Journal of Global Antimicrobial Resistance 19 (2019) 22–27

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Prevalence, characteristics and antimicrobial susceptibility patterns of *Clostridioides difficile* isolated from hospitals in Iran

Mojtaba Alimolaei[a,b,*], Hamid-Reza Rahimi[c], Majid Ezatkhah[d], Mehrdad Shamsaddini Bafti[d], Sadegh Afzali[b]

[a] *Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran*
[b] *Department of Research and Technology, Kerman Branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Kerman, Iran*
[c] *Department of Toxicology and Pharmacology, School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran*
[d] *Department of Anaerobic Bacterial Vaccine Research and Production, Kerman Branch, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Kerman, Iran*

ARTICLE INFO

Article history:
Received 19 August 2018
Received in revised form 13 February 2019
Accepted 19 February 2019
Available online 27 February 2019

Keywords:
Clostridioides difficile
Clostridium difficile
Prevalence
Antimicrobial susceptibility
Iran
EIA
PCR

ABSTRACT

Objectives: *Clostridioides* (previously *Clostridium*) *difficile* is a major growing cause of nosocomial diarrhoea known as *C. difficile* infection (CDI). This study investigated the prevalence and antimicrobial resistance patterns of *C. difficile* isolated from patients suffering from diarrhoea in Iran between 2016–2018.
Methods: A total of 151 stool specimens were collected and were screened for the presence of *C. difficile*. Specimens were examined for toxins by culture, enzyme immunoassay (EIA) and PCR. Antimicrobial susceptibility testing was performed for 12 antibiotics (metronidazole, vancomycin, clindamycin, tetracycline, erythromycin, ciprofloxacin, levofloxacin, moxifloxacin, fusidic acid, piperacillin, piperacillin/tazobactam and rifampicin) by the disk diffusion method according to the guidelines of the CLSI, EUCAST and CA-SFM.
Results: Of 151 stool specimens, 66 (43.7%) were positive for *C. difficile* by PCR, whereas 2 (1.3%) were only positive for *C. difficile* toxins based on EIA. A total of 292 clostridial isolates were obtained from specimens by culture, of which 133 (45.5%) were finally confirmed as *C. difficile* by PCR. Of 121 isolates resistant to at least one antibiotic, 107 (88.4%) were resistant to three or more antimicrobials and thus were defined as multidrug-resistant (MDR). Different and diverse resistance patterns to the antimicrobial drugs were seen among the isolates.
Conclusion: This is the first report of the isolation of *C. difficile* from different governmental hospitals of Iran and indicates that CDI might be an important nosocomial infection in different hospital wards. Moreover, this study provides a comprehensive picture of the MDR phenotype characteristics of *C. difficile* isolates in Iran.

1. Introduction

Clostridium difficile has recently been reclassified and renamed as *Clostridioides difficile* [1]. It is an obligate, anaerobic, spore-forming bacillus present in 2–5% of healthy adults but in 10–20% of the elderly population as a part of the gut flora. *Clostridioides difficile*, a nosocomial enteric pathogen, is the major infectious cause of antibiotic-associated diarrhoea, antibiotic-associated colitis, colon dilation, pseudomembranous colitis, toxic megacolon, sepsis and even death, collectively known as *C. difficile* infection (CDI), among hospitalised patients [2–4].

Clostridioides difficile-associated diarrhoea is common in hospitals. CDI is recognised as the main cause of nosocomial infectious diarrhoea, and its international spread has made it more challenging than ever. The increased morbidity and mortality among hospitalised patients has had a huge economic burden on healthcare systems worldwide [5,6]. Outbreaks of severe CDI have been caused by the hypervirulent strain *C. difficile* PCR ribotype 027 (CD027) in Canada [7] and European countries [8]. Two major toxins, namely enterotoxin A (TcdA) and cytotoxin B (TcdB), as well as the binary toxin (CDT) mediate the pathogenesis of CDI. TcdB is

* Corresponding author. Present address: Post Box 76175-359, 7636161313, Kerman, Iran.
E-mail address: m.alimolaei@rvsri.ac.ir (M. Alimolaei).

https://doi.org/10.1016/j.jgar.2019.02.013
2213-7165/

considered more important than TcdA and is essential for *C. difficile* virulence [6,9]. Production of the major toxins by CD027 strains can be approximately 20 times higher than that in common *C. difficile* strains [9].

There are multiple risk factors for the development of CDI, including antibiotic therapy and its duration, administration of multiple antibiotics, increasing age, severity of underlying diseases, non-surgical gastrointestinal procedures, presence of a nasogastric tube, anti-ulcer medications, duration of hospital stay, and stay in the intensive care unit (ICU) [10]. *Clostridioides difficile* spores are resistant to heating, drying and many commonly used disinfectants, thus they can survive in the hospital setting for >6 months and can spread in hospitals via medical devices and staff [11,12].

Antibiotic treatment is the most important risk factor for CDI, which commonly occurs after long antibiotic therapies in healthcare facilities [13]. Multidrug-resistant (MDR) strains are considered to be important in *C. difficile* persistence and their spread in hospital facilities. Over the years, metronidazole and vancomycin have been the standard antimicrobial therapies for CDI [14,15], however there have been reports of recurrence with these and other antibiotics as well as reduced rates of susceptibility [16–20]. Fusidic acid has been suggested as an alternative drug [21]. This highlights the need for new antimicrobial agents as well as antimicrobial susceptibility testing of *C. difficile* strains. Thus, surveillance of the antibiotic resistance profiles and diagnosis of MDR *C. difficile* strains appears to be of significant importance.

Laboratory determination of *C. difficile* and its related toxins is highly desirable both for epidemiological studies and for the diagnosis of CDI and is associated with better prognosis [6]. Clinicians can prescribe appropriate therapies and deploy adequate measurements to control the nosocomial spread of *C. difficile* with the use of an accurate and rapid diagnostic test. For this reason, various methods such as tissue culture assay, stool culture, toxin enzyme immunoassay (EIA) and PCR-based methods may be used. Culture assay for cytotoxin B is the 'gold standard' for diagnosis of *C. difficile*. Others are used as alternative methods. Bacteriological stool culture requires special media and appropriate methods for isolation of *C. difficile*. It is recommended by some authors because of its advantages, including detection of toxigenic isolates in culture-negative stool specimens as well as the availability of isolates suitable for toxigenic typing, determination of toxinogenesis (toxigenic culture), molecular analysis and determination of antimicrobial susceptibility. EIAs are also available to detect TcdA and/or TcdB in stool specimens. Moreover, molecular identification of *C. difficile* by various PCR techniques has been used frequently [22–24].

The current study was performed to evaluate the prevalence of *C. difficile* in stool specimens of hospitalised patients and outpatient cases suffering from diarrhoea in Iranian governmental hospitals. The methodological approaches were bacteriological stool culture and toxin A/B EIA test, followed by PCR targeting a species-specific 16S rRNA internal fragment to confirm the isolate identity. The aim was also to assess the antimicrobial resistance patterns and to detect MDR *C. difficile* strains. We believe this study is an initial step towards the introduction of proper interventions to control CDI in Iran.

2. Materials and methods

2.1. Patients

During the 2-year study period (March 2016 to March 2018), a total of 151 stool specimens from inpatients with suspected CDI (137 cases) in different hospital wards (infectious diseases, paediatrics, general, surgery and ICU/CCU) as well as outpatients (14 cases) suffering from diarrhoea were collected from six governmental hospitals and one medical laboratory in Kerman, Iran. Among the 151 patients, 67 (44.4%) were male and 84 (55.6%) were female. Diarrhoea was defined as two or more watery, loose or unformed stools per 24 h for ≥2 days [25]. The frequency of investigated patients from the different locations was as follows: 120 (79.5%) infectious diseases; 11 (7.3%) paediatrics; 2 (1.3%) general; 1 (0.7%) surgery; 3 (2.0%) ICU/CCU; and 14 (9.3%) outpatients. Patients were stratified into four age groups as follows: <18 years (paediatrics); 18–44 years (adults); 45–64 years (elderly adults); and ≥65 years (geriatric). The frequency of investigated patients in the different age groups was as follows: 23 (15.2%) paediatrics; 102 (67.5%) adults; 20 (13.2%) elderly adults; and 6 (4.0% geriatric. Demographic and clinical information, e.g. long hospitalisation (>5 days), clinical symptoms, antibiotic use and underlying conditions, were collected for all patients from their medical record. Patient complaints included fever, abdominal pain, nausea, vomiting, colitis, diarrhoea and dysentery. Faecal samples were screened for the presence of *C. difficile* and its toxins by bacteriological culture, toxin EIA and PCR tests. The protocol for this study was approved by the local ethics committee Kerman University of Medical Sciences (Kerman, Iran).

2.2. Toxin diagnosis

Fresh faecal specimens were immediately transported to the laboratory and were stored at −20 °C until they were tested for qualitative determination of toxin A and toxin B (enterotoxin and cytotoxin) of *C. difficile*. Samples were analysed using a RIDASCREEN® *Clostridium difficile* Toxin A/B EIA kit (R-Biopharm, Darmstadt, Germany; Art. No. C0801). The assay was performed and interpreted according to the manufacturer's instructions for sample storage and methods. Of the 151 human stool samples, 94 (corresponding to the first 55 patients positive for *C. difficile* and the first 39 patients negative for *C. difficile*) were tested by EIA.

2.3. Stool culture and isolation of Clostridioides difficile

Fresh faecal specimens of patients were processed and cultured for pathogenic bacteria using standard techniques. Briefly, an approximately 1 mL aliquot of stools was thoroughly mixed and was treated with an equal volume of 95% ethanol. Following incubation of the suspension at room temperature (20–25 °C) for 15 min, the suspension was inoculated on a *C. difficile* selective agar plate (QUELAB Laboratories Inc., Montreal, Quebec, Canada) supplemented with D-cycloserine (250 mg/L) and cefoxitin (8 mg/L). Plates were incubated in an anaerobic chamber with atmosphere inclusion of 0% O_2, 10% H_2, 10% CO_2 and 80% N_2 at 37 °C for 48 h. All plates were monitored daily up to 5 days. *Clostridioides difficile* colonies were presumptively identified on the basis of their characteristic morphology on selective agar plates, specific horse-stable odour, Gram stain and green-chartreuse fluorescence under ultraviolet light. Samples confirmed as *C. difficile* were stored in cryopreservative beads (Microorganism Preservation System-Protect; Technical Service Consultants Ltd., Lancashire, UK) at −70 °C for further molecular identification.

2.4. Confirmation of Clostridioides difficile isolates by PCR

Isolates were then confirmed as *C. difficile* by PCR for the 16S rRNA gene (a species-specific gene for internal PCR control). DNA was extracted using the protocol provided in the SinaPure DNA Gram-positive Bacteria Kit (EX6021; SinaClon Bioscience, Karaj, Iran). The PCR assay was performed in a total reaction volume of 25 μL with the following reagents: 2.5 μL of extracted DNA with 12.5 μL of ready-to-use PCR Master Mix 2× (MM2011;

SinaClon Bioscience), 0.7 μL (100 pmol/μL) of specific forward (5′-CCTCCTCAAGTACCGTCATTATC-3′) and reverse (5′-CAAAGGTGAGCCAGTACAGGA-3′) primers (SinaClon Bioscience) and dH_2O up to a volume of 25 μL. Amplicons were obtained as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 45 s, annealing at 51 °C for 45 s and extension at 72 °C for 40 s; followed by a final extension at 72 °C for 4 min. Amplification products were fractionated by electrophoresis through 1.5% standard agarose gel.

2.5. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing to 12 antibiotics (metronidazole, vancomycin, clindamycin, tetracycline, erythromycin, ciprofloxacin, levofloxacin, moxifloxacin, fusidic acid, piperacillin, piperacillin/tazobactam and rifampicin) was carried out for isolated *C. difficile* strains by the disk diffusion method on Mueller–Hinton agar (HiMedia Laboratories, Maharashtra, India) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [26,27], the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [28] and the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) [29] as well as data that were recently published by Erikstrup et al. [30]. Antimicrobial disks were purchased from Padtan-teb Co. (Tehran, Iran), Rosco Diagnostica A/S (Taastrup, Denmark) and Bioanalyse® (Ankara, Turkey).

2.6. Statistical analysis

Data analysis was performed using IBM SPSS Statistics v.21.0 (IBM Corp., Armonk, NY). The χ^2 test and Fisher's exact test were applied to compare differences among categorical variables and different groups. Statistically significant differences were defined as a *P*-value of <0.05.

3. Results

3.1. Diagnostic results

During the 2-year study period, a total of 151 stool specimens from patients suspected of having CDI were tested. Of the 151 patients who met the study inclusion criteria, 66 (43.7%) had toxigenic *C. difficile* isolates recovered from their samples confirmed by PCR and 85 (56.3%) gave rise to negative results for *C. difficile* isolation (Table 1); 2 samples (1.3%) were only positive for *C. difficile* toxin by the EIA method ($P < 0.05$). Both positive cases that were identified as CDI-positive were from the infectious diseases ward and were in the adult (18–44 years) age group.

3.2. *Clostridioides difficile* isolation and confirmation by PCR

All 151 faecal specimens were processed and cultured on selective agar plates and were monitored daily. One to three colonies were selected from each plate based on colony morphology and specific bacterial characteristics. A total of 292 clostridial isolates were recovered and were subjected to PCR to confirm *C. difficile* isolates. A total of 133 isolates (45.5%) were positive for the 16S rRNA control gene and were confirmed as *C. difficile* strains. They were obtained from stool specimens of 66 CDI-suspected patients (2 *C. difficile* isolates from each faecal sample, approximately). Among the investigated patients, 34.8%, 49.0%, 30.0% and 33.3% of cases in the paediatric, adult, elderly adult and geriatric age groups, respectively, showed positive results for *C. difficile* isolation by PCR (Table 2). PCR-confirmed isolation rates of *C. difficile* in male and female patients were 46.3% and 41.7%, respectively. These rates did not differ significantly between sex ($P > 0.05$) and age groups ($P > 0.05$).

3.3. Antibiotic resistance profiles

Of the 133 investigated *C. difficile* strains, 121 (91.0%) were resistant to at least one antibiotic, of which 2 (1.7%) were resistant to only one antibiotic, 12 (9.9%) were resistant to two antibiotics and 107 (88.4%) were resistant to at least three antibiotics and thus were defined as MDR. Resistance to erythromycin, fusidic acid and rifampicin, followed by clindamycin, was observed most frequently (Table 3). Multiple diverse resistance patterns to the antimicrobial drugs were seen among the isolates (Table 4).

4. Discussion

Clostridioides difficile is the leading cause of nosocomial infections and represents a great threat to public health. In this study, the prevalence of *C. difficile* was determined in Iranian hospitalised patients and outpatients as 43.7% (66/151). Identification of CDI patients in governmental hospitals was performed and the presence of *C. difficile* was confirmed in stool specimens of investigated patients by culture, toxin A/B EIA and PCR-based diagnostic assays.

Rapid diagnosis of *C. difficile* is important for clinicians to choose the best and appropriate therapy for CDI and to take appropriate measures to control its nosocomial spread [31]. It is thought that nutritional conditions, age, genetic factors, immunological alterations, diseases and antimicrobial therapy can interfere with *C. difficile* isolation [32]. *Clostridioides difficile* was isolated from 43.7% of patients with nosocomial diarrhoea in the current study. According to a previous study, *C. difficile* was isolated from 6.1% of Iranian patients with nosocomial diarrhoea and 4% of children with diarrhoea [33]. A rate of 4.9% was reported for Turkish diarrhoeic patients [34]. Furthermore, EIAs for toxins A/B are commercially available and are currently used most frequently by clinical laboratories to diagnose CDI [35]. In view of the current results, PCR showed rapid and reliable results as an initial diagnostic tool for *C. difficile*.

CDI as a major nosocomial infection has recently risen in medical centres and hospital wards following antibiotic therapy. This study was undertaken to determine the prevalence of CDI in patients with nosocomial diarrhoea in Iranian governmental

Table 1
Rate of *Clostridioides difficile* positivity by PCR in suspected *C. difficile* infection (CDI) patients from different hospital wards.

Positivity	*n* (%)						Total	*P*-value
	Paediatrics	ICU/CCU	ID	General	Surgery	O/P		
Positive	3 (2.0)	2 (1.3)	53 (35.1)	1 (0.7)	1 (0.7)	6 (4.0)	66 (43.7)	>0.05
Negative	8 (5.3)	1 (0.7)	67 (44.4)	1 (0.7)	0 (0.0)	8 (5.3)	85 (56.3)	
Total	11 (7.3)	3 (2.0)	120 (79.5)	2 (1.3)	1 (0.7)	14 (9.3)	151 (100)	

ICU, intensive care unit; CCU, critical care unit; ID, infectious diseases; O/P, outpatient.

Table 2
Clostridioides difficile isolation rates from investigated patients based on sex and age group.

Group	Positive		Negative		Total		95% CI for positive samples	*P*-value
	n	%	*n*	%	*n*	%		
Sex								
Male	31	46.3	36	53.7	67	100	34.3–58.2	0.344
Female	35	41.7	49	58.3	84	100	31.1–52.2	
Total	66	43.7	85	56.3	151	100	35.8–51.6	
Age group								
<18 years	8	34.8	15	65.2	23	100	15.3–54.2	
18–44 years	50	49.0	52	51.0	102	100	39.3–58.7	0.295
45–64 years	6	30.0	14	70.0	20	100	9.9–50.1	
≥65 years	2	33.3	4	66.7	6	100	−4.4 to 71.0	
Total	66	43.7	85	56.3	151	100	35.8–51.6	

CI, confidence interval.

Table 3
Antimicrobial agents, zone diameters and resistance rates of *Clostridioides difficile* isolates ($n = 133$).

Antimicrobial agent (concentration)	Diffusion zone diameter (mm)			Resistance		
	S	R	Standard guideline	S (*n*)	R (*n*)	RR (%)
Metronidazole (5 μg)	≥15	<15	EUCAST	123	10	7.5
Levofloxacin (5 μg)	≥17	≤13	EUCAST	102	31	23.3
Moxifloxacin (5 μg)	≥21	<18	EUCAST	101	32	24.1
Piperacillin/tazobactam (100/10 μg)	≥21	≤17	CA-SFM	87	46	34.6
Piperacillin (100 μg)	≥21	≤17	CA-SFM	84	49	36.8
Ciprofloxacin (5 μg)	≥21	≤14	CLSI	79	54	40.6
Vancomycin (30 μg)	≥19	<19	EUCAST	77	56	42.1
Tetracycline (30 μg)	≥19	<17	CA-SFM	66	67	50.4
Clindamycin (2 μg)	≥15	<15	CLSI	51	82	61.7
Rifampicin (5 μg)	≥20	≤16	CLSI	42	91	68.4
Fusidic acid (10 μg)	≥26	≤22	CLSI	36	97	72.9
Erythromycin (15 μg)	≥22	<17	CLSI	35	98	73.7

S, susceptible; R, resistant; EUCAST, European Committee on Antimicrobial Susceptibility Testing; CA-SFM, Comité de l'Antibiogramme de la Société Française de Microbiologie; CLSI, Clinical and Laboratory Standards Institute; RR, resistance rate.

hospitals. The results showed that many of patients with diarrhoea carried *C. difficile* in their stools, which differed significantly between hospital wards ($P = 0.027$). CDI was confirmed in only 1.3% of hospitalised patients suffering from diarrhoea. This was lower than a previous study in Iran that detected CDI in 5.3% of patients with gastrointestinal complaints [35].

Risk factors for CDI have been described previously [36]. Length ofhospitalisation, type and duration of antimicrobial therapy, patient age and severity of underlying illnesses are some of the predisposing factors that may influence the incidence rate of CDI. All 151 investigated patients with *C. difficile*-positive stool samples had received antibiotics at the time of or prior to sampling. These results are in accordance with another study in Iran [37] but are in contrast to another report that only 4.6% of patients with toxigenic *C. difficile* were receiving antibiotics at the time of sampling and 96.4% of them had not used antibiotics [35].

Antimicrobial therapy, as the most important risk factor for *C. difficile* colonisation, plays a principal role in CDI [38]. Administration of clindamycin, fluoroquinolones, third-generation cephalosporins and penicillins have the greatest risk for CDI [38]. In the current study, *C. difficile*isolates showed the highest antibiotic resistance rates to erythromycin, fusidic acid, rifampicin and clindamycin. The observed resistance patterns against the tested antibiotics were diverse (72 different patterns). Of the 121 isolates resistant to at least one antibiotic, 107 (88.4%) showed MDR characteristics, and many of them were resistant to different classes of antibiotics, including fluoroquinolones, lincosamides, macrolides, ansamycins, fusidanes, tetracyclines, nitroimidazoles, β-lactam/β-lactamase inhibitors and glycopeptides. MDR phenotypes were recently observed in 67.3% of Iranian *C. difficile* isolates [39]. Multidrug resistance may be caused by accumulation of multiple resistance genes typically on resistance plasmids or by high expression of genes coding for multidrug efflux pumps [40]. Characterisation of MDR *C. difficile* isolates shows the potential advantages of this pathogen over the co-resident gut flora.

Metronidazole, vancomycin and, alternatively, fusidic acid have been suggested for CDI treatment. Full susceptibility of *C. difficile* isolates to metronidazole and vancomycin have been reported [14]. However, recent studies have reported failure of metronidazole to treat CDI [41,42]. Resistance to metronidazole has gradually increased in different countries and the rate is up to 9% of isolates [18]. In a recent study in Iran, 67.4% of 46 *C. difficile* isolates showed resistance to metronidazole [39]. In the current study, 10 *C. difficile* isolates (7.5%) were resistant to metronidazole. This may be due to differences in the number of investigated isolates. Metronidazole resistance of *C. difficile* isolates in Iran may be generated by excessive use of this antibiotic to treat CDI [43]. In addition to the available treatments for CDI, other options such as faecal microbiota transplantation, probiotic therapy, monoclonal antibodies, vaccines and novel antibiotics are needed to control and treat CDI [44].

This study has a number of limitations. First, the EIA test used has a low sensitivity (89.7%) and acceptable specificity (96.8%) to detect *C. difficile* toxins A/B. Second, molecular typing and ribotyping of *C. difficile* isolates were not performed in this part of the study.

Table 4
Different resistance patterns among *Clostridioides difficile* isolates (*n* = 133).

Phenotype	Resistance patterns	*n*	%
S	Full susceptibility	12	9.0
R	Full resistance	10	7.5
P1	TET, CLI, RIF, FUS, ERY	5	3.8
P2	PIP, TZP, VAN, TET, CLI, RIF, FUS, ERY	5	3.8
P3	PIP, TZP, VAN, CLI, RIF, FUS, ERY	4	3.0
P4	PIP, TZP, VAN, TET, RIF, FUS, ERY, MFX, CIP	3	2.3
P5	VAN, TET, CLI, RIF, FUS, ERY, MFX, CIP	3	2.3
P6	VAN, RIF, FUS	3	2.3
P7	CLI, CIP, VAN, TET, RIF, FUS, ERY, LVX	3	2.3
P8	CLI, FUS, ERY	3	2.3
P9	CLI, FUS	3	2.3
P10	CLI, ERY	3	2.3
P11	CLI, RIF, FUS, ERY	3	2.3
P12	CLI	2	1.5
P13	MFX, CIP, VAN, TET, CLI, RIF, ERY, LVX	2	1.5
P14	MFX, CIP, VAN, CLI, FUS, ERY	2	1.5
P15	MFX, CIP, TET, CLI, RIF, FUS, ERY	2	1.5
P16	MFX, CIP, RIF, FUS, ERY, PIP, TZP, LVX	2	1.5
P17	TET, RIF, ERY	2	1.5
P18	TET, RIF, FUS	2	1.5
P19	VAN, TET, CLI, RIF, FUS, ERY	2	1.5
P20	VAN, CLI, FUS, ERY	2	1.5
P21	VAN, TET, RIF, FUS, ERY	2	1.5
P22	CIP, RIF, ERY	2	1.5
P23	CIP, VAN, TET, RIF, FUS, ERY	2	1.5
P24	CIP, VAN, CLI, RIF, FUS, ERY, LVX	2	1.5
P25	CIP, TET, CLI, RIF, FUS, ERY, PIP, TZP	2	1.5
P26–P70	Other phenotypes	45[a]	33.8
Total		133	100

TET, tetracycline; CLI, clindamycin; RIF, rifampicin; FUS, fusidic acid; ERY, erythromycin; PIP, piperacillin; TZP, piperacillin/tazobactam; VAN, vancomycin; MFX, moxifloxacin; CIP, ciprofloxacin; LVX, levofloxacin.
[a] One phenotype for each isolate (data not shown).

5. Conclusions

To our knowledge, this study is the first report of the isolation of *C. difficile* from different governmental Iranian hospitals. The results indicate that CDI might be an important nosocomial infection in hospitalised patients in different hospital wards. There is little epidemiological information about CDI in Iran and it is important to pay attention to this disease. The performance of PCR was proven to be more superior to that of EIA in the diagnosis of *C. difficile*. EIA can be used as a screening test but cannot be used as the sole laboratory method to detect *C. difficile*. Further research is needed to characterise the toxigenic *C. difficile* strains and types that are distributed in this region. Moreover, this study provides a good comprehensive picture of the MDR phenotype characteristics of *C. difficile* isolates in Iran and represents an important source of data for future comparative studies.

Funding

This study was supported by Kerman University of Medical Sciences (Kerman, Iran) [grant no. 940447].

Competing interest

None declared.

Ethical approval

The protocol for this study was approved by the local ethics committee of Kerman University of Medical Sciences [IR.KMU.REC.1394.695].

References

[1] Lawson PA, Citron DM, Tyrrell KL, Finegold SM. Reclassification of *Clostridium difficile* as *Clostridioides difficile* (Hall and O'Toole 1935) Prévot 1938. Anaerobe 2016;40:95–9, doi:http://dx.doi.org/10.1016/j.anaerobe.2016.06.008.

[2] Poutanen SM, Simor AE. *Clostridium difficile*-associated diarrhea in adults. CMAJ 2004;171:51–8, doi:http://dx.doi.org/10.1503/cmaj.1031189.

[3] Hurley BW, Nguyen CC. The spectrum of pseudomembranous enterocolitis and antibiotic-associated diarrhea. Arch Intern Med 2002;162:2177–84.

[4] Jones A, Kuijper E, Wilcox M. *Clostridium difficile*: a European perspective. J Infect 2013;66:115–28, doi:http://dx.doi.org/10.1016/j.jinf.2012.10.019.

[5] Kelly CP, LaMont JT. *Clostridium difficile*—more difficult than ever. N Engl J Med 2008;359:1932–40, doi:http://dx.doi.org/10.1056/NEJMra0707500.

[6] Leffler DA, Lamont JT. *Clostridium difficile* infection. N Engl J Med 2015;372:1539–48, doi:http://dx.doi.org/10.1056/NEJMra1403772.

[7] Pépin J, Saheb N, Coulombe M-A, Alary M-E, Corriveau M-P, Authier S, et al. Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort study during an epidemic in Quebec. Clin Infect Dis 2005;41:1254–60, doi:http://dx.doi.org/10.1086/496986.

[8] Kuijper E, Barbut F, Brazier J, Kleinkauf N, Eckmanns T, Lambert M, et al. Update of *Clostridium difficile* infection due to PCR ribotype 027 in Europe, 2008. Euro Surveill 2008;13:18942.

[9] Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, et al. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet 2005;366:1079–84, doi:http://dx.doi.org/10.1016/S0140-6736(05)67420-X.

[10] Bignardi GE. Risk factors for *Clostridium difficile* infection. J Hosp Infect 1998;40:1–15.

[11] Dubberke ER, Reske KA, Noble-Wang J, Thompson A, Killgore G, Mayfield J, et al. Prevalence of *Clostridium difficile* environmental contamination and strain variability in multiple health care facilities. Am J Infect Control 2007;35:315–8, doi:http://dx.doi.org/10.1016/j.ajic.2006.12.006.

[12] Vonberg RP, Kuijper EJ, Wilcox MH, Barbut F, Tüll P, Gastmeier P, et al. Infection control measures to limit the spread of Clostridium difficile. Clin Microbiol Infect 2008;14(Suppl 5):2–20, doi:http://dx.doi.org/10.1111/j.1469-0691.2008.01992.x.

[13] Wiegand PN, Nathwani D, Wilcox M, Stephens J, Shelbaya A, Haider S. Clinical and economic burden of *Clostridium difficile* infection in Europe: a systematic review of healthcare-facility-acquired infection. J Hosp Infect 2012;81:1–14, doi:http://dx.doi.org/10.1016/j.jhin.2012.02.004.

[14] Debast S, Bauer M, Kuijper E. European Society of Clinical Microbiology and Infectious Diseases: update of the treatment guidance document for *Clostridium difficile* infection. Clin Microbiol Infect 2014;20(Suppl 2):1–26, doi:http://dx.doi.org/10.1111/1469-0691.12418.

[15] Schmidt C, Löffler B, Ackermann G. Antimicrobial phenotypes and molecular basis in clinical strains of Clostridium difficile. Diagn Microbiol Infect Dis 2007;59:1–5, doi:http://dx.doi.org/10.1016/j.diagmicrobio.2007.03.009.

[16] Baines SD, O'Connor R, Freeman J, Fawley WN, Harmanus C, Mastrantonio P, et al. Emergence of reduced susceptibility to metronidazole in Clostridium difficile. J Antimicrob Chemother 2008;62:1046–52, doi:http://dx.doi.org/10.1093/jac/dkn313.

[17] Freeman J, Vernon J, Morris K, Nicholson S, Todhunter S, Longshaw C, et al. Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes. Clin Microbiol Infect 2015;21(248):e9–e16, doi:http://dx.doi.org/10.1016/j.cmi.2014.09.017.

[18] Pelaez T, Alcala L, Alonso R, Rodriguez-Creixems M, Garcia-Lechuz J, Bouza E. Reassessment of *Clostridium difficile* susceptibility to metronidazole and vancomycin. Antimicrob Agents Chemother 2002;46:1647–50, doi:http://dx.doi.org/10.1128/AAC.46.6.1647-1650.2002.

[19] Tenover FC, Tickler IA, Persing DH. Antimicrobial-resistant strains of *Clostridium difficile* from North America. Antimicrob Agents Chemother 2012;56:2929–32, doi:http://dx.doi.org/10.1128/AAC.00220-12.

[20] Dong D, Zhang L, Chen X, Jiang C, Yu B, Wang X, et al. Antimicrobial susceptibility and resistance mechanisms of clinical *Clostridium difficile* from a Chinese tertiary hospital. Int J Antimicrob Agents 2013;41:80–4, doi:http://dx.doi.org/10.1016/j.ijantimicag.2012.08.011.

[21] Zar FA, Bakkanagari SR, Moorthi K, Davis MB. A comparison of vancomycin and metronidazole for the treatment of *Clostridium difficile*-associated diarrhea, stratified by disease severity. Clin Infect Dis 2007;45:302–7, doi:http://dx.doi.org/10.1086/519265.

[22] Lister M, Stevenson E, Heeg D, Minton NP, Kuehne SA. Comparison of culture based methods for the isolation of *Clostridium difficile* from stool samples in a research setting. Anaerobe 2014;28:226–9, doi:http://dx.doi.org/10.1016/j.anaerobe.2014.07.003.

[23] Carson KC, Boseiwaqa LV, Thean SK, Foster NF, Riley TV. Isolation of *Clostridium difficile* from faecal specimens—a comparison of chromID *C. difficile* agar and cycloserine–cefoxitin–fructose agar. J Med Microbiol 2013;62:1423–7, doi:http://dx.doi.org/10.1099/jmm.0.056515-0.

[24] Rodriguez C, Van Broeck J, Taminiau B, Delmée M, Daube G. *Clostridium difficile* infection: early history, diagnosis and molecular strain typing methods. Microb Pathog 2016;97:59–78, doi:http://dx.doi.org/10.1016/j.micpath.2016.05.018.

[25] Fenner L, Frei R, Gregory M, Dangel M, Stranden A, Widmer A. Epidemiology of *Clostridium difficile*-associated disease at University Hospital Basel including molecular characterisation of the isolates 2006–2007. Eur J Clin Microbiol Infect Dis 2008;27:1201–7, doi:http://dx.doi.org/10.1007/s10096-008-0564-9.

[26] Clinical and Laboratory Standards Institute (CLSI). Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard—seventh edition. CLSI document M11-A7. Wayne, PA: CLSI; 2007.
[27] Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; twenty-second informational supplement. CLSI document M100-S22.. Wayne, PA: CLSI; 2012.
[28] European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Version 7.1, 2017. EUCAST; 2017.
[29] Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM). Recommandations 2013. CA-SFM; 2013.
[30] Erikstrup LT, Danielsen T, Hall V, Olsen K, Kristensen B, Kahlmeter G, et al. Antimicrobial susceptibility testing of *Clostridium difficile* using EUCAST epidemiological cut-off values and disk diffusion correlates. Clin Microbiol Infect 2012;18:266–72, doi:http://dx.doi.org/10.1111/j.1469-0691.2012.03907.x.
[31] Fekety R. Guidelines for the diagnosis and management of *Clostridium difficile*-associated diarrhea and colitis. Am J Gastroenterol 1997;92:739–50.
[32] Ferreira CE, Nakano V, Durigon EL, Avila-Campos MJ. Prevalence of *Clostridium* spp. and *Clostridium difficile* in children with acute diarrhea in Sao Paulo city, Brazil. Mem Inst Oswaldo Cruz 2003;98:451–4.
[33] Sadeghifard N, Salari MH, Ghassemi MR, Eshraghi S, Harati FA. The incidence of nosocomial toxigenic *Clostridium difficile* associated diarrhea in Tehran tertiary medical centers. Acta Med Iran 2010;48:320–5.
[34] Söyletir G, Eskitürk A, Kiliç G, Korten V, Tözün N. *Clostridium difficile* acquisition rate and its role in nosocomial diarrhoea at a university hospital in Turkey. Eur J Epidemiol 1996;12:391–4.
[35] Nazemalhosseini-Mojarad E, Azimirad M, Razaghi M, Torabi P, Moosavi A, Alebouyeh M, et al. Frequency of *Clostridium difficile* among patients with gastrointestinal complaints. Gastroenterol Hepatol Bed Bench 2011;4:210–3.
[36] Ananthakrishnan AN. *Clostridium difficile* infection: epidemiology, risk factors and management. Nat Rev Gastroenterol Hepatol 2011;8:17–26, doi:http://dx.doi.org/10.1038/nrgastro.2010.190.
[37] Sadeghifard N, Salari M, Ghassemi M, Shirazi M, Feizabadi M, Kazemi B, et al. Prevalence of *Clostridium difficile*-associated diarrhea in hospitalized patients with nosocomial diarrhea. Iran J Public Health 2005;34:67–72.
[38] Owens Jr. RC, Donskey CJ, Gaynes RP, Loo VG, Muto CA. Antimicrobial-associated risk factors for *Clostridium difficile* infection. Clin Infect Dis 2008;46 (Suppl 1):S19–31, doi:http://dx.doi.org/10.1086/521859.
[39] Baghani A, Ghourchian S, Aliramezani A, Yaseri M, Mesdaghinia A, Douraghi M. Highly antibiotic-resistant *Clostridium difficile* isolates from Iranian patients. J Appl Microbiol 2018;125:1518–25, doi:http://dx.doi.org/10.1111/jam.14035.
[40] Nikaido H. Multidrug resistance in bacteria. Annu Rev Biochem 2009;78:119–46, doi:http://dx.doi.org/10.1146/annurev.biochem.78.082907.145923.
[41] Musher DM, Aslam S, Logan N, Nallacheru S, Bhaila I, Borchert F, et al. Relatively poor outcome after treatment of *Clostridium difficile* colitis with metronidazole. Clin Infect Dis 2005;40:1586–90, doi:http://dx.doi.org/10.1086/430311.
[42] Pépin J, Alary M-E, Valiquette L, Raiche E, Ruel J, Fulop K, et al. Increasing risk of relapse after treatment of *Clostridium difficile* colitis in Quebec, Canada. Clin Infect Dis 2005;40:1591–7.
[43] Goudarzi M, Goudarzi H, Alebouyeh M, Rad MA, Mehr FSS, Zali MR, et al. Antimicrobial susceptibility of *Clostridium difficile* clinical isolates in Iran. Iranian Red Crescent Med J 2013;15:704–11, doi:http://dx.doi.org/10.5812/ircmj.5189.
[44] Goudarzi M, Seyedjavadi SS, Goudarzi H, Mehdizadeh Aghdam E, Nazeri S. *Clostridium difficile* infection: epidemiology, pathogenesis, risk factors, and therapeutic options. Scientifica (Cairo) 2014;2014:916826, doi:http://dx.doi.org/10.1155/2014/916826.