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Prevalence of *Clostridium difficile* in raw beef, cow, sheep, goat, camel and buffalo meat in Iran

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Abstract

Background: *Clostridium difficile* has been shown to be a nosocomial pathogen associated with diarrhoea and pseudomembranous colitis in hospitalised patients and the infection is believed to be acquired nosocomially. Recent studies have shown the occurrence of *C. difficile* in food animals which may act as a source of infection to humans. The aim of this study was to determine the occurrence of *C. difficile* in retail raw beef, cow, sheep, goat, camel and buffalo meat in Iran.

Method: From April to October 2012, a total of 660 raw meat samples from beef, cow, sheep, goat, camel and buffalo were purchased from 49 butcheries in Isfahan and Khuzestan provinces, Iran, and were evaluated for the presence of *C. difficile* using a method including selective enrichment in *C. difficile* broth, subsequent alcohol shock-treatment and plating onto *C. difficile* selective medium. *C. difficile* isolates were tested for the presence of toxin genes and were typed using PCR ribotyping.

Results: In this study, 13 of 660 meat samples (2%) were contaminated with *C. difficile*. The highest prevalence of *C. difficile* was found in buffalo meat (9%), followed by goat meat (3.3%), beef meat (1.7%), cow (0.94%) and sheep meat (0.9%). Seven of the 13 *C. difficile* strains (53.9%) were positive for *tcdA*, *tcdB* and *cdtB* toxin genes and were classified as ribotype 078. Four strains (30.8%) were positive *tcdA*, and *tcdB*, and one strain (7.7%) was possessed only *tcdB*. The remaining isolate was non-toxicogenic. Susceptibilities of 13 *C. difficile* isolates were determined for 11 antimicrobial drugs using the disk diffusion assay. Resistance to clindamycin, gentamycin, and nalidixic acid was the most common finding.

Conclusions: To our knowledge, the present study is the first report of the isolation of *C. difficile* from raw buffalo meat. This study indicates the potential importance of food, including buffalo meat, as a source of transmission of *C. difficile* to humans.

Keywords: *Clostridium difficile*, Raw meat, Camel, Buffalo, Beef, Antimicrobial resistance

Background

Clostridium difficile is a Gram-positive, anaerobic, spore-forming bacterium that has come to the forefront as an important human pathogen. It was initially dismissed as commensal in healthy infants, but was recognized as an important cause of antimicrobial-associated with diarrhoea in the 1970s. It is, now, the most commonly diagnosed cause of antimicrobial-associated and hospital-associated diarrhoea, and the cause of virtually all cases of pseudomembranous colitis [1]. *C. difficile* infection (CDI) more recently was described in non-hospitalized patients without

underlying disease or a predisposing risk factor such as antimicrobial exposure, advanced age or significant comorbidities [2,3].

C. difficile also appears to be an important cause of enteric disease or a commensal in a wide variety of animal species [4-6]. Food animals are an important source of enteropathogens, and *C. difficile* has been isolated from food animals such as poultry and sheep [4-7], pigs [8,9], chickens, goats and cattle [6] and calves [10]. The types of *C. difficile* found in animals and humans are often indistinguishable [10-12] raising concerns that *C. difficile* might be a zoonotic pathogen [9,11]. In particular, ribotype 078 is commonly found in food animals [5,13] and an increasingly reported cause of community-associated CDI in humans [5,14].

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The epidemiology of CDI in Iran is poorly understood. The recent finding of ribotype 078 as the leading ribotype in a small study of CDI in humans in Iran [14] raised concern about the potential for food as a source of infection, but the prevalence of *C. difficile* in food in Iran has never been reported. The aim of this study was to determine the occurrence of *C. difficile* in retail raw beef, cow, sheep, goat, camel and buffalo meat in Iran.

Methods

Sample collection

From April to October 2012, a total of 660 raw meat samples from beef (young cattle) (n = 121), cow (adult dairy cow) (n = 106), sheep (n = 150), goat (n = 92), camel (n = 124) and buffalo (n = 67) were purchased unpacked from 49 butcheries in Isfahan and Khuzestan provinces, Iran. These cities are the most prominent national cultural and tourist centers located in the center and south of the country, respectively. From each city 40–55 samples (about 0.5 kg / sample; two sections of meat (10 cm × 10 cm × 3 cm) from neck of each carcasses were aseptically removed) were purchased monthly. All samples were placed in separate sterile plastic bags to prevent from spilling and cross contamination and were immediately transported to the laboratory in a cooler with ice packs and processed within 6 h.

Isolation and identification of *C. difficile*

The samples were processed immediately upon arrival using aseptic techniques. The detection and isolation method used were based on the method described by Rodriguez-Palacios et al. [15] and de Boer et al. [16]. Briefly, 5 g of each sample was transferred to 20 mL of *C. difficile* broth (CDB; Oxoid SR0048) containing 40 g/l proteose peptone, 5.0 g/l, disodium hydrogen phosphate, 0.1 g/l magnesium sulphate, 2.0 g/l sodium chloride, 6.0 g/l fructose and 1.0 g/l sodium taurocholate supplemented with *C. difficile* selective supplement (Oxoid, UK, Code: SR0173) and 5% (v/v) defibrinated sheep blood. After incubation at 37°C for 10 to 15 days under anaerobic conditions 2 mL of the enrichment broth was added to 2 mL of 96% ethanol in a centrifuge tube and homogenized for 50 min on a shaker at room temperature. After centrifugation (3800 × g for 10 min), a loopful of the sediment was streaked onto *C. difficile* agar base (Oxoid, UK, Code: CM0601) supplemented with an antibiotic supplement for the selective isolation of *C. difficile* (Oxoid, UK, Code: SR0173) and 7% (v/v) defibrinated sheep blood and the plates were incubated for 48 h at 37°C, under anaerobic conditions. Three colonies per plate were subcultured onto tryptone soya agar (Oxoid, UK, Code: CM0131) and tested by standard microbiological and biochemical procedures including odour, Gram stain morphology and L-proline

aminopeptidase test [4]. Crudely extracted DNA [boiling method: One colony was suspected in 500 µl distilled water and after heating for 10 min at 95°C, the suspension was centrifuged (5 min, 10000 × g)] was used for PCR confirmation (*tpi* gene detection), determination of toxin gene (*tcdA*, *tcdB* and *cdtB*), and PCR ribotyping of isolates as performed in previous studies [17,18]. For assurance managed at the lab positive and negative controls were included in each batch.

The limitations of the study include the small number of *C. difficile* isolates which were analysed in the study and the impossibility of sampling from other areas of Iran.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was done by the Kirby–Bauer disc diffusion method using Mueller–Hinton agar (HiMedia Laboratories, Mumbai, India) according to the Clinical Laboratory Standards Institute (CLSI) [19] as has been previously described [4]. The antimicrobial agents tested and their corresponding concentrations were as follows: nalidixic acid (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), tetracycline (30 µg), doxycycline (30 µg), gentamicin (10 µg), metronidazol (5 µg), ampicillin (10 µg), chloramphenicol (30 µg), vancomycin (30 µg), and clindamycin (2 µg). After incubating the inoculated plate for 48 h at 37°C, under anaerobic conditions, the susceptibility of the *C. difficile* to each antimicrobial agent was measured and the results were interpreted in accordance with interpretive criteria provided by CLSI [19].

Results and discussion

Table 1 shows the prevalence of *C. difficile* isolated from beef, cow, sheep, goat, camel and buffalo meat in two provinces, Iran. *C. difficile* was isolated 13/660 meat samples (Table 1). There were no significant differences ($P > 0.05$) in the frequency of positive samples among the meat samples or between Isfahan (4/315, 1.3%) and Khuzestan (9/355, 2.3%) ($P > 0.05$).

The highest prevalence of *C. difficile* was found in buffalo meat samples (6/67), followed by goat (3/92), beef (2/121) and sheep (1/150) (Table 1). All the camel meat samples found to be negative. Toxigenic *C. difficile* strains (*tcdA*, *tcdB* and *cdtB* toxin genes) were detected in 12/13 of isolates. Seven of the 13 *C. difficile* strains (53.9%) were positive for *tcdA*, *tcdB* and *cdtB* toxin genes. Four strains (30.8%) were positive *tcdA*, and *tcdB*, and one strain (7.7%) was possessed only *tcdB*. The remaining isolate was non-toxigenic. Our finding of *C. difficile* and its toxigenic strains in meat are supported by similar reports from other countries [9,15,16,20–23]. The low prevalence of *C. difficile* in cow, beef, goat and sheep meat samples are comparable with those reported by others [16,20–23]. However, higher contamination rates (20% to 50%) have also been reported [9,15]. In contrast, Von Abercron et al. [24] did not detect

Table 1 Prevalence of *Clostridium difficile* detected in beef, cow, sheep, goat, camel and buffalo meat samples in Iran

Meat sample	No. of samples	No. of <i>C. difficile</i> -positive samples	No. of isolates positive for toxins			Ribotype 078
			<i>tcdA</i>	<i>tcdB</i>	<i>cdtB</i>	
Beef	121	2 (1.65%)	2	2	2	2
Cow	106	1 (0.94%)	1	1	-	-
Sheep	150	1 (0.67%)	1	1	0	-
Goat	92	3 (3.26%)	2	2	2	2
Buffalo	67	6 (8.96%)	5	6	3	3
Camel	124	0 (0.00%)	-	-	-	-
Total	660	13 (1.97%)	11	12	7	7

C. difficile in meat samples other than beef. Whether this reflects a true different prevalence or is due to differences in sampling techniques employed (meat sample, carcass swab or carcass rinse fluid sample), seasonal effects [20] and/or laboratory methodologies employed in different studies is not clear.

The source of *C. difficile* in food products is unclear. Contamination of meat might be due to *C. difficile* residing in the gastrointestinal tract of animals, but could also originate from the hands of personnel working in the slaughterhouse, meat processing equipment or the slaughterhouse environment during the slaughtering process [5,25,26]. The prolonged survival of *C. difficile* spores in the environment increases the possibilities for contamination of animals and foods. Another potential source of infection that requires investigation is the presence of *C. difficile* spores in healthy muscle tissue in living animals [1].

Seven of the 13 *C. difficile* strains were positive for *tcdA*, *tcdB* and *cdtB* toxin genes and were classified as ribotype 078. The predominance of ribotype 078 is consistent with other studies of food animals and food [8,23,27]. Given the presence of this strain in humans in the same region with CDI, consideration must be given as to whether food might be the source. However, further study is required to determine whether food is a reasonable source of infection.

Antimicrobial susceptibility data are presented in Table 2. Resistance of *C. difficile* to clindamycin, gentamycin, nalidixic acid, ciprofloxacin, erythromycin, ampicillin, and tetracycline was high. These results are comparable to those reported by other investigators [6,22,28]. All the *C. difficile* isolates were susceptible to metronidazole, and vancomycin as was observed in other studies [6,15,22]. These two drugs are the most commonly used to treat *C. difficile* diarrhea in humans but are not used in food animals. The results of antimicrobial resistance found in this study are correlated with antibiotics usage to treat infections in food animals in Iran. In contrast,

Table 2 Antimicrobial resistance of 13 *Clostridium difficile* isolated from beef, cow, sheep, goat, camel and buffalo meat in, Iran

Antimicrobial agent	Sensitive	Intermediate	Resistant
Ampicillin	3 (23.08%)	3 (23.08%)	7 (53.85%)
Chloramphenicol	11 (84.62%)	2 (15.38%)	0 (0.0%)
Ciprofloxacin	1 (7.69%)	2 (15.38%)	10 (76.92%)
Clindamycin	0 (0.0%)	1 (7.69%)	12 (92.31%)
Doxycycline	10 (76.92%)	3 (23.08%)	0 (0.0%)
Erythromycin	3 (23.08%)	2 (15.38%)	8 (61.54%)
Gentamicin	0 (0.0%)	0 (0.0%)	13 (100%)
Metronidazole	13 (100%)	0 (0.0%)	0 (0.0%)
Nalidixic acid	0 (0.0%)	0 (0.0%)	13 (100%)
Tetracycline	5 (38.46%)	4 (30.77%)	4 (30.77%)
Vancomycin	13 (100%)	0 (0.0%)	0 (0.0%)

many of the drugs to which the isolates were resistant (i.e. gentamicin) are commonly used in food animals.

Conclusions

This study indicates that the potential importance of food, including buffalo meat, as a source of transmission of *C. difficile* to humans. Slaughterhouses can be heavily contaminated with foodborne pathogens [29-31], the maintenance of slaughter hygiene, regular microbiological monitoring of carcasses, implementation of good manufacturing practices and a food safety system such as the HACCP system are essential to minimize the risk to the consumer. To the author's knowledge, the present study is the first report of the isolation of *C. difficile* from raw beef, cow, goat, sheep and buffalo meat in Iran. Further studies are required to determine the prevalence of *C. difficile* in meat in Iran and to explore the potential risk of human infection with *C. difficile* via consumption of meat.

Abbreviations

C. difficile: *Clostridium difficile*; CDI: *C. difficile* infection; PCR: Polymerase chain reaction; CLSI: Clinical laboratory standards institute.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The supporting of project were performed by ER, MJ and JSW, DNA extraction, PCR techniques were performed by ER and MJ and samples collection, culture, statistical analysis and writing of manuscript were performed by ER. All authors read and approved the final manuscript.

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