AN UNUSUAL CASE OF CLOSTRIDIUM DIFFICILE INFECTION IN TRINIDAD & TOBAGO

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ABSTRACT

AIM: To report the first fatal case of a toxin A positive toxin B gene positive strain of Clostridium difficile that caused pseudomembraneous colitis in a patient living in Trinidad & Tobago.

Presentation of case: Patients admitted in a tertiary hospital with history of diarrhea were being investigated and reviewed. A case of a 47-year old Trinidadian of African descent presenting with diarrheal stool at a public hospital in Trinidad and Tobago. The identified patient had his stool sample analyzed using conventional and molecular microbiology procedures to identify the bacteria, toxins and genes produced by the microorganism. The antimicrobial susceptibility was also determined using agar dilutions according to CLSI guidelines.

DISCUSSION: The patient's stool sample produced positive results for Clostridium difficile toxin. The Quick Check Complete® kit demonstrated the production of toxin and the glutamate dehydrogenase enzyme. This strain exhibits the toxin A and B gene.

CONCLUSION: Clostridium difficile isolated from this patient revealed the presence of toxin A and B gene. This gave a 1266bp and 204bp DNA fragment corresponding to the toxin A and B gene respectively, based on the primers used. The isolated strain was resistant to meropenem, ampicillin, ceftriaxone, cefotaxime and ciprofloxacin. This organism seems to have been responsible for the formation of psuedomembranous colitis in the studied patient. Further

research is needed on similarly detected strains, to better understand their significance in Inflammatory Bowel Diseases and their prevalence in our geographical location.

KEYWORDS

Clostridium difficile, Pseudomembraneous colitis, Polymerase Chain Reaction, Trinidad & Tobago

INTRODUCTION

Clostridium difficile is an anaerobic gram positive bacilli which is the major cause of psuedomembraneous colitis [1]. Infection with this organism may result in a range of presentations including asymptomatic carriage, mild diarrhea, psuedomembraneous colitis and toxic megacolon [2]. Various researches have shown that Clostridium difficile is present in approximately 10% of the normal healthy adult population and is implicated in 20-30% of nosocomial diarrhea.This organism is also the major role player implicated in antibiotic associated diarrhea [3-5].

Acquisition of Clostridium difficile infection often occurs in conjunction with disruption of the normal colonic microflora. Causes for such disruption may include antibiotic overexposure and underlying disease [6].Clostridium difficile spores and vegetative cells are brought into the body via ingestion. The vegetative cells are destroyed by the low pH of the stomach, however the spores continue on to the small intestine where they germinate upon exposure to bile acids [7]. The proliferating cells move into the colon where they attach themselves, being adequately protected by a polysaccharide layer. In a patient with good IgG response, this colonization leads to asymptomatic carriage; however in patients with poor IgG response toxin production may lead to manifestations of disease [6].

Two major toxins-toxin A and toxin B are produced by Clostridium difficile vegetative cells [8]. Both toxins attach themselves to epithelial cells of the colon and enter the cells via endocytosis. The resulting vacuole has a pH which facilitates the glycosyltransferase portion of the toxin to be released into the cytoplasm. This glycosyltransferase moiety facilitates the transfer of a glucose molecule from uridine diphosphate to Rho, Rac or Cdc-42 molecules which leads to inactivation of these molecules [9]. These molecules are responsible for actin formation, regulation of apoptosis and transcriptional regulation [9]. The mechanism for both toxins is the same; however there are differences in the effect on intestinal cells. Toxin A is enterotoxic; and causes neutrophil infiltration, substance P production, chemokine production, disruption of tight

junctions and apoptosis. Toxin B has more direct (cytotoxic) effects causing disruption of tight junctions and apoptosis. The overall effects are edema and an inflammatory response [9].

There are now evidences of a third toxin known as a binary toxin that is produced by various Clostridium difficile strains; one of such is the NAP 1 strain [10]. This new strain is an actin-specific ADP-ribosyltransferase toxin, that has two dependent proteins; CDTa, which is the catalytic component and CDTb which is the binding component [11]. It is widely accepted that certain strains of Clostridium difficile have the propensity to cause outbreaks, including multiple-state outbreaks in healthcare facilities [12]. Even though toxigenic strains have been sensitive to the widely accepted cytotoxicity assay, its use has become limited and methods such as Enzyme Linked Immuno-Sorbent Assay (ELISA) testing are now routinely used [13].

Molecular analysis has now been added to the algorithm for Clostridium difficile detection. This includes toxin gene detection via simple PCR analysis [14, 15]. This enables researchers and health-care workers to understand the genetic aspect of the organism and how it may be related to where the organism was found. The species-specific internal gene fragment (tpi) for Clostridium difficile, as well as the gene for toxin A and B can efficiently be tested for using specific primers; which in turn can show the pathogenic nature of Clostridium difficile [13].

PRESENTATION OF CASE

A 47 year-old Trinidadian male of African descent was admitted to the Eric Williams Medical Sciences Centre (EWMSC) in Mount Hope, Trinidad and Tobago as a referral from his family doctor where he had been seeking medical attention for the past 5 months. He presented with complaints of fever, abdominal cramps, generalized swelling and moderate diarrhea. He had no history of travel abroad. However, he had a 6 month history of rheumatoid arthritis. (Rheumatoid Factor was positive at 720 IU/ml). While in the care of his family physician, he received steroids for persistent swelling of his upper and lower limbs. According to the patient, he visited his family physician because he was having diarrhea and fever after he and a relative consumed substantial amounts of seafood at a weekend social gathering. He stated that they both became sick with symptoms of diarrhea and malaise following the meal. Although his relative recovered, the patient remained ill and was then referred to the medical center by his family physician.

On admission to the EWMSC he was treated for arrhythmia with adenosine; and empirically with ceftriaxone for suspected septic arthritis. Steroid therapy was continued his symptoms dramatically improved and he was subsequently discharged. Four months later he was readmitted with similar complaints – diarrhea, fever, malaise. On physical examination the patient appeared very ill, pale and dehydrated. He was febrile, blood pressure was 115/81 mm/hg and pulse rate was 125 beats/min. He was in mild respiratory distress and had generalized abdominal

tenderness and swelling. The right inguinal lymph node was soft, mobile and enlarged. Rectal examination showed the presence of soft stool mixed with bright red blood on the examining finger.

Blood, stool and urine samples were taken for laboratory investigations. Stool was submitted specifically for identification of ova, cyst and parasite (OCP); as well as for culture. His hemoglobin was 11.1g/dl and his white blood cell count was 25.6 x 109/dl. Blood and urine cultures came back negative; stool was also negative for OCP and culture with Cycloserine Cefoxitin Fructose Agar (CCFA) media. Flexible sigmoidoscopy revealed a pale mucus membrane and edema in the left transverse colon. There were no mucosal lesions visible. Computed Tomography (CT) of the abdomen/pelvis showed a hypodense mass in inguinal canal adjacent to the right iliopsoas muscle. Unfortunately, surgical intervention was not carried out to remove this mass as patient was too ill. The patient was treated with amantadine, levofloxacin, ibuprofen, prednisolone, Celebrex®, Panadol® and metronidazole 500mg intravenously.

His diarrhea persisted, and twelve days later the white blood cell count was 30 x 109/l. A repeat CT scan showed a wedged shaped area in the periphery of the spleen, diffused thickening and mucosal enhancement of the distal ilium and the colon. Flexible sigmoidoscopy was repeated and demonstrated inflammation, jelly-like mucin, an enhanced, yellowish membrane and few mucosal lesions in the transverse colon. Biopsy of the region ruled out malignancy and a diagnosis of pseudomembranous colitis was made. Vancomycin was subsequently added to the treatment regimen; and was given orally at 500mg doses every 6 hours each day. The patient did not respond to treatment and eventually died before surgical procedures were carried out.

METHOD

Prior to the patient's death, a repeat stool samples were collected and tested for the organism using the C.diff Quick Check Complete® from Tech Labs. The stool sample was positive for the Clostridium difficile toxin however the ELISA kit used above did not indicate which toxin was present. The stool sample was cultured anaerobically using Cycloserine Cefoxitin Fructose Agar (CCFA) for 96 hours at 37°C. Culture revealed flat, ground- glass shaped greenish colonies which produced a horse scent; gram stain showed gram positive bacilli. The organism was confirmed as Clostridium difficile using Polymerase Chain Reaction to test for the organism's house-keeping gene. No additional biochemical tests were carried out in Trinidad & Tobago. This isolate was further analyzed at Foothills laboratory of the University of Calgary, Calgary Alberta, Canada. Here, the isolate was re-cultured and incubated on a CCFA plate where positive growth was obtained (Figure 1). This isolate was also subjected to the Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) test method which is a mass spectrometry

technique that allows for DNA, peptides, proteins and sugars to be analyzed further confirmed the isolate as C.difficile. The ATCC 9686 control strains was analyzed with PCR and similar DNA fragments were obtained.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial susceptibility of C. difficile was determined by minimum inhibitory concentration obtained by agar dilution method; as recommended by the Clinical Laboratory Standard Institute (CLSI) [16]. A 105 bacteria inocula was prepared by the direct suspension of the bacterial colonies, into 10 ml peptone water, equivalent to a 0.5 McFarlane standard (Biomerieux Vitek Inc, Hazel Hood Missouri). Fresh bacterial colony was taken from that which was grown for a 48-72 hour period on the CCFA agar. The suspended colonies were then inoculated 105 bacteria per spot on the Muller Hinton supplemented with 7% horse blood and a 2 fold increase in varying antibiotic concentration. Antibiotics used were: Metronidazole, Ampicillin, Pipercillin-Tazobactam, Meropenem, Penicillin G, Ceftriaxone, Cefotaxime and Ciprofloxacin. A total of 5 concentrations were used for each antibiotic according to the CLSI standard for anaerobes. Inoculated plates were incubated at 37°C for 48 hours.

PCR ANALYSIS

PCR analysis for the toxin A gene was carried out using the NK11 (5_TGATGCTAATAATGAATCTAAAATGGTAAC-3_) and NK (5_CCACCAGCTGCAGCCATA_3). For toxin B gene NK104 (sequence-5_-GTGTAGCAATGAAAGTCCAAGTTTACGC-3_) and NK105 (sequence-, 5 CACTTAGCTCTTTGATTGCTGCACCT-3_), were used as previously reported [17].The house keeping gene (tpi) which is specific for Clostridium difficile was also assessed to further confirm the organism using the primer set tpi-F 5 AAAGAAGCTACTAAGGGTACAAA-3 and tpi-R 5-CATAATATTGGGTCTATTCCTAC-3 [14].

PCR conditions were as follows: the PCR mixtures were denatured (3 minutes at 95 °C), and then a touchdown procedure was implemented, consisting of 30 seconds at 95 °C, annealing for 30 seconds at temperatures decreasing from 65 °C to 55 °C during the first 11 cycles (with a 1 °C decremental steps in cycles 1 to 11), and a final extension step of 72 °C for 30 seconds. A total of 45 cycles were performed following methods previously described by [13]. PCR was performed using Promega GoTaq Green® master mix.

RESULTS

Patient stool C.difficile isolate produced positive PCR result for toxin A, B and the house-keeping gene. The C.difficile isolate was subjected to antimicrobial susceptibility testing using agar dilution and revealed that the isolate was resistant to ampicillin, ceftriaxone meropenem, cefotaxime and ciprofloxacin. However it was sensitive to metronidazole, pipercillin-tazobactam, and penicillin G.

The Quick direct kit demonstrated the production of toxin and the glutamate dehydrogenase enzyme. All test procedures (isolation, ELISA and molecular) were repeated before making any conclusions. However, no toxinotyping, ribotyping or cytotoxicity tests were carried out. The ATCC 9686 C. difficile strain reveals the presence of two major toxin genes A and B, (figure not shown) and was used as a positive control. The DNA fragment from the isolate is shown in lane 1 of figures 2 and 3, both toxin A and B gene fragments were shown respectively; a 1% agar gel and 1kb ladder was used for analysis.

DISCUSSION

Clostridium difficile has two major virulent factors toxin A and toxin B [18]. Clostridium difficile is the main etiological agent linked to psuedomembranous colitis, and is responsible for over 90% of all cases [19]. Patients usually present with fever, abdominal pain, diarrhea, loss of appetite and fatigue [20]. Clostridium difficile-associated diarrhea seems to be on the increase and is implicated in 20-30% of all antibiotic associated diarrheal cases [21]. Toxin A and B have enterotoxic and cytotoxic effects respectively; which have now been reported to show synergistic capabilities in their virulent processes [9]. These may lead to fluid infiltration, inflammation, disruption of tight junctions, psuedomembrane and mucus formation in the colon [5].

Psuedomembranous colitis is a mucosal disease, recognizable by numerous yellow plaques which are 0.2-2.0 cm in diameter; these attach themselves to varying lengths of the colon [22]. Patients with psuedomembranous colitis are normally treated based on the stage of the disease. Metronidazole and vancomycin are the two major drugs used [23]. In mild to moderate cases, supportive therapy along with the withdrawal of the offending drug is the first step to recovery [23].

For patients with severe psuedomembranous colitis, vancomycin is the preferred treatment and is given at 125 mg qds for 10-14 days [24]. A colectomy is carried out for patients who have megacolon consisting of a diameter >10 cm [25].

In this case the patient proved to be positive on culture which is indicated by the presence of the organism in an amount greater than 2000 of 10 X 1010 bacteria present per gram of wet feces. The isolated organism produced the enzyme glutamate dehydrogenase and a toxin, both of which were detected using the C.diff Quick Check Complete Kit® (Tech Labs). However this test was unable to differentiate if the toxin produced was A, B or both. The test is described to be 87.8% sensitive and 99.4% specific [26].

The DNA from the organism was positive for the confirmatory housekeeping tpi gene (not shown). Previous studies have shown strains of similar gene fragment lengths [10]. Non-toxigenic Clostridium difficile strains have been shown to adhere far less frequently to intestinal mucosa than toxigenic strains [19]. It has also been shown that the adhesions produced by toxigenic strains binds to gut, mucosa and cells more frequently than non-toxigenic strains [19]. This ability of the cell may cause increased colonization and an enhanced ability to carry out its toxigenic effects.

As stated in the case presentation, the patient clearly stated during interview that he had consumed substantial amounts of seafood at a weekend social gathering. This indicates that the patient may have been infected in a community setting after ingesting contaminated seafood. Re-infection may have occurred later at a similar event since seafood is a popular dish in Trinidad & Tobago. Consideration should also be given to the patient's past medical history which includes a diagnosis of rheumatoid arthritis. It is known that increased risk factors for the acquisition of C.difficile associated diarrhea include the severe underlying diseases and a faulty response to the toxins [27]. This has important implications for this patient because of the production of abnormal immunoglobulin G and formation of immune complexes in rheumatoid arthritis. Patients at greatest risk for severe disease are those who have had surgical intervention and or have been treated with immunosuppressive therapy since such patients cannot mount an adequate response [27]. This patient, while having not received surgical intervention, had been maintained on steroid therapy for rheumatoid arthritis.

CONCLUSION

This case demonstrated that the toxin A and B positive strain have a role in the formation psuedomembranous colitis and that possible resistant strains are present in the population. Further research is needed on similarly detected strains, to better understand their significance in Inflammatory Bowel Diseases.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

Both authors designed the study. LB collected, analyzed the data and drafted the manuscript. PEA supervised and coordinated the project. Both authors read and approved the final manuscript.

CONSENT

Authors declare that written informed consent was obtained from the patient and hospital authority for publication of this case report. A copy of the written consent is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal.

ETHICAL APPROVAL

The ethics committee of the Faculty of Medical Sciences, the University of the West Indies, St. Augustine gave approval for this study.

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FIGURE 1

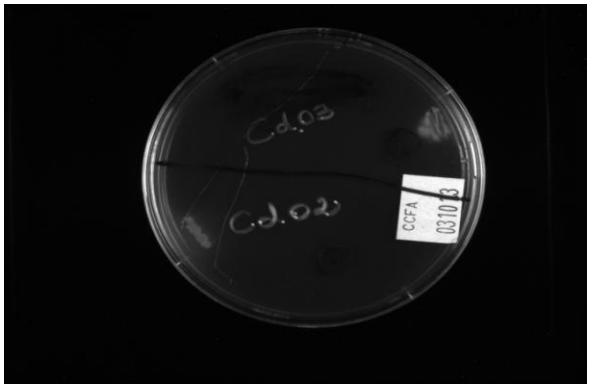

Figure 1 A fatal case of Clostridium difficile infection fromTrinidad & Tobago.Isolate was grown on a Cycloserine Cefoxitin Fructose Agar (CCFA) plate before being subjected to Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) test and it confirmed the isolate as Clostridium difficile.

FIGURE 2

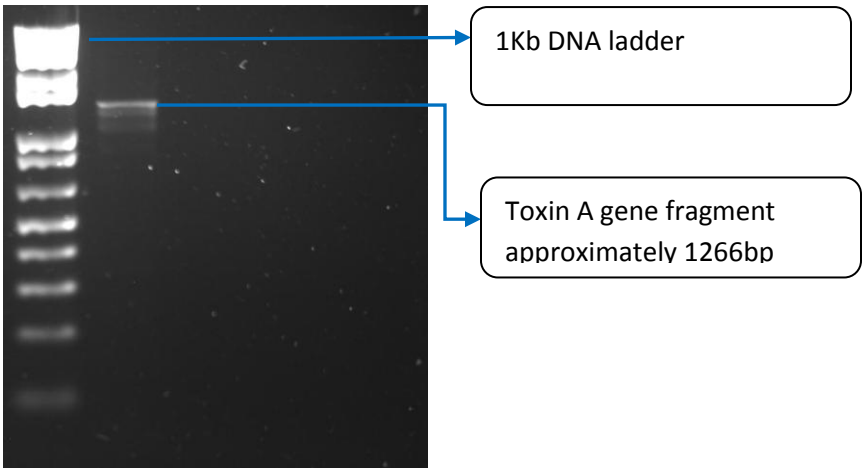

Figure 2 of PCR products of A fatal case of Clostridium difficile infection fromTrinidad & Tobago. The isolated strain revealed the presence of the toxin A genes as indicated in lane 1. Lane. The patient produced a 1266bp band on a 1% agar gel, using a 1kb ladder.

FIGURE 3

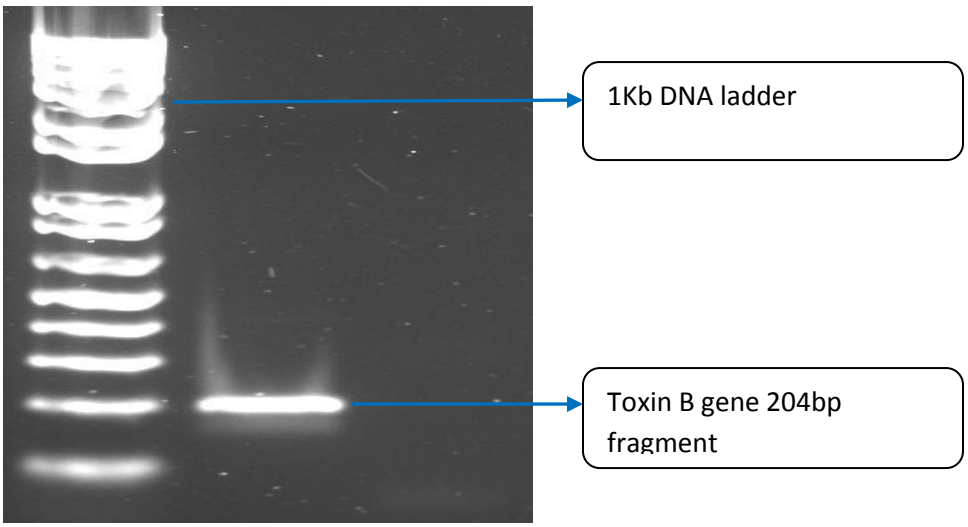

Figure 3 of PCR products of A fatal case of Clostridium difficile infection fromTrinidad & Tobago. The isolated strain revealed the presence of the toxin B genes as indicated in lane 1. Lane. The patient produced a 204bp band on a 1% agar gel, using a 1kb ladder.