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Intensive Care Unit Inpatients Complicated with Diarrhea Mediated by Toxigenic Clostridium Difficile: Rapid Diagnosis and Efficient Treatment

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ABSTRACT

Identifying infections in a cerebrovascular accident (CVA) is challenging due to involvement of tracheal tubes and invasive catheters. Prophylactic antibiotic therapy may prevent some infections, but may predispose CVA patient to toxigenic Clostridium *difficile* diarrhea. Ninety-eight CVA patients were studied who suffered from diarrhea during antibiotic treatment. Toxigenic culture, cytotoxicity assays and multiplex Real-time PCR results yielded 26 (5.3%) toxigenic C. *difficile* among which 22 cases were positive for both A and B toxin genes (*tcd*A, *tcd*B) and four were positive for *tcd*B. C. *difficile* infection was controlled through prescribed oral vancomycin as the first-line and intravenous metronidazole as the second-line antibiotic-therapy.

KEYWORDS: Clostridium *difficile*; CVA; real-time PCR

INTRODUCTION

Cerebrovascular accident (CVA) patients have baseline characteristics, including acute physiology and chronic health evaluation II (APACHE II) score, age, sex, history of drug use and antibiotic therapy (defences against co-infecting pathogens). Dysphagia occurs in most CVA inpatients. thus prophylactic antibiotics commonly decrease the risk of post-pneumonia in critically ill patients. However, antibiotic prophylaxis may increase the risk of developing Clostridium *difficile* mediated antibiotic-associated diarrhoea during hospitalization. Due to the severity and unclear comorbidities in acute stroke patients, prior antibiotic therapy in CVA patients suffering from underlying diseases, including pulmonary disease, diabetes, renal failure and coronary heart disease, may worsen patients' health condition drastically [1, 2].

Prophylactic antibiotic therapy may start 48hrs before or after ICU admission considering the high rate of dysphagia and pneumonia

in post stroke CVA patients. Multidrug infections are globally the major cause of mortality in CVA patients and inadequate diagnosis often leads to inappropriate antibiotic prescriptions [3].

A highly common infection in these patients is C. *difficile* infection (CDI), with rates ranging from 14% to 71.6%. The pathogenicity of toxigenic types of C. *difficile* is mainly mediated by the production of toxins A and B (*Tcd*A and *Tcd*B). Thus, it is important to be aware of the accuracy and routine detection of toxin genes in samples from patients suffering from diarrhea [4].

The main objective of the present study was to evaluate the CDI rates among CVA patients in the ICU ward. We used a two-step diagnostic model of a rapid and sensitive multiplex Real-time PCR assay to detect toxigenic C. *difficile* combined with toxigenicity assessment following the effective treatment of the CDI cases.

POPULATIONS

We investigated ICU-admitted CVA patients from two major general hospitals in Tehran from December 2017 to November 2018. A total of 489 patients were admitted to the intensive care unit with CVA as the main complication and hospitalized for more than 5 days.

Ninety-eight adult CVA patients (20%), receiving antibiotics and having diarrhea (3< unformed stools a day) were investigated for toxigenic C. *difficile* infections.

Based to the development of febrile periods mostly due to relevant infections, 95 per cent of the patients received antibiotic therapy as follows: Carbapenems (39%), Vancomycin (32%), and Cephalosporin, Penicillin groups, Metronidazole, Polymyxin (14%) and Quinolones, Clindamycin (9%). The history of antibiotic therapy starts from one day to one month before diarrhoea happening. The Faculty of Medical Sciences Ethics Committee at Tarbiat Modares University (IR.TMU.REC.1395.403) and Digestive Disease Research Institute, Tehran University of Medical Sciences, approved this survey.

MULTIPLEX REAL-TIME PCR

Total DNA from stool samples was extracted by QIAamp® DNA Stool Mini Kit (Qiagen) and spiked with the internal control of RealStar® C. *difficile* PCR Kit (altona), as instructed by the manufacturer. The presence of the toxin genes (*tcd*A and *tcd*B) was examined simultaneously by RealStar® C. *difficile* PCR Kit using both LightCycler@96 (Roche) and Rotor-gene Q (Qiagen) machines.

Multiplex Real-time PCR assay carried out with the following reaction mixture: RealStar® master mix A (5µL/reaction), master mix B (15µL/reaction) and eluted nucleic acid extraction (10µL/reaction) with timing procedure of 2 min at 95°C, followed by 45 cycles consisting of 15 sec at 95°C and 45 sec at 58°C. The RealStar® C. *difficile* PCR assay carried out in the total combination of amplification and detection time of 90 min.

Specific C. *difficile* toxin gene probes (*tcd*A, *tcd*B) and kit`s internal control were labelled Cy®5, FAM and JOE fluorophores respectively. The qualitative aspect of the real-time PCR defines a sample positive if the Cy®5 (*tcd*A), FAM (*tcd*B) and JOE (Positive control) channels are positive, negative if the Cy®5 and FAM channel are negative and the JOE channel is positive, and undetermined and retest needed if all channels are negative.

Qualitative multiplex Real-time PCR assay detected 26 toxigenic C. *difficile* (5.3%) among which 22 (4.5%) were positive for both *tcd*A and *tcd*B and four (0.8%) were only positive for *tcd*B.

CYTOTOXIGENIC CULTURE TESTS

Direct Toxigenic Culture

The stool samples were separated in two spikes (~1g), one spike was transferred into the Clostridium *difficile* Brucella broth (CDBB) for 1h under strict anaerobic condition [5]. Another part was treated with alcohol shock for 1h. Treated and non-treated samples were inoculated onto enriched Cycloserine - Cefoxitin Fructose Agar by vitamin K1 (1µg/mL) and hemin (5µg/mL) and incubated anaerobically for 2-5 days at 37°C. C. *difficile* colonies were identified by Gram stain, appearance, and odour. DNA from isolates were extracted and purified by QIAamp® DNA Mini Kit (Qiagen) and confirmation of C. *difficile* species-specific glutamate dehydrogenase (GDH) gene (gluD) fulfilled by PCR [6].

Cytotoxicity Assay

Monolayer cultures of Hep-2 cells were prepared in 96-well plates (Jet-Biofil®) using RPMI (Gibco®) supplemented with 5% FBS (Gibco®), non-essential amino acids (Gibco®) and penicillin-streptomycin (Gibco®) [7]. Three colonies from GDH C. *difficile* isolates were grown in 30 mL of TSB media (Trypticase Soy Broth) under anaerobic condition for 72h. Cell-free media were subsequently prepared by centrifuging cultures at 8000×g for 20 min at 4°C. The cultures were then passed through 0.22 µm membrane filters. Monolayer Hep-2 cell cultures were directly exposed to C. *difficile* supernatants for 30, 60 and 120 min and cell viability was measured using the MTT test. Briefly, culture supernatants were removed and the wells were washed with fresh RPMI medium. Then, complete RPMI media (above) that contained MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5mg/mL) was added to all wells and incubated for 4h. Plates were then centrifuged (8000×g) to remove media and the purple formazan crystals were dissolved by adding 100µL of DMSO (Sigma-Aldrich) to each well. The absorbance measured at 570 nm using mQuant plate reader (Bio-Tek Instrument) and cell viability was calculated according to the following equation [8].

$$\text{Cell viability (\%)} \frac{((\text{absorbance of treated well}))}{((\text{absorbance of control well}))} \times 100$$

RESULTS

The *tcd*A and *tcd*B genes were investigated by RealStar® C. *difficile* PCR Kit as described above. GDH-PCR assay confirmed 29 (5.9%) isolates from anaerobic culture as C. *difficile*. Multiplex real-time PCR results demonstrated 3 (A-B-; 0.6%) non-toxigenic isolates, 26 (A+B+; 5.3%) were positive for *tcd*A and *tcd*B toxin genes which confirmed through direct toxigenic culture and cytotoxicity assay.

No significant correlation between CDI rate and antibiotic-therapy was shown by pairwise regression analysis of our results obtained from each run on both machines. The results were analysed through one-way analysis of variance (ANOVA), pairwise two-tailed correlation and regression with SPSS Version 25.0 (IBM® SPSS® Statistics, USA).

ANTIBIOTIC THERAPY

Oral vancomycin was administered to patients as the first-line treatment for mild or moderate diarrhea, on the same day of obtaining positive results of the real-time PCR [9]. Intravenous Metronidazole was administered for at least 5 days as the second-line antibiotic treatment for severe or complicated diseases to for treating the clinical signs with higher success in controlling toxigenic C. *difficile* infection during this study.

DISCUSSION AND CONCLUSION

C. *difficile* infections are a pressing problem in the developed world as well as in developing countries. Nevertheless, a lack of effective diagnostic procedures make it difficult to control the disease in less developed areas [9].

CVA group is the most significant population among the ICU patients with a high susceptibility to several types of infections. Over recent years, there has been an increasing rate of C. *difficile* infection in ICU patients. Frequently, critically ill patients are difficult to treat due to complicated diseases, ranging from mild or moderate to lethal forms. CVA patients are exposed to many risk factors making them susceptible to CDI such as multiple antibiotic therapy, immunosuppression and underlying morbidities. Likewise, treatment failure is frequent in intensive care units [10].

CDI outcomes are reported as symptomatic cases (0.4-4%) or asymptomatic ones (10-20%). Distinguish the occurrence of the infection in patients.

Present study covers 1 year on the ICU patients with CVA. Most patients aged over 50 with the history of multiple antibiotic therapies including carbapenems, vancomycin and metronidazole and other broad-spectrum antibiotics (cephalosporin, penicillin groups, Polymyxin, quinolones, and clindamycin).

Our study showed that 5.3% of CVA patients were infected by the toxigenic C. *difficile*. The infection rate increased to 5.9% if three non-toxigenic C. *difficile* isolates were included.

The cytotoxicity assay based on the MTT method confirmed 26 isolates as toxin-producing C. *difficile*. The toxigenicity of the isolated strains was investigated by exposing Hep-2 cells directly to the bacterial broth-culture filtrates.

The phenotypic evaluation of toxin production is time-consuming and necessitates specialized laboratory equipment. Multiplex real-time PCR method can quantify toxigenicity of the isolates and distinguish between low and high toxin-producing organisms, a characteristic that may contribute to their pathogenicity.

In our work, the length of hospital stay was more than 9 days for all the patients, with the history of antibiotic therapy ranging from 1 week to two months. Being subject to antibiotic therapy was the main risk factor of developing C. *difficile* infection, we did not detect C. *difficile* through Cytotoxigenic culture or multiplex real-time PCR less than a week.

However, we did not find any correlation between antibiotic treatment and converting of colonized C. *difficile* to pathogenic toxin-producing forms, especially toxins A and B. Most of the diagnosed cases (26/29) were toxigenic. The presence of toxins is believed to be significant for the prognosis of CDI occurrence in critically ill ICU patients. Therefore, we detected toxin genes through multiplex Real-time PCR followed by the assessment of cytotoxigenic culture (both anaerobic culture and cytotoxicity assay).

Monitoring further epidemiological investigations, tracing toxigenic C. *difficile* is necessary for optimal antibiotic treatments to decrease CDI rates within CVA patients [11].

The lower prophylactic ratio (9-14%) prescribed, concerning reduce using the antibiotics higher associated with C. *difficile* infection rates, as “4C” (clindamycin, cephalosporin, co-amoxiclav, and ciprofloxacin). Cases of co-infections with multidrug-resistant bacteria were mostly treated with carbapenems [12]. Oral vancomycin or metronidazole was the favoured treatment for the resolution of diarrhea as the first antibiotic monotherapy. Intravenous metronidazole was administered in the case of non-prescription oral drugs for the intestinal impairments and also for severe or complicated disease [13]. The treatment was administered after collecting diarrheal stool samples.

Although, various diagnostic methods have been used to detect C. *difficile* toxins rapidly in stool specimens, real-time PCR assay was the most sensitive, specific, rapid and user-friendly.

Toxin gene detection was achieved in less than 4 hours. Antibiotic therapy to the patients was optimized using these the test results. Nevertheless, to prevent co-infection of high risk ICU patients, it is essential to screen the symptomatic infections with the precise assay.

Finally, we established multiplex real-time PCR as a reliable, time-saving method with acceptable sensitivity and specificity to

monitor CDI in antibiotic-associated diarrhea clinical fecal samples.

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