**Incidence and Profiles of Antibiotic Resistance and Putative Genes of the *Clostridium difficile* Recovered From Fish**

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**Introduction**

*Clostridium difficile* (*C. difficile*) is a toxigenic bacterium with emergence of antibiotic resistance accountable for incidence of food poisoning. The purpose of this survey was to examine the antibiotic resistance profile and incidence of toxigenic genes amid the *C. difficile* bacteria recovered from dissimilar varieties of fish samples. One-hundred and eighty-four fish samples were obtained and examined by culture technique. *C. difficile* isolates were confirmed another time using the polymerase chain reaction. PCR and disk diffusion techniques were applied for detection of putative genes and phenotypic profile of resistance. Eleven out of 184 (5.97%) fish samples harbored *C. difficile*. Common carp (17.50%) had the uppermost incidence of *C. difficile*, though *Scomberomorus guttatus* (2.50%) had the lowermost. There were no positive results for *Scomberomorus commerson* and barracuda fish samples. *TcdA* (45.45%) was the most generally perceived toxigenic genes, though *tcdC* (18.18%) was the less frequently. There were no perceived *cdtA* and *cdtB* toxigenic genes. *C. difficile* bacteria displayed the uppermost incidence of resistance toward amoxicillin (63.60%), ampicillin (54.54%), moxifloxacin (54.54%) and piperacillin (54.54%). *C. difficile* bacteria displayed the uppermost incidence of susceptibility toward meropenem (90.90%), vancomycin (90.90%) and metronidazole (72.72%). Common carp, rainbow trout and *Scomberomorus guttatus* may be reservoirs of *C. difficile*. Boostincidence of toxigenic and resistant *C. difficile* pose an imperative health threatening issue rendering the consumption of raw fish samples.

**Keywords:** *Clostridium difficile*, Toxigenic genes, Antibiotic resistance, Fish.

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sistance of C. difficile toward antibiotic agents, particularly carbapenems, quinolones, penicillins, aminoglycosides, macrolides, fluoroquinolones, cephalosporins, tetracyclines and sulfonamides [11].

Rendering to an unspecified person of C. difficile in seafoods and lack of epidemiological surveys in Iran, an existing enquiry was addressed to assess the incidence rate and toxin and antibiotic resistance profiles of C. difficile bacteria covered from fish in Isfahan, Iran.

Materials and Methods

Samples

Fish samples were collected amid October and March 2018. A convenience sample of 184 fish samples including Cyprinus carpio (common carp) (n= 40), Oncorhynchus mykiss (Rainbow trout) (n=40), Scomberomorus commerson (S. commerson) (n=32), Barracuda (n= 32) and Scomberomorus guttatus (S. guttatus) (n= 40) were purchased from marketing places of Isfahan, Iran. Fish species were identified by an expert professors of the field of aquaculture. Samples were obtained in distinct sterile belongings to avert falling and cross contamination. Ice packs were applied for samples transmission.

Isolation of Clostridium difficile

C. difficile isolation was performed rendering the protocol described beforehand [12,13]. C. difficile broth (CDB; Oxoid, UK) supplemented with different growth stimulators and antibiotics [12,13] was applied for this goal. Media were incubated at 37°C for 10 to 15 days on anaerobic circumstances. C. difficile agar base (Oxoid, UK) was applied for specific isolation of bacteria. Definitive identification was performed using the biochemical tests [12,13].

PCR procedure

Incubated media contained C. difficile isolates on the C. difficile broth were applied for DNA extraction rendering the protocols of the producing factory (Thermo Fisher Scientific, Germany). Extracted DNA samples were subjected to quantification by NanoDrop device (NanoDrop, Thermo Scientific, Waltham, USA), qualification (2% agarose gel) and purity checking (A260/A280). TPI specific gene of the C. difficile bacteria was perceived by PCR rendering the technique labeled beforehand [14].

Phenotypic profile of antibiotic resistance

Phenotypic profile of antibiotic resistance of C. difficile isolates were examined by disk diffusion. Mueller–Hinton agar (Merck, Germany) media were applied for this goal. Protocols of the Clinical and Laboratory Standards Institute (CLSI) were applied for this goal [15]. Diverse antibiotic disks (Oxoid, UK) were applied for this goal.

PCR detection of toxigenic genes

Table 1 significeth the PCR circumstances applied for detection of toxigenic genes [10]. A programmable DNA thermo-cycler (Eppendorf, Germany) was applied for this goal. Fifteen microliters of the PCR products were electrophoresed using 1.5% agarose gel[10]. Both negative and positive controls were applied for this goal.

Numerical examination

Data gotten from the experimentations were classified in the Excel software. SPSS/21.0 software was accompanied for numerical examination. Chi-square and Fisher’s tests were accompanied to measure any noteworthy association. Arithmetical denotation was determined at a P< 0.05.

Results

Table 2 discloses the incidence of C. difficile in dissimilar varieties of fish samples. Eleven out of 184 (5.97%) fish samples were positive for C. difficile. All isolates were also confirmed by PCR detection of tpi specific gene of the C. difficile. Common carp was the most frequently contaminated fish samples (17.50%). Incidence of C. difficile in S. guttatus samples was lower (2.50%). Additionally, there were no positive results for S. commerson and barracuda fish samples. Arithmetic momentous variances were gotten amid kinds of samples and incidence of C. difficile (P<0.05).

Table 3 discloses the incidence of toxigenic genes amid the C. difficile bacteria covered from dissimilar varieties of fish samples. TcdA (45.45%) had the uppermost incidence amid all perceived toxigenic genes, though tcdC (18.18%) had the lowermost. None of C. difficile bacteria covered from fish samples were not positive for cdtA and cdtB toxigenic genes. Arithmetic momentous variances were gotten amid kinds of samples and incidence of toxigenic genes (P<0.05).

Table 4 embodies the profile of antibiotic resistance of C. difficile bacteria. C. difficile bacteria harbored the uppermost incidence of resistance toward amoxicillin (63.60%), ampicillin (54.54%), moxifloxacin (54.54%) and piperacillin
TABLE 1. Target genes, oligonucleotide primers and PCR conditions used for detection of antibiotic resistance genes in the *C. difficile* bacteria recovered from various types of fish samples.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5'-&gt;3')</th>
<th>Primer concentration (µM)</th>
<th>PCR product (bp)</th>
<th>PCR programs</th>
<th>PCR volume (25 µL)</th>
</tr>
</thead>
</table>
| TcdA        | F: GCATGATAAGGCAACTTCAGTGTA  
             | R: AGTCCCTCCTGCTCCATCAAATG | 0.6              | 629           | 1 cycle: 94°C  
             |                         |               | 35 cycle: 94°C  
             |                         |               | 54°C  
             |                         |               | 72°C  |
| TcdB        | F: CCAAARTGAGTGGTTACAAACAGGTG  
             | R: GCATTCTCCATTCTCAGCAAAGTA | 0.4              | 410           | 1 cycle: 94°C  
             |                         |               | 35 cycle: 94°C  
             |                         |               | 54°C  
             |                         |               | 72°C  |
| TcdC        | F: AAAAGGGAGATTGATTATGTTTTC  
             | R: CAATAACTTAGTTACCTCAGACCTCA | 0.2              | 475           | 1 cycle: 94°C  
             |                         |               | 35 cycle: 94°C  
             |                         |               | 54°C  
             |                         |               | 72°C  |
| CdtA        | F: GGGAAGCACTATATAAAGCGACAAGC  
             | R: GGGAACATTATATAAAGCGACAAGC | 0.05             | 221           | 1 cycle: 72°C  
             |                         |               | 3 min |
| CdtB        | F: TTGACCCAAAGTTGAGTGCTGATTG  
             | R: CGGATCTCTTGCTCTAGTTTATAG | 0.1              |               |                 |
TABLE 2. Incidence of *C. difficile* bacteria recovered from dissimilar varieties of fish samples.

<table>
<thead>
<tr>
<th>Types of samples</th>
<th>No samples collected</th>
<th>N (%) of <em>C. difficile</em> positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common carp</td>
<td>40</td>
<td>7 (17.50)</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>40</td>
<td>3 (7.50)</td>
</tr>
<tr>
<td><em>S. commerson</em></td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>Barracuda</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td><em>S. guttatus</em></td>
<td>40</td>
<td>1 (2.50)</td>
</tr>
<tr>
<td>Total</td>
<td>184</td>
<td>11 (5.97)</td>
</tr>
</tbody>
</table>

TABLE 3. Toxigenic gene profile of *C. difficile* bacteria recovered from different types of shellfish samples.

<table>
<thead>
<tr>
<th>Types of samples (N samples positive for <em>C. difficile</em>)</th>
<th>N (%) isolates harbor each gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>TcdA</em></td>
</tr>
<tr>
<td>Common carp (7)</td>
<td>2 (28.57)</td>
</tr>
<tr>
<td>Rainbow trout (3)</td>
<td>2 (66.66)</td>
</tr>
<tr>
<td><em>S. guttatus</em> (1)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Total (11)</td>
<td>5 (45.45)</td>
</tr>
</tbody>
</table>

TABLE 4. Antibiotic resistance pattern of *C. difficile* bacteria recovered from different types of fish samples.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Antibiotic resistance pattern of 11 <em>C. difficile</em> bacteria recovered from fish samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>2 (18.18)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>2 (18.18)</td>
</tr>
<tr>
<td>Cefaroline</td>
<td>3 (27.27)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>4 (36.36)</td>
</tr>
<tr>
<td>Linezolid</td>
<td>3 (27.27)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>10 (90.90)</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>8 (72.72)</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>-</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>1 (9.09)</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>3 (27.27)</td>
</tr>
<tr>
<td>Penicillin</td>
<td>2 (18.18)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>10 (90.90)</td>
</tr>
</tbody>
</table>

(54.54%). Moreover, *C. difficile* bacteria harbored the uppermost incidence of susceptibility toward meropenem (90.90%), vancomycin (90.90%) and metronidazole (72.72%). The uppermost incidence of intermediate resistance was seen toward penicillin (63.63%) and linezolid (54.54%).

**Discussion**

Thus far, threatened evidences are obtainable on the incidence of *C. difficile* in seafood, particularly fish. An existing survey was accompanied to

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bacteria are also perhaps of our study were bivalve species obtained and C. difficile tcdC and bacteria recovered from animal sources were bacteria of the current survey. Opportunity of and This toxinotype is chiefly tcdA bacteria was and bacteria recovered putative genes in and bacteria were 45.45%, toxigenic genes and also Farsani (2014) [26] described that the incidence of toxin A and B positive Bacci et al. (2011) [25] conveyed boost incidence of C. difficile bacteria was also conveyed in surveys conducted on Czech Republic [16], France [19], Italy [20], Iran [21], Canada [9], Slovenia [22], Spain [23] and Brazil [24]. The C. difficile bacteria of our study were chiefly toxin A+ B+. This toxinotype is chiefly accompanying with severe clinical infections. Bacci et al. (2011) [25] conveyed boost incidence of toxin A and B positive C. difficile bacteria amongst the clinical cases. Doosti and Mohktari-Farsani (2014) [26] described that the incidence of tcdA, tcdB, cdtA and cdtB toxigenic genes and also tcdA+ tcdB+ cdtA+ cdtB and tcdA+ tcdB combined putative genes amid the C. difficile ebacteriae covered from animal sources were 8.80%, 17.70%, 8.80% and 15.50% and 1.10% and 2.20%, respectively.

C. difficile bacteria of the current survey harbored the high incidence of resistance toward routinely used antibiotics, particularly amoxicillin, ampicillin, moxifloxacin and piperacillin. Meropenem, vancomycin and metronidazole were found to be more efficient than other tested antibiotic agents on C. difficile bacteria. As majority of used antibiotic agents were human-based antimicrobials, thus it is more prone to concluded that C. difficile bacteria were transmitted from the infected hunter of hard-shells and also staffs of harbors. The statement may indirectly approve that the C. difficile bacteria are also perhaps transferred from human-based sewage depleted to sea water. High incidence of resistance toward amoxicillin-clavulanate, penicillin, ampicillin, moxifloxacin and piperacillin antibiotic agents was also conveyed in the C. difficile bacteriae covered from samples collected from Iran [27, 28], Netherlands [29], Spain [30], Italy [31], and Slovenia [32]. Hampikyan et al. (2018) [33] conveyed that incidence of antibiotic resistance in the C. difficile bacteriae recovered from meat samples in Turkey toward ampicillin, cefotaxim, clindamycin, amoxicillin-clavulanic acid, imipenem, metronidazole, tetracycline and vancomycin antibiotic agents were 6.80%, 1.20%, 12.40%, 87.0%, 24.90%, 1.90%, 3.10% and 97.50%, respectively. Rahimi et al. (2015) [28] described that the incidence of antibiotic resistance of C. difficile bacteriae recovered from ready-to-eat food samples toward ampicillin, chloramphenicol, ciprofloxacin, clindamycin, doxycycline, erythromycin, gentamicin, metronidazole, nalidixic acid, tetracycline and vancomycin antibiotic agents were 20%, 0%, 80%, 100%, 0%, 40%, 80%, 0%, 100%, 40% and 0%, respectively which was fairly similar to our findings. Comparable discoveries were also conveyed by Tenover et al. (2012) [34] and Goudarzi et al. (2013) [35].

Conclusions

To sum it up, we acknowledged a noteworthy incidence of resistant and putative C. difficile infish samples obtained from the retail centers of Isfahan, Iran. Common carp had the uppermost incidence of C. difficile amid all studied fish samples. Additionally, C. difficile bacteria exhibited the uppermost incidence of resistance toward amoxicillin, ampicillin,
moxifloxacin and piperacillin. Reversely, *C. difficile* bacteria were relatively susceptible to meropenem vancomycin and metronidazole. *TcdA*, *tdcB* and *tdcC* toxigenic genes were also found in the *C. difficile* bacteria recovered from fish samples. Concurrent attendance of multiple putative genes and attendance of resistance toward several kinds of antibiotic agents in the *C. difficile* bacteria postulate an imperative public health risk rendering the raw or undercooked consumption of fish samples. Moreover, high incidence of antibiotic resistance raised concerns rendering transmission risk of antibiotic resistant bacteria following the consumption of fish samples harbored these bacteria. Supplementary enquiry is obligatory to confirm an existing introductory information and to clarify the public health implication of seafood contamination by *C. difficile*.

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**Conflict of interest**

The authors declared that no conflict of interest.

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**References**


