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Antimicrobial Susceptibility Profile of Environmental *Clostridium difficile* in Yola Adamawa State, Nigeria

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ABSTRACT

Clostridium difficile is a common cause of antibiotic associated diarrhea with increased role of environment as a source of contamination. Clostridium difficile spores are resistant to disinfectant surviving for months or years on contaminated surfaces. Presence of C. difficile in the environment by enrichment and culturing of soil samples on Cycloserine Cefoxitin fructose medium (CCFM) was carried out. Ten (10) soil samples each were collected from different refuse dump sites in schools, markets, residential wards and hospitals within Yola North Local Government Area of Adamawa State, Nigeria. Out of the 40 samples, 25% (10) harbored C. difficile and 37.5% (15) yielded growth of unidentified bacteria. Hospital environments had the highest prevalence of 50% and school environments were found to be free of C. difficile spores. Prevalence of C. difficile is significantly (P=0.05,) associated with environment-based activities. Minimum inhibitory concentrations of isolates using five antibiotics showed that only 60% of the hospital-based isolates were susceptible to tetracycline and the rest resistant to the other four antibiotics used. C. difficile isolated from market dump sites were susceptible to all antibiotics tested. None of the residence-based isolates were resistant to metronidazole; however, 66.7% were susceptible to erythromycin and tetracycline. This study indicates that C. difficile has become an emerging pathogen in Nigeria. Increased understanding of the factors leading to outbreaks of C. difficile is partinent.

Keywords

Bacillus, *Clostridium difficile*, Diarrhea, Environment, Prevalence, Spores.

Introduction

Clostridium difficile is a spore forming, anaerobic Gram-positive bacillus measuring about 0.3-2.0 by1.5-2.0 µm. This organism naturally inhabits intestinal tract of humans and animals as well as soils where they live as saprophytes. It is motile with peritrichous flagella and produces spherical endospores and exotoxin. These endospores and toxins when ingested, can lead to asymptomatic carriage or clinical disease which ranges from mild diarrhea to life threatening pseudomembranous colitis [1]. *Clostridium difficile* has also been described as one of the leading cause of nosocomial diarrhea and is responsible for an increase in hospital stays with high healthcare and economic repercussions [2].

A remarkable change in epidemiology of *Clostridium difficile* infection has been encountered over the years with increasing incidence, mortality and relapse rates in human [3,4]. Additionally, while classically a hospital-associated pathogen predominantly affecting elderly individuals, there are increasing reports of community-associated *Clostridium difficile* infection including young individuals and people with few or no risk factors [5]. Several studies have revealed the widespread presence of the organism in hospital wards and on the hands of nursing personnel and infections are generally believed to be nosocomial [6].

Fewer studies have been directed at the environment outside hospitals and reports of *C. difficile* in the environment suggest either that its geographical distribution may be patchy, or that the different methodologies used were responsible for different isolation rates. Hafiz and Oakley [7] from Sheffield, England reported the organism in soil and a similar research in Korea [8] has also found it in soil. However, a study of 20 random soil samples from Michigan, USA gave negative results. Little is known

about the prevalence of *C. difficile* in the domestic environment [9]. The source of infection for community-associated cases of *Clostridium difficile* infection remains uncertain; however, Jhung et al. [10] suggested food borne infection. *Clostridium difficile* spores are resistant to disinfectant and can survive for months or years on contaminated surfaces [11-13]. In this study, a survey and antimicrobial susceptibility profile of *Clostridium difficile* from some refuse dump sites was carried out.

Materials and Methods

Sampling Method

The study site includes refuse dumps located at schools, hospitals, markets and wards within Yola North. Clustered random sampling technique was used to achieve randomness and this was carried out by grouping the communities within Yola North into four categories based on their population density. Within each category the various names of wards were written on pieces of paper, folded and non-participants of the research were asked to select any five from each cluster and refuse dumps site within selected wards were sampled.

Collection of Samples

A total of 40 samples, 10 each from refuse dump sites were collected by random sampling using simple hand auger at a depth of 50 cm and at different points aseptically. The auger was cleansed at intervals to avoid contamination of the sample. The various samples collected were put together in sterile polythene and properly mixed to form composite sample which was then transported to the laboratory for analysis.

Isolation and identification of *Clostridium difficile*

Isolation was done based on method described by Costa et al. [14]. Approximately 2 g of soil sample was inoculated in to 9 ml of modified Cycloserine Cefoxitin fructose enrichment broth (CCFB), where Cefoxitin was replaced with Cefixime and supplemented with 0.1% sodium taurocholate. The broth was incubated at 37 o C in an anaerobic jar for 7 days. After incubation, 2 ml of the broth was then withdrawn and added to 2 ml ethanol in a test tube and incubated once again at ambient temperature for 30 min and then centrifuged at 3500 rpm for 10 min. The pellet was retained and inoculated on to 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 at pH 7.4 with added selective supplement containing 250 mg/ml D-Cycloserine, 400 mg/ml Cefixime, 1% neutral red and 0.1% sodium taurocholate. It was then anaerobically incubated at 37°C for 48 h. Negative cultures were further re-incubated. Isolates were then identified by morphological characteristics, odor, biochemical test (catalase and oxidase) and Gram stains. A single colony of each identified isolate was sub cultured and stored at 4°C prior to further analysis.

Antibiotic Susceptibility Testing

The Minimum inhibitory concentration (MICs) of five (5) antibiotics against the isolates was determined by agar well diffusion method as described by Brook et al. (2013). The microbial inoculum of each isolate was prepared by inoculating each isolate in 2 ml of normal saline and incubated anaerobically at 37ºC for 2 h. The antibiotics and concentrations used included: metronidazole (0.5-32 µg/ml), erythromycin (0.5-32 µg/ml), Tetracycline (1-64 µg/ml), Ciprofloxacin (0.5-32 µg/ml), and Clindamycin (0.5-32 µg/ml), in accordance with the CLSI standard [15]. The various concentrations were prepared by serial dilution from a stock solution (prepared by dissolving each tablet of the antibiotics in 100 ml sterile distilled water). Mueller-Hinton agar supplemented with 0.1% sodium taurocholate was inoculated with the microbial inoculum by spreading 1 ml over the entire agar surface. Holes (6 mm diameter) were punched aseptically onto the agar plates using a sterile Cork borer and 20 µl of each concentration was introduced into the well. The plates were then incubated anaerobically at 37°C for 48 h. The MIC (read as the concentration with zones of inhibition) was recorded.

Statistical Analysis

To compare occurrence rates with demographic factors and bacterial isolates obtained, the mean, standard deviation as well as, Chi-square (χ^2) test were performed using SPSS 20 statistical software for windows and a probability value of 0.05 or less was considered to be significant.

Results

The 40 soil samples enriched in CCFB and cultured on CCFA yielded growth of 10 bacterial isolates which were identified to be *Clostridium difficile*. Morphologically, the isolates were pleomorphic, about 3 mm in diameter, yellow to grey in color and are associated with a distinctive odor. Gram staining reaction of isolates revealed that they were Gram positive clustered rods and biochemical test has shown that they are both oxidase negative and catalase negative.

Out of the 40 samples collected only 25% were positive for *C. difficile*, about 37.5% had no growth and 37.5% yielded unidentified bacterial growth. The results also showed that hospital refuse dump sites has the highest prevalence rate of 50 %, followed by Residential wards with a prevalence of 30% and Market sites with a prevalence of 20% (Figure 1).

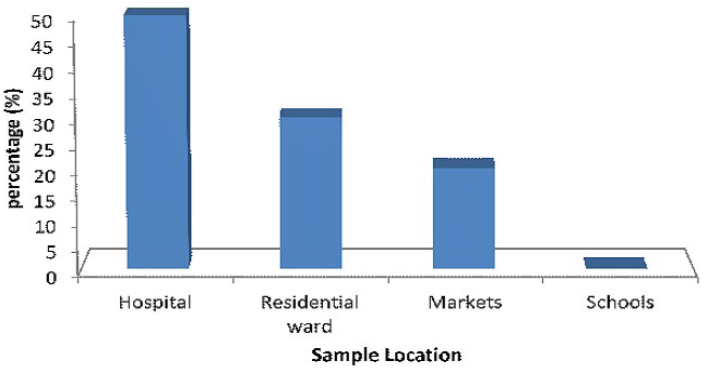

Figure 1: Percentage occurrence of *Clostridium difficile* with respect to sample location.

Results of antibiotic susceptibility test in this study showed that only 60% of hospital isolates were susceptible to Tetracycline (MIC range ≥ 32µg/ml), however they were resistant to Ciprofloxacin (MIC range ≥ 32µg/ml to 128µg/ml), Clindamycin (MIC range ≥ 32µg/ml to 256µg/ml), Erythromycin (MIC range ≥ 64µg/ml to128µg/ml) and Metronidazole (MIC range ≥ 64µg/ml to128µg/ml) (Table 1). *Clostridium difficile* isolates obtained showed a relatively high multi drug resistance (Table 1). The minimum inhibitory concentration (MIC) value of the isolates obtained from hospitals showed that all isolates were resistant against Ciprofloxacin, Clindamycin, Erythromycin and Metronidazole, only 60% of the isolates were susceptible to Tetracycline. On the other hand all the residential ward isolates were susceptible to Metronidazole, 33.3% were resistant to Erythromycin and Tetracycline while resistance against ciprofloxacin and clindamycin was 100%. There was no resistance amongst isolates obtained from market refuse dump.

Table 1: Range of minimum inhibitory concentration (MIC) and resistance rate of isolates.

Antibiotic	MIC Range (µg/ml)	H1	H5	H7	H9	H10	W5	W7	W8	M2	M5	% Resistance
Ciprofloxacin	0.5-32	128	128	128	128	128	64	64	64	32	32	80
Clindamycin	0.5-32	256	64	128	128	128	64	64	64	32	32	80
Erythromycin	0.5-32	128	64	128	128	128	64	32	32	32	16	60
Metronidazole	0.5-32	128	64	64	128	64	32	32	32	32	16	50
Tetracycline	1.0 – 64	64	32	128	128	64	64	32	64	32	16	60

Key: H – Hospitals W- Wards M- Markets.

Discussion

Clostridium difficile is ubiquitous in the environment and a common cause of antibiotic associated diarrhea. The increase in *C. difficile* infection related to hospitalization and case fatality rate has been reported [16] and this indicates its importance as an emerging infectious agent. The environment has been demonstrated to be an important source of contamination and that it enhances the potential for the spread of the infection [17] with the spores being capable of persisting on surfaces for prolonged periods. In this study, prevalence of *C. difficile* in environment soils showed an increase of 25% in comparison with the reports of the research carried out in Ohio-USA which had 6.5% prevalence rate [18] which is contrary to the reports of a study carried out in Lagos-Nigeria which had 0% prevalence of *C. difficile* in the soils. This indicates that *C. difficile* has become an emerging pathogen in Nigeria and therefore further standard approaches to the investigation of *C. difficile* are essential in order to increase understanding of the factors leading to outbreaks of *C. difficile*, its preventive and control measures.

The increased prevalence rate of *C. difficile* in the hospital environment in this study predisposes the human population to infection. Besides, it is reported that the hospital environment refuse dump had the highest concentration of spores [18]. The study revealed that prevalence rate of *C. difficile* accounted for 50% in hospital sites, 30% in the populated neighborhood and 20% in markets sites. Contamination of healthcare workers' hands can lead to and result from contamination of the environment. Thus, the prevalence of healthcare worker hand contamination with *C. difficile* correlates with the level of environmental contamination [19].

However, proof that reducing environmental *C. difficile* can decrease the incidence of infection is lacking. This study further indicated that school refuse dumps were free of *C. difficile* spores and this findings is similar to the reports of Tariq et al. [18]. Results from this study showed that the presence of *C. difficile* is associated with specific activity carried out in an environment. Tariq et al. [18] had earlier reported that presence of *C. difficile* is not associated with soil type while the spores can infest all soil types [18].

Results of antibiotic susceptibility test in this study showed that 60% of the hospital isolates were susceptible to only Tetracycline, but resistant to Ciprofloxacin, Clindamycin, Erythromycin and Metronidazole. The resistance could be associated with over exposure of the isolates to varouis antibiotics and disinfectants in the hospital environment. *C. difficile* has been reported to be suceptible in previous studies [20]. Furthermore this study has shown that all *C. difficile* isolates obtained from Market sites were susceptible (MIC range ≥ 16µg/ml to 32µg/ml) to all five antibiotics tested. Lack of resistance amongst these isolates is due to favourable growth conditions and unlikely exposure to the antibtiotics. The environmental conditions might also have been favourable for growth [22]. Metronidazole remains the most potent drug against the isolates although still with 50% rate of resistance. A study of prophylaxis with oral metronidazole or vancomycin against *C. difficile* infection, concluded that neither was effective at preventing asymptomatic faecal excretion of *C. difficile* [21]. Two studies have claimed evidence that Metronidazole, given as surgical prophylaxis to surgical patients, may reduce the risk of postoperative *C. difficile* infection [1]. However, in one of these, the comparator group with more cases of *C. difficile* infection received clindamycin (a high-risk antibiotic) instead of Metronidazole as anti-anaerobic cover. In another study, a non-significant difference was seen in the incidence of *C. difficile* colonization in patients receiving a non-standard prophylaxis regimen [1]. Thus; there is no sound evidence of a benefit of using metronidazole to prevent *C. difficile* infection. There are few proven therapeutic regimens for *C. difficile* infection. First-line treatment should, where possible, involve discontinuation of the precipitating antibiotic(s), although in many patients this is not possible. The fundamental principle in the control of *C. difficile* infection is the control of antimicrobial prescribing, and there are numerous examples of restrictive antibiotic policies associated with reduction in rates of *C. difficile* associated diarrhea [22].

Conclusion

The Result from this study showed that *C. difficile* could remain viable in public outdoor environments and support the suggestion of human-to-human transmission within the community. The work showed a 25% occurrence rate of *C. difficile* in refuse dump sites of Yola North LGA in Adamawa State which is an indication of the prevalence of these bacteria in the hospital environment and other environments studied. Metronidazole remains the most potent drug against the isolates although with 50% rate of resistance. This also emphasized the importance of soil and public environment in acquisition of *C. difficile* in the community. The research also presented a reliable tool for the screening of *C. difficile* in the environment that can easily be adopted to monitor environmental contamination and provide information to assess the risk of obtaining *C. difficile* in public places. *C. difficile* is an organism of increasing importance, especially in the elderly in the hospital setting. Although its incidence may be controlled somewhat by careful antibiotic prescribing, it is not going to disappear. However, it will be of great help if novel methods, whether chemical, probiotic or immunological, should be developed in order to control the organism and the diseases it causes.

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