Screening of *Clostridium difficile* Isolates for Metallo-Resistant Gene and Extended Spectrum Beta-Lactamases

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Abstract *Clostridium difficile* naturally inhabits intestinal tract of animals including humans. Spores of *C. difficile* are known contaminant in the Environment. Indiscriminate use of antibiotics has led to the emergence of a powerful resistance determinant (known as Metallo resistant genes) in many bacteria worldwide. Multidrug resistant bacteria (MRB) pose threats to health globally. This study aims to determine the distribution of Metallo resistant *Clostridium difficile* in hospital environment within Yola. A total of 150 surface samples from the hospital wards were collected from Federal Medical Centre, Yola (FMC) and Specialist hospital, Yola (SHY) Adamawa state, Nigeria. *Clostridium difficile* were recovered by inoculating samples in modified Cycloserine-cefoxitin fructose broth (CCFB) and Cycloserine-cefoxitin fructose agar (CCFA). Screening of isolates for extended spectrum β -lactamase (ESBL) and metallo- β -lactamase enzymes was done by double disc diffusion method and Imipenem-EDTA combined disc test respectively. A total of 18 *C. difficile* isolates were obtained and found to be extended spectrum β -lactamase (ESBL) positive. 16 (88.89 %) of the *Clostridium difficile* were found to be metallo- β -lactamase positive. Polymerase chain reaction showed that 11 (61.1%) of the isolates possess *bla*-IMP metallo resistant gene. This study suggests that multidrug resistant *C. difficile* with metallo resistant genes are wide spread in hospital settings and there is need for relevant authorities to improve on the

sanitization of environment as well as continuous surveillance to hinder *C. difficile* transmission.

Keywords *C. difficile*, Hospital Wards, Metallo Resistant Gene, Extended Beta Lactamase

1. Introduction

Clostridium difficile infection has been regarded as a nosocomial infection in human especially in patient receiving prolonged antibiotic therapy [1]. Infection with *Clostridium difficile* causes various outcome, ranging from asymptomatic colonization to pseudomembranous colitis (PMC), which may result in death if untreated [2]. The most important determinants of *Clostridium difficile* infection are exposure to the organism and treatment with antibiotic [3]. The use of antibiotics leads to killing of antibiotic-sensitive organisms and colonization by *C. difficile* leading to clinical conditions [4]. The role of the environment as a potential reservoir for MBL *C. difficile* and its significance in infection remains unclear in most African countries.

Bacteria with multidrug resistant gene pose an impending threat to health [5]. The widespread, indiscriminate and increasing use of antibiotic is the notable cause of the emergence of multidrug resistant

bacteria. β -Lactamases are enzymes which hydrolyze the β -lactam ring of Penicillins, Cephalosporins, Monobactams and Carbapenems. This is an important mechanism of microbial resistance to β -lactam antibiotic [6]. Extended spectrum Beta-lactamases are major mechanism of resistance to the second, third and fourth generation Cephalosporins [7]. These enzymes hydrolyze and confer resistance to modern cephalosporin. Infections with metallo- β -lactamases producing bacteria are difficult to manage because of their zinc-catalyzed mechanism of hydrolysis [8]. Genes encoding for MBL are located on the integron structures that resides on transposons or plasmids [9] thus enhancing dissemination. They are unique in their ability to hydrolyze carbapenems and be inhibited by EDTA chelators of Zn^{2+} [10]. *Clostridium difficile* have several MBL genes among which are beta-lactamase like protein, beta-lactamase like hydrolase, beta-lactamase inhibitor protein II, metallo-beta lactamase superfamily protein, metallo-beta lactamase superfamily hydrolase, metallo-beta lactamase superfamily exported protein, cell surface protein penicillin-binding protein amongst others [11]. *bla*-IMP and *bla*-VIM are the most common MBL genes described throughout the world [12]. *bla*-AIM, *bla*-GIM, *bla*-SPM, *bla*-SIM, NDM-1 are the other types of MBL genes found only sporadically in some geographical regions [13]. Difficulty in management and increased hospital stay of patients with serious infections due to MBL organism are attributed to continuous treatment with antibiotic to which complete resistance has developed [14]. Therefore, detection of MBL producing organisms especially *C. difficile* is highly significant for optimal treatment and controlling the spread of drug resistance organism.

2. Methodology

2.1. Sample Collection

One hundred and fifty surface samples were collected, 75 samples each from Federal Medical Centre Yola (FMC) and Specialist Hospital Yola (SHY). Using sterile moistened swab, samples were collected from medical ward, intensive care unit (ICU) and female surgical wards. Patient's bed sheet, bed railings, table tops, toilets and windows were specific sites sampled.

2.2. Isolation of *Clostridium Difficile*

The swab containing the specimen was immersed in Cycloserine-Cefoxitin Fructose broth (CCFB) supplemented with taurocholate to enhance recovery of spore and it was then incubated anaerobically at 35°C for 7 days. (CCFB). A loop full of cultured broth was then inoculated onto Cycloserine-Cefoxitin fructose Agar (CCFA) composed of 4 % proteose peptone, 0.01 % $MgSO_4$, 0.2 % NaCl, 0.5 % Na_2HPO_4 , 0.1 % KH_2PO_4 ,

0.6 % fructose, 1.5 % agar at pH 7.4 supplemented with 250 mg/ml, D-Cycloserine and 8 mg/ml cefoxitin [15]. It was then incubated anaerobically at 37°C for 48 hours. The isolates obtained were identified by morphological characteristics, odour, biochemical test (catalase, oxidase), motility test and Gram stain. Pure isolates were stored in CCFB and Brain heart infusion broth (BHI) at -20°C for further studies.

2.3. Antimicrobial Susceptibility Testing (MIC)

The inoculum of isolates was prepared from overnight cultures of organisms. A sterilized wire loop was used to emulsify a colony of the test organism in 4 ml of sterile nutrient broth which was then incubated anaerobically for 2 hours at 37 °C. The nutrient's broth turbidity was matched to 0.5 McFarland Standard [16]. The Minimum inhibitory concentration (MIC) was determined following the Clinical and Laboratory Standard Institute [17] using the well diffusion method. The antibiotics used includes Metronidazole (0.5-32 µg/ml), Ciprofloxacin (0.5-32 µg/ml), Erythromycin (0.5-32 µg/ml), Tetracycline (1-64 µg/ml) and Clindamycin (0.5-32 µg/ml). Varying concentrations were prepared by serial dilution from stock solution that was prepared by dissolving each tablet of the antibiotic in 100 ml of distilled water in a sterile container. The test organism was inoculated unto Mueller Hinton agar using a sterile cotton swab to streaked evenly over the surface of the agar [18]. Five wells were made on each of the streaked plates corresponding to the five antibiotics to be used using a sterile cork borer. 20 µl of the various antibiotic concentration was dispensed into each of the well and was allowed to diffuse for three hours on the bench. All the inoculated plates were incubated anaerobically at 37°C for 48 hours in an anaerobic jar. The MIC was recorded for each isolate after 48 hours [19].

2.4. Phenotypic Detection of Extended Spectrum β -Lactamase (ESBL)

The phenotypic detection of ESBL was performed on Mueller Hinton agar using Kirby-Bauer double disk diffusion method according to technique described by Wayne [20]. Amoxicillin-clavulanic acid and Ceftazidime disc were used. Inoculum was prepared from overnight cultures of the test organism. Using a sterile swab, test organisms were inoculated onto Mueller Hinton agar. A disc containing 30 µg of Ceftazidime was placed on the agar at a distance of 20 mm from the Amoxicillin-clavulanic acid (30 µg) disc. The plates were incubated anaerobically at 37 °C for 24 hours in an anaerobic jar. The inhibition zones were observed and recorded.

2.5. Phenotypic Detection of Metallo β -Lactamases

The production of metallo beta lactamase enzyme was

determined following the method described by Yong *et al.*, [21]. The Imipenem combine disc synergy was done by placing two (10 µg) Imipenem disc at a distance of 20 mm from each other and 10 µl of EDTA solution was added to one of them to the desired concentration of 750 µg on the Mueller Hinton agar inoculated with test organism. The inhibition zones of the Imipenem and Imipenem-EDTA disc were compared after 18-24 hours of anaerobic incubation at 37 °C. An increased inhibition zone with the Imipenem and EDTA disc >7 mm than the Imipenem disc alone was considered as metallo-β-lactamase positive [22].

2.6. Molecular detection of *bla-IMP* gene

2.6.1. DNA Extraction

DNA extraction was performed using ZR Bacterial DNA MiniPrep™ kit following the manufacturers instruction.

2.6.2. Polymerase Chain Reaction (PCR)

Metallo resistant gene sequence of *C. difficile* was obtained from NCBI and primer pairs were designed using primer blast tool of NCBI to amplify a 597 bp fragment of the *bla-IMP* gene. The primer sequences obtained are forward-(5'AGGTGGCACGAATACAGGAA-3') and reverse-(5'ACTTGCTTGCCATGCCCTAA-3'). The Polymerase chain reaction (PCR) analysis was performed as described by Indra *et al.*, [23]. The PCR reaction was performed in a final volume of 25 µl containing the following: 12.5 µl HosterTaq PCR master mix (Qiagen, Germany), 5.0 µl Q solution (Qiagen, Germany), 2.5 µl Coral load (Qiagen, Germany), 2 µl each (0.6 µM) of forward and reverse primer and 1 µl of DNA sample. PCR amplification was performed as follows: one cycle of initial denaturation at 94 °C for 30 seconds; 35 cycles of denaturation at 94°C for 1 minute; Annealing at 48°C for 1 minute; Extension at 72 °C for 30 seconds and final extension at 72 °C for 10 minutes. Amplicons were analyzed by agarose gel electrophoresis using 1.5 % agarose gel for 60 minutes at 100 volts.

3. Results

3.1. Isolation of *Clostridium Difficile*

Eighteen (12%) positive isolates of *C. difficile* were obtained with a distribution of 8 and 10 from Specialist Hospital Yola and the Federal Medical Centre, Yola respectively. The colonies are approximately 4 mm in diameter, yellow and ground glass-like with a slightly filamentous edge and distinctive horse urine odor. Gram stain showed that isolates were gram positive bacilli in cluster. Biochemically the isolates were catalase and oxidase negative and were motile.

3.2. Antimicrobial Susceptibility Testing

Antibiotic susceptibility tests reveal that *Clostridium difficile* isolated were resistant to an array of antibiotics. The isolates were found to be resistant to Ciprofloxacin (MIC >64 µg/ml), Erythromycin (MIC >64 µg/ml), Metronidazole (MIC 64 µg/ml), Tetracycline (MIC > 128 µg/ml) and clindamycin (MIC > 64 µg/ml).

3.3. Phenotypic Detection of Extended Spectrum β-Lactamase (ESBL) and Metallo Beta Lactamase

ESBL detection showed that all the 18 (Eighteen) isolates were ESBL positive. After anaerobic incubation at 37°C for 48 hours, the inhibition zone around Amoxicillin-clavulanic acid discs showed a 5 mm differentiation (21 mm) as against the zone of inhibition around the Ceftazidime disc (16 mm) alone (Plate I). Similarly, the phenotypic detection of metallo-β-lactamase by Imipenem-EDTA combined disc test, showed that 16 (88.89%) out of the 18 isolates were positive. The inhibition zone around Imipenem-EDTA disc was >7mm (23 mm) than the inhibition zone around the Imipenem disc (16 mm) alone (Plate II). Eleven (11) out of the 18 isolates were positive for *bla-IMP* gene (Plate III).

Plate I. ESBL positive showing Amoxicillin- Clavulanic acid disc (right) with increased zone of inhibition than ceftazidime disc (left)

Plate II. Metallo β -lactamase positive, IMP-EDTA increased clear zone of inhibition on the right (upper). Metallo β -lactamase negative (Both IMP-EDTA and IMP disc have increased zones of inhibitions (lower)

ATCATGCTCAGATGAACGCTGGCGGCGTGCCTAACACATGCAAGTTGAGCGATTTACTTCGGTAAAGAGC
GGCGGACGGGTGAGTAACGCGTGGGTAACCTACCCTGTACACACGGATAACATACCGAAAGGTATGCTA
ATACGGGATAATATATTTGAGAGGCATCTCTTGAATATCAAAGGTGAGCCAGTACAGGATGGACCCGCGT
CTGATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATCAGTAGCCGACCTGAGAGGGTGATCG
GCCACATTGGAAGTGAACGCGGTCCAACTCTACGGGAGGCGAGCAGTGGGGAATATTGCACAATGGGC
GAAAGCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAACTCTGTCCTCAAGGAA
GATAATGACGGTACTTGAGGAGGAAGCCCCGGCTAACTACGTGCCANCAGCCCCGGTAATACGTAGGGGG
CTAGCGTTATCCGGATTTACTGGGCGTAAAGGGTGCGTAGGCGGTCTTTCAAGTCAGGAGTGAAAGGCTA
CGGCTCAACCGTAGTAAGCTCTTGAAACTGGGAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCTAGT
GTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCAGTTGCGAAGGCGGCTCTCTGGACTGTAACGTGAC
GCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGAG
TACTAGGTGTTCGGGGGTTACCCCTTCGGTGCCGCACTAACGCATTAAGTACTCCGCTGGGAAGTACGCT
CGCAAGAGTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGTAGCGGAGCATGTGGTTTAATTCGAA
GCAACGCGAAGAACCTTACCTAAGCTTGACATCCCAATGACATCTCCTTAATCGGAGAGTTCCCTTCGGG
GACATTGGTGACAGGTGGTGATGGTTGTCGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAAC
GAGCGCAACCCTTGCTCTTTAGTTGCCATCATTAAGTTGGGCACTCTGAGAGACTGCCAGGGGATAACCTGG
AGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGCTTAGGGCTACACACGTGCTACAATGGGTA
GTACAGAGGGTTGCCAAGCCGTAAGGTGGAGCTAATCCCTTAAAGCTACTCTCAGTTCGGATTGTAGGCT
GAAACTCGCCTACATGAAGCTGGAGTTATATAATCGCAATCAAATGCTGCGGTGAATGCGTTCCCGGGG
TCTTTACACACCGCCCGTCACACCCGGGAGTTGGAAACGCCGAAGCCGAATTATCTAACCTTTTGGAAN
AATCTCGAAAGTGGAATCAATAACTTGGGTAATCGTAACAAGGTAACCGTATCGGAAGGT

Figure 1. 16S RNA sequence of *Clostridium difficile*

Plate III. 1% agarose gel showing 597 bp *Bla* protein-like amplicons Lane M: DNA ladder (1Kb), Lanes 4,5,7,8,10,11,12,13,14,16 and 17 is positive for *Bla* protein-like metallo resistant gene. Lanes 1,2,3,6,9,15 and 18 are negative for *Bla* protein-like metallo resistant gene

4. Discussion

The results in this research were not unexpected as *C. difficile* has been reported to be a nosocomial pathogen. *C. difficile* infected patient are reservoirs as well as disseminators as can excretes are more than 10^5 *C. difficile* per gram of feces. *C. difficile* spores are highly resistant and can survive in the environment for months [24]. This explains why the toilets (38.9%) are the most contaminated sites as observed in this study. Research has shown that *C. difficile* spores are found in 34-58% of sites in hospital environment [25], including after disinfection [26] and commodes, bed frames, toilets floors are the most contaminated sites. A study by Fawley and Wilcox [25] showed that *C. difficile* remained viable in one third of surface samples taken from the hospital rooms of pseudomembranous colitis patients with (PMC). Spores on equipment's and hands of health workers facilitates spread of *C. difficile* and hence are potential vehicle in cross contamination.

Studies conducted by Adegoyega, [27], Titov, *et al.*, [28] and Fawley and Wilcox [25] had similar findings. The hospital, particularly the ICU harbors strains of *C. difficile* which may have been introduced by hospitalized patients or staff and easily transmitted to other patients as well as the environment. Adegoyega, [27] also reported 11.7% recovery rate of *C. difficile* in medical wards. Another study with 12.5% rate from the Lagos University Teaching Hospital (LUTH) environment depicts that the source of transmission of this organism may be hospitalized patients. The problems faced with ESBL screening and identification as ESBL pathogens by laboratories in underdeveloped countries have brought about variations in the *in vitro* pattern of resistant to β -lactam agents. World Health Organization (WHO) and the Centre for Disease Control (CDC) have raised concerns about the inability of many laboratories to detect ESBL-producing microorganism [29]. *C. difficile* is a multidrug resistant bacterium [30] and research has shown that *C. difficile* carries a *cfr* protein like gene that confers multiple antibiotic resistance by the same mechanism as *cfr* gene in *Enterobacteriaceae*. Two separate studies carried out by Stiefel and Nerandzic in 2014, also revealed that *Clostridium difficile* are Extended Spectrum Beta-lactamases (ESBL) producers [31]. Standardized method for screening metallo beta-lactamase (MBL) in clinical isolates has not been recommended. However, different studies have reported the use of methods like Imipenem-EDTA combined disc test, double-disc synergy test using Imipenem and EDTA, E-test and modified Hodges test [22]. This study showed the reliability of combined disc diffusion test method (CDDT). Previous studies have shown that more organism was found to be positive for MBL by Imipenem-EDTA disc than Ceftazidime disc. MBL producing organism might have alternative mechanism of resistance and Behera *et al.*, [22]

suggest that using Ceftazidime instead of Imipenem disc will not show MBL production. Yan *et al.*, [32] also affirms that the Imipenem-EDTA combined disc test was the most sensitive for phenotypic detection of metallo beta-lactamase production.

In contrast to phenotypic screening of isolates that depicts sixteen (16) isolates as positive for MBL production, the molecular screening indicates that the *bla-IMP* gene was not the only metallo resistant gene that are harbored by *Clostridium difficile* but seem to be the dominant, even though the primer used was meant to specifically amplify the *bla IMP* gene segment and lack of amplifying the whole genome precludes other genes. Nonetheless, the result of this study confirms the presence of metallo resistant gene in *Clostridium difficile* as previously reported by Manot *et al.*, [11].

5. Conclusions

The study confirmed the occurrence of *C. difficile* in the hospitals studied. The *C. difficile* are ESBL producers with approximately 69% harboring the *bla-IMP* metallo-resistant gene. All isolates obtained demonstrated a significant level of resistance to the five antibiotics tested. The result depicts that multidrug resistant *C. difficile* is an emerging concern within the hospitals sampled in Adamawa State, Nigeria. This finding poses a threat to the cost associated with *Clostridium difficile* treatment and may increase a patient's hospital stay. Further studies on potential contamination of surfaces by *C. difficile* needs to be conducted. Similarly, current disinfection techniques need to be re-evaluated regarding the survival of spore.

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