Detection of cytolethal distending toxin (cdt) genes of Campylobacter Jejuni and Coli in fecal samples of pet birds in Iran

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Abstract:

BACKGROUND: Campylobacteriosis is one of the most common bacterial intestinal disorders of humans in many countries. A wide range of domestic and wild bird species have been identified as natural reservoirs. OBJECTIVES: The aim of this study was to determine the Campylobacter spp from pet birds and screen the determined Campylobacters for presence of virulence cytolethal distending toxin (cdt) genes. METHODS: A total of 660 fecal samples from 32 different species of pet birds were taken and examined for detection of Campylobacter spp. and were investigated for presence of cdt genes. All the samples were collected from clinically healthy birds that were kept in cage, zoological parks, and/or in zoo of Tehran, the capital of Iran. RESULTS: In total, 20 thermophilic Campylobacter were detected from 8 different avian species. From 20 confirmed Campylobacter spp., 16 samples (80%) were C. jejuni positive and 4 (20%) were C. coli in species-specific PCR test. Furthermore, out of 20 detected Campylobacter, 13 (65%) harbored the various subunits of cdtA, cdtB and cdtC genes, and 7 (35%) were negative for all tested cdt genes. CONCLUSIONS: Our findings indicate that the carriage rate of Campylobacter in different species of cage and/or in zoo birds is high and confirm that cdt genes may frequently be present in campylobacter spp.

Introduction

Thermophilic Campylobacter has emerged as a leading bacterial cause of foodborne gastroenteritis in humans around the world, with most cases being linked to the handling or consumption of contaminated and undercooked poultry products (Hanninen et al., 1998; Waldenstrom et al., 2005). Campylobacters are not significant pathogens for poultry, but are important to food safety and public health, with C. jejuni being responsible for the majority of human campylobacteriosis, followed by C. coli, and rarely by C. lari. The C. jejuni and C. coli, adapt well to the bird’s intestinal tract as they usually could have prominent coloniza-
tion and pathogenicity factors is necessary. A comprehensive review of the colonization factors in chickens was published recently and the list of genes contributing to the infection will probably continue to grow with the advance of genomics and functional genomics (Hermans et al., 2011). Many bacterial factors contribute to the colonization of Campylobacter in poultry, and therefore a number of putative virulence and toxin genes have been studied. The genes include flaA, CiaB, cadF and some other genes which are involved in adhesion and colonization of the host’s intestine; however, the specific virulence mechanisms are not fully elucidated (Konkel, 1999; Nuijten et al., 2000; Hermans et al., 2011). Previous reports have indicated that toxicity may be an important virulence factor, with the cytolethal distending toxin (cdt) genes being the principal toxicity determinant (Pickett and Whitehouse, 1999; Asakura et al., 2007; Rizal et al., 2010). Nevertheless, different species of Campylobacter seem to share cdt genes. Cytolethal distending toxin gene cluster consists of three adjacent genes, including cdtA, cdtB and cdtC. The subunit of cdtB is recognized as the enzymatically active subunit, and the two heterodimeric subunit cdtA and cdtC are responsible for the holotoxin binding to cell membrane (Lara-Tejero, and Galan, 2001).

Detection of cytolethal distending toxin

Campylobacter isolation: First, the swabs samples were immediately streaked on Campylobacter Charcoal Differential Agar (CCDA) plate as direct culture (Funbacmedia, 23122, Tehran, Iran). Then, these swabs were numbered and kept in normal saline serum for DNA extraction. The plate was incubated in micro-aerobic incubator with 8% of CO2 at 42°C for 24-48 hour. Colonies suspected as being Campylobacter species were examined for cell-morphology by optical microscope. Single colonies were picked and characterized to species level by their oxidase and catalase reactions and their ability to hydrolyze hippurate and indoxyl acetate according to standard procedures (On and Holmes, 1992). Isolates were stored with glycerol 15% at -70°C until required for further investigations (Bang et al., 2001).

DNA extraction: A- DNA was extracted from isolates, and a loop full of Campylobacter colonies from the CCDA was transferred and suspended in 300 μL of ultrapure water then heated at 100 °C for 10 min and centrifuged for 10 min at 12000 rpm. The supernatant fluid was used for DNA extraction and frozen at –20 °C until needed further use.

B- DNA was also extracted from swabs samples that Campylobacters could not be isolated by classic culture-based procedures. Therefore, normal saline of each tube (pooled swabs) was centrifuged. Sediment pellet was placed into microcentrifuge tube for DNA extraction by using the DNeasy Tissue kits (Qiagen GmbH, Hiden, Germany), according to the manufacturer’s instructions.

Identification of Campylobacter species using multiplex -PCR: For species identifi-
cation, PCR was performed initially with the universal Campylobacter 16s rRNA primer (Linton et al., 1996). All PCR positive samples were then subjected to a second PCR for differentiation of C. jejuni from C. coli (Table 1). For CampF/CampR primer (Sinaclon, Tehran, Iran), the reaction was performed in a 25 μl solution, containing 5.0 ng DNA, 0.1 μM each of CampF and CampR primers, 2 units of Taq DNA polymerase (Sinaclon, Tehran, Iran), 0.2 mM of each dNTP, 10 mM Tris-HCl and 2.5 mM MgCl2. The amplification reactions were carried out using a Perkin Elmer 9600 thermo cycler with the following program: one cycle of 10 min at 95°C, 35 cycles of 30 s at 95°C, 1.5 min at 59°C, 1 min at 72°C and a final extension step of 10 min at 72°C. For Campylobacter species used 0.2 μM of MDmapA1/MDmapA2 and COL3/ MDCOL2 primers. The amplification reactions were carried out using a Perkin Elmer 9600 thermo cycler with the same program described above. For the visualization of PCR products, 10 μl aliquots were subjected to electrophoresis in 1.5% Agarose gel, stained with ethidium bromide for 1.5 hour at 100 V and viewed under UV light (Fig. 1).

Detection of cdt genes from Campylobacter isolates by multiplex PCR: The primers and multiplex PCR were used to detect the cdt genes of C. jejuni and C. coli are summarized in table 2 (Asakura et al., 2008; Findik et al., 2011). The PCR protocol of Findik et al. (2011) was modified as described below. Our result proves that the concentration of the primer sets (Sinaclon, Tehran, Iran) in the multiplex PCR reactions was appropriate, 40 μl of PCR reaction mix contained 0.2 mM dNTP mix and 1.0 U of Ex Taq DNA polymerase. PCR products were analyzed by 1.5% agarose gel electrophoresis. Bands were visualized and images were captured.

Results

In this study, Campylobacter was isolated from 13 out of 660 individual swab samples collected from 32 different avian species. All isolates were oxidase and catalase positive; this finding indicates that they were thermophilic campylobacters. Based on microbiological and biochemical investigations, e.g. hippurate hydrolysis studies on these 13 isolates, 10 isolates were C. jejuni and 3 isolates were C. coli. All these findings were confirmed by PCR assay (Fig. 1). From swab samples that campylobacter had not been isolated by culture-based procedures, 7 campylobacter spp. were identified by PCR test and 6 of them were C. jejuni and 1 was C. coli.

In total, from 20 confirmed Campylobacter spp., identified by PCR test, 16 samples (80%) were C. jejuni positive and 4 (20%) were C. coli positive. (Table 3).

PCR method was used not only for confirmation of genus and species but also for detecting cdt genes using three different primer sets in two different combinations (Table 2, Figs. 2 and 3). In this study, from the 16 detected C. jejuni, 5 (31%) had cdt ABC, 2 (13%) had cdt AB, 4 (25%) had cdt BC genes, and 5 (31%) had non cdt present. Furthermore, out of 4 Campylobacter coli detected, 1 had cdt ABC genes, 1 had cdt AB, and 2 had no cdt genes (Table 3).

Discussion

Campylobacter spp. is a zoonotic bacteria and is recognized as important enteric pathogens in humans. A wide range of domestic and wild animal species, including birds, have been identified as natural reservoirs of Campylobacter in industrialized as well as in developing countries, and they could be a source of contamination for human beings (Jeffrey et al., 2001). The role of feral and domestic birds in the transmission of organisms to poultry and especially to humans has been well documented by many authors (Jeffrey et al., 2001; Waldenstrom et al., 2002; Abulreesh et
Many studies have been conducted in Europe, the United States, North America, and developing countries which reported Campylobacter positive poultry flocks ranging from 3% to 97% (Newell and Wagenaar, 2000; Newell and Fearnley, 2003). In Iran, Ansari-Lari et al. (2011) studied the prevalence of Campylobacter infection and showed that 76% of broiler flocks were positive for thermophilic Campylobacter spp. The contamination of

<table>
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<th>Target</th>
<th>Primer Name</th>
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<th>PCR Product Size</th>
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Table 1. Primers used for identification genus and species of Campylobacter by polymerase chain and multiplex polymerase chain reaction.

Table 2. Characteristics of PCR primers used for cdt genes in the study. (*= C. jejuni; **= C. coli).

Table 3. Molecular confirmation and the occurrence of cdt gene subunits in 20 Campylobacter species detected in different birds.
turkey carcasses by *Campylobacter* spp. at slaughterhouses of Iran was evaluated by using PCR method, and the results revealed 62% infection rate in commercial turkeys (Rahimi et al., 2010). A recent study by Mirzaie et al. (2011) in Iran indicated that 55% of samples taken from turkeys and 22% samples taken quails flocks were positive for *Campylobacter* spp., respectively.

The percentage of positive cloacal or faecal samples of thermophilic campylobacters in diverse wild birds from a wide range of geographical locations has been reviewed by Abulreesh et al. (2007). In this report, the prevalence of *Campylobacter* spp. of waterfowl were 11% in UK, 42% in USA, 44% in Taiwan, and 5% in Sweden, respectively; while, in feral pigeons (*Columba livia*), the percentage was 26% in Spain, 14% in Croatia, and 4% in USA. Other reports indicate that the isolation rates of house sparrow was 33% in Chile, starling was 3%, and blackbird was 6% in Sweden; while, in France, *Campylobacter jejuni* was isolated from 106 of 200 (53%) in domestic pigeon feces samples (Megraud, 1987).

Generally, the carriage rate of Campylobacter in domestic poultry was found to be much higher than that of wild birds (Yogasundram et al., 1989; Shane, 1992). This finding is probably due to the high bird density in commercial poultry houses, which facilitates the spread of Campylobacter between birds. In our study, the rate of thermophilic Campylobacter isolation in pet or companion birds was much lower (Table 3) compared to those reports in wild birds and was more obvious when compared with the prevalence of Campylobacter in industrial poultry of Iran. The use of antibiotics for disease control, which were implemented in cage condition, may have positive effects on lowering infection rate of these birds with Campylobacter. However, this method is not recommended for the prevention strategy due to emergence of antimicrobial resistance and public health concerns for humans.
One of the main characterized Campylobacter pathogenic markers is the *cdt* gene which may be intimately involved in successful enterocyte colonization processes and in the ability of the organism to cause inflammatory diarrhea in humans (Nuijten et al., 2000; Pasquali et al., 2011). In the present study, three *cdt* genes were followed and with regard to the PCR test, out of 20 *C. jejuni* and *C. coli* detected in the current study, 6 (30%) possessed all three toxin gene subunits. Three (15%) of the detected Campylobacter had both *cdtA* and *cdtB* markers and 4 of them showed both the *cdtB* and *cdtC* markers. However, 7 (35%), out of 20 Campylobacters tested, did not have any detectable *cdt* subunits. Until now, there is no information concerning the prevalence of the *cdtA*, *cdtB*, and *cdtC* genes published on Campylobacter isolates from pet birds and on the isolates of Iran either. However, we recently studied the virulence markers of *cdt* in industrial poultry (broiler chickens, turkeys, and quail). In this study, regardless of avian sample type, only 66.4% of all *C. jejuni* isolates and 43.6% *C. coli* isolates had all three types of *cdt* genes (A, B and C) and the rest of the isolates showed one or two subunits of *cdt* genes. The percentage of toxin-positive Campylobacter examined by other authors was different (Lara-Tejero and Galán, 2001,2002; Lutful Kabir et al., 2011). These differences illustrate that the variations of subunits *cdt* genes have a wide range and depend on the samples from birds and also the geographical locations. The complete role of *cdt* in campylobacteriosis is unknown, nevertheless, Lee et al. (2003) reported that *cdtA* and *cdtC* subunits have roles in binding to the host cell, and the *cdtB* subunit has a nuclease activity. Abuoun et al. (2005) reported that the levels of toxin expressed might be strain dependent, since some strains have no detectable *cdt* activity. Additionally, these authors explained that *cdt*-negative strains were also obtained from patients having symptoms of campylobacteriosis (Abuoun et al., 2005). Based on this information, it could be suggested that other pathogenicity genes e.g. flaA, cadF or factors such as invasion might also be important for campylobacter pathogenesis and survival within epithelial cells (Hickey et al., 2005).

This is the first molecular investigation on campylobacter spp. in pet birds, especially with the presence of *cdt* genes in *Campylobacter* spp. The results obtained here reveal that many companion birds carry campylobacter that could be recognized as reservoir and potential for human pathogens; therefore, handling these birds could be a risk for public health in society. Additionally, our results confirm that *cdt* genes may be frequently present in *Campylobacter* spp.; however, the role of those in campylobacteriosis is still unclear. Further study is needed to improve the understanding of the role of CDT in the pathogenesis of campylobacteriosis

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**References**


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