

# Isolation of *Brachyspira pilosicoli* from Gastrointestinal Tract of Commercial Chickens in Khuzestan, South West Iran

Leila Alavi<sup>1</sup>, Mahmoud Jamshidian<sup>2</sup>, Masoud Reza Seifi-Abadshapuri<sup>2</sup>, Mansour Mayahi<sup>2</sup>, Seyed Mohammad Alavi<sup>3,\*</sup>

<sup>1</sup>Department of Food and Drug Deputy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran

<sup>2</sup>Veterinary School, Shahid Chamran University, Ahvaz, IR Iran

<sup>3</sup>Infectious and Tropical Disease Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran

\*Corresponding author: Seyed Mohammad Alavi, Infectious and Tropical Disease Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran. Tel: +98 6113387724, E-mail: alavi.seyedmohammad@yahoo.com

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**Background:** *Brachyspira pilosicoli* is an intestinal *spirochete* which colonizes in cecum, colon and rectum of the birds. This infection in broiler and laying chicken may cause disorders such as: diarrhea, reduced egg production, pasty vents, fecal staining of egg shells, poor shell quality, pale egg yolks, increased feed consumption, slower growth and poor feed digestion.

**Objectives:** The aim of this study was to isolate and identify *B. pilosicoli* from broiler and laying chicken.

**Materials and Methods:** This study was conducted by using bacteriological examination and PCR in Khuzestan located in the South West of Iran. Totally 150 samples of broiler chicken feces and 100 samples of laying chicken feces were collected. The samples were cultured on blood agar containing three antibiotics (vancomycin 12.5 µg/mL, spiramycin 25 µg/mL and spectinomycin 400 µg/mL). The colonies of intestinal *spirochetes* were studied by PCR.

**Results:** Intestinal *spirochetes* were isolated in 21 out of 150 fecal samples of broiler chicken and in 9 out of 100 fecal samples of laying chicken. Species identification of isolates was done by PCR using 16S rRNA gene specific for *B. pilosicoli*. *B. pilosicoli* was isolated in 13 out of 21 positive broilers and in 4 out of 9 positive laying chickens.

**Conclusions:** This study showed that *B. pilosicoli* exists in the broiler and laying chicken in the region. This intestinal pathogen may be considered as a major cause of production threats and economic losses in chicken farms in the region of study.

**Keywords:** *Spirochetes*; Gastrointestinal Tract; Isolation and Purification

## 1. Background

Each year millions of dollars damages arising from the loss of poultry due to diarrhea or reduced production of poultry, especially in broiler chicken in the world (1, 2). Due to the diversity of the causes of these conditions, identifying the responsible pathogens in some cases does not happen (3, 4). Unknown pathogens, the incidence of new microbial species, lack of access to new and advanced technology for identifying pathogens that are not easily removed by conventional methods, neglect and lack of awareness of new emerging infectious agents are the main causes of this phenomenon (5). The bacteria that causes diarrhea and loss of production over the last century have been introduced are intestinal *spirochete* (5-10). Establishment of intestinal *spirochete* in most cases is asymptomatic (1, 3, 5). In various forms of mild to severe cases symptoms may fluctuate. Infection with these bacteria may cause an intestinal disorder called sub-acute to chronic intestinal spirochetosis that is associated with

clinical signs such as diarrhea and a drop in egg production in laying hens. In some cases, severe disease is associated with high mortality rates (2, 5, 6, 8, 9, 11).

Despite the fact that the intestinal spirochetosis has been present since the last time, but for various reasons such as the lack of specific clinical signs, technical difficulties such as unavailability of specific culture medium, no growth in conventional culture media for the isolation of microbes, lack of awareness of veterinary and medical practitioners to these microorganisms, they were not considered (4). Over the last few years with the advent of selective media for the microbial isolation, several species of intestinal *spirochete* isolated from humans and birds that have been proven to be pathogenic (5). Except for *Hyodysenteriae* that causes severe dysentery with high mortality in pigs, humans and birds, other species known to be pathogenic for chickens are more or less in the direction of a veterinarian are important (5, 6, 12-15). Another species of the *spirochete* named *Brachyspira pilosicoli* is an enteric pathogens for poultry and humans

### Implication for health policy/practice/research/medical education:

This study is the first of its kind in the field of fecal *B. pilosicoli* isolates from broiler chickens in poultry industry in the country and the presence of these bacteria is a subject that should be considered. The results of this study can be used by researchers in various fields of health and poultry production.

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(6, 8, 16, 17). *B. pilosicoli* is an intestinal *spirochete* which colonized in cecum, colon and rectum of the birds. This infection in broiler and laying chicken may cause disorders such as: reduced egg production, pasty vents, fecal staining of egg shells, poor shell quality, pale egg yolks, increased feed consumption, slower growth and poor feed digestion (3, 5, 9, 17). Since, there were no reports on isolation of *B. pilosicoli* from broiler and laying chickens in Ahvaz, this investigation was done.

## 2. Objectives

The aim of this study was to isolate and identify *B. pilosicoli* from broiler chickens as well as laying chicken in Ahvaz with a large number of poultry.

## 3. Materials and Methods

### 3.1. Sample Collection

At first, the gastrointestinal (GI) tract of industrial broiler chicken was studied. From October 2007 to May 2008, A total of 150 fowl colon samples of poultry slaughterhouses over 7 times collected and transferred to the Bacteriology Laboratory of Veterinary College of Shahid Chamran University in Ahvaz, Iran. Since the presence and persistence of bacteria in the digestive tract of laying hens are longer than the broilers, GI samples of industrial laying hens were also obtained. In the same period, 208 fresh feces samples of industrial laying poultry was collected in three times and transferred to the microbiology laboratory. Of 208 samples only 100 samples were investigated. Another 108 samples due to sampling error or fungus growth were excluded.

### 3.2. Order to Build Primer

Due to the very similar biochemical properties to the intestinal, yet certain biochemical techniques that distinguish of *brachyspira* species from other *spirochete* species is not exist. Today a common technique for species identification in all laboratories and research centers are molecular methods. In this study, the sequences of the primers R and F for the identification of *B. pilosicoli* from suspected isolates was made based upon the Internet and primer 3 output Web guidance, and the complete gene was extracted from the bacterial species. Then the building for primer was ordered to Sinagen Company. The following sequences of the primers were used:

5'-AGA GGA AAG TTT TTT CGC TTC 3' F  
5'-AAC GGC TTC AGG TAA AAC 3' R

### 3.3. Sampling Method

To collect samples from the slaughterhouse; near the end of the slaughter line chain, after opening the abdominal cavity and before discharge its contents, about 20

to 25 cm of the colon and cloaca (fast piece of intestine), cut with scissors and were placed in a plastic bag (freezer bags). At each visit, 15 to 20 samples were taken. Samples were taken randomly without any preconditions. Each bag containing the sample was tied and completely blocked, and all collected samples were placed in a larger bag and transported to the laboratory. Since killing the hens was done on the night, the samples were kept in the refrigerator for several hours until the next morning to be cultured in previously prepared media. For taking fecal samples of laying hens, we referred to local farms on several occasions and fresh feces excreted by birds were randomly and without any preconditions collected by spoon samplers and placed in special disposable plastic containers. Cans containing feces were packed in plastic bags and in the least possible time were transported to the bacteriology laboratory. Fecal samples as slaughterhouse samples as soon as possible were cultured in a medium that had been previously prepared.

### 3.4. Medium

#### 3.4.1. Preparation of Selective Medium Containing Antibiotics

To isolate an intestinal *spirochete*; *Brachyspira* genus, we used selective blood agar medium containing antibiotics. To prepare this medium, after autoclaving blood agar nutrient medium and slightly before full cooling it, we added spectinomycin 400 µg/mL, spiramycin 25 µg/mL, vancomycin 12.5 µg/mL. Then 5 to 7 percent defibrinated sheep blood, as well as the ratio of 5/1 of cholesterol was added to the medium in order to better growth of the *spirochete*. The prepared medium, near the flame and under strict observance of sterile conditions were distributed in disposable sterile plates. In order to control the contamination, prepared plates were kept overnight in the incubator (16).

#### 3.4.2. Preparation of Milk Scheme Broth

One hundred grams of powdered Milk Scheme was dissolved in 1 liter of distilled water and was placed on a magnetic heater for 2-1 minutes to boil. Then 3 ml of liquid in each tube cork and was autoclaved.

### 3.5. Culture and Isolation Methods

In order to facilitate uniform distribution of the bacterial in culture media, small amount of distilled water was added to each sample and was somewhat diluted. By using loops and near the flame, samples were placed in four parts in the previously prepared selective blood agar medium. Because *Brachyspira* colonies are beta-type hemolysis, and deep colonies show better hemolysis, therefore we placed the loops in the depth

of medium. The culture plates in an anaerobic jar incubated at 37 °C and were maintained for 10 days. Ten days after incubation jar lid opens and the culture plates were examined. From the irregular colonies, grown on blood agar and beta-hemolytic colorless suspected to brachyspira, wet preparation was prepared and examined by phase contrast microscopy. With the observation of spiral-shaped bacteria and their motion that indicate the existence of intestinal *spirochete*, the colonies cultured in micro tubes Liquid Milk Scheme and held in the freezer -20 °C. Micro tubes in turn of sampling were numbered, and profile of each sample appeared to be positive was recorded in the office. Micro tubes containing culture samples suspected of Brachyspira were stored in this condition (-20 °C) until the end of the collection period. In cases where no bacteria were grown in agar plates containing the antibiotic and in other cases, that the bacteria had no resemblance to the *spirochete* colonies were grown, the samples were excluded. In cases where other bacteria along with *spirochete* was grown in culture, in order to extract the pure *spirochetes*, suspected colonies in an anaerobic jar were kept for another 8-10 days. These purified samples by passage, like the previous examples in Milk Scheme micro tubes were suspended and stored.

### 3.6. Identification of Isolates

Because biochemical reactions in many different species of brachyspira are similar, biochemical methods for species identification of bacteria is not trusted. Since PCR method for the identification of isolates is mainly used. We also used this method.

#### 3.6.1. Preparation of Isolates for PCR Testing

*Spirochete* suspected strains that previously were isolated based on morphology, colony and microscopic aspects and were kept in micro tubes containing Scheme Milk in the freezer -20 °C, removed from freezer on several occasions, and re-inserted in the agar medium containing antibiotic, and were incubated in anaerobic conditions and proper temperatures for 10 days in order to extract pure enough isolates.

#### 3.6.2. Preparation of Bacterial Suspension for PCR

After examining the culture plates, irregular hemolytic colonies were placed in test tubes containing 1-2 mL of sterile distilled water, mixed and relatively concentrated suspension of bacteria was prepared. The numbers on each sample tube was inserted. Then by using a sampler 30-50 mL of bacterial suspension was removed and placed in small micro tubes specific for PCR. Sample number was written on the door of micro tubes.

#### 3.6.3. DNA Extraction

Micro tubes containing bacterial suspension were

placed in a thermo cycler with temperature of 99 °C for 3 min. Bacteria statue was destroyed and its contents were discharged into the environment. Then the micro tubes were centrifuged for 5 min with 13 thousand rounds to sediment bacteria and DNA components to be suspended in solution. Then supernatant containing DNA was collected in another micro tube and precipitated was out.

#### 3.6.4. Amplification of Isolates SrRNA16 Gene by PCR

In this study, PCR component with the exact values of each component was added in the Master Mix micro-tubes. These values were for testing on isolates. For doing 30 PCR, that amount was 30 times. Values of the master mix was prepared as follows:

Buffer 10X, 5 µl  
dNTP mix (10 mM) 2 µL  
MgCl<sub>2</sub> (50 mM) 2 µL  
Primer F (50 pmol/µL) 30X, 1 µL  
Primer R (50 pmol/µL) 1 µL  
Polymerase Taq enzyme (5 U /µL) 0.5 µL  
MQ (distilled water) 35.5 µL  
Total 47 µL

Equal to the number of suspected *B. pilosicoli* isolates already their DNA been extracted, microtubes was placed. In each microtube 47 microliters of master mix and 3 ml of sample were poured. All work related to the preparation of the master mix and transferred to microtubes were done under the hood. For prevention of contamination, 20 minutes before starting the work, hood table was completely disinfected with 70% alcohol and UV lights were turned on. UV lights were turned off at the start of work.

#### 3.6.5. Thermo Cycler Schedule to Conduct PCR

Mix Master Content was placed in thermo cycler to the following schedule (Table 1):

**Table 1.** Thermo Cycler Schedule

°C	Min
95	03
95	01
48	1 × 35
72	5.1
72	10

After a total of 35 cycles for each PCR test lasted about 2.30 hours, the micro-tubes were removed from the machine.

#### 3.6.6. Electrophoresis of PCR Product

##### 3.6.6.1. Agarose Gel Preparation

First 0.3 g of agarose powder in 30 mL of TAE buffer was heated in a microwave to boil for one minute to evenly agarose dissolved and a clear solution was obtained.

Then 30  $\mu$ L ethidium bromides were added to the agarose. Then the agarose was poured in tray electrophoresis apparatus with wrapping tape around it. For the hole into the gel a shoulder was placed in it.

### 3.6.6.2. Electrophoresis Methods

After solidification of the agarose gel and removing the comb and the tape from the tray, the tray was placed in the electrophoresis apparatus. So a tray was placed in the electrophoresis buffer to cover the holes in the agarose gel. The first hole was added 2 mL marker. The second hole was add 2 mL loading buffer and 10 mL of the positive control, and to the subsequent cavities 2 mL loading buffer and 10 mL of a PCR product from each sample was added. Then power supply device turned on to move samples on agarose gels. Voltage was set to 100 volts, which would take about 30 minutes to move from the negative pole to the positive pole. After this period, electrophoresis apparatus off and compliance with all safety aspects agarose gel was removed from the trays and were placed

on the trans eliminator lid containing the UV rays. And after closing the protective device door, lamp of device switched on and in the dark room, generated bands were analyzed. If the bands were formed, a formed band was compared with positive control sample, the bands along with control band, were considered as positive samples.

## 4. Results

21 samples were suspected of intestinal *spirochete* (Table 2) out of total 150 intestinal samples from broiler chickens cultured in blood agar containing antibiotics. Of total 100 fecal samples from laying chickens cultured in blood agar containing antibiotics, 9 samples were suspected of intestinal *spirochete* (Table 3). Using PCR and primers specific SrRNA16 for identification *B. pilosicoli*, of 21 broiler chicken intestine specimens that were culture positive, 13 samples were containing the *B. pilosicoli* (Figure 1 & Table 2). 4 samples were containing the *B. pilosicoli* out of nine fecal specimens from laying chicken that were culture positive, (Figure 1 and Table 3).

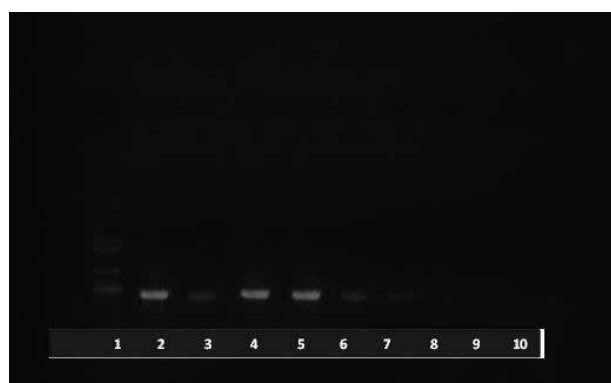
**Table 2.** The Results of Bacteriological Tests in Culture Media of Poultry Samples in Khuzestan

Bacteriologic Evaluation			
Sample sources	Number	Positive, No. (%)	Negative, No. (%)
Broiler chicken	150	21 (14)	129 (86)
Laying chicken	100	09 (9)	91 (91)

**Table 3.** The Results of PCR Tests of Culture Products of Poultry Samples in Khuzestan

PCR Evaluation			
Sample Sources	Number	Positive, No. (%)	Negative, No. (%)
Broiler chicken	21	13 (61.9)	08 (39.1)
Laying chicken	09	04 (44.4)	05 (55.6)

**Figure 1.** The Results of PCR Test 1



DNA ladder, 2: positive control, 3, 4, 5, 6, 7: positive samples, 8, 9: negative samples, 10: negative control

## 5. Discussion

*Brachyspira* species are anaerobic *spirochete* that colonized the gastrointestinal tract of humans and animals and clinical presentation is mostly diarrhea (2, 5, 6, 8, 9, 11). One species of this genus which is pathogenic in poultry, swine and human is the *B. pilosicoli* (6, 8, 16, 17). This study showed that the genus of *Brachyspira* is identifiable from the colon and feces of laying and broilers hens. Fourteen percent of broiler and 9% of laying hens showed *spirochete* colonies. Several studies on isolation and pathogenicity of this microorganism in various countries including Australia, America and European countries, especially England, has been published. To now, there is no published report except the study by Jamshidi et al. (18) about the isolation of intestinal *spirochete* in Iran. The results of our study are similar with the unpublished study of Biranovand (Doctoral dissertation, Veterinary school of University of Shahid Chamran, Ahvaz). Biranovand showed that

11.6% of domestic poultry around the city of Ahvaz regardless of clinical symptoms has been infected with bowel *spirochetes*. Stephens et al. (5) in their study on the infectious *spirochete* in poultry showed that over 40% of the laying chickens were infected with the bowel *spirochetes* that about half of them were *B. Pilosicoli* or *B. intermediate*. But the pollution was not seen in broiler flocks. It is not clear that why the *spirochete* isolated from egg laying hens stood is less than of the results reported by Stephens, but it may be due to differences in sampling and sanitary conditions of poultry are kept. Bowel *spirochetes* from the fecal samples of broiler chickens in Stephens study have not been isolated whereas in the present study *spirochetes* were mostly isolated from broiler chickens. The reason for this discrepancy is not clear and other studies have also made no mention. Therefore, further studies are needed to clarify the issues and to be more precise.

*Brachyspira* is a fragile and anaerobic organism; its isolation from the sample depends on their number in culture media. This suggests that why only 21% of the samples showed *spirochete* colonies. Another reason could be that the medium may be paired with other *spirochete* which growth conditions in this study were not available. In previous studies *Brachyspira* have been frequently isolated the birds with symptoms of diarrhea or reduced production (19). In this study, samples were prepared without regard to the clinical signs and symptoms; therefore, this may be the reason for a small number of infected hens in our study.

The results of this study showed that *B. pilosicoli* is identifiable from more than half of the colonies from blood agar medium contains three antibiotics using PCR and SrRNA16 primers. These results are similar to other studies that *B. pilosicoli* have been successfully isolated by using PCR techniques (3, 5, 7, 14). Phillips and Hampson (10) in his study for the identification of intestinal *spirochete* from intestinal isolates used PCR and sequencing SrRNA 16. Using this method, *B. pilosicoli* was detected in 3.1% of *spirochetes* culture positive samples (3). Feberwee et al. (9) in a study on laying hens flocks with diarrhea or reduced production isolated *Brachyspira* from fecal samples. In this study using PCR and SrRNA16, *B. pilosicoli* was the most common identified species (9).

The present study revealed that 8.6% of broiler chickens and 4% of laying chickens are infected with *B. pilosicoli*. In previous studies *B. pilosicoli* have been isolated from colon or feces of animal species, including pigs, dogs, egg laying hens, broiler breeder and seabirds (6, 8, 16, 17). Jamshidi et al. (16) has isolated *B. Pilosicoli* from laying hens flocks in Mashhad by using culture and molecular methods (18).

Most studies in dealing with *B. pilosicoli* isolation have been done on laying hens and broiler breeder and there are no further studies about isolating *B. pilosicoli* from the broiler chicken (6, 8, 16, 17). The present study showed the presence of the *B. pilosicoli* in the feces of broiler chicken

industry in the region of study. This study is the first of its kind in the field of fecal *B. pilosicoli* isolates from broiler chickens in poultry industry in the country and the presence of these bacteria is a subject that should be considered. The results of this study can be used by researchers in various fields of health and poultry production.

The results of this study indicate *B. pilosicoli* contamination in the poultry industry (both in broiler chicken and laying chicken) in Khuzestan may be an important health and economic problem. Pathogenicity of bowel *spirochetes* in human and animals alarms the health and economic policymakers and veterinary officials to think more about the hazards of these organisms and search practical ways to fight against *B. pilosicoli* and other bowel *spirochetes*.

The study was limited by restriction to one central laboratory in the university. Most of industry poultry farms were located in cities far away the laboratory that might impact on sampling and transporting them to laboratory where examination were done. To control these effects we did our work by very exact performance and transported the samples as soon as possible to the lab.

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## Author Contributions

All authors have participated equally.

## Financial disclosure

There is no conflict of interest.

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