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The emergence of metronidazole and vancomycin reduced susceptibility in *Clostridium difficile* isolates in Iran

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ABSTRACT

Objectives: Clostridium difficile (*C. difficile*) is the main causative agent of antibiotic-associated diarrhoea (AAD) and pseudomembranous colitis. The accumulation of antimicrobial resistance in *C. difficile* strains can drive *C. difficile* infection (CDI) epidemiology. This study was undertaken to evaluate the antimicrobial resistance patterns of toxigenic *C. difficile* isolates cultured from diarrhoeal stool samples of hospitalised patients with suspected CDI in three tertiary care hospitals in Tehran, Iran.

Methods: Two hundred and fifty diarrhoeal stool samples were investigated by toxigenic culture using cycloserine-cefoxitin-fructose agar and the VERO cell line. Antimicrobial susceptibility to metronidazole, vancomycin, clindamycin, tetracycline, and moxifloxacin was performed by disk diffusion and Etest methods on Brucella Blood Agar supplemented with hemin and vitamin K.

Results: Thirty-five stool samples (14.0%) proved positive using *C. difficile* toxigenic culture. According to Clinical and Laboratory Standards Institute breakpoints, the following resistance was identified in *C. difficile* isolates: metronidazole (2 of 35); moxifloxacin (7 of 35); clindamycin (18 of 35); and tetracycline (5 of 35). Using European Committee on Antimicrobial Susceptibility Testing breakpoints, three of 35 isolates showed reduced-susceptibility for vancomycin and 14 of 35 for metronidazole. In addition, the results showed a good correlation between the inhibition zone diameter (disk diffusion) and MIC values (Etest); Pearson correlation coefficient 0.7400.95 ($P < 0.001$).

Conclusions: Multidrug resistance was observed in Iranian clinical toxigenic *C. difficile* isolates, including reduced susceptibility to first-line CDI treatment drugs. In addition, disk diffusion can be used as a cost-effective option for the antimicrobial susceptibility testing of *C. difficile* isolates.

1. Introduction

Clostridium difficile (*C. difficile*) is a Gram-positive rod, spore-forming, obligate anaerobe, and has been associated with antibiotic-associated diarrhoea and pseudomembranous colitis [1]. A dramatic change in the epidemiology of *C. difficile* infection (CDI) has been noted in the last two decades. The emergence and spread of hypervirulent *C. difficile* strains are probably responsible for the increased morbidity and mortality of CDI [2]. The main virulence factors in the pathogenesis of CDI are the production of two large *C. difficile* toxins – A and B – whose genes are located in a 19.6 kb chromosomal region called the pathogenicity locus (PaLoc) [3]. In addition, a limited number of isolates have also been found to produce a third binary toxin encoded by the genes *cdtA* and *cdtB* [[3],4].

Several laboratory methods have been used to diagnose CDI, including: enzyme immunoassay-based methods or immunochromogenic assays for toxins A and/or B and glutamate dehydrogenase

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detection; cell culture based-methods such as cell cytotoxin neutralization assay or toxigenic culture; and real-time polymerase chain reaction methods [5]. However, the gold-standards for diagnosing CDI are toxigenic culture and cell cytotoxin neutralization assay [5]. Due to the limited laboratory diagnostic capacity in Iran and a low awareness of CDI, CDI cases are often underdiagnosed.

Chemotherapy is the most common risk factor for CDI [6] and almost all antibiotics can cause *C. difficile*-associated diarrhoea. Antimicrobial susceptibility testing (AST) for *C. difficile*, is labour-intensive and too expensive for a routine clinical laboratory procedure. However, AST is important for detecting emerging hypervirulent strains that are demonstrating increasing resistance – notably to quinolone antibiotics. Additionally, the eradication of resistant isolates in hospital settings could help to assess the effectiveness of infection control practices [1].

2. Materials and methods

2.1. Collection of stool specimens

A total of 250 unformed (n = 71) and liquid (n = 179) stools specimens were collected from hospitalised patients suspected of having CDI (90 females and 160 males with an age range 50–87 years; mean, 59 years) between June 2016 and November 2017 at three tertiary hospitals: Firouzabadi, Firouzgar and Rasoul Akram in Tehran, Iran. The clinical and personal data were collected, including: clinical symptoms, previous antibiotic treatment and any underlying conditions.

2.2. Identification of toxigenic Clostridium difficile isolates

2.2.1. Toxigenic culture

Stool specimens were subjected to alcohol shock for 1 h at room temperature followed by culture on selective agar plates (cycloserine-cefoxitin-fructose agar) (Himedia Laboratories) supplemented with 10% defibrinated sheep blood and selective components (8 mg/L cefoxitin and 250 mg/L cycloserine) in anaerobic conditions (Whitley Jar Gassing System) at 37 °C for 48 h.

The suspected colonies were characterised as *C. difficile*, based on characteristic phenotype (circular yellow or grey-white colonies with raised centres with irregular filamentous or opaque edges, Gram staining, and typical odour: horse barn) and prolin-aminopeptidase test [7]. Five colonies of *C. difficile* were inoculated on to Brain Heart Infusion (BHI) (HiMedia Laboratories) broth and anaerobically incubated for 5 days. Cultured BHI broths were centrifuged for 10 min at 4000 × *g*. Supernatants were filtered (0.22-μm pore size) and used for cell-toxicity assay. Filtrated supernatants with two dilutions 1:2 and 1:10 were added in triplicate onto VERO cell monolayers (96-well microtitre plate; SPL life sciences, Korea), followed by incubation for 48 h at 35 °C in 5% CO_2 and then examined using an inverted microscope after 24 and 48 h for cytopathic effect characteristic of *C. difficile* toxins. A positive result was defined as the presence of cytopathic effect in at least 50% of the cell monolayer [7].

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing to vancomycin, metronidazole, moxifloxacin, clindamycin, and tetracycline antibiotics was performed using the gradient Etest (bioMérieux) and disc diffusion method (Becton Dickinson). The Etest strips ranged from 0.016 μg/mL to 256 mg/L and antimicrobial disks had the following concentrations: metronidazole (5 μg), vancomycin (5 μg), clindamycin (2 μg), moxifloxacin (5 μg), and tetracycline (30 μg).

Cultured *C. difficile* isolates were suspended in BHI to a density of 1.0 McFarland (~ 3.0 × 108 CFU/mL) and inoculated into Brucella agar (HiMedia Laboratories) supplemented with defibrinated sheep blood (5% v/v) and selective components (hemin 5 mg/L, vitamin K1 1 mg/L) in bacterial suspension density of 1.0 McFarland ~ 3.0 × 108 CFU/mL and anaerobically incubated (Whitley Jar Gassing System) at 37 °C for 48 h [6]. *Clostridium difficile* ATCC 700057 was used as quality control strain for AST.

According to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI) released in 2018 [8], the minimal inhibitory concentration (MIC) determination of the following breakpoints were used: metronidazole ≥32 mg/L, clindamycin ≥8 mg/L, tetracycline ≥16 mg/L, and moxifloxacin ≥8 mg/L. The MIC interpretive breakpoints for vancomycin and metronidazole were based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values (ECOFFs): MIC ˃2 mg/L [9]. Diameters of the inhibition zones were interpreted based on the CLSI guidelines [8].

2.4. Statistical analysis

A Pearson correlation test was used to evaluate the correlation between two methods of Etest and disk diffusion on different antibiotics. Distribution curves were plotted for all tests. All data were analysed using StataCorp software, 2007 (Stata Statistical Software: Release 10. College Station, TX: StataCorp LP). The level of statistical significance was defined as $P < 0.05$.

3. Results

3.1. Clostridium difficile toxigenic culture and patient data

Out of 250 diarrhoeal stool samples, 35 isolates (14.0%) were identified as toxigenic by toxigenic culture. Clinical and demographic characteristics of the 35 CDI patients are shown in Table 1. The average age was 59 years (range 51–87), the median was 63

Table 1
Demographic characteristics of 35 patients with *Clostridium difficile* infection.

Characteristic	N (%)
Gender	
Male	22 (62.85)
Female	13 (37.15)
Age, years	
51–68	32 (91.43)
>68	3 (8.57)
Hospital ward	
Internal medicine	6 (17.14)
Intensive care unit	7 (20.00)
Infectious ward	12 (32.28)
Surgical ward	3 (8.57)
Gastroenterology	5 (14.28)
Other	2 (5.71)
Laboratory parameters	
Neutropenia	4 (11.43)
Leucocytosis	17 (48.57)
Blood in stool	5 (14.30)
Clinical parameters	
Fever	21 (68.57)
Abdominal pain	19 (54.30)
Previous use of antibiotics	
Penicillin	20 (57.14)
Cephalosporin	18 (51.42)
Clindamycin	10 (28.00)
Aminoglycoside	7 (20.00)
Fluoroquinolones	5 (14.30)
Metronidazole	4 (11.43)
Vancomycin	4 (11.43)
Other	6 (17.14)

years; 62.9% of patients were male. The majority of CDI patients (32.3%) were hospitalised in an infectious disease ward.

3.2. Antibiotic susceptibility of toxigenic Clostridium difficile isolates

Thirty-five toxigenic *C. difficile* isolates were tested for susceptibility to vancomycin, metronidazole, moxifloxacin, clindamycin, and tetracycline. Susceptibility and resistance rates, MIC ranges, and MIC_{50}, and MIC_{90} values for five antimicrobials are displayed in Table 2. Fig. 1 depicts the scattergram of MICs and zone diameters for all five tested antimicrobials. Resistance to metronidazole was observed for two (5.72%) isolates with MIC 32 mg/L using CLSI breakpoints and reduced susceptibility for 14 (40.0%) according to EUCAST ECOFF values with MICs 3–32 mg/L (Table 2). The breakpoint of 23 mm for metronidazole disc diffusion was proposed by Erikstrup et al. [10]. A correlation coefficient of 0.91 ($P < 0.001$) was derived between agar dilution and disk diffusion methods (Fig. 1A). Three isolates (8.57%) had an MIC >2 mg/L for vancomycin, which were classified as 'with reduced susceptibility' using the EUCAST breakpoint, and these isolates had a zone diameter of ≤14 mm. Thirty-three isolates with an inhibition zone diameter of >19 mm had an MIC value ≤2 mg/L, the calculated ECOFF value (Fig. 1B). The correlation coefficient for vancomycin was 0.95 ($P < 0.001$).

Among the vancomycin reduced-susceptibility isolates, one isolate with MIC 3 mg/L was cultured from a 72-year-old patient who had undergone pseudomembranous colitis; another patient with CDI caused by a vancomycin reduced-susceptibility isolate (MIC 4 mg/L) developed gastrointestinal malignancy. All *C. difficile* isolates with reduced susceptibility to metronidazole and vancomycin were derived from stool samples of patients who had symptoms of fever and abdominal pain, and were hospitalised in the intensive care unit, infectious disease and/or gastroenterology wards. In addition, these patients had a history of taking different classes of antibiotic such as beta-lactams, aminoglycosides and fluoroquinolones.

Except for CDI treatment drugs, 18 (51.4%) and five (14.3%) isolates revealed resistance to clindamycin and tetracycline, respectively. Additionally, 5.71% of the total 35 isolates were shown to be intermediate susceptible (4 mg/L) to clindamycin and 17.1% to be intermediate susceptible (8 mg/L) to tetracycline. Additionally, for clindamycin and tetracycline, 51.4% and 14.3% of the isolates had inhibition zone diameters of ≤16 mm and ≤22 mm with MICs values ≥8 mg/L and ≥32 mg/L, respectively (Fig. 1C and E). The correlation coefficient for clindamycin was 0.74 ($P < 0.001$), and for tetracycline it was 0.86 ($P < 0.001$). Twenty-eight isolates were susceptible to moxifloxacin (MICs ≤2 mg/L), but the remaining seven strains demonstrated resistance with MIC values ≥8 mg/L (Table 2). For moxifloxacin, 28 (80.0%) of the isolates had an inhibition zone diameter of ≥20 mm and an MIC >2 mg/L (Fig. 1D). The correlation coefficient for moxifloxacin was 0.90 ($P < 0.001$).

Multidrug-resistance (MDR) was defined as resistance to at least three or more antibiotics. Of the 35*C. difficile* isolates tested, nine (25.7%) were MDR. In addition, 27 isolates (77.2%) were resistant to at least one or more antimicrobial agent.

4. Discussion

Clostridium difficile infection is a growing concern for global public health. The prevalence of CDI is varied and depends on the geographic region, sampling collection method, infection control policies, antibiotic stewardship, and diagnostic techniques [1]. There is little available information about the true prevalence of CDI and also on the antimicrobial resistance profiles of *C. difficile* isolates circulating in developing Asian countries; however, previous limited studies have indicated that CDI is a significant nosocomial pathogen that also has a comparatively high prevalence rate [1].

In the present study, from the 250 stool samples collected, the prevalence of CDI in three Iranian tertiary-care hospitals was found to be 14.0%. This observation is comparable with data from a single Iranian centre study performed between January 2011 and August 2011, where *C. difficile* was detected in 18.1% (19 of 105) of patient stool samples [11], but contrasts with a previous Iranian study (2002–2006) where a lower CDI incidence (6.1%, 57 of 942) was reported [12].

Several studies have also reported on the prevalence of CDI in the Middle East. In Lebanon, for instance, in a single centre study, *C. difficile* was detected in 65.2% of 88 stool samples collected between September 2011 and April 2012 [13], which was comparable with the current study and is a 4.6-fold higher incidence of CDI. In contrast, lower CDI incidence rates were observed in Qatar between 2011–2012, and a CDI prevalence of 7.9% was reported among 1532 patients from two hospitals [14]; in Kuwait, the prevalence of CDI was 10.5% (73 of 697) between 2003–2005 [15].

The rapid evolution of antibiotic resistance plays an important role in driving *C. difficile* epidemiology [16]. The current study evaluated the antimicrobial resistance profile of 35 toxigenic *C. difficile* strains using agar dilution and the disk diffusion method. Five antimicrobials of various classes were used and 25.7% of the *C. difficile* isolates displayed MDR phenotypes, which is lower than the recently reported Iranian data on antimicrobial resistance in *C. difficile* where the MDR phenotype was observed in 67.3% and 48.0% of tested isolates [17,18].

As was shown in the current study, an inhibition zone diameter of 23 mm for a 5-μg metronidazole disk was an indicator of the susceptibility of isolates to metronidazole. The current findings are in agreement with Erikstrup et al., who proposed that the zone diameter breakpoints for metronidazole should be ≥23 mm for wild-type *C. difficile* strains [10], although the Fraga et al. recommend a zone diameter of ≥30 mm as the breakpoint [19]. The different findings between the studies of Fraga et al. and Erikstrup et al. could be explained by the different numbers of tested isolates [19]. However, the current authors believe that different ribotype distribution in the Danish and Brazilian studies could also explain the differences between observed zone diameters [10,19]. Nevertheless, Fraga et al. evaluated the AST results on metronidazole, using the breakpoint of 23 mm, and no change in the number of susceptible isolates was found [19].

Table 2
Antimicrobial susceptibilities of 35 toxigenic *Clostridium difficile* isolates to five antimicrobial agents.

Antimicrobials	MIC (mg/L)			N (%) of isolates		
	MIC_{50}	MIC_{90}	Range	Susceptible	Intermediate	Resistant
Metronidazole	0.25	8	0.125–32	33 (94.28)	–	2 (5.72)
Vancomycin	0.38	2	0.125–4	32 (91.43)	–	3 (8.57)
Clindamycin	8	32	1.5–>256	15 (42.86)	2 (5.71)	18 (51.43)
Moxifloxacin	2	16	1.5–32	28 (80.00)	–	7 (20.00)
Tetracycline	0.75	32	0.19–48	24 (68.56)	6 (17.14)	5 (14.30)

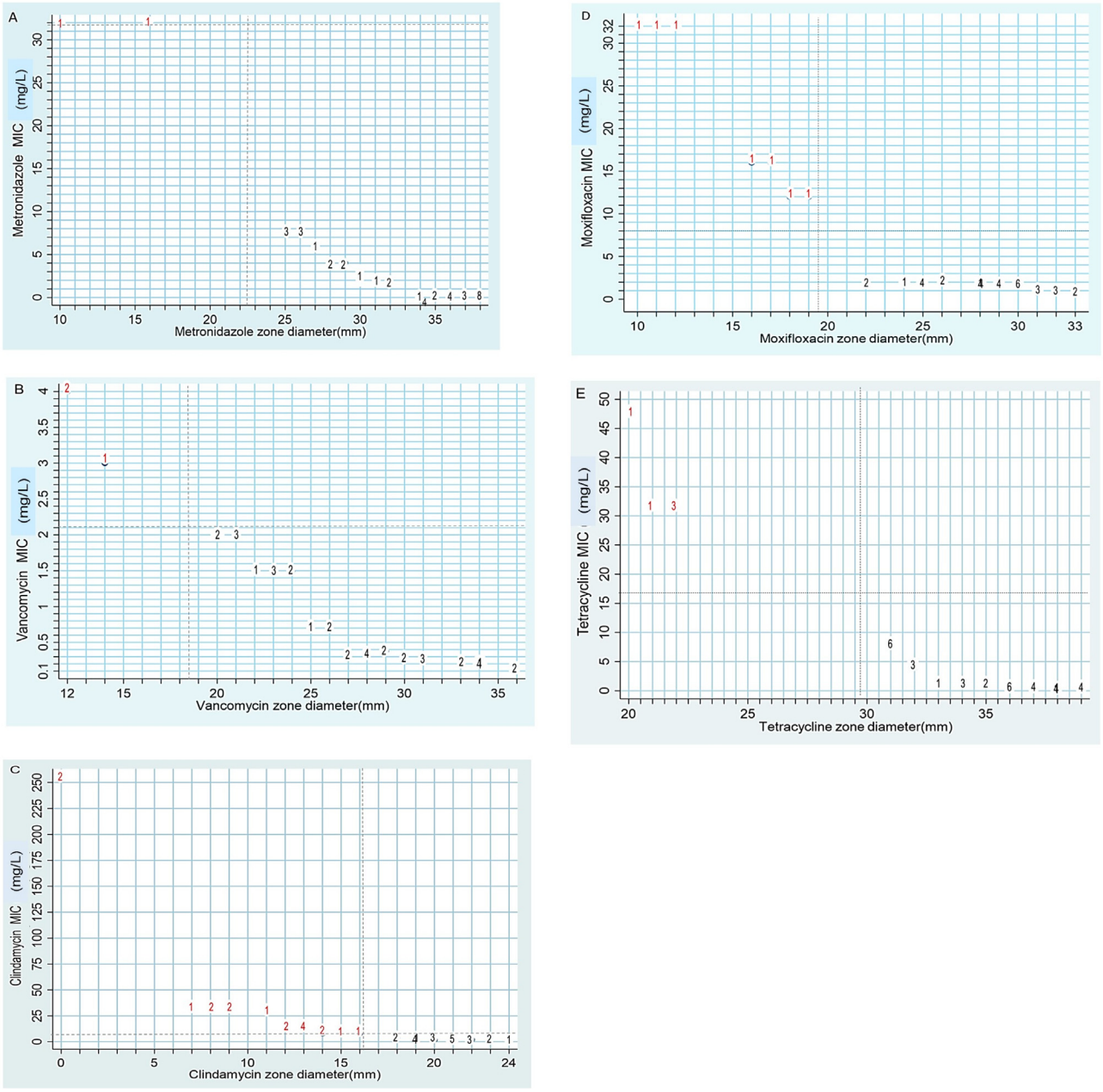

Fig. 1. Scattergrams comparing inhibition zone diameters (mm) with gradient test MICs (mg/L) tested against 35 clinical isolates of *Clostridium difficile*. The solid horizontal line represents the MICs breakpoint established by Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing. The solid vertical line represents the proposed zone diameter breakpoint. Isolates with known resistance towards five antibiotics are marked with red.

In general, MTZ-resistant *C. difficile* strains have very rarely been reported but are gradually emerging [20]; moreover, several studies have indicated high rates of treatment failures following metronidazole administration [21,22]. In the current study, two MTZ-resistant isolates, with MIC 32 mg/L, were detected (5.71%) but when the EUCAST breakpoint for metronidazole was applied, the metronidazole reduced-susceptibility rate increased to 40.0% of isolates. Similar differences between the interpretation of MIC results were observed in the study by Shayganmehr et al., where the metronidazole resistance rate was 5% using a breakpoint of MIC ≥32 mg/L; however, the majority of isolates revealed MIC_{50} and MIC_{90} of ≤8 mg/L [23]. In Iran, the high rate of metronidazole resistance accounting for 67.4% of isolates was observed in the samples collected between 2010-2016 [17]; however, in contrast, a low resistance rate to metronidazole (5.3%) was reported by Goudarzi et al. [18] in 2010–2011. Although the distribution of metronidazole resistant isolates in the 6-year period from the study by Baghani et al. is unknown [17], the occurrence and possible spread of such a high percentage of metronidazole-resistant *C. difficile* isolates in Iran may be cause for concern.

Excluding Iran, metronidazole resistance has also been reported in Iraq [24], Canada [25], Spain [26,27,28], and Texas [29].

Vancomycin is the first-line drug that is often used for moderate to severe CDI [20] and the growth of a majority of the isolates in the current study (n = 32, 91.43%) was inhibited by ≤2 mg/L of vancomycin. Three isolates that exceeded the ECOFF for vancomycin had MICs 3 mg/L (n = 1), and 4 mg/L (n = 2). Similar MICs were observed in two studies conducted in the United States during 2014-2015 [30,31] and in Scotland [32], where the occurrence of

vancomycin-resistant isolates with MICs of 4 mg/L dramatically increased from 2.7% to 21.6% (1999–2000) in 2005.

It has been suggested that moxifloxacin resistance drives the spread of certain *C. difficile* strains in healthcare settings [33]; however, in the present study, higher resistance rates to clindamycin and moxifloxacin (51.43% vs 20%) were observed. This is in agreement with data on European *C. difficile* isolates collected between 2011 and 2014, where the proportion of clindamycin resistance versus moxifloxacin resistance was 56.6% vs. 35.8% [34].

In Iran, the study by Baghani et al. [17] investigated the susceptibility of isolates to moxifloxacin and, in comparison with the current study, an almost four-fold higher rate of moxifloxacin-resistant isolates was observed (78.3%). Data on clindamycin resistance from the study by Sadeghifard et al. showed similar higher rates of resistant isolates (61.4% and 89.3%, respectively) [12]. These observations call for an improvement in the antimicrobial stewardship for quinolones and the MLSB group of antimicrobials in Iranian healthcare settings.

The acquisition of tetracycline resistance in C. difficile has been suggested to more likely occur through agricultural use rather than human healthcare [35]. The current study found five isolates resistant to tetracycline, but when the CLSI breakpoint from 2007 was applied (8 mg/L), the resistance rate increased from 14.3% to 31.4%. Unfortunately, the susceptibility of isolates to tetracycline was not tested in the European pan-European longitudinal surveillance of antibiotic resistance. However, whole genome sequencing of isolates from another large European study identified a link between the carriage of the tetW gene; a gene that encodes the protein protecting the ribosome from the translation inhibition of tetracycline in ribotype 078 isolates [36]. This ribotype was the most prevalent ribotype found in raw beef, cow, sheep, goat, camel and buffalo meat in Iran [37]. In addition, ribotype 126, which shared the same sequence type and clade (11 and 2), was the most common ribotype (21.7%) identified in a single-centre Iranian study [11]. In order to prevent the spread of tetracycline-resistant strains of ribotypes 078/126 from agriculture to the community, the 'One health' approach of *C. difficile* occurrence should be implemented in Iran.

5. Conclusion

This study found a strong correlation between the inhibition zone diameters of disk diffusion and MICs determined by Etest. Therefore, the disk diffusion method is proposed as a cost-effective option for antimicrobial susceptibility testing. In addition, multi-drug resistance was observed in Iranian clinical *C. difficile* isolates, including resistance to first-line CDI treatment drugs. These observations highlight the need to implement antimicrobial stewardship in hospital settings and to monitor the occurrence of *C. difficile* by the 'One *Clostridium difficile* health' approach.

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Competing interests

There are no conflicts of interest.

Ethical approval

This project was approved by the Iran University Human Ethics committee (Ethical code: IR.IUMS.FMD.REC 1394.26960).

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