

Polymerase chain reaction assay targeting *nox* gene for rapid identification of *Brachyspira canis* in dogs

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Article Info	Abstract
<p>Article history:</p> <p>Received: 30 July 2017 Accepted: 12 December 2017 Available online: 15 March 2019</p> <p>Key words:</p> <p><i>Brachyspira canis</i> Dog Iran <i>nox</i> gene Polymerase chain reaction</p>	<p>Genus <i>Brachyspira</i>, as Gram negative anaerobic bacteria, colonize in dogs intestine. The aim of the current study was to determine the prevalence of <i>Brachyspira</i> spp. for the first time in Iran and rapid identification of <i>Brachyspira</i> spp. in dogs by a new designment of a species-specific primer set for <i>B. canis</i>. One hundred fifty-one fecal samples were obtained from dogs by rectal swab. Twenty dogs suffered from diarrhea and 131 of them were healthy. In 9.27% (14/151) of samples, spirochaetes were detected on primary cultures by weak hemolysis and positive Gram staining and then <i>Brachyspira</i> genus was confirmed by NADH oxidase (<i>nox</i>) gene via polymerase chain reaction. Among 14 isolates, twelve isolates were <i>B. canis</i>, one isolate was <i>B. intermedia</i> and another one was non-typeable. From 12 <i>B. canis</i>, only eight isolates were detected by designed specific primers. Ten <i>Brachyspira</i> spp. were isolated from dogs \leq 1 year old (10/67, 14.92%) and 4 isolates were from $>$ 1 year old dogs (4/84, 4.76%). The isolation rates from healthy and diarrheic dogs were (12/131, 9.16%) and (2/20, 10.00%), respectively. A statistically significant association was observed between the presence of <i>Brachyspira</i> spp. and the age under one year. Based on our findings, the <i>nox</i> gene in <i>B. canis</i> might have more sequence variability compared to other <i>Brachyspira</i> spp.</p> <p>© 2019 Urmia University. All rights reserved.</p>

سنجش واکنش زنجیره ای پلیمرز با هدف گیری ژن *nox* برای شناسایی سریع براکی اسپیرا کنیس در سگ

چکیده

جنس براکی اسپیرا، به عنوان باکتری های گرم منفی بی هوازی در روده سگ ها استقرار می یابد. هدف مطالعه حاضر، تعیین شیوع گونه های براکی اسپیرا برای اولین بار در ایران و شناسایی سریع گونه های براکی اسپیرا در سگ با پرایمر جدید طراحی شده اختصاصی براکی اسپیرا کنیس بود. صد و پنجاه و یک نمونه مدفوع از سگ ها به وسیله سواب رکتومی اخذ گردید. بیست سگ از اسهال رنج می بردند و ۱۳۱ قلاده از آن ها سالم بودند. از بین این نمونه ها، ۹/۲۷ درصد (۱۴/۱۵۱) اسپروکت در کشت های اولیه با همولیز ضعیف و رنگ آمیزی گرم مثبت شناسایی شدند و سپس تأیید جنس براکی اسپیرا با واکنش زنجیره ای پلیمرز ژن *nox* (NADH oxidase) انجام شد. در بین این ۱۴ جدایه، ۱۲ جدایه براکی اسپیرا کنیس، یک جدایه براکی اسپیرا اینترمدیا و دیگری غیر قابل تایپ بودند. از ۱۲ جدایه براکی اسپیرا کنیس، تنها هشت جدایه به وسیله پرایمرهای طراحی شده شناسایی شدند. تعداد ۱۰ گونه براکی اسپیرا از سگ هایی با سن یکسال و کمتر (۱۰/۶۷، ۱۴/۹۰ درصد) و چهار جدایه از سگ های بزرگتر از یکسال (۴/۸۴، ۴/۷۶ درصد) جداسازی شدند. میزان جداسازی از سگ های سالم و مبتلا به اسهال به ترتیب (۱۲/۱۳۱، ۹/۱۶ درصد) و (۲/۲۰، ۱۰/۰۰ درصد) بود. بین حضور براکی اسپیرا و سن زیر یک سال، ارتباط آماری معنی دار وجود داشت. بر اساس یافته های این مطالعه، ژن *nox* در براکی اسپیرا کنیس ممکن است از تنوع توالی بیشتری نسبت به سایر گونه های براکی اسپیرا برخوردار باشد.

واژه های کلیدی: ایران، براکی اسپیرا کنیس، ژن *nox* سگ، واکنش زنجیره ای پلیمرز

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Introduction

Genus *Brachyspira* contains several Gram-negative, oxygen-tolerant and anaerobic intestinal spirochete species colonizing in the large intestine of humans, birds (water birds, laying hens and corvid birds) and mammals (pigs, dogs, and wild rodents). Sixteen different species of *Brachyspira* have been identified up to now including *B. aalborgi*, *B. alvinipulli*, *B. canis*, *B. corvi*, *B. hamptonii*, *B. hyodysenteriae*, *B. ibaraki*, *B. innocens*, *B. intermedia*, *B. murdochii*, *B. muridarum*, *B. muris*, *B. pulli*, *B. rattus*, *B. suanatina*, and *B. pilosicoli*.¹⁻⁴

Human intestinal spirochetosis (HIS) is spread worldwide and its prevalence (2.50 to 62.50%) is higher among sensitive groups such as male homosexuals and human immunodeficiency virus-positive persons. The HIS is a condition defined by the presence of a layer of spirochetes attached by one cell end to the colorectal epithelium. Although the pathologic significance of HIS is uncertain, it has been linked to weight loss, chronic diarrhea, rectal bleeding, and other abdominal complaints. There are two *Brachyspira* species in humans including *B. pilosicoli* and *B. aalborgi*.⁵⁻⁷

Brachyspira pilosicoli is a zoonotic agent that was first described as a cause of porcine intestinal spirochaetosis and pigs colitis leading to diarrhea and dysentery.⁸⁻¹⁰

The prevalence rate of *Brachyspira* spp. in dogs is about 5.00% of the canine population and the organisms are transmitted between dogs by the fecal-oral route. Species which have been identified in dogs include *B. pilosicoli*, *B. canis*, *B. intermedia* and *B. alvinipulli*.¹¹⁻¹³

Reports of the presence of spirochaetes in dog's feces can be traced back more than 100 years. Canine intestinal spirochetes were first described in mature dogs with normal feces, but Duhamel *et al.* have later documented intestinal spirochetes in a 3-month-old beagle pup with diarrhea that had concurrent infection with *Giardia* spp. Likewise, Turek and Meyer have isolated spirochetes from beagle pups.¹³⁻¹⁶

Rapid identification of canine intestinal spirochaetes has been accomplished by determination of few biochemical properties¹⁷ and polymerase chain reaction (PCR) based on *16S rDNA* sequence.^{11,18} Additional methods used for identification of intestinal spirochaetes from other hosts include phylogenetic analysis based on *16S rDNA* sequences, multilocus enzyme electro-phoresis,^{11,18,19} pulse field gel electrophoresis,²⁰ random amplification of polymorphic DNA,²¹ duplex PCR^{22,23} and reverse transcription-PCR.²⁴

In previous studies, *B. pilosicoli* was detected by a molecular method with *B. pilosicoli*-specific primers for *nox* and *16s rDNA* gene,^{4,25} but *B. canis*-specific primers have not been designed up to this time. The purposes of this study were to isolate and rapid identification of *Brachyspira* spp. in dogs by designing a species-specific primer set for *B. canis* and also to discover the prevalence of *Brachyspira* spp. in dogs for the first time in Iran.

Materials and Methods

Sampling. A total of 151 fecal samples were collected from 151 dogs that were referred to the Veterinary Hospital of the Ferdowsi University of Mashhad, Iran (during 2014 to 2015). The owners were asked to sign consent before sampling. 131 of dogs were apparently healthy (routine checkup) or without any history of diarrhea and antibiotics therapy; however, 20 dogs had diarrhea as a main clinical sign. The samples were obtained directly from the rectum of the dogs using sterile swabs. Samples were collected from 91 female and 60 male dogs aging three months to 11 years old.

Bacterial culture. Fecal specimens were cultured on the selective tryptose soy agar (Merck, Darmstadt, Germany) supplemented with 5.00% sheep blood and the antibiotic supplement was composed of three antibiotics of the following final concentrations: spectinomycin 400 µg mL⁻¹, vancomycin 25.00 µg mL⁻¹ and colistin 25.00 µg mL⁻¹.²⁶ Sheep blood agar plates were incubated in an anaerobic jar at 37 °C for five days. Plates being weakly beta-hemolytic were checked for the presence of *Brachyspira* spp. by Gram staining. Two or three passages were done to obtain pure culture in positive samples.

DNA extraction and PCR. The PCR preparation kit (Denazist Asia, Mashhad, Iran) was used for DNA extraction according to the manufacturer's instructions. Briefly, a single colony from pure culture was chosen and then subjected to DNA extraction procedure. At first, a genus-specific primer pair was used for confirmation by targeting conserve segment of the NADH oxidase (*nox*) gene.²⁷ The *nox* gene was chosen as a target gene because its sequence is less conserved than *16S* or *23S rDNA* gene sequences. The DNA extracts were used for species-specific primer sets targeting *B. pilosicoli*, *B. intermedia*, *B. innocens*, *B. merdochii* and *B. hyodysenteriae* (Table 1).²⁷

***Brachyspira canis*-specific *nox* PCR designment.** The only two *nox* gene sequences belonged to *B. canis* obtained from public databases with the accession number of EF436590 and EU819071 and both of them were analyzed by CLC Genomics workbench (version 7.0; CLC Bio, Aarhus, Denmark). Based on multiple sequence alignments, highly conserved regions of *nox* gene were chosen and PCR primers were obtained from these regions. Oligonucleotide primers were synthesized by Macrogen Inc., Seoul, Korea. The PCR primers used in this study are listed in Table 1.

The PCRs were run in total reaction volumes of 25.00 µL containing the primers. The materials used in the PCR reaction were provided by Ampliqon (Odense, Copenhagen, Denmark). Amplification reactions were carried out in a 50 µL reaction volume containing 5 µL 10x PCR buffer, 5 mM dNTPs, 25.00 mM MgCl₂, 5.00 U of Taq DNA polymerase and the primers (Table 1), at the given concentrations. The thermocycler conditions were an initial denaturation for 15 min at 95 °C and the reaction

Table 1. Oligonucleotide primers used for genus- and species-specific *Brachyspira* polymerase chain reaction.

Name	Sequence 5' to 3'	Product size	Temperatures (°C)	Comment
<i>BrNOX2-F</i>	C(AT)GGTTCTTGGCCTGTAAC	250bp	55	Detects all <i>Brachyspira</i> species
<i>BrNOX2-R</i>	(CG)CCATAACTCT(AT)GGCA(AT)AGC			
<i>Hyo-F1</i>	AGGTGACTGTGCTACTGT	320bp	62	Specific for <i>B. hyodysenteriae</i>
<i>Hyo-R1</i>	AACGTCTGCTGCCTTCTT			
<i>Pilo2004-F1</i>	TGAAATCTTCTAAAGATGAG	96bp	55	Specific for <i>B. pilosicoli</i>
<i>Pilo2004-R1</i>	TAGCTAAAGCAATATATTCA			
<i>Interm2004-F2</i>	TTGCCTAGAGTTATGGGTAAT	182bp	55	Specific for <i>B. intermedia</i>
<i>Interm2004-R2</i>	GACATAACTACCATATCTACT			
<i>Innoc-F1</i>	ATGGTGTATAAAAAGTAGAC	249bp	55	Specific for <i>B. innocens</i>
<i>Innoc-R1</i>	ACCAACCAGTAGAAGCCATG			
<i>Murd-F1</i>	GAATACTGCGTACTCAAGGA	260bp	55	Specific for <i>B. murdochii</i>
<i>Murd-R1</i>	GAGAATGCGTGAATAGCTTCG			
<i>Canis-F</i>	AGGAAATCATTGATGAGTTG	439bp	56	Specific for <i>B. canis</i>
<i>Canis-R</i>	ATATAGTCCTGAGCTCCG			

mixture was subjected to 40 cycles of heat denaturation at 94 °C for 30 sec. The temperatures for each primer annealing are shown in Table 1 which were 30 sec for all the primers, and DNA extension was done at 72 °C for 1 min which was completed by a final extension of further 10 min at 72 °C.²⁷ Following PCR, 10.00 µL of the amplification products were electrophoresed in a 2.00% Tris acetate EDTA-agarose gel. The gel was stained with ethidium bromide and the bands were visualized under ultraviolet light. All PCR reactions were carried out in duplicate with negative and positive controls. For positive controls, DNA extracts which have been confirmed by 16S rDNA sequencing and biochemical scheme analysis in previous studies were used.^{4,28,29}

Evaluation of sensitivity and specificity of the *B. canis* specific primers. To determine the primers specificity for *B. canis*, PCR assay with specific primers *B. canis* forward and reverse (BCF) and *Brachyspira canis* reverse (BCR) was performed on the DNA extracted from *B. pilosicoli*, *B. intermedia*, *B. innocens*, *B. murdochii*, *Clostridium perfringens*, *C. difficile* and *Escherichia coli*. In order to obtain the lowest amount of DNA for PCR primers amplification and detection, concentration of a sample of extracted DNA was determined by spectrophotometer device ranging from 10⁻² to 10⁻⁷ copies/reaction. The PCR assay was eventually performed on each sample.

Statistical analysis. Data analysis was performed using SPSS software (version 21.0; IBM, Chicago, USA). The relationships between bacteria and the presence of diarrhea, bacteria, and gender, bacteria and age were assessed by Pearson Chi-square. A *p*-value < 0.05 was considered significant.

Results

A total of 151 fecal samples were collected throughout the study, 131 from clinically healthy non-diarrheic and 20 from diarrheic dogs. From these samples, 14 (9.27%) spirochaetes were detected on primary cultures by weak hemolysis and positive Gram staining and then

Brachyspira genus was confirmed by NADH oxidase (*nox*) gene PCR. The median age of non-diarrheic and diarrheic dogs included in this research was not statistically different which was two years (from 1 month to 10 years) and 2.50 years (from 5 months to 7 years). Eighty-one female and 50 male dogs were in the non-diarrheic group. Diarrheic group consisted of 10 female and 10 male dogs. Ten *Brachyspira* spp. isolates were from dogs ≤ 1 year old (10/67, 14.92%) and four isolates from > 1-year-old dogs (4/84, 4.76%). The isolation rate from healthy and diarrheic dogs was 9.16% (12/131) and 10.00% (2/20) respectively. Out of 91 female dogs, 11 (12.00%) and from 60 male dogs, three dogs (5.00%) were positive in culturing identification method and PCR.

A total of 14 isolates in species-specific PCR of *Brachyspira* had shown *nox* gene with 250 bp (Fig. 1) and in order to identify the *Brachyspira* species (such as *B. pilosicoli*, *B. intermedia*, *B. innocens*, *B. murdochii*, *B. hyodysenteriae* and designed *B. canis*; Fig. 2), species-specific PCR primer was performed. Among these 14 isolates, 12 isolates were *B. canis*, one isolate was *B. intermedia* and another one was non-typeable due to loss of DNA extract.

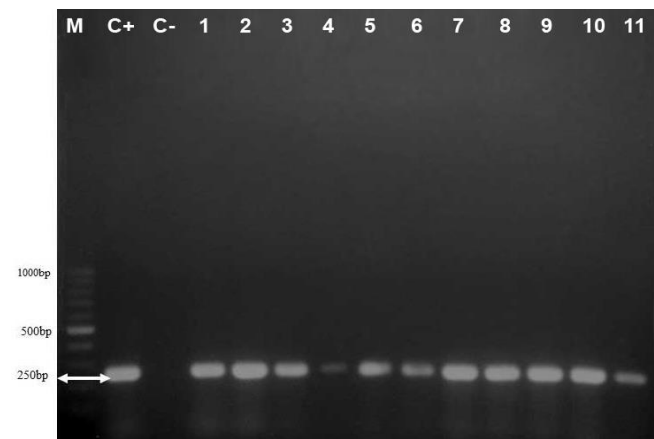


Fig. 1. The *nox* gene polymerase chain reaction. M: 100 bp DNA marker; C+: Positive control 250 bp band; C-: Negative control; Lanes 1-11: *Brachyspira* genus isolates.

From 12 *B. canis*, only eight isolates were detected by specific primers designed in this study, while the other four isolates were confirmed by the School of Veterinary and Life Sciences, Murdoch University, Perth, Australia after full sequencing of *16S rDNA* and NADH oxidase. However, one isolate was found to be negative by genus-specific *16S rDNA* and genus-specific *nox* PCRs in the study conducted by Murdoch University. This could be due to the fact that the sample had insufficient DNA. Accession numbers of four isolates based on *16S rDNA* gene deposited in the GenBank are as follows: KT381873, KT381872, KT381871, and KT381870.

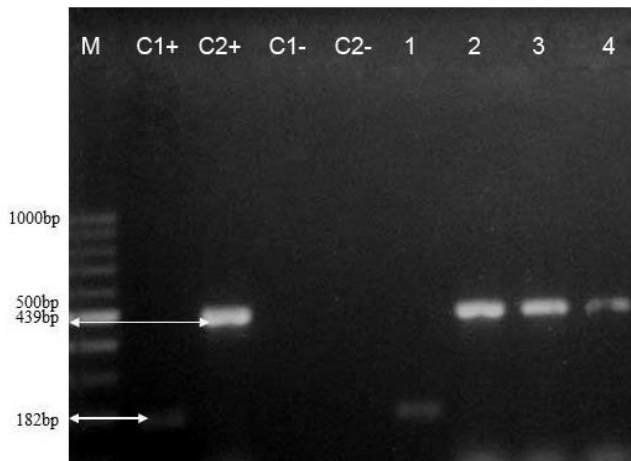


Fig. 2. *Brachyspira* species- specific primer. M: 100 bp DNA marker; C1+: Control positive for *B. intermedia*; C2+: Control positive for *B. canis*; C1-: Control negative for *B. intermedia*; C2-: Control negative for *B. canis*; Lane 1: *B. intermedia* isolate; Lanes 2-4: *B. canis* isolates.

No significant association was observed between the presence of *Brachyspira* spp. with diarrhea and between the presence of *Brachyspira* spp. with age ($p > 0.05$), but a statistically significant association was observed between the presence of *Brachyspira* spp. and ≤ 1 -year-old dogs ($p < 0.05$). Characterization of *Brachyspira* spp. isolated from dogs is shown in Table 2.

Table 2. Characterization of *Brachyspira* spp. isolated from dogs.

<i>Brachyspira</i> spp.	Gender	Age (months)	Presence of diarrhea
<i>B. canis</i>	Female	12	+
	Female	36	+
	Female	5	-
	Female	12	-
	Female	12	-
	Female	12	-
	Female	12	-
	Female	12	-
	Female	24	-
	Female	36	-
	Male	12	-
	Male	5	-
	<i>B. intermedia</i>	Female	12
<i>Non-typable</i>	Male	24	-

Sensitivity assay showed a range threshold for detection of microbial DNA at 10^{-2} to 10^{-7} copy number (*B. canis*).

Discussion

Genus *Brachyspira*, as anaerobic intestinal spirochaetes, is known worldwide to colonize in the dogs intestine, which has been seen in dogs feces and colons for decades; however, inconsistent reports regarding its role in the emergence of the diseases have made it difficult to determine its pathogenetic significances. The *B. pilosicoli* is a zoonotic agent with a human public health risk. There have been previous studies reporting the isolation of *B. pilosicoli* from dogs and human confirming the possibility of inter-species transfer in various geographical areas.^{23,30}

There has been no report regarding the distribution or prevalence of *Brachyspira* spp., although more people tend to keep dogs as pets in their homes in Iran. The current study was performed to determine the prevalence of cultivable anaerobic intestinal spirochetes in dogs population.

Few studies have been conducted on *Brachyspira* spp. in Iran,^{4,28,29} the focus is usually on poultry and related industry. This study was the first investigation of *Brachyspira* spp. in dogs population in Iran. The 9.00% prevalence rate obtained in this study was almost similar to the 11.00% prevalence rate of *Brachyspira* spp. of a recently reported research on dogs in Thailand.³¹ On the other hand, this rate is less than some other studies including 18.70% in Australia,³¹⁻³² 17.60% in Swedish pet dogs¹⁷ and 40.80% in pet shop puppies in Australia.¹¹

In a study performed in Australia, of 40.80% ($n = 20$) *Brachyspira* spp. isolated from pet shop puppies by primary culture, but in PCR method, 14.20% ($n = 5$) were *B. canis* and 4.10% ($n = 2$) were *B. pilosicoli*. Major of isolates were *B. canis* (5/7, 71.42%). All *B. canis* were isolated from healthy dogs.³⁰ In Thailand, 10.59% (16/151) *Brachyspira* spp. were isolated. The isolation rate from puppies (< 1 year) was 13.180% (12/91) and the rate from adults was 6.70% (4/60). There was no significant association between *Brachyspira* spp. and the presence of diarrhea.²⁰

Šperling *et al.* had only reported 10 *Brachyspira* spp. positive out of 1139 dogs being studied in the Czech Republic, 9 isolates were *B. pilosicoli* and one isolate was *B. hyodysenteriae*,²³ which was not compatible with the results obtained in our study.

In Spain, three species of *Brachyspira* were identified in 13.29% (41/311) dogs. The highest prevalence belonged to *B. canis* (8.00%, 25/311). The prevalence rates of *B. pilosicoli* and *B. intermedia* were 4.82% (15/311) and 0.30% (1/311), respectively.²⁵ In that study, a statistically significant association between *B. pilosicoli* and the presence of diarrhea was established in dogs for the first time.

Our study was similar to this study in terms of *B. canis* (12/151, 7.90%) and *B. intermedia* (1/151, 0.60%) while there was no significant association between *Brachyspira* spp. and diarrhea in current study.

In a recent study in Thailand, out of 151 dogs, 17 (11.30%) had fecal shedding of *Brachyspira* spp. including *B. canis* (9/151, 6.00%), *B. pilosicoli* (4/151, 2.60%), *B. pulli* (3/151, 2.00%) and *B. intermedia* (1/151, 0.70%). Spirochaetes were isolated from 6 of the 47 (12.80%) healthy dogs and from 11 of the 104 (10.60%) dogs with diarrhea. The *Brachyspira* spp. were more common among the dogs younger than one year old (12/91, 13.20%) compared to adults (5/60, 8.30%), but this difference was not significant. In the current study, the percentages of isolation of *B. canis* and *B. intermedia* as well as the isolation of *Brachyspira* spp. from dogs \leq 1-year-old were similar; however, we found a significant association between *Brachyspira* spp. and age.

The majority of the isolates (12/14; 85.70%) were identified as *B. canis*, which has already been reported in other studies in different geographical regions such as Australia, Thailand and Spain. In another word, *B. canis* is considered as the most common *Brachyspira* spp. in dogs stool.^{11,25,31} The *B. canis* is a common commensal species of dogs.³¹

In the current study, *B. intermedia* contamination rate was very low (1/151, 0.60%), which was similar to the studies conducted in Spain and Thailand. The prevalence of *B. intermedia* was 0.60% and 0.30% in Thailand and Spain, respectively.^{25,31} Sampled dogs from Iran and Spain were healthy, but Thai dogs had bloody diarrhea. As *B. intermedia* could be generally found in chicken colon, dogs infected by *B. intermedia* had probably consumed contaminated chicken meat or chicken carcasses.^{31,33}

Unlike other studies, we could not find any *B. pilosicoli* isolate. The small number of samples with diarrhea in our study might be the reason for low levels of *B. pilosicoli* detection because a recent study has demonstrated a significant relationship between diarrhea and the presence of *B. pilosicoli*.²⁵

To the best of our knowledge, there are no available species-specific PCR assays for *B. canis* identification.²⁵ Detection of *B. canis* based on biochemical test and specific gene sequencing is time-consuming and it is not cost-effective. In our study, 8 of 12 (67.00%) *B. canis* were identified using species-specific primers.

Continued research in detecting target genes and designing specific primers of *B. canis* appears fully justified so that the researchers and clinicians could differentiate and determine pathogenic species of diarrhea in dogs from non-pathogenic ones. Although the designed primer seems to have proper specificity and sensitivity to detect *B. canis*, the failure to identify four isolates and the importance of full sequencing of *nox* gene and *16S rDNA* have indicated that firstly, designing primer with high detection range for

this species requires more data and secondly, unlike other *Brachyspira* spp. of birds and other animals, the *nox* gene in *B. canis* has more sequence variability. Relatively, the *B. canis*-specific primers introduction and detection require further investigations using of modified oligonucleotides.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

1. Backhans A, Johansson KE, Fellstrom C. Phenotypic and molecular characterization of *Brachyspira* spp. isolated from wild rodents. *Environ Microbiol Rep* 2010; 2(6): 720-727.
2. Hampson DJ, La T, Phillips ND. Emergence of *Brachyspira* species and strains: Reinforcing the need for surveillance. *Porcine Health Manag* 2015;1(8):1-6.
3. Hafstrom T, Jansson DS, Segerman B. Complete genome sequence of *Brachyspira intermedia* reveals unique genomic features in *Brachyspira* species and phage-mediated horizontal gene transfer. *BMC Genomics* 2011; 12(395): 1-13.
4. Razmyar J, Peighambari SM, Barin A. Isolation and characterization of *Brachyspira* species based on biochemical scheme and 16S rDNA partial sequencing. *Iran J Vet Med* 2011; 5(4): 204-210.
5. Hampson DJ, Oxberry SL, La T. Potential for zoonotic transmission of *Brachyspira pilosicoli*. *Emerg Infect Dis* 2006; 12(5): 869-870.
6. Mikosza AS, Hampson DJ. Human intestinal spirochetosis: *Brachyspira aalborgi* and/or *Brachyspira pilosicoli*? *Anim Health Res Rev* 2001; 2(01): 101-110.
7. Rojas P, Petrich A, Schulze J, et al. Distribution and phylogeny of *Brachyspira* spp. in human intestinal spirochetosis revealed by FISH and *16S rRNA*-gene analysis. *Anaerobe* 2017; 47: 25-32.
8. Taylor DJ, Simmons JR, Laird HM. Production of diarrhoea and dysentery in pigs by feeding pure

- cultures of a spirochaete differing from *Treponema hyodysenteriae*. *Vet Rec* 1980; 106(15): 326-332.
9. Trivett-Moore NL, Gilbert GL, Law CL, et al. Isolation of *Serpulina pilosicoli* from rectal biopsy specimens showing evidence of intestinal spirochetosis. *J Clin Microbiol* 1998; 36(1): 261-265.
 10. Trott DJ, Huxtable CR, Hampson DJ. Experimental infection of newly weaned pigs with human and porcine strains of *Serpulina pilosicoli*. *Infect Immun* 1996; 64(11): 4648-4654.
 11. Oxberry SL, Hampson DJ. Colonisation of pet shop puppies with *Brachyspira pilosicoli*. *Vet Microbiol* 2003; 93(2): 167-174.
 12. Johansson KE, Duhamel GE, Bergsjö B, et al. Identification of three clusters of canine intestinal spirochaetes by biochemical and 16S rDNA sequence analysis. *J Med Microbiol* 2004; 53(4): 345-350.
 13. Duhamel GE. Intestinal spirochaetosis in non-production animals. In: Hampson DJ, Stanton TB, (Eds.). *Intestinal spirochaetosis in domestic animals and humans*. Wallingford, UK: CAB International 1997; 301-320.
 14. Turek JJ, Meyer RC. Studies on a canine intestinal spirochete: Scanning electron microscopy of canine colonic mucosa. *Infect Immun* 1978; 20(3): 853-855.
 15. Duhamel GE, Hunsaker BD, Mathiesen MR, et al. Intestinal spirochetosis and giardiasis in a beagle pup with diarrhea. *Vet Pathol* 1996; 33(3): 360-362.
 16. Turek JJ, Meyer RC. Studies on a canine intestinal spirochete. I. Its isolation, cultivation and ultrastructure. *Can J Comp Med* 1977; 41(3): 332-337.
 17. Fellstrom C, Pettersson B, Zimmerman U, et al. Classification of *Brachyspira* spp. isolated from Swedish dogs. *Anim Health Res Rev* 2001; 2(01): 75-82.
 18. Duhamel GE, Trott DJ, Muniappa N, et al. Canine intestinal spirochetes consist of *Serpulina pilosicoli* and a newly identified group provisionally designated *Serpulina canis* sp. nov. *J Clin Microbiol* 1998; 36(8): 2264-2270.
 19. Lee JJ, Hampson DJ, Lymbery AJ, et al. The porcine intestinal spirochaetes: identification of new genetic groups. *Vet Microbiol* 1993; 34(3): 273-285.
 20. Atyeo, RF, Oxberry SL, Hampson DJ. Pulsed-field gel electrophoresis for sub-specific differentiation of *Serpulina pilosicoli* (formerly "Anguillina coli"). *FEMS Microbiol. Lett* 1996; 141(1): 77-81.
 21. Jansson DS, Johansson K-E, Olofsson T, et al. *Brachyspira hyodysenteriae* and other strongly β -hemolytic and indole-positive spirochaetes isolated from mallards (*Anas platyrhynchos*). *J Med Microbiol* 2004; 53(4): 293-300.
 22. La T, Phillips ND, Hampson DJ. Development of a duplex PCR assay for detection of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* in pig feces. *J Clin Microbiol* 2003; 41(7): 3372-3375.
 23. Šperling D, Cada F, Cizek A. Isolation and characterization of *Brachyspira* spp. from dogs in the Czech Republic. *Acta Vet Brno* 2010; 79(3): 437-442.
 24. Westerman LJ, Stel HV, Schipper ME, et al. Development of a real-time PCR for identification of *Brachyspira* species in human colonic biopsies. *Plos One* 2012; 7(12): e52281. doi: 10.1371/journal.pone.0052281.
 25. Hidalgo A, Rubio P, Osorio J, et al. Prevalence of *Brachyspira pilosicoli* and *Brachyspira canis* in dogs and their association with diarrhoea. *Vet Microbiol* 2010; 146(3-4): 356-360.
 26. Jenkinson SR, Wingar CR. Selective medium for the isolation of *Treponema hyodysenteriae*. *Vet Rec* 1981; 109(17): 384-385.
 27. Weissenböck H, Maderner A, Herzog AM, et al. Amplification and sequencing of *Brachyspira* spp. specific portions of nfx using paraffin-embedded tissue samples from clinical colitis in Austrian pigs shows frequent solitary presence of *Brachyspira murdochii*. *Vet Microbiol* 2005; 111(1-2): 67-75.
 28. Bassami MR, Jamshidi A, Kasaei AK, et al. Isolation and Identification of *Brachyspira pilosicoli* from laying hens flocks, using conventional culture and molecular methods in Mashhad, Iran. *Iran J Vet Sci Technol* 2014; 4(1): 29-36.
 29. Zarabi M, Jamshidi A, Khanzadi S, et al. Intestinal colonization of different *Brachyspira* spp. in laying hens. *Iran J Vet Med* 2014; 8(3): 213-218.
 30. Oxberry SL, Hampson DJ. Colonization of pet shop puppies with *Brachyspira pilosicoli*. *Vet. Microbiol* 2003; 93(7): 167-174.
 31. Prapasarakul N, Lugsomya K, Disatian S, et al. Faecal excretion of intestinal spirochaetes by urban dogs, and their pathogenicity in a chick model of intestinal spirochaetosis. *Res Vet Sci* 2011; 91(3): e38-e43.
 32. Lee J, Hampson D. The prevalence of intestinal spirochaetes in dogs. *Aust Vet J* 1996; 74(6): 466-467.
 33. Stephens CP, Hampson DJ. Prevalence and disease association of intestinal spirochaetes in chickens in eastern Australia. *Avian Pathol* 1999; 28(5): 447-454.