Intestinal spirochaetes (Brachyspira spp.) colonizing flocks of layer and breeder chickens in Malaysia

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Abstract

Avian intestinal spirochaetosis causes problems including delayed onset of lay and wet litter in adult chickens, and results from colonization of the caecae/rectum with pathogenic intestinal spirochaetes (genus *Brachyspira*). Because avian intestinal spirochaetosis has not previously been studied in South East Asia, this investigation was undertaken in Malaysia. Faecal samples were collected from 25 farms and a questionnaire was administered. *Brachyspira* species were detected by polymerase chain reaction in 198 of 500 (39%) faecal samples from 20 (80%) farms, including 16 (94%) layer and four (50%) breeder farms. Pathogenic *Brachyspira pilosicoli*was identified in five (29%) layer and two (25%) breeder farms whilst pathogenic *Brachyspira intermedia* was detected in nine (53%) layer and one (12.5%) of the breeder farms. Twelve (80%) layer farms had egg production problems and 11 (92%) were positive for *Brachyspira*: three (25%) for *B. pilosicoli* and six (50%) for *B. intermedia*. Of three breeder farms with egg production problems, one was colonized with *B. pilosicoli.* Three of ten layer farms with wet litter were positive for *B. pilosicoli* and six for *B. intermedia.* Of four breeder farms with wet litter, one was colonized with *B. pilosicoli* and one with *B. intermedia*. No significant associations were found between colonization and reduced egg production or wet litter, perhaps because so many flocks were colonized. A significant association ($P = 0.041$) occurred between a

high prevalence of colonization and faecal staining of eggs. There were significant positive associations between open-sided housing ($P = 0.006$), and flocks aged >40 weeks ($P < 0.001$) and colonization by pathogenic species.

Introduction

Anaerobic intestinal spirochaetes of the genus *Brachyspira* colonize the large intestine of various mammalian and avian species, and include both pathogenic and non-pathogenic species. *Brachyspira intermedia* and *Brachyspira pilosicoli* are the two most common pathogenic species found in adult poultry (McLaren *et al*., 1997), with pathogenic *Brachyspira alvinipulli* and *Brachyspira hyodysenteriae* being isolated infrequently (Feberwee *et al*., 2008). The disease complex associated with colonization with pathogenic species is known as avian intestinal spirochaetosis (AIS) (Hampson, 2013; Mappley *et al*., 2014), and may include signs such as a delayed onset of egg laying, wet litter, faecal staining of eggshells, reduced egg weights and reduced carotenoid content of eggs (Davelaar *et al*.,; Griffiths *et al 1987*; Dwars *et al*., 1989, 1992; Swayne *et al*., 1995; Trampel *et al*., 1994). Experimental infection of chickens with avian isolates of *B. intermedia* and *B. pilosicoli* have resulted in reduced egg production and increased faecal water content (Hampson & McLaren, 1999; Stephens & Hampson, 2002).

AIS is still poorly recognized and not investigated in most countries, particularly as the signs of the disease are non-specific (Stephens & Hampson, 2001). Studies to date mainly have been restricted to some European and Scandinavian countries, the USA, Canada and Australia (e.g., Davelaar *et al*., 1986; Griffiths *et al*., 1987; Swayne *et al*., 1992; McLaren *et al*., 1996; Stephens & Hampson, 1999; Bano *et al*., 2008; Jansson *et al*., 2008; Myers *et al*., 2009; Medhanie *et al*., 2013). In these studies around 70% of layer flocks and 50% of breeder flocks have been positive for spirochaetes, with around two-thirds of the spirochaetes being known pathogenic species (Hampson, 2013).

Currently no publications are available relating to epidemiological studies on avian intestinal spirochaetes in South East Asia, despite the large poultry populations in countries in the region. Accordingly, the aims of this study were to determine the occurrence and prevalence of *Brachyspira* species in layer and broiler breeder flocks in Malaysia, to look for associations with disease, and to identify risk factors for colonization.

Materials and methods

Study design

The study was conducted in 2007 in the five States of Perak, Penang, Negeri Sembilan, Melaka and Selangor on the west-coast side of peninsular Malaysia. Twenty-five farms were selected based on availability and the willingness of the owners/managers to cooperate with the study. The farms consisted of eight broiler breeder and 17 layer farms; 11 were in Perak, eight in Penang, three in Negeri Sembilan, two in Melaka and one in Selangor. Three types of housing system were in use: open-sided houses (where there were no external walls, but the chickens were in cages under a roofed structure), enclosed houses, and a combination of the two. The open-sided housing system usually was used for layer chickens while the closed house system was used for both layer and broiler breeder chickens.

Questionnaires and interviews with farmers

Prior to collecting samples, the farmers were interviewed to elicit information about their farms. A questionnaire was prepared that included specific questions related to farm management, production and disease problems, disinfectants, antibiotics and anti-stress medications used, as well as the biosecurity measures they undertook in their farms. The interviews were conducted in Malaysian and English languages, but whenever farmers were having problems in understanding questions they were given a Chinese written version of the questionnaire to facilitate understanding of the questions.

Sample collection

A total of 500 faecal samples were collected from the 25 farms. The chickens sampled were between 15 and 84 weeks of age. Samples were from two age groups on the farms: group 1 were chickens aged ≤40 weeks (n = 239) and group 2 were chickens aged >40 weeks (n = 261). Freshly passed caecal faeces samples that were soft and yellowish to brown in colour were collected from the floor of the layer and broiler breeder houses. Sampling was conducted randomly throughout the houses, with five to 10 samples collected per house. Clean wooden spatulas were used to collect approximately 4 g faeces into a sterile 20 ml container. The containers were kept on ice to maintain the temperature at 4°C during transport to the laboratory. Upon arrival, about 2 g faeces were separated from each of the samples and placed into 10 ml centrifuge tubes for DNA extraction, while the remainder was used for water content determination.

Faecal water determination

Approximately 2 g each faeces sample was weighed, dried to a constant weight in a hot-air oven, and the percentage water content was calculated.

DNA extraction

The methods used for DNA extraction were based on those recommended by the manufacturer of the QIAamp DNA Stool Mini Kit (QIAGEN, GmbH, Hilden, Germany), with some modifications for avian faeces as described by Phillips *et al*. (2006). Briefly, approximately 2 g faecal samples were washed in 10 ml sterile phosphate-buffered saline, and after centrifugation the top layer of the pellet was subjected to DNA extraction.

Polymerase chain reaction analysis

The extracted DNA was subjected to polymerase chain reaction (PCR) analysis using one PCR for the detection of the genus *Brachyspira*, and then individual species-specific PCRs for *B. pilosicoli* and *B. intermedia* on samples that were positive in the first PCR (Phillips *et al*., 2006). In the first PCR, primers designed to amplify 1307 base pairs of the 16S rRNA gene region of members of the genus *Brachyspira* were used. For *B. pilosicoli* another pair of 16S rRNA gene primers was used, with a predicted amplified product size of 823 base pairs in the region from base position 209 to 1032.

The *B. intermedia* primers were designed from the NADH oxidase (*nox*) gene, with a predicted product size of 567 base pairs from base position 517 to 1083 (Phillips *et al*., 2005).

Data analysis

The data were analysed using SPSS version PASW Statistic Grad Pack 18 (SPSS Inc., Chicago, Illinois, USA). The prevalence of spirochaetes of the genus *Brachyspira*, *B. pilosicoli* and/or *B. intermedia* in layer and broiler breeder farms and in different age groups were compared using chi-square analysis for independence, or the Fisher's exact test. Associations with reported reduced egg production and occurrence of wet litter similarly were compared in the colonized and non-colonized flocks. The median within-farm prevalence for spirochaetes of the genus *Brachyspira* was determined. Farms that had an in-farm prevalence greater than or equal to the median within-farm prevalence were classified as high-prevalence farms, and those less than the median were classified as low-prevalence farms. Associations between farms with high prevalence of pathogenic species (*B. pilosicoli* and/or *B. intermedia*) and potential risk factors were determined by univariate analysis using chi-square analysis or the Fisher's exact test, whenever appropriate. The odds ratios of the factors were also determined. Factors that were significant at $P \leq 0.25$ were offered to a binary logistic regression model. Backward elimination was used to remove factors from the model. The significance of the factors selected to remain in the final model was set at 5%.

Results

Descriptive analysis of the chicken farms

Amongst the farms sampled, the most commonly used housing system was the open-sided house system (46.2%) and the least common was the combination of open-sided and closed house system (11.5%). The largest farm visited had a total population of 1.5 million chickens and the smallest had 8000 chickens (median = 50,000; standard deviation = 342,134). The greatest number of chicken sheds per farm was 128, and the least was two sheds (median = 10; standard deviation = 32). Nine

(36%) of the farms practiced all-in all-out management, with litter disposal at the end of the cycle, while the other 16 (64%) farms disposed of their chicken litter at regular intervals, varying from daily to twice per week. All of the farms left the chicken sheds vacant after emptying, with the longest vacancy being 18 weeks and the shortest 2 weeks (median = 4; standard deviation = 3.8).

Only one farm reported having poor growth rates in their chickens at 16 weeks of age. Fifteen (60%) farms reported poor egg production in chickens between 20 and 60 weeks of age. Fourteen (56%) farms reported having a diarrhoea problem that occurred as early as 20 weeks up to 60 weeks of age. Ten (40%) farms reported having problems with faecal staining of eggs, and the chickens were between 24 and 60 weeks of age. Seven (28%) farms noticed an increased number of dirty eggs in their farms over the past 12 months. Only one farm had had a recent change in chicken feed when the study took place. Eight (32%) farms reported other disease problems, but only two infections had been confirmed by laboratory testing. One farm was diagnosed with infectious coryza and the other with infectious bronchitis.

Twenty-three (92%) farms reported using disinfectants, with three types of disinfectants being used in four (16%) farms and only one disinfectant being used in seven (28%). The disinfectant most commonly used was glutaraldehyde (56%) while the least commonly used were acetic acid, hydrogen peroxide and chloroxylenol (each 4%). Twenty-three (92%) farms reported using antibiotics, with the maximum of four antibiotics being used in two (8%) farms and only one antibiotic being used in seven (28%) farms. Most farms (44%) used two antibiotics concurrently. Tylosin was the most commonly used antibiotic (48%), while the antibiotics that were least commonly used were trimethoprim and streptomycin (each 4%). Twenty-one (84%) farms used anti-stress medication in their flocks, with a maximum of six medications incorporated into one formulation that was used in one farm, while most farms used only one medication per rearing. The most commonly used medication was multivitamins (44%), while the least used were vitamin E and sodium bicarbonate (each 4%).

Fourteen (56%) farms practised disinfectant spray/dip for vehicles prior to them entering the farms. Twelve (48%) farms required the use of dedicated boots while 10 (40%) farms required workers and

visitors to wear specific protective clothes before entry. Disinfectant showers were available in eight (32%) farms. Control of wild birds and rodents was practised in 19 (76%) farms, and five (20%) farms practised insect control. Of the 25 farmers/managers interviewed, only four (16%) claimed to have heard about AIS.

Polymerase chain reaction analysis and prevalence

Brachyspira species were detected in 198 (39%) faecal samples from 47 (57%) sheds on 20 (80%) farms, of which 16 (94%) were layer farms and four (50%) were breeder farms. Significantly more layer than breeder farms were colonized ($\chi^2 = 6.62$; $P = 0.023$).

Sixteen (64%) of the farms were colonized with the pathogenic species *B. pilosicoli* and/or *B. intermedia*. *B. pilosicoli* was detected in 67 of the 198 re-tested samples (13.4% overall, 29.6% of the 198 positive samples). These were recovered from 14 sheds from seven (28%) farms, of which five were layer farms and two were breeder farms. *B. intermedia* was detected in 62 samples (12.4% overall and 31.3% of the 198 positive samples). These were recovered from 23 sheds from 10 (40%) farms, of which nine (53%) were layer farms and one (12.5%) was a breeder farm. One farm had chickens colonized with both *B. pilosicoli* and *B. intermedia*, with 20 samples being positive for *B. pilosicoli* and 10 positive for *B. intermedia* (none were positive for both). There were no significant differences in colonization rate between farm types for either of the pathogenic species.

Reported production problems and colonization

Egg production problems were reported in 15 (60%) farms, including 12 (80%) layer farms and three (37.5%) breeder farms. Of these, 11 (92%) layer farms were positive for *Brachyspira*species: three of the 12 (25%) were positive for *B. pilosicoli* and six (50%) were positive for *B. intermedia.* Only one of the three breeder farms was colonized, and this was colonized with *B. pilosicoli*.

Wet litter problems were reported in 14 (56%) of the 25 farms, of which 10 (71%) were layer farms and four (50%) were breeder farms. Amongst these 10 layer farms, three (30%) were positive for *B. pilosicoli* and six (60%) for *B. intermedia*. Two of the four breeder farms with wet litter were positive, one each with *B. pilosicoli* and *B. intermedia* respectively.

No significant associations were found between the presence of spirochaetes, including pathogenic species, and egg production problems or wet litter.

Housing systems and colonization

There was a significant association ($P = 0.006$) between colonization with the two pathogenic *Brachyspira* species and open-sided housing systems compared with closed housing systems.

Prevalence in different age groups

Spirochaetes were not isolated from chickens under 20 weeks of age, and colonization rates tended to increase with the age. Amongst chickens aged under 40 weeks, *Brachyspira* species were detected in 47 (20%) faecal samples, of which 16 (7%) were *B. pilosicoli* and 17 (7%) were *B. intermedia*. Amongst chickens >40 weeks of age, *Brachyspira* species were detected in 151 (58%) samples, of which 51 (20%) were *B. pilosicoli* and 45 (17%) were *B. intermedia*. Chickens >40 weeks of age were significantly more likely to be colonized with the genus *Brachyspira* ($\chi^2 = 76.1$; $P < 0.001$), *B. pilosicoli* ($\chi^2 = 17.7$; $P = 0.001$) and *B. intermedia* ($\chi^2 = 11.8$; $P < 0.001$) than younger flocks.

Faecal water contents

There were no significant differences in the faecal moisture contents between colonized and non-colonized chickens and between the two age groups (Table 1).

Univariate analysis

The median within-farm prevalence of colonization by spirochaetes was 35%. Twelve (48%) farms were classified as high prevalence (above the median), and 13 (52%) farms were classified as low prevalence. Chi-square analysis revealed significant positive associations between high prevalence and sheds with <7000 chickens ($P = 0.001$) and faecal staining of eggs ($P = 0.041$). Farm factors significantly associated with the presence of pathogenic species included open-sided housing systems ($P = 0.011$), having <7000 chickens per shed ($P = 0.031$), not using disinfectant spray/dip for vehicles ($P = 0.033$), not using boots ($P = 0.041$), and not using specific protective clothing ($P = 0.009$).

Logistic regression analysis

Logistic regression analysis did not reveal any significant association between the farm factors amongst the high-prevalence farms colonized with the genus *Brachyspira* or with the farms colonized with the pathogenic species *B. pilosicoli* and/or *B. intermedia.* This is likely to have been contributed to by the small number of farms involved in the study, and the high flock prevalence.

Discussion

In this study, PCR methodology was used to detect *Brachyspira* spp. and the two main avian pathogenic species *B. intermedia* and *B. pilosicoli* in the faeces of Malaysian chickens. Similar methods have been used in some of the more recent studies on AIS conducted in other countries, since it avoids the need to culture and then identify these fastidious and slow-growing anaerobes (Myers *et al*., 2009). It was recognized that this approach had limitations, including only focusing on two of the pathogenic species, but it served the purpose of determining whether these spirochaetes were colonizing Malaysian chickens.

The first main finding was that the prevalence of intestinal spirochaetes in Malaysian chicken farms was high, with 80% of the 25 farms testing positive. The rate in breeder farms (50%) was broadly similar to those found in previous studies in other countries outside the region, but the rate amongst the layer farms (94%) was even higher than has been reported elsewhere. In part this may be attributed to the fact that many of the chickens in the layer farms were housed in open-sided sheds, where they would be freely exposed to wild birds, insects and various domesticated and feral mammalian species that might act as vectors (Hampson, 2013). Consistent with this, there was a significant association between colonization and the use of open-sided houses.

Another significant finding was that 64% of the farms were colonized with the pathogenic species *B. pilosicoli* and/or *B. intermedia*. As in studies in other parts of the world, *B. intermedia*was detected more frequently than *B. pilosicoli* at the farm and shed level, although both species were detected at a

similar chicken (sample) level (12.4% and 13.4% of the 500 samples, respectively). Somewhat unexpectedly given the high overall prevalence, only one (5%) of the 20 colonized farms was PCR-positive for both species.

As in previous studies in other countries, spirochaete colonization was significantly more common amongst older flocks (>40 weeks) than younger flocks (Stephens & Hampson, 1999; Bano *et al*., 2008; Myers *et al.,* 2009). This may be attributed to an increased cumulative opportunity for infection to be introduced into a house and to spread amongst chickens.

A significant association was found overall between faecal staining of eggshells and a high prevalence of spirochaete colonization, but no significant association was found between farms with or without egg production and/or wet litter problems and colonization with spirochaetes, including pathogenic species. The lack of association is different from findings in some previous studies, and may be due to a number of factors. Firstly, disease was self-reported by the farmers/managers, and it is possible that the health status was incorrectly understood or stated. Secondly, the high overall prevalence meant that achieving significant differences was not possible with the relatively small number of farms sampled. Thirdly, there was a high rate of reported disease, and some of this was likely to be due to other causes. Fourthly, antibiotics were widely used by the farms surveyed, and it is possible that these helped to control disease signs. Fifthly, the extent of colonization in individuals was not measured, and the weight of infection is likely to influence disease expression. Measuring this would require the use of diagnostic techniques such as quantitative PCR (Song & Hampson, 2009). Despite these possible explanations for a lack of association, it was interesting that there also were no significant differences in the measured water content between colonized and non-colonized faecal samples, or in chickens ≤40 weeks or >40 weeks of age. This is in contrast with previous studies that showed significant associations between increased faecal water contents and spirochaete colonization (Hampson & McLaren, 1999; Stephens & Hampson, 1999). In part this may be due to the targeted selection of samples (yellowish–brown caecal faeces with soft consistency) that probably contributed to the overall high (~80%) mean water content of the faeces.

The study demonstrated that the use of good farm biosecurity measures can play a significant role in preventing spirochaete colonization at the farm level. The following measures were all associated with reduced occurrence of pathogenic spirochaetes, and can be recommended to poultry farmers: using closed houses rather than open-sided houses; using disinfectant spray/dips for vehicles; and providing boots and protective clothing for workers. Avian intestinal spirochaetes are known to be rapidly inactivated by common disinfectants, and do not persist in the environment for long periods (Phillips *et al*., 2003). This message needs to be disseminated to poultry farmers in the region as it demonstrates the value of investing in simple biosecurity measures to prevent entry of these and other pathogens into farms.

In conclusion, pathogenic *Brachyspira* species colonize chickens in many layer and breeders farms in Malaysia. This information should increase awareness of AIS in the region, and should encourage farmers and veterinarians to consider this condition in their differential diagnosis and to establish appropriate diagnostic and biosecurity procedures to assist with control.

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Table 1. Percentage water content of faecal samples in chickens either colonized or not colonized with intestinal spirochaetes, and in two different age groups.

Group	Number	Minimum	Maximum	Mean	Standard deviation	*t* value	*P* value
Colonization							
Positive	197	68.8	86.3	80.4	2.9	0.926	0.355
Negative	303	55.6	89.4	80.1	3.8		
Age group							
≤40 weeks	239	55.6	89.4	80.1	3.7	0.672	0.502
>40 weeks	261	68.8	88.8	80.3	3.3		