of 95%, power of 90%, average cluster size of two flocks per farm, and intra-class correlation of 0.5.

The sample size calculation for demonstrating flock-level freedom from disease using pooled testing was based on a pool size of five, a conservative estimate of pool-level test sensitivity of 50%, required flock-sensitivity of 95%, and an expected between-flock prevalence as low as 28% in flocks without a dirty egg problem (McLaren et al., 1996). Therefore, a minimum of six pooled faecal samples were tested for each flock.
2.2. Flock recruitment

Flocks were recruited by the two egg grading companies in Ontario, which each comprise approximately 50% of the market share of Ontario table eggs. A list of flocks with a dirty egg problem and flocks without a problem was provided by each company based on the percentage of eggs graded ‘B’ each week for four consecutive weeks before the day of sampling, in conjunction with the company’s knowledge of each flock. Accordingly, a flock was designated as ‘a flock with a dirty egg problem’ if \( \geq 0.5\% \) of the eggs were graded ‘B’ at least one week during the indicated time period, or the grading company was aware that the flock had a history of a dirty egg problem. Flocks were recruited in this manner because the study was initiated to address the concern of the grading companies and the Canadian Food Inspection Agency to minimize dirty egg problem in Ontario layer chickens.

2.3. Questionnaire

A questionnaire to assess potential risk factors for the presence of \textit{Brachyspira} species was developed and pre-tested on four layer farms after approval by the University of Guelph Research Ethics Board (REB # 10MY009). The questionnaire was revised based on comments from producers and industry collaborators. Most questions were closed (Appendix). Egg producers purposively recruited by study collaborators representing the two major egg grading companies in Ontario were contacted by telephone to confirm their willingness to participate and to schedule an interview and sample collection. Farm visits were conducted from the end of August 2010 to the end of February 2011 by visiting each farm once. Upon agreement to participate, an e-mail was sent to voluntary participants to inform them of the farm records required for the interview. Flock production, barn temperature, and feed and vaccination records were some of the records requested to address the study objective. Flock status (whether the
flock had a dirty egg problem or not) was known to the researchers prior to the interview and sample collection; however, the methods did not differ by flock status. The interview pertained to general flock information, management, biosecurity, feed, and other related management practices.

The questionnaire covered nine key areas: 1) farm characteristics, including the number of farms owned by the producer, whether the farm had multiple flocks of different ages, and whether employees were specific to each barn, the presence of other animals on the farm, and the number of poultry and swine farms near the farm; 2) flock characteristics, including age, breed, and size of the flock, egg colour, history of disease and mortality; 3) water, including source, and water treatment and frequency; 4) housing, including housing type, ventilation, bird density, barn age, age of cages, barn size, and building materials; 5) manure disposal, including method of disposal, how often manure was disposed of, and storage and removal from the farm; 6) cleaning and disinfection, including the length of the rest period before the flock was placed; 7) biosecurity, including general hygienic practices on the farm, clothing and footwear; 8) pest control, including rodent and fly control; and 9) feed and vaccinations, including feed and water additives and medications, and pullet vaccination history.

2.4. Flock sampling

Random selection of faeces was not possible; however, fresh (moist) faecal samples, approximately six to eight grams each, were purposively collected from different sections of the barn or manure belt. Pooled faecal samples (six pools of five) from each flock were submitted to the AHL for testing by real-time polymerase chain reaction (Chapter 2). A coded number was assigned to farms and flocks to maintain confidentiality. All flocks on a farm were sampled, with the exception of six farms whose owners were not willing to allow access to more than one barn.
Sampling all the flocks on a farm and collecting corresponding risk factors data was considered important, as flocks on the same farm can be of different ages or breeds, or have a different flock status, housing system, feeding practices, or manure disposal. A flock was considered positive for *Brachyspira* species if at least one of the pooled samples tested positive on the real-time polymerase chain reaction.

### 2.5. Statistical model building

The dependent variable was the presence or absence of *Brachyspira* at the flock level. Independent variables included categorical, continuous, and ordinal farm and flock-level variables. STATA Intercooled 11 statistical software was used for data analysis (Stata Corp. LP, College Station, TX).

The association between each independent variable and the outcome was first screened using a univariable logistic regression analysis with a random effect for farm at $\alpha = 0.3$ (two tailed). Linearity between the log odds of the outcome and each continuous variable was assessed using the lowess smoother graphic method. If the linearity assumption was not met, quadratic terms and log and natural log transformations were assessed, followed by categorization as required (Dohoo *et al.*, 2003). Assessment of collinearity among significant variables was conducted using Spearman’s rank correlation statistics. If the correlation coefficient between variables was ($r \geq |0.7|$) (Mason and Perreault, 1991), only one of the variables was included in the multivariable analysis.

Significant variables from the univariable analysis were next included in a main effects multivariable logistic regression model with a random effect for farm. A manual forward stepwise selection method was used to build the model with $\alpha = 0.05$ indicating statistical significance. Possible confounders were assessed one at a time in the main effect model to assess
whether they changed the coefficient by $\geq 20\%$. A priori confounders, such as breed, feed consistency and flock age, were specifically assessed for confounding after the final model was built.

Two-way interactions between significant variables in the main effects model were tested; interactions that were not significant at ($\alpha = 0.05$) were removed. The significance of categorical variables was tested using a likelihood ratio test. Deviance and Pearson residuals were assessed at the farm level. All outliers ($\geq |3|$ SD’s) were investigated by examining the covariate patterns for possible data entry errors. Normality of best linear unbiased predictors (BLUPs) of the random effect were graphically assessed. The odds ratio and $P$-value were computed for the predictors in the final multi-level logistic model. Intra-class correlation coefficients (ICCs) were estimated using the latent variable technique (Dohoo et al., 2003). All tests, unless stated otherwise were two-tailed with $\alpha = 0.05$.

3. RESULTS

3.1. Description of general farm practices

Faecal samples were collected from 89 flocks on 58 farms. 63.5% of flocks with a dirty egg problem and 23.5% of flocks without a dirty egg problem were positive for *Brachyspira* species, and *B. pilosicoli* was the only pathogenic species detected (11 flocks). Seven flocks positive for *B. pilosicoli* were from flocks with a dirty egg problem. Flocks were placed in the layer barns between 16 and 20 weeks of age (mean = 18.7). Biosecurity practices and rodent control were similar among the flocks because of the industry’s Hazard Analysis and Critical Control Points program. Rodent traps and rodent bait stations were used by 98% and 96% of the farms, respectively. All barns were cleaned and disinfected before flock placement. Detergents were used for 85% of the farms (71% of the flocks) and disinfectants were used for all farms and
flocks. None of the producers allowed visitors without proper biosecurity clothing and foot wear. Twenty-one farms had more than one flock and all were multi-age farms (multiple flocks of different ages on the same farm); of these, four had barn-specific employees. None of the flocks showed signs of illness, and no antimicrobials were given within four weeks prior to sampling. Only 18 flocks from four farms had been vaccinated with enteric vaccines for Salmonella and/or Escherichia coli, in addition to the standard vaccines administered as pullets.

Although the questionnaire was designed to gather detailed information about feeds and additives administered throughout the production period, layer feed is changed frequently (average every two weeks). Therefore, it was not possible to document all the feeds for some of the flocks. However, all flocks, except for four that were fed producer-formulated rations, were on commercially-prepared diets provided by feed suppliers. All flocks, including those that were fed producer-formulated rations, were given corn-based feed. Commercial feeds also consisted of 7 to 15% wheat. Twenty-three percent of the flocks were fed pellets, 25.8% were fed coarse crumbs, 44.9% were fed crumbs, and 6.7% were fed mash.

3.2. Description of variables included in the analysis

Descriptive statistics of continuous variables describing farm, barn, and flock characteristics are presented in Table 3.1. Ten of these variables (barn age, flock age, flock size, number of barns on the farm, number of employees per barn, rest period prior to flock placement, water line flushing frequency, water testing frequency, barn temperature, and bird density) were eligible for inclusion in the final model. However, none of them fulfilled the assumption of linearity on lowess smoother graphic assessment, except for flock size and number of employees per barn, for which quadratic terms were included in the model. Barn age and flock age were categorized based on a discussion with industry collaborators, whereas all other
continuous variables were categorized into quartiles to obtain maximum information from the
categories (Brown et al., 1994). Barn age and flock age remained statistically significant after
categorization and were included in the multivariable model as categorical variables. However,
rest period, water testing frequency, water line flushing frequency, barn temperature, and bird
density were not significant in the univariable analysis after being categorized; thus, they were
not included in the multivariable model. Variables significant in the univariable analysis at \( P \leq 0.3 \) that were included in the multivariable model are shown in Table 3.2.

Housing type and manure disposal system were both significant on univariable analysis;
however, the variables were highly correlated \( (r = 0.99) \). Housing type was selected for inclusion
in the multivariable model based on the value of information that it could provide, especially in
terms of exposure of the birds to faecal material given that the route of transmission for
\textit{Brachyspira} is faecal-oral (Jordan and Hampson, 2008).

Three flocks housed in free-run barns with manure belts and one flock housed in a free-run
barn with access to pasture were excluded from the analyses due to insufficient sample size in
these categories. Housing type was categorized into cage-stacked, A-frame with manure curtains
(including high-rise and low-rise barns), and A-frame without manure curtains (Table 3.2).

The variable multi-age farm was correlated with the number of barns on the farm \( (r = 0.73) \)
and whether employees were specific to the barn \( (r = 0.71) \). Multi-age farm was selected for
inclusion in the multivariable analysis due to the stronger association observed in the univariable
analysis. It was also found to be more explanatory because all farms with more than one barn
were also multi-age farms. The variables farm-specific boots and farm-specific clothing were
correlated \( (r = 0.91) \); farm-specific boots was selected for inclusion in the multivariable analysis
because of the stronger association in the univariable analysis.
Although breed and categorized flock age were not significant in the univariable analysis, they were included in the multivariable model due to specific interest by industry, and to assess their confounding effect on other variables in the model. Breed (Chapter 2) was re-grouped into four categories based on discussions with industry collaborators (Table 3.2).

### 3.3. Final multi-level logistic model

Flock age, housing type, barn age, and multi-age farm were significantly associated with presence of *Brachyspira* species in the final multi-level model (Table 3.3). Flocks from multi-age farms, and flocks ≥ 60 weeks of age, were positively associated with the presence of *Brachyspira* species, whereas flocks housed in barns ≥ 30 years of age was negatively associated. The odds of *Brachyspira* species for flocks housed in A-frame cages with manure curtains were significantly higher than for flocks housed in stacked cages. There was no significant difference on the presence of *Brachyspira* species between flocks housed in A-frame cages with manure curtains and those in A-frame cages without manure curtains (OR = 12.1; 95% CI: 0.6 – 226.1; \( P=0.095 \))

Breed was neither a confounder nor had any significant interaction effect; thus, it was excluded from the model. Moreover, there were no significant two-way interactions between any of the variables in the final model. Flock 72 (standardized Pearson residual = -4.14), flock 29 (standardized Pearson residual = -3.08) and flock 47 (standardized Pearson residual = 3.16) were identified as outliers. Flock 72 and 29 were from multi-age farms housed in A-frame cages with manure curtains, in which the barns were less than 15 years of age. Flock 72 (age = 53 weeks) from farm 58, and flock 29 (age = 34 weeks) from farm 25 were the only *Brachyspira*-negative flocks from their respective farms. Flock 47 (age = 54 weeks) from farm 44 was from a single-age farm, housed in stacked cages in a barn 28 years of age. This flock was positive for
Brachyspira. Re-running the model without flocks 72 and 29 affected neither the \( P \)-values nor the coefficients of the fixed effects in the final model; however, rerunning the model without flock 47 changed the \( P \)-value for the barn age category 15 – 29 year old barns from non significant \( (P = 0.088) \) to significant to \( (P = 0.036) \). Although flock 47 was an influential observation, we did not find a valid reason to remove it from the model; thus, the final model included these observations. On visual assessment of the BLUPs, the residuals were diverted from the normality line at the extremes. The ICCs for the null and final models were 0.67 and 5.6 x \( 10^{-14} \), respectively (Table 3.3).

4. DISCUSSION

This is the first study to investigate multiple risk factors for the presence of Brachyspira species in chickens, while assessing for confounding and interaction between factors, and controlling for clustering by farm. Further, it is the first epidemiological study conducted in Canada on Brachyspira species in chickens. A separate model could not be constructed for \( B. pilosicoli \) due to its low prevalence.

We found that the odds of Brachyspira was higher in flocks \( \geq 60 \) weeks of age compared to flocks \( \leq 34 \) weeks of age, which is in agreement with previous studies (Myers et al., 2009; Phillips et al, 2005; Stephens and Hampson, 1999). Faecal contamination of the water source by water fowl could be a source of infection to chickens (Jordan and Hampson, 2008). The water sources for 84.3% and 3.4 % of the flocks in this study were well and surface water respectively. There was a positive association between the presence of water fowl on or near the farm, and Brachyspira species in the univariable analysis. Once the bacterium is introduced into a flock, the chance of it spreading within the flock increases through attending personnel, equipment (Phillips et al., 2005), and possibly aerosol (Jordan and Hampson, 2008).
Our study has shown that the odds of *Brachyspira* species for flocks housed in stacked cages were lower than for flocks housed in A-frame cages with manure curtains. This could be due to contamination with faecal material from the upper tiers to lower tiers in A-frame cages. High rise barns, which were included in this category, are likely to have higher fly and rodent infestations due to the manure that is deposited and stored in the barn, usually for the life of the flock. Flies and rodents serve as transmitters of *Brachyspira* species (Hirsh, 2004). In addition, the manure disposal system for all the cage-stacked houses in our study were conveyor belts, which have been associated with a reduced occurrence of *Brachyspira* species (Bano et al., 2008; Burch et al., 2006). Although it has been reported that layer flocks housed in cages had a lower prevalence of *Brachyspira* species than free range flocks (Bano et al., 2008; Burch et al., 2006), to-date, this the first study to compare the prevalence of *Brachyspira* species among different types of cages.

The higher odds of *Brachyspira* species for flocks from multi-age farms compared to single-age farms suggest inter-flock transmission within a farm. Similar observations were documented by other studies (Bano et al., 2008; Burch et al., 2006; Trampel et al., 1994). The current finding was not surprising because the majority of multi-age farms did not have employees designated to a specific barn. Further, most of the multi-age farms were within a single complex where personnel could access all barns once a biosecurity procedure at the main entrance was followed. Although all barns were disinfected and were given downtime of at least 24 hours between flocks, the bacterium is more likely to be maintained within multi-age farms. On univariable analysis, the odds of *Brachyspira* species increased as the number of barns on a farm increased, also suggestive of inter-flock transmission.
It is unclear why there was an increased occurrence of *Brachyspira* species in newer barns. We noted differences in the building materials in barns of different ages, especially between barns less than 15 years old and those greater than 30 years old. For example, plastic walls and ceilings were used only in barns less than 15 years old, whereas barns older than 30 years had predominantly wooden walls. The floor material for all barns older than 15 years was concrete. Perhaps some barns are more difficult to clean than others, or this finding might be related to differences in barn cleaning and disinfecting procedures or products. It has been suggested that *Brachyspira* species might not be acquired from the environment provided that barns are cleaned and disinfected properly (Jordan and Hampson, 2008; Phillips et al., 2003). Thorough investigation and further research might help to understand the observed protective effect of older barns.

Due to the large number of variables investigated, a forward stepwise manual variable selection method was chosen over a backward elimination method. The number of variables that could be included in the model was limited by the number of flocks in the study; therefore, it was not possible to include all the variables significant on univariable analysis in the model and eliminate them one-by-one. The disadvantage with this approach is that there is a possibility that potential confounders, distorter, and suppressor variables were missed. However, biologically-plausible and *a priori* confounders were assessed during the model-building process. The non-normal distribution of the BLUPs at the extremes for the farm-level residuals was likely due to the fairly small number of farms included in the analysis. There were six farms with multiple flocks in which only one flock was included in the study. Further, not all of the producers contacted by the egg grading company representatives participated in the study. This could have led to non-response bias; however, to our knowledge, the lack of participation was associated
neither with the exposures nor the outcome. The primary reason for declining to participate was time constraint; the questionnaire would take 30 to 45 minutes of their time. The lack of blinding of the researchers to the flock status could also have introduced bias into the study; however, the researchers made every effort to administer the questionnaire and collect samples exactly the same in both groups.

The farm-level variance for the null model was relatively high; however, it decreased substantially when the fixed effects were included in the model, suggesting that the farm-level variable (multi-age farm) included in the final model accounted for most of the farm-to-farm variation in *Brachyspira* presence. Therefore, it is recommended that strict biosecurity be followed on farms with multiple flocks of different ages to avoid transmission of the bacteria between flocks.

**5. CONCLUSIONS**

For farms experiencing dirty eggs problems, older flocks could be tested routinely for early detection of *Brachyspira* species and treated if necessary, in consultation with a veterinarian. For new farms, stacked cages, rather than A-frame cages, are recommended. Further research is required to understand the effect of barn age on the presence of *Brachyspira* species.

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This project was supported by the OMAFRA-University of Guelph Agreement through the Animal Health Strategic Investment fund managed by the Animal Health Laboratory of the University of Guelph. The authors would like to thank Dr. David Pearl, Dr. Marina Brash, Dr. Mike Petrik, Dr. Agnes Agunos, Jennifer Zoethout, and Amanda Drexler. We appreciate the
cooperation of the Egg Farmers of Ontario and the participating producers. The authors wish to thank the Public Health Agency of Canada for providing supplies for field work.

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Table 3.1. Descriptive statistics of continuous variables describing farm, barn, and flock characteristics in Ontario layer chicken flocks (n = 89 flocks and 58 farms) in a study investigating risk factors for *Brachyspira* species, August, 2010 to February, 2011

<table>
<thead>
<tr>
<th>Variable</th>
<th>Range</th>
<th>Median</th>
<th>Mean</th>
<th>S.D.</th>
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</thead>
<tbody>
<tr>
<td>Age of cages (years)</td>
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<td>12</td>
<td>7.7</td>
</tr>
<tr>
<td>Barn age (years)&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>20</td>
<td>13.7</td>
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<td>21 - 79</td>
<td>46</td>
<td>47</td>
<td>15.6</td>
</tr>
<tr>
<td>Flock size&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4,740 – 115,050</td>
<td>24,930</td>
<td>31,729</td>
<td>23,855.9</td>
</tr>
<tr>
<td>No. of barns on the farm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 - 10</td>
<td>2</td>
<td>4</td>
<td>2.9</td>
</tr>
<tr>
<td>No. of employees per barn&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 - 50</td>
<td>4</td>
<td>10</td>
<td>15.2</td>
</tr>
<tr>
<td>No. of farms owned</td>
<td>1 - 5</td>
<td>1</td>
<td>2</td>
<td>1.1</td>
</tr>
<tr>
<td>No. of poultry farms within 2 km of the farm</td>
<td>0 - 10</td>
<td>1</td>
<td>1</td>
<td>2.3</td>
</tr>
<tr>
<td>No. of swine farms within 2 km of the farm</td>
<td>0 - 10</td>
<td>0</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>No. of tiers of cages</td>
<td>1 - 8</td>
<td>4</td>
<td>4</td>
<td>1.4</td>
</tr>
<tr>
<td>Rest period prior to flock placement (days)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 - 30</td>
<td>7</td>
<td>7</td>
<td>4.2</td>
</tr>
<tr>
<td>Water line flushing frequency (days)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 - 360</td>
<td>7</td>
<td>63</td>
<td>113.5</td>
</tr>
<tr>
<td>Water testing frequency per year&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 - 2</td>
<td>2</td>
<td>1.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Barn temperature (°C)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>11.1 – 29.4</td>
<td>23.6</td>
<td>23.8</td>
<td>2.4</td>
</tr>
<tr>
<td>Bird density (cm&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>329.0 - 516.1</td>
<td>440.6</td>
<td>458.1</td>
<td>152.3</td>
</tr>
<tr>
<td>Size of barn (m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>929.0 – 3,251.6</td>
<td>1,021.9</td>
<td>1,110.7</td>
<td>729.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Continuous variables eligible for inclusion to the main effects model at $P \leq 0.3$

<sup>b</sup>Barn temperature within two weeks of sampling
Table 3.2. Variables significantly \((P \leq 0.3)\) associated with the presence of *Brachyspira* species in univariable logistic regression analyses with a random effect for farm and then subsequently included in a multivariable main effects model \((n = 89\) flocks and 58 farms)

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breed(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lohman white (43)</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovan white (22)</td>
<td>0.7</td>
<td>0.13 – 4.30</td>
<td>0.744</td>
</tr>
<tr>
<td>Other white (11)</td>
<td>0.4</td>
<td>0.05 – 3.31</td>
<td>0.397</td>
</tr>
<tr>
<td>Brown (13)</td>
<td>0.2</td>
<td>0.02 – 1.34</td>
<td>0.092</td>
</tr>
<tr>
<td>Flock age (weeks)(^ab)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\leq 34) (22)</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35 – 46 (22)</td>
<td>1.9</td>
<td>0.2 – 15.9</td>
<td>0.549</td>
</tr>
<tr>
<td>47 – 59 (20)</td>
<td>1.7</td>
<td>0.2 – 12.9</td>
<td>0.593</td>
</tr>
<tr>
<td>(\geq 60) (25)</td>
<td>6.8</td>
<td>0.6 – 78.3</td>
<td>0.125</td>
</tr>
<tr>
<td>Enteric vaccines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (71)</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (18)</td>
<td>26.0</td>
<td>0.6 – 1155.1</td>
<td>0.094</td>
</tr>
<tr>
<td>Housing type(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cage stacked (63)</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-frame with manure curtain (17)</td>
<td>7.6</td>
<td>1.5 – 38.5</td>
<td>0.014</td>
</tr>
<tr>
<td>A-frame without manure curtain (5)</td>
<td>3.3</td>
<td>0.3 – 41.1</td>
<td>0.351</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
<td><strong>Odds ratio</strong></td>
<td><strong>95% CI</strong></td>
<td><strong>P-value</strong></td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>----------------</td>
<td>---------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Pullet housing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cage stacked (68) Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Floor (8)</td>
<td>0.9</td>
<td>0.1 – 9.8</td>
<td>0.914</td>
</tr>
<tr>
<td>A-frame (13)</td>
<td>11.9</td>
<td>0.6 – 218.7</td>
<td>0.096</td>
</tr>
<tr>
<td>Barn age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 14 (40) Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 – 29 (27)</td>
<td>1.0</td>
<td>0.23 – 4.73</td>
<td>0.966</td>
</tr>
<tr>
<td>≥ 30 (22)</td>
<td>0.2</td>
<td>0.04 – 1.18</td>
<td>0.076</td>
</tr>
<tr>
<td>Barn cleaning detergent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (26) Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (63)</td>
<td>0.2</td>
<td>0.02 – 2.25</td>
<td>0.197</td>
</tr>
<tr>
<td>Garbage removal from barn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As needed (49) Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weekly (40)</td>
<td>3.7</td>
<td>0.4 – 31.9</td>
<td>0.235</td>
</tr>
<tr>
<td>Pre-soaking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (32) Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (57)</td>
<td>0.2</td>
<td>0.02 – 1.89</td>
<td>0.163</td>
</tr>
<tr>
<td>Multi-age farm&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (36) Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (53)</td>
<td>14.0</td>
<td>1.6 – 131.1</td>
<td>0.017</td>
</tr>
<tr>
<td>Risk factors</td>
<td>Odds ratio</td>
<td>95% CI</td>
<td>P-value</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>------------</td>
<td>-------------</td>
<td>---------</td>
</tr>
<tr>
<td>Dogs owned</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (65)</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (24)</td>
<td>0.1</td>
<td>0.01 – 1.31</td>
<td>0.085</td>
</tr>
<tr>
<td>Water fowl on/near farm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (72)</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (17)</td>
<td>10.0</td>
<td>0.7 – 143.7</td>
<td>0.085</td>
</tr>
<tr>
<td>Farm-specific boots</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (45)</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (44)</td>
<td>0.1</td>
<td>0.01 – 1.37</td>
<td>0.084</td>
</tr>
<tr>
<td>Feed spill</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No (73)</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (16)</td>
<td>4.9</td>
<td>0.4 - 65.5</td>
<td>0.232</td>
</tr>
<tr>
<td>Flock size&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.000096</td>
<td>1.000002 – 1.000191</td>
<td>0.046</td>
</tr>
<tr>
<td>Flock size sq.</td>
<td>1.00</td>
<td>1.00 – 1.00</td>
<td>0.089</td>
</tr>
<tr>
<td>No. of employees per barn&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.35</td>
<td>0.93 – 1.95</td>
<td>0.113</td>
</tr>
<tr>
<td>No. of employees per barn sq.</td>
<td>0.99</td>
<td>0.99 – 1.00</td>
<td>0.179</td>
</tr>
</tbody>
</table>

<sup>a</sup>Breed and flock age were not significant at (P ≤ 0.3); however, they were included in the multivariable model to assess their confounding effect on the other variables.

<sup>b</sup>Descriptive statistics of variable prior to categorization (i.e. as a continuous variable) are provided in Table 3.1.

<sup>c</sup>Housing type: four flocks housed in free run barns were excluded from the analyses.
d Multi-age farm: flock was from a farm in which there were multiple flocks of different ages
Table 3.3. Final logistic regression model with a random effect for farm, of variables significantly ($P < 0.05$) associated with the presence of *Brachyspira* species in Ontario layer chicken flocks ($n = 85$ flocks and 57 farms)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flock age (weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\leq$ 34</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35 - 46</td>
<td>1.0</td>
<td>0.2 – 5.1</td>
<td>0.960</td>
</tr>
<tr>
<td>47 - 59</td>
<td>1.9</td>
<td>0.3 – 10.7</td>
<td>0.477</td>
</tr>
<tr>
<td>$\geq$ 60</td>
<td>9.3</td>
<td>1.5 – 55.2</td>
<td>0.014</td>
</tr>
<tr>
<td>Housing type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cage-stacked</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-frame with curtains</td>
<td>20.0</td>
<td>2.9 – 139.6</td>
<td>0.002</td>
</tr>
<tr>
<td>A-frame without curtains</td>
<td>1.7</td>
<td>0.2 – 16.9</td>
<td>0.672</td>
</tr>
<tr>
<td>Barn age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\leq$ 14</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 – 29</td>
<td>0.3</td>
<td>0.1 – 1.2</td>
<td>0.088</td>
</tr>
<tr>
<td>$\geq$ 30</td>
<td>0.1</td>
<td>0.01 - 0.34</td>
<td>0.002</td>
</tr>
<tr>
<td>Multi-age farm$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8.5</td>
<td>2.3 – 31.1</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Variance components**

<table>
<thead>
<tr>
<th></th>
<th>Variance</th>
<th>SE</th>
<th>ICC$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm (with fixed effects)</td>
<td>$1.8 \times 10^{-13}$</td>
<td>$6.3 \times 10^{-7}$</td>
<td>$5.6 \times 10^{-14}$</td>
</tr>
<tr>
<td>Farm (null model)</td>
<td>6.8</td>
<td>6.3</td>
<td>0.67</td>
</tr>
</tbody>
</table>

$^a$Multi-age farm: flock was from a farm in which there were multiple flocks of different ages
bICC: intra class correlation calculated using the latent variable technique
CHAPTER FOUR
Conclusions and Recommendations

In 2010, 636.3 million dozen eggs were produced in Canada, and Ontario contributed 38 % of the total production. Statistics Canada data indicate that the production of eggs has been increasing steadily over the last 10 years. However, almost two percent (12 million dozen) of total egg production were rejects (Statistics Canada, 2011). Faecal stained egg shells are the most common reason for rejection and down grading of eggs (Scott Houghton, Leanne Cooley, 2009, personal communication). The objectives of this thesis were to fill the knowledge gaps on the presence of Brachyspira species in Ontario layer chicken flocks, its association with the dirty egg problem in Ontario chicken layers, and risk factors associated with its presence. This is the first study in Canada to determine the role of Brachyspira in the dirty egg problem and the risk factors associated with its presence in layer chicken flocks.

The findings suggest the importance of Brachyspira as a potential cause of dirty egg problem. The higher prevalence of Brachyspira species and B. pilosicoli in the dirty flocks from this study is consistent with previous studies that showed strong associations of this bacterium with avian intestinal spirochetosis (Dwars et al., 1989; Stephen and Hampson, 1999; Bano et al., 2008). This finding might alert producers and concerned parties to consider Brachyspira as a potential determinant of high number of dirty eggs in a flock. Although determining the prevalence of Brachyspira was not a primary objective of this research, the 47% prevalence reported shows that the presence of this bacterium is relatively common in Ontario layers.

A multilevel logistic regression model indicated a positive association between the presence of Brachyspira and dirty eggs, flock age, multi-age flocks and A-frame housing, whereas there was a negative association between barn age and presence of Brachyspira species.
For farms with more than one flock, positive and negative flocks were observed within the same farm, supporting the conclusion that proper sanitation might confine and prevent transmission between barns. All producers in our study reported that they administered a strict rule against letting visitors into barns without proper hygienic precaution; however, this practice might not routinely apply to farm personnel. According to our observation, intra-farm contamination was more difficult to control in some farms than the others. For instance, most multi-age farms were located in one complex where employees have access to all the flocks within the complex. Biosecurity measures that are used to prevent the introduction of infection from other poultry farms or other external sources should also be applied to prevent within farm spread.

There are some limitations to this research. Multiple variables were eligible for inclusion in to the mains effect model on the multilevel univariable analysis; however, only four of these factors were found to be significant in the final multilevel logistic model. In addition, all the interaction factors were found not significant. This could be due to low number of observations within specific categories. To address this problem, we combined categories in a biologically sensible way in order to increase power within categories, but this was not possible in all instances in order to avoid misclassification bias.

Lists of egg producers were provided by the major egg grading companies in Ontario. However, not all flocks recruited by the grading companies participated in the study, and this might lead to non-response bias. This was due to reluctance of owners to participate. Because studies on *Brachyspira* have been emphasized only recently, participating (and non-participating) producers were unlikely to be familiar with this bacterium. The potential for non-response bias was minimized by involvement of the two major grading companies that
encouraged their clients to participate. The information from this study might create awareness that could encourage producers to participate in future studies.

One of the strengths of face-to-face interview format was relatively few missing data. However, we acknowledge that some of the responses could be subjective, which might have resulted in inconsistent or disparate responses to similar or comparable questions. Because of the time constraints producers face, especially during planting and harvesting seasons, the interview was at times brief. Therefore, some of the questions that required detailed information or specifications might not have been adequately addressed or at times were incomplete. Thus, these variables (e.g. feed additive) were not studied. In addition, despite assurances of confidentiality, producers sometimes appeared not to disclose actual practices. For instance, the responses regarding hand washing and sanitizer usage were invariably affirmative, but we observed that this was not always the case on some farms.

**Future Research Recommendations**

We were not able to assess all potential risk factors for *Brachyspira*, so additional research is needed. For example, seasonal factors may be important, by affecting the presence of waterfowl and wild birds around the farm. In addition, flies, rodents, and wild birds are known to transmit *Brachyspira* within a farm (Joens and Kinyon, 1982; Phillips *et al*., 2005), and their levels of activity and presence may vary by season. A year round sampling with fairly equivalent distribution of samples is recommended for future study.

The shedding pattern of *Brachyspira* by chickens is not clearly understood. Faecal samples were collected only once; hence, this study does not inherently disclose any information on the duration of the infection or colonization. Previous experimental studies showed that under laboratory conditions, infected chickens could shed the bacteria starting 7 to 21 days after
infection (Dwars et al., 1992; Trott et al., 1995). Dwars et al (1990) reported spirochetes in the caeca of experimentally infected chickens after 9 months; however, the authors did not indicate whether bacterial shedding was intermittent or continuous. Repeated sampling at specific times along a flock’s life time might prove a more rigorous approach to avoid potential bias associated with bacterial shedding dynamics. This information might help producers develop a more targeted approach for intervention.

Conclusion

This study has shown that Brachyspira species is a prevalent bacterium in layer flocks, and is significantly associated with the dirty egg problem in Ontario layer flocks. The relatively high variation in the prevalence of Brachyspira species among farms, on the multi-level logistic regression that determine the relationship between flock status and presence of Brachyspira, indicates that identification of risk factors and interventions to decrease the dirty egg problem should be aimed primarily at the farm level. For farms experiencing dirty eggs problems, older flocks should be tested routinely for early detection of Brachyspira species and treated if necessary, in consultation with a veterinarian. For new farms, stacked cages, rather than A-frame cages, are recommended. Strict on-farm biosecurity is needed to prevent the spread of the bacterium between flocks for farms with multi-age farms. Further research is required to understand the effect of barn age on the presence of Brachyspira species.

REFERENCES


APPENDIX 1

Questionnaire for *Brachyspira* in Ontario Layers project

**FARM CHARACTERISTICS**

Farm name____________________________________

Briefly describe your operation in terms of the number of farms (F), number of barns (B), and number of employees (E), including all-in-all-out practices (AIAO)?

____________________________________________________________________________________________________________
____________________________________________________________________________________________________________

If more than one barn, is this a multi-age farm? _____ Yes  _____ No

If more than one barn, is your farm considered to be one complex with biosecurity before entering the complex? _____ Yes  _____ No

If more than one barn, are employees specific to each barn?

_____ Yes

_____ No

_____ No – however maintenance personnel work in all barns

_____ No – however maintenance personnel work in all barns however change clothes/footwear between barns

**FLOCK CHARACTERISTICS**

Barn number  _________________________________
Date of flock placement ____________________ Age of flock when placed ____________ weeks

Current age of flock ____________ weeks

How were the pullets housed? ____ Floor barn
   ____ Cage barn – traditional (stacked)
   ____ Cage barn – A-frame

Name of pullet grower _______________________________________

Name of hatchery _________________________________________

Breed of flock ____ Bovan brown  ____ Bovan white  ____ Other (specify _____________________________)
   ____ Lohman brown  ____ Lohman white
   ____ ISA brown  ____ Shaver white
   ____ Hyline brown

Egg colour  ____ White
   ____ Brown

Flock size _______________ birds

Obtain the following information from the flock records:
Flock production at 20 weeks _______ %
Flock production at 21 weeks _______ %
Flock production at 22 weeks _______ %
Flock production at 23 weeks _______ %
Flock production at 24 weeks _______ %
Flock production at 25 weeks _______ %
Flock production at 26 weeks _______ %
Flock production at 27 weeks _______ %
Flock production at 28 weeks _______ %

Flock production within the last week (1 to 7 days ago) ____________ %
Flock production 2 weeks ago (8 to 14 days ago) ____________ %
Within the last week (1 to 7 days ago), the maximum barn temperature was ________°C or ________°F
Within the last week (1 to 7 days ago), the minimum barn temperature was ________°C or ________°F

Two weeks ago (8 to 14 days ago), the maximum barn temperature was ________°C or ________°F
Two weeks ago (8 to 14 days ago), the minimum barn temperature was ________°C or ________°F

Do you monitor barn humidity? _____ Yes     _____ No

If yes, what was the average humidity in the:
- Winter (October to March) _____ %
- Summer (April to September) _____ %

Within the last month, has the flock experienced any of the following infectious disease problems?
- No disease problems
- Necrotic enteritis
- Coccidiosis challenge
- Infectious bronchitis break
- Other (specify ____________________________)

If yes, describe signs (and veterinary diagnosis, if applicable) ____________________________________________________________________________
____________________________________________________________________________________

Have the flock’s weights been stable from month to month?
- Yes
- No
- Uncertain
- N/A – do not weigh the birds
- N/A – birds are still growing (i.e. < 30 weeks)

Within the last month, flock mortality has been:
- Lower than expected (Number of birds that died ____________)
- Normal (Number of birds that died ____________)
- Higher than expected (Number of birds that died ____________)

WATER
What is the water source for the flock?  
_____ Surface water (e.g. reservoir, pond, lake, river, or rainwater collection)  
_____ Well  
_____ Municipal water

How often do you test the flock’s drinking water for bacterial contamination?  
_____ Once per year  
_____ Twice per year  
_____ More frequent than twice per year  
_____ Never

Where in the system do you test the flock’s drinking water?  
_____ At the source  
_____ In the lunch room or work room  
_____ At the beginning of the water line  
_____ At the end of the water line  
_____ At the farm house  
_____ Other (________________________________________________)

Did you treat the flock’s drinking water?  
_____ Yes  
_____ No  
_____ N/A – water is treated by municipality

*If yes, describe water treatment and frequency, including prior to pullet placement and during the life of the flock (Check all that apply)*

<table>
<thead>
<tr>
<th>Type of water treatment</th>
<th>Continuous treatment</th>
<th>Intermittent treatment (specify how often)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse osmosis</td>
<td></td>
<td></td>
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<tr>
<td>Ultraviolet light</td>
<td></td>
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<tr>
<td>Chlorine</td>
<td></td>
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<tr>
<td>Hydrogen peroxide</td>
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<tr>
<td>Acid</td>
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<tr>
<td>Other (specify__________)</td>
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</tbody>
</table>

Did you flush the water lines?  
_____ Yes  
_____ No

*If yes, when did you flush the lines, what products did you use, and what water pressure did you use (high/low)? (List each cleaning, including prior to pullet placement and during the life of the flock)*
HOUSING AND BIRD DENSITY

Type of housing system:

- Cages – stacked
  Manufacturer and year ______________________________
- Cages – air dry system (e.g. pipe or whisk)
  Manufacturer and year ______________________________
- Cages – A-frame with plastic manure curtains
  Manufacturer and year ______________________________
- Cages – A-frame with wooden manure curtains
  Manufacturer and year ______________________________
- Cages – A-frame without manure curtains
  Manufacturer and year ______________________________
- Free run – with manure belts
- Free run – single level with all wire/slats
- Free run – single level with combination of wire/slats and scratch area
- Organic – access to pasture only
- Organic – access to pasture and an enclosed area with concrete floor
- Other (specify _________________________________________________________________________________________)

For cage housing systems, how many tiers of cages are there? _________________________

For cage housing systems, what is the actual cage floor space per bird? _____________ cm² or _______________ in²

For floor housing systems, what is the actual floor space per bird? _____________ cm² or _______________ in²

Age of the barn _______ years or, Year that the barn was built __________

Barn construction where hens are housed: (Check all that apply)
<table>
<thead>
<tr>
<th>Floor Material of Barn</th>
<th>Floor Material of Manure Pit (ONLY if high-rise building)</th>
<th>Wall Material (inside barn)</th>
<th>Ceiling Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>_____ Wood – solid or plywood</td>
<td>_____ Wood – solid or plywood</td>
<td>_____ Wood – solid or plywood</td>
<td>_____ Wood – solid or plywood</td>
</tr>
<tr>
<td>_____ Concrete or cement</td>
<td>_____ Concrete or cement</td>
<td>_____ Concrete or cement only</td>
<td>_____ Concrete or cement</td>
</tr>
<tr>
<td>_____ Other</td>
<td>_____ Other</td>
<td>_____ Concrete at bottom, wood at top</td>
<td>_____ Plastic</td>
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<td></td>
<td></td>
<td>_____ Plastic only</td>
<td>_____ Met al</td>
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<tr>
<td></td>
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<td>_____ Met al only</td>
<td>_____ Other</td>
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<td></td>
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<td></td>
<td>_____ Other</td>
</tr>
</tbody>
</table>

How **wide** is the barn in the area where the hens are housed?  ________ m or  ________ ft

How **long** is the barn in the area where the hens are housed?  ________ m or  ________ ft

Describe the air inlets on the barn in terms of their size and distribution around the barn (e.g. 16 inch baffle board with a 12 inch opening along the entire length of one wall)?

____________________________________________________________________________________________________________
____________________________________________________________________________________________________________

Describe the exhaust fans on the barn in terms of the number of fans of each diameter and their location around the barn (e.g. five 36 inch diameter fans, and ten 24 inch diameter fans located along the side wall of the barn)?

____________________________________________________________________________________________________________
____________________________________________________________________________________________________________
Are there any circulating fans in the barn?  
_____ Yes (number, fan size and location in barn_______________________________)  
_____ No

For this flock, did you use misters in the barn?  
_____ Yes  
_____ No

**MANURE DISPOSAL**

How often is manure removed from the barn? (*Check appropriate row once*)

<table>
<thead>
<tr>
<th>Type of manure disposal</th>
<th>Twice daily</th>
<th>Daily</th>
<th>Every other day, on average</th>
<th>Weekly</th>
<th>Less frequent than once weekly</th>
<th>Yearly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belt system / stacked manure system</td>
<td></td>
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<td></td>
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<tr>
<td>Skid steering</td>
<td></td>
<td></td>
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<tr>
<td>High-rise A-frame with curtains (in barn manure storage)</td>
<td></td>
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<tr>
<td>Low-rise A-frame with curtains (scraper system)</td>
<td></td>
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<tr>
<td>Low-rise A-frame without curtains</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Free-run or organic</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Free-run or organic with belts</td>
<td></td>
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<td></td>
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<tr>
<td>Free-run or organic with scraper under slats</td>
<td></td>
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</tr>
<tr>
<td>Organic with enclosed area with concrete floor</td>
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</tr>
</tbody>
</table>
If free-run with a scratch area, how often do you clean out (e.g. shovel) the scratch area? ______________________________

Where do you dispose of manure? (Check all that apply)  _____ Manure storage within barn  
_____ Manure storage attached to barn (direct manure conveyor)  
_____ Manure storage unattached to barn (storage facility on farm)  
_____ Manure removed from barn under nutrient management plan  
_____ Compost on farm  
_____ Spread manure on fields  
_____ Other (specify__________________________)

If contracted manure removal, how far is the manure shipped away from the farm?

km, or ______ miles, or ______ hours away

Uncertain

CLEANING AND DISINFECTION

Obtain HACCP records or invoices for HACCP records for C&D products used

Prior to flock placement, how long was the period of:

- No activity ____ days
- Dry cleaning/blow down stage ____ days
- Washing stage ____ days
- Disinfection stage ____ days
- Drying stage ____ days

If you washed the barn with water prior to flock placement, describe how you cleaned the barn, in terms of:

- Water temperature ______ Cold
  ______ Hot
Water pressure  
_____ Low  
_____ High (i.e. power washer)  

Cleaning products (soaps, detergents, sanitizers, NOT disinfectants)  
_____ Yes (products ________________________)  
_____ No  

Pre-soaking  
_____ Yes  
_____ No  

*If you disinfected the barn* prior to flock placement, describe how you disinfected the barn in terms of type of products and method of application:  
_____ Spray (products ____________________________________________)  
_____ Foam (products ____________________________________________)  
_____ Fog/fumigation (products ______________________________________)  

Did you use chemical insect foggers or sprays prior to pullet placement?  
_____ Yes (products ______________________________)  
_____ No  

**BIOSECURITY**  
Do you have any dogs or cats on the farm?  
_____ Yes  
_____ No  

*If yes, where are they allowed to go on the farm?*  
Dogs  
_____ Allowed on farm however not inside the barn  
_____ Allowed in the pack room however not in the barn  
_____ Allowed inside the barn  
_____ No dogs  

Cats  
_____ Allowed on farm however not inside the barn  
_____ Allowed inside the barn  
_____ Allowed inside the barn however not outside the barn  
_____ No cats  

Do you have any other domestic animals on the farm, such as rabbits, guinea pigs, turtles, or pet birds?  
_____ Yes
Do you raise livestock other than layers on this farm or at another location?  

<table>
<thead>
<tr>
<th>Livestock</th>
<th>At another location (specify distance)</th>
<th>On the same farm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep / Goats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deer / Llamas / Other</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Do you raise other types of poultry on this farm or at another location?  

<table>
<thead>
<tr>
<th>Poultry</th>
<th>At another location (specify distance)</th>
<th>On the same farm however in a different barn</th>
<th>On the same farm and in the same barn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Layer pullets / Layer breeders</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broilers / Broiler breeders / Broiler breeder pullets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turkeys / Turkey breeders</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ducks / Geese</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigeons</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Emu / Ostrich</td>
<td></td>
<td></td>
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<tr>
<td>Quail / Pheasants / Other</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
If you raise other types of poultry at another location, do you have premise-specific employees? _____ Yes  
_____ No

If you raise other types of poultry on the same farm however in a different barn, do you have barn-specific employees? _____ Yes  
_____ No

Are there waterfowl (wild ducks or geese) living on or near the premises (e.g. in fields or ponds)? _____ Yes  
_____ No

How many poultry farms or small flocks (backyard flocks) are within 2 km of your farm? __________

How many swine farms are within 2 km of your farm? __________

Is there a distinct clean area and dirty area immediately outside the entrance to the barn or complex? _____ Yes  
_____ No

If yes, describe the barrier used to distinguish the clean side from the dirty side, and how often you used it?

_____ Painted line  (_____ Always  _____ Sometimes)
_____ Step-over (e.g. rope, wooden board)  (_____ Always  _____ Sometimes)
_____ Bench (e.g. wooden/plastic bench)  (_____ Always  _____ Sometimes)
_____ Straw bale  (_____ Always  _____ Sometimes)
_____ Door  (_____ Always  _____ Sometimes)
_____ Other (______________________________)  (_____ Always  _____ Sometimes)

If no, what measures did you use to prevent bringing infectious agents into the barn?
____________________________________________________________________________________________________________  ____________________________________________________________________________________________________________

Do you or your employees manage or work on another poultry farm?  
_____ Yes (___________________________________________________________________________________________)  
_____ No (Check N/A in table for farm-specific clothing/footwear in table below)

Which of the following biosecurity protocols do you use when entering the barn and how often do you use them?
<table>
<thead>
<tr>
<th>Protocol</th>
<th>Producer/Employees</th>
<th>Visitors (e.g. neighbor, marketing board person)</th>
<th>Pullet delivery</th>
<th>Service personnel (e.g. electrician)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm (premise)-specific boots</td>
<td></td>
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<td></td>
<td>Yes</td>
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<td>No</td>
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<td></td>
<td>N/A</td>
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<tr>
<td>Farm (premise)-specific clothing</td>
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<td></td>
<td>Yes</td>
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<td>No</td>
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<td></td>
<td>N/A</td>
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<tr>
<td>Dedicated boots</td>
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<td>Yes</td>
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<td>N/A</td>
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<tr>
<td>Disposable boots</td>
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<td>Yes</td>
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<td>Uncertain</td>
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<tr>
<td>Boots dips (if yes, go to end of table)</td>
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<tr>
<td>e.g. basin/tray with dip solution or lye powder, foam</td>
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<td></td>
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<tr>
<td>system, sprays, granular/crunch products</td>
<td>Yes</td>
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<td>No</td>
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<tr>
<td>Dedicated clothing</td>
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<tr>
<td>Disposable clothing (PPE)</td>
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<td>Yes</td>
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<td>Uncertain</td>
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<tr>
<td>Washing hands with soap and water</td>
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<td></td>
<td>Yes</td>
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<tr>
<td>Hand sanitizers</td>
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<td>Yes</td>
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<tr>
<td>Protocol</td>
<td>Producer/Employees</td>
<td>Visitors (e.g. neighbor, marketing board person)</td>
<td>Pullet delivery</td>
<td>Service personnel (e.g. electrician)</td>
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<td>----------------------------------------------</td>
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<tr>
<td>Disposable gloves</td>
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<td>Yes</td>
<td>Yes</td>
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<td>Uncertain</td>
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<td>Other</td>
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<td>Yes</td>
<td>Yes</td>
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<td>Uncertain</td>
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<td>Uncertain</td>
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</tbody>
</table>

*If other, describe the protocol*  
______________________________________________________________________________  
______________________________________________________________________________  

*If boot dips are used, how often is the dip changed?*  
  
  1. Daily  
  2. Every other day, on average  
  3. Twice per week  
  4. Once per week  
  5. Less frequent than once per week

*If boot dips are used, what product(s) do you use and describe how you use them (e.g. rotate products every month)?*  
______________________________________________________________________________  
______________________________________________________________________________

Do egg or feed truck drivers spray their tires upon entering your farm?  
  
  1. Yes – producer has a tire sprayer on the farm  
  2. Yes – the truck has a built-in sprayer  
  3. No

*If yes, describe how and when the sprayers are used (e.g. only the feed truck has a built-in sprayer and the driver uses it every time)*  
______________________________________________________________________________  
______________________________________________________________________________

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PEST CONTROL
Obtain HACCP records or invoices for HACCP records for pest control products used

For this flock, how often do you clean entrance rooms and work rooms?  
- Twice daily
- Daily
- Every other day, on average
- Weekly
- Less frequent than once weekly

How often do you dispose of garbage from the barn, egg room, work room and lunch room?

Did you have a feed spill inside or outside the barn that would attract rodents?  
- Yes
- No

If yes, was it cleaned up immediately?  
- Yes
- No (How long before clean up)

Are there any areas outside the barn that have or had stagnant water (including potholes)?  
- Yes
- No

Was there water seepage into the barn?  
- Yes
- No

For high-rise barns, was there condensation on the walls that wept into the manure pit?  
- Yes
- No

Were there holes in the barn walls, roof or doors?  
- Yes
- No

If yes, were they repaired?  
- Yes
- No

Were there cracks in the barn floor?  
- Yes

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If yes, were they repaired?  _____ Yes
              _____ No

How often do you cut the grass around the barn?  _____ Always (__________________________)
              _____ Sometimes (__________________________)
              _____ Never
              _____ N/A – concrete or gravel or crushed rock around barn

Are there barriers (e.g. screens) covering the air inlets to prevent entry of wild birds?  _____ Yes
              _____ No

If yes, describe the barriers?  _____ Screens
              _____ Other (____________________________________________________________)

Are there screens or guards covering the exhaust fans to prevent entry of wild birds?  _____ Yes
              _____ No

Do you use rodent traps (e.g. tin cats)?  _____ Yes
              _____ No

If yes, how and when are they set?  _____ Set at all times
              _____ Intensified just before shipment of previous flock in barn
              _____ Set immediately before pullet placement
              _____ Set after pullet placement
              _____ Periodically throughout the year
              _____ Other (specify __________________________________________________________)

Do you use rodent bait stations (rodenticides)?  _____ Yes (products ____________________________________________)
              _____ No

If yes, how and when are they set?  _____ Set at all times
              _____ Intensified just before shipment of previous flock in barn
              _____ Set immediately before pullet placement
              _____ Set after pullet placement
_____ Other (specify _________________________________)

Do you use rodenticides in the shavings storage area?  _____ Yes (products _________________________________)
_____ No
_____ N/A - bedding not stored on farm
_____ N/A - caged housing or non-litter floor housing

Do you have wild bird control in shavings storage area?  _____ Yes (______________________________)
_____ No
_____ N/A - bedding not stored on farm
_____ N/A - caged housing or non-litter floor housing

Do you have any lights outside the barn to deter pests or intruders at night?  _____ Yes
_____ No

*If yes, what type of lights do you have?  _____ Flood lights
_____ Motion-activated lights
_____ Dawn-to-dusk-activated lights

Do you use fly traps (e.g. sticky papers)?  _____ Yes
_____ No

Do you have screens on barn doors and windows to keep flies out of the barn?  _____ Yes
_____ No

Did you use chemical insecticide powder along wall-floor junction prior to pullet placement or during the life of the flock?  _____ Yes (products _________________________________)
_____ No

Do you have an insect zapper (UV light)?  _____ Yes
_____ No

Do you use fly bait?  _____ Yes (products _________________________________)
_____ No

Do you use fly fungus?  _____ Yes
_____ No
If yes, how and where is it applied?  

During the preceding month, the fly numbers (e.g. fly problem) on the farm were  

- None  
- Low  
- Moderate  
- High  

During the life of the flock, have you observed any wild animals (e.g. skunks, raccoons, possums) in or near the barn?  

- Yes  
- No  

What do you do with mortalities?  

- Compost  
- Incinerate  
- Put in freezer then send or have picked up for rendering  
- Other (__________________________________________________________________)  

What do you do with dirty eggs? (Check all that apply)  

- Ship to grading station  
- Throw away  
- Compost  
- Manure pile  
- On-farm washing  
- Other (________________________________________________)  

If you have more than one flock, are the eggs mixed in the egg room before you check for dirts, cracks, etc.?  

- Yes  
- No  

Do you separate dirty eggs from cracked or broken eggs before putting them in the pail?  

- Yes  
- No  

If yes, how many dirts do you remove per day?  

- ¼ pail (size of pail _________ L or _________ gal)  
- ½ pail (size of pail _________ L or _________ gal)  
- ¾ pail (size of pail _________ L or _________ gal)  
- 1 pail (size of pail _________ L or _________ gal)  
- Other (size of pail _________ L or _________ gal)  

If no, what percentage of your pail are dirts?  

- % (size of pail _________ L or _________ gal)
Or, how many eggs are dirt?

______ Number of eggs
FEED, FEED ADDITIVES, AND FEED MEDICATIONS
(Note to researcher – do not include additives or medications given at pullet farm)
(Additives include shell additives, vitamins, acids)
(Medications include antimicrobials, anticoccidials)

Describe your feeding program for the flock, beginning from the day the hens entered the barn until now. Include all feeds given. (Researcher to look at feed label or records and then call feed mill to find out major ingredients (e.g. wheat, corn) and protein formulation (e.g. amino acids, crude protein))

<table>
<thead>
<tr>
<th>Supplier (or indicate if home mixed)</th>
<th>Name of feed (be specific)</th>
<th>Additive or medication / Dosage</th>
<th>Start date, or Age when first given</th>
<th>Feed consistency (e.g. pellet, mash, crumb)</th>
<th>Reason for administration (medications only)</th>
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WATER ADDITIVES AND WATER MEDICATIONS
(Note to researcher – do not include additives or medications given at pullet farm)
(Additives include shell additives, vitamins, acids)
(Medications include antimicrobials, anticoccidials)

99
Did you administer any water additives or water medications to the flock?  

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<th>Yes</th>
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If yes, provide the following information:

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<th>Name of additive or medication (trade or brand name)</th>
<th>Dosage</th>
<th>Start date, or Age when first given</th>
<th>Duration of administration</th>
<th>Reason for administration</th>
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**Pullet Vaccination History**

*Obtain pullet vaccination records from producer or call pullet grower or hatchery*
<table>
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<tr>
<th>Age (weeks)</th>
<th>Name of vaccine and/or pathogens vaccinated against</th>
<th>Method of administration (water, spray, eye drop, wing web, SQ, IM, other)</th>
<th>Dosage</th>
<th>Date when vaccine was given</th>
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