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

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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*bacteriarecovered from dissimilarvarieties 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 uppermostincidence 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 generallyperceived toxigenic genes, though*tcdC* (18.18%) was the less frequently. There were no perceived *cdtA* and *ctdB* 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*bacteria. Boostincidence of toxigenic and resistant *C. difficile* pose an imperative health threating issue rendering the consumption of raw fish samples.

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

Introduction

Clostridium difficile (*C.difficile*) is toxigenicbacterium with boostability to occurrence of both human and animal infections.*C. difficile* is an imperativegastricbacterium accountable fordiarrheain human and animal (1,2). *C. difficile* infections (CDIs) have been increased both in hospital and community (3). It is also recovered fromdiverse kinds of food samples, particularly, foods with animal origins[1]. A total of 3 million hospitalizations has been conveyed due to the CDIs in the United States in each year[1, 2]. Food samples, particularly foods with animal origin have been measured as one of the imperative sources for CDIs [3]. Portion of meat [4], milk[5], vegetable [6], salad [7], water [8] and marine foods [9] as sources for transmission of CDIs to human has been identified.

Some toxins are accountable for occurrence of CDIs. *TcdA* enterotoxin and *tcdB* cytotoxin had the upper most importance in CDIs. They belong to PaLoc operon which also includes *tcdR*, *tcdE* and *tcdC* toxins. *TcdC* is a negative regulator of *tcdA* and *tcdB* toxins [10]. *C. difficile* binary toxin (CDT) is another imperative enzymatic component with boost clinical importance [10].

CDIs are difficult to treat because of boost re-

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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 anunspecifiedperson of *C. difficile* in seafoods and lack of epidemiological surveys in Iran, anexistingenquiry was addressed to assess the incidence rate and toxin and antibiotic resistance profiles of *C. difficile*bacteriare 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 marketingplaces of Isfahan, Iran. Fish species was identified by an expert professors of the field of aquaculture. Samples were obtained in distinct sterile belongings to avertfalling 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 signifiesthe 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. Arithmeticmomentous variances were gottenamid kinds of samples and incidence of *C. difficile* ($P<0.05$).

Table 3 discloses the incidence of toxigenic genes amid the *C. difficile* bacteriare covered from dissimilar varieties of fish samples. *TcdA* (45.45%) had the uppermostincidenceamid all perceived toxigenic genes, though *tcdC* (18.18%) had the lowermost. None of *C. difficile* bacteriare covered from fish samples were not positive for *cdtA* and *ctdB* 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.

Target gene	Primer sequence (5'-3')	Primer concentration (µM)	PCR product (bp)	PCR programs	PCR volume (25 µL)
TcdA	F: GCATGATAAGGCAACTTCAGTGGTA R: AGTTCCTCCTGCTCCATCAAATG	0.6 0.6	629	1 cycle: 94 ^{0C} ---------- 10 min. 35 cycle: 94 ^{0C} ---------- 50 s 54 ^{0C} ---------- 40 s 72 ^{0C} ---------- 50 s 1 cycle: 72 ^{0C} ---------- 3 min	1X PCR buffer 50 mM Tris-HCl 10 mM KCl 5 mM $(NH4)_2SO_4$, pH 8.3 2.6 mM $MgCl_2$ 260 lM each of dATP, dCTP, dGTP and dTTP 1.25 U of Taq polymerase (Thermo Fisher Scientific, St. Leon-Rot, Germany) each primer
TcdB	F: CCAAARTGGAGTGTTACAAACAGGTG R: GCATTTCTCCATTCTCAGCAAAGTA	0.4 0.2	410		
TcdC	F: AAAAGGGAGATTGTATTATGTTTTC R: CAATAACTTGAATAACCTTACCTTCA	0.2 0.2	475		
CdtA	F: GGGAAGCACTATATTAAAGCAGAAGC R: GGGAAACATTATATTAAAGCAGAAGC	0.05 0.05	221		
CtdB	F: TTGACCCAAAGTTGATGTCTGATTG R: CGGATCTCTTGCTTCAGTCTTTATAG	0.1 0.1			

(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, threatenedevidencesareobtainableon the incidence of *C. difficile* in seafood, particularly fish. Anexisting survey was accompanied to

TABLE 2. Incidence of *C. difficile* bacteria recovered from dissimilar varieties of fish samples.

Types of samples	No samples collected	N (%) of *C. difficile* positive samples
Common carp	40	7 (17.50)
Rainbow trout	40	3 (7.50)
S. commerson	32	-
Barracuda	32	-
S. guttatus	40	1(2.50)
Total	184	11 (5.97)

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

Types of samples (N samples positive for *C. difficile*)	N (%) isolates harbor each gene				
	TcdA	***TcdB***	***TcdC***	***CdtA***	***CtdB***
Common carp (7)	2 (28.57)	3(42.85)	2 (28.57)		
Rainbow trout (3)	2 (66.66)	1 (33.33)	-		
S. guttatus (1)	1 (100)	-	-	-	-
Total (11)	5 (45.45)	4 (36.36)	2 (18.18)		

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

Antimicrobial agent	Antibiotic resistance pattern of 11 *C. difficile* bacteria recovered from fish samples (%)		
	Susceptible	**Intermediate**	**Resistant**
Amoxicillin	2 (18.18)	2 (18.18)	7 (63.63)
Ampicillin	2 (18.18)	3 (27.27)	6 (54.54)
Ceftaroline	3 (27.27)	5 (45.45)	3 (27.27)
Clindamycin	4 (36.36)	5 (45.45)	2 (18.18)
Linezolid	3 (27.27)	6 (54.54)	2 (18.18)
Meropenem	10 (90.90)	1 (9.09)	-
Metronidazole	8 (72.72)	2 (18.18)	1 (9.09)
Moxifloxacin	-	5 (45.45)	6 (54.54)
Piperacillin	1 (9.09)	4 (36.36)	6 (54.54)
Ticarcillin	3 (27.27)	5 (45.45)	3 (27.27)
Penicillin	2 (18.18)	7 (63.63)	2 (18.18)
Vancomycin	10 (90.90)	1 (9.09)	-

examine the incidence rate and profiles of toxin and antibiotic resistance of *C. difficile* bacteriare covered from common carp, Rainbow trout, *S. commerson*, *Barracuda S. guttatus* fish samples collected from marketingplaces of the Isfahan, Iran. Absolutely, 5.97% of fish samples were contaminated with*C. difficile*. Opportunity of cross contamination's occurrence for examined samples by infected persons is a crediblemotive for boostincidence of *C. difficile*. Pasquale et al. (2011) [16] conveyed that shellfish, seawater and zooplankton samples obtained from Czech Republic were contaminated with toxin producing strains of *C. difficile* bacteria. Metcalf et al. (2011) [9] conveyed that the incidence of*C. difficile*in fish samples obtained from the Canada 4.80%. Pasquale et al. (2012) [17] conveyed that the incidence of *C. difficile* amongst bivalve molluscs was 49%. They determined that the incidence of bacteria in *Mytilusgalloprovincialis* and *Tapesphilippinarum* bivalve species obtained from the Italy was 48% and 53%, respectively. Close incidence rate to our survey was also described from Texas (4.50%)[18]. Deprived hygienic circumstances of fishing and marketing places is the chiefmotiveof boostincidence of *C. difficile* bacteria. A probable reason for the higher incidence of *C. difficile* in the common carp fish samples is its mode of life which increased fish exposure with *C. difficile* bacteria presented in the sediments and soils of the sea floor.

Furthermore, attendance of unalike toxigenic genes, exclusively *tcdA*, *tcdB* and *tcdC*was additional significant finding of our survey. Incidence of *tcdA*, *tcdB* and *tcdC* putative genes amid the *C. difficile* bacteria were 45.45%, 36.36% and 18.18%, respectively. Thus far, anexisting survey is an initialdescription of detection of *tcdA*, *tcdB* and *tcdC* putative genes in *C. difficil e*bacteriare covered from fish samples in Iran. Boostincidence of C. difficile putative genes 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*bacteriaof our study were chiefly toxin *A+ B+*. This toxinotypeischiefly accompanying with severe clinical infections. Bacci et al. (2011) [25] conveyedboost incidence of toxin A and B positive *C. difficile* bacteria amongst the clinical cases. Doosti and Mokhtari-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. difficil e*bacteriare 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, particularlyamoxicillin, 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* bacteriare covered from samples collected from Iran [27, 28], Netherlands [29], Spain [30], Italy [31], and Slovenia [32].Hampikyana et al. (2018) [33]conveyed that incidence of antibiotic resistance in the *C. difficile*bacteriarecovered from meat samples in Turkey towardampicillin, 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*bacteriarecovered 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. Comparablediscoveries were also conveyed by Tenover et al. (2012) [34] and Goudarzi et al. (2013) [35].

Conclusions

To sum it up, we acknowledged a noteworthyincidence of resistant and putative *C. difficile* infishsamples obtainedfrom the retail centers of Isfahan, Iran.Common carp had the uppermost incidence of *C. difficile*amid all studied fish samples. Additionally, *C. difficile*bacteriaexhibited the uppermost incidence of resistance toward amoxicillin, ampicillin,

moxifloxacin and piperacillin. Reversely, *C. difficile*bacteria were relatively susceptible to meropenem vancomycin and metronidazole. *TcdA*, *tcdB* and *tcdC* toxigenic genes were also found in the *C. difficile*bacteriarecovered from fish samples. Concurrentattendance of multipleputative genes and attendance of resistance toward several kinds of antibiotic agents in the *C. difficile*bacteriaposture an imperative public health riskrendering 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. Supplementaryenquiry es are obligatory to confirm anexisting introductoryin formation 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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