Isolation and Identification of Brachyspira pilosicoli from laying hens flocks, using conventional culture and molecular methods in Mashhad-Iran

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Abstract

The purpose of this study was to investigate the prevalence of the anaerobic intestinal Brachyspira spp among laying hens flocks located in Mashhad suburb of Iran. In the first stage, as a pilot study, number of 50 rectal swabs sample from five laying hen flocks (10 samples from each flock), were collected. Samples were cultured anaerobically on selective agar and confirmed primarily as intestinal spirochaete by it's spirochaetal form under phase contrast microscopy. Final confirmation as Brachyspira spp performed by PCR amplification with specific primers, nested-PCR and finally sequencing the amplified PCR product. The first isolate were used as positive control during the study. With 4% prevalence of infection in pilot study, the sample size was determined. In the second stage, number of 130 rectal swab samples from 13 flocks was collected, and Brachyspira spp were isolated by conventional culture and confirmed by PCR and nested-PCR. The prevalence of Brachyspira spp infection in laying hens determined as 5%. In this study 16.7% of flocks were determined as infected with Brachyspira spp. This is the first report of the isolation of Brachyspira spp from laying hens in Iran. Further studies are needed to clarify the sensitivity and specificity of this test, and also determining the species and presence of infection in human populations.

Key words: Brachyspira, laying hens, culture, PCR.

Introduction

The intestinal spirochaetes are included in the genus Brachyspira (1). To date, seven species of Brachyspira spp have been described: B. hyodysenteriae, B. intermedia, B. murdochii, B. innocens, B. pilosicoli,B. alvinipulli and B. aalborgi. The significance and consequences of Brachyspira infections in pigs (8) have been recognized worldwide for decades, Brachyspira spp. have been isolated from pigs, birds, dogs, humans, non-human primates, guinea pigs, opossums and wild rodents(5). In chicken and pullets, e.g, B. intermedia, B. pilosicoli and B. alvinipulli have been associated with retarded growth, diarrhea, delayed onset of egg production and intestinal lesions. During recent years, research on the intestinal spirochaetes has been focused on classification, diagnostics, epidemiology and pathogenesis. Substantial achievements have been made due to new technologies based on molecular biology, eg DNA–DNA reassociation, polymerase chain reaction (PCR), DNA sequencing, pulsed-field gel electrophoresis (PFGE) and recombinant DNA-techniques. A number of new spirochaetal species has been recognized which may be identified on diagnostic laboratories by PCR-systems combined with traditional culture technique and biochemical tests(6,7). Improved knowledge on the genetic organization, the ultrastructure and virulence factors has been gained through the use of the mentioned techniques.
The purpose of this study was to investigate the prevalence of the anaerobic intestinal Brachyspira spp amongst laying hens flocks located in Mashhad suburb of Iran using PCR method.

Materials and Methods

1. Sampling:

Number of 10 laying hens flock were selected randomly in Mashhan suburb (with 10-20 months average age and apparently healthy). From each flock number of 10 rectal swabs were obtained.

2. Culture of faecal samples:

The rectal swabs plated onto Trypticase Soy agar supplemented with 5% defibrinated bovine blood, 400 µg mL-1 spectinomycin, 25 µg mL-1 of vancomycin and 25 µg mL-1 of colistin. Plates incubated at 37°C in anaerobic jars in an atmosphere of 94% H2 and 6% CO2 for 15 days. Spirochaete growth indicated by low flat confluent growth surrounded by areas of weak β-haemolysis. The presence of spirochaetes confirmed by examining bacterial growth resuspension in phosphate buffered saline under a phase contrast microscope.

3. DNA extraction:

Chromosomal DNA was extracted and purified from the cultured samples on Trypticase Soy agar plates using phenol-chloroform procedure. The final pellet of DNA were resuspended in 100-150 µL TE buffer and stored in -20 °c. The extracted DNA was used as template for PCR amplification.

4. First Polymerase chain reaction:

Suspected colonies on selective media employed as templates for PCR assay. A 648 base pair sequence of the 16S rRNA gene of Brachyspira spp, was targeted for PCR amplification. The designated F-brachy-16S and brachy-R primers (F-brachy-16S: 5-GCAGTCATCTGGGCATTT-3, and R-brachy-16S: 5-CGTATTCACCGTAGCGTTCT-3) were used in this study. For PCR reaction, the amplification mixtures consisted of a 25 µL reaction mix of 2.5µL PCR buffer(10X), 0.5µL of dNTPs mix(10mM), 1µL of MgCl2(50mM), 0.2µL DNA Polymerase(5U/µL), 12.5 pmol of each primer, 2µL DNA sample, and 16.3µL of DW. Cycling Amplification conditions involved a 5 min denaturing step at 95°C, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and a primer extension at 72°C for 90 s. After the last cycle the product incubated at 72°C for 10 min. The PCR products were subjected to electrophoresis in 1% (w/v) agarose gels in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The current for electrophoresis set at 0.60 Volts for 50 min. The bands stained by emersion in 0.5 µg ml-1 ethidium bromide for 30 min, and the gels viewed over ultraviolet light.

5. Nested-PCR:

Positive samples in the first PCR employed as templates for nested-PCR assay. Primers for nested-PCR amplification, was F-brachy-nest (5-GCAGTCATCTGGGCATTT-3) and R-brachy-nest (5 TCCTCAGGCGGTACACTT-3). The amplification mixtures of PCR reaction and cycling conditions were the same as described previously. The PCR products were subjected to electrophoresis and stained with ethidium bromide and viewed under UV light.

Results and Discussion

From 180 rectal swab samples that were collected randomly from different laying hens flocks in Mashhad in two different steps, after enrichment and selective plating number of 24 suspected colonies were isolated, which were confirmed as Brachyspira spp by phase contrast microscope. In PCR assay, using specific primers for 16S rRNA gene, number of 9 samples confirmed to be contaminated with Brachyspira spp (5%) (Figure1). Positive rectal swab samples were belonged to three of 18 flocks, so 16.7% of flocks were determined as contaminated with this group of bacteria.

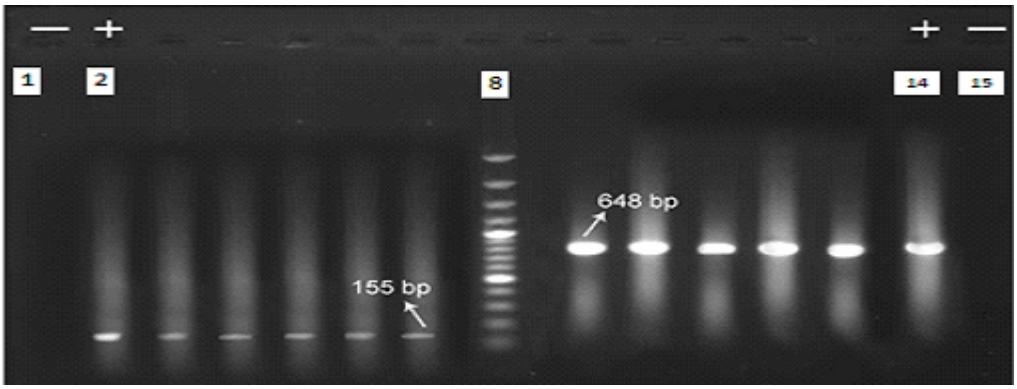

Figure 1: Detection of B.pilosiloli in faecal samples by PCR assay, amplifying 649 bp segment of 16SrRNA gene, and nested-PCR that amplifying 155 bp. Lane: (1 & 15) negative control, Lane(2 & 14) positive control, Lane(8) 100 plus bp markers. Lanes 3, 4,5, 6, and 7 positive samples in nested-PCR and lanes 9, 10, 11, 12, and 13 in first PCR assay.

In this study, the method of isolation, was base on surface culture of rectal swabs on CVS– TSA plates which is selective media for isolation of Brachyspira spp (3), and we didn't use direct PCR on feacal samples, because it has been reported that direct PCR (6,7) is not a successful method due to low PH, presence of uric acid and other inhibitors of PCR assays, in bird fecal samples. For positive control of our test, first we performed a pilot study on birds flocks to determine the prelevance of infection. After the primarily identification of bacteria and final confirmation as Brachyspira spp, by sequencing the PCR product, we use it as positive control. The prevalence of Brachyspira spp infection in laying hens by conventional culture, PCR and nested-PCR assays was determined as 5%. In this study, 16.7% of laying hens flocks determined as contaminated with Brachyspira spp. The sensitivity of detection of Brachyspira spp by faecal culture is not particularly high [>5 x 10^4 cells (g faeces)–1], and it is possible that the other birds may have been colonized with lower population of the bacteria. Comparing with other reports (4.4% in Netherlands), this high level of colonization in laying hens may be due to higher antibiotic prescription in Iran. It is assumed that antimicrobial agents disrupt the normal caecal microflora, reducing colonization resistance and hence enhancing spirochaetal colonization, besides considering the potential of transferring the bacteria between human population and birds flocks, it has been reported that the infection with Brachyspira spp, being common (10-30%) in developing countries, but rare in the general population in developed countries(4,3)

In this study we only proved the presence of contamination in laying hens with Brachyspira spp that have not been reported previously.

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جداسازی و بررسی میزان آلودگی گله های طیور تخمگذار در شهرستان مشهد به باکتری برکیسپایرا پیلوسی کولای با استفاده از روش توام کشت و PCR

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چکیده

هدف از انجام این مطالعه بررسی شیوع آلودگی گله های مرغ تخمگذار به باکتری جنس برکیسپایرا در اطراف شهرستان مشهد بوده است. در اولین مرحله تعداد ۵۰ سواب رکتال از پنج گله(۱۰ نمونه از هر گله) اخذ گردید. شناسائی اولیه بوسیله کشت در محیط انتخابی در شرایط بیهوازی و مشاهده باکتری با میکرسکوپ فاز-کنتراست و تایید نهائی با تست PCR، nested PCR و نهایتا با تعیین توالی صورت گرفت. اولین مورد تایید شده به عنوان کنترل مثبت در نظر گرفته شد و با تعیین میزان شیوع اولیه که ۴٪ بود، حجم نمونه برآورد گردید. در مرحله دوم تعداد ۱۳۰ سواب رکتال از ۱۳ گله مرغ تخمگذار جمع آوری گردید. آلودگی نمونه ها به باکترهای جنس برکیسپایرا، پس از کشت ، تست PCR و nested-PCR مورد تایید قرار گرفت. در این بررسی میزان آلودگی مرغان تخمگذار۵٪ و میزان آلودگی گله ها ۱۶,۷٪ تعیین گردید.

مطالعات بیشتر جهت تعیین حساسیت و ویژگی تست و نیز تعیین گونه های جداسازی شده و همچنین تعیین چگونگی حضور عفونت در انسان توصیه می گردد.

واژه‌های کلیدی: جنس برکیسپایرا، مرغان تخمگذار، روش کشت و PCR