**Brachyspira intermedia** and **Brachyspira pilosicoli** Are Commonly Found in Older Laying Flocks in Pennsylvania

Suzanne E. Myers, A Patricia A. Dunn, A Nyree D. Phillips, B Tom La, B and David J. Hampson BC

AAnimal Diagnostic Laboratory, Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, PA 16802-1110

BAnimal Research Institute, School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia

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SUMMARY. Anaerobic intestinal spirochetes (genus *Brachyspira*) include several species that are recognized as pathogens of poultry. Surveys undertaken in Europe and Australia have shown that layer and breeder flocks are often colonized by the pathogenic species *Brachyspira intermedia* and *Brachyspira pilosicoli*, but similar surveys have not been conducted in the United States. In the current study, DNA was extracted from fecal samples (n = 50) collected from each of 21 flocks of laying hens >40 wk of age in Pennsylvania, and this material was tested for *B. intermedia* and *B. pilosicoli* using a duplex PCR. Negative samples also were tested using a *Brachyspira* genus-specific PCR. The consistency of the feces was observed, and manure handling systems and medication histories were recorded. *Brachyspira intermedia* was detected in 662 (63.1%) samples from 17 (81%) flocks, with a within-flock prevalence of 10%–100%. *Brachyspira pilosicoli* was detected in 112 (10.7%) samples from 5 flocks (23.8%), with a within-flock prevalence of 8%–82%. Four of the flocks had both pathogenic species present, three had no pathogenic species detected, and two had no *Brachyspira* species detected. Nine flocks had many fecal samples with a wet appearance and/or a caramel color, and all of these were colonized with one or the other of the two pathogenic species. Nine of 12 flocks with manure that was mainly dry also were colonized. Differences in colonization rates between flocks with or without wet manure were not significant. Colonization with pathogenic *Brachyspira* species, and particularly *B. intermedia*, occurs very commonly in layer flocks >40 wk of age in Pennsylvania. The significance of this high rate of colonization requires further investigation.

RESUMEN. Las espiroquetas *Brachyspira intermedia* y *Brachyspira pilosicoli* se encuentran comúnmente en parvadas de aves de postura de mayor edad en el estado de Pennsylvania.

Las espiroquetas intestinales anaeróbicas del género *Brachyspira* incluyen varias especies que están reconocidas como patógenos en la avicultura. Los muestreos llevados a cabo en Europa y Australia ha demostrado que las parvadas de postura y de reproductoras están colonizadas por las especies patógenas *Brachyspira intermedia* y *Brachyspira pilosicoli*, pero no se han realizado muestreos similares en los Estados Unidos. En el presente estudio, se extrajo ADN de muestras de excretas (n = 50) recolectadas de 21 parvadas de aves de postura mayores de 40 semanas de edad en Pennsylvania, este material se analizó para detectar la presencia de *B. intermedia y B. pilosicoli* mediante el uso de un método PCR para detectar simultáneamente ambas especies. Las muestras negativas también fueron analizadas usando un método de PCR específico para el género *Brachyspira*. Se observó la consistencia de las excretas, y se registraron los sistemas de manejo de la cama y la historia de medicaciones. Se detectó *Brachyspira intermedia* en 662 muestras (63.2%) de 17 (81%) parvadas, con prevalencia dentro de las parvadas y entre las parvadas de 10% a 100%. Se detectó *Brachyspira pilosicoli* en 112 muestras (10.7%) de cinco parvadas (23.8%), con prevalencia dentro y entre las parvadas de 8% a 82%. Cuatro de las parvadas presentaban ambas especies patogénicas, tres parvadas no presentaron especies patógenas y en dos parvadas no se detectó alguna especie de *Brachyspira*. Nueve parvadas presentaron varias muestras de excretas con apariencia húmeda y un color tipo caramel, todas estas parvadas estuvieron colonizadas con alguna de las especies patogénicas. Nueve de doce parvadas con cama que estaba principalmente seca también presentaron colonización. No se observaron diferencias significativas en los porcentajes de colonización entre las parvadas que tenían camas húmedas o más secas. La colonización con especies patogénicas de *Brachyspira* y particularmente *B. intermedia*, se presenta comúnmente en parvadas de aves de postura mayores de 40 semanas en Pennsylvania. La significancia de este alto porcentaje de colonización requiere de mayor investigación.

Key words: avian intestinal spirochetosis, *Brachyspira*, spirochete, layers, staining of eggshells, wet manure

Abbreviations: AIS = avian intestinal spirochetosis
Epidemiological studies on AIS in laying chickens first were conducted in the Netherlands, where it was shown that colonization with unidentified spirochetes occurred commonly, and was associated with enteritis (2,6). Surveys in Australia also found that spirochetes commonly occurred in laying and breeder flocks, and that there was a significant association between infections with *B. intermedia* and *B. pilosicoli* and disease symptoms such as diarrhea and reduced egg production. The rate of infection gradually increased with increasing age of the flocks (14,23,26). Subsequent surveys conducted in Italy (1), the Netherlands (7), and Sweden (13) have confirmed the frequent occurrence of *Brachyspira* species in adult chickens, and *B. intermedia* and *B. pilosicoli* were the two pathogenic species most commonly identified. In the United States there have been several case reports of AIS in layers (3,27,29), but no detailed surveys have been reported.

The current study was designed to determine the prevalence of *B. intermedia* and *B. pilosicoli* in older layer flocks in Pennsylvania and to look for associations with disease.

### MATERIALS AND METHODS

**Collection of fecal samples.** The survey was conducted in 2008, with 50 fecal samples collected from each of 21 flocks on 20 laying hen farms in Pennsylvania. Flocks T and H were from the same farm; flock H was selected by the owner because it had a problem with loose sticky feces, whereas flock T had no such problems identified. Apart from the situation on this farm, the only criteria for selection of flocks was that they be >40 wk of age. Information on the age of the flock, breed, diet type, medications used in the last 3 mo, whether there was wet manure and caramel feces, and whether there was fecal staining of eggs was sought from the owner/manager. The housing system, the manure handling system, and the state of the flocks also were recorded by attending veterinarian at the time of sampling. The droppings were subjectively recorded as either being dry or wet, and the presence of caramel-colored feces was noted. The number of wet droppings was recorded as a few or many.

Freshly dropped cecal feces were collected from under the cages or the floor at random positions throughout each shed, and immediately transported to the Animal Diagnostic Laboratory at the Pennsylvania State University for processing.

**DNA extraction and PCRs.** The methods for DNA extraction and PCR amplification were based on those previously described (18), with modifications. Briefly, approximately 1 g of feces was washed once in 50 ml of sterile phosphate-buffered saline, and after centrifugation the surface layer was used for genomic DNA extraction using the QIAamp DNA Stool Mini Kit (QIAGEN, GmbH, Hilden, Germany). The extracted DNA was amplified using a single-step nonnested duplex-PCR system, which simultaneously detected the presence of *B. intermedia* and *B. pilosicoli* in the sample (18). The *B. intermedia* forward primer “Int 1” was modified to 5'-AGAGTTTGAAGACACTTATGAC-3' in the current assay, because this has been shown to improve the performance of the original *B. intermedia* PCR (Phillips, unpubl. data). Samples that were negative for *B. intermedia* and *B. pilosicoli* were further analyzed using a *Brachyspira* genus-specific PCR using the 16S rRNA gene as a target, as previously described (17,18).

**Statistical analysis.** Management systems in flocks with or without one or the other of the two pathogenic species, or both, were compared using Fisher’s exact probability test. This test also was applied to investigate possible connections between colonized flocks and symptoms that could be ascribed to AIS (wet manure, caramel-colored feces, stained eggs).

### RESULTS

The results of the study are summarized in Table 1. The flocks ranged in age from 46 wk to 2 yr. A variety of management styles, housing, breeds, feeds, medications, flock sizes, and locations in the state were represented. Flock A was a small cage-housed research flock with 1500 birds. Apart from this, flock sizes ranged from ~62,000 to 130,000 in caged system houses, and from ~7,000 to 16,000 in noncaged system housing. The chickens in seven of the flocks were Hyline, eight flocks had Lohman birds, and six had Bovan birds. Diets were corn- and soybean-based, except for flock S where the diet also contained a little wheat. Five flocks were fed organic crumble and the others received conventional mash diets appropriately formulated for their stage in the laying cycle.

*Brachyspira intermedia* was detected in 662 (63.1%) samples from 17 (81%) flocks, with a within-flock prevalence of 10%–100%. *Brachyspira pilosicoli* was detected in 112 (10.7%) samples from five flocks (23.8%), with a within-flock prevalence of 8%–82%. Four of the flocks had both pathogenic species present, three had no pathogenic species detected, and two had no *Brachyspira* species detected at all. The three flocks that were negative for both pathogenic species had the lowest number of birds per flock, but there was no overall correlation between flock size and extent of colonization. Of the seven flocks for which the managers reported using bacitracin and/or tetracycline currently or within 3 mo prior to the sampling date, all were positive for *B. intermedia*, and one was also positive for *B. pilosicoli*.

Nine flocks had many fecal samples that were observed to have a wet appearance and/or a caramel color, and all six for which records were available reported having eggs that had fecal staining. All nine of these flocks with clinical signs consistent with AIS were colonized with one or the other of the two pathogenic species. Nine of the 12 (75%) flocks with feces that were observed to be mainly dry also were colonized with one or the other of the pathogenic species. For eight flocks (A, D, G, J, L, M, N, and R) there were anomalies between what the producer recorded and the observed state of the manure. For example, in flocks B and M the manure appeared wet and the producers reported the presence of fecal-stained eggs, but neither reported the occurrence of wet manure in their flocks. On farm R, the producer reported wet manure and stained eggs, but the manure appeared to be dry with only a few caramel-colored droppings when inspected by the veterinarian. Five flocks were reported to have reduced egg production, two of which also had wet manure. Four of these flocks had high rates of colonization with *B. intermedia*, but one did not.

No significant differences were found between colonization and any of the categories that were analyzed.

### DISCUSSION

This study has demonstrated that colonization with *B. intermedia*, and to a lesser extent *B. pilosicoli*, is very common in older laying flocks in Pennsylvania. Within-flock prevalence also is very high. Colonization rates were even higher than those previously reported in layer flocks in Europe and Australia, and to an extent this might be attributable to the use of direct fecal PCR in the current study, rather than initial anaerobic culture followed by PCR. In addition, older flocks were targeted in the current study, because at the time the project was planned it was uncertain whether many spirochetes would be found. Previous studies have indicated that colonization is significantly more common in flocks >40 wk than in younger flocks (1,23). In future studies in Pennsylvania it would be useful also to examine flocks <40 wk of age, to confirm the existence of such age-related trends. Other *Brachyspira* species also were present in samples from 14 of the 16 flocks that were tested, but, given the already high prevalence of the two main pathogenic species, further work was not
Table 1. Management, clinical signs, and presence of *Brachyspira* species in fecal samples from 21 layer flocks in Pennsylvania.

<table>
<thead>
<tr>
<th>Flock</th>
<th>Age in weeks</th>
<th>Medications</th>
<th>Housing and manure system</th>
<th>Observed condition of manure</th>
<th>Questionnaire responses</th>
<th>Number and % positive for <em>Brachyspira</em> spp. in PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>104</td>
<td>None</td>
<td>Caged, belt</td>
<td>Dry; a few wet droppings</td>
<td>No</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>B</td>
<td>64</td>
<td>Lactobacillus, BMD-60</td>
<td>Caged, pit</td>
<td>Many wet droppings</td>
<td>No</td>
<td>24 (48%)</td>
</tr>
<tr>
<td>C</td>
<td>92</td>
<td>Neoterramycin, BMD</td>
<td>Caged, pit</td>
<td>A few wet droppings</td>
<td>Some</td>
<td>47 (94%)</td>
</tr>
<tr>
<td>D</td>
<td>59</td>
<td>Bacitracin, probiotic</td>
<td>Caged, pit</td>
<td>Dry</td>
<td>No</td>
<td>50 (100%)</td>
</tr>
<tr>
<td>E</td>
<td>60</td>
<td>Bacitracin, Neoterramycin</td>
<td>Caged, pit</td>
<td>Many wet droppings</td>
<td>Yes</td>
<td>50 (100%)</td>
</tr>
<tr>
<td>F</td>
<td>102</td>
<td>Bacitracin</td>
<td>Caged, pit</td>
<td>Dry</td>
<td>No</td>
<td>NT</td>
</tr>
<tr>
<td>G</td>
<td>80</td>
<td>None</td>
<td>Caged, belt</td>
<td>Many wet droppings, caramel</td>
<td>No</td>
<td>46 (92%)</td>
</tr>
<tr>
<td>H</td>
<td>60</td>
<td>Wazine 34</td>
<td>Floor, slats</td>
<td>Many sticky wet caramel droppings</td>
<td>Yes</td>
<td>35 (70%)</td>
</tr>
<tr>
<td>I</td>
<td>98</td>
<td>None</td>
<td>Caged, pit</td>
<td>Many wet sticky droppings</td>
<td>Yes</td>
<td>50 (100%)</td>
</tr>
<tr>
<td>J</td>
<td>46</td>
<td>None</td>
<td>Floor, litter and slats</td>
<td>A few wet droppings</td>
<td>Yes</td>
<td>46 (92%)</td>
</tr>
<tr>
<td>K</td>
<td>86</td>
<td>None</td>
<td>Caged, belt</td>
<td>Many wet droppings</td>
<td>Yes</td>
<td>50 (100%)</td>
</tr>
<tr>
<td>L</td>
<td>58</td>
<td>None</td>
<td>Floor, litter and slats</td>
<td>A few wet droppings</td>
<td>Yes</td>
<td>NT</td>
</tr>
<tr>
<td>M</td>
<td>67</td>
<td>None</td>
<td>Floor, litter and slats</td>
<td>Many wet droppings</td>
<td>Yes</td>
<td>6 (33%)</td>
</tr>
<tr>
<td>N</td>
<td>67</td>
<td>None</td>
<td>Caged, pit</td>
<td>Many caramel droppings</td>
<td>No</td>
<td>44 (88%)</td>
</tr>
<tr>
<td>O</td>
<td>46</td>
<td>Probiotic</td>
<td>Floor, litter and slats</td>
<td>A few caramel droppings,</td>
<td>Some</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>P</td>
<td>66</td>
<td>None</td>
<td>Caged, pit</td>
<td>Dry</td>
<td>No</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>Q</td>
<td>56</td>
<td>None</td>
<td>Floor, litter and slats</td>
<td>A few wet caramel droppings</td>
<td>Some</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>R</td>
<td>58</td>
<td>BMD</td>
<td>Caged, pit</td>
<td>Dry; some caramel droppings</td>
<td>Yes</td>
<td>36 (72%)</td>
</tr>
<tr>
<td>S</td>
<td>76</td>
<td>Neoterramycin</td>
<td>Caged, pit</td>
<td>Many caramel droppings</td>
<td>Yes</td>
<td>29 (58%)</td>
</tr>
<tr>
<td>T</td>
<td>73</td>
<td>None</td>
<td>Floor, slats</td>
<td>A few caramel droppings</td>
<td>No</td>
<td>21 (66%)</td>
</tr>
<tr>
<td>U</td>
<td>48</td>
<td>CuSO₄, lactobacillus</td>
<td>Caged, pit</td>
<td>A few caramel droppings</td>
<td>No</td>
<td>29 (58%)</td>
</tr>
</tbody>
</table>

A Flocks in boldface type were observed to have many droppings that were wet and/or caramel in color, and were recorded as being diseased.
B Medications in the last 3 mo. BMD = bacitracin methylene disalicylate. Wazine 34 is a treatment for roundworms. Lactobacillus is a probiotic species used to promote intestinal health.
C 'Don't know' response was given because eggs were sent to a breaker.
D Only samples negative in the *B. intermedia* and *B. pilosicoli* PCRs were tested. NT = not tested.
done to try to identify these to species level. Consequently their exact identity and significance remain uncertain.

Interestingly, there was a high prevalence of *B. intermedia* in all seven flocks that reported treatment with bacitracin and/or tetracyclines in the 3 mo preceding the sampling. It was not known why these treatments had been used, but the owners of six of the flocks reported having dirty eggs, and one did not know if their eggs were dirty because they went to a breaker. Because tetracycline has been shown to temporarily remove *Brachypia* species from an infected flock (23), and zinc bacitracin inhibits proliferation of *B. intermedia* in experimentally infected chickens (10), it is unclear why these medications appeared to be ineffective in these flocks. On the other hand, zinc bacitracin has been shown to enhance infection with *B. pilosicoli* in experimentally infected laying chickens (12), and no increase in this species was found in the flocks receiving bacitracin. Diet is also known to influence infections with *Brachypia* species; for example, wheat-based diets have enhanced experimental infection with *B. intermedia* (19). The flocks in the current survey generally had similar diets, which did not contain wheat, so the diet was unlikely to account for the high rate of colonization seen among the flocks.

Despite all the nine flocks with wet manure being colonized with *B. intermedia*, or *B. pilosicoli* in one case, it was not possible to establish a causal relationship because these spirochetes also were detected in nine of the 12 flocks that did not appear to have wet manure. In part this might be explained by the difficulties in defining the clinical problem. In a number of cases there were disagreements between what the visiting veterinarian observed and recorded, and what the producer reported in relation to wet manure, caramel-colored feces, and stained eggs. Most producers observed the general condition of the manure pack in the pit, floor, or litter, which is influenced by the ventilation and other house-specific conditions, rather than the appearance of the freshly voided droppings from individual birds. In future studies it would be advisable to use less-subjective recording of clinical signs that may relate to AIS; for example, the manure samples that were tested for spirochetes could be individually measured for moisture and fat content, and records of stained eggs from the whole house could be examined independently.

Another possible reason for discrepancies between colonization and disease could relate to the number of spirochetes present in a positive sample, and/or the presence of different strains of the species in different flocks. For example, when examining flocks T and H on the same farm, flock T, with clinical problems, had a higher within-flock prevalence of *B. intermedia* colonization than did flock H, which did not have clinical problems. There may also have been differences in numbers of spirochetes within individual fecal samples (and hence in the ceca). In future studies it would be useful to use techniques such as quantitative PCR (22) to investigate any such differences that might account for whether or not individual colonized chickens develop signs of disease. Furthermore, within the spirochete species it is known that many individual strains exist, and this may add to the complexity of the analysis. For example, in an Australian study 24 isolates of *B. intermedia* from one layer farm were divided into four pulsed-field gel electrophoresis types (17), and in another study four isolates from a layer farm were divided into four electrophoretic patterns in multilocus enzyme electrophoresis (26). Furthermore, isolates of both *B. intermedia* and *B. pilosicoli* from various farms were all different from each other (26). It is likely that these different strains vary in their biological properties, potentially including their virulence, and this might explain why the presence of these species on some farms but not on others is associated with disease. Further more-detailed studies, including strain typing and pathogenicity testing of isolates from different flocks of known health status, are required to more fully understand the significance of finding “pathogenic” spirochete species in chicken flocks.

The possibility of interaction between spirochete species, or between other potential pathogens and the spirochetes in the etiology of clinical problems also requires further investigation. The situation in the field is likely to be complex, with infectious agents, environmental factors, and stressors interacting to create clinical problems. Additional studies are needed in North America and elsewhere to further define the epidemiology and possible clinical, subclinical, and economic significance of these spirochetes in poultry.

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