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 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*bacteria. 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.

### Introduction

*Clostridium difficile* (*C. difficile*) is toxigeniebacterium with boostability to occurrence of both human and animal infections. *C. difficile* is an imperative gastricbacterium accountable fordiahrhean human and animal (1,2). *C. difficile* infections (CDIs) have been increased both in hospital and community (3). It is also recovered from diverse 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 uppermost 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-
sistance of *C. difficile* toward antibiotic agents, particularly carbapenems, quinolones, penicillins, aminoglycosides, macrolides, fluoroquilones, cephalosporins, tetracyclines and sulfonamides [11].

Rendering to an unspecifed person of *C. difficile* in seafood and lack of epidemiological surveys in Iran, an existing ng inquiry 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 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 averfalling 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 signifies 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. 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* bacteria covered from dissimilar varieties of fish samples. TcdA (45.45%) had the uppermost incidence of *C. difficile* bacteria covered from fish samples, though tcdC (18.18%) had the lowermost. None of *C. difficile* bacteria covered from fish samples were not positive for cdxA and cdxB 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                    | 629              | 1 cycle: 94³⁰C ------------ 10 min.  
                             35 cycle: 94³⁰C ------------ 50 s  
                             54³⁰C ------------ 40 s  
                             72³⁰C ------------ 50 s  
                             1 cycle: 72³⁰C ------------ 3 min  | 1X PCR buffer  
                             50 mM Tris-HCl  
                             10 mM KCl  
                             5 mM (NH₄)₂SO₄, pH 8.3  
                             2.6 mM MgCl₂  
                             260 µM each of dATP, dCTP, dGTP and dTTP  
                             1.25 U of Taq polymerase  
                             (Thermo Fisher Scientific, St. Leon-Rot, Germany) each primer |
| *TcdB*      | F: CCATAARTGGATGTGTTACAAGACGGTG  
             R: GCATTTCCTCTGATCTCAGCAAGTA | 0.4                    | 410              | 1 cycle: 94³⁰C ------------ 10 min.  
                             35 cycle: 94³⁰C ------------ 50 s  
                             54³⁰C ------------ 40 s  
                             72³⁰C ------------ 50 s  | 1X PCR buffer  
                             50 mM Tris-HCl  
                             10 mM KCl  
                             5 mM (NH₄)₂SO₄, pH 8.3  
                             2.6 mM MgCl₂  
                             260 µM each of dATP, dCTP, dGTP and dTTP  
                             1.25 U of Taq polymerase  
                             (Thermo Fisher Scientific, St. Leon-Rot, Germany) each primer |
| *TcdC*      | F: AAAAGGGAGATGTGATTGTTTTTC  
             R: CAATAACTGAATAACCTTACCTCA | 0.2                    | 475              | 1 cycle: 94³⁰C ------------ 10 min.  
                             35 cycle: 94³⁰C ------------ 50 s  
                             54³⁰C ------------ 40 s  
                             72³⁰C ------------ 50 s  | 1X PCR buffer  
                             50 mM Tris-HCl  
                             10 mM KCl  
                             5 mM (NH₄)₂SO₄, pH 8.3  
                             2.6 mM MgCl₂  
                             260 µM each of dATP, dCTP, dGTP and dTTP  
                             1.25 U of Taq polymerase  
                             (Thermo Fisher Scientific, St. Leon-Rot, Germany) each primer |
| *CdtA*      | F: GGGAACCATATATAAAGCAGAAGC  
             R: GGGAAACATTATATAAAGCAGAAGC | 0.05                   | 221              | 1 cycle: 94³⁰C ------------ 10 min.  
                             35 cycle: 94³⁰C ------------ 50 s  
                             54³⁰C ------------ 40 s  
                             72³⁰C ------------ 50 s  | 1X PCR buffer  
                             50 mM Tris-HCl  
                             10 mM KCl  
                             5 mM (NH₄)₂SO₄, pH 8.3  
                             2.6 mM MgCl₂  
                             260 µM each of dATP, dCTP, dGTP and dTTP  
                             1.25 U of Taq polymerase  
                             (Thermo Fisher Scientific, St. Leon-Rot, Germany) each primer |
| *CdtB*      | F: TTGACCCAAAGTTGAAGTCTGATTG  
             R: CGGATCTCTTGTGCTCAGTCTTTATA | 0.1                    | 221              | 1X PCR buffer  
                             50 mM Tris-HCl  
                             10 mM KCl  
                             5 mM (NH₄)₂SO₄, pH 8.3  
                             2.6 mM MgCl₂  
                             260 µM each of dATP, dCTP, dGTP and dTTP  
                             1.25 U of Taq polymerase  
                             (Thermo Fisher Scientific, St. Leon-Rot, Germany) each primer |
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  | CdtB  |
| 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) | -        |

Discussion

Thus far, threated evidences are obtainable on the incidence of *C. difficile* in seafood, particularly fish. An existing survey was accompanied to...
bacteria 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 bacteria 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 bacteria was 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 infishsamples obtained from the retail centers of Isfahan, Iran. Common carp had the uppermost incidence of C. difficile among 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, tcdB and tcdC 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 es are obligatory to confirm an existing introductory formation and to clarify the public health implication of seafood contamination by C. difficile.

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Conflict of interest
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References
1. Zhang, S., Palazuelos-Munoz, S., Balsells, E.M., Nair, H., Chit, A. and Kyaw, M.H., Cost of hospital management of Clostridium difficile infection in United States—a meta-analysis and modelling study. BMC Infect. Dis., 16(1), 447 (2016).
2. Freeman, J., Bauer, M., Baines, S.D., Corver, J., Fawley, W., Goorhuis, B., Kuijper, E. and Wilcox, M., The changing epidemiology of Clostridium difficile infections. Clin.Microbiol.Rev., 23(3), 29-549 (2010).
3. Hensgens, M.P., Keessen, E.C., Squire, M.M., Riley, T.V., Koene, M.G., de Boer, E., Lipman, L.J. and Kuijper, E.J., Clostridium difficile infection in the community: a zoonotic disease? Clin.Microbiol. Infect., 18(7), 635-645 (2012).
4. Visser, M., Sepehrim, S., Olson, N., Du, T., Mulvey, M.R. and Alfa, M.J., Detection of Clostridium difficile in retail ground meat products in Manitoba. Canadian Journal of Infect. Dis. Med. Microbiol., 23(1), 28-30 (2012).
5. Bandelj, P., Briski, F., Mrle, O., Rataj, A.V., Rupnik, M., Ocepek, M. and Vengust, M., Identification of risk factors influencing Clostridium difficile prevalence in middle-size dairy farms. Vet.Res., 47(1), Article 41, pp.1-11 (2016). doi: 10.1186/s13567-016-0326-0.
6. Metcalf, D., Costa, M., Dew, W. and Weese, J., Clostridium difficile in vegetables. Canada. Lett. Appl.Microbiol., 51(5), 600-602 (2010).
7. Bakri, M.M., Brown, D.J., Butcher, J.P. and Sutherland, A.D., Clostridium difficile in ready-to-eat salads, Scotland. Emerg.Infect. Dis., 15(5), 817-818 (2009).
8. Kotila, S.M., Pitkänen, T., Brazier, J., Eerola, E., Jalava, J., Kuusi, M., Köönnönen, E., Laine, J., Miettinen, I.T. and Vuento, R., Clostridium difficile contamination of public tap water distribution system during a waterborne outbreak in Finland. Scand. J. Publ. Health., 41(5), 541-545 (2013).
9. Metcalf, D., Avery, B.P., Janecko, N., Matic, N., Reid-Smith, R. and Weese, J.S., Clostridium difficile in seafood and fish. Anaerobe, 17(2), 85-86 (2011).
10. Persson, S., Torpdahl, M. and Olsen, K., New multiplex PCR method for the detection of Clostridium difficile toxin A (tcdA) and toxin B (tcdB) and the binary toxin (cdtA/cdtB) genes applied to a Danish strain collection. Clin. Microbiol. Infect., 14(11), 1057-1064 (2008).
11. Peng, Z., Jin, D., Kim, H.B., Stratton, C.W., Wu, B., Tang, Y.-W. and Sun, X., Update on antimicrobial resistance in Clostridium difficile: resistance mechanisms and antimicrobial susceptibility testing. J.Clin.Microbiol., 55(7), 1998-2008 (2017).
12. Harvey, R.B., Norman, K., Andrews, K., Hume, M.E., Scanlan, C.M., Callaway, T.R., Anderson, R.C. and Nisbet, D.J., Clostridium difficile in poultry and poultry meat. Foodborne Pathog.Dis., 8(12), 1321-1323 (2011).
13. Rodriguez-Palacios, A., Staempfl, H.R., Duffield, T. and Weese, J.S., Clostridium difficile in retail ground meat, Canada. Emerg.Infect.Dis., 13(3), 485-487 (2007). doi: 10.3201/eid1303.060988.
14. Lemee, L., Dhalluin, A., Testelin, S., Mattrat, M-A., Maillard, K., Lemeland, J-F. and Pons, J-L., Multiplex PCR targeting tpi (ribose phosphate isomerase), tcdA (Toxin A), and tcdB (Toxin B) genes for toxigenic culture of Clostridium difficile. J.Clin.Microbiol., 42(12), 5710-5714 (2004).
15. CLSI, Performance Standards for Antimicrobial Susceptibility Testing; CLSI document M100. Wayne PA: 19087. (2017).

16. Pasquale, V., Romano, V.J., Rupnik, M., Dumontet, S., Čižnár, I., Albieri, F., Mauri, F., Saggionio, V. and Krovecak, K., Isolation and characterization of Clostridium difficile from shellfish and marine environments. *Folia Microbiol.*, **56**(5),431-737. (2011). doi: 10.1007/s12233-011-0068-3.

17. Pasquale, V., Romano, V., Rupnik, M., Capuano, F., Bove, D., Albieri, F., Krovecak, K. and Dumontet, S., Occurrence of toxigenic Clostridium difficile in edible bivalve molluses. *Food Microbiol.*, **31**(2),309-312 (2012).

18. Norman, K.N., Harvey, R.B., Andrews, K., Hume, M.E., Callaway, T.R., Anderson, R.C. and Nisbet, D.J., Survey of Clostridium difficile in retail seafood in College Station, Texas. *Food Addit. Contam. Part A Chem Anal. Control Expo. Risk Assess.*, **31**(6),1127-1129 (2014).

19. Barbut, F., Decre, D., Lalande, V., Burghoffer, B., Noussair, L., Gigandon, A., Espinasse, F., Raskine, L., Robert, J. and Mangeol, A., Clinical features of Clostridium difficile-associated diarrhoea due to binary toxin (actin-specific ADP-ribosyltransferase)-producing strains. *J. Med. Microbiol.*, **54**(2),181-185 (2005).

20. Spigaglia, P. and Mastrantonio, P., Comparative analysis of Clostridium difficile clinical isolates belonging to different genetic lineages and time periods. *J. Med. Microbiol.*, **53**(11),1129-1136 (2004).

21. Esfandiar, Z., Jalali, M., Ezzatpanah, H., Weese, J.S. and Chamani, M., Prevalence and characterization of Clostridium difficile in beef and mutton meats of Isfahan region, Iran. *Jundishapur J. Microbiol.*, **7**(8),e16771 (2014).DOI: 10.5812/jjm.16771

22. Rupnik, M., Widmer, A., Zimmermann, O., Eckert, C. and Barbut, F., Clostridium difficile toxino-type V, ribotype 078, in animals and humans. *J. Clin. Microbiol.*, **46**(6),2146-2146 (2008).

23. Alonso, R., Martin, A., Pelaye, T., Marin, M., Rodriguez-Creixems, M. and Bouza, E., Toxigenic status of Clostridium difficile in a large Spanish teaching hospital. *J. Med. Microbiol.*, **54**(2),159-162 (2005).

24. Silva, R.O.S., Santos, R.L.R., Pires, P.S., Pereira, L.C., Pereira, S.T., Duarte, M.C., Assis, R.A.d. and Lobato, F.C.F., Detection of toxins A/B and isolation of Clostridium difficile and Clostridium perfringens from dogs in Minas Gerais, Brazil. *Braz. J. Microbiol.*, **44**(1),133-137 (2013).

25. Bacci, S., Molbak, K., Kjeldsen, M.K. and Olsen, K.E., Binary toxin and death after Clostridium difficile infection. *Emerg. Infect. Dis.*, **17**(6),976-982(2011). doi: 10.3201/eid/1706.101483.

26. Doosti, A. and Mokhtari-Farsani, A., Study of the frequency of Clostridium difficile tcdA, tcdB, cdtA and cdtB genes in feaces of Calves in south west of Iran. *Ann. Clin. Microbiol. Antimicrob.*, **13**(1),Article 21, pp.1-6(2014). doi: 10.1186/1476-0711-13-21.

27. Rahimi, E., Jalali, M. and Weese, J.S., Prevalence of Clostridium difficile in raw beef, cow, sheep, goat, camel and buffalo meat in Iran. *BMC Public. Health*, **14**(1):119, pp.1-4,(2014). doi: 10.1186/1471-2458-14-119.

28. Rahimi, E., Afzali, Z.S. and Baghbdorani, Z.T., Clostridium difficile in ready-to-eat foods in Isfahan and Shahrekord, Iran. *Asian Pacific. J. Trop. Biomed.*, **5**(2),128-131 (2015).

29. Keessen, E.C., Hensgens, M.P., Spigaglia, P., Barbanti, F., Sanders, I.M., Kuijper, E.J. and Lipman, L.J., Antimicrobial susceptibility profiles of human and piglet Clostridium difficile PCR-ribotype 078. *Antimicrob. Res. Infect. Control.*, **2**(1),Article 14, pp.1-6 (2013).DOI: 10.1186/2047-2994-2-14

30. Peláez, T., Alcalá, L., Blanco, J.L., Álvarez-Pérez, S., Marin, M., Martín-López, A., Catalán, P., Reigadas, E., García, M.E. and Bouza, E., Characterization of swine isolates of Clostridium difficile in Spain: a potential source of epidemic multidrug resistant strains? *Anaerobe*, **22**,45-49 (2013).

31. Spigaglia, P., Drigo, I., Barbanti, F., Mastrantonio, P., Bano, L., Bacchin, C., Puiatti, C., Tonon, E., Berto, G. and Agnoletti, F., Antibiotic resistance patterns and PCR-ribotyping of Clostridium difficile strains isolated from swine and dogs in Italy. *Anaerobe*, **31**,42-46 (2015).
32. Pirš, T., Avberšek, J., Zdovec, I., Krt, B., Andlovic, A., Lejko-Zupanč, T., Rupnik, M. and Ocepek, M., Antimicrobial susceptibility of animal and human isolates of Clostridium difficile by broth microdilution. J. Med. Microbiol., 62(9), 1478-1485 (2013).

33. Hampikyan, H., Bingol, E.B., Muratoglu, K., Akkaya, E., Cetin, O. and Colak, H., The prevalence of Clostridium difficile in cattle and sheep carcasses and the antibiotic susceptibility of isolates. Meat Sci., 139, 120-124 (2018).

34. Tenover, F.C., Tickler, I.A. and Persing, D.H., Antimicrobial-resistant strains of Clostridium difficile from North America. Antimicrob. Agent. Chemother., 56(6), 2929-2932 (2012).

35. Goudarzi, M., Goudarzi, H., Alebouyeh, M., Rad, M.A., Mehr, F.S.S., Zali, M.R. Aslani, M.M., Antimicrobial susceptibility of Clostridium difficile clinical isolates in Iran. Iran Red. Cres. Med. J., 15(8), 704-711 (2013). doi: 10.5812/ircmj.5189.