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The Prevalence of Extended Spectrum Beta Lactamase Producing Citrrobacter Freundii and Pprovidencia Stuartii from Medical Laboratories in Parts of Lagos State, Nigeria

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Abstract
Citrobacter freundii and Providencia stuartii are members of the family Enterobacteriaceae which are emerging as important Extended Spectrum Beta Lactamase (ESBL) producing pathogens. This study was aimed to determine the prevalence of ESBL and carbapenemase production in C. freundii and P. stuartii. from laboratories in the northwest of Lagos. A total of 134 isolates were obtained from laboratories. Isolate identification, antibiotics susceptibility testing, and phenotypic detection of ESBL production were done using standardized procedures. Multiplex polymerase chain reaction (PCR) was performed on ESBL producing isolates to detect bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{KPC}, bla_{VIM}, bla_{IMP} and bla_{OXA}. Out of 134 isolates 15 (11.2%) were Citrobacter freundii and only 2 (1.5%) were of the Providencia genus both of which were Providencia rettgeri and Providencia stuartii. A total of 9 (60%) tested positive for the production of Extended Spectrum Beta Lactamase (ESBL) enzyme while only Providencia stuartii of the Providencia genus also produced ESBL. Also, 8 (47%) of the initial Citrobacter and Providencia isolates produced carbapenemase Molecular characterization showed that 4 (44.4%) and 1 (100%) of the Citrobacter freundii and Providencia stuartii isolates respectively, displayed the presence of CTX-M gene while 2 (20%) of all ESBL producing isolates also possessed SHV gene including 1 (11.1%) of ESBL producing Citrobacter freundii and 1 (100%) of ESBL producing Providencia stuartii. Antibiotic susceptibility testing showed that Citrobacter freundii displayed the highest resistance to Ceftazidime and Cefotaxime at 71.4% and the least resistance to Amoxicillin-Clavulanate at 28.6%. Providencia stuartii also displayed absolute resistance (100%), to cefotaxime and ciprofloxacin and absolute susceptibility (100%) to Ceftazidime. Also, Citrobacter freundii showed the same resistance patterns with 57.1% resistance to Gentamicin, Cefuroxime and Ceftriaxone antibiotics while Providencia stuartii showed varied resistance patterns with absolute resistance (100%), absolute susceptibility (100%) and absolute resistance (100%) to Gentamicin, Cefuroxime and Ceftriaxone antibiotics respectively. In conclusion, the Multiple Antibiotic Resistance Index values of both bacterial genera showed that 70.6% were from High-Risk sources indicating that the majority of bacteria analysed were from environments where they have been subjected to a high burden of antibiotic exposure.
Keywords: *Extended Spectrum Beta Lactamase, Carbapenemase, Citrobacter freundii, Providencia stuartii, antibiotics.*

INTRODUCTION

Extended Spectrum Beta Lactamases (ESBLs) are a group of Beta Lactamases which hydrolyse oxyimino-cephalosporins and have been found worldwide in many different genera of the family Enterobacteriaceae (Bradford, 2001):. ESBLs have become a serious health concern all over the world due to the indiscriminate use of antibiotics, poor sanitation in hospitals and unhealthy lifestyles. Carbepenemases are also a serious cause of concern as they hydrolyze 'carbapenems' which are from the last line of drugs in the fight against infections. *Citrobacter freundii* and *Providencia stuartii* are members of the family Enterobacteriaceae which are emerging as important ESBL and carbapenemase producing pathogens (Tumbarello *et al*., 2004; Liu *et al*., 2017). This study was carried out to determine the antibiotic susceptibility patterns and the ESBL and carbapenemase producing prevalence of *C. freundii* and *P. stuartii* in Festac Town, Lagos State.

METHODS

A total of 134 non-duplicatebacterial isolates obtained from urine, wound, high vaginal swab (HVS) and stool samples, were subject to Standard and Rapid methods such as the Gram Stain technique and the Microbact™ system for the identification of isolates obtained.

Antibiotic sensitivity of the identified isolates was determined by the Kirby Bauer Disk Diffusion method on Muller-Hinton agar using commercially available paper disks (Kirby *et al*.,1966). The antibiotic disks used were in the following concentrations: Amoxillin-clavulanate (10 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Ciprofloxacin (30 µg), Gentamicin (30 µg), and Meropenem (10 µg).

The double disk synergy test (DDST) method was employed to phenotypically determine the production of

Extended Spectrum Beta Lactamase. An enhancement of the zone of inhibition on Mueller-Hinton Agar from third generation Cephalosporins such as Ceftazidime and Cefotaxime towards Amoxicillin-Clavulanate antibiotic disks on an incubated culture of the isolate being analyzed was indicative of ESBL production. Production of Carbapenemase was also phenotypically determined using CLSI breakpoints for Meropenem antibiotic disks (CLSI, 2000).

Gene amplification using Polymerase Chain Reaction as well as the electrophoresis of amplification product was carried out to confirm the presence of ESBL and Carbapenemase.

EXTENDED SPECTRUM BETA-LACTAMASE TEST:
The double synergy test (DDST) method described by CLSI (2000) was employed. Standardized inoculum of the bacteria was inoculated on Mueller Hinton Agar with turbidity adjusted to 0.5 MacFarland standard (Oxoid, United Kingdom) using sterile swab sticks. Amoxicillin/ Clavulanic acid disc (10microgram, Oxoid, UK) was placed at the centre of the inoculated Mueller Hinton Agar. Ceftazidime and Cefotaxime (30 micrograms, Oxoid Ltd., United Kingdom) were placed 25mm centre to centre away from the Amoxicillin/Clavulanic acid disc. The plates were incubated at 37^{o}C for 24 hours. After incubation enhancement of Zone of inhibition of either or both the Ceftazidime and Cefotaxime discs towards the Amoxicillin /Clavulanic acid discs is indicative of ESBL production.

INVESTIGATION FOR CARBAPENEMASE PRODUCTION:
Meropenem discs of 10microgram was used for the physical detection of Carbapenemase enzyme production. Discs were placed at the centre of already prepared Mueller Hinton Agar plates on which bacteria of interest has already been swabbed. The plates were thereafter incubated at 36.5^{o}C for 24 hours. Zone of inhibition was thereafter recorded and using CLSI standards of 2017 for Meropenem susceptibility; susceptible, resistant and intermediate bacteria were identified. According to the standards, bacteria that show zones of inhibition less than or equal to 18mm are Resistant, between 20-22mm are intermediate and 23mm and above are susceptible for most bacteria. These standards however do not hold for microorganisms such as Pseudomonas and a few others.

MULTIPLE ANTIBIOTICS RESISTANCE (MAR) INDEXING
It is calculated as the number of antibiotics to which test isolate displayed resistance divided by total number of antibiotics to which the test organism has been evaluated for sensitivity (Downing *et al*., 2011). The value of MAR index 0.2 differentiates the low and high risk. MAR index greater than 0.2 implies that the strain of such bacteria has been subjected either actively or passively to several antibiotics (Jacoby and Medeiros,1991).

DNA EXTRACTION (BY BOILING)
Isolates of interest were cultured on Nutrient Agar and incubated at 37^{o}C for 24hrs. This was done to provide freshly cultured bacteria for accurate results. Bacterial colonies of interest were then put in 1000µL of Sterile water contained in Eppendorf bottles and homogenized using a Vortex Mixer (Phoenix Instrument, United States). The samples were then centrifuged at 10,000 revolutions per minute for 5mins. The supernatant was eliminated and the pellet was suspended in Eppendorf bottles containing 200µl and re-centrifuged for another 5mins. After discarding the supernatant, the pellet was then collected and boiled at 100^{o}C for 10mins. The Eppendorf bottles were then cooled on ice for 5mins. It was then centrifuged at 10,000rpm for about 5mins and the supernatant is transferred fresh Eppendorf tubes and then stored at -20^{o}C in a deep freezer.

MULTIPLEX PCR (POLYMERASE CHAIN REACTION) AMPLIFICATION
A multiplex PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 20 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 2.0 mM $MgCl_2$, 200µM of each deoxynucleoside triphosphates (dNTP) (Solis Biodyne), 20pMol of each primer (Jena Bioscience, Germany), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in a Pielter Thermal Cycler PTC 100 (MJ Research Series) for an initial denaturation of 95°C for 5 minutes followed by 35 amplification cycles of 30 seconds at 95°C; 1 minute at 59°C and 62°C and then 1 minute 30 Seconds at 72°C. This was followed by a final extension step of 10 minutes at 72°C.

AGAROSE GEL ELECTROPHORESIS OF AMPLIFICATION PRODUCT
The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100bp DNA ladder was used as DNA molecular weight standard. The Agarose gel was prepared by adding 1gram

of Agarose powder with 100 µL of TBE (Tris Borate EDTA) buffer. The solution was boiled and allowed to cool to 70 ºC. 10 µL of 0.1 % Ethidium bromide was added and mixture was swirled gently. Once it had cooled down, it was poured in an electrophoresis tray to make a cast. The comb was inserted and allowed to gelatinize. After it had gelled, it was submerged in TBE buffer inside the electrophoresis tank. The samples were loaded with the DNA marker (lambda DNA/ Hind 111) and it was allowed to run at 100 volts.

PRIMERS USED FOR THE STUDY

GENE	PRIMER (5'-3')	BASE PAIR (bp)
CTX-M	**F:**TTTGCGATGTGCAGTACCAGTAA **R:**CGATATCGTTGGTGGTGCCATA	544
SHV	**F:** ATGCGTTATATTCGCCTGT **R:** TGCTTTGTTATTCGGGCCAA	753
TEM	**F:** GTATGGATCCTCAACATTTCCGTGTCG **R:** ACCAAAGCTTAATCAGTGAGGCA	862
IMP	**F:** GTTTATGTTCATACATCG **R**: GGTTTAACAAAACAACCAC	448
OXA-48	**F:** TTGGTGGCATCGATTATCGG **R:** GAGCACTTCTTTTGTGATGGC	744
OMP- 35	**F:** CAGACACCAAACTCTCATCAAGGG **R:** AGAATTGGTAAACGATACCCACG	1000
OMP- 36	**F:** CAGCACAATGAATATAGCCGAC **R:** GCTGTTGTCGTCCAGCAGGTTG	1115
OMP-36 N	**F:** CTGCGGCTGACCTGTCGCGTAAC **R:** CGGTCAGCTGGTCGTTGATCTGG	1024
KPC	**F:** GATACCACATTCCGTCTGG **R:** GCAGGTTCCGGTTTTGTCTC	246

RESULTS

Out of a total of 134 isolates obtained in this study, 15 (11.2%) were *Citrobacter freundii* and only 2 (1.5%) were of the *Providencia* genus both of which were *Providencia rettgeri* and *Providencia stuartii*. The bacteria were tested for their antibiotic susceptibility, their production of Extended Spectrum Beta-lactamases and Carbapenemases, whether they are High Risk or Low Risk (through their Multiple Antibiotic Resistance index values) and their patterns across gender and age. With the Double Disk Synergy Test (Plate 1) out of 15 *Citrobacter freundii* isolates, 9 (60%) tested positive for the production of Extended Spectrum Beta Lactamase (ESBL) enzyme while only *Providencia stuartii* (accounting for 50%) of the *Providencia* genus, tested positive for the enzyme. Together, 10 (58.8%) from all *Citrobacter freundii* and *Providencia* species were positive producers of ESBL. Phenotypic testing for the production of the carbapenemase enzyme concluded that out of the 9 *Citrobacter freundii* isolates that tested positive for ESBL production, 7 (77.7%) also tested positive for Carbapenemase production. The 1 (100%) *Providencia* isolate which tested positive for the production of ESBL was also positive for the production of carbapenemase. Thus together, 8 (80%) of the ESBL positive bacteria also produced carbapenemase. This also means that 8 (47%) of all initial *Citrobacter and Providencia* isolates produced the carbapenemase enzyme (Table 1). Molecular techniques also utilized involved the investigation of CTX-M, TEM and SHV genes for ESBL producing isolates, as well as OXA-48 and IMP genes for the carbapenemase producing isolates. Of these, 4 (44.4%) and 1 (100%) of the *Citrobacter freundii* and *Providencia stuartii* isolates respectively, displayed the presence of CTX-M gene. Thus together, 5 (50%) of all ESBL isolates analysed, possessed the CTX-M gene and 2(20%) of all ESBL producing isolates also displayed the presence of SHV gene including 1 (11.1%) of ESBL producing *Citrobacter freundii* and 1 (100%) of ESBL producing *Providencia stuartii.* The two isolates harboring the SHV gene also possessed the CTX-M gene, indicating that 2 (20%) of ESBL producing isolates, jointly possessed CTX-M and SHV genes including 1 (11.1%) from the *Citrobacter freundii* and 1 (100%) from the *Providencia stuartii* isolates. None of the ESBL producing isolates possessed the TEM gene. None of the isolates displayed OXA-48 and IMP mediated carbapenemase production with PCR (Figure 1).

Antibiotic susceptibility profile of carbapenemase producing isolates (Plate 2) showed that *Citrobacter freundii* and *Providencia stuartii* exhibited 4 (57.1%) and absolute resistance to Gentamicin; 5 (71.4%) and absolute susceptibility to Ceftazidime; 3 (42.8%) and absolute resistance to Ciprofloxacin; 4 (57.1%) and absolute susceptibility to Ceftriaxone; 5 (71.4%) and absolute resistance to Cefotaxime; 2 (28.6%) and absolute susceptibilty to Amoxicillin-Clavulanate and 4 (57.1%) as well as absolute resistance to Cefuroxime antibiotics respectively. (Figure 2).

The Multiple Antibiotics Resistance (MAR) Indexing values for *Citrobacter freundii* and *Providencia* species revealed that 12 out of the 17 (70.6%) isolates were from High Risk sources, with 5 isolates (29.4%) coming from Low Risk sources.

Table 1: Distribution of ESBL and Carbapenemase producers amongst the Citrobacter spp. and Providencia spp.

Bacteria	*Citrobacter* spp.	*Providencia* spp.	Total
ESBL +ve (58.8%)	9 (60%)	1 (50%)	10
ESBL –ve (41.2%)	6 (40%)	1 (50%)	7
Carbapenemase +ve (47%)	7 (46.7%)	1 (50%)	8
All Isolates (100%)	15 (100%)	2 (100%)	17

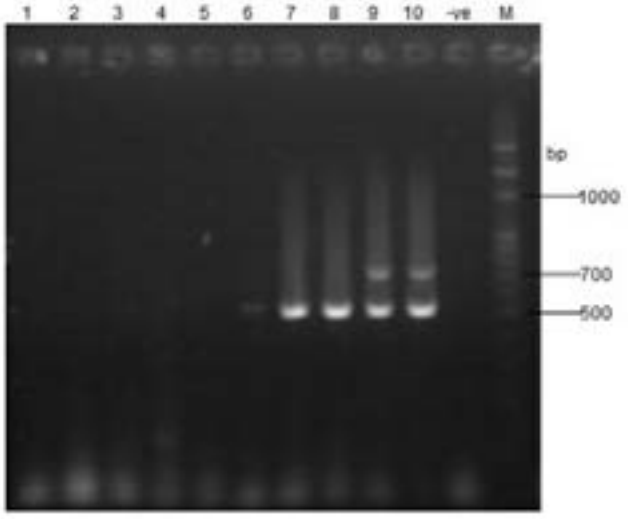

Figure 1: blaCTX-M gene at 544bp and blaSHV gene at 753bp detected by PCR and visualized by agarose gel electrophoresis with ethidium bromide staining.

KEY
- Lane M : DNA marker
- Lane -ve : Negative control
- Lane 1 to 9 : Citrobacter freundii
- Lane 10 : Providencia stuartii
- Lane 6,7,8, 9 and 10 : Positive for blaCTX-M
- Lane 9, 10: Positive for blaSHV

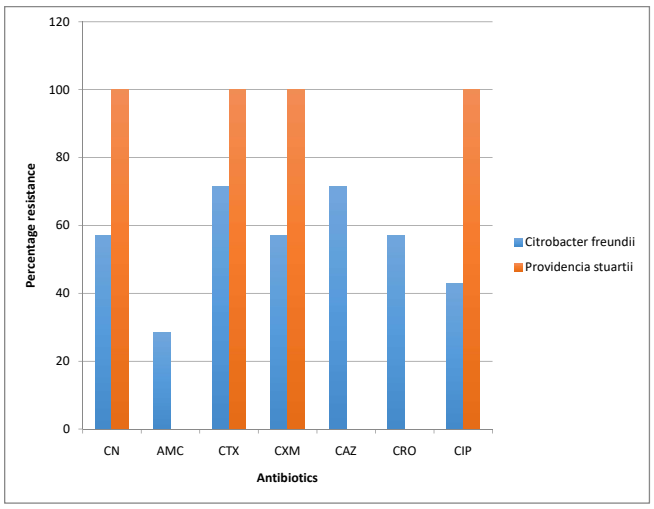

Figure 2: Antibiotic susceptibility profile of Carbapenemase producers to various antibiotics.

KEY
AMC = Amoxicillin-Clavulanate
CTX = Cefotaxime
CXM = Cefuroxime
ATM = Aztreonam
CAZ = Ceftazidine
CRO = Ceftriaxone
CN = Gentamicin
CIP = Ciprofloxacin

PLATE 1: ESBL Positive Isolate cultured on Mueller Hinton Agar showing synergistic action between Amoxicillin-Clavulanate and Ceftriaxone antibiotic discs.

PLATE 2: Antibiotic Suceptibility Testing of a bacterium on Mueller Hinton Agar showing the widest susceptibility to Meropenem antibiotic disc.

KEY: AMC=Amoxicillin-Clavulanate, CTX = Cefotaxime, CXM = Cefuroxime, CAZ = Ceftazidine, CRO = Ceftriaxone, CN = Gentamicin and CIP = Ciprofloxacin

DISCUSSION

The growing trends of antibiotic resistance and the potential harm that accompanies it has been well established in bacteria such as *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas* spp*., and Staphylococcus aureus;* and as such, has got a large chunk of scientists' attention. However, other bacteria are now beginning to prove to be of equal importance such as the likes of *Citrobacter freundii* and *Providencia stuartii* as recognized by Metri *et al.,* (2013) and (Sabri *et al.,* 2012). This study examined *P. stuartii* and *C. freundii* in this light to investigate the extent of this emerging problem in Northwest, Lagos; by determining their antibiotic resistance patterns, ESBL and carbapenemase production.

Prevalence of ESBL production was 60% for *C. freundii* in this study. This is very similar to 61.6% ESBL prevalence in *C. freundii* reported by Ashish and co-workers (2012). These values confirmed that ESBL production has become common to the bacteria and that necessary checks will have to be put in place so that such resistance does not become more perverse. All *P. stuartii* isolates also produced ESBL which does not agree with the findings of Tumbarello and colleagues, (2004) where 52% of *P. stuartii* isolates produced ESBL. This difference may be due to the fact that this study analysed only one *P. stuartii* which may tend to conflate the result in favour of the properties of the only bacterium analysed. In either case, both scenarios confirmed that ESBL production is not uncommon in *P. stuartii.* Making it important in the discourse about antibiotic resistance control. Thus, generally, both bacteria are significant ESBL producers and was confirmed by their combined ESBL prevalence of 58.8%. this combined prevalence is slightly similar to Nanon-Veneza *et al.* (2003) report of 42.5% ESBL prevalence. A higher prevalence value in this study may be due to the specificity of this study on only two species of bacteria as opposed to the several species analysed by Nanon-Veneza *et al.* (2003).

SHV and CTX-M mediated resistance was found to be the principal cause of ESBL production in this study. This was not in tandem with (Liu *et al.,*2017) which found ESBL production resistance to be mediated by SHV, CTX-M and TEM genes with TEM-1 responsible for 16.7% of all cases, while SHV and CTX-M accounted only for 5.6% of total ESBL production. In another study, TEM-52 was responsible for 87% of ESBL producing isolates. Such disagreement may be because other studies cited, were conducted on isolates obtained from Intensive Care or patients using catheters. This as a result, would promote a wider diversity for ESBL mediated genes due to the heavy use of antibiotics and the ease of horizontal gene transfer in such settings. Also 65% of ESBL producing *P. stuartii* was mediated by *bla*CTX-M gene according to (Sabri *et al.,* 2012) This is lower than the total mediation by CTX-M in this study, probably due to the small size of test isolates analyzed in this study. This study agrees with that conducted by Metri and co-workers (2013) in that all *C. freundii* isolates were obtained from urine sources. This was however different from 3.2% of *C. freundii* obtained from urine samples according to Ashish and colleagues (2012). The former results confirm that the majority of these *C. freundii* are implicated in urinary tract infections (UTIs). The discrepancy in the latter result may be due to genetic factors which conferred innate immunity or diets which help to reduce the occurrence of UTIs in Taiwan, where this study was conducted. A total of 71% of bacteria analyzed had high MAR (Multiple Antibiotic Resistance) index values which signifies a high level of MDR (Multi-drug resistance). For instance, the *P. stuartii* isolate analyzed was multi-drug resistant with its resistance to four antibiotics. This agrees with (Tumbarello *et al.,* 2004) which found all *P. stuartii* analysed to be multi-drug resistant. This MDR as displayed by *C. freundii* also agree with the worrisome results of (Liu *et al.,* 2017) and the conclusions of Ashish and co-workers (2012), that multi-drug resistance is high amongst emerging *Citrobacter* spp. infections. This may be due to horizontal genetic transfer of resistance genes as well as the continuous use and abuse of antibiotics.

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Consent for publication: All authors participated and agreed to the publication of this work.

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Authors' contributions

Egwuatu, T.O.G. designed the study, supervised the experimental work, participated in data analysis, and wrote the manuscript. Agbaje, A.O. performed the experimental work, Osuagwu, C.S provided laboratory support and edited the manuscript for content. All authors read and approved the final manuscript

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