Research Article

Clostridioides difficile in Foods with Animal Origins; Prevalence, Toxigenic Genes, Ribotyping Profile, and Antimicrobial Resistance

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Clostridioides difficile is an important nosocomial pathogen and is considered as a reason of diarrhea and gastrointestinal infections. As a majority of community-originated C. difficile cases are not related to antibiotic prescription and hospitalization, the food portion as a vector of infection transmission has been raised. An existing survey was aimed evaluating the prevalence, antimicrobial resistance, profile of toxigenic genes, and ribotypes of C. difficile isolated from raw meat and carcass surface swab samples. In total, 485 raw meat and carcass surface swab samples were collected. C. difficile was isolated via culture and a diverse biochemical examination. The assessment of minimum inhibitory concentration (MIC) was addressed to evaluate the antibiotic resistance of isolates. Toxin genes detection and ribotyping were used for isolates characterization. The prevalence of C. difficile contamination in all examined samples was 3.71%. The bacterium was detected in 2.91% of raw meat and 4.48% of carcass surface swab samples. Raw sheep meat (5%) and sheep carcass swab (7.50%) samples harbored the highest C. difficile prevalence. The highest rate of antibiotic resistance was observed toward clindamycin (38.88%), ciprofloxacin (38.88%), metronidazole (44.44%), erythromycin (72.22%), and tetracycline (77.77%). C. difficile bacteria showed the minimum rate of resistance meropenem (16.66%) and chloramphenicol (16.66%). TcdA, tcdB, cdtA, and cdtB toxigenic genes were detected in 22.22%, 44.44%, and 16.66% of isolates, respectively. TcdB + tcdA (27.77%) were the most prevalent combined toxigenic gene profile. Both 027 and 078 ribotypes were identified in C. difficile isolates. The role of raw meat and carcass surface swab samples as toxigenic and antibiotic-resistant C. difficile strains vectors was signified. This study authorizes that food animals, particularly sheep and cattle, are C. difficile carriers at slaughter stages and ribotypes are equal in human cases. Subsequently, contamination of carcasses occurs inside the slaughterhouse.

1. Introduction

Clostridioides difficile (C. difficile), an anaerobe Gram-positive spore-bearing bacterium, is the causative bacterium of dangerous diseases, including toxic megacolon, pseudomembranous colitis, and diarrheal syndromes after antibiotic prescription [1]. In severe cases, the disease can lead to death [2]. There were about 15, 512 C. difficile cases in 2017 in the United States [3]. C. difficile infections (CDIs) prevalence amongst the some Asian countries was 12.40% according to a meta-analysis conducted on September 30, 2021 [4].

CDIs are more prevalent amongst hospitalized patients due to the contaminated environment and antibiotic therapies [5]. However, there are records of genetic intersection amid C. difficile strains of animals and humans, signifying the CDI transmission zoonotic feature to the human population [6]. In this regard, three main issues have increased concern rendering the C. difficile potential to be a food-borne disease, including the cumulative recognition of community-associated CDI, previous investigations identifying C. difficile in food and food animals, and some similarities in the genetic bases, toxigenic profile, antimicrobial resistance, and ribotyping pattern of C. difficile.
isolates from animals, food, and humans [7, 8]. Investigations have revolved around the _C. difficile_ assessment on the animal species carcasses at slaughter [9, 10]. These researches displayed that animal species are _C. difficile_ carriers and contamination of livestock carcasses occur during the slaughter stages and also at the time of meat supply, signifying a retail meat contamination potential risk [11, 12]. Previous studies have shown the _C. difficile_ presence in bovine, swine, ovine, buffalo, caprine, camel, and poultry [13–16] meat samples, which confirm the possible role of meat in bacterial transmission.

Pathogenesis of CDI relies on the presence of some virulence and toxigenic factors. Enterotoxin (Toxin A) and cytoxin (Toxin B) encoded _tcdA_ and _tcdB_ genes can directly affect the host epithelial cells [17]. Binary toxins divided into enzymatic (_cdtA_) and binding (_cdtB_) components, caused increased bacterial adherence to the intestinal epithelium. _C. difficile_ virulence is mostly related to the presence of these toxins [18]. Some _C. difficile_ hypervirulent ribotypes, such as 078 and 027 are considered as highly toxin producers and sporulation attributes [19]. These two ribotypes are accompanied by severe CDI cases in the human population and are also related to those isolated from food animals and food samples [20, 21].

However, antibiotic therapy may cause CDI development, but antibiotic prescription can diminish the signs of CDIs. Additionally, determination of the _C. difficile_ antibiotic resistance may help a lot in finding the best therapeutic approaches and changing the infection epidemiology. In CDI development after antibiotic therapy, clindamycin, moxifloxacin, and tetracycline are considered high-risk antimicrobial agents [22]. In CDI treatment, metronidazole, fidaxomicin, and vancomycin are recommended [22]. In keeping with this, recent studies showed that food-originates _C. difficile_ harbored a high rate of resistance against varied types of antimicrobial agents, such as tetracyclines, fluoroquinolones, macrolides, and penems [23, 24]. Thus, it is essential to assess the changes that occurred in the _C. difficile_ pattern of antibiotic resistance.

There are scarce data available on the _C. difficile_ prevalence, antibiotic resistance, and molecular properties of food in Iran [4]. Moreover, the number of studies performed on antibiotics susceptibility testing, polymerase chain reaction (PCR)-ribotyping, and the toxigenic gene profile for _C. difficile_ is restricted in Iran. It would seem, this is the first research to study the rate of raw meat and carcass surface swab samples contamination with _C. difficile_ in Iran, and describe the toxigenic profiles, antibiotic resistance, and PCR-ribotyping.

2. Materials and Methods

2.1. Ethics. Ethically, all aspects of the survey were approved by related Committee. Information of managers, workers, staffs, and owners of livestock were kept undisclosed.

2.2. Study Area. Figure 1 shows the map of the study area. The study was performed from November 2021 to March 2022 in Mazandaran and Golestan Province, North of Iran. Golestan Province is located at 55.1376°E and 37.2898°N at 3820 m. a. s. l. average altitude and 510 km far from Tehran. The province climate is mainly humid and rainy. The mean annual rainfall and temperature of Golestan Province are 609 mm and 17.6°C, respectively. Mazandaran Province is located at 36.2262°N, 52.5319°E at 1500 to 3000 m. a. s. l. average altitude (depends on location) and 189.2 km far from Tehran. The province’s climate is mainly humid and rainy. The mean annual rainfall and temperature of Mazandaran Province are 977 mm and 18.3°C, respectively.

2.3. Samples. A total of 485 samples, including raw cattle (_n_ = 80), sheep (_n_ = 80), goat (_n_ = 80) meat and cattle (_n_ = 85), sheep (_n_ = 80), and goat (_n_ = 80) carcasses surface swab samples were randomly collected from animals referred to slaughterhouses in North Iran. The targeted organ for the raw meat sampling was the tight muscle. For that, 100 g of muscle was collected using forceps and was placed on sterile plastic bags. Collected samples were transferred quickly (within 2 h) in refrigerated condition to the laboratory. Sample transportation and processing were done within 2 h after collection. Carcass swab samples were taken from a 20 cm² area of the tight muscle after bleeding, skinning, eviscerating, and washing stages using the swabbing technique. At first, swab samples were got wet using buffered peptone water contained cysteine 0.5% (Oxoid, UK).

2.4. _C. difficile_ Isolation and Identification. Twenty-five grams of collected raw meat samples were added to 25 ml of phosphate-buffered saline (PBS; Merck, Germany) in a sterile bottle. After that, 1 ml of the PBS-and-meat mixture and also 1 ml of the swab solutions were removed and inoculated into 9 ml of _C. difficile_ broth (CDB; Oxoid SR0048, UK), which contained disodium hydrogen phosphate (5.0 g/l), protease peptone (40 g/l), sodium chloride (2.0 g/l), fructose (6.0 g/l), magnesium sulfate (0.1 g/l), and sodium taurocholate (1.0 g/l). Cultures were supplemented with _C. difficile_ selective supplement (Oxoid SR0173, UK) and defibrinated sheep blood (5% (v/v)). Cultures were anaerobically incubated at after incubation at 37°C for 10 to 15 days. At that time, 2 ml of the incubated broth were added to 2 ml of ethanol (96%, Merck, Germany) and homogenized for 50 min using a shaker. The centrifugation procedure was done for 10 min at 3800 × g. At that point, a loopful of the sediment was streaked onto _C. difficile_ agar base (Oxoid CM0601, UK) supplemented with antibiotic (Oxoid SR0173, UK) and defibrinated sheep blood (7% (v/v)). At that point, the plates were incubated anaerobically at 37°C for 48 h [25]. Three colonies per plate were subcultured onto tryptone soya agar (TSA; Oxoid CM0131, UK) and tested by standard microbiological and biochemical procedures including Gram staining, odor, colony morphology, and L-proline aminopeptidase production [26].

2.5. _C. difficile_ Antibiotic Resistance. For this goal, the minimum inhibitory concentrations (MICs) were
determined using the principles suggested by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (https://www.eucst.org) and the Clinical and Laboratory Standard Institute (CLSI) M11-A8 and M100-S28) [27–29]. As a result, the Epsilon test (E-test strips, BioMérieux, France) was performed. Eleven diverse antimicrobial agents, including vancomycin (resistance ≥2 µg/mL), rifampin (resistance ≥4 µg/mL), erythromycin, moxifloxacin, levofloxacin, ciprofloxacin, clindamycin (resistance ≥8 µg/mL), tetracycline, meropenem (resistance ≥16 µg/mL) chloramphenicol, and metronidazole (resistance ≥32 µg/mL) were applied. For this goal, the Brucella agar media (Oxoid, UK) were applied. Media were complemented with 5 mg/L chlorhematin, 1 mg/L vitamin K1, and 5% defibrinated sheep blood (Oxoid, UK).

2.6. DNA Extraction. First of all, isolates were cultured on Tryptone Soya broth (TSB; Merck, Germany). After incubation for 48 h at 37°C, DNA was extracted from the bacteria using the DNA extraction kit (Thermo Fisher Scientific, St. Leon-Rot, Germany). All principles were followed in rendering the guidelines of the kit’s manufacture. The quality and quantity for DNA were checked (by gel electrophoresis and NanoDrop, respectively).

2.7. Detection of Toxigenic Genes. Table 1 displays the primers, reaction volumes, and thermal programs in all PCR reactions [30]. A Eppendorf Mastercycler device (5330, Eppendorf, Germany) was applied for the thermal procedure. Electrophoresis on 2.5% agarose gel stained with

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![Study area map](image-url)
<table>
<thead>
<tr>
<th>Target genes</th>
<th>Housekeeper genes</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>Thermal procedure</th>
<th>Volumes (50 μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tcdA</td>
<td>Toxin A, enterotoxin effects on epithelial cells</td>
<td>GCATGATAAGGCAACTTCAGTGGTA AGITCTCCTGCTGCCATCAATG CCAARTGGAGTGTACCAACAGGTG GCATTCTCCATTCTACGCAAAGTA GGAAAGACATATATGAAGACAGAA GC</td>
<td>629</td>
<td>1 cycle: 94°C—10 min</td>
<td>5 μL PCR buffer 10x 2 mM MgCl₂ 150 μM dNTP 0.75 μM of each primer F &amp; R 1.5 U FastStart Taq polymerase (Roche Diagnostics, Mannheim, Germany)</td>
</tr>
<tr>
<td>tcdB</td>
<td>Toxin B, cytotoxic effects on epithelial cells</td>
<td>GCATTTCTCCATTCTACGCAAAGTA GC</td>
<td>410</td>
<td>35 cycles: 94°C—50 s</td>
<td></td>
</tr>
<tr>
<td>cdtA</td>
<td>The binary toxin, enzymatic component of binary toxin</td>
<td>TTGGATTTACAGGTACCATTTTACGACCA TGGACCCAAAGGTGATGTCGTGATTG CGGATCTCTGCTCCAGTCTTTATAG</td>
<td>221</td>
<td>1 cycle: 72°C—50 s</td>
<td></td>
</tr>
<tr>
<td>cdtB</td>
<td>The binary toxin, binding component of binary toxin</td>
<td>TTGGATTTACAGGTACCATTTTACGACCA TGGACCCAAAGGTGATGTCGTGATTG CGGATCTCTGCTCCAGTCTTTATAG</td>
<td>262</td>
<td>1 cycle: 72°C—3 min</td>
<td></td>
</tr>
</tbody>
</table>
0.4 μg/ml ethidium bromide at 120 V/208 mA was done to assess PCR products. The visualization was done using the UV light (Grade GB004, Jencons PLC, London, UK). Positive (C. difficile (ATCC 43255)) and negative (PCR-grade water (Thermo Fisher Scientific, Germany)) controls were also applied.

2.8. PCR-Ribotyping. PCR-ribotyping was done by amplification of ribosomal (16S and 23SrRNA) genes. For this purpose, specific primers of 16SrRNA (3′-GTGCGG CTGGATCACCTCCT-5′) and 23SrRNA (3′-CCCTGC ACCCTTAATAACTTGACC-5′) [31] were used. Amplification reactions were made in 100 μl volume containing KCl (50 mM), Tris-HCl (10 mM, pH 8.8), MgCl2 (1.5 mM), primer F (50 pmol) primer R (50 pmol), dXTP (200 μM), FastStart Taq DNA polymerase (2.5 units, Roche Diagnostics, Mannheim, Germany), and DNA sample (10 μl with 50 ng concentration) (or distilled water as a negative control). Additional or removal of dimethyl sulfoxide (5%, DMSO, Merck, Germany) was considered a comparison. Thermal cycling included a cycle of 95°C for 2 min, 35 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a cycle of 72°C for 5 min. Electrophoresis using 10% polyacrylamide gel at 20 W for 6 h using a 24 cm distance amid electrodes was applied. Gel was stained by ethidium bromide and after that results were visualized by UV light. Figure analyzing was done by Image Master™ software (Bio-Rad). 027 and 078 ribotypes profiles recorded in reference laboratories were used for comparison [31].

2.9. Statistical Analyses. SPSS Statistics 21.0 (SPSS Inc., Chicago, IL, USA) software was applied for data analysis. Two qualitative tests, the chi-square and Fisher’s exact two-tailed, were applied for determination of significance amid obtained data. Statistically significant level was determined as P value < 0.05.

3. Results and Discussion

3.1. C. difficile Prevalence amongst the Examined Samples. Table 2 shows the C. difficile prevalence amongst the examined raw meat and surface carcass swab samples. Eighteen out of 485 (3.71%) examined raw meat and surface carcass swab samples were contaminated with C. difficile. C. difficile prevalence amongst the raw meat and surface swab samples collected from animal species was 2.91% and 4.48%, respectively. A significant difference was obtained for the C. difficile prevalence between raw meat and surface swab samples (P < 0.05). Amongst the samples of raw meat, raw sheep meat harbored the highest C. difficile prevalence (5%). Additionally, amongst the samples of the surface swab, sheep carcasses harbored the highest C. difficile prevalence (7.50%). Significant differences were obtained between C. difficile prevalence and sample type (P < 0.05).

CDI is progressively documented as a diarrheal cause in outpatients and people with no contact with health care centers. Community-associated infections have been distinguished in peoples conventionally measured to be low risks, such as the young and those without antibiotic exposures. Food has been theorized as a likely C. difficile source in the community, but incomplete evidence is available to refute or confirm it [32]. In food animals, C. difficile is considered both a gut colonizer and a diarrheal cause. Existing research studies have isolated C. difficile from retail foods envisioned for human consumption in different countries, including Canada, the United States, and Europe [33]. An enhanced thought of the association between C. difficile human and animal strains will help to assess the possibility of bacterial food-borne transmission and animal-human contact role in C. difficile epidemiology. However, there are large data gaps on C. difficile epidemiology in foodstuff. In the present study, 2.91% of raw meat samples and 3.71% of carcass surface swab samples were contaminated with C. difficile spores. In comparison with previous records [34–36], our study reported lower C. difficile prevalence in raw meat samples and carcass surface swabs. Nevertheless, the reported prevalence rate in the contemporary research was higher than those reported in previous investigations [37, 38]. A Korean study [39] showed that the C. difficile prevalence amongst the raw chicken, pork, and beef meat samples was 16.40%, 8.30%, and 6.80%, respectively. In another Iran research [40], the C. difficile prevalence amongst the raw buffalo, goat, beef, cow, and sheep meat samples was 9%, 3.30%, 1.70%, 0.94%, and 0.90%, respectively. Bakri [41] stated that the C. difficile prevalence amongst the raw cow and sheep meat samples collected from Saudi Arabia was 3.50% and 1%, respectively. In a Turkish study [42], C. difficile prevalence in cattle and sheep carcass surface samples was 33.60% and 25.30%, respectively. In Belgium [43], the contamination rate of cattle and pig carcasses with C. difficile was 7.90% and 7%, respectively. However, in Australia [44], the contamination rate of cattle carcasses with C. difficile was 25.30%. Put together, C. difficile contamination rate in retail meat has varied extensively, ranging from 0 to 43% [39]. Though, C. difficile did not identify in raw meat samples of animal species, particularly those other than cattle [41, 45, 46]. The prevalence of C. difficile amongst the poultry meat in Egypt [16], red (beef and mutton) meat in Iran [34, 47], cattle meat in Turkey [37], and meat samples in Japan [48], and South Korea [39] were 0.00%, 2.00 to 30.00%, 2.00 to 6.90%, 21.00%, 10.80%, and 29.90%, respectively [4]. Prevalence variations reported in diverse surveys may be owing to variances in geographical region, method of isolation, and levels of antibiotic administration.

Our finding showed that raw sheep meat and also sheep carcass surface swab samples harbored a higher C. difficile contamination rate than other animal species. Our finding of C. difficile higher prevalence in raw sheep meat samples is reinforced by alike investigations from other counters [25, 49]. However, some investigations determined a higher prevalence of C. difficile in bovine meat samples and carcasses [40, 42]. The chief cause of the advanced C. difficile prevalence in sheep than in cattle and goats is unclear. However, close contact between sheep with farmers and the use of human leftover food as their feed
may be possible reasons for the higher contamination rate of sheep meat and carcasses. Goat samples had the lowest contamination rate, which was similar to the findings of Rahimi et al. [40] and Bakri [41]. Goats frequently live in mountain areas where they pass at liberty with no human contact. This could explain the lower contamination rate of goat samples with \textit{C. difficile}. Carcass surface swab samples had a higher \textit{C. difficile} prevalence than raw meat. The reason for this finding is probably the contamination of the carcass surface of the animals due to manipulation and contact with the contaminated slaughterhouse environment. To put it in a nutshell, animal’s gastrointestinal tract and the occurrence of cross-contamination from the slaughters handling to animal carcasses are a major source of \textit{C. difficile} in examined samples.

### Table 2: \textit{C. difficile} prevalence amongst the examined raw meat and surface carcass swab samples.

<table>
<thead>
<tr>
<th>Types of samples</th>
<th>N. collected samples</th>
<th>N (%) positive for \textit{C. difficile}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw cattle meat</td>
<td>80</td>
<td>2 (2.50)</td>
</tr>
<tr>
<td>Raw sheep meat</td>
<td>80</td>
<td>4 (5)</td>
</tr>
<tr>
<td>Raw goat meat</td>
<td>80</td>
<td>1 (1.25)</td>
</tr>
<tr>
<td>Total</td>
<td>240</td>
<td>7 (2.91)</td>
</tr>
<tr>
<td>Cattle carcass surface swab</td>
<td>85</td>
<td>3 (3.52)</td>
</tr>
<tr>
<td>Sheep carcass surface swab</td>
<td>80</td>
<td>6 (7.50)</td>
</tr>
<tr>
<td>Goat carcass surface swab</td>
<td>80</td>
<td>2 (2.50)</td>
</tr>
<tr>
<td>Total</td>
<td>245</td>
<td>11 (4.48)</td>
</tr>
<tr>
<td>Total</td>
<td>485</td>
<td>18 (3.71)</td>
</tr>
</tbody>
</table>

### 3.2. \textit{C. difficile} Antibiotic Resistance Pattern

Table 3 displays the antibiotic resistance based on the MIC levels of \textit{C. difficile}. \textit{C. difficile} isolates from raw meat and surface carcass swab samples harbored the uppermost resistance rates against tetracycline (77.77%), erythromycin (72.22%), metronidazole (44.44%), ciprofloxacin (38.88%), and clindamycin (38.88%). The lowest resistance rate was obtained against chloramphenicol (16.66%) and meropenem (16.66%). \textit{C. difficile} isolates of raw meat samples harbored a higher resistance rate toward antibiotic agents than those of carcass surface swab samples \((P < 0.05)\). Additionally, from a statistical view, significant differences were obtained between \textit{C. difficile} resistance rate and sample type \((P < 0.05)\).

\textit{C. difficile} strains harbored a boost resistance toward some of the basic therapeutic options mainly used in CDI, particularly erythromycin, tetracycline, ciprofloxacin, metronidazole, and clindamycin. Unauthorized and indecorous antibiotic administration, antibiotics and disinfectant overdoing, and self-treatment with antibiotics can be imaginable details for the high antibiotic resistance. Antibiotic resistant strains may originate from the slaughterhouse environment and contaminated staff. In studies conducted in China [50], Saudi Arabia [51], and Iran [52], \textit{C. difficile} strains harbored a high resistance rate toward metronidazole, erythromycin, tetracycline, clindamycin, and ciprofloxacin. However, other studies conducted in Korea [39], Turkey [37], Manitoba [53], and Italy [54] showed the high susceptibility of \textit{C. difficile} strains toward metronidazole and rifampicin. Taha [55] stated that \textit{C. difficile} strains isolated from raw meat samples of animal species were susceptible to vancomycin (100%), metronidazole (100%), tetracycline (66.70%), clindamycin (73.30%), and moxifloxacin (40%). Han et al. [56] stated that the antibiotic resistance of \textit{C. difficile} strains isolated from food samples against metronidazole, vancomycin, clindamycin, erythromycin, and cefotaxime were 100%, 100%, 9.80%, 100%, and 36.60%, respectively. In a similar study, Tsuchiya et al. [57] reported that \textit{C. difficile} antibiotic resistance against clindamycin, ceftazidime, metronidazole, tetracycline, ceftriaxone, and vancomycin were 86.40%, 63.60%, 22.70%, 40.90%, 9.10%, and 13.60%, respectively. Rahimi et al. [40] reported that the prevalence of resistance against ampicillin, chloramphenicol, ciprofloxacin, clindamycin, doxycycline, erythromycin, gentamicin, metronidazole, nalidixic acid, tetracycline, and vancomycin was 53.85%, 0%, 76.92%, 92.31%, 0%, 61.54%, 100%, 0%, 100%, 30.77%, and 0%, respectively. The alterations stated in the antibiotic resistance in numerous investigations are undoubtedly because of the obtainability or nonobtainability of antibiotics, severe rules level in antibiotic prescribing, and doctors and veterinaries opinion on antibiotic prescription. Additionally, there were diverse methods (MIC and simple disc diffusion) in antibiotic resistance identification amongst the \textit{C. difficile} strains. Meropenem and chloramphenicol rates of resistance were low. It is because of the forbidden condition of chloramphenicol administration and also hospital route of meropenem.

### 3.3. Distribution of Toxigenic Genes

Table 4 shows the \textit{C. difficile} toxigenic gene profile. \textit{tcdA}, \textit{tcdB}, \textit{cdtA}, and \textit{cdtB} distribution amongst the \textit{C. difficile} isolates of raw meat and surface carcass swab samples were 61.11%, 22.22%, 44.44%, and 16.66%, respectively. Amongst the combined toxigenic genes, \textit{tcdB+tcdA} (27.27%) were the most prevalent. However, \textit{tcdA+tcdB} and \textit{tcdB+cdtB} (18.18%) harbored the lowest distribution. Significant differences were obtained between \textit{C. difficile} toxigenic genes and sample type \((P < 0.05)\).

\textit{C. difficile} strains in the current RESEARCH harbored all \textit{tcdB}, \textit{tcdA}, \textit{cdtB}, and \textit{cdtA} toxigenic genes. Some strains harbored two toxicigenic genes simultaneously. Our findings on the presence of toxicigenic genes are supported by similar reports from other counters [16, 40, 58, 59]. The distribution of \textit{tcdA} and \textit{tcdB} toxicigen genes amongst the \textit{C. difficile} isolates of raw meat in Brazil was 28.75% and 6.20%, respectively [36]. Wu et al. [36] stated that \textit{tcdA+tcdB+cdtB+cdtA} \textit{C. difficile} strains were detected in 48% of samples collected from animal carcasses in a slaughterhouse and 17% of raw meat samples in retail markets in Taiwan. In \textit{C. difficile} strains isolated from meat products [60], the total distribution of \textit{tcdA}, \textit{tcdB}, and \textit{cdtA/B} toxicigenic genes were 100%, 100%, and 86.40%, respectively. In previous studies, all \textit{tcdA}, \textit{tcdB}, \textit{cdtA}, and
<table>
<thead>
<tr>
<th>Samples (N. positive)</th>
<th>C30</th>
<th>Er</th>
<th>Cip</th>
<th>Tet</th>
<th>Mer</th>
<th>Cln</th>
<th>Met</th>
<th>Mox</th>
<th>Rif</th>
<th>Van</th>
<th>Lev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw cattle meat (2)</td>
<td>—</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>2 (100)</td>
<td>—</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Raw sheep meat (4)</td>
<td>1 (25)</td>
<td>3 (75)</td>
<td>2 (50)</td>
<td>3 (75)</td>
<td>1 (25)</td>
<td>2 (50)</td>
<td>2 (50)</td>
<td>1 (25)</td>
<td>2 (50)</td>
<td>1 (25)</td>
<td>2 (50)</td>
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<tr>
<td>Raw goat meat (1)</td>
<td>—</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>—</td>
<td>—</td>
<td>1 (100)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Total (7)</td>
<td>1 (14.28)</td>
<td>5 (71.42)</td>
<td>4 (57.14)</td>
<td>6 (85.71)</td>
<td>1 (14.28)</td>
<td>3 (42.85)</td>
<td>4 (57.14)</td>
<td>2 (28.57)</td>
<td>3 (42.85)</td>
<td>2 (28.57)</td>
<td>3 (42.85)</td>
</tr>
<tr>
<td>Cattle carcass surface swab (3)</td>
<td>—</td>
<td>2 (66.66)</td>
<td>1 (33.33)</td>
<td>2 (66.66)</td>
<td>1 (33.33)</td>
<td>2 (66.66)</td>
<td>2 (66.66)</td>
<td>1 (33.33)</td>
<td>1 (33.33)</td>
<td>1 (33.33)</td>
<td>1 (33.33)</td>
</tr>
<tr>
<td>Sheep carcass surface swab (6)</td>
<td>1 (16.66)</td>
<td>4 (66.66)</td>
<td>1 (16.66)</td>
<td>5 (83.33)</td>
<td>1 (16.66)</td>
<td>1 (16.66)</td>
<td>1 (16.66)</td>
<td>1 (16.66)</td>
<td>1 (16.66)</td>
<td>1 (16.66)</td>
<td>2 (33.33)</td>
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<tr>
<td>Goat carcass surface swab (2)</td>
<td>1 (50)</td>
<td>2 (100)</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>—</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>—</td>
</tr>
<tr>
<td>Total (11)</td>
<td>2 (18.18)</td>
<td>8 (72.72)</td>
<td>3 (27.27)</td>
<td>8 (72.72)</td>
<td>2 (18.18)</td>
<td>4 (36.36)</td>
<td>4 (36.36)</td>
<td>3 (27.27)</td>
<td>3 (27.27)</td>
<td>3 (27.27)</td>
<td>3 (27.27)</td>
</tr>
<tr>
<td>Total (18)</td>
<td>3 (16.66)</td>
<td>13 (72.22)</td>
<td>7 (38.88)</td>
<td>14 (77.77)</td>
<td>3 (16.66)</td>
<td>7 (38.88)</td>
<td>8 (44.44)</td>
<td>6 (27.77)</td>
<td>6 (27.77)</td>
<td>5 (27.77)</td>
<td>6 (33.33)</td>
</tr>
</tbody>
</table>

*N (%): isolates resist each antibiotic

toxigenic genes had an active presence in the C. difficile strains isolated from human clinical infections [61, 62]. Due to the presence of toxigenic genes in C. difficile strains isolated from meat and carcasses of livestock, these samples can be known as possible sources of transmission of virulent C. difficile strains to human communities. However, further studies are needed to substantiate this claim. In keeping with this, thorough cooking of meat before consumption can prevent the transmission of virulent strains of C. difficile. A study in Belgium [43], similarly reported that C. difficile strains isolated from animal meat and carcass samples harbored tcdA, tcdB, cdtA, and cdtB toxigenic genes.

3.4. Ribotype Distribution. Figure 2 shows a sample of 027 and 078 PCR-ribotyping of C. difficile strains.

Figure 3 shows the ribotype distribution amongst the C. difficile isolates of raw meat and surface carcass swab samples. Amongst the examined samples, C. difficile isolates of raw cattle meat (50%), raw goat meat (100%), and goat carcass surface swab (50%) samples only harbored 078 ribotypes. The distribution of 027 and 078 ribotypes amongst the raw sheep meat, cattle carcass surface swab, and sheep carcass surface swab samples were 25% and 50%, 33.33% and 66.66%, and 33.33% and 50%, respectively. Furthermore, significant differences were obtained between C. difficile ribotype distribution and sample type (P < 0.05).

PCR-ribotyping is the preferred method in C. difficile typing, because of its reproducibility, discriminatory power, and simplicity. The final section of the present study showed the presence of both 027 and 078 amongst the isolated C. difficile strains. Ribotype 078 had the highest distribution amongst the examined isolates. Ribotype 027 was only detected amongst the C. difficile isolates from raw sheep meat and carcass and also cattle carcass samples. The presence of both 027 and 078 ribotypes has been described in C. difficile strains isolated from human clinical infections [63, 64]. The presence of both 027 and 078 ribotypes has also been reported in C. difficile strains isolated from a wide range of foods, particularly meat [38, 39, 43, 65–68], milk [69], vegetable [70, 71], and ready-to-eat food [72, 73] samples. Ribotype 078 was also detected in C. difficile isolates of raw meat in the Netherlands [25]. Ribotype 027 was also detected in C. difficile isolates of raw meat in Belgium [43].

The Table 4: Toxigenic genes distribution amongst the C. difficile strains.

<table>
<thead>
<tr>
<th>Samples</th>
<th>N (%). isolates harbored each toxigenic genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tCD A</td>
</tr>
<tr>
<td>Raw cattle meat (2)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Raw sheep meat (4)</td>
<td>3 (75)</td>
</tr>
<tr>
<td>Raw goat meat (1)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Total (7)</td>
<td>5 (71.42)</td>
</tr>
<tr>
<td>Cattle carcass surface swab (3)</td>
<td>2 (66.66)</td>
</tr>
<tr>
<td>Sheep carcass surface swab (6)</td>
<td>3 (50)</td>
</tr>
<tr>
<td>Goat carcass surface swab (2)</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Total (11)</td>
<td>6 (54.54)</td>
</tr>
<tr>
<td>Total (18)</td>
<td>11 (61.11)</td>
</tr>
</tbody>
</table>
isolates harbored a high resistance rate against C. difficile. Swab samples harbored the highest contamination rates. Surface swab samples. Sheep meat and carcass surface isolated from cattle, sheep, and goat meat and carcass 027 was reported in studies conducted on Iran [75, 76] and Israel [77]. Additionally, amongst the Middle- and Far-East countries, ribotype 078 was reported in studies conducted on Iran [78], Korea [66], Taiwan [36], Japan [48], and China [34]. Therefore, paying special attention to the ribotyping pattern and toxigenic gene profile of C. difficile and examining the differences between food isolates and clinical specimens can greatly help determine the role of food in the transmission of C. difficile strains.

4. Conclusions

In conclusion, toxigenic, and antibiotic-resistant strains of C. difficile attributed to ribotypes 027 and 078 were isolated from cattle, sheep, and goat meat and carcass surface swab samples. Sheep meat and carcass surface swab samples harbored the highest contamination rates. C. difficile isolates harbored a high resistance rate against metronidazole, tetracycline, ciprofloxacin, erythromycin, and clindamycin antibiotic agents. TcdA, tcdB, cdtA, and cdtB toxigenic genes were found in the majority of isolates. Additionally, some C. difficile isolates harbored two toxigenic genes simultaneously, which shows their high pathogenicity. PCR-ribotypes 027 and 078 were predominant among isolates. Livestock carcasses swabs and raw meat samples were contaminated with two PCR-ribotypes which may show their common source of contamination and suggest a slaughterhouse environmental contamination. The role of raw meat and carcass surface swab samples in the transmission of toxigenic, virulent, and antibiotic-resistant C. difficile to the human community was also determined. Animals are C. difficile carriers at slaughter and carcass and meat contamination occur inside the slaughterhouse. Further investigations should be performed to compare the antibiotic resistance pattern, toxigenic gene profile, and ribotyping of C. difficile strains isolated from meat and humans to find the exact role of food animals in the transmission of bacterium.

Data Availability

The data are made available upon request from the corresponding author upon request.

Conflicts of Interest

The authors declare they have no conflicts of interest regarding the publication of this article.

Authors’ Contributions

Mohammad Ahmadi was involved in the conceptualization, designing, and support of the study and was responsible for writing, reviewing, and revising the manuscript. Hadi Bacheno was involved in data curation, investigation, sampling, culture, antimicrobial resistance, and molecular analysis. Fatemeh Fazeli was involved in statistical analysis. Peiman Ariaii was involved for the investigation and Methodology. All authors read and approved the final manuscript.

Acknowledgments

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