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Characterization of Toxigenic Clostridium Difficile from Soil in Yola, Nigeria

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Abstract:- *Clostridium difficile* toxins plays a major role in diarrhea and colitis associated with the organism. The bacterium is mostly known for its production of two major toxins: a potent enterotoxin (toxin A) and a cytotoxin (toxin B). Characterization of toxigenic *C. difficile* was carried out by a polymerase chain reaction (PCR) assay. Two sets of primer pairs sequences which are specific for toxin A (*tcdA*) and toxin B (*tcdB*) genes were designed and used to amplify a 1988-bp and 1936-bp DNA fragments respectively. The PCR products (amplicons) were visualized on a 2% agarose gel electrophoresis stained with ethidium bromide. A total of forty (40) soil samples were collected from refuse dump sites located in schools, markets, residential areas and hospitals within Yola North local government, Adamawa state. Out of the 40 samples 25% (10) were harboring *C. difficile*. Molecular characterization of isolates showed that 40% (4) were toxin A^-B^+ strains, 40% (4) were toxin A^+B^+ while the remaining 20% (2) were non toxigenic strains. Distribution of toxigenic strains of *C. difficile* isolates from all different sample sites studied showed that the hospital sites contained the highest number (60%) of isolates with toxin A^+B^+ strains while residential ward sites harbors only toxin A^-B^+ strains. It is apparent that toxigenic *Clostridium difficile* is present in Yola North and its prevalence is environmentally influenced. The study also shows the usefulness of PCR methodology in characterizing *C. difficile*

Contribution/Originality: The results of this studies showed up to 80% of *C. difficile* isolated from environment are toxigenic.

I. INTRODUCTION

The *Clostridium* genus is a member of the family Clostridiaceae and it contains about 203 species however only a few species are being pathogenic to humans. The bacterial capsule, hydrolytic enzymes and fimbriae are some of the several virulence factors attributed to *C. difficile*. These virulence factors are mostly notable in highly toxigenic strains [1]. The most important virulence factors of *C. difficile* are its ability to sporulate and produce toxins. Toxigenic *C. difficile* are known to have a Pathogenicity Locus that encodes the genes for toxins, however non-toxigenic strains lacks the PaLoc entirely [2]. *C. difficile* produces two major toxins. One of these toxins, toxin A, causes an accumulation of fluid in ligated intestinal loops and has been referred to as the enterotoxin. The other toxin B, is a potent cytotoxin which in an experimental cell research has been shown to cause rounding of cells [3]. The *tcd*A gene encodes a 308 kDa protein and *tcd*B gene encodes 270 kDa proteins which are glucosyltransferases, which inactivate Rho, Rac and Cdc42 within targeted cell [4]. Initially, toxin A was believed to be the most important virulence factor. However, the view that toxin A was the important virulence factor was challenged when clinical reports provided evidence of disease causing strains that did not produce toxin A. Further studies by Lyerly and colleagues showed that a certain strain is a naturally occurring toxin A^-, toxin B^+ strain which is capable of producing infection [5]. Additionally, A^-B^+ strains were reported to be responsible for fatal human epidemics [6]. Hence both toxins contribute to pathogenicity of *C. difficile* though toxin B makes a larger contribution to the virulence. It is likely though that the contribution of toxins to virulence is not yet well understood.

Separate from the PaLoc, *C. difficile* encodes an additional toxin called binary toxin [7]. This Binary toxin is also referred to as ADP ribosylates toxin and it is produced in two individual fragments, *CdtA* and *CdtB*, which are secreted separately however join extracellularly to form a functional toxin. *CdtA* is the enzymatic fragment, while *CdtB* is responsible for binding and translocation [4]. Receptor-mediated endocytosis is the method by which host cells take up binary toxin. The existence of binary toxin within a can lead to the degradation of the cytoskeleton [8]. it is not all *C. difficile* strains that possesses binary toxin but it has been found in strains that do not contain the PaLoc. Strains that contains no toxins A and B are able to colonize animals, but do not cause any disease symptoms [8]. A recent report mentioned that binary toxin plays a vital role in increasing the adherence of C. *difficile* [10].

The increase in *Clostridium difficile* infection occurrence is associated with the emergence of a hyper virulent strain of *Clostridium difficile* [11]. Certain *Clostridium difficile* ribotype has been shown to produce a large quantity of toxins A and B when compared to other ribotype [12]. In addition, ribotype that produces both binary toxin and toxin A/B are associated with increased virulence [13]. This toxin is only found in 6-12.5% *C. difficile* [11]. According to the findings of Wilcox and colleagues the predominating ribotype 027 prevalence decreased from 55% in 2007-2008 to 21% in 2009-2010 and this is as a result of active surveillance [14]. Investigating and understanding the strain in an environment will guide in policy direction for the control and prevention of infections associated with these group of infectious agents.

II. MATERIALS AND METHOD

2.1 Isolation of *Clostridium difficile*

Clustered random sampling technique was used to achieve randomness. A total of 40 samples, 10 from each refuse location were collected by random sampling using simple hand auger at a depth of 30 cm. The various samples collected at random positions at a refuse dump was put together in sterile polythene and transported to the laboratory for analysis. Isolation was done based on method described by Costa *et al,* (2012) [16]. Approximately 2 g of soil sample was inoculated in to 9 ml of Cycloserine Cefoxitin fructose broth (CCFB) which contains 0.1 % sodium taurocholate. It was incubated at 37° C in an anaerobic jar for 7 days. 2 ml of the cultured broth was then added to 2 ml ethanol which was incubated at ambient temperature for 30 minutes. The broth was then centrifuged at 3500 rpm for 10 minutes. The pellet was then inoculated onto modified Cycloserine Cefoxitin fructose agar (CCFA) consisting of 4 % proteose peptone, 0.5 % Na_2HPO_4, 0.1 % KH_2PO_4, 0.01 % $MgSO_4$, 0.2 % NaCl, 0.6 % fructose, 1.5 % agar, 250 mg/ml D-Cycloserine, 400 mg/ml cefixime, 1 % neutral red and 0.1 % sodium taurocholate at pH 7. It was then anaerobically incubated at 37°C for 48hrs. Negative cultures were further incubated. Isolates were then identified by morphological characteristics, smell, biochemical test and gram stains. A single colony of each isolate was sub cultured and stored at 4° C before molecular analysis.

2.2 Detection of C. *difficile* Toxins by PCR

DNA extraction was carried out using commercial kit (Zymo Research, USA) following the manufacturer's instructions. The PCR was performed using two primers targeting *tcdA* and *tcdB* genes. The primers were designed on National Centre for Biotechnology Information database (NCBI) using the primer design tool and the following are sequences of the primers *tcdA*-F-5'TTGGTGGGAAACTGGAGCAG3',*tcd*A-R-5'CTCCCAACTGCATCAACCA3'and *tcd*B-F-5'GGGAAACAGGATGGACACCA3,*tcdB*-R5'CCAATTGAAGCAGCTCCACC3'.The procedure involves adding 12.5 µl of HotStarTaq Plus Master Mix (QIAGEN, Hilden, Germany), 2 µl of each primer, 5 µl of Q-solution (QIAGEN, Hilden, Germany) and 2.5µl of Coral load into a PCR tube which was then followed by addition of 1 µl of DNA sample properly mixed and placed into the thermo cycler. The reaction mixture was subjected to amplification in a Techine 3 prime thermo cycler with the following PCR conditions: 15 minutes at 95˚C for initial enzyme activation step. It was then followed by 40 cycles of 30 seconds at 94 ºC for denaturation, 30 seconds at 55 ˚C for annealing and 30 seconds at 72 ˚C for elongation, plus an additional 10 minutes at 72 ˚C for final elongation step.

2.2.1 *PCR Product analysis*

The PCR products were analyzed on 2 % agarose gel electrophoresis. The gel was prepared by adding 2g of agarose powder in 100ml of 1X T.E buffer and it was heated the powder completely dissolved. It was then allowed to cool to 60ºC and 10ml of Ethidium bromide was added then poured into a gel tray. After solidification the tray was then transferred into the tank and 1X T.E buffer was added into the tank until the gel is completely covered. The gel was then loaded with 10 µl of each sample along with a 1 kb marker (Gene ruler) which was then set to electrophoresis for 60 minutes at 100 volts. After electrophoresis the gel was then placed on a UV Trans illuminator and the bands were viewed.

III. RESULTS

Out of the 40 samples collected only 25 % (10) were positive for *C. difficile.* The results also showed that hospital refuse dump sites have the highest prevalence rate of 50 %, followed by Residential wards with a prevalence of 30 % and Market sites with a prevalence of 20 %. In this study, the ten (10) isolates of *C. difficile* obtained eight (80%) has demonstrated the presence of toxin genes. The toxin typing of *C. difficile* isolates by PCR is shown on fig.1 and fig.2. The ethidium bromide stained agarose gel shows the amplicons for toxin A and B genes with 1988 bp and 1936 bp respectively. Lane M and lane C are DNA marker and negative control respectively. On figure1 it is shown that lane H7, H9, H10 and M2 are the isolates with toxin A gene and on figure 2 only lanes W5 and W8 are lacking toxin B gene. The results also showed that 40% (4) were toxin A^-B^+ strains, 40% (4) were toxin A^+ B^+ while the remaining 20% (2) were non toxigenic strains of *C. difficile*. Distribution of toxigenic strains of *C. difficile* isolates from all different sample sites studied showed that the hospital sites contained the highest number of isolates with toxin A^+ B^+ strains while residential ward sites harbors only toxin A^-B^+ strains C. *difficile* (Fig.3).

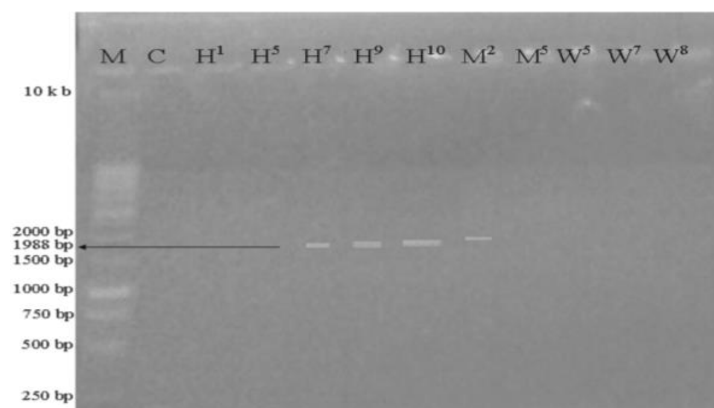

Fig.1 Agarose gel electrophoresis depicting amplified *tcdA* gene fragments(≅1988 bp) in *Clostridium difficile* using 2% agarose gel. M:1Kb DNA ladder (Gene Ruler), C:control,lanes H7,H9,H10 and M2 represents fragment of targeted gene

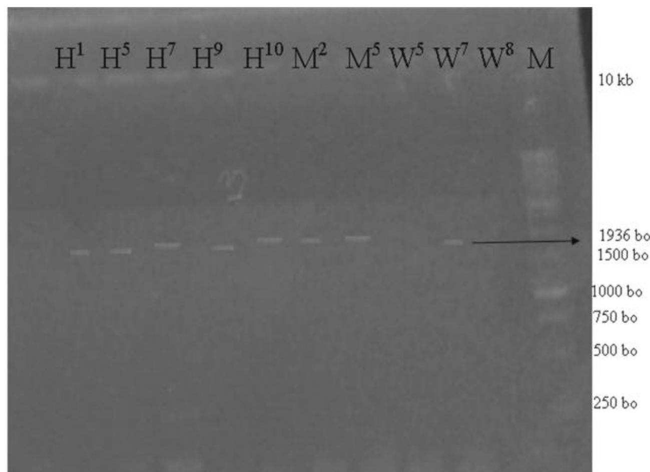

Fig. 2 Agarose gel electrophoresis depicting amplified *tcdB* gene fragments (≅1936 bp) in *Clostridium difficile* using 2% agarose gel. M: 1Kb DNA ladder (Gene Ruler), lanes W^5 and W8 are the only isolates lacking the targeted gene

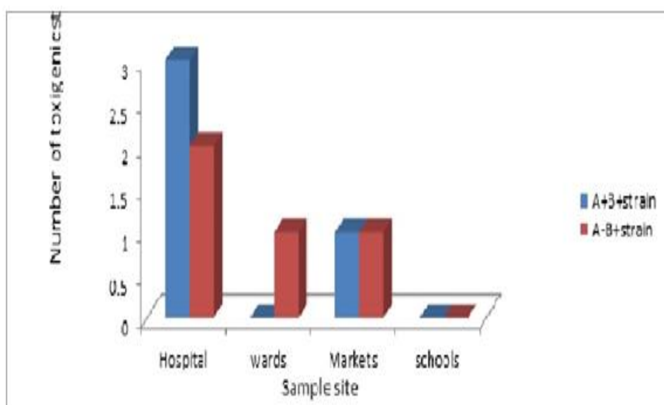

Fig.3 Distribution of toxigenic strains of *C. difficile* isolates from different sample site

IV. DISCUSSION

This study depicts that the utilization of PCR in determining the toxigenic potential of *C. difficile* can overcome the initial time-consuming cell culture assay used in identifying toxigenic *C. difficile*. The overall occurence rate of *C. difficile* (25%) in this present study is higher when in comparison with the reports of the research carried out in Ohio-USA which had 6.5% prevalence rate [17]. Furthermore other studies have reported a high prevalence (30–68%) of *C. difficile* in the household environment and bio solid treatment plants [18]. The reason for the higher prevalence of *C. difficile* is not clear, even though *C. difficile* can multiply to any great extent when germinated spores are exposed to nutritious environment or light. Another contributing factor to increased prevalence could be the ability of *C. difficile* spores can survive up to ~5 months in the environment [19]. Therefore, the difference in prevalence could be explained by environmental factors over time and contact with animals which could help disperse the spore particularly on the surface [20]. Molecular characterization of isolates showed that the hospital sites mostly harbored toxin $A^+ B^+$ strains of *C. difficile* and only two hospital isolates were toxin A^-B^+ strains. A single market site isolate has also been shown to be a toxin A^+B^+ strains. These findings can be explained by the fact that *C. difficile* is a common nosocomial pathogen and mostly associated with adults. The prevalence of toxin A^+B^+ strains is 40% which when compared to previous studies with prevalence rate of 63% indicates a decrease in predominance by toxin $A^+ B^+$ strains *C.difficile* [21]. It is also evident that of all the isolates obtained from residential ward refuse dump sites only one isolate was toxin A^-B^+ strains of *C. difficile.* The presence of this toxin type has been attributed to a deletion in the *tcdA* gene with functional t*cdB* gene [22]. which also has the potential to cause disease. However the remaining two isolates were non toxigenic strains. The prevalence of toxin A^-B^+ strains *C. difficile* is 40 % which is similar to the prevalence rate of 39% as described in a Japanese study [23].). However this contradicts the findings of Ackerman *et al.* (2001), which reported that most *C. difficile* isolates recovered from public places, produces no toxins [24].

It would be interesting to follow up the toxigenic strains in public environment to monitor the effect of environmental condition and antibiotic exposure on toxin production and possible emergence of hyper virulent strains in public places. The limitation of this study is the inability to detect the hyper virulent strain of *C. difficile* known as NAPI/BI/027 which had been implicated in outbreaks with increase morbidity and mortality since early 2000s [25]. Similarly the actual production of toxin A and B by the cultured *C.difficile* was not investigated. Furthermore, results from this study showed that the presence of *C.difficile* is associated with specific activity carried out in an environment. the reports of a study conducted in Ohio states that the presence of *C. difficile* is not associated with soil type and the spores can infest all soil type [17].

V. CONCLUSION

This study has demonstrated the presence of both toxins $A^+ B^+$ and toxin A^-B^+ strains of *Clostridium difficile* in the environment with a prevalence rate of 25%. The hospital environment has the highest occurrence of 50% while school environment are free from spores of *C. difficile*.It will recommended that further research should be conducted on a larger scale which will provide necessary information on the increasing prevalence of *C. difficile* infection and management measures

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