DETECTION OF *CLOSTRIDIUM DIFFICILE* FROM FAECAL SPECIMENS OF HOSPITALIZED PATIENTS AT THEODOR BILHARZ RESEARCH INSTITUTE

Thesis

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ABSTRACT

Introduction: *Clostridium difficile* is the leading cause of healthcare-associated diarrhea in Western and industrialized countries. Aim of the work: The purpose of this study was to investigate the prevalence of *C. difficile* colonization among patients in the hospital setting at Theodor Bilharz Research Institute (TBRI) and its role in antibiotic-associated diarrhea; the study also aimed at developing a multistep algorithm that can provide a specific and cost-effective approach to the laboratory detection of toxigenic *C. difficile*. **Materials and Methods:** Eighty seven specimens were subjected to screening for the presence of *C. difficile* by anaerobic culture on CCFA, GDH detection by EIA and real time PCR for genes of *C. difficile*; toxin production was also tested for by EIA for *C. difficile* toxins and real time PCR for detection of *C. difficile* toxins genes. **Results:** In this study, using PCR, the prevalence of *C. difficile* was 21.8% (19 out of 87 specimens); 15 of them were in group A and 4 in group B representing 27.8% and12.1% of each group, respectively. Out of the 19 *C. difficile* positive specimens detected by PCR in this study, 17 harboured non-toxigenic organism representing 19.5% of total specimens (87). Two specimens out of the 19 positive specimens were found by real-time PCR to harbour toxigenic *C. difficile*. They belonged to group A; thus the prevalence of CDI in our study was 2.3%. In the present study faecal specimens were inoculated on cycloserine cefoxitin fructose agar (CCFA). Very low values were recorded as regards sensitivity and positive predictive value (26.3% and 31.3%, respectively); the specificity and negative predictive value were 83.8% and 80.3 %, respectively. As regard the sensitivity of EIA for GDH it was found to be 47.4% and the specificity 83.8%. Combining results of both culture and EIA for GDH has improved the specificity from 83.8% to 94.1% and the positive predictive value from 31.3% to 50%; however, the values of both the sensitivity and the negative predictive value declined from 47.4% to 21.1% and 85.1% to 81%, respectively. High sensitivity (100%) and negative predictive value (100%) were recorded when EIA for toxins was compared to PCR. However, the very small number of the supposedly true toxigenic isolates (2) as detected by PCR doesn't allow the accurate estimation of the sensitivity and the negative predictive value of EIA test for *C. difficile* toxins. The specificity of EIA for toxin detection was 87.1% compared to PCR. An important limitation in the present study was the small sample size of the diagnosed CDI; statistical evaluation was not applicable regarding the use of combination of different methods employed for diagnosis of CDI. In this study, 36.4% of patients with a hospital stay of more than 10 days, 25% of those previously hospitalized and 26.5% of those who had comorbidity were colonized by *C. difficile*. However, no statistical significance was found for these predictors. *C. difficile* infection was found in 2 patients who were among group A (had diarrhea with unidentifiable cause), were on antibiotic therapy (cefoxitin) and harboured toxin-producing *C. difficile* as detected by PCR. The 2 patients had additional risk factors which were previous hospitalization, hospital stay more than 10 days as well as comorbidity in the form of liver cirrhosis and ascitis for one patient and hepatocellular carcinoma for the other**. Conclusions:** *C. difficile* colonization is not uncommon among the hospital patients of Theodor Bilharz Research Institute. The use of EIA for GDH for screening for presence of *C. difficile* in faecal specimens followed by real-time PCR for presence of toxins genes in the samples provides a convenient, rapid and specific strategy for diagnosis of CDI. However, the sensitivity is not satisfactory.

Key words: *C. difficile*, Quik Chek Complete, Toxins A/B, Cycloserine-cefoxitin-fructose agar, RIDA PCR.

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LIST OF ABBREVIATIONS

B1/NAP1/027	Ribotype 027
CCFA	Cycloserine, cefoxitin, fructose agar
CCNA	Cell cytotoxicity neutralization assay
CDAD	*C. difficile* associated diarrhea
CDI	*C. difficile* infection
CFA	Cellular fatty acid
EIAs	Enzyme immune-assay
FMT	Faecal micribiota transplantation
GDH	Glutamate dehydrogensae
IVIG	Intravenous immunoglobulin
NAATs	Nucleic acid amplification tests
RR	Relative risk
SLPs	Surface layer proteins
TcdA	*C. difficile* toxin A
TcdB	*C. difficile* toxin B
US	United states
VRE	Vancomycin resistant enterococci

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INTRODUCTION

Clostridium difficile (*C. difficile*) is a Gram-positive, spore-forming rod that grows anaerobically **(Van den Berg *et al.*, 2007).** Strains of *C. difficile* can be toxigenic or nontoxigenic; however, only toxigenic strains produce disease. The two main virulence factors are toxin A, an enterotoxin with some cytopathic effects and toxin B, a potent cytotoxin that affects various tissue cell lines *in vitro* and inhibits bowel motility *in vivo* **(Reller *et al.*, 2007).** Toxin B is ten times more potent than toxin A. Toxin B was identified as the virulence factor necessary for full expression of *C. difficile* infection (CDI) **(Lyras *et al.*, 2009).**

The two toxins, TcdA and TcdB, are encoded on a pathogenicity locus along with negative and positive regulators of their expression. Following expression and release from the bacterium, TcdA and TcdB translocate to the cytosol of the target cells and inactivate small GTP-binding proteins, which include Rho, Rac and Cdc42. TcdA and TcdB inactivate these substrates through monoglucosylating small GTPases, causing actin condensation and cell rounding due to disassembly of actin microfilaments leading to impairment of tight junctions in human colonocytes **(Voth and Ballard, 2005).**

Infection with *C. difficile* can cause asymptomatic colonization reaching 20-30% in acute care hospitals and may be as high as 50% in long-term care facilities **(Bartlett, 2008)** or a spectrum of clinical manifestations ranging from mild diarrhea to severe colitis, the latter often resulting in life threatening complications such as pseudomembrane formation, toxic megacolon and sepsis **(Stamper *et al.*, 2009).** *C. difficile* is the most commonly identified cause of antibiotic-associated diarrhea, accounting for 15% to 25% of cases **(El-Defrawi and Fahkri, 2001). Redelings *et al.* (2007)** stated that the number of deaths from CDI exceeds that of all other intestinal infections. **Kyne *et al.* (2002)** found that nosocomial infection by *C. difficile* increases the cost of hospitalization by 54% and the length of stay

by 3.6 days. One study estimated that the annual cost for management of CDI in the United States was $3.2 billion **(O'Brien *et al.*, 2007).**

The epidemiology of *C. difficile* infection has changed over the past decade due to the emergence of hypervirulent *C. difficile* epidemic strains which produce excess toxin. This strain (BI/NAPI/027) has been characterized as "BI" by restriction enzyme analysis (REA), "NAPI" (North American pulsed field type 1) by pulsed-field gel electrophoresis, "027" by PCR ribotyping. It causes severe illness, which often requires colectomy for control and results in increased mortality. This strain is more likely to spread in the hospital environment due to antimicrobial resistance and enhanced spore formation **(Sloan *et al.*, 2008).**

Potential factors contributing to the increased frequency and severity of CDI are an aging population, hospitalized patients with numerous comorbidities, excess antibiotic use and emergence of a more virulent strain of *C. difficile* **(Salkind, 2010).**

While most cases of CDI occur in hospitalized patients, community–acquired infections are recognized. Antibiotic exposure and age above 65 years are common risk factors for the acquisition of CDI in the hospitalized patient, but these factors may be absent in individuals with community–acquired CDI (CA-CDI). Use of proton pump inhibitors and concurrent diagnoses of renal failure, inflammatory bowel disease, irritable bowel disease and peripartum women were also related to an increased risk of CA-CDI. Possible community sources for *C. difficile* are soil, household pets, food and exposure to hospitalized patients **(Pituch, 2009).**

Early recognition of *C. difficile* infection has a profound effect on proper disease management. A rapid yet sensitive and specific diagnostic assay would be advantageous to clinicians for the early recognition of disease and to infection

control practitioners for swift implementation of control measures **(Larson *et al.*, 2010).**

A variety of diagnostic methods exist for the detection of *C. difficile* in faecal samples. A cell culture cytotoxicity neutralization assay (CCNA) is generally considered the optimal gold standard for the detection of toxigenic *C. difficile.* However, CCNA is labour-intensive, subjective and time-consuming and therefore is not an ideal standard **(Delmee *et al.*, 2005).**

Anaerobic culture for *C. difficile* using a selective medium is the most sensitive method but is time consuming and requires confirmation of the toxigenicity of isolates by another method such as CCNA or molecular detection of toxin regulating genes (toxigenic culture); several days are required to complete all testing **(Sloan *et al.*, 2008).**

Traditional enzyme-immunoassays (EIAs) for the detection of toxins A and B are relatively quick but lack sensitivity ranging from 32 to 79% when used alone and often lack specificity **(Gilligan, 2008)**.

An additional assay detects the "common antigen" of *C. difficile* (glutamate dehydrogenase [GDH]). GDH EIAs have been reported to be highly sensitive for *C. difficile* detection, allowing same–day reporting of negative results, but positive results must be followed by another test to differentiate between toxigenic and non-toxigenic strains. However, performance of a two-step algorithm often delays detection, has been reported to be variable in some institutions and may be impacted by staffing and financial constraints **(Larson *et al.*, 2010).**

An alternative, highly sensitive method to detect toxigenic *C. difficile* is real-time PCR with sensitivity values ranging from 83.6% to 93.4% and specificity from 93.9% to 98.2% respectively, when compared to toxigenic culture. Real-time PCR can be completed on the day of specimen submission, thus providing same-day results. However, PCR techniques have not been widely used

for faecal specimens due to primarily budgetary issues as well as the challenge of extracting nucleic acids from faeces and separating template DNA from potentially interfering substances **(Sloan *et al.*, 2008).**

Thus, the optimal strategy to provide timely, cost-effective and accurate results remains a subject of controversy **(Larson *et al.*, 2010).**

AIM OF THE WORK

The purpose of this study was to:

1. Investigate the prevalence of *C. difficile* colonization among patients in the hospital setting at Theodor Bilharz Research Institute (TBRI) and its role in antibiotic-associated diarrhea.
2. Develop a multistep algorithm that can provide a specific and cost-effective approach to the laboratory detection of toxigenic *C. difficile*.

CHAPTER I: CLOSTRIDIA

Historical Review

The development of the field of anaerobic bacteriology is usually attributed to Pasteur, who in 1861 discovered anaerobiosis by observing that butyric acid fermentation occurred in the absence of oxygen. Pasteur noted that the anaerobic fermentation was due to a rod shaped organism that he called *'Vibrion butyrique'* which likely corresponds to the organism *Clostridium butyricum*, the type species of the genus *Clostridium.* Endospores were discovered independently by Ferdinand Cohn and Robert Koch in 1876, soon after Pasteur has made microbiology famous. The clostridia were initially classified in the genus *Bacillus* because of their cylindrical shape and formation of endospores, but in 1880 a new genus, *Clostridium*, was proposed by Prazmowski for the anaerobic spore-forming organisms **(Johnson, 2005).**

Taxonomy

The genus *Clostridium* comprises obligately anaerobic (or occasionally aerotolerant), gram-positive rods. The number of clinically significant clostridia from human infections is limited **(Johnson *et al.*, 2007) (Table 1).**

Description of the Genus

Morphological and phenotypic properties that have traditionally been used to define the genus include (i) the formation of endospores, (ii) anaerobic energy metabolism, (iii) an inability to reduce sulfate to sulfide, and (iv) a gram-positive cell wall structure **(Johnson, 2005).**

TABLE 1 Characteristics of *Clostridium* species of clinical significance[a] **(Johnson *et al.*, 2007)**

Species	Gelatin hydrolysis	Lecithinase	Lipase	Indole	Esculin hydrolysis	Nitrate	Milk digestion	Fermentation of:			Fermentation of:										Spore location	Metabolic end products from PYG
								Glucose	Arabinose	Cellobiose	Fructose	Lactose	Maltose	Mannitol	Mannose	Melibiose	Ribose	Salicin	Sucrose	Xylose		
Saccharolytic, proteolytic																						
C. bifermentans[b]	+	+	−	+	+$^{-}$	−	+	+	−	−	−^{w}	−	w^{-}	−	−^{w}	−	−	−	−	−	ST	A (iv, ic, p, ib, b, l, s)
C. botulinum[c]																						
Types A, B, and F	+	−	+	−	+	−	+	+$^{-}$	−	−	−^{w}	−	−^{w}	−	−	−	−	−	−	−	ST	A, B, IV, ib (ic, v, p)
Types B, E and F[d]	+	−	+	−	−	−	−	+	w^{-}	−	+^{w}	−	+$^{-}$	−^{w}	+^{w}	−^{w}	v	−	+^{w}	−	ST	B, A (1)
Types C and D	+	−$^{+}$	+	−$^{+}$	−	−	+	+	−	−	v	−	v	−	v	−^{w}	v	−	−	−	T	B, P, A (v, l, s)
C. cadaveris	+	−	−	+	−	−	+	+	−	−	v	−	−	−	−^{w}	−	−	−	−	−	T	B, A
C. difficile[e]	+	−	−	−	+	−	−	+^{w}	−	v	+^{w}	−	−	w^{+}	v	−	−^{w}	−^{w}	−	−^{w}	ST^{T}	B, A, ic, iv, ib (v, l)
C. novyi A	+	+	+	−	−	−	−	+	−	−	−^{w}	−	v	−	−	−^{w}	v	−^{w}	−	−	ST	A, B, P
C. perfringens	+	+	−	−	v	v	+	+	−	−$^{+}$	+	+	+	−	+	v	v	−$^{+}$	+	−	ST	A, B, L (p, s)
C. putrificum	+	−	−	−	−$^{+}$	−	+	+^{w}	−	−	−^{w}	−	−^{w}	−	−	−	−	−	−	−	TST	A, B, ib, iv (p, ic, v, l, s)
C. septicum[f]	+	−	−	−	+	v	+	+	−	+^{w}	+	+	+	−	+	−	v	v	−	−	ST	B, A (p, l)
C. sordellii[b]	+	+	−	+	−$^{+}$	−	+	+	−	−	v	−	w	−	−^{w}	−	−^{w}	−	−	−	ST	A (IC, p, ib, iv, l)
C. sporogenes[f]	+	−	+	−	+	−	+	+	−	−^{w}	−	−	−^{w}	−	−	−	−	−	−	−	ST	A, B, iv, ib (p, ic, v, l, s)
Saccharolytic, nonproteolytic																						
C. baratii	−	+	−	−	+	+$^{-}$	−	+	−	+	+	w^{+}	w^{+}	−	+	−^{w}	w^{-}	+$^{-}$	+	−	ST	B, A, L (p, s)
C. bolteae[g]	−	−	−	−	−$^{+}$	−	−	+	+	−$^{+}$	+	−	+	−	+		−	−$^{+}$	+	+	ST	A (l)
C. butyricum	−	−	−	−	+	−	−	+	+$^{-}$	+	+	+	+	−^{w}	+	+^{w}	+^{w}	+^{w}	+	+	ST	B, A (l, s)
C. carnis[h]	−	−	−	−	+	−	−	+	−	w^{+}	v	v	w^{+}	−	w^{+}	−	−	w	w^{+}	−	ST	B, A, L (s)
C. clostridioforme[g]	−	−	−	−	+	−	−	+	+	+	+^{w}	+	+	−	+	v	v	+	+^{w}	+^{w}	ST	A (l)
C. glycolicum	−	−	−	−	−$^{+}$	−	−	+	−	−	+^{w}	−	v	−	−	−	−	−	−	+$^{-}$	ST	A, IV, IB (p, l, s)
C. hathewayi[g]	−	−	−	−	+^{w}	−	−	+	V	+	+	+	+	−	+	+	+	+	+	+	ST	A (l)
C. indolis	−	−	−	+	+	+$^{-}$	−	+	w^{-}	+^{w}	w^{+}	w^{+}	w^{+}	−^{w}	−^{w}	−^{w}	−^{w}	w^{-}	V	v	T	A
C. innocuum[e]	−	−	−	−	+	−	−	+	−$^{+}$	+	+	−^{w}	−	+^{w}	+	−	v	+^{w}	+^{w}	−^{w}	T	B, L, a (s)
C. paraputrificum	−^{w}	−	−	−	+	−$^{+}$	−	+	−	+	+^{w}	+	+	−	+	−	w^{-}	+	+	−	TST	B, A, L (s)
C. ramosum	−	−	−	−	+	−	−	+	−	+	+	+	+	+$^{-}$	+	+$^{-}$	v	+	+	−^{w}	T	A, l (s)
C. sphenoides	−	−	−	+	+	+$^{-}$	−	+	−^{w}	+^{w}	+^{w}	w^{+}	+^{w}	w^{+}	+^{w}	v	−^{w}	v	w^{-}	v	ST^{T}	A (l, s)
C. symbiosum	−^{w}	−	−	−	−	−	−	+^{w}	V	−	+	−$^{+}$	−	−	v	−	−	−	−	−	ST	A, B, L
C. tertium[h]	−	−	−	−	+	+$^{-}$	−	+	−	+^{w}	+	+	+	w^{+}	+^{w}	+^{w}	+^{w}	+^{w}	+	v	T	A, B, L
Asaccharolytic																						
C. argentinense	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	ST	A, b, ib, iv (l)
C. hastiforme	+	−	−	−	−	−$^{+}$	−$^{+}$	−	−	−	−	−	−	−	−	−	−	−	−	−	T	A, B, iv, ib(p, ic)
C. histolyticum[h]	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	ST	A (l, s)
C. limosum	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	ST	A (l, s)
C. subterminale	+	−$^{+}$	−	−	−$^{+}$	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	ST	A, B, IV, ib(p, ic, l, s)
C. tetani[f]	+	−	−^{w}	v	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	T	A, B, p (l, s)

[a] +, positive reaction; −, negative reaction; v, variable reaction; w, weakly positive reaction; ST, subterminal; T, terminal. A superscript indicates rare variabili Boldface type indicates key reactions. Capital letters indicate major metabolic products from PYG, lowercase letters indicate minor products, and parentheses indic a variable reaction for fatty acids as follows: A, acetic; P, propionic; IB, isobutyric; B, butyric; IV, isovaleric; V, valeric; IC, isocaproic; L, lactic; S, succinic; and P. phenylacetic.

[b] *C. bifermentans* is urease negative, and *C. sordellii* is urease positive. *C. bifermentans* usually forms chalk-white colonies on egg yolk agar.

[c] A toxin neutralization test is required for identification. Send suspected isolates or *C. botulinum*-containing material to the appropriate local or state public health agency

[d] Nonproteolytic.

[e] L-Proline aminopeptidase differentiates *C. difficile* and *C. innocuum*. *C. difficile* is positive, and *C. innocuum* is negative.

[f] Swarming.

[g] Cigar shaped. *C. bolteae* is lactose and β-NAG negative, *C. clostridioforme* is lactose positive and β-NAG negative, and *C. hathewayi* is lactose and β-NAG positive.

[h] *C. tertium*, *C. carnis*, and most *C. histolyticum* isolates grow aerobically.

Morphology

Vegetative cells of *Clostridium* species are pleomorphic, rod shaped, and arranged in pairs or short chains; the cells have rounded or sometimes pointed ends. Rods may join to form tight coils or spiral configurations in species such as *C. cocleatum* and *C. spiroforme*. Clostridia stain gram positive in early stages of growth, although some species, such as *C. clostridioforme*, *C. hathewayi*, *C. innocuum*, and *C. ramosum*, may appear gram negative. Several species (e.g., *C. tetani*) appear gram negative by the time that spores have formed. Endospores are often wider than the vegetative organisms, imparting characteristic spindle shapes to clostridia. Most strains are motile by means of peritrichous flagella. Nonmotile species include *C. perfringens*, *C. ramosum*, and *C. innocuum* **(Stackebrandt and Rainey, 1997).**

Metabolism

Clostridium species are metabolically diverse. As currently designated, most species are chemoorganotrophic; some species may be chemoautotrophic and chemolithotrophic. They can be saccharolytic, proteolytic, neither, or both; they do not carry out sulfate reduction. They usually produce mixtures of organic acids and alcohols from carbohydrates, proteins and peptides, or purines and pyrimidines **(Johnson *et al*., 2007).**

Culture

Most of the clostridia grow best at 37°C, between pH 7.0-7.4 under anaerobic conditions, though many of them are capable of growing at temperatures of 20°C and even lower. On solid media, growth is relatively slow and sometimes takes the form of a thin, effuse, often spreading film, which may be difficult to distinguish from the underlying medium. Film formation is promoted by moisture. The spreading of clostridia is inhibited by certain chemicals. Some