EVALUATION OF ANTIMICROBIAL SUSCEPTIBILITY PATTERNS AND β-LACTAMASE PRODUCTION BY *CLOSTRIDIUM SPECIES* ISOLATED FROM FOOD AND FAECAL SPECIMENSIN LAGOS, NIGERIA

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ABSTRACT

The susceptibility patterns of anaerobic bacteria have become increasingly unpredictable challenging the concept of reliable anaerobic therapy and empirical treatment. Resistance to even the most active drugs, such as amoxicillin and metronidazole, has been reported. These factors emphasize the need for antimicrobial susceptibility testing of anaerobes as well as periodic surveillance to detect geographic or temporal trends. We evaluated the invitro antimicrobial susceptibility of 56 Clostridium speciesisolated from food commodities and faecal specimens in Lagos State to five antibiotics. Testing was done by agar dilution method on Wilkins-Chalgren agar supplemented with hemin (5 µg/ml) and vitamin K (1 µg/ml) as recommended by Clinical Laboratory Standard Institute. Bacteria strains resistant to amoxicillin were tested for the Production of β-lactamase by nitrocefin disk assay method. The antibiotics used were amoxicillin, clindamycin, erythromycin, tetracycline and metronidazole. The strains were more susceptible to metronidazole (96.4%). This was followed by amoxicillin (91.1%)) and tetracycline (80.4%). High level of resistance was observed with clindamycin (33.9%) and erythromycin (28.6%). The MIC range of metronidazole was 0.125-32 µg/ml with MIC_{50} at 1.0 µg/ml and MIC_{90}at 8.0 µg/ml respectively. The MIC_{90} for tetracycline, erythromycin, clindamycin and amoxicillin are 8.0µg/ml, 64 µg/ml, 64 µg/ml and 0.5 µg/ml respectively. This result shows that food products sold in Lagos State may serve as possible vehicles for the transmission of drug-resistant strains of Clostridium species. Metronidazole remains the drug of choice for the treatment of clostridial infections and indeed anaerobic infections. However, the presence of resistant strains should not be overlooked.

Keywords: Anaerobic bacteria, Empirical treatment, Resistance, Antimicrobial susceptibility, *Clostridiumperfringens.*

INTRODUCTION

Species of the genus *Clostridium* are Gram-positive and have the ability to form spores. Currently there are known to be about 130 species of clostridia (Murray *et al.,* 2003).Members of the genus are naturally found in soil, sewage, marine sediments, and the intestinal tracts of humans and other animals (Murray *et al*., 2003; Montso and Ateba, 2014).*Clostridium* infections can be severe and life-threatening and studies have demonstrated that failure to direct appropriate therapy leads to poor clinical response (Lassmann *et al.*, 2007; Goldstein *et al*., 2011). Anaerobic sample collection, transport, and manipulation of culture isolates can be time-consuming and difficult to perform correctly. As a result, empirical broad-spectrum antimicrobial therapies for anaerobic infections are often being instituted long before results of susceptibility testing are available. However, antimicrobial bacterial resistance among anaerobic bacteria is increasing globally, as demonstrated by numerous surveys in Europe, the United States, Canada, and New Zealand (Roberts *et al*., 2006; Snydman *et al.*, 2010; Nagy *et al*., 2011; Karlowsky *et al.,* 2012). Differences in resistance patterns may be due to the variety of susceptibility testing methodology used, the selective antibiotic pressures associated with antimicrobial usage, and the lack of uniformity in adoption of interpretive breakpoints.

Susceptibility testing is performed to obtain information on the predicted response of the bacteria to antibiotics in the form of minimum inhibitory concentration (MIC), which is defined as the lowest concentration of the antibiotic that inhibits growth of organisms (Schuetz, 2014). MICs may be obtained by utilizing the Clinical and Laboratory Standards Institute (CLSI)–defined agar or broth microdilution methods or by using a variety of commercially available methods (Brook *et al*., 2013; Schuetz, 2014). Commercial methods include Etest strips, M.I.C. Evaluator strips, and Sensititre panels. Etests and M.I.C. Evaluator strips are gradient diffusion methods. Agar and broth microdilution methods are usually employed by research or reference laboratories (Brook *et al*., 2013).

Anaerobes manifest three major mechanisms of resistance to β-lactam antibiotics namely: inactivating enzymes, mainly beta-lactamases (penicillinases and cephalosporinases); low-affinity penicillin-binding proteins (PBPs); and decreased permeability through alterations in the porin channel (Wexler, 1991;

Schuetz*et al.*, 2014). The production of beta-lactamases is the most common mechanism of resistance to β-lactam antibiotics in anaerobes (Mishra *et al*., 2016). Of the clostridial species, *Clostridium perfringens* is one of the most susceptible to penicillin, though clindamycin resistance is increasing (Roberts *et al*., 2006; Tyrrell *et al.*, 2006). Drugs that have reportedlymaintained activity against non-perfringens*Clostridium* species include piperacillin, carbapenems, metronidazole, and vancomycin (Stevens *et al*., 2011; Schuetz *et al.*, 2014). However, there has been a recentupsurge in antimicrobial resistance among anaerobic bacteria globally and resistance to even the most active drugs, has been reported prompting the need for periodic surveillance to detect any geographic or temporal trends. This study therefore evaluates the *invitro* antimicrobial susceptibility of *Clostridium* speciesisolated from food commodities and faecal specimens in Lagos State to five commonly used antibiotics.

MATERIALS AND METHODS

Sample Collection

The samples were collected within a period of one year from May 2014 to April 2015. Study sites comprisedsix randomly selected local governments in Lagos State including Epe, Shomolu, Mushin, Ikeja, Surulere and Lagos Island. Food samples were collected randomly from different open markets, supermarkets, vendors and cafeteria and restaurants while stool samples were collected from General hospitals in these local governments.All samples were collected into a transport medium (Thioglycollate broth) or sterile plastic bag (food samples) and transported to the laboratory for processing within 2 hours of collection.

Bacteria Isolation and identification

Bacterial strains were isolated using selective media (Reinforced Clostridial Agar media). Identification was performed using morphological appearance, API 20A kit (bioMerieux, France) and confirmed by Polymerase Chain Reaction (PCR)using oligonucleotide primers *Clos58 F:* 5'- AAA GGA AGA TTAA TACC GCA TAA- 3' and C*los780 R* 5'- ATC TTG CGA CCG TAC TCCCC -3' as previously described by Mirhosseini *et al*. (2010). The amplification reaction was carried out using a thermocycler (Eppendorf master cycler gradient, Germany) to detect *Clostridium* species. The cycling parameter consisted of an initial denaturation step at 95 ^{o}C for 3 minutes; this was followed by 30cycles of denaturation at 94 ^{0}C for 30 seconds, a primer annealing step at 54 ^{0}C for 30 seconds, an extension step at 72 ^{0}C for 90 seconds and final extension step at 72 ^{0}C for 3 minutes. The PCR products were separated using a 1.5% agarose gel

electrophoresis performed in an electrophoretic tank at 90 volts for 2.5 hours (Sigma Chemical Company), stained with 0.5 µg/ml ethidium bromide and photographed under UV transilluminator by using a digital camera (Kodak Digital System DC-120).The expected amplicon length for *Clostridium specie* was 722 bp.

The Isolates were stored in triplicate in skimmed milk, Thioglycollate broth with 50% glycerol to prevent contamination and Brain heart Infusion broth at - 80^0C.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of fifty-six *Clostridium* isolates to 5 antibiotics was determined by agar dilution method as recommended by Clinical Laboratory Standard Institute (CLSI, 2014). An inoculum was grown in brain heart infusion broth supplemented with hemin and menadione for 24hours and the turbidity was adjusted to 0.5 McFarland standards. The antibiotics were reconstituted according to the manufacturer's instructions and serial two-fold dilutions (ranging from 0.125-64 µg/ml) were prepared on the day of the test and added to Wilkins-Chalgren agar supplemented with hemin (5 µg/ml), vitamin K (1 µg/ml). The antibiotics used include: amoxicillin, clindamycin, erythromycin, tetracycline (LuperInd Farm Ltd, Brazil) and metronidazole (Aventis Farm Ltd, Brazil). A final inoculum of 1.5×10^5cfu/spot was delivered using a Steers replicator (Cefar Ltd, Brazil). Control plates without drugs were inoculated before and after each drug containing plates. All the plates were incubated in anaerobiosis (90 % N_2, 10 % CO_2) at 37 ^{0}C for 48 hours. A Reference strain *Clostridium perfringens* ATCC 13124 was included in each experiment to assess the reliability of the method.

Determination of MIC

The Minimum inhibitory concentration (MIC) were recorded as the lowest concentration of the antibiotic in the medium that inhibited bacteria growth, gave a faint haze of growth or with not more than one discrete bacterial colony. The MIC's were interpreted as being resistant or sensitive by applying the breakpoints that were proposed by Clinical Laboratory Standard Institute (CLSI, 2014).

Beta-lactamase (BLA) Detection

Bacteria strains resistant to amoxicillin were tested for the production of β-lactamase by nitrocefin disk assay method according to the method of Bridson (1998) using DryslideNitrocefin (Cefinase; BBL, Cockeysville, Md) as described by Clinical Laboratory Standard Institute (CLSI, 2014). The reaction area was moistened with deionised water using a sterile Pasteur pipette. A sweep of the

pure colony from blood agar was collected using a sterile wire loop and the specimen was smeared onto the moistened area of the Nitrocefin disk. The reaction was observed for the appearance of colour change from yellow to pink within 5-30 minutes.

RESULTS

Fifty-six (56) strains of *Clostridium*species were obtained and included in this study comprising *C. perfringens* 38 (67.9 %), *C. dificile*5(8.9%), *C. botulinum*2(3.6 %) and non- *C. perfringens*, non- *C.dificile*, non- *C. botulinum*, *Clostridium* species 11(19.6 %). They were tested for their susceptibility to five commonly used antibiotics (metronidazole, amoxicillin, erythromycin, clindamycin and tetracycline). Two (3.6%) strains (both *C. botulinum*) were resistant to metronidazole. Five (8.9 %) strains were resistant to amoxicillin including one *C. perfringens* strain and four *C.difficile*strains. It was observed that the MICvalues of each antimicrobial agent studied varied with the isolates tested with MIC range between 0.125 – 64 µg/ml (Table 1). Among the antimicrobial agents tested, the strains were most susceptible to metronidazole (96.4%), followed by amoxicillin (91.1%) and tetracycline (80.4%). High level of resistance was observed with clindamycin (33.9%) and erythromycin (28.6%). The MIC range of metronidazole was 0.125 - 32 µg/ml with MIC_{50} at 1.0 µg/ml and MIC_{90} at 8.0 µg/ml respectively. The MIC_{90} for tetracycline, erythromycin, clindamycin and amoxicillin were8.0 µg/ml, 64 µg/ml, 64 µg/ml and 0.5 µg/ml respectively. The two *C. botulinum* strains isolated were susceptible to amoxicillin, tetracycline and erythromycin (100%) but resistant to metronidazole and clindamycin (100%). Furthermore, the five *C.difficile* isolated were resistant to clindamycin (100%) and amoxicillin (80%) but susceptible to metronidazole, tetracycline and erythromycin. All (100%) *C. perfringens* isolates tested were susceptible to metronidazole but showed varying degrees of resistance to amoxicillin (3.3%), tetracycline (16.7%), erythromycin (33.3%) and clindamycin (36.6%) (Figure 1).

The five strains (four *C. difficile* isolates and one *C. perfringens* isolate) resistant to amoxicillin were tested for β- lactamase production. However, all were negative for β- lactamase production.

Table 1: Susceptibility pattern of isolated*Clostridium species* to antibiotics

Antibiotics	CLSI MIC (µg/ml)	Range (µg/ml)	MIC $_{50}$	MIC $_{90}$	Resistance (%)	Susceptible (%)
Amoxicillin	≥2	0.125 – 32	0.25	0.5	8.9	91.1
Metronidazole	≥32	0.125 – 32	1.0	8	3.6	96.4
Clindamycin	≥8	0.25 - ≥64	1.0	64	33.9	66.4
Tetracycline	≥16	0.125 - 64	2.0	8.0	19.6	80.4
Erythromycin	≥8	0.125 – 64	1.0	64	28.6	71.4

Key:
CLSI: Clinical Laboratory Standard Institute
MIC: Minimum Inhibitory Concentration
MIC$_{50}$: MIC at which growth of 50% of the isolates were inhibited
MIC$_{90}$: MIC at which growth of 90% of the isolates were inhibited

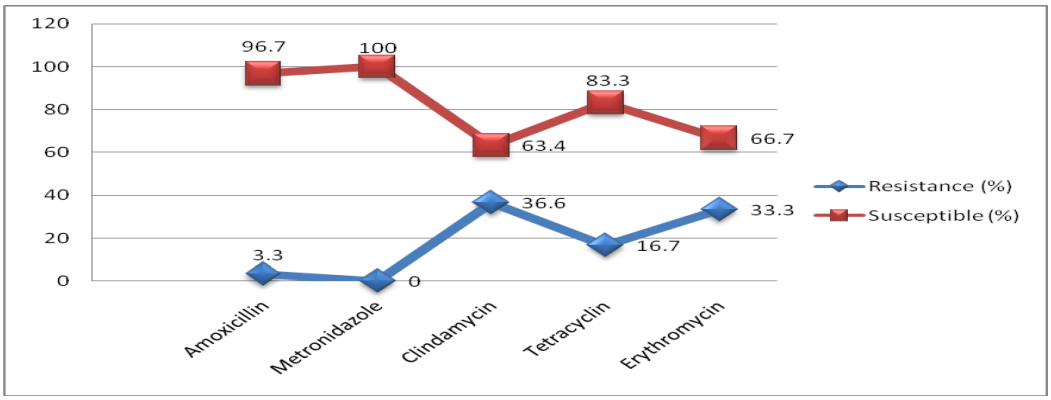

Figure 1: Resistance pattern of *Clostridiumperfringens* species to five commonly used antibiotics

DISCUSSION

The result of this study showed an encouraging susceptibility pattern for metronidazole (96.4%) and amoxicillin (91.1%) which are the drugs of choice for treating clostridial infections (Table 1). These results are in agreement with the findings of Kouassi and colleagues (2014) who reported metronidazole and penicillin G as the most potent agents against *Clostridium perfringens* and *Clostridium difficile* isolated from cooked beef sold in Cote d'Ivoire. It should also be noted that certain degrees of resistance (3.6%) and (8.9%) was observed in metronidazole and amoxicillin respectively in this study and should not be overlooked.Tetracycline showed moderate susceptibility (80.4%). Nevertheless,

given the present result, tetracycline cannot be depended upon in clostridial infections without *in-vitro* testing. Tetracycline resistance has been documented in up to 75% of *C. perfringens* isolates obtained from commercial poultry, but data in humans are few (Johansson *et al*., 2004; Hecht, 2006). The activities of tetracycline against Clostridialspecies have not been well documented (Hecht, 2006). Therefore empirical use of tetracycline for Clostridial infections is greatly discouraged. At present, there are numerous genes associated with resistance to tetracycline in Gram-positive bacteria and it remains to be established which ones are present in these*Clostridium* species isolated from food products and faecal specimens in Lagos State.

High level of resistance was observed with clindamycin (33.9%) and erythromycin (28.6%). This is consistent with the findings of previous researchers (Drummond *et al*., 2003; Kouassi *et al*.,2014). Although clindamycin is considered to be a gold standard for treatment of anaerobic bacterial infection since the 1960s, antibiotic resistance to clindamycin among these pathogens has increased steadily over the past 15 years (Schuetz, 2014).

All (38) *C. perfringens* isolates tested in this study were susceptible to metronidazole but showed varying degrees of resistance to amoxicillin (3.3%), tetracycline (16.7%), erythromycin (33.3%) and clindamycin (36.6%) (Figure 1). Furthermore, the five *C. difficile* isolated were susceptible to metronidazole (100%), tetracycline (100%) and erythromycin (100%) but resistant to clindamycin (100%) and amoxicillin (80%). A similar result was noted for clinical isolates of *C. difficile* in a study (Huang *et al.*, 2009) and isolates from broiler chickens in another study (Simango and Mwakurudza, 2008).

The two *C. botulinum* strain isolated were 100 % susceptible to amoxicillin, tetracycline and erythromycin but 100 % resistant to metronidazole and clindamycin. This data are in accordance with the study by Koluman *et al*. (2013) where susceptibility to tetracycline (68%) and penicillin G (78.9%) was recorded. There are limited data about the antibiotic susceptibility of *C. botulinum*strains. This is largely because the use of antibiotic drugs is discouraged in food-borne and infant botulism management. However, antibiotics are administered only for treatment of secondary infections, taking into consideration that these drugs would not cause a worsening of neurological condition (Koluman *et al*., 2013).

The five strains (one *C. perfringens* and four *C. difficile*) resistant to amoxicillin were tested for β- lactamase production. Surprisingly, all tested negative for β-

lactamase. The production of BLAs is the most common mechanism of resistance to β-lactam antibiotics in anaerobes. With some exceptions, strains of *Clostridiumspecies*, have also been found to express resistance by one or more of the β-lactamases (Hetch, 2004). A recent study reported beta-lactamase producing *C. perfringens* bacteremia in an elderly man with acute pancreatitis (Mishra *et al*., 2016).Be that as it may, the strains that were resistant to amoxicillin in this study were not beta-lactamase producers. Thus, theirmechanism of resistance was not by beta-lactamase production but may be through alternative mechanism.

CONCLUSION

The findings of this study revealed that food products sold in Lagos State may serveas possible vehicles for transmission of drug-resistant *Clostridium* species. Adequate strategy to reduce microbial contamination of these foods is therefore strongly recommended. Metronidazole remains the drug of choice for the treatment of clostridial infections and indeed anaerobic infections. However, the presence of resistant strains should not be overlooked. Beta-lactamase production was not observed in this study, though *invitro* resistance to amoxicillin was noted in five isolates. There is need to investigate on a larger scale the mechanism of resistant of *Clostridium* species to beta-lactam antibiotics.

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REFERENCES

Bridson, E.Y.(1998). The Oxoid manual.Oxoid.Basingstoke England.5-4.

Brook, I., Wexler, H.M and Goldstein, E.J. (2013).Antianaerobic antimicrobials: spectrum and susceptibility testing. *ClinMicrobiol Rev*. **26**:526–546.

Clinical Laboratory Standard Institute (2014). Performance Standard for Antimicrobial Susceptibility Testing. Twenty fourth informational supplement. M100- S24.

Drummond, L.J., McCoubrey, J., Smith, D.G., Starr, J.M andPoxton, I.R. (2003).Changes in sensitivity patterns to selected antibiotics in

Clostridium difficile in geriatric in-patients over an 18-month period.*J Med Microbiol*.**52**:259–263.

Goldstein, E.J., Solomkin, J.S., Citron, D.M and Alder, J.D.(2011). Clinical efficacy and correlation of clinical outcomes with in vitro susceptibility for anaerobic bacteria in patients with complicated intra abdominal infections treated with moxifloxacin. *Clin Infect Dis*.**53**:1074–80.

Hecht, D.W. (2004).Prevalence of Antibiotic Resistance in Anaerobic Bacteria: Worrisome Developments. *CID***. 39:** 92-97

Hecht, D.W. (2006). Anaerobes: antibiotic resistance, clinical significance, and the role of susceptibility testing. *Anaerobe*.**12:**115–121.

Johansson, A., Greko, C., Engström, B.E andKarlsson, M (2004).Antimicrobial susceptibility of Swedish, Norwegian and Danish isolates of Clostridium perfringens from poultry, and distribution of tetracycline resistance genes.*Vet Microbiol*. **99**:251–257.

Karlowsky, J.A.,Walkty, A.J., Adam, H.J., Baxter, M.R., Hoban, D.J andZhanel, G G (2012). Prevalence of antimicrobial resistance among clinical isolates of *Bacteroidesfragilis* group in Canada in 2010-2011: CANWARD surveillance study. *Antimicrob Agents Chemother*,**56**:1247–52.

Koluman, A.,MelikoğluGölcü, B., Derin, O., Özkök, S andAnniballi, F.(2013).*Clostridium botulinum*in honey: prevalence and antibiotic susceptibility of isolated strains. *Turk J. Vet. Anim.Sci*,**37**: 706-711.

Kouassi, K.A., Dadie, A.T., N'Guessan, K.F., Dje, K.M andLoukou, Y.G.(2014).*Clostridium perfringens* and *Clostridium difficile* in cooked beef sold in Cote d'Ivoire and their *a*ntimicrobial susceptibility. *Anaerobe*.**28**: 90-94.

Lassmann, B., Gustafson, D.R., Wood, C.M and Rosenblatt, J.E. (2007).Reemergence of anaerobic bacteremia.*Clin Infect Dis*. **44**:895–900.

Mirhosseini, S.Z., Seidavi, A., Shivazad, M., Chamani, M., Sadeghi, A.A andPourseify, R. (2010).Detection of *Clostridium* sp. and its Relation to Different Ages and Gastrointestinal Segments as Measured by Molecular Analysis of 16S rRNA Genes.*Braz. Arch. Biol. Technol*. 53 (1): 69-76.

Mishra, R., Sinha, N andDuncalf, R. (2016).Beta Lactamase Producing *Clostridium perfringens*Bacteremia in an Elderly Man with Acute Pancreatitis. Case Reports in Critical Care.4 pages http://dx.doi.org/10.1155/2016/7078180.

Montso, K.P. andAteba, C.N.(2014). Molecular Detection of *Clostridium* Species in Beef Obtained from Retail Shops in North West Province, South Africa. *J Food Nutr Res.* **2**(5): 236-243.

Murray, P.R., Baron, E.J., Jorgensen, J.H., Pfaller, M.A andYolken, R.H. (2003).*Manual of Clinical Microbiology,* 8th ed. ASM Press, Washington, D.C.

Nagy, E., Urbán, E., Nord, C.E. (2011). ESCMID Study Group on Antimicrobial Resistance in Anaerobic Bacteria. Antimicrobial susceptibility of *Bacteroidesfragilis* group isolates in Europe: 20 years of experience. *ClinMicrobiol Infect*. **17**:371–379.

Roberts, S.A., Shore, K.P., Paviour, S.D., Holland, D and Morris, A.J. (2006). Antimicrobial susceptibility of anaerobic bacteria in New Zealand: 1999-2003. *J AntimicrobChemother*.**57:**992–998.

Schuetz, A.N.(2014).Antimicrobial Resistance and Susceptibility Testing of Anaerobic Bacteria.*Clin Infect Dis*. **59**(5):698–705.

Simango, C andMwakurudza, S. (2008). *Clostridium difficile* in broiler chickens sold at market places in Zimbabwe and their antimicrobial susceptibility. *Int J FoodMicrobiol*; **124**:268e70.

Snydman, D.R., Jacobus, N.V., McDermott, L.A., Golan, Y., Hecht, D.W., Goldstein, E.J., Harrell, L., Jenkins, S., Newton, D., Pierson, C., Rihs, J.D., Yu, V.L., Venezia, R., Finegold, S.M., Rosenblatt, J.E., Gorbach, S.L. (2010). Lessonslearned from the anaerobe survey: historical perspective and review ofthe most recent data (2005-2007). *Clin. Infect. Dis.***50**(Suppl 1):S26–S33.

Stevens, D.L., Bryant, A.E., Berger, A., von Eichel-Streiber., C (2011). Clostridium. In: Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW. Manual of clinical microbiology.Vol 1. 10th ed. Washington, DC: ASM Press. 834–857.

Tyrrell, K.L., Citron, D.M.,Warren, Y.A., Fernandez, H.T., Merriam, C.V and Goldstein, E.J. (2006). In vitro activities of daptomycin, vancomycin, and penicillin against Clostridium difficile, C. perfringens, Finegoldia magna, and Propionibacterium acnes.*Antimicrob Agents Chemother*.**50**: 2728–2731.

Wexler, H.M. (1991). Susceptibility testing of anaerobic bacteria: myth, magic, or method? *Clin.Microbiol.Rev*. **4:**470–484.