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Research Article

Prevalence, Antibiotic Resistance, and Genetic Diversities of *Clostridium difficile* in Meat Nuggets from Various Sources in Isfahan, Iran

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Clostridium difficile is recognized as one of the leading causes of diarrhea and is responsible for almost all cases of pseudomembranous colitis. The main aim of this study was to determine the prevalence and genetic diversities of *Clostridium difficile* contamination in meat nuggets in Isfahan (Iran). To achieve this, 100 samples of chicken, ostrich, quail, shrimp, fish, and beef nuggets were collected by the simple random sampling method from the market of Isfahan from July 2018 to July 2019. Microbiological analyses were performed on each collected sample to isolate and identify *C. difficile* strains. Susceptibility of the isolated strains to antibiotics was assessed by the Kirby–Bauer method. The PCR technique was used to identify the genes responsible for producing toxins. Of the 600 food samples, 7 *C. difficile* strains were found, representing a prevalence of 1.17%. Based on the obtained antibiogram results, the highest resistance was related to ampicillin (100%) and then amoxicillin (85.72%), and the highest susceptibility was related to vancomycin (100%) and metronidazole (85.72%). Concerning the identification of the genes responsible for the production of the toxins, it appears that que 4 samples (57.14%) had *tcdA* genes, 2 samples (14.57%) had *tcdC* genes, and 7 samples (100%) had *tcdB* genes. The *cdtA* and *cdtB* genes were observed in only one positive sample on beef. This study showed the presence of *C. difficile* in the analyzed food products. The isolated strains are toxigenic and resistant to antibiotics, except for vancomycin. A mutation would be the basis for the absence of the *tcdC* gene in the genome of all isolates.

1. Introduction

Clostridioides (*Clostridium difficile* or *C. difficile*) is a Gram-positive, spore-forming, strictly anaerobic, cytotoxin-producing bacterium. Their optimal growth temperature is between 35 and 40°C [1–3]. The prevalence of *C. difficile* in the intestinal tract of healthy individuals is 2–3% and 40% in newborns [4]. This pathogen is recognized as one of the main causes of infectious diarrhea [1]. This pathogen and *Clostridium perfringens* are responsible for almost all cases of pseudomembranous colitis [4]. Moreover, people suffering

from inflammatory bowel disease and cancer, those taking immunosuppressive drugs, or those taking antibiotics during treatment may have an increased risk of developing *C. difficile* infection [5]. This bacterium came into the limelight in 1978 as the leading cause of diarrhea caused by antibiotics, called "Antibiotic-Associated Diarrhea." Also, this disease was introduced as the prime cause of pseudomembranous colitis and patient mortalities, particularly in the elderly [6]. Toxin A (*tcd A*) and toxin B (*tcdB*) are two virulence factors associated with *C. difficile* infection, which are enterotoxin and cytotoxin, respectively [7].

C. difficile was formerly known as an essential nosocomial pathogenic bacterium, with healthcare facility environments considered the most important sites of infection [7]. Since 2003, the severity and mortality rate of "*C. difficile* nosocomial infection" has increased significantly in North America and many European countries [5]. Furthermore, several changes in bacterial epidemiology have been observed, including "community-acquired *C. difficile* infection," the occurrence of the disease in young people without risk factors, the emergence of highly invasive strains, the emergence of fluoroquinolone-resistant strains, increase in disease incidence, mortality, and similarities between *C. difficile* isolated from humans and animal feces [5]. Also, in 2015, *Clostridium difficile* infection (CDI) was responsible for approximately 453,000 cases of diarrhea and 29,000 deaths in the United States [8]. The increase in infections has been primarily associated with the emergence of highly virulent strains of *C. difficile* [9]. In particular, ribotype 027 strains are responsible for severe *C. difficile* infections, characterized by a high recurrence rate, mortality, and refractoriness to traditional treatments [9].

The treatment of *C. difficile* infections is based on antibiotic therapy [10, 11]. Antibiotic resistance increases the risk of *C. difficile* infection [10, 11]. One theory proposed to explain the significant outbreaks reported is that the fluoroquinolone-resistant strain 027 was contaminated when fluoroquinolone was used every day in hospitals. Standard antimicrobial therapy for *C. difficile* infections has changed little since the disease was first described [10, 11]. Vancomycin (VAN) is a primary treatment [10, 11]. The bacteria can be transmitted to humans through food [12]. The high genotypic similarity between strains isolated from patients, food, and animals has raised questionsabout the possibility that food may be a vehicle for the transmission of *C. difficile* to humans [13]. The contamination of food for human consumption by *C. difficile* strains varies from 1.6% to 15.4% [13].

In Iran, *C. difficile* strains of ribotype 078 were identified as the most frequent isolate in hospitalized diarrhea patients tested in the Isfahan Region [14]. However, the status of *C. difficile* in raw meat and human consumption food is unknown due to the lack of scientific data on the problem. In this context, the present study was conducted to sensitize the local population on the respect of hygiene rules and the health actors regarding the prescription of antibiotics to limit or eradicate diarrheal diseases linked to this pathogen. This study aimed to determine the prevalence and genetic diversities of *C. difficile*-contaminated meat nuggets in Isfahan (Iran).

2. Materials and Methods

2.1. Study Procedures.
In this cross-sectional descriptive study, 600 samples of chicken, ostrich, quail, shrimp, fish, and beef nuggets (100 samples each) were collected by the simple random sampling method from the market of Isfahan from July 2018 to July 2019 and transported on ice to the Research Center for Nutrition and Organic Products, Islamic Azad University, Shahrekord Branch, Iran.

2.2. Microbiological Analysis.
In order to isolate *C. difficile*, 5 grams of meat and feces samples of native birds were enriched in 45 ml of *C. difficile* broth (CDB) and were anaerobically incubated at 37°C for 10–15 days. The samples were cultured on *C. difficile* Moxalactam-Norfloxacin (CDMN) agar. Multiple colonies from each sample were identified by phenotypic experiments including colony morphology, gram staining, colony odor, and L-proline aminopeptidase disk. The DNA of colonies identified by the classical method was extracted by E.Z.N.A.® Stool DNA kit

2.3. Molecular Analysis.
Multiplex PCR was used to detect the *tcdA*, *tcdB*, *tcdC*, *cdtA*, and *cdtB* genes of toxigenic *C. difficile* isolates. In brief, the PCR mixture was consisting of $2.5\,\mu L$ of PCR buffer, $2\,\mu L$ of each deoxynucleotide triphosphates (dNTP) at a concentration of 10 mM; 1 unit of single DNA polymerase enzyme; $5\,\mu L$ template DNA and $0.1\,\mu L$ of each primer including *tcdA*, *tcdB*, *tcdC*, *cdtA*, and *cdtB*; and sterilized deionized water. The thermal cycle involved the following steps: "initial denaturation" at 94°C for one minute, annealing at 94°C for 45 seconds, annealing at 52°C for 60 seconds, and extension at 72°C for 80 seconds, based on the method introduced by Torki Baghbaderani et al. [15]. The PCR products were visualised by electrophoresis on 1.5% agarose gel for 1 hour at 80 V. The gel was stained with ethidium bromide solution, and isolated bands were observed using UV-doc [16]. For PCR-ribotyping reaction, it was performed in a total volume of $100\,\mu L$ containing $200\,\mu M$ of each dNTPs mix, 1.5 mM MgCl2, 2.5 U of Taq DNA polymerase, $50\,\mu l$ of each primer, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, and $10\,\mu L$ of DNA extract. The amplification was 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, and 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 [16]. In the molecular tests, the strains of *C. difficile* ribotypes 027 and 078 received from Guelph University in Canada were used as positive controls.

2.4. Antibiotic Resistance Analysis.
Antimicrobial susceptibility testing to different antibiotics was performed using the gradient Etest (bioMérieux) and disc diffusion (Kirby–Bauer method). According to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI) released in 2018, the minimal inhibitory concentration (MIC) determination of the following breakpoints was used. The MIC interpretive breakpoints for vancomycin were based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Diameters of the inhibition zones were interpreted based on the CLSI guidelines for the disc diffusion method. In this method, antibiotic resistance was measured using standard disks of such antibiotics as amoxicillin, ampicillin, ceftaroline, clindamycin, linezolid, meropenem, metronidazole, moxifloxacin, penicillin, pyracylene, tetracycline, and vancomycin on the Mueller–Hinton agar medium according to the relevant protocols [17, 18]. The diameter of

TABLE 1: Examining the infection and genetic diversities of *C. difficile* strains in nuggets.

Types of nuggets	Number of nuggets samples	Number of samples infected with *C. difficile* of nuggets	Test based on L-aminopeptidase	Test based on PCR gel	Toxin genes				
					tcdA	*tcdB*	*tcdC*	*cdtA*	*cdtB*
Chicken	100	1	1	1	+1	+1	-	-	-
Ostrich	100	-	-	-	-	-	-	-	-
Quail	100	-	-	-	-	-	-	-	-
Shrimp	100	2	2	2	+2	+2	+1	-	-
Fish	100	1	1	1	-	+1	-	-	-
Beef*	100	3	3	3	+1	+3	+1	+1	+1
Total	600	7	7	7	4	7	2	1	1

* Ribotypic 027 strain related to beef nuggets were reported.

TABLE 2: Examining resistance and susceptibility of *C. difficile* strains in chicken, duck, partridge, and quail meat.

Antibiotics	Range	Concentration of antibiotics	Resistance	Susceptible (percentage)	Intermediate (percentage)	Resistance (percentage)
Amoxicillin	1–10	2	Resistant	0	1 (14.28)	6 (85.72)
Ampicillin	5–25	5	Resistant	0	0	7 (100)
Ceftaroline	6–64	52	Intermediate	2 (28.75)	3 (42.85)	2 (28.75)
Clindamycin	1–16	1	Resistant	1 (14.28)	1 (14.28)	5 (71.42)
Linezolid	1–10	4	Intermediate	5 (71.42)	1 (14.28)	1 (14.28)
Meropenem	5–25	17	Intermediate	2 (28.75)	3 (42.85)	2 (28.75)
Metronidazole	0.125–80	80	Susceptible	6 (85.72)	1 (14.28)	0
Moxifloxacin	1–10	6	Intermediate	2 (28.75)	3 (42.85)	2 (28.75)
Penicillin	1–10	4	Resistant	0	2 (28.75)	5 (71.42)
Pyracylene	—	12	Intermediate	2 (28.75)	3 (42.85)	2 (28.75)
Tetracycline	—	19	Intermediate	1 (14.28)	5 (71.42)	1 (14.28)
Vancomycin	0.25–4	3	Susceptible	7 (100)	0	0

the zone of inhibition was read and interpreted after 48 hours of anaerobic incubation at 37°C. To measure antibiotic resistance, one single colony of each strain resistant to aforementioned antibiotics was examined. These discs include amoxicillin (10 μg), ampicillin (25 μg), ceftaroline (64 μg), clindamycin (16 μg), linezolid (10 μg), meropenem (25 μg), metronidazole (8 μg), moxifloxacin (10 μg), penicillin (10 μg), pyracylene (16 μg), tetracycline (30 μg), and vancomycin (4 μg). Based on the specification of the disks, the antibiogram test report for each antibiotic was characterized as susceptible, resistant, and intermediate.

2.5. Statistical Analysis. The different data obtained were entered into Excel (Microsoft Corp., Redmond, WA, USA) before being analyzed. The SPSS software (SPSS Inc., Chicago, IL, USA) was used for the different statistical tests performed.

3. Results

Based on the morphological examination of the obtained colonies on each plate, 7 samples (1.17%) contained *C. difficile*. White-gray, opaque, circular, and slightly raised colonies indicated the presence of *C. difficile*. In addition, the gel PCR test was carried out on all samples to confirm the diagnosis. To identify and detect the *tcdA*, *tcdB*, *tcdC*, *cdtA*, and *cdtB* genes, positive samples were evaluated using Multiplex PCR. The use of this method revealed that 4 samples (57.14%) had *tcdA* genes, 2 samples (14.57%) had

tcdC genes, and 7 samples (100%) had *tcdB* genes. The *cdtA* and *cdtB* genes were observed in only one positive sample on beef. Among them, one ribotypic 027 strains (14.29%) related to beef nuggets were reported (Table 1).

In this study, in the antibiotic resistance test, when the antibiotic concentration inhibits the growth of *C. difficile* by more than 70%, it is considered susceptible; when the antibiotic concentration inhibits the growth of *C. difficile* between 30% and 70%, it is considered intermediate; and when the antibiotic concentration inhibits the growth of *C. difficile* less than 30%, it is considered resistant. Based on the obtained antibiogram results, the highest resistance was related to ampicillin (100%) and then amoxicillin (85.72%), and the highest susceptibility was related to vancomycin (100%) which is an effective drug to treat *C. difficile* infection (Table 2).

4. Discussion

Surveillance of *C. difficile* using phenotypic and genotypic approaches is a critical element in the strategy to understand and reduce the impact of *Clostridium difficile* infections on global health systems. The main aim of this study was to determine the prevalence and genetic diversities of *Clostridium difficile*-contaminated meat nuggets in Isfahan (Iran).

The accumulation of resistance mechanisms gives an advantage to *C. difficile*, as the disease can develop after using antimicrobials due to alteration of the gut microbiota

[1]. To achieve this, 600 samples of chicken, ostrich, quail, shrimp, fish, and beef nuggets (100 samples each) were collected by the simple random sampling method from the market of Isfahan from July 2018 to July 2019. Microbiological analyses were performed on each collected sample to isolate and identify *C. difficile* strains.

Of the 600 food samples, 7 species of *Clostridium difficile* were found, representing a prevalence of 1.17%. It has been noted through some of the work carried out that the identification rate of the bacterium (*C. difficile*) is generally lower than 9%, especially in beef and chicken [19–21]. On the other hand, a high prevalence of up to 42% has been determined in several studies [19, 22]. In Europe, low prevalence rates of up to 4.3% were reported, while in North America, reported prevalence rates were higher (44%) [23]. The differences obtained would be related to the different methods used to isolate and identify *C. difficile*. According to Lund and Peck [23], this bacterium has several enrichment and isolation media. The different prevalence obtained could also be justified by the difference between the matrices (biological material) and the collection technique and hygiene of each site. Indeed, the reservoir of *C. difficile* is digestive, with a sometimes-asymptomatic carriage in animals [19, 21]. The bacterium is transmitted to humans through contaminated food and meat. The triggering factor of the infection is a modification of the digestive flora, most often linked to the administration of antibiotics, especially those with an excellent activity on the anaerobic flora (penicillins, cephalosporins, and lincosamides) [19, 22]. The disruption of the natural barrier effect of the digestive flora favors the implantation and proliferation of *C. difficile*. The clinical manifestations are linked to the production of the two toxins A and B, which act in synergy [19, 22].

Based on the obtained antibiogram results, the highest resistance was related to ampicillin (100%) and then amoxicillin (85.72%), and the highest susceptibility was related to vancomycin (100%) and then metronidazole (85.72%), which is an effective drug to treat *C. difficile* infection. Metronidazole and vancomycin are the antibiotics recommended for treating simple and severe infections due to *C. difficile* [24]. According to Ersöz and Coşansu [24], resistance to metronidazole and vancomycin is rare, but a decrease in sensitivity is emerging. This situation was observed in this study. Indeed, a sensitivity of 85.72% was observed for metronidazole, but a sensitivity of 100% was observed for vancomycin. In addition, Varshney et al. [25] achieved 100% sensitivity to vancomycin among *C. difficile* strains isolated from meat samples. In opposite, according to a study by Saha et al., the resistance of *C. difficile* to vancomycin is on the increase, with a smaller, declining resistance to metronidazole. We also observed resistance to tetracycline, clindamycin, and moxifloxacin in the present study that was recorded in an earlier study [26]. The importance of antibiotic resistance in *C. difficile* was highlighted by the epidemic in the early 2000s. The widespread use of fluoroquinolones led to the emergence of the resistant ribotype 027 strain, which contributed to the epidemic [26]. Antibiotic resistance in *C. difficile* may create a survival advantage for resistant strains causing therapeutic failure

and increasing chances of recurrence. These effects may be amplified by the effect of the drug on the gut microflora. The past two decades have seen a rise in the clinical failure rates with metronidazole and vancomycin, raising concerns that *C. difficile* could be developing antibiotic resistance, which is leading to clinical failures [26]. Owing to low cure rates with metronidazole, the current Infectious Diseases Society of America (IDSA) guideline recommends vancomycin or fidaxomicin as the antibiotic of choice for an initial episode of CDI [26].

With regard to the identification of the genes responsible for the production of the toxins, it appears that que 4 samples (57.14%) had *tcdA* genes, 2 samples (14.57%) had *tcdC* gene, and 7 samples (100%) had *tcdB* genes. The *cdtA* and *cdtB* genes were observed in only one positive sample on beef. The virulence of *C. difficile* has been linked to the production of two toxin molecules: toxin A and toxin B. They are encoded in the pathogenicity locus (PaLoc). Indeed, these toxins cause intestinal damage and ultimately clinical disease. Toxin A binds to a specific glycoprotein receptor on the brush border, resulting in an influx of polynuclei to the *lamina propria* and an intense inflammatory response. Toxin B, which has no direct effect on the intestinal epithelium, then exerts its strong cytotoxic activity on the digestive mucosa previously injured by toxin A. Both toxins have the same enzymatic activity. Upon entering the intestinal epithelial cells, they catalyze the transfer of glucose onto the Rho family of GTPases, resulting in a reorganization of the actin cytoskeleton, a complete rounding of the cells, and destruction of the intestinal barrier function. This causes diarrhea and, in some cases, can lead to a severe inflammatory response and pseudomembranous colitis. Among all *C. difficile* strains, a ribotypic 027 strain, linked to beef nuggets, was reported. This was the only strain out of all the strains isolated. Compared to other studies in this region, the prevalence of ribotypic 027 has decreased. Recent studies indicate that toxigenic *C. difficile* may be present in food and meat products, usually at low levels [20, 21, 24]. Two strains of toxigenic *C. difficile* (toxin producers A and B) isolated from chicken and fish samples lacked the *tcdC* gene. This result could be related to a mutation of the gene that did not allow the primer used to identify this regulatory gene. Indeed, the *tcdC* gene in the *C. difficile* pathogenicity locus encodes the putative negative regulator of toxin A and B production. A base pair deletion (in particular 18 bp) in the *tcdC* gene of the strain was initially considered to be responsible for the toxin hyperproduction. It has recently been reported that a single nucleotide mutation at position 117, causing a frameshift that introduces a stop codon resulting in truncation of the *tcdC* gene product, is the most likely mechanism for this hyperproduction. Given the existence of toxigenic strains, along with the opportunistic nature of the disease induced by *C. difficile*, the role of epidemiologic studies in determining the possible causes of diseases is significant. Also, to limit the implications of contaminated meat, many food treatment procedures should be taken into account, although the spore-forming nature of *C. difficile* and the heat tolerance of spores make it difficult to disinfect food through food preparation. So, the existence of toxigenic

and antibiotic-resistant strains of *C. difficile* in food is a real public health problem. This situation deserves special attention from the authorities in order to take necessary and effective measures to limit or eradicate the virulent and resistant strains of *C. difficile* responsible for food poisoning and infectious diarrhea.

5. Conclusions

The control of infectious diarrhea due to virulent strains of *C. difficile* must be based on a better understanding of the contamination of food products. The results of this study confirm the existence of *C. difficile* in the food products analyzed. The isolated strains are toxigenic and resistant to some antibiotics. The consumption of this group of animals is the favorite of Iranian people. Therefore, appropriate cooking of these animals is recommended. More studies are suggested to understand the different aspects of the epidemiology of *C. difficile* in Iran.

Data Availability

The data that support the findings of this study are available from the corresponding author upon request.

Disclosure

The funders had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

Conflicts of Interest

The author declares that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

Authors' Contributions

Formal analysis, methodology, investigation, writing—original draft, and writing—review and editing were done by Parvin Ghorbani. Supervision, writing—original draft, and writing—review and editing by Amir Shakerian. Supervision and writing—review and editing by Ebrahm Rahimi. Supervision, resources, project administration, funding acquisition, validation, visualization, methodology, and writing—review and editing by Zahra Esfandiari.

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