

Intestinal colonization of different *Brachyspira* spp. in laying hens

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Abstract:

BACKGROUND: Avian intestinal spirochetosis (AIS) is caused by spiral-shaped Gram-negative *Brachyspira* spp. in poultry. It is known as a cause of diarrhea, low egg production, and increased occurrence of dirty eggs in layer hens. **OBJECTIVES:** In this study, the presence of some *Brachyspira* spp. was investigated in laying hens. **METHODS:** A total of 100 cloacal swab samples were individually collected from 20 laying hen flocks showing fecal egg staining in northeast of Iran. **RESULTS:** Using culture and morphologic examination, 41 samples (41%) from 20 flocks were positive; however, by using genus-specific PCR, only 37 (37%) samples were confirmed as *Brachyspira* spp. Using species-specific primers, single colonization was identified in 18 samples associated with *B. pilosicoli* (48.6%), while single colonization with *B. intermedia* was found in only two samples (5.4%). Simultaneous colonization by *B. intermedia* and *B. murdochii* was detected in 3 samples (8.1%). *B. pilosicoli* was the most prevalent species in concurrent colonization in 11 cases (29.7%). Finally, co-colonization by *B. intermedia* and *B. innocens* was identified in 3 samples (8.1%). **CONCLUSIONS:** The results of this study showed the colonization of different species of *Brachyspira* with dominance of *B. pilosicoli* in layer hens.

Introduction

Intestinal spirochetosis is caused by spiral-shaped Gram-negative *Brachyspira* spp. The bacteria are isolated from human and various animal species; some of them have zoonotic potential (Jamshidi and Hampson, 2003). *Brachyspira* colonization in human causes weight loss, abdominal pain, chronic diarrhea, and rectal bleeding and is known as a cause of pseudo-appendicitis (Jamshidi and Hampson, 2003). *Brachyspira* spp. is Gram-negative, loosely coiled, aerotolerant bacteria that colonize the gastrointestinal (GI) tract of mammals and birds, but varies in pathogenicity (Mapple et al., 2012).

According to the morphological characteristics of the bacteria including spiral form and their motility, they could be easily identified by phase contrast or "dark field" microscopy (Peghini et al., 2000). Despite the development of conventional culture methods, many *Brachyspira* spp. are fastidious and are not easy to culture (Hampson and Ahmed, 2009; Rasback et al., 2007). Molecular techniques have been used for classification of *Brachyspira* spp. (Brooke, 2003). They are reported mainly from adult laying hens and breeders with diarrhea, decreased in egg production and increased numbers of dirty eggs (Hampson and Swayne, 2006). Ever since, fifteen species has been officially or provisionally reported as *Brachyspira* spp., but it seems that the only three species (*B.*

pilosicoli, *B. intermedia*, and *B. alvinipulli*) are pathogenic in chickens (Smit et al., 1998). Outcomes of clinical colonizations with *Brachyspira* spp. depend on the age and species of the bird and potential pathogenicity of particular *Brachyspira* spp., the degree of colonization and probably many other predisposing factors (Hampson, and Swayne, 2006).

Pathological and histological lesions are non-specific and therefore not routinely used for AIS diagnosis. Therefore, the diagnosis is usually confirmed by various phenotypic and genotypic methods (Razmyar et al., 2011). The purposes of this study were to confirm the possibility of colonization of laying hens in Iran with more than one *Brachyspira* spp. and to identify the isolates at the species level.

Materials and Methods

Sampling: Sampling was performed from 20 laying hen flocks less than 40 weeks of age, laying stained eggs, in different areas of Razavi Khorasan province located in northeastern of Iran (five cloacal swabs were taken from chicken in each house). Totally, 100 samples were taken randomly from birds showing clinical conditions such as diarrhea and dirty vents in different locations in the house.

Voluntary participants were scheduled for cloacal sample collection from their flocks. Designation of flocks as high and low dirty flock was based on the percentage of dirty eggs each day for one week before the day of sampling. Accordingly, a flock was designated as 'high dirty flock' if more than 5% of the eggs were dirty and 'low dirty flocks' if < 5% of the eggs were dirty. Flocks without a problem (< 0.5% of the dirty eggs) were assumed as clean flocks. Approval for the study was obtained from the Research Ethics Committee at the Ferdowsi University of Mashhad, Iran.

Culture conditions: At the farm, the cloacal swabs were plated onto Trypticase Soy agar (TSA) supplemented with 5% defibrinated sheep blood, 400 µg mL⁻¹ spectinomycin and 25 µg mL⁻¹ vancomycin. Plates were transferred to anaerobic jars in an atmosphere of 94% N₂ and 6% CO₂ generated by GasPack A (Merck, Germany). Jars containing inoculated plates were transferred to the laboratory and incubated at 40°C for a period of 7-10 days. Bacterial growth was confirmed by the presence of

typical growth with weak beta-haemolysis and confirmed by phase contrast microscopy (Weissenböck et al., 2005; Fellstrom et al., 2001).

PCR tests: The DNA was extracted according to Phillips et al.'s (2006) instruction, from all positive samples by the TE boiling method (Aldus et al., 2005). For genus identification, a pair of genus-specific primers, and for identification at the species level, five species-specific primers were used (Table 1). These primer sets were selected according to Weissenböck et al. (2005). Annealing temperatures for each primer pair are shown in Table 1. The other thermocycler conditions were set according to Weissenböck et al. (2005). All PCR reactions were carried out in duplicate with negative and positive controls. For positive controls, we used the DNA extracts from our previous studies which has been confirmed by 16S rRNA sequencing and biochemical scheme analysis (Razmyar et al., 2011; Bassami et al., 2012).

Results

Out of one hundred samples, 41 (41%) were positive in culture and morphologic examination. Genus-specific PCR confirmed only 37 (37%) samples as *Brachyspira* genus. 73.8% of positive samples were from flocks showing diarrhea and dirty eggs (more than 5%). By using species-specific primers, 18 cases were infected with *B. pilosicoli* (48.6%), while *B. intermedia* colonization was found in only two cases (5.4%). Simultaneous colonization with *B. intermedia* and *B. murdochii* were detected in 3 cases (8.1%) and co-colonization with *B. innocens* and *B. intermedia* in 3 cases (8.1%). *B. pilosicoli* was the most common *Brachyspira* species in concurrent colonizations: *B. pilosicoli* with *B. innocens* in 3 cases (8.1%), with *B. murdochii* in 2 cases (5.4%), with *B. intermedia* in 5 cases (13.5%) and triple colonization with *B. intermedia*, *B. pilosicoli* and *B. murdochii* in one case (2.7%). Diarrhea and increased in the number of dirty eggs was higher in single and multiple colonizations with *B. pilosicoli* and milder symptoms were observed in colonizations with *B. intermedia*. Table 2 shows the detailed results. As the *nox* (NADH oxidase) gene specific primers were not successful in detecting positive control of *B. pilosicoli*, in this study, 16S rRNA gene specific

Table 1. Characteristics of primers used in this study.

Name	Sequence 5' to 3'	Product length	Annealing T(°C)	Target Gene	comment	References
BrNOX2-F	CATGGTTCTTGGCCTGTAAC	250	55	nox	Detects all <i>Brachyspira</i> species	Weissenbocket al. (2005)
BrNOX2-R	CGCCATAACTCTATGGCAATAGC					
Pilo-F1	TGAAATCTTCTAAAGATGAG	96	52	nox	Specific for	
B.pilosicoli	Weissenbocket al. (2005)					
Pilo-R1	TAGCTAAAGCAATATATCA					
F-Acoli1	AGA GGA AAG TTT TTT CGC TTC	439	51	S rRNA16	Specific for	
B.pilosicoli	Mikosza et al (1999)					
R647	CCC CTA CAA TAT CCAAGACT					
Interm-F2	TTGCCCTAGAGTTATGGGTAAT	182	55	nox	Specific for <i>B.intermedia</i>	Weissenbocket al. (2005)
Interm-R2	GACATAACTACCATATCTACT					
Innoc-F1	ATGGTGCTATAAAAGTAGAC	249	55	nox	Specific for	
B.innocens	Weissenbocket al. (2005)					
Innoc-R1	ACCAACCAGTAGAAGCCATG					
Murd-F1	GAATACTGCGGTACTCAAGGA	260	57	nox	Specific for <i>B.murdochii</i>	Weissenbocket al. (2005)
Murd-R1	GAGAATGCGTGAATAGCTTCG					

Table 2. Results of species-specific PCR on nox and 16S rDNA genes and clinical manifestations in laying hens. ^(a) Represents samples from flocks with more than 5% , ^(b) Represents less than 5% of clinical manifestations (dirty eggs and diarrhea).

Positive no.(%)	Diarrhea	Dirty eggs	<i>B. hyodysenteriae</i>	<i>B. innocens</i>	<i>B. murdochii</i>	<i>B. intermedia</i>	<i>B. pilosicoli</i>
18(48.6%)	a	a	-	-	-	-	+
3(8.1%)	a	a	-	+	-	-	+
2(5.4%)	b	a	-	-	+	-	+
1(2.7%)	b	b	-	-	+	+	+
5(13.5%)	a	a	-	-	-	+	+
3(8.1%)	a	b	-	-	+	+	-
3(8.1%)	b	a	-	+	-	+	-
2(5.4%)	a	b	-	-	-	+	-

primers were used for the diagnosis of this species (Mikosza et al., 1999).

Discussion

In our study, isolation method was on the basis of culturing cloacal swabs on selective agar plates at the farm and quickly transferring them for growth in anaerobic jars. This method prevents losing these susceptible bacteria upon transportation to the laboratory. Although this practice might not be perfect, it could reduce the risk of missing some positive samples.

Different antibiotics have been used for selective *Brachyspira* isolation, including colistin, spectinomycin, vancomycin, and rifampicin. As using all antibiotics simultaneously might limit growth for some species of *Brachyspira*, in this study only spectinomycin and vancomycin were used (Brooke, 2003; Razmyar et al., 2011).

Direct PCR assays for the detection of *Brachyspira* species have been conducted on DNA extracted from human and pig fecal material (Mikosza et al., 1999; Choi et al., 2002; La et al., 2003; La et al., 2006); however, according to the literature, direct PCR on chicken fecal samples has not been successful (Brooke, 2003). The reason is likely to be associated with the low pH of chicken feces, and the presence of uric acid and other PCR inhibitors; therefore, direct PCR on fecal samples was not employed in our study. Washing processes may be effective for removing potential PCR inhibitors from chicken feces (Phillips et al., 2006).

In this study, PCR tests targeting the nox gene were used to differentiate *Brachyspira* species. It has been shown that the nox gene has less homology in nucleotide sequence identity among the species of *Brachyspira* (86.3%), compared to 16S rRNA and 23SrRNA genes that have only 2-3% sequence variation (Atyeo et al., 1999). The PCR method based on the nox gene has been used to identify *B.*

intermedia, *B. aalborgi*, *B. pilosicoli*, *B. hyodysenteriae*, *B. innocens*, and *B. murdochii* (Rhode et al., 2002; Ogata et al., 2010; Backhans et al., 2011). Studies have shown that the *nox* gene is a suitable target for PCR-based differentiation of *Brachyspira* spp., although for *B. pilosicoli* the sensitivity is low (Atyeo et al., 1999). On the basis of the *nox* gene sequence, all *Brachyspira* spp. isolates in another study shared a homology of 83-93% with all known *Brachyspira* spp., whereas on the basis of the 16S rDNA gene sequence, all *Brachyspira* spp. isolates showed homology of 96-99.4% with other known *Brachyspira* isolates (Chander et al., 2012).

Although, among the diagnostic methods, microbial culture of *Brachyspira* spp. is considered as a standard method but regarding concurrent colonization with two or more species based on our finding and other reports (Westerman et al., 2012) and also the possibility of colonization with non-pathogenic species, it is better not to emphasize only the culture results. It is advisable to continue identification using molecular methods to ensure the identity of the isolates at species levels as a rapid and sensitive diagnostic tool (Myers et al., 2009).

Jansson et al. (2008), made the assumption that birds could be an important source of *Brachyspira* spp. by using phenotypic, molecular (PCR, RADP, PFGE, sequencing 16S rRNA and *nox* gene), and phylogenetic analyses. They isolated pathogenic (*B. intermedia* and *B. alvinipulli*), and non-pathogenic species (*B. innocens* and *B. murdochii*), and those that were not attributable to any of the known species in laying hens (Jansson et al., 2008). *B. alvinipulli* was not investigated.

In another study, 165 fecal samples from commercial laying hens, broilers, broiler breeder, turkeys, and ostriches were examined for the presence of *Brachyspira* spp. (Razmyar et al., 2011). A total of 16 out of the 165 samples were positive by conventional culture, and 66% of the positive samples belonged to laying hens. In their study, PCR test and 16S rRNA gene sequencing confirmed all isolates as *B. pilosicoli* (Razmyar et al., 2011). The results of the present study also indicated the dominance of *B. pilosicoli* (64.8%) in Iran. On the other hand, a study carried out by Backhans et al. (2011) in laying hens in Sweden found that *B. murdochii* and *B. innocens* were the most common

isolates, while in the present study the simultaneous presence of these two species was not detected. An interesting finding in this study was that both of these non-pathogenic species were detected simultaneously with the pathogenic species *B. intermedia* and *B. pilosicoli*. This difference could be due to the sampling from hen without AIS symptoms in the study of Backhans et al. (2011). Myers et al. (2009) examined 21 flocks of laying hens of at least 40 wk of age in Pennsylvania and reported (81%) and (23.8%) of flocks infected with *B. intermedia* and *B. pilosicoli* respectively. In contrast in our study *B. pilosicoli* was dominant (78.3%), and the rate of colonization with *B. intermedia* was different (37.8%) which can be due to the flock's age and ecological and geographical differences. Also in the study of Bano et al. (2008) in northeastern Italy 450 feces samples from 45 houses in 29 laying hen farms, feces were cultured for the presence of intestinal spirochetes. In 19 houses from 12 farms, layer hens showed similar symptoms of colonization with intestinal spirochetes including decreased egg production, wet litter and pasty vent and dirty eggshells. Using PCR assays, 27.5% *B. intermedia*, 13.8% *B. pilosicoli*, 49.7% *B. innocens* and *B. murdochii* were detected and 9.6% of the samples remained unknown. Although their findings are different from our results, the variety of species is the same. Regarding that four isolate did not confirm by genus-specific PCR, it is possible that selected primers could not detect these kinds of spirochetes.

According to reports concerning the role of *Brachyspira* spp. in dirty egg and low egg production syndrome in laying hens, as well as the potential of *B. pilosicoli* as a zoonotic agent (Jamshidi and Hampson, 2003; Jamshidi and Hampson, 2005) and the results of this study, it is recommended to implement monitoring and control programs in Iranian laying farms. This is the first report of the concurrent colonization of pathogenic and non pathogenic *Brachyspira* species in poultry in Iran.

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عفونت روده ای با گونه های مختلف اسپیروکت در مرغ های تخمگذار

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چکیده

زمینه مطالعه: اسپيروكتوز روده ای پرندگان توسط براکیسپیراهای گرم منفی ماریچی شکل در پرندگان ایجاد شده و منجر به اسهال، کاهش تولید تخم مرغ و آلودگی سطح تخم ها به مدفوع می گردد. **هدف:** در این مطالعه جهت بررسی تنوع گونه ای براکیسپیراها در گله های مرغ تخمگذار بود. **روش کار:** در این مطالعه تعداد ۱۰۰ سواب کلوکاز ۲۰ گله تخمگذار در شمال شرقی ایران جمع آوری گردید. **نتایج:** پس از کشت و آزمایشات مرفولوژیک ۴۱ نمونه (۴۱٪) مثبت ارزیابی گردید، ولی در آزمایش PCR اختصاصی جنس تعداد ۳۷ (۳۷٪) نمونه به عنوان جنس براکیسپیرا مورد تأیید قرار گرفت. در آزمایش PCR با پرایمرهای اختصاصی گونه، آلودگی انفرادی در تعداد ۱۸ نمونه (۴۸/۶٪) به براکیسپیرا پیلوسی کولای و تنها در دو نمونه (۵/۴٪) به براکیسپیرا اینترمدیا تشخیص داده شد. آلودگی همزمان در تعداد ۳ نمونه (۸/۱٪) به براکیسپیرا اینترمدیا و براکیسپیرا مردوخی، و در تعداد ۳ نمونه به براکیسپیرا اینترمدیا و براکیسپیرا اینوسنس تشخیص داده شد و براکیسپیرا پیلوسی کولای دارای بیشترین میزان آلودگی همزمان در ۱۱ مورد (۲۹/۷٪) به همراه سایر گونه های مورد بررسی بود. **نتیجه گیری نهایی:** شدت علائم اسپيروكتوز روده ای در عفونت همزمان با گونه های پاتوژن و غیر پاتوژن کاهش نشان می داد که می تواند نشان دهنده نقش رقابتی سویه های غیر پاتوژن در جلوگیری و کاهش کلونیزه شدن سویه های پاتوژن باشد.

واژه های کلیدی: اسپيروكتوز روده ای، براکیسپیرا، مرغ تخمگذار، PCR

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