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Toxigenic *Clostridium difficile* in retail packed chicken meat and broiler flocks in northeastern Iran

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Summary

This study was designed to evaluate the occurrence of *Clostridium difficile* in both broiler chicken farms and packed chicken parts sold at market places in Mashhad, the second most popular Islamic pilgrimage city after Mecca in northeastern Iran. The fresh faecal samples were obtained from broiler farms, while the chicken packs were purchased from retail outlets across the city at market places and samples were obtained from the necks, thighs, and wings. The selective culture was used for isolation of *C. difficile*. Out of 40 pooled fresh faecal and 65 packed chicken samples, 14 (35%) and 10 (15.3%) samples were positive, respectively. Some of the *C. difficile* isolates from chickens packs (7 out of 10, 70%) and faecal samples (5 out of 14, 36%) were detected as toxigenic (A, B and binary toxins) using molecular identification. From 14 isolates of the faecal samples, five isolates were tcdA and tcdB positive, and none was binary toxin positive. The results of the present study suggest that broiler chickens are a potential source of *C. difficile*, which may infect humans through contact or consumption of chicken meat, although the significance of food contamination is entirely unclear, the role of poultry products as a potential source of the infection should be investigated.

Key words: Broiler chickens, *Clostridium difficile*, Iran, Packed chicken meat

Introduction

Clostridium difficile is an important enteropathogen in humans and some other animal species. Recently, changes in the epidemiology of *C. difficile* infection (CDI) in people and the severity of the disease associated with toxigenic *C. difficile* appear to be increasing. Such increases may be due to the emergence of a new strain of toxigenic *C. difficile* (ribotype 027/NAP1/toxinotype III) that exhibits increased levels of resistance, virulence, and toxin production (McDonald *et al*., 2005). Various strains of *C. difficile*, including NAP1, toxin type III, can be isolated from food animals and meat (Songer and Anderson, 2006; Weese *et al*., 2009). However, the dominant strains from food animals are ribotype 078/NAP7-8/toxinotype V strains (Jung *et al*., 2010). Because food animals can be colonized by *C. difficile*, and the bacterium has been isolated from retail meats and poultry, some researchers speculate that *C. difficile* is a food-associated organism and the consumption of contaminated meat or even the exposure to the contaminated faecal material could be responsible for the increased community-associated CDI (Songer and Anderson, 2006; Jung *et al*., 2010; Harvey *et al*., 2011a, b).

No data has yet been available in chickens as a source of *C. difficile* contamination in Iran. Here, we have determined the occurrence and characteristics of *C. difficile* in retail chicken meat and faecal samples of the broilers in northeastern Iran.

Materials and Methods

Samples collecting

In 2014, a total of 105 samples were collected which included 40 pooled fresh faecal samples (five individual samples from a different location in each house (each house was divided into five approximately equal sectors and each pooled sample represents one house in the farm) from 15 flocks (with one to three house/farm) with average population of 20000 birds/flock, 30 to 45-day-old broiler chickens) and 65 chicken packed portions (necks, thighs, and wings) from various meat markets in northeastern Iran.

To isolate *C. difficile* strains, equal volumes of faecal samples were mixed with 96% ethanol (v/v); after incubation for 30 min at room temperature, all the samples were cultured on the selective Columbia agar supplemented with 5% sheep blood (Merck, Germany) and cefoxitin-cycloserine egg yolk agar (CCEY HiMedia, India). These plates were incubated anaerobically at 37°C for 48-72 h (Pituch *et al*., 2002).

A total of 65 chicken neck, thighs and wings (3 necks and 3 wings in one pack were considered as one sample) were purchased from retail stores in different regions of Mashhad. Samples were shipped immediately to our laboratory in sterile packages of ice and were processed within the 24 h of receipt. The sample pieces were added to 50 ml phosphate-buffered saline (PBS) and were massaged thoroughly, then were placed on the shaker for 10 min. After the alcoholic shock, one hundred

microliters was then inoculated onto the selective Columbia agar supplemented with 5% sheep blood (Merck, Germany) and cefoxitin-cycloserine egg yolk agar (CCEY HiMedia, India). Identification of *C. difficile* was based on the morphological characteristics including the shape of the colony and Gram staining appearance with typical spore and horse-manure (Fedorko and Williams, 1997). Two or three passages were conducted to obtain a pure culture. Reference strains of *C. difficile* (kindly which were provided by Research Center of Gastroenterology and Liver Diseases, Shahid Beheshti University, Tehran, Iran) were used as positive controls. For DNA extraction, a single colony of each positive sample was processed using commercial DNA extraction kit (Bioneer, South Korea) according to manufacturer instruction.

Typing by multiplex and single polymerase chain reaction (PCR)

A 5-plex PCR was performed for the detection of tcdA (toxin A), tcdB (toxin B), cdtA, cdtB (binary toxin) and 16S rDNA based on Persson *et al*. (2008). The tcdC analysis was performed based on Antikainen *et al*. (2009). Polymerase chain reaction products were fractionated by electrophoresis on 1.5% agarose gels, and the target was detected by staining ethidium bromide and UV transillumination. DNA molecular weight marker 100 bp (Dena Zist Asia, Iran) was used as molecular weight markers.

Results

According to the data achieved from 105 samples which were analyzed based on conventional bacteriological methods and confirmed by 16S rRNA species-specific PCR, 24 (23.8%) samples were detected as positive. From 40 pooled fresh faecal samples, 14 (35%) were *C. difficile* positive, from these isolates, five (36%) were tcdA and tcdB positive but none of them were binary toxin positive (Table 1). Out of 65 packed chicken samples, 10 (15.3%) samples were positive for *C. difficile*. 7 (10.7%) samples were tcdA and tcdB positive, one sample had only showen harboring tcdA, and two (3%) of them were binary toxin positive. The details are provided in Table 1.

Discussion

It has been reported that less than 5% of the adults and approximately 29% of neonates were infected with *C. difficile*, in which most cases show no symptoms (Viscidi *et al*., 1981; Bartlett, 1992). Recently, *C. difficile* has been isolated from foods in the developed countries (Gould and Limbago, 2010), however there are few reports from developing countries, especially Middle East region.

In the present study, 22.8% of the total samples, in which 10/65 (15.3%) belonged to packed chicken portions and 14/40 (35%) pooled faecal samples were *C. difficile* positive, using conventional culture method and confirmation by PCR assay. In our study, the confirmation was performed by using a PCR assay which targeted genus-specific 16S rDNA. In studies from Zimbabwe, approximately the same contamination rate of *C. difficile* was reported (17-29%) from chicken faeces samples (Simango, 2006; Simango and Mwakurudza, 2008).

According to Weese *et al*. (2010), and Guran and Ilhak (2015), *C. difficile* was isolated from 12.8% and 8.06% of chicken portions, respectively. Based on our results and the result of other studies, it may be suggested that poultry meat contamination is originated in the faecal material. *Clostridium difficile* was isolated from faecal samples in 62% of the chickens from a single poultry farm in Slovenia (Zidaric *et al*., 2008). Harvey *et al*. (2011a) reported *C. difficile* was obtained from 2.3% of faecal samples from marketed age broilers, and 12.5% from poultry meat samples. The results of the present study are in agreement with other published reports of poultry meat contamination (range = 8-15%), however, the faecal contamination rate in our study is, higher than in other reports that could be due to pool sampling or different shedding age.

Pathogenic strains of *C. difficile* produce TcdA (enterotoxin) and/or TcdB (cytotoxin) encoded by *tcdA* and *tcdB*, respectively. Several strains were shown to harbor the genes encoding the binary toxin CDT, expressed from the cdtA (enzymatic component) and cdtB (binding component) operon (Popoff *et al*., 1988; Dupuy *et al*., 2008). Therefore, *tcdA*, *tcdB*, *cdtA*, and *cdtB* were investigated in the present study, as they were considered the most common genes for toxin typing of *C. difficile*. The results showed that 36% of faecal isolates were $tcdA^+$ and $tcdB^+$, whereas in packed chicken isolates, 50% harbored the same toxins and one isolate was $tcdA^+$ and $tcdB^-$. Only in two packed chicken isolates (20%) was the binary toxin detected ($tcdA^+$, $tcdB^+$, $cdtA^+/cdtB^+$). Based on Weese *et al*. (2010) all of the isolates from chicken portions (12.8%) possessed genes encoding toxins A, B, and CDT. Although isolates could be typed by PCR ribotyping as it has already been described by Bidet *et al*. (1999), similar to our study, genes encoding production of toxins A (*tcdA*) and B

Table 1: Prevalence of different patterns of toxin-related genes in this study based on proportion to all samples

Samples	Gene's profile						Total
	16s rDNA, $tcdA^+$, $tcdB^+$, $tcdC^+$ (139 bp), $ctdAB^-$	16s rDNA, $tcdA^+$, $tcdB^+$, $tcdC^+$ (85 bp), $ctdAB^-$	16s rDNA, $tcdA^+$, $tcdB^+$, $tcdC^+$ (85 bp), $ctdAB^+$	16s rDNA, $tcdA^-$, $tcdB^-$, $tcdC^-$, $ctdAB^-$	16s rDNA, $tcdA^+$, $tcdB^-$, $tcdC^-$, $ctdAB^-$	16s rDNA, $tcdA^-$, $tcdB^-$, $tcdC^+$, $ctdAB^-$	
Faecal	3 (7.5%)	2 (5%)	0	8 (20%)	0	1 (2.5%)	14/40 (35%)
Packed chicken	5 (7.67%)	0	2 (3.06%)	2 (3.06%)	1 (1.53%)	0	10/65 (15.3%)
Total	8 (7.6%)	2 (1.9%)	2 (1.9%)	10 (9.5%)	1 (0.95%)	1 (0.95%)	24/105 (22.8%)

(*tcdB*) has been evaluated using PCR by other researchers (Kato *et al*., 1998; Lemee *et al*., 2004). Based on our findings, four of the 24 *C. difficile* strains (16.6%) were tcdA, tcdB positive, and cdtAB toxin genes which were classified as ribotype 078. Contrary to studies on other food animals and food sources, our results showed that this ribotype was not predominant in poultry (Indra *et al*., 2008; Songer *et al*., 2009; Curry *et al*., 2012).

In addition, detection of CDT (binary toxin) genes has been performed using PCR targeting cdtB, the binding component of (Stubbs *et al*., 1999) *C. difficile*. In the present study, we used multiplex PCR which has targeted both cdtA and cdtB. In the present study, 14/40 (35%) of faecal samples were determined as *C. difficile* using conventional and molecular methods, in which 5/14 (35.7%) of them were toxigenic. We have detected *C. difficile* in 10/65 (15.3%) of packed chicken samples, and the compelling finding was the fact that 7/10 (70%) of the isolates showed a toxigenic pattern.

Some studies have detected increasing levels of *tcdc* and *tcdd* production at the same time, and some have found a high variability in toxin expression levels among hypervirulent *C. difficile* strains with a mutation in *tcdC* (Curry *et al*., 2007; Merrigan *et al*., 2010; Vohra and Poxton, 2011). Bakker *et al*. (2012) designed a study to clarify the role of *tcdC* in regulating toxin expression in *C. difficile*, and they did not report a significant difference in toxin expression between the wild type of strains and the mutant CT: the *tcdC* strain that they designed (Bakker *et al*., 2012). In our study 16 isolates were *tcdC* positive (135 bp PCR product, indicating the presence of intact gene) and 8 isolates harbored the incomplete gene (85 bp). Contrary to isolates with a positive pattern for A and B toxins, all of the negative tcdA and tcdB isolates were tcdC negative (20 isolates).

Our results suggest that consumption of chicken meat, which could have been contaminated directly through the faeces or the environment, could be considered a source for human infection. As in previous studies, *C. difficile* has been isolated from food animals such as poultry, sheep, pigs, chickens, goats, dogs and cattle (Rupnik, 2007; Ghavidel *et al*., 2016).

Considering the high percentage of toxigenic strains in packed chicken sample isolates (70%), along with the opportunistic and spore-forming nature of the disease induced by *C. difficile*, the role of epidemiologic surveys in discovering the potential source of the infection becomes significant. Also, to limit the implications of contaminated raw meat, many food handling and cooking protocols are designed, but the spore-forming nature of *C. difficile* and the heat tolerance of spores (Rodriguez-Palacios *et al*., 2007) make it difficult to decontaminate food through cooking or routine kitchen cleaning practices (Weese *et al*., 2009). The fact that a ribotype isolated from the faeces of Austrian patients was similar to the ones isolated from ground meat implies zoonotic potential of the disease. However, contamination during food processing by human shedders is also a possibility. *Clostridium difficile* colonizes the human intestinal tract in 3% of healthy adults and up to 80% of healthy newborns and infants (Viscidi *et al*., 1981; Bartlett, 1992).

The results of the present study suggest that broiler chickens are considered a potential source of *C. difficile*, which may infect humans through contact or consumption of the chicken meat, especially because of the presence of the strains harboring A and B toxins in this study.

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Conflict of interest

No competing financial interests exist.

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