Diagnostic deficiencies of *Clostridioides difficile* infection among patients in a tertiary hospital in Saudi Arabia: A laboratory-based case series

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**A B S T R A C T**

*Clostridioides difficile* infection (CDI) has become a threatening public health problem in the developed world. In the kingdom of Saudi Arabia, prevalence of CDI is still unknown due to limited surveillance protocols and diagnostic resources. We used a two-step procedure to study and confirm *C. difficile* cases. We also studied toxin profiles of these isolates.

Stool samples were collected from symptomatic patients and clinically suspected of CDI for almost 12 months. Isolates were confirmed by culture method followed by 16S rRNA sequencing. Multiplex PCR was performed for the identification of toxin A, toxin B and binary toxin genes and compared to Gene Expert results.

Out of the 47 collected samples, 27 were successfully grown on culture media. 18 samples were confirmed as *C. difficile* by both culture and 16S rRNA sequencing. Interestingly, the rest of the isolates (9 species) belonged to different genera. Our results showed 95% of samples were positive for both toxin A and B (tcdA, tcdB) and all samples exhibited the toxin gene regulator tcdC. All samples were confirmed negative for the binary toxin gene cdtB and 11% of the isolates were positive for cdtA gene. Interestingly, one isolate harbored the binary toxin gene (cdtA") and tested negative for both toxins A and B.

We believe that combining the standard culture method with molecular techniques can make the detection of *C. difficile* more accurate.

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1. Introduction

*Clostridioides difficile* is a known pathogen discovered in 1935 and recognized as a major cause of antibiotic-associated diarrhea (AAD) and Pseudomembranous colitis in the 1970s (Heinlen and Carroll, 2013). It is a Gram positive strictly anaerobic, spore-forming bacillus that can live in the gastrointestinal tract of humans or animals with thousands of different microbial species and then shed spores into their feces. These spores (metabolically inactive) can be spread to humans through hands, water and food. *C. difficile* exists in toxigenic form where pathogenicity can be mainly caused by two virulent genes *TcdA* and *TcdB* producing toxin A and toxin B respectively. A third and binary toxin has been shown to enhance *C. difficile*’s virulence and cause a variety of cytotoxic mechanisms leading to cell death (Schwan et al., 2009; Pruitt and Lacy, 2012). Other nontoxic virulent factors such as Putative type IV pilus, capsule and flagella play a role in bacterium pathogenesis (Carey-Ann and Carroll, 2013).

*Clostridioides difficile*-associated Disease (CDAD) appear mostly in old, hospitalized patients (more than 65 years old) with dimin-
ished immune response and recent antibiotic exposure associated with treatment failure (Lessa et al., 2012; Burke and Lamont, 2014; (CDC, 2021). In the last several years, the incidence and severity of CDI has been remarkably raised as one of the most common hospital-acquired infection (Peery et al., 2012; Wiegand et al., 2012; Hensgens et al., 2013; Aldeyab et al., 2014). This infection has become a threatening public health problem in the developed world, with significant high rate of morbidity and mortality reported since the early 2000 s. The centers for disease control and prevention (CDC) declared 223,900 cases of CDI in the United States in 2017 of which 12,800 patients died (Centers for Disease Control and Prevention (U.S.), 2019). European surveillance of CDI started in 2016 with 7711 cases including 5756 (74.6%) healthcare-associated cases (European Centre for Disease Prevention and Control, 2016).

Studies suggested similar rates of CDI in Asia compared to Europe and North America with estimated prevalence in the middle east around 11% (Borren et al., 2017). Although the increasing number of CDI reported worldwide, only few studies has been done in developing countries to evaluate the extent of this infection and the problem may be still underestimated (Cheng et al., 2016a, 2016b; Curcio et al., 2019).

In the Eastern Province of Saudi Arabia, there is a growing concern on the C. difficile infections in hospitals (Hudhaiah and Elhadi, 2019; Al-Tawfiq et al., 2020). C. difficile toxin was detected in 9.5% of patients diagnosed with gastroenteritis at a major referral center in Saudi Arabia in 1994 (Akbst et al., 1994). A more recent study done in 2007–2008 estimated the annual incidence rate of CDI to be around 2.4 and 1.7 per 10,000 patient days respectively (Al-Tawfiq and Abed, 2010). However, prevalence of CDI in the Kingdom of Saudi Arabia is still unknown where difficulties in estimating and comparing the incidence of CDI to the international rates comes from the inconsistent diagnostic, limited surveillance protocols and diagnostic resources.

The techniques that are most often used to detect C. difficile include culture methods, enzyme immunoassay (EIA) and nucleic acid amplification tests (NAATs) (Xiao et al., 2020). PCR is known to have higher sensitivity for toxin genes testing than the toxin EIA routinely used (de Jong et al., 2012). Establishing a routine detection method that is sensitive and fast is essential for the clinical management of patients with CDI (Tenover et al., 2012). The call for the current study was recommended by an earlier study which reported that in a recent study last year investigates the prevalence and genotype of nosocomial infection of C. difficile in Eastern Province as there is lack and urgent need to develop a diagnostic protocol for rapid identification of virulent strains of C. difficile (Al-Tawfiq et al., 2020).

Since a fast and accurate laboratory diagnostic is crucial to evaluate the extent of CDI problem in developing countries, we used a two-step procedure to study and confirm C. difficile isolates. The culture method was used followed by 16S rRNA sequencing. Molecular detection was also performed for the identification of toxin A, toxin B and binary toxin genes.

2. Materials and methods

2.1. Sample collection

Patients recruited for the study have been consented as per the Institutional Review Board (IRB) regulations and the proposed project has been submitted for the ethical approval by the ethical committee of Imam Abdulrahman Bin Faisal University (IRB-2017–338-Dent) and all procedures performed with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Stool samples were collected from patients exhibiting diarrhea and clinically suspected of C. difficile infection from Feb 2017 till May 2018.

All patients enrolled in the study had a history of recent antibiotic exposure, the majority of the samples isolated from male patients (88%) and the average age was 54.8 years. Patients showed different diagnosis such as, chronic diarrhea, ulcerative colitis, fistula of intestine, decompensated liver cirrhosis, urosepsis, acute pancreatitis, recurrent urinary tract infection, end stage renal failure on peritoneal dialysis and cauda equina syndrome.

2.2. C. Difficile isolates toxigenic culture study and bacterial culture

Frozen stool samples have been received from King Fahad University of the Province (Imam Abdulrahman Bin Faisal University, Dammam), symptomatic patients who were positive for both toxin A and toxin B by GeneXpert were proceeded for bacterial culture. Identification of C. difficile was performed by boiling fecal samples in PBS (phosphate-buffered saline) with pH 7.4, then cultured on the selective medium, cycloserine cefoxitin fructose agar medium (CCFA) in 86% N2, 7% H2 and 7% CO2 atmosphere at 37°C for 48 h.

Preliminary identification of C. difficile growth showed shiny, grayish, and flat colonies on the selective medium under anaerobic conditions with a horse manure odor and microscopically gram-positive rods.

Isolated pure colonies were prepared for DNA extraction. The DNA was extracted right away from the pure colonies.

2.3. Genetic study

2.3.1. Confirmation of C. Difficile isolates by 16S rRNA sequencing

DNA was extracted using Centra Puregene Yeast/Bact. Kit (Qiagen, Hilden, Germany) according to the manufacturer protocol. The extracted DNA was washed and purified three times before the final elution in 100 μl of elution buffer. DNA concentrations were measured by spectrophotometer (nanodrop 2000) and the extracted DNA was stored at –80 °C for further molecular analysis. In order to confirm C. difficile isolates, DNA from all the individual colonies were PCR amplified for 16S rRNA gene, sequenced and analysed as we described earlier (Rehman et al., 2019).

Nucleotide sequence accession numbers. 16S rRNA sequences of C. difficile and other detected bacteria were submitted to the GenBank database under the following accession numbers; MK909920-MK909925, MN080438, MN053902, MN049824, MN049823, MN049821, MN049793, MN049583, MN049570, MN049549, MN049543, MK791717, MK791684.

2.3.2. Virulence genes identification using multiplex PCR

A multiplex PCR was established using single tube PCR reaction for the identification of the virulent genes. A total of 12 primers were used for the detection of 16S rDNA, tcdA, tcdB, ctdB, ctdA and tcdC genes with amplicon size 1062, 629, 410, 262, 221 and 475 bp respectively (Table 1). Except tcdC gene, all the other genes were used identifying single tube multiplex PCR (Persson et al., 2008).

The Taq polymerase mediated amplifications were carried out in total volumes of 25 μl with the following recipe: 1 × PCR buffer, 2.6 mM MgCl2, 260 μM each of dNTP and 1.25 U of Taq polymerase (MoleQue-ON, New Zealand), and each primer (Table 1). Thermocycler profile: 10 min at 94°C; 35 cycles of 94°C for 50 s, 54°C for 40 s and at 72°C for 50 s, and a final extension at 72°C for 3 min. Similar protocol was applied for a single ampiclon for PCR of tcdC (475 bp) gene with 2 primers.
Erysipelatoclostridium ramosum (MN049820), perfringens MK791686), Terrisporobacter petrolearius have different toxin profiles of (MK793698), Enterococcus faecalis agents.

Gentamicin were the most prevalent predisposing antimicrobial agents.

Clostridium difficile A and toxin B. However, only 27 samples were successfully grown for 3 months. Those samples were positive by GeneXpert for both toxin tcdA and tcdB accounting for 89% of the strains with tcdA+.

The results of multiplex PCR analysis showed different grouping of samples (Table 2, Fig. 1). All samples were revealed toxigenic based on the above results comparing two different methods for detecting tcdA, tcdB accounting for 89% of the strains with tcdA+. The two remaining strains (MD2505, MD1742) harbored the binary toxin gene cdtA accounting for 11% of the strains. These two latter strains had different toxin profiles of (tcdA + B + C+, cdtA+) and (cdtA+, tcdC+) respectively (see Fig. 2).

### 3. Results

#### 3.1. Confirmation of C. difficile by 16S rRNA sequencing

A total of 47 samples were collected over a period of twelve months. Those samples were positive by GeneXpert for both toxin A and toxin B. However, only 27 samples were successfully grown on the selective media. A total of 18 sequences were related to Clostridioides difficile strains using 16S rRNA and 9 samples belonged to other bacterial strains as showed out of the 27 isolates grown successfully on the culture medium.

Eleven antimicrobial agents have been associated with C. difficile infection, ciprofloxacin followed by ceftriaxone, tazocin and gentamicin were the most prevalent predisposing antimicrobial agents.

#### 3.2. Virulence genes identification using multiplex PCR

The results of multiplex PCR analysis showed different grouping of samples (Table 2, Fig. 1). All samples were revealed toxigenic where the majority tested positive for the two toxin genes tcdA, tcdB accounting for 89% of the strains with tcdC+. The two remaining strains (MD2505, MD1742) harbored the binary toxin gene cdtA accounting for 11% of the strains. These two latter strains had different toxin profiles of (tcdA + B + C+, cdtA+) and (cdtA+, tcdC+) respectively (see Fig. 2).

### 4. Discussion

In this research work, we investigated a group of 47 samples that were collected from February 2017 for a period of one year in the Eastern Province of Saudi Arabia. We used a two-step test to isolate C. difficile from stool samples; culture followed by 16S rRNA sequencing. We also studied toxin profiles of these isolates. Only few studies were done in this field in our region despite the high mortality rates and financial burden encountered worldwide due to hospital-acquired infections including CDI (Al-Tawfiq et al., 2020; Alasmari et al., 2014; Aldeyab et al., 2014; Shajan et al., 2014; Alzoubay et al., 2020). Our study had limitations where only 47 samples were studied. However, this small sample size does not invalidate our interesting results as discussed below.

Samples were taken from patients exhibiting diarrhea and clinically suspected of C. difficile infection. Detection of Clostridioides in the medium studied (Lessa et al., 2012; Cadnum et al., 2014; Fang et al., 2017). 16S rRNA sequencing was used to confirm our culture results and only 18 samples were confirmed as C. difficile out of the 27 isolates grown successfully on the culture medium. The rest of 9 isolates belonged to other bacterial strains as showed in the results.

Terrisporobacter glycolicus and Erysipelatoclostridium ramousum are part of Clostridioides cluster XI and XVIII clostridial cluster, respectively. Terrisporobacter glycolicus was initially classified as Clostridium glycolicum and it is placed within the family Peptostreptococcaceae along with Clostridioides difficile (Cheng et al., 2016a; Ohashi and Fujisawa, 2019). Erysipelatoclostridium ramosum belongs to the human gut microbiota. It is a Gram-positive anaerobic enteric bacterium most widely known as Clostridioides ramosum (Zakham et al., 2019). Hence, the growth of these two bacteria on the culture medium and their disapproval as Clostridium difficile (Cheng et al., 2016a; Ohashi and Fujisawa, 2019). Erysipelatoclostridium ramosum was isolated from stool samples on the selective media and under the appropriate growth requirements. This standard method showed more sensitivity than PCR and EIA tests used in the few studies done in our region (Al-Tawfiq et al., 2020). These two latter techniques have shown low sensitivity to detect low concentrations of Clostridioides in the medium studied (Lessa et al., 2012; Cadnum et al., 2014; Fang et al., 2017). 16S rRNA sequencing was used to confirm our culture results and only 18 samples were confirmed as C. difficile out of the 27 isolates grown successfully on the culture medium. The rest of 9 isolates belonged to other bacterial strains as showed in the results.

Enterococcus faecalis is an enterococcus, a nosocomial pathogen that has similar risk factors as C. difficile including antibiotic treatment and hospitalization (Özsoy and Ilki, 2017). This Vancomycin-Resistant Enterococcus faecalis (VRE) is often detected in the C. difficile positive samples (Rodriguez et al., 2016). Therefore, based on the above results comparing two different methods for detecting C. difficile, we are reporting herein the recovery of nine bacterial species belonging to different genera on the C. difficile culture medium. Despite the negative impact that wrong diagnosis might have putting the patients at risk, delaying the treatment and transmitting the infection, few studies have shed the light on this area in the developing countries leading to the underestimation of CDI.

The 18 samples confirmed as C. difficile by both culture and 16S rRNA sequencing were also subject to toxinotyping by GeneXpert test and Multiplex PCR to identify the virulence genes tcdABC and cdtAB. The predisposing antimicrobial agents that showed the highest association with C. difficile infection were ciprofloxacin and tazocin.

These findings coincide with the meta-analytic study by Brown et al who noted that tetracyclines and penicillin were associated with the lowest risk, while fluoroquinolones, clindamycin, and expanded spectrum cephalosporins were associated with the highest risk of CDI. It also concurs with that literature reported that the excessive use of cephalosporins is related to the occurrence of CDI than other antibiotics (Brown et al., 2013; Czeple et al., 2019).
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...exhibited the toxin gene regulator \( \text{tcdC} \). All samples were positive for both toxin A and B. The presence of binary toxin genes is significantly related to the region corresponding to the reverse primer sequence and consequently led to the false negative results in the multiplex PCR. The primers sequences were examined and sequencing was negative for \( \text{rRNA} \) in the multiplex PCR. The primers sequences were examined and sequencing was negative for \( \text{rRNA} \) in the multiplex PCR. The primers sequences were examined and sequencing was negative for \( \text{rRNA} \) in the multiplex PCR. The primers sequences were examined and sequencing was negative for \( \text{rRNA} \) in the multiplex PCR. The primers sequences were examined and sequencing was negative for \( \text{rRNA} \) in the multiplex PCR.

**Table 2**

Identification of toxin genes in *Clostridioides difficile* using multiplex PCR.

| Sample No. | 16S rDNA | tcdA | tcdB | cdtA | cdtB | tcdC |
|------------|----------|------|------|------|------|------|
| MD837      | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MD500      | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MD895      | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MD949      | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MD1400     | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| CDT25      | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MD814      | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MD991      | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MD1346     | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MD2136     | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MD1904     | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| CDT18      | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| 95,731     | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| 6603       | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MRN121781  | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MRN66943   | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MD2505     | (+)      | (+)  | (+)  | (−)  | (−)  | (+)  |
| MD1742     | (−)      | (−)  | (−)  | (−)  | (−)  | (−)  |

(+) indicates positive for the corresponding toxin gene, (−) indicates negative for the corresponding toxin gene.

![Image](170x347 to 206x359)
![Image](170x372 to 206x384)
![Image](170x410 to 206x422)
![Image](170x423 to 206x435)
![Image](170x360 to 408x372)
![Image](170x385 to 442x397)
![Image](170x398 to 338x410)
![Image](170x448 to 375x460)
![Image](170x461 to 442x473)

**Fig. 1.** Representative gel pictures of PCR amplicons of toxic genes in *Clostridioides difficile*. A: Multiplex PCR for 16S rDNA (1062 bp) tcdA (629 bp) tcdB (410 bp) cdtA (221 bp) cdtB (221 bp) and cdtA (221 bp) genes using the following primers: \( \{(\text{PS13, PS14}) - (\text{F3345, R3989}) - (\text{F5670, R6079A, R6079B}) - (\text{F617, R878}) - (\text{F739A, F739B, R958})\} \), respectively. L: 100 bp ladder; Lane 1 to 18: *C. difficile* strains; Lane 14 and 15: *Enterococcus faecalis*; Lane 19: Internal *C. difficile* control. B: Representatives of single amplicon for PCR of tcdC (475 bp) gene by F (17), R (+462) primers. L: 100 bp ladder; Lane 1 to 13: *C. difficile* strains; Lane 14 and 15: *Enterococcus faecalis*; Lane 16: Internal *C. difficile* control. Lane 17: Negative PCR control.

In this current study, all patients with positive Gene Expert test were having symptoms, as this expensive test is not performed for screening. According to the recommendations for the first line of treatment, both metronidazole and vancomycin were used and patients with asymptomatic colonization should not be treated (McDonald et al., 2018). The laboratory investigations to identify *C. difficile* are still critical (Carey-Ann and Carroll, 2013).

Our results showed that GeneXpert test is a reliable diagnostic test to identify the toxins, yet it can be associated with other bacteria. The major virulence factors of *C. difficile* are toxins A and B but, the presence of binary toxin genes is significantly related to the threat of mortality (Berry et al., 2017). Although the role of binary toxin genes has been shown to cause severe pathogenicity, the role of this toxin remains unclear. Our results showed 95% of samples were positive for both toxin A and B (tcdA, tcdB) and all samples exhibited the toxin gene regulator tcdC. Recent studies reported that tcdB could provoke the characteristics of CDI without tcdA and consequently, they are focusing on the toxin-mediated treatments to broaden the options for eliminating the risk of CDI (Chandrasekaran and Lacy, 2017; Czepiel et al., 2019). All samples were confirmed negative for the binary toxin gene cdtB and 11% of the isolates were positive for cdtA gene.

Many studies have used NAAT to study CDI and many of them reported false positive results for colonized patients without disease (Fang et al., 2017). Interestingly, one of the samples (MD1742, Table 2) that has been confirmed as *C. difficile* by 16S rRNA sequencing was negative for 16S rDNA gene testing included in the multiplex PCR. The primers sequences were examined and compared to the sequence of the *C. difficile* obtained by 16S rRNA sequencing. Only forward primer sequence matched with the bacterium sequence, while no match was found with the reverse primer region. We suggest that this can be the result of a mutation in the region corresponding to the reverse primer sequence and consequently led to the false negative results in the multiplex PCR. This same isolate (MD1742) harbored the binary toxin gene (cdtA’), and tested negative for both toxins A and B. However, most of the binary toxin-producing *C. difficile* strains reported to date also produced TcdA and/or TcdB.

To conclude, our study has many salient features which are crucial to explore this life threatening infection in the Eastern province. The present study sheds the light on the importance of the methods of detection in the *C. difficile* study. Improper detection leads to wrong diagnosis, treatments delay and ineffective infection control measures. We believe that combining the standard culture
method with 16SrRNA sequencing can make the detection of *C. difficile* more accurate. In addition, our results confirm the presence of locus of pathogenicity with 5 toxin genes in *C. difficile* isolates from the patients. The locus of pathogenicity in the isolates clearly signifies the multidrug resistance of the isolates and the clinical implication of the patients. These observations strongly suggest that nation-wide large-scale studies are necessary on the locus of pathogenicity and clinical severity for the *C. difficile* infection to identify the most appropriate drug targets and to design more sensitive diagnostics tools and appropriate preventive measures.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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