Short Communication

Ostrich as a possible source of pathogenic strains of *Clostridium difficile*

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Abstract

Clostridium difficile (*C. difficile*) is known as the most important causative agent of antibiotic-associated pseudomembranous colitis in human. According to recent studies, food animals, especially poultry, are known as carriers for toxin harboring *C. difficile*. Since ostrich farming is a growing industry in Iran, the characterization and genotyping of *C. difficile* isolates in ostriches are very important for public health. A total of 100 fecal samples were collected from 10 ostrich farms located in the north-east of Khorasan Razavi province, Iran. The samples were cultured anaerobically. Multiplex PCR was applied in order to detect *tcdB*, *tcdA*, *cdtA* and *cdtB* genes and *16S rDNA* sequence, while single PCR was used to detect *tcdC* gene. Eleven fecal samples (11%) were suspected to contain *C. difficile* by growth pattern, colony morphology and Gram stain. Ten of the 11 suspected samples were later confirmed by 16s rDNA sequence PCR. In terms of toxin profile, five isolates (50%) were *tcdA* and *tcdB* positive, two (20%) were *tcdA* negative and *tcdB* positive, and the remaining three isolates were *tcdA* and *tcdB* negative. Also, 70% of the total isolates were *tcdC* positive and it was found that only strains harboring *tcdA* or *tcdB* were *tcdC* positive. In addition, all isolates lacked CDT producing gene (*cdtA* and *cdtB*). The findings of this study showed that ostrich might be a potential reservoir of *C. difficile* infection for humans.

Keywords: *Clostridium difficile*, ostrich, antibiotic-associated pseudomembranous colitis

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Introduction

Clostridium difficile was first isolated from stool samples of healthy breast-fed infants by Holland and O'Toole in 1935 (Kachrimanidou and Malisiovas, 2011; Arroyo et al., 2005). However, *C. difficile* had not been known as a significant pathogen until 1977 when it was found as the main source of antibiotic-associated pseudomembranous colitis (Bartlett, 1994). Among factors affecting the virulence of *C. difficile*, toxin A (*tcdA* gene) and toxin B (*tcdB* gene) along with the proteins expressed by *tcdR*, *tcdE* and *tcdC* genes are considered the most important ones. All of these genes are located on the Pathogenecity Locus (PaLoc) in genome of the bacterium (Stabler et al., 2006; Dupuy and Sonenshein, 1998). Another toxin found in *C. difficile* is binary toxin or cytolethal distending toxin (CDT) which is encoded on bacterial chromosome but separately from PaLoc. It includes three genes of *cdtB*, *cdtA* and *cdtR* (Kachrimanidou and Malisiovas, 2011). According to several researches in this area, toxin A and toxin B are the main causes of *C. difficile*-associated diseases and their related symptoms (Cohen et al., 2000). It has been hypothesized that mutations in *tcdC* may result in a loss of negative regulatory function, leading to increased toxin production and virulence (McDonald et al., 2005). Moreover, pets and food animals could be potential sources of the infection in humans (Arroyo et al., 2005). *C. difficile* isolates from pets and food animals were closely similar to those isolated from human infections in terms of genetic characteristics (Dingle et al., 2011). In a recent study on ostrich meat, researchers reported 9.16% isolation rate of *C. difficile* (Hasanzade and Rahimi, 2013). In other studies, *C. difficile* was also considered as a pathogen causing hepatitis, enteritis and even death in several ostriches (Frazier et al., 1993; Shivaprasad, 2003).

This study was conducted to investigate the probability that ostriches might be the potential reservoirs of pathogenic strains of *C. difficile* for humans involved in ostrich farming and related professions.

Materials and Methods

Sampling: Ten ostrich farms with the average population of 100 productive ostriches were randomly selected and a total of 100 fecal samples were collected. In each flock, three groups of ostriches were sampled including day old to 3 months old, 3-12 months old, and productive adults above 3 years old.

Bacterial culture: The fecal samples were obtained by sterile cotton swab and transferred into 5 ml normal saline. One ml of feces was mixed with an equal volume of absolute ethanol, vortexed and left at room temperature for 1 hr. The supernatant was discarded and an aliquot of the resulting pellet was inoculated onto Colombia agar medium containing 5-7% sheep blood agar and incubated for 48-72 hrs under anaerobic conditions at 37°C (Alvarez-Perez et al., 2009). The samples were evaluated in terms of morphological characteristics (shape, color and consistency of the colony; bacterial growth pattern, unique odor, Gram staining and bacterial or their spore observation). *C. difficile*-suspected isolates were kept at -80°C in 50% glycerol until further use.

Polymerase Chain Reaction (PCR): DNA was extracted from a single colony using an extraction kit (Bioneer, South Korea) according to the manufacturer's instructions. A multiplex PCR was carried out for the detection of *tcdA*, *tcdB*, *cdtA*, *cdtB* and 16S rDNA as described by Persson et al. (2008). Amplification reactions were prepared in a 50-µl reaction volume containing 5 µl 10x PCR buffer, 5 mM dNTPs, 25 mM $MgCl_2$, 5 U of *Taq* DNA polymerase and the required concentration of each primer (Table 1). Amplification was programmed in a thermocycler (Techne TC 3000, England) as follows: 94°C for 10 min followed by 35 cycles of 94°C for 50 sec, 54°C for 40 sec, 72°C for 50 sec and a final extension at 72°C for 3 min (Persson et al., 2008). The materials used in PCR reaction were provided by Ampliqon (Odense, Denmark).

Detection for *tcdC* gene was performed in single PCR as described by Antikainen et al. (2009). Briefly, amplification reactions were prepared in a 25-µl reaction volume containing 12.5 µl Ampliqon Red MasterMix, 2.0 mM $MgCl_2$, 5 µl template DNA, 1 µl (10 pm/µl) from each of forward and reverse primers and 5.5 µl deionized water. PCR was initiated with a denaturation step at 94°C for 5 min followed by 36 cycles of 98°C for 10 sec, 60°C for 20 sec, 72°C for 20 sec and a final extension at 72°C for 10 min.

The amplified products were detected on ethidium bromide-stained (Cinnagen, Tehran, Iran) 1.5% agarose gel after electrophoresis and ultraviolet illumination. DNA molecular weight markers 100 bp (Dena Zist Asia, Iran) were used as molecular weight markers.

Table 1 Results of *Clostridium difficile* PCR in ostrich fecal samples in terms of age group

Group	No. of samples	*No. of *16s rDNA*+ isolates	No. of non-toxigenic isolates	No. of *tcdA*+ isolates	No. of *tcdB*+ isolates	No. of *tcdA+ and tcdB*+ isolates	**No. of *tcdC*+ isolates
1 day to 3 months old	30	4 (13.3%)	1 (3.3%)	0	1 (3.3%)	2 (6.6%)	3 (10%)
3 to 12 months old	40	5 (12.5%)	2 (5%)	0	1 (2.5%)	2 (5%)	3 (7.5%)
productive adults above 3 years old	30	1 (3.3%)	0	0	0	1 (3.3%)	1 (3.3%)
Total	100	10 (10%)	3 (3%)	0	2 (2%)	5 (5%)	7 (7%)

*All *16s rDNA* positive isolates lacked *cdtA* or/and *cdtB* (binary toxin).
**All strains harboring *tcdA* or *tcdB* were *tcdC* positive.

Results and Discussion

Of the 100 fecal samples cultured, 11 samples (11%) were positive based on morphological features, but in species-specific PCR for *16S rDNA* gene of *C. difficile* only 10 samples were confirmed. The prevalence of *C. difficile* fecal infection in birds has been estimated approximately 20-60% (Harvey et al., 2011; Zidaric et al., 2008; Simango, 2006; Simango and Mwakurudza, 2008). In one study, samples obtained from chicks under 15 days and 18 weeks old had the prevalence of 100 and 40.9%, respectively, of *C. difficile* (Zidaric et al., 2008). These findings are comparable to those found in other species such as horses (Båverud, 2002), cows (Rodriguez-Palacios et al., 2007; Hammitt et al., 2008) and pigs (Alvarez-Perez et al., 2009) in which the isolation of *C. difficile* was much higher in young animals.

The number and percentages of PCR-positive samples for the three age groups of 1-90 days, 3-12 months and older than 3 years old were 4 out of 30 (13.3%), 5 out of 40 (12.5%) and 1 out of 30 (3.3%), respectively. As a result, 90% (9/10) of the isolates were obtained from ostriches below 12 months old and only one isolate was obtained from adult ostriches above 3 years old. These results are in agreement with results achieved in previous studies. *C. difficile* was isolated from the intestinal tracts of 30-70% of infants, in contrast to 5% in healthy adults (Kachrimanidou and Malisiovas, 2011).

In the present study, 70% of the isolates were toxigenic and found to harbor toxin A and toxin B producing genes. In terms of toxin profile, multiplex PCR revealed that out of the 10 positive isolates, five (12.5%) were *tcdA* and *tcdB* positive, two (20%) were *tcdA* negative and *tcdB* positive and three isolates lacked the detected genes, also no *cdtA* and/or *cdtB* (binary toxin) were detected in any of the 10 isolates.

The multiplex PCR results are summarized in Table 1. Based on previous studies, *C .difficile* isolates carry a wide range of toxigenic genes (20-100%) in different animal species (Stubbs et al., 1999; Simango and Mwakurudza, 2008; Weese et al., 2010; Warny et al., 2005). In our study, there was no significant correlation between the frequency of toxigenic isolates and age-related groups, which might be due to the small amount of sample size.

In this research, 70% of the total isolates were *tcdC* positive and it was found that only strains harboring *tcdA* or *tcdB* were *tcdC* positive. Single PCR results are summarized in Table 1. Hypervirulent strains have been described as high expressors of toxins A and B. This is associated with the point mutation in *tcdC* (Stabler et al., 2006). It has been revealed that various strains of *C. difficile* possess different forms of PaLoc genes which include *tcdC* sequences leading to PaLoc gene polymorphism. To date, 24 toxin profiles of this bacterium have been studied by PCR methods. In this study, all the isolates containing toxin A or B had intact *tcdC* gene. McCannell et al. (2006) found that the removal of one nucleotide in *tcdC* gene might lead to the production of TcdC defective protein. In other words, TcdC's defect could cause high level of toxin production (Warny et al., 2005). However, several studies suggest a wide variety of toxin producing level in hypervirulent strains and in strains with mutant *tcdC* sequence (Curry et al., 2007; Merrigan et al., 2010; Vohra and Poxton, 2011). In a recent study that aimed to give a comprehensive account of this gene's role in toxin producing arrangement of the bacterium, no significant difference was detected between wild strains' toxin producing level and that of the mutant strains (Bakker et al., 2012).

Toxigenic strains of *C. difficile* were isolated from cases with antibiotic-associated diarrhea (10-25%) and cases with pseudo-membranous colitis (95%) (Persson et al., 2008; Bidet et al., 1999). The prevalence of *C. difficile*-associated infections had an increasing trend from 1987 to 2001 in North America. Other studies reported an increase in *C. difficile*-associated deaths from 1999 to 2004 (Oughton and Miller, 2008). Pets and food animals are considered among significant sources of animal-to-human transmission of *C. difficile* (Arroyo et al., 2005). The *C. difficile* isolates from pets and food animals have shown very strong genetic similarity to those isolated from human infections (Dingle et al., 2011). The findings of this study and previous investigations on the occurrence of *C. difficile*-associated enteritis and deaths in ostriches (Shivaprasad, 2003; Frazier et al., 1993) and the high mortality rate of ostrich chicks in the first 3 months after hatching (Shivaprasad, 2003) emphasized that the role of *C. difficile* on the output and future prospects of ostrich farming in Iran should be taken into account.

In previous studies, *C. difficile* was isolated from 12-13% of chicken samples (Harvey et al., 2011; Weese et al., 2010). In addition, 9.16% of the samples obtained from ostrich meat in Iran were *C. difficile* positive (Hasanzade and Rahimi, 2013). It can be speculated, from the presence of toxigenic strains of *C. difficile* in ostriches' fecal samples found in this study, that there is the possibility of fecal infection in packet ostrich products and thus its transmission to humans. The possibility of fecal contamination of ostrich meat with *C. difficile* in processing stages and its possible transmission to humans is a matter of public health concern.

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บทคัดย่อ

นกกระจอกเทศ อาจเป็นแหล่งสะสมของเชื้อ *Clostridium difficile* ก่อโรค

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Clostridium difficile (*C. difficile*) เป็นที่รู้จักว่าเป็นสาเหตุสำคัญในการก่อโรค antibiotic-associated pseudomembranous colitis ในคน จากการศึกษาพบว่าสัตว์ปีก เป็นสัตว์พาหะของเชื้อ *C. difficile* ที่ผลิตท็อกซิน ในประเทศอิหร่านปัจจุบันอุตสาหกรรมการเลี้ยงนกกระจอกเทศได้ขยายตัวมากขึ้น ดังนั้นการศึกษาลักษณะทางพันธุกรรมและจีโนไทป์ของเชื้อ *C. difficile* จากนกกระจอกเทศจึงมีความสำคัญต่อสุขภาพของประชาชน การศึกษาครั้งนี้ได้เก็บตัวอย่างอุจจาระ 100 ตัวอย่าง จากฟาร์มเลี้ยงนกกระจอกเทศจำนวน 10 ฟาร์ม ที่ตั้งอยู่ในภาคตะวันออกเฉียงเหนือของจังหวัด Khorasan Razavi ประเทศอิหร่าน และเพาะเลี้ยงเชื้อแบบไม่ใช้ออกซิเจน จากนั้นใช้วิธี multiplex-PCR เพื่อตรวจหายีน *tcdB, tcdA, cdtA, cdtB* และ *16S rDNA* และวิธี PCR เพื่อตรวจหายีน *tcdC* ผลการศึกษาพบตัวอย่างอุจจาระ 11 ตัวอย่าง (11%) มีเชื้อ *C. difficile* ด้วยวิธีตรวจตามรูปแบบการเจริญเติบโต สัณฐานวิทยาของโคโลนีและ การย้อมสีแกรม และพบว่า 10 ตัวอย่างสามารถยืนยันเชื้อ *C. difficile* ด้วยวิธี 16s rDNA-PCR ในแง่การศึกษาเกี่ยวกับท็อกซินยีนพบว่ามี 5 ตัวอย่าง (50%) ให้ผลบวกต่อ *tcdA* และ *tcdB* และ 2 ตัวอย่าง (20%) ให้ผลลบต่อ *tcdA* และ *tcdB* และอีก 3 ตัวอย่างให้ผลลบต่อ *tcdA* และ *tcdB* โดยรวมพบว่า 70% ของตัวอย่างทั้งหมดให้ผลบวกต่อ *tcdC* และพบว่ามีเพียงหนึ่งตัวอย่างที่พบยีน *tcdA* หรือ *tcdB* ร่วมกับยีน *tcdC* และพบว่าทุกตัวอย่างไม่พบยีนที่สร้าง CDT (*cdtA* และ *cdtB*) ผลการศึกษาครั้งนี้สรุปได้ว่า นกกระจอกเทศอาจเป็นแหล่งสะสมของเชื้อ *C. difficile* ที่อาจก่อให้เกิดโรคในคน

คำสำคัญ: *Clostridium difficile* นกกระจอกเทศ antibiotic-associated pseudomembranous colitis

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