　　Research Article

Prevalence and Characterization of *Clostridium difficile* in Beef and Mutton Meats of Isfahan Region, Iran

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Background: *Clostridium difficile* (*C. difficile*) is a frequent cause of nosocomial infections. During last few years, the mortality rate of *C. difficile* infection (CDI) increased in healthcare facilities. This organism has become a major public health concern in developed nations. Because of the increasing incidence of acquired-CDI (CA-CDI) and notable genetic overlap between *C. difficile* isolates from animals and humans, meat has defined as one of the probable transmission route of *C. diffiicle* to humans.

Objectives: This study was performed to determine the prevalence of toxigenic *C. difficile* in beef and mutton meats consumed as human food in Isfahan, central part of Iran. Furthermore the polymerase chain reaction (PCR)-ribotyping employed to compare the genetic pattern of positive isolates in meat with clinical ones.

Materials and Methods: A total of 200 raw meat samples (81 beef and 119 mutton) were purchased from meat packaging plants. The samples were anaerobically cultured in *C. difficile* moxalactam norfloxacin (CDMN) broth and plated on selective enrichment medium. The suspicious colonies were recultured on blood agar anaerobically. All *C. difficile* isolates identified by morphological and biochemical testing were screened by PCR for the presence of genes encoding the triose phosphate isomerase (*tpi*), toxin A (*tcdA*), toxin B (*tcdB*) and binary toxin (*cdtB*). The genomes of extracted isolates were analyzed by 16S-23S rRNA-based PCR ribotyping.

Results: The overall prevalence of *C. difficile* with two toxigenic genes including *tcdA* and *tcdB* was estimated at 4.0%. *C. difficile* was detected in 2.8%, 2.1%, 3.6% and 6.2% of chopped beef, ground beef, chopped mutton and ground mutton, respectively. The *cdtB* gene was not found in positive isolates. Eight different ribotypes were found in isolated strains that were not identical with those belonging to patients with CDI.

Conclusions: The results of PCR-ribotyping indicate that no relationship exists between clinical and meat isolates. We therefore conclude that other sources than meat may function as a vector for CA-CDI.

Keywords:Clostridium difficile; Meat; PCR; Iran

1. Background

Clostridium difficile is a Gram-positive, spore-forming and obligate anaerobic bacterium (1). CDI was considered a cause for symptoms of infectious diarrhea in patients after hospitalization and antibiotic treatment back in the 1970's (2). The CDI associate with two virulence factors including toxin A (*tcdA*) and B (*tcdB*) (3). Some strains of this organism have the ability to produce another toxin called binary toxin (*cdt*) with a yet unclear role for CDI (4). *C. difficile* has become an important health concern due to its high mortality, particularly in the hospitalized individuals in developed nations (5). The epidemiology of CDI in humans have been changed as a consequence of the emergence and dissemination of new strains of *C. difficile* called ribotype 027 and 078. The infection occurred in young individuals and other patients that were at low risk. In fact, the major risk factors for CA-CDI were not related with advanced age, antibiotic therapy or duration of hospitalization (6).

The source and transmission of *C. difficile* to humans altered from healthcare facilities to the outside of these places. The researchers stated that the bacterium can be transmitted via food based on the recognition of CA-CDI, detection of *C. difficile* in food animals as well as on the genetic similarities observed between *C. difficile* isolates from food animals, food and humans (7). Some previous reports pointed out that meat can very likely be a vector of *C. difficile* to human (8-10). In Iran, *C. difficile* strains of ribotype 078 was identified as the most common isolate in diarrheic hospitalized patients tested in Isfahan region. The existence of this ribotype referred to the probable incidence of *C. difficile* in food and food animals in the community of Isfahan (11). However, the situation of *C. difficile* in raw meat for human consumption is poorly understood in Iran.

2. Objectives

The aim of this surveillance study was to assess the prevalence of toxigenic *C. difficile* in beef and mutton meats demanded from meat packaging plants by PCR in Isfahan, Central part of Iran. In addition, PCR-ribotyping of toxigenic isolates was investigated to determine whether CA-CDI might be transmitted from consumption of meat.

3. Materials and Methods

3.1. Sampling, Preparation and C. difficile Identification

A total of 200 samples including beef and mutton were randomly purchased from meat packaging plants during an 8-month period. The examined samples were consisted of 35 (17.5%) chopped and 46 (23%) ground beef, 55 (27.5%) chopped and 64 (32%) ground mutton. The samples were transferred to the Infectious Disease and Tropical Medicine Research Center in Isfahan University of Medical Sciences in portable insulated cold boxes and analyzed in the same day of collection. Around 5 grams of each sample was cultured in 25 mL of *Clostridium difficile* moxalactam norfloxacin (CDMN) (Oxoid SR0048) and fortified with *C. difficile* selective supplement (Oxoid, SR0173) including 500 mg cysteine hydrochloride, 12 mg norfloxacin and 35 mg moxalactam per liter. The samples were incubated anaerobically for 7 days at 37°C. Subsequently, 2 mL of enriched culture were added to 2 mL of 98% ethanol (Merck, Germany) and kept for 2 h at room temperature. The tubes were centrifuged for 10 min at $10000*g$. The sediment was streaked onto CDMN agar and then incubated under anaerobic conditions at 37°C for 24-48 hours. All *C. difficile* isolates were confirmed by morphology and L-proline- aminopeptidase test (prodisk, hardy diagnostics, Santa Maria, USA). Positive strains were recultured on blood agar and incubated at 37°C for 36 h in anaerobic conditions (8, 12).

3.2. DNA Extraction

A full loop of *C. difficile* grown in blood agar was suspended in 100 μL of sterile distilled water, boiled at 95°C for 3 minutes and then centrifuged at $3000*g$ for 15 minutes. The supernatant was collected for use as a template for amplification reaction in a thermocycler PCR (T-cY, Netherlands) (13).

3.3. Multiplex Polymerase Chain Reaction for Detection of tpi, tcdA and tcdB

The volumes of the reactions for the detection of genes encoding *tpi*, *tcdA*, *tcdB* of extracted DNA were based on the method described previously (13). The multiplex PCR procedure was carried out under the following conditions: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing for 30 sec-

onds at temperature decrease from 65 to 55°C in 11 cycles and extension at 72°C for 30 seconds. The PCR products were then assayed by electrophoresis on 1.5% agarose gel stained with ethidium bromide (13). All PCR materials were purchased from SinaClon BioScience Company, Iran.

3.4. Identification of cdtB by Polymerase Chain Reaction

All conditions including volumes and temperatures of the reactions for detection of *cdtB* were performed from the method described by Stubbs et al. (14).

3.5. Polymerase Chain Reaction -Ribotyping of C. difficile isolates

PCR-ribotyping reaction was performed in a total volume of 100 μL containing 200 μM of each dNTPs mix, 1.5 mM MgCl2, 2.5 U of Taq DNA polymerase, 50 μl of each primer, 10 mM Tris-HCl (pH 8.8), 50 mM KCl and 10 μL of DNA extract. Amplification programmed for 30 cycles consisting of 95°C for 6 minutes in initial denaturation, 92°C for 60 seconds in denaturation, 55°C for 60 seconds in annealing, 72°C for 6 minutes in extension steps. Amplicon product was loaded on 1.5% agarose gel for 6 hours at 80 V. Scanning by UV light was done after staining with ethidium bromide (15). The patterns of ribotype were visually compared with over 3000 isolates from humans and animals which gathered in the international collections of the University of Guelph, Canada. For ribotype pattern included in the collection, international name were choosen. Otherwise an internal nomenclature selected for unavailable ribotype patterns in the collections (11).

3.6. Bacteria

The strain of *C. difficile* ribotype 027 for microbiological analysis kindly obtained from the database of the University of Guelph, Canada.

3.7. Oligonucleotide Primers

The primers for the amplification of the tpi housekeeping, tcdA, tcdB and cdtB genes provided by Metabion International AG, Martinsried, Germany has listed in Table 1. The primer sequences of PCR-ribotyping were 5'-GTGCGGCTGGATCACCTCCT-3' and 5'-CCCTGCACCCTTAATAACTTGACC-3' for 16s and 23s, respectively (15).

4. Results

The morphology of *C. difficile* strains subcultured on CDMN agar is presented in Figure 1.

L-proline aminopeptidase test by the proline disk was used for the detection of *C. difficile* (Figure 2). This test is used to examine enzymatic hydrolysis of L-proline-β-naphthylamide extant in the disc based on releasing free β-naththylamine by the color changes after addition PEP

(Para-dimethyl amino-cinnamaldehyle in a weak hydrochloric acid solution) reagent. The aforementioned test is approved especially useful in screening for *C. difficile*.

We isolated strain of *C. difficile* with *tpi* housekeeping gene (230 bp) and two toxigenic genes including *tcdA* (369 bp) and *tcdB* (160 bp) in 8 (4%) of the 200 samples of beef and mutton meats for human consumption as follows: one (2.8%) in the 35 chopped beef, one (2.1%) in the 46 ground beef, two (3.6%) in the 55 chopped mutton and four (6.2%) in the 64 ground mutton samples (Figure 3).

Table 1. The Primers Used for Identification of Toxigenic *C. difficile* in This Study

| Gene	Sequence (5'→3')	Product Size, bp	Reference
tpi		230	(13)
Forward	AAAGAAGCTACTAAGGGTACAAA		
Reverse	CATAATATTGGGTCTATTCCTAC		
tcdA		369	(13)
Forward	AGATTCCTATATTTACATGACAATAT		
Reverse	GTATCAGGCATAAAGTAATATACTTT		
tcdB		160	(13)
Forward	GGAAAAGAGAATGGTTTTATTAA		
Reverse	ATCTTTAGTTATAACTTTGACATCTTT		
CdtB		510	(14)
Forward	CTTAATGCAAGTAAATACTGAG		
Reverse	AACGGATCTCTTGCTTCAGTC		
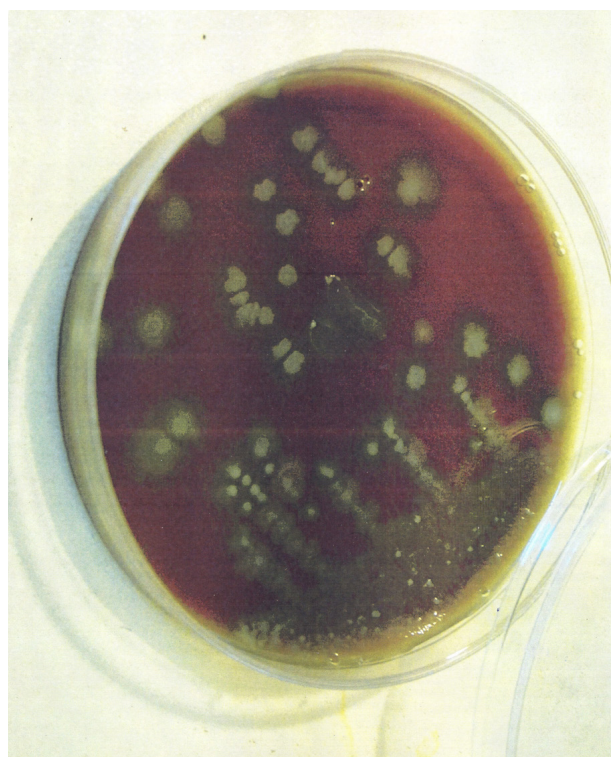
Figure 1. Irregular and Grey Colony with Opaque Appearance of *C. difficile*
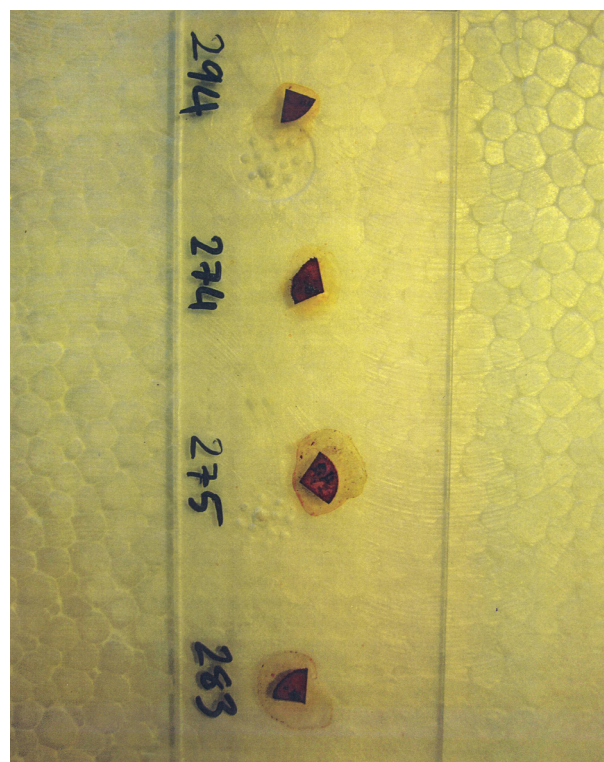
Figure 2. Proline Disk Staining of *C. difficile* From Meat Samples

Figure 3. The Result of the Multiplex PCR for *C. difficile* in Agarose Gel Electrophoresis
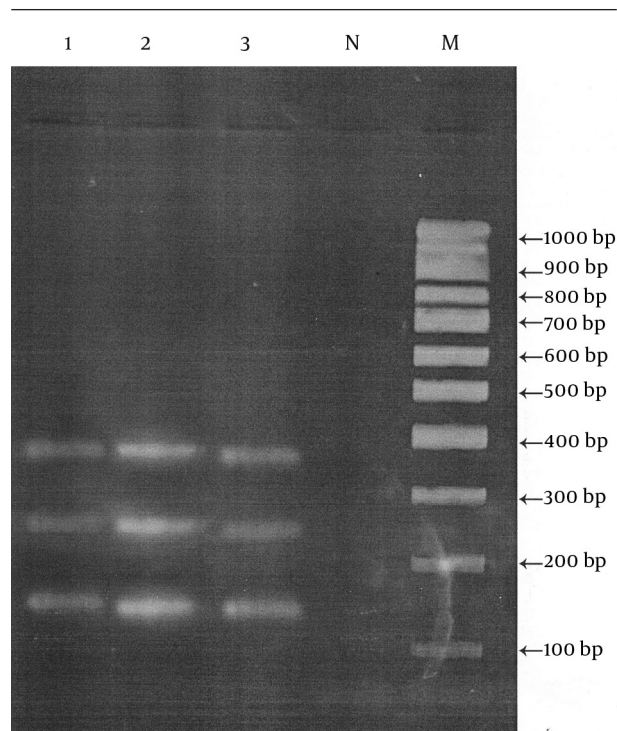
Lane M, marker 100-1000 bp; Lane N, negative control; Lane 1 and 3, *tpi*, *tcdA* and *tcdB* fragments of isolated *C. difficile* in meat samples; Lane 2, *C. difficile* strandard ribotypye 027.

Table 2. Ribotype Data for Isolates of *C. difficile* Strains From Beef and Mutton Samples

Sample Code	Meat Type	Ribotype
1	Chopped beef	IR11
2	Ground beef	IR12
3	Chopped mutton	IR13
4	Chopped mutton	IR14
5	Ground mutton	IR15
6	Ground mutton	IR16
7	Ground mutton	IR17
8	Ground mutton	IR18

All isolated colonies were identified in different carcasses collected in different dates. No *cdtB* gene was found in positive isolates of toxigenic *C. difficile*. In the present study, a series of eight different ribotypes were identified that genetic overlap were not observed with those belonging to animal and patients with CDI (Table 2).

5. Discussion

The present study evaluated the prevalence of *C. difficile* in beef and mutton collected from the meat packaging plants in an attempt to show how the Isfahan community may be exposed to this organism by consumption of meat. The prevalence of *C. difficile* considered in our study was much lower than several of the previous studies. A relatively high incidence of *C. difficile* contamination was observed in the United States, where 37 (42%) of 88 different retailed meat products and raw meats were found to be contaminated with this organism (9). Another study in the United States showed *C. difficile* prevalence in four (8%) out of 50 retailed ground meat samples (16). *C. difficile* has also been detected in experiments run in Canada with an incidence of 20% (12 out of 60) meat samples analyzed by Rodriguez-Palacios et al. (8). Afterwards, the aforementioned authors identified *C. difficile* in 13 (6.1%) out of 214 samples of ground and chopped beef meat (17). Similarly, *C. difficile* was isolated in 28 (12%) out of 230 meat samples submitted to analysis in another Canadian study (18). However, lower incidence rates of *C. difficile* have been reported in the studies led by other authors.

In a survey in Costa Rica, 200 meat samples were tested, out of which four (2%) were contaminated with *C. difficile* similarly to a study in the United States where 2% (2 out of 102) of the samples were contaminated (19, 20). Furthermore, the contamination rate of *C. difficile* was reported 2% in 13 out of 660 different meat samples tested in recent survey in Iran (10). In Sweden, France, Austria and the Netherlands, incidence rates of *C. difficile* contamination were 2.4%, 1.9%, 3% and 1.6% in the meat samples analyzed, respectively (6, 21-23). The results of the present investigation indicated a higher percentage of toxigenic strains of *C. difficile* in ground meats than in chopped ones (5% vs. 3%). This cannot be regarded as an unusual event because of further handling of minced meats. Our findings are in close agreement with the evidences of other authors that indicate the persistence of *C. difficile* spores which may exist in the environment and facilities of processing plants (16).

Care must be taken when interpreting available studies because the different data about the frequency of *C. difficile* are mainly affected by variable numbers of samples with different sampling, isolation methods and variations in regions. Nonetheless, the finding of present study would contribute to the international data of *C. difficile* prevalence in raw meat. In accordance to the results of the present survey concerning the ribotyping, the study led by de Boer et al., showed that around 80% of the isolated strains were not identical with the ribotypes found in patients with CDI in the Netherlands (6).

A Canadian report managed to evidence a collection of 12 ribotypes from *C. difficile* isolates in meat samples through PCR-ribotyping. Overall, eight (67%) of the 12 isolates hadn't previously been identified and designated as belonging to the ribotype M31. The other identified ribotypes were 014, 077 and M26 (8). Furthermore, these authors cited the genetic diversity of *C. difficile* in retail raw meat in another study, including ribotypes 014, 077, M26, C, F, H, K and J (17). In contrast, some different studies showed a link between the presence of similar ribotypes in meat samples and clinical ones. For example, ribotype

012, 027, 078 identified in meat samples in Canada, Iran, USA and Europe (9, 10, 18, 20, 22). Unfortunately, no comprehensive surveys or reference laboratory data exist in Iran. It seems that establishment of an association to gather routine information about *C. difficile* as a causative agent of CDI in Iranian hospitals is necessary by regulatory agencies. Not having adequate information on the prevalence of *C. difficile* in food and food animal in Iran and the geographical aspects constitute the limitations of this study.

It is well accepted that the presence of *C. difficile* could result from different factors such as pollutants in environment, slaughtering process, poor operational and operators' hygiene. This is an issue rarely subjected to serious studies; therefore, comprehensive surveys are recommended in this regards. Overall, the results of the current study confirm the existence of *C. difficile* in beef and mutton meats mostly in their ground form in Iran which could be related to the wide distribution of *C. difficile* spores in the environment of meat packaging plants especially in meat grinder. No relationship was observed between meat isolates and clinical ones based on PCR-ribotyping results. Regarding with our finding, it suggests further investigations on other food matrices in Iran to determine the real situation of *C. difficile* contamination.

Acknowledgements

Authors' contributions

The project was performed by ZE, MJ, HE, JSW and MC. DNA extraction, PCR techniques were performed by ZE. Sample collection, culture, statistical analysis and manuscript writing were performed by ZE, MJ, HE and JSW. All authors read and approve the final manuscript.

Funding/Support

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