Isolation and characterization of *Brachyspira* species based on biochemical scheme and 16S rDNA partial sequencing

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Abstract:

Avian intestinal spirochetosis (AIS) is a disease of birds characterized by a marked colonization of the cecum and/or rectum with anaerobic intestinal bacteria of the genus *Brachyspira*. The purpose of the study was to determine the occurrence of *Brachyspira* spp from avian sources in Iran and to characterize selected isolates by biochemical and molecular methods. Out of 165 fecal samples obtained from commercial layer, broiler and broiler breeder chickens, turkeys and ostriches, 16 samples were positive in phase contrast microscopy examination and selective culture media. Biochemical scheme, polymerase chain reaction (PCR), and sequencing of partial sequence of 16S rDNA, revealed that all of isolates were *Brachyspira pilosicoli*, a broad spectrum host member of this genus that infects both humans and animals. This is the first report on isolation and identification of *Brachyspira pilosicoli* from birds in Iran.

Introduction

Avian intestinal spirochetosis (AIS) is an infection of birds associated with anaerobic intestinal spirochetes. AIS has emerged as a widespread and economically important problem in both the egg and chicken meat industries in many parts of the world (Stephens and Hampson, 2001; Bano et al., 2008; Hampson and Swayne, 2008). The causative agent is now named *Brachyspira*, a genus containing more than 10 official and proposed species. The organism is weakly Gram negative, anaerobic, and spiral shaped with a diameter that ranges from 0.25 to 0.6 μm and in length from 3 to 19 μm. Light, dark field and phase contrast microscopy examinations allow an unequivocal diagnosis but tentative confirmation should be achieved by isolation of bacteria, demonstration of spirochete antigens or through visualization of periplasmic flagella by ultramicroscopy, and recently by polymerase chain reaction (PCR) assay based on genes such as 16S rDNA, 23S rDNA, *nox*, or *tly* (Suriyaarachchi et al., 2000; Jansson et al. 2004). Species and strain typing can provide significant data to help devise controlling measures. Various techniques have been used to type these microorganisms such as multilocus enzyme electrophoresis, pulse field gel electrophoresis, random amplified polymorphism of DNA, and determination of biochemical schemes using indole, hippurate hydrolysis, α- glucosidase, β- galactosidase, and hemolysis pattern (Fellström et al., 1997; Stephens et al., 2005; Townsend et al., 2005; Råsbäck et al., 2007).

In the present study, the occurrence of

Brachyspira spp. in samples from avian sources in Iran was investigated for the first time in Iran and the isolated species were characterized using biochemical and molecular methods.

Materials and Methods

Samples: In total, 165 fecal samples were obtained from commercial layers, broiler and broiler breeder chickens, turkeys, and ostriches during the years 2005-2008 (Table1). Samples were characterized as "symptomatic" or "asymptomatic", i.e. symptomatic samples originated from flocks with dirty vent or eggs, or from individuals with loose and watery feces. Samples were transferred to the laboratory in cold condition in sterile tubes that contained transport media (2 ml brain heart infusion broth containing: spectinomycin 500 mg/L, colistin and vancomycine each 25 mg/L).

Bacteriological procedures: In the laboratory, the sample tubes were shaken to achieve homogeneity, and then a wet smear of each sample was prepared to detect the spirochetes using phase contrast or dark field microscopy (x400). Isolation procedure was performed as described previously (Fellström et al., 1995) with minor modifications, i.e. incubation temperature was set at 40°C and the spectinomycin concentration in the selective medium was at 500 µg/ml.

Biochemical identification: β-Hemolysis was classified as strong or weak. Indole production was performed by both spot inoculation test and tube test (BHI broth containing 2 % tryptophan, 5% heat inactivated horse serum) as described previously (Fellström et al., 1995). α-Galactosidase and β-glucosidase activity were determined using commercial tablets (Diatabs®, Rosco Diagnostic, Denmark).

Polymerase chain reaction (PCR): To extract bacterial DNA, the method of Fellström et al. (1997) was used with some modifications. Briefly, each bacterial isolate was cultured on a tryptic soy agar (TSA) plate for 3-7 days, then harvested, suspended in 0.5 ml phosphate-buffered saline (PBS), washed two times, re-suspended in 0.2 ml double distilled water, and finally boiled for 15 min. The suspension was chilled at -20°C for 10 min, centrifuged at 12,000x g, the supernatant was removed and used as template DNA in PCR reactions. The supernatant was stored at -20°C for further use.

To choose a suitable primer, the sequences of the 16S rDNA (rRNA) from different *Brachyspira* spp. previously deposited in GenBank by other researchers (Table 2) were selected and aligned. Based on this sequence data analysis, a set of primers were designed to amplify a segment of the gene that contained the most variable part of the 16S rDNA (rRNA) among all species. Finally, the sequences of primers used in this study for the amplification of a 230-bp product were as follows: forward (Br-109): 5'-ATAACCCATGGAAACATGGAC-3' and reverse (Br-129): 5'-TCCATTGTGGAAGATTCTCAG-3'. Amplification reactions were carried out in a 50-µl reaction volumes that contained 5 µl 10x PCR buffer, 1 µl dNTPs mixture (10 mM/µl), 2 µl of each primer (10 pmol/µl), 0.3 µl Taq DNA polymerase (5 U/µl), 2µl $MgCl_2$ (50 mM/µl), 5 µl template DNA, and dH_2O up to a 50 µl final volume. In all PCR reaction sets, negative controls (dH_2O instead of template DNA) were included. Amplification was programmed in a thermocycler (Gradient Mastercycler, Eppendorff, Germany) as follows: 35 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 25 s and extension at 72°C for 30 s. The amplification products were detected by gel electrophoresis in 1.5% agarose gel in 1 TAE buffer followed by ethidium bromide staining (Sambrook and Russell, 2001). The primers and other materials used in PCR reaction were provided from CinnaGen Inc. (Tehran, Iran).

Sequencing and phylogenetic analysis: Four isolates were selected for sequence analysis of partial 16S rDNA (230 bp). PCR products were purified using the Roche purification kit (Roche Molecular Biochemicals, Germany) and submitted for automated sequencing in both directions at the MWG

Table 1: Sources of samples and culture results. Pos.= Positive, Symp.= Symptomatics, Asymp. = Asymptomatic.

Host	Population	Pos./Samples	.qmγS\.soq	Pos./Asymp.
Caged layer	50000	6/30	5/15	1/15
Caged layer	20000	3/20	3/15	-
Caged layer	10000	1/10	1/10	-
Broiler breeder	20000	0/20	-	-
Broiler	50000	0/20	-	-
Broiler	30000	0/10	-	-
Broiler	20000	0/10	-	-
Turkey (backyard)	10	1/10	1/10	-
Turkey (commercial)	1000	0/10	-	-
Ostrich	50	0/5	-	-
Ostrich	110	5/6	5/6	-
Ostrich	100	0/4	-	-
Total	201270	16/165	15/56	1/15

Table 2: List of isolates and strains used for comparison in this study.

No.	Strain	Accession no.	Source
1	SRBP5IR (*B. pilosicoli*)	EF436593	Layer
2	SRBP17IR (*B. pilosicoli*)	EF436591	Ostrich
3	SRBP20IR (*B. pilosicoli*)	EF436592	Ostrich
4	SRBP22IR (*B. pilosicoli*)	EF436595	Turkey
5	*B. hyodysenteriae* B78	U14930	Pig
6	*B. hyodysenteriae* B2	EF517540	Pig
7	*B. hyodysenteriae* B204	U14932	Pig
8	*B. intermedia* PWS/A	U23033	Pig
9	*B. suanatina* AN3949: 2/02	AY352290	Mallard
10	*B. suanatina* AN4859/03	DQ473575	Pig
11	*B. pilosicoli* P43/6/48	U14927	Pig
12	*B. innocens* B256	U14920	Pig
13	*B. murdochii* C301	U14917	Pig

Co. (Germany) using PCR primers as sequencing primers. Nucleotide sequence data were aligned with the Clustal alignment algorithm. Phylogenetic analysis based on nucleotide sequences was conducted using a distance method (UPGMA) by calculating bootstrap values for 1000 replicates in CLC Sequence Viewer 6 program (CLC Bio). The sequence data were submitted to GenBank under the accession numbers shown in Table 2. The accession numbers for other known *Brachyspira* spp. sequences used for multiple alignment analysis are also shown in Table 2.

Results

Bacteriological findings: Sixteen of the 165 fecal samples were positive for growth of *Brachyspira* spp by selective culture confirmed by phase contrast microscopy (Table 1). Two thirds of positive samples were isolated from layers. A strong epidemiological relation to diarrhea in layers was found since 15 of the 65 "symptomatic" but only one of the 15 "asymptomatic" samples taken was positive for *Brachyspira* growth. No broiler or broiler breeder farms were found to be positive. An interesting finding was the isolation of B. pilosicoli from recently imported common ostriches (*Struthio*

Table 3: Biochemical and partial and complete sequencing findings. α-gal = α-galactosidase, β-glu = β-glucosidase, ND = Not Done , D = Done, C = Complete sequencing, P = Partial sequencing, L = both bacteria were lost during sub-culturing and no further analysis was possible.

Designation	Origin	Hemolysis	α-gal	β-glu	Indole	Hippurate	PCR16S rDNA(Comp. Seq.)	PCR16S rDNA(Partial seq)	Indicated species
SRBP5IR	Layer	+	+	-	-	Weak	D	D	*B. Pilosicoli*
SRBP17IR	Ostrich	+	+	-	-	Weak	D	D	*B. Pilosicoli*
SRBP18IR	Ostrich	+	+	+/-	-	Weak	D	D	L
SRBP20IR	Ostrich	+	+	-	-	Weak	D	D	*B. Pilosicoli*
SRBP22IR	Turkey	+	+	-	-	Weak	D	D	*B. Pilosicoli*
SRBP25IR	Ostrich	+	+	+/-	-	Weak	ND	ND	L

camelus) in a herd that was suffering from severe diarrhea and mortality (Table 1).

Biochemical identification: All isolates recovered were weakly hemolytic. According to the performed biochemical tests, all available isolates from different hosts demonstrated identical patterns in phenotyping determination (Table 3).

PCR, sequencing, and phylogenetic analysis: PCR and sequencing of partial sequence of 16S rDNA, revealed that all of isolates were *Brachyspira pilosicoli*, a broad spectrum host member of this genus (Fig. 1). Different reactions in β-glucosidase test in isolate (SRBP18IR) and results from complete sequencing performed by Karl-Erik Johansson (Division of Bacteriology and Food Safety, Department of Biomedical Sciences and Veterinary Public Health, The Swedish University of Agricultural Sciences, Uppsala, Sweden) revealed the contamination of SRBP18IR culture with *Ralstonia picketti*, a member of the family *Burkholderiaceae*. In addition to our partial sequencing, the complete sequencing was performed on isolates 5, 17, 18, 20, and 22 by the above laboratory in Sweden for re-confirmation of our results. The isolate SRBP25IR was lost during sub-culturing and no further analysis was done on this isolate. The 16S rDNA sequencing of isolate SRBP5IR with a length of 1431 nucleotides resulted in no ambiguous positions. It was very similar to the type strain P43/6/48 of *Brachyspira pilosicoli* with only two nucleotide differences. The sequences of other isolates (SRBP17IR, SRBP20IR, and SRBP22IR) were identical to that of isolate SRBP5IR.

Discussion

In this study, for the first time in Iran, the occurrence of *Brachyspira* spp. in birds was demonstrated. Out of 165 fecal samples obtained from different types of birds, 16 samples were found to be positive. Biochemical scheme and sequencing of partial sequences of 16S rDNA identified all isolates as *B. pilosicoli*, a broad spectrum host member of this genus that infects both humans and animals.

Avian intestinal infection has been reported in different countries. In a report by Stephens and Hampson (2001), they noted a high rate of infection (> 40%) in 136 layer and broiler breeder flocks that were examined for the intestinal spirochetes. Myers et al. (2009) reported *Brachyspira pilosicoli* prevalence in laying flocks in Pennsylvania, USA. They detected 10.7% positive samples from five flocks (23.8% of flocks) and a within-flock prevalence of 8%-82%. The high prevalence (100%) of intestinal spirochetes in caged laying hens in our study may be due to their prior infection during the growing period on the floor. Poor staining characteristics of spirochetes and difficulties in their isolation procedures might be the reason for the less attention of poultry veterinarians and researchers to this anaerobic pathogen. Failure of demonstration and isolation of bacteria in breeder farms in our study may have been affected by interval usage of tylosin phosphate programs to control *Mycoplasma gallisepticum* infection in the majority of Iranian breeder farms or administration of high biosecurity measures in these

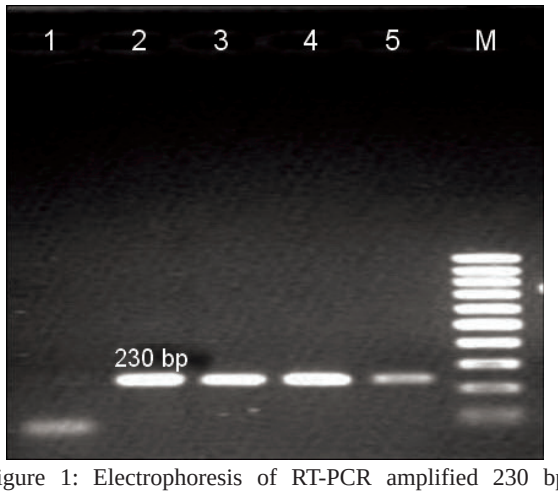

Figure 1: Electrophoresis of RT-PCR amplified 230 bp products of the 16S rDNA gene on 1.5% agarose gel and stained with ethidium bromide.. Lane 1, negative control; Lanes 2-5, positive samples; M, 100 bp ladder.

types of farms. The negative results in broiler farms corresponded with findings of Stephens and Hampson (1999) in eastern Australia that *Brachyspira* spp. were not isolated from broiler farms. However, they reported a 42.9% and a 68.2% positive presence in broiler breeder and layer flocks, respectively. There are many reports of severe necrotic typhilitis in common rheas (*Rhea Americana*) induced by B. hyodysenteriae (Sagartz et al., 1992, Buckles et al., 1997). In our study, the presence of *Brachyspira* was demonstrated in one of three ostrich farms that were sampled.

Fellström et al. (1996) emphasized that β-hemolysis criteria were only able to differentiate strongly β-hemolytic B. hyodysenteriae from other weak β-*hemolytic* intestinal spirochetes (WBHIS) and that additional criteria are required for distinguishing among WBHISs. They indicated that there was a possible presence of several biochemical variants of WBHISs in the same herd of pigs that supports our results in ostrich farm, in which we observed two different patterns. Based on the conventional biochemical tests, all of our isolates were identified as B. pilosicoli.

The Sequencing of partial sequence of 16S rDNA revealed that all of our isolates were *Brachyspira pilosicoli* confirming the biochemical patterns of the isolates. However, the agreement between the biochemical pattern and the genotypic profile has been disputed among researchers. Pettersson et al. (1996) reported 99% homology in 16S rRNA sequencing but interestingly the results were in agreement with the biochemical groups reported by Fellström and Gunnarsson (1995) in porcine intestinal spirochetes (PIS). Fellström et al. (1997) represented a specific PCR for the biochemical group IV of PISs (*B. pilosicoli*) and emphasized on this opinion that classification according to biochemical tests reflected the genotypes based on 16S rRNA. Fisher et al. (1997) also reported the similar classification schemes using *flaA*1 RFLP patterns, MEE, and biochemical tests. Fellström and Gunnarsson (1995) presented a series of biochemical tests (hemolysis, indole production, hippurate hydrolysis, β-glucosidase and β-galactosidase production) for phenotyping of 163 porcine isolates and placed them in four main groups and six subgroups. However, Rohde et al. (2002) compared molecular and conventional biochemical tests and indicated that because of variation in biochemical tests, genotyping must be preferred to phenotyping. Fossi and coworkers (2004) reported the presence of hippurate-negative isolates of B. pilosicoli that shared 99.16% homology in 16S rDNA sequence with hippurate-positive ones. Råsbäck et al. (2006) compared biochemical test and a duplex-PCR and concluded that culture and biochemical test were more sensitive than PCR for detection of *β. hyodysenteriae* but that the conclusion was opposite for *β. pilosicoli*. However, interestingly, they reported an isolate phenotypically as *β. hyodysenteriae* but genetically distinct from this species that was in contrast with their final conclusion. Based on earlier works even emphasis on genotyping methods alone could not yield a proper classification in this genus of bacteria. High homology in 16S rDNA (rRNA) sequence may reflect the recent evolution of this bacterial species.

In conclusion, this study documents the first report of *Brachyspira* spp. isolation and identification from poultry flocks in Iran. It appears that the proper classification of *Brachyspira* spp. requires the use of all available methods in specialized laboratories to establish a new robust

method. Moreover, the use of nonspecies-specific primers and sequencing, instead of species-specific and RFLP patterns that undergo point mutation changes by point mutations, are preferred. The study of pathogenicity and the determination of drug resistance patterns in our *Brachyspira* isolates are the future goals in our laboratory.

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