Research Article

Phenotypical and Genotypical Comparison of Clostridium difficile Isolated from Clinical Samples: Homebrew DNA Fingerprinting versus Antibiotic Susceptibility Testing (AST) and Clostridial Toxin Genes

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Background. Clostridium (Clostridioides) difficile is recognized as the major cause of healthcare antibiotic-associated diarrhea. We surveyed a molecular epidemiological correlation between the clinical isolates from two general hospitals in Iran through clustering toxigenic types and antibiotic susceptibility testing (AST) accuracy. Methods. Study population included 460 diarrhoeic specimens from inpatients with a history of antibiotic therapy. All samples underwent enriched anaerobic culture, confirmed by detection of gluD gene with PCR. Toxin status and AST were assessed by the disk diffusion method (DDM) and minimal inhibitory concentrations (MICs) of metronidazole, vancomycin, and rifampin. C. difficile outbreak was analyzed through conventional PCR by tracing toxin genes and Homebrew pulsed-field gel electrophoresis (PFGE) for characterizing isolates within our healthcare systems. Results. A total of 29 C. difficile strains were isolated by enriched anaerobic culture from the clinical samples. Among them, 22 (4.8%) toxigenic profiles yielded toxins A and B (tcdA, tcdB) and binary toxins (cdtA, cdtB). The minimum inhibitory concentration (MIC) was 18.1% and 9% for vancomycin and metronidazole, and all isolates were susceptible to rifampin and its minimum inhibitory concentration was at <0.003 μg/mL. The most dominant toxigenic and antibiotic-resistant "pulsotype F" was detected through PFGE combined with multiple Clostridial toxigenic pattern and AST. Conclusions. DNA fingerprinting studies represent a powerful tool in surveying hypervirulent C. difficile strains in clinical settings. Resistance to vancomycin and metronidazole, as first-line antibiotics, necessitate accomplishment of proper control strategies and also prescription of tigecycline as a more appropriate option.

1. Introduction

Clostridium difficile (new taxonomic name: Clostridioides difficile) is a Gram-positive, spore-forming, obligate anaerobe and recognized as the most common cause of nosocomial and gastrointestinal infections such as mild diarrhea, severe pseudomembranous colitis, and toxic megacolon. The pathogenicity of this bacterium is related to the toxin production of A and/or B and binary toxins which are encoded by tcdA, tcdB, and cdtA and cdtB genes [1, 2].

C. difficile infection (CDI) is initiated following antimicrobial consumption and eventuates in disruption of the normal colon microflora. Antibiotic therapy may also cause C. difficile antibiotic resistance in patients suffering from CDI and is a source of high morbidity and mortality worldwide.

Epidemiological studies of C. difficile in European countries have shown an increase in infection rate over time. CDI incidence in the United States in 2011 was more than 400,000 cases and resulted in 29,000 deaths in patients mostly aged above 65 years. CDI mortality rate before the
year 2000 was low with a rate of less than 2%; however, it has since increased to 16.7% [3, 4].

The severity of CDI in the world is influenced by overuse of antibiotics (especially fluoroquinolones, macrolides, and β-lactams), prolongation of hospitalization, and increase in the aging population [5, 6].

The easy transmission of C. difficile through the oral-fecal route results in high persistence in the environment and is a major hospital problem. Early detection of CDI is important to prevent the transmission of the organism in clinical settings, as well as in managing the prescription of antibiotics [7].

Although the first-line treatments for CDI are metronidazole and vancomycin, fidaxomicin is also considered a complementary therapy. However, several cases of resistance to metronidazole and vancomycin have been reported around the world [8, 9]. Determination of antimicrobial resistance patterns is critical for both patient treatment and epidemiological studies.

For instance, reports of outbreaks from Canada, the United States, Asia, and the UK confirmed that fluoroquinolone resistance was related to the emergence of C. difficile NAP1/027/BI [5, 10].

According to studies from Iran by Goudarzi et al., the resistance to metronidazole and vancomycin was 5.3% and 8%, respectively [11]. However, Shoaei et al. reported 100% of isolates to be susceptible to metronidazole and 11.7% of isolates to be resistant to rifampicin in 2019 [10]. Unlike the several epidemiological investigations reported from Europe, North America, and Australia, there are limited studies in Middle East Asia [12].

Metronidazole (nonsevere CDI) and vancomycin (severe CDI) are the first-line treatments of CDI. Although vancomycin was more effective than metronidazole for chemotherapy of severe and mild/moderate CDI, several cases of resistance to metronidazole and vancomycin have been reported from around the world [8, 9, 13].

Some antibiotics that are mostly related to C. difficile-associated disease (CDAD) are clindamycin, ampicillin, and cephalosporin, which may contribute as important risk factors for the progress of CDAD [12, 14, 15]. Therefore, the emergence of metronidazole-nonsusceptible C. difficile is a serious concern in clinical settings [16].

Furthermore, multidrug-resistant (MDR) strains have emerged owing to the uncontrolled usage of antibiotics. Hence, disclosure of susceptibility profile is a strategy toward lowering the increasing antibiotic resistance trend.

In the present study, molecular epidemiology of C. difficile infection in the two general hospitals of Tehran was characterized by pulsed-field gel electrophoresis (PFGE), possession of A and B toxin and binary toxin genes, and also antimicrobial susceptibility pattern.

2. Materials and Methods

2.1. Study Population. A total of 460 clinical stool samples were collected during the years 2017 to 2019. Diarrhoeic stool samples belonged to adult patients with a history of antibiotic therapy from 2 to 8 weeks, followed by symptomatic antibiotic-associated diarrhea [17].

2.2. Enriched Toxigenic Culture and Identification C. difficile. The fecal specimens were transported at room temperature and cultured anaerobically within 8 hr of collection or stored at 4°C for no more than 48 hr. Toxigenic culture was performed after isolation of C. difficile [18]. One portion of each sample was cultured regularly, and the rest was exposed to alcoholic shock for 1 hr before being cultured to inhibit the growth of other bacteria in feces [19].

Treated and untreated samples were inoculated onto the cycloserine-cefoxitin fructose agar, enriched with vitamin K1 solution (1 mg/mL and hemin solution 5 mg/mL), placed in anaerobic jars (Merck) with a Gas Pack Anaerocult® A (Merck, Germany), and incubated at 37°C for 2–5 days [20]. To optimize the growth of C. difficile, suspicious colonies were subcultured under anaerobic condition into BHI agar supplemented with 5% (v/v) sheep blood and incubated at 37°C for 24 hr. BHI agar was used for investigating colony characteristics (flat, horse barn odor, and Gram staining) and DNA extraction. Molecular identification of C. difficile was performed by conventional PCR of specific gene glutamate dehydrogenase (gldD). Confirmed colonies were preserved at −80°C for long-term storage.

2.3. DNA Extraction. Preserved C. difficile isolates were transferred with an inoculating loop into a 1.5 mL microcentrifuge tube containing 200 mL of sterile PBS buffer. Total bacterial DNA was extracted by using the QIAamp kit.
denaturation at 94°C for 10 min, 30 cycles of 94°C for 50 s, and
72°C for 50 s, with a final extension at 73°C for 10 min [21–23].

2.5. Antibiotic Disks. Disk diffusion was performed with the following material: antibiogram disks obtained from Merk, Germany. Disk diffusion was performed with the described previously (tested for epidemiological purpose only) [28].

2.6. Antibiotic Susceptibility Test. Antibiotic susceptibility to vancomycin (VAN), metronidazole (MTZ), rifampicin (RA), tigecycline (TIG), ciprofloxacin (CP), erythromycin (E), clindamycin (CC), amoxicillin-clavulanate (AMC), tetracycline (TET), meropenem (MEN), imipenem (IMI), and chloramphenicol (C) was determined using the disk diffusion method as per clinical laboratory standards and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints 2021. Results classified isolates as susceptible, intermediate, and resistant strains.

MIC of vancomycin was determined by the agar dilution method and MIC for metronidazole was determined by the test strips (Liofilechem, Italy) as recommended by the manufacturer’s instructions.

MIC values were tested using the following MIC ranges: vancomycin > 2 mg/L and metronidazole > 2 mg/L, based on the EUCAST breakpoint [25]. An agar plate without any antimicrobial agent was permanently incubated as growth control. An isolated C. difficile colony was tested for susceptibility to vancomycin and metronidazole by the agar dilution method [26]. Double dilution of each antibiotic was conducted in 1280 μg/mL of stock solution, and it was added to enriched Brucella agar with 5 μL/mL hemin, 1 μL/mL vitamin K₁, and 5% (v/v) sheep blood.

Plates with double concentration of antibiotics were prepared, namely, 0.25–16 μg/mL for vancomycin, 0.0002–32 μg/mL for rifampicin, and 0.12–64 μg/mL for metronidazole. The suspension equivalent of C. difficile 0.5 McFarland standard was prepared in Brucella broth. The MIC results were read after 48 hr of incubation at 37°C under anaerobic condition (Gas Pack Anaeroecult® A Merk, Germany).

Antibiotic susceptibility was defined as vancomycin breakpoint, > 2 μg/mL based on the EUCAST guideline, > 2 μg/mL for metronidazole breakpoint based on EUCAST breakpoints [27], and 0.004 μg/mL for rifampicin as described previously (tested for epidemiological purpose only) [28].

2.7. DNA Fingerprinting. Fingerprinting of DNA was performed by the pulsed-field gel electrophoresis (PFGE) technique to discriminate between strains. Firstly, bacteria were cultured in BHI agar with 5% (v/v) sheep blood under anaerobic condition, overnight at 37°C. A suspension of bacteria was made in cell suspension buffer (100 mM Tris, 100 mM EDTA, pH 8.0) with the absorbance range (optical density) of 1.4–1.7. The fresh bacterial cells were harvested into 3 mL of Gram-positive lysis buffer with RNase (1 mg/mL) and incubated overnight at 37°C in the anaerobic jar (Gas Pack Anaeroecult® A Merk, Germany) [24].

MIC values were tested using the following MIC ranges: vancomycin > 2 mg/L and metronidazole > 2 mg/L, based on the EUCAST breakpoint [25]. An agar plate without any antimicrobial agent was permanently incubated as growth control. An isolated C. difficile colony was tested for susceptibility to vancomycin and metronidazole by the agar dilution method [26]. Double dilution of each antibiotic was conducted in 1280 μg/mL of stock solution.
patients were hospitalized in the gastroenterology ($n = 11$), intensive care unit (ICU) ($n = 9$), bone marrow transplantation ($n = 3$), pulmonary disease ($n = 3$), cardiac surgery ($n = 2$), and renal disorder ($n = 1$) wards (Figure 1).

Of 29 positive cultures, 16 patients were female (55.1%) and 13 were male (44.8%) and the average age was 54.3 years. Analysis of patient history demonstrated that the medium hospitalization duration was 17.1 days and 60% of patients used at least 3 antibiotics before sampling. The frequent antibiotics administered were meropenem (79.3%) and vancomycin (48.2%) (Table 2).

### 3.2. Toxigenic Profile

Detection of toxin A (tcdA) and toxin B (tcdB) genes was performed by conventional PCR. In total, 29 (6.3%) *C. difficile* isolates were yielded through anaerobic culture, in which 22 (4.8%) were toxigenic, 20 isolates were (tcdA+, tcdB+), 2 (0.4%) isolates were (tcdA−, tcdB+), and also 7 (1.5%) isolates were nontoxigenic (tcdA−, tcdB−). Furthermore, 3 (0.7%) isolates possessed binary toxin genes (cdTA and cdTB) (Table 3).

### 3.3. Antibiotic Susceptibility Tests

We used the agar dilution method to assess the minimum inhibitory concentration of vancomycin, metronidazole, and rifampicin in toxigenic isolates. The MIC50 and MIC90 results for the three antibiotics are demonstrated in Table 4. Metronidazole and vancomycin resistance was shown in 9% and 18.1% of isolates, respectively, while all isolates were susceptible to rifampicin and the minimum inhibitory concentration was at <0.003 μg/mL.

In addition, the disk diffusion method for AST showed that most isolates were susceptible to tigecycline (100%), metronidazole (83.3%), vancomycin (77.7%), and rifampicin (75%). The susceptibility rates for other antibiotics included chloramphenicol (88.8%), tetracycline (52.7%), amoxicillin-clavulanate (38.8%), imipenem (25%), meropenem (21.1%), clindamycin (13.8%), ciprofloxacin (13.8%), and erythromycin (8.3%).

### 3.4. Multidrug Resistance (MDR)

MDR indicates resistance to one agent in three or more antibiotic classes. High-level resistance to ciprofloxacin was detected in most of the *C. difficile* isolates. Two (5.5%) isolates were MDR and exhibited resistance to vancomycin, metronidazole, and ciprofloxacin.

### 3.5. PFGE

A dendrogram, produced from PFGE data, demonstrated 22 toxigenic isolates divided into 11 clusters and 13 subtypes (based on a similarity value of 0.80) (Figure 2). The most dominant type was pulsotype F which was identified in 3 (13.6%) isolates from gastroenterology and ICU wards. Pulsotype F was toxigenic with (tcdA+/ tcdB+) and binary toxin (cdTA and cdTB) genes. Twenty (tcdA+/tcdB+) isolates had 10 different pulsotypes and 12 subtypes. These pulsotypes were identified in gastroenterology and ICU wards in both hospitals. All of the isolates that distinguished into 4 pulsotypes were screened in the

#### Table 2: Percentage of antibiotic classes used for treatment in 2–8 weeks prior sampling.

| Antibiotics | % |
|-------------|---|
| β-Lactams   |   |
| Meropenem   | 27.4 |
| Imipenem    | 15.5 |
| Ceftriaxone | 9.5  |
| Cefepime    | 2.3  |
| Ceftazidime | 1.2  |
| Ampicillin/sulbactam | 1.2 |
| Piperacillin| 1.2  |
| Glycopeptides| 16.6 |
| Vancomycin  | 15.5 |
| Metronidazole| 4.8 |
| Colistin    | 3.6  |
| Lincosamides|   |
| Clindamycin |   |
| Fluoroquinolone| 1.2 |
| Ciprofloxacin|   |

#### Table 3: Distribution of toxigenic profile of comparison isolates together with antibiotic susceptibility.

| Pulsotype | Toxigenic profile genes | Antibiotic susceptibility |
|-----------|-------------------------|--------------------------|
|           | tcdA | tcdB | cdTA | cdTB | Van | Mtz | Rif |
| A1        | +    | −    | −    | −    | S    | S   | S   |
| A2        | +    | −    | −    | −    | S    | S   | S   |
| B1        | +    | +    | −    | −    | S    | S   | S   |
| B2        | +    | +    | −    | −    | S    | S   | S   |
| C1        | +    | +    | −    | −    | R    | S   | S   |
| C2        | +    | +    | −    | −    | R    | R   | S   |
| D         | +    | +    | −    | −    | S    | S   | S   |
| E1        | +    | +    | −    | −    | S    | S   | S   |
| E2        | +    | +    | −    | −    | S    | S   | S   |
| F1        | +    | +    | +    | +    | S    | S   | S   |
| F2        | +    | +    | +    | +    | S    | S   | S   |
| F3        | +    | +    | +    | +    | S    | S   | S   |
| G1        | +    | +    | −    | −    | S    | S   | S   |
| G2        | +    | +    | −    | −    | S    | S   | S   |
| H1        | +    | +    | −    | −    | S    | S   | S   |
| H2        | +    | +    | −    | −    | S    | S   | S   |
| I1        | +    | +    | −    | −    | S    | S   | S   |
| I2        | +    | +    | −    | −    | S    | S   | S   |
| J1        | +    | +    | −    | −    | S    | S   | S   |
| J2        | +    | +    | −    | −    | S    | S   | S   |
| K1        | +    | +    | −    | −    | R    | S   | S   |
| K2        | +    | +    | −    | −    | R    | R   | S   |

*Van: vancomycin, Mtz: metronidazole, and Rif: rifampicin.*

angle = 120 [29]. The gels were analyzed with Bionumerics software (Applied Maths, GelCompar II, Belgium) to develop a dendrogram.

### 3. Results

#### 3.1. Clinical Data of Patients

During this study, we screened 460 patients who were suspicious of CDI. Twenty-nine (6.3%) stool samples were positive in culture and confirmed as *C. difficile* through endpoint PCR for *gluD*. Intended
ICU ward. Two (tcdA−/tcdB+) isolates showed the same pulsotype which belonged to the ICU ward. Both types C1 and K2 showed concurrent resistance to metronidazole and vancomycin; these types were isolated from gastroenterology and ICU wards.

3.6. Statistical Analysis. The results were analyzed through one-way analysis of variance (ANOVA) and pairwise two-tailed correlation with SPSS Version 25.0 (IBM® SPSS® Statistics, USA).

4. Discussion

In the last decade, with increasing nosocomial diarrhea among people in North America and Europe, CDI has become a major problem [30]. However, the epidemiology of CDI is less known in Asia in general and the Middle East, in particular [31]. In this study, 460 suspicious patients were evaluated for C. difficile infection, antibiotic resistance pattern, and molecular characteristics. PFGE was performed to demonstrate the epidemiological characteristics of C. difficile isolates in our local health systems.

CDI prevalence in our study was 4.8% (22/460), comparable to the studies from the United States and Europe [4]. The prevalence of CDI in Kuwait and Qatar was reported to be 7.2% and 7.9%, respectively [32, 33]. In a survey performed in Saudi Arabia, the incidence of CDI was 1.7 per 10,000 patients [34]. The annual CDI prevalence in Iran in the years 2017 and 2019 was 18.1% and 11.4%, respectively [10, 33].

The risk factors associated with CDI include old age (≥65 years), antibiotic consumption, hospitalization, and exposure to healthcare systems [35]. Our sample population had been exposed to antibiotics for 2 months prior to the study (Table 2), and the mean duration of hospitalization was 17.1 days. Analysis of the patients’ history demonstrated that...
beta-lactams were the most common antibiotics before the occurrence of CDI. Our study reported antibiotics therapy panels including beta-lactams, fluoroquinolones, and lincosamides.

Although metronidazole and vancomycin are the current choices for treatment of mild-to-moderate CDI and severe infection, susceptibility to these antibiotics has been gradually decreasing [13, 36, 37]. In a study conducted in Israel, the susceptibility to metronidazole and vancomycin among ribotype 027 was 44.6% and 87.7%, respectively [38]. A study of antimicrobial resistance among toxigenic C. difficile isolates in Iran in 2013 showed resistance to metronidazole and vancomycin to be 5.3% and 8%, respectively [11]. Also, current studies in Iran showed a susceptibility decrease to all antibiotics [10].

In our study, the resistant phenotype was observed in 5.5% isolates. The MIC90 for metronidazole was 1 mg/L. However, 77.2% of isolates were inhibited in <1 mg/L concentration of metronidazole and 2 isolates were resistant to >256 mg/L of metronidazole. According to data from the present study, up to 81% of isolates were inhibited with 1 mg/L of vancomycin. However, 4 isolates were resistant to vancomycin (MIC was 4 mg/L for two isolates and 8 mg/L for two isolates).

The percentage of MDR C. difficile ranges from 2.5% to 66% in various countries. Noticeably, resistance to vancomycin and metronidazole is a great concern that necessitates a proper consumption route.

Previous studies from Iran’s neighboring countries report low resistance to metronidazole and vancomycin as assessed by disk diffusion assay and MIC. In addition, in East Asian and European countries, the rate of resistance to these antibiotics has been low (0–6.3%) as confirmed by various methods. Owing to the high-level metronidazole resistance, its prescription and consumption should be confined.

Based on disk diffusion assays, all isolates were susceptible to tigecycline. The majority of isolates were susceptible to commonly prescribed agents based on both the antibiotic susceptibility test and MIC results.

In the present study, the MIC50 for vancomycin was 1 mg/L and MIC90 was 8 mg/L, breakpoint to vancomycin was MIC >2 mg/L, and 4 isolates were vancomycin-resistant. The MIC50 and MIC90 of metronidazole were 0.5 mg/L that was significantly lower than the susceptibility category breakpoint of ≥32 mg/L. Only two isolates were resistant to metronidazole with MIC ≥265 mg/L.

Toxigenic and drug-resistant C. difficile has been reported in various regions of the world. Accordingly, an urgent antibiotic susceptibility test report is essential alongside pathogenicity assessment to avoid the selection of nonsusceptible isolates.

Therefore, based on previous research studies on susceptibility to metronidazole and vancomycin, a subinhibitory concentration of these antibiotics can promote the production of biofilms and the resistance to metronidazole and vancomycin in C. difficile isolates. In case of failure of antibiotic therapy, tigecycline has been proved highly effective [39, 40]. Furthermore, resistance to metronidazole and vancomycin may be due to overuse of these antibiotics in patients. According to our study, 16.6% and 15.5% of patients had a history of usage metronidazole and vancomycin, respectively, and generally, 60% of the patients used at least three prior antibiotic therapies.

Our dendrogram analysis showed that PFGE type F was the most common pulsotype identified (13.6%). All the patients harbouring pulsotype F were positive for binary toxins (cdtA and cdtB) and also tcdA and tcdB genes with a high genetic correlation. These patients were hospitalized in two different wards in the same hospital.

In the present study, genetic diversity among 22 toxigenic C. difficile strains was high and isolates had a low genetic correlation with each other. In addition, both pulsotypes C and K (4 isolates) were vancomycin-resistant types, but they had a low genetic correlation. Isolates in pulsotypes C and K were detected in different wards in a hospital, namely, gastroenterology, ICU, and BMT wards. A−B+ toxigenic genotype was observed in 2 isolates, belonging to pulsotype A, and these were obtained from the ICU. This pulsotype was completely susceptible to metronidazole, vancomycin, and rifampicin. It is noteworthy that pulsotypes with A−B+ toxin gene were different in our study from that of Goudarzi et al. [11].

5. Conclusion

Our study of adult inpatients covered antibiogram pattern and showed low correlation genetic diversity in the C. difficile toxin profile. Our findings highlight the necessity for continuous monitoring of the clinical history of the inpatients and antibiotic treatment procedures. It is noteworthy that our analysis was limited by the lack of strain diversity and could be improved by including more hospitals. Furthermore, the assumption of clonal transmission between present pulsotypes proved false. Finally, high susceptibility to tigecycline could prove useful for CDI treatment and must be investigated as an alternate therapy.

Data Availability

Further information is available from the corresponding author upon request (Ashraf Mohabati Mobarez (Ph.D) Professor of Bacteriology, Department of Bacteriology, Faculty of Medical Sciences, Tarbiat Modares University Al-e Ahmad Exp. Tehran, Iran; PO box: 14115-111; mmmobarez@modares.ac.ir; office tel/fax: +98 21 8288 3862).

Ethical Approval

This survey was approved by the Ethics Committee of Tarbiat Modares University (IR.TMU.REC.1395.403).

Disclosure

Javid Sisakhtpour and Fatemeh Savaheli Moghadam are the first author jointly.
Conflicts of Interest
The authors declare that they have no conflicts of interest.

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References
[1] V. Zidaric and M. Rupnik, “Sporulation properties and antimicrobial susceptibility in endemic and rare Clostridium difficile PCR ribotypes,” Anaerobe, vol. 39, pp. 183–188, 2016.
[2] A. Camacho-Ortiz, D. Lopez-Barrera, R. Hernandez-Garcia et al., “First report of Clostridium difficile NAP1/027 in a Mexican hospital,” PLoS One, vol. 10, no. 4, Article ID e0122627, 2015.
[3] M. P. Bauer, D. W. Notermans, B. H. van Benthem et al., “Clostridium difficile infection in Europe: a hospital-based survey,” Lancet (London, England), vol. 377, no. 9759, pp. 63–73, 2011.
[4] F. C. Lessa, Y. Mu, W. M. Bamberg et al., “Burden of Clostridium difficile infection in the United States,” New England Journal of Medicine, vol. 372, no. 9, pp. 825–834, 2015.
[5] G. Terhes, A. Maruyama, K. Latkoczy et al., “In vitro antibiotic susceptibility profile of Clostridium difficile excluding PCR ribotype 027 outbreak strain in Hungary,” Anaerobe, vol. 30, pp. 41–44, 2014.
[6] J. H. Kwon, M. A. Olsen, and E. R. Dubberke, “The morbidity, mortality, and costs associated with Clostridium difficile infection,” Infectious Disease Clinics of North America, vol. 29, no. 1, pp. 123–134, 2015.
[7] C. E. F. Castro and L. S. Munoz-Price, “Advances in infection control for Clostridiodes (formerly Clostridium) difficile infection,” Current Treatment Options in Infectious Diseases, vol. 11, no. 1, pp. 12–22, 2019.
[8] F. Mathias, C. Curti, M. Montana, C. Bornet, and P. Vanelle, “Management of adult Clostridium difficile digestive contaminations: a literature review,” European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology, vol. 38, no. 2, pp. 209–231, 2019.
[9] T. Pelaez, R. Alonso, C. Perez, L. Alcala, O. Cuevas, and E. Bouza, “In vitro activity of linezolid against Clostridium difficile,” Antimicrobial Agents and Chemotherapy, vol. 46, no. 5, pp. 1617–1618, 2002.
[10] P. Shoaei, H. Shojaei, F. Khorvash et al., “Molecular epidemiology of Clostridium difficile infection in Iranian hospitals,” Antimicrobial Resistance and Infection Control, vol. 8, p. 12, 2019.
[11] M. Goudarzi, H. Goudarzi, M. Alebouyeh et al., “Antimicrobial susceptibility of Clostridium difficile clinical isolates in Iran,” Iranian Red Crescent Medical Journal, vol. 15, no. 8, pp. 704–711, 2013.
[12] L. C. McDonald, D. N. Gerding, S. Johnson et al., “Clinical practice guidelines for Clostridium difficile infection in adults and children: 2017 update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA),” Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, vol. 66, no. 7, pp. e1–e48, 2018.
[13] E. Mylonakis, E. T. Ryan, and S. B. Calderwood, “Clostridium difficile-associated diarrhea: a review,” Archives of Internal Medicine, vol. 161, no. 4, pp. 525–533, 2001.
[14] J. Pépin, N. Saheb, M.-A. Coulombe et al., “Emergence of fluoroquinolones as the predominant risk factor for Clostridium difficile-associated diarrhea: a cohort study during an epidemic in Quebec,” Clinical Infectious Diseases, vol. 41, no. 9, pp. 1254–1260, 2005.
[15] F. A. Zar, S. R. Bakkanagari, K. Moorthy, and M. B. Davis, “A comparison of vancomycin and metronidazole for the treatment of Clostridium difficile-associated diarrhea, stratified by disease severity,” Clinical Infectious Diseases, vol. 45, no. 3, pp. 302–307, 2007.
[16] S. D. Baines, R. O’Connor, J. Freeman et al., “Emergence of reduced susceptibility to metronidazole in Clostridium difficile,” Journal of Antimicrobial Chemotherapy, vol. 62, no. 5, pp. 1046–1052, 2008.
[17] P. Spigaglia, F. Barbanti, and P. Mastrantonio, “Multidrug resistance in European Clostridium difficile clinical isolates,” Journal of Antimicrobial Chemotherapy, vol. 66, no. 10, pp. 2227–2234, 2011.
[18] M. E. Reller, C. A. Lema, T. M. Perl et al., “Yield of stool culture with isolate toxin testing versus a two-step algorithm including stool toxin testing for detection of toxigenic Clostridium difficile,” Journal of Clinical Microbiology, vol. 45, no. 11, pp. 3601–3605, 2007.
[19] J. A. Sorg and S. S. Dineen, “Laboratory maintenance of Clostridium difficile,” Current Protocols in Microbiology, Wiley, Hoboken, NJ, USA, 2009.
[20] P. Spigaglia and P. Mastrantonio, “Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among Clostridium difficile clinical isolates,” Journal of Clinical Microbiology, vol. 40, no. 9, pp. 3470–3475, 2002.
[21] T. T. Tian, J. H. Zhao, J. Yang et al., “Molecular characterization of Clostridium difficile isolates from human subjects and the environment,” PLoS One, vol. 11, no. 3, Article ID e0151964, 2016.
[22] A. M. McGovern, G. O. Androga, D. R. Knight et al., “Prevalence of binary toxin positive Clostridium difficile in diarrheal humans in the absence of epidemic ribotype 027,” PLoS One, vol. 12, no. 11, Article ID e0187658, 2017.
[23] S. Khodaparast, A. M. Mobarez, and M. Saberifirooz, “A two-step approach for diagnosing glutamate dehydrogenase genes by conventional polymerase chain reaction from Clostridium difficile isolates,” Middle East Journal of Digestive Diseases, vol. 11, no. 3, p. 135, 2019.
[24] J. B. Patel, Performance Standards for Antimicrobial Susceptibility Testing, Clinical and Laboratory Standards Institute, Wayne, PA, USA, 2017.
[25] O. Shifman, T. Aminov, M. Aftalion et al., “Evaluation of the European committee on antimicrobial susceptibility testing guidelines for rapid antimicrobial susceptibility testing of Bacillus anthracis-,-,Verminia pestic- and Francisella tularensis-positive blood cultures,” Microorganisms, vol. 9, no. 5, p. 1055, 2021.
[26] C. J. Hastey, S. E. Dale, J. Nary et al., “Comparison of Clostridium difficile minimum inhibitory concentrations obtained using agar dilution vs. broth microdilution methods,” Anaerobe, vol. 44, pp. 73–77, 2017.
[27] European Committee on Antimicrobial Susceptibility Testing, Breakpoint Tables for Interpretation of MICs and Zone Diameters, European Committee on Antimicrobial Susceptibility Testing, Växjö, Sweden, 2018.
F. Barbut, P. Mastrantonio, M. Delmee, J. Brazier, E. Kuijper, and I. Poxton, “Prospective study of Clostridium difficile infections in Europe with phenotypic and genotypic characterisation of the isolates,” Clinical Microbiology and Infection, vol. 13, no. 11, pp. 1048–1057, 2007.

K. Jordan and M. Dalmasso, Pulse Field Gel Electrophoresis: Methods and Protocols, Springer, Berlin, Germany, 2015.

J. S. Martin, T. M. Monaghan, and M. H. Wilcox, “Clostridium difficile infection: epidemiology, diagnosis and understanding transmission,” Nature Reviews Gastroenterology & Hepatology, vol. 13, no. 4, pp. 206–216, 2016.

F. K. Berger, S. S. Rasheed, G. F. Araj et al., “Molecular characterization, toxin detection and resistance testing of human clinical Clostridium difficile isolates from Lebanon,” International Journal of Medical Microbiology, vol. 308, no. 3, pp. 358–363, 2018.

A. A. Al-Thani, W. S. Hamdi, N. A. Al-Ansari, S. H. Doiphode, and G. J. Wilson, “Polymerase chain reaction ribotyping of Clostridium difficile isolates in Qatar: a hospital-based study,” BMC Infectious Diseases, vol. 14, p. 502, 2014.

M. Azimirad, M. Krutova, O. Nyc et al., “Molecular typing of Clostridium difficile isolates cultured from patient stool samples and gastroenterological medical devices in a single Iranian hospital,” Anaerobe, vol. 47, pp. 125–128, 2017.

J. A. Al-Tawfiq and M. S. Abed, “Clostridium difficile-associated disease among patients in Dhahran, Saudi Arabia,” Travel Medicine and Infectious Disease, vol. 8, no. 6, pp. 373–376, 2010.

J. A. Cecil, “Clostridium difficile: changing epidemiology, treatment and infection prevention measures,” Current Infectious Disease Reports, vol. 14, no. 6, pp. 612–619, 2012.

P. Spigaglia, “Recent advances in the understanding of antibiotic resistance in Clostridium difficile infection,” Therapeutic Advances in Infectious Disease, vol. 3, no. 1, pp. 23–42, 2016.

M. R. Seo, J. Kim, Y. Lee, D. G. Lim, and H. Pai, “Prevalence, genetic relatedness and antibiotic resistance of hospital-acquired Clostridium difficile PCR ribotype 018 strains,” International Journal of Antimicrobial Agents, vol. 51, no. 5, pp. 762–767, 2018.

A. Adler, T. Miller-Roll, R. Bradenstein et al., “A national survey of the molecular epidemiology of Clostridium difficile in Israel: the dissemination of the ribotype 027 strain with reduced susceptibility to vancomycin and metronidazole,” Diagnostic Microbiology and Infectious Disease, vol. 83, no. 1, pp. 21–24, 2015.

T. Ethapa, R. Leuzzi, Y. K. Ng et al., “Multiple factors modulate biofilm formation by the anaerobic pathogen Clostridium difficile,” Journal of Bacteriology, vol. 195, no. 3, pp. 545–555, 2013.

Z. Peng, D. Jin, H. B. Kim et al., “Update on antimicrobial resistance in Clostridium difficile: resistance mechanisms and antimicrobial susceptibility testing,” Journal of Clinical Microbiology, vol. 55, no. 7, pp. 1998–2008, 2017.