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Prevalence and molecular detection of extended spectrum β-lactamase producing *Burkholderia species* from clinical and environmental sources in Abeokuta, Ogun State, Nigeria.

[*1]Ifeoma C. Umeokoli, [1]Balogun A. Saka, [1]Ike E. Williams and [2]Ojo E. Olufemi.

[1]Department of Microbiology, College of Biological Sciences, Federal University of Agriculture Abeokuta, Ogun State Nigeria.

[2]Department of Veterinary Microbiology, College of Veterinary Medicine, Federal University of Agriculture Abeokuta, Ogun State Nigeria.

ABSTRACT

Background: Extended Spectrum Beta-Lactamase producing bacteria is on the increase across the globe. This study investigated the prevalence and molecular detection of ESBL producing *Burkholderia* sp from clinical and environmental sources in Abeokuta.

Methods: The clinical (135) and environmental (53) samples were examined. Clinical samples were obtained from diabetic (71) and HIV (64) patients, while effluent sources from various units of the hospital. Samples were cultured on *Burkholderia cepacia* selective agar. Isolates were identified using biochemical procedures and confirmed with 16SrRNA pair of primer set (1500 bp). Antimicrobial susceptility test was performed by disc diffusion method. Production of ESBL were determined using double disc synergy method. Gene encoding CTX-M was probed by PCR and gel electrophoresis using bla_{CTX-M} pair of primer set (650 bp). Fifteen isolates (8%) were identified as *Burkholderia* sp, using biochemical procedure and confirmed by 16SrRNA gene sequencing.

Results: All the 15 (8%) isolates were resistant to cefuroxime, augumentin (100), ceftazidime (93.3 %), gentamycin (60 %), ofloxacin, cefixime and ciprofloxacin (53.3 %). However, least resistance was recorded against nitrofurantoin (26.7 %). Ten (66.7 %) isolates were multidrug resistant. Five (33.3 %) of the isolates exhibited ESBL production. Three (20 %) of the isolates harbor CTX-M gene. Highest prevalence of *Burkholderia* sp was recorded against age group 46-55 (13.3 %). Similarly, higher prevalence of *Burkholderia* sp was recorded in females 9(6.7 %) than in males 4(3 %). No prevalence was recorded for age group 6-15 and 86-95.

Conclusion: This study revealed that *Burkholderia* sp were present, isolates obtained were multidrug resistant and harbor bla_{CTXM} in clinical and environmental sources in Abeokuta.

Key words: *Burkholderia cepacia, CTX-M,* Extended Spectrum β-Lactamase, Multidrug resistance.

1. INTRODUCTION

Report reviewed that the prevalence of ESBLs is on the increase across the globe, varying according to geographic location and has been linked to use and abuse of antibiotic [1]. Extended spectrum β lactamase isolates were first detected in Western Europe in the mid-1980s. Since then, their incidence has been increasing steadily. ESBLs are defined as enzymes produced by certain bacteria that are able to hydrolyze cephalosporin as enzymes but not the cephamycins (cefoxitin) or carbapenems by -lactamase inhibitors, that is, clavulanic acid [2]. Among all the types of ESBLs described in a variety of pathogens, CTXM, TEM, and SHV types proved to

[*] Corresponding author: E-mail: umeokoliifeoma@gmail.com Tel: +2348065463592

be the most efficacious in terms of promiscuity and dissemination across various epidemiological niches [2]. *Burkholderia cenocepacia* isolates produced chromosomally encoded TEM-116 extended-spectrum β-lactamase (ESBL). Analysis of outer membrane proteins revealed that shrink expression of a 36-kDa protein could join a high level of β-lactam resistance in the isolates [3]. Ferreira et al., [4] Reported that the most frequent ESBL producing bacteria was *B. cepacia*. Resistant bacteria are approaching worldwide as a menace to the favorable outcome of common infections in hospital settings. Multidrug resistance in *Burkholderia cepacia complex* can cause a wide range of infections, including pneumoniae, urinary tract infection and bacteremia which can lead to substantial morbidity and mortality. These *Burkholderia* species also show resistance to many common antibiotics; this feature, along with facile aerosol transmission of the pathogenic species and no availability of effective vaccines, forms the basis for their categorization as influential bioterror agents [5]. During the last few decennium, occurrence of microbial resistance has increased which led to generation of multi-drug-resistance (MDR) organisms. Although MDR development is a natural phenomenon, extensive rise in the number of immunocompromised has led to examination and elucidation of the molecular mechanism of organism during infection [6]. In addition, *B. cepacia* is very difficult to treat due to its highly resistant pattern against available antibiotics. Antibiotic-resistant bacteria cause 700,000 deaths worldwide each year, and a UK government surmised that this number could rise to 10 million by 2050 [7]. If superbugs are allowed to disseminate, we may reach a point where it is too perilous to conduct surgeries such as C-sections and transplants because of the danger of superbug infection; which would have colossal implications for the health of people around the world [7]. *Burkholderia cepacia complex* (Bcc) belong to Betaproteobacteria family. It is gram-negative, heterotroph, non-fermenting, motile and aerobic bacillus [8]. This genus now consists of more than 60 Gram-negative bacterial species, which fit into two clades called *Paraburkholderia*: the plant-associated beneficial and environmental one or the pathogenic one [9]. The *Burkholderia* pathogenic clade comprises of plant, animal and human pathogens separated in two well-known groups: the "pseudomallei" group (*B. pseudomallei*, *B. mallei* and the environmental strain and study model *B. thailandensis*) and the opportunist pathogen species forming the *Burkholderia cepacia complex* (Bcc) [10]. There are plethora of information on *B. cepacia* in other part of Africa like Egypt, however, little or none has been reported in Nigeria [11]. The capability of the mobile resistant elements to circulate within the environment, water, wild animals and from food animals to human makes the epidemiology of ESBL quite complicated [2]. Reports on ESBL producing *B. cepacia* from Nigeria are sparse and rudimentary; little is known about the prevalence and mechanism of antimicrobial resistance due to ESBL. This study therefore was conducted to determine the prevalence and detect ESBL production in *B. cepacia* from clinical and Environmental sources in Abeokuta, Ogun State, Nigeria.

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Sample collection

Clinical samples from 135 patients and 53 from environmental sources collected and maintain on ice packs contained in a flask were taken immediately to the research laboratory for microbiological analysis. The clinical samples were obtained from diabetic (71) and HIV (64) patients, while environmental samples were obtained from various units of the hospital at Sacred Heart Hospital Lantoro, State Hospital Ijaiye and Federal Medical Centre Idi-Aba. Clinical samples were sputum, urine, and wound swab. Environmental sample was sewage water.

2.2 Methods

2.2.1 Ethical approval

Clearance for research involving human subjects was granted by the Ethics and Research Committee of the Federal Medical Centre, Sacred Heart Hospital Lantoro and State Hospital Ijaiye Abeokuta Ogun State, Nigeria for this study.

2.2.2 Study Setting and Design

This is a prospective study of patients who were presented with *Burkholderia cepacia* at Sacred Heart Hospital Lantoro, Abeokuta Ogun State Hospital, Ijaiye and Federal Medical Centre Idi-Aba all in Abeokuta. The study was conducted at the inpatient and outpatient departments of all the Hospitals listed above. Abeokuta is the capital and largest city in Ogun State, Southwest Nigeria.

2.2.3 Isolation and identification of Burkholderia sp

Employing aseptic technique, pure cultures were obtained using *B.cepacia* selective agar (OXOID, UK). The plates were incubated at 37 °C aerobically for 72 hours, after which they were checked for bacterial growth. All isolates were identified by their colony morphology, staining characters, pigment production, motility and use of standard biochemical procedures [12].

2.2.4 Antibiotic susceptibility test

The 15 isolates identified by 16SrRNA as *Burkholderia* sp were tested for antimicrobial susceptibility using Kirby Bauer disc diffusion method on Mueller Hinton agar. The isolates were tested against gentamicin, ciprofloxacin, ceftriaxone, ceftazidime, cefuroxime, cefixime, ofloxacin, augumentin and nitrofurantoin. Zone of inhibition was interpreted according to Clinical and Laboratory Standards Institute guidelines [12].

2.2.5 Screening for ESBLs production in Burkholderia sp using Double Disc Synergy Test (DDST)

Isolates resistant to third-generation cephalosporins were tested for ESBLs production by CLSI double disc synergy test (DDST). Further confirmation was done by double disc approximation test in which a disc containing ceftriaxone-clavulanic acid was placed on the center of Muller-Hinton agar medium plate and another disc containing ceftriaxone alone was placed 15 mm from the first disc. Enhancement of the zone of inhibition of ceftriaxone+clavulanic acid 5 mm more than ceftriaxone disc alone was considered a positive result. *E.coli* ATCC 25922 was used as negative quality control.

2.2.6 Burkholderia spp genomic DNA analysis

The molecular identification of *B.cepacia* was confirmed by bacteria genomic DNA using Agarose gel electrophoresis, DNA amplification and 16SrRNA primer set (1500bp) [13]. Tris-EDTA boiling extraction method was used as described by Yang [14]. Briefly, an overnight culture of each bacterial isolate in Mueller Hinton broth was centrifuged at 10000 rpm for 5 min to obtain bacterial cell pellet. The pellet was processed by suspension in 200 µL of TE buffer (10 mM Tris-HCl (pH 8.0), 1mM EDTA). The mixture was briefly mixed on vortex mixer and then placed on heating block at 100oC for 10 min. This was followed by centrifugation at 10,000 rpm for 5 min. A 100 µL of supernatant was transferred to a sterile tube and stored at -20 ºC until PCR testing.

2.2.7 Molecular detection of blaCTX-M genes by PCR

The eleven ESBL producing isolates detected were subjected to molecular detection of blaCTX-M genes which was performed using Polymerase Chain Reaction (PCR) machine Promega Corp (Germany). The primer used for detection of the genes is shown in Table 1

2.2.8 Detection of ESBL genes

This was done by separate polymerase chain reaction (PCR), using primers indicated in Table 1. The primers were synthesized and supplied by Promega Corp (Germany).

Table 1: Primers used for PCR amplification of *bla* genes

Target	Primer name	Primer sequence (5′-3′)	Product size (bp)	Reference(s)
blaCTX-M	*CTX*-F	TCTTCCAGAATAAGGAATCCCG	650	[15]
	CTX-R	CCGTTTCCGCTATTACAAACGG		

Each PCR reaction is a 20-µL volume, comprising 1 x PCR buffer (pH 8.3), 1.5 mM of MgCl2, 200 nM each of the deoxynucleotide triphosphates (dNTPs), 40 picomole each of the forward primer and reverse primers indicated in Table 1. One microlitre (1 µL) each of genomic DNA template (~100 ng) and 1.25U of Taq polymerase (Promega, Germany). The PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 25 cycles of DNA denaturation at 94 °C for 30s primer annealing at 50 ºC for 1 min for blaCTX primer extension at 72 °C for 1 min. After the last cycle, a final extension step at 72 °C for 7 min was added. Five-microliter aliquots of PC PCR product were analyzed by gel electrophoresis with 2 % agarose (Sigmaaldrich, USA). Gels were stained with ethidium bromide at 0.5 µg/ml and visualized by UV transillumination. A 100-bp DNA ladder (Fermentas, Canada) was used as marker to extrapolate the 650 bp PCR product. The PCR reaction was done, using TC-312 thermal cycler (Techne, Netherlands). The eleven ESBL producing isolates detected were taken for molecular detection of blaCTX-M genes which was performed using uniplex Polymerase Chain Reaction (PCR).

2.3 Statistical Analysis

Data were subjected to statistical analysis of variance (ANOVA) using software package for social sciences (SPSS) version 20 (Armonk,New York: IBM Corp).

3. RESULTS

Out of one hundred and eighty, eight (188) samples, the prevalence of *Burkholderia* sp was 15 (8 %). Out of the 15 isolates, 13 (87 %) were from clinical samples while 2 (13 %) were from environmental sources. Table 2 shows prevalence of *Burkholderia* sp isolate from clinics according to age the highest prevalence 4(13.3 %) was recorded among patients of age group 46-55 years while no prevalence was recorded among patients of age group 86-95 years. Table 3 shows the *Burkholderia* sp in relation to sex. It shows that more females 9(6.7 %) were infected than males 4(3 %). In the hospital environment, the ICU had the highest prevalence of 10 % (Table 4). Antibiotic susceptibility pattern of *Burkholderia* sp isolated from clinical samples showed that out of (15) isolates 64.1 % were resistance to ceftazidime (93.3 %), cefuroxime (100 %), gentamycin (60 %), augumentin (100 %), ofloxacin (53.3 %), cefixime (53.3 %) and ciprofloxacin (53.3 %; Table 5). Table 6 shows the ESBL reaction to *Burkholderia* sp isolates that were resistant to ceftriaxone. 15 of the total isolates were subjected to ESBL test. 5(33.3 %) of the ESBL isolate were positive to double disc diffusion synergy.

Table 2: Prevalence of *Burkholderia* sp isolate according to age distribution of patient from clinics

Age group (year)	N	*Burkholderia* sp positive n_1 (%)
6-15	2	0(0)
16-25	10	1(10)
26-35	20	2(10)
36-45	30	2(6.7)
46-55	30	4(13.3)
56-65	30	2(6.7)
66-75	10	1(10)
76-85	2	1(50)
86-95	1	0(0)
Total	135	13(9.6)

KEY: N= Total number of isolate

Table 3: Prevalence of *Burkholderia* sp isolates in relation to sex

Sex	N	*Burkholderia* sp positive n_1 (%)
Female	100	9 (6.7)
Male	35	4(3)
Total	135	13 (9.6)

KEY: N= Total number of isolates in male and female; n_1=number of isolate Positive,

Table 4: Prevalence of *Burkholderia sp* in Environmental samples

Sources	N	*Burkholderia* sp positive n_1 (%)
Female ward	12	1(8.3)
Male ward	9	0(0)
Surgical unit	12	0(0)
ICU	10	1(10)
Outpatient unit	10	0(0)
Total	53	2(3.8)

KEY: N= Total number of isolate; n_1=number of isolate Positive

Table 5: Antimicrobial Susceptibility patterns of *Burkholderia spp.* from Clinical and Environmental sources.

Antibiotics	N	Resistance (%)	Sensitivity (%)
CAZ	15	14(93.3)	1(6.7)
CRX	15	15(100)	0(0)
GEN	15	9 (60)	6(40)
CXM	15	8 (53.3)	7(46.7)
OFL	15	8 (53.3)	7(46.7)
AUG	15	15(100)	0(0)
NIT	15	4(26.7)	11(73.3)
CPR	15	8(53.3)	7(46.7)

KEY: N= Total number of isolate; CAZ= ceftazidime; CRX= cefuroxime; GEN= gentamycin; CXM= cefixime; OFL= ofloxacin; AUG= augumentin; NIT= nitroflaxacin; CPR= ciprofloxacin.

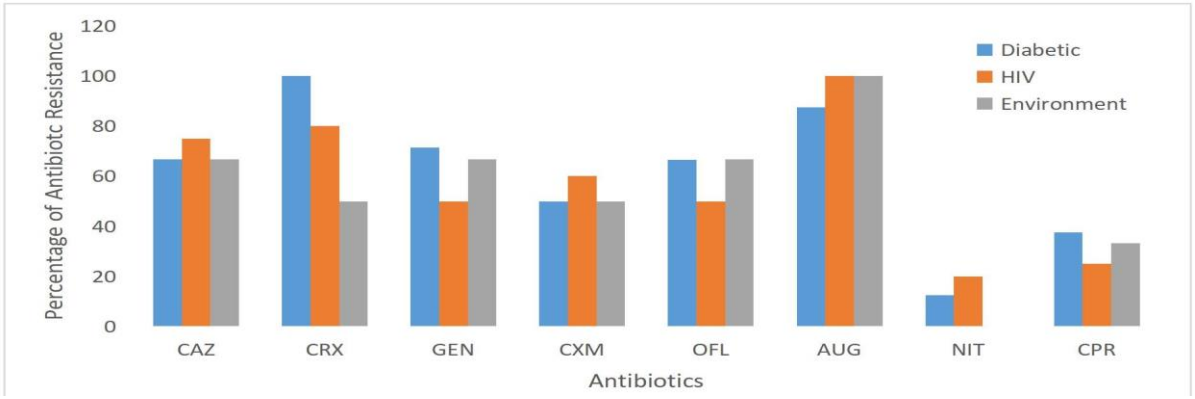

Figure 1: Percentage comparative of Antibiotic resistance among Clinical and Environmental isolates

KEY: CAZ= ceftazidime; CRX= cefuroxime; GEN= gentamycin; CXM= cefixime; OFL= ofloxacin; AUG= augumentin; NIT= nitroflaxacin; CPR= ciprofloxacin

Table 6: Isolates, Sources, ESBL production, Identification and bla$_{CTX-M}$Gene

S/N	Isolates		Sources	ESBL Production	Isolates 16SrRNA Identification	Presence of CTX-M Gene
1	*B. specie*	*SA1*	Sputum	Positive	*B.cepacia*	Present
2	*B. specie*	*SA3*	Sputum	Negative	*B. cepacia*	Nil
3	*B. specie*	*SB2*	Sputum	Negative	*B. cepacia*	Nil
4	*B. specie*	*SF4*	Sputum	Positive	*B.cenocepacia*	Present
5	*B. specie*	*SF1*	Sputum	Positive	*B.cepacia*	Present
6	*B. specie*	*SF6*	Sputum	Negative	*B. cepacia*	Nil
7	*B. specie*	*UF10*	Urine	Negative	*B.cepacia*	Nil
8	*B. specie*	*UF11*	Urine	Negative	*B.cepacia*	Nil
9	*B. specie*	*UF12*	Urine	Negative	*B. cenocepacia*	Nil
10	*B. specie*	*UF13*	Urine	Positive	*B. pseudomallei*	Present
11	*B. specie*	*SF2*	Sputum	Negative	*B.cepacia*	Nil
12	*B. specie*	*SF5*	Sputum	Negative	*B.cepacia*	Nil
13	*B. specie*	*SB18*	Sputum	Negative	*B.cenocepacia*	Nil
14	*B. specie*	*WSW1*	Wastewater	Positive	*B.mallei*	Present
15	*B. specie*	*WSW 2*	Wastewater	Negative	*B.pseudomallei*	Nil

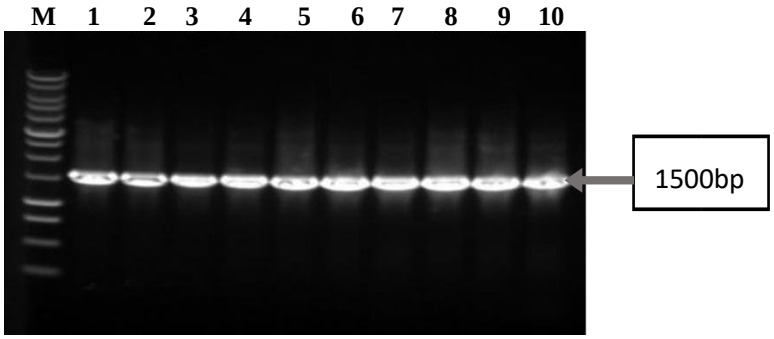

Figure 2: Gel Electrophoresis of selected *Burkholderia cepacia isolates*
KEY: Lane M= Molecular Ladder, *1= B. cepacia ATCC® 17759*, 2 to 10=*Burkholderia species.*

I. Extended-spectrum β-lactamases

The production of extended-spectrum β-lactamases (ESBLs) was phenotypically tested, and the presence of the ESBL encoding genes was confirmed by polymerase chain reaction (PCR) amplification and DNA sequencing.

The plate for the agarose gel for the molecular detection of *blaCTX- M* gene is shown in Figure 2. The result showed that 3(20%) of *Burkholderia cepacia* were detected to be *bla CTX- M* gene carrier. This study also revealed that 3(20%) of the *Burkholderia cepacia* isolates habour *blaCTX-M* genes.

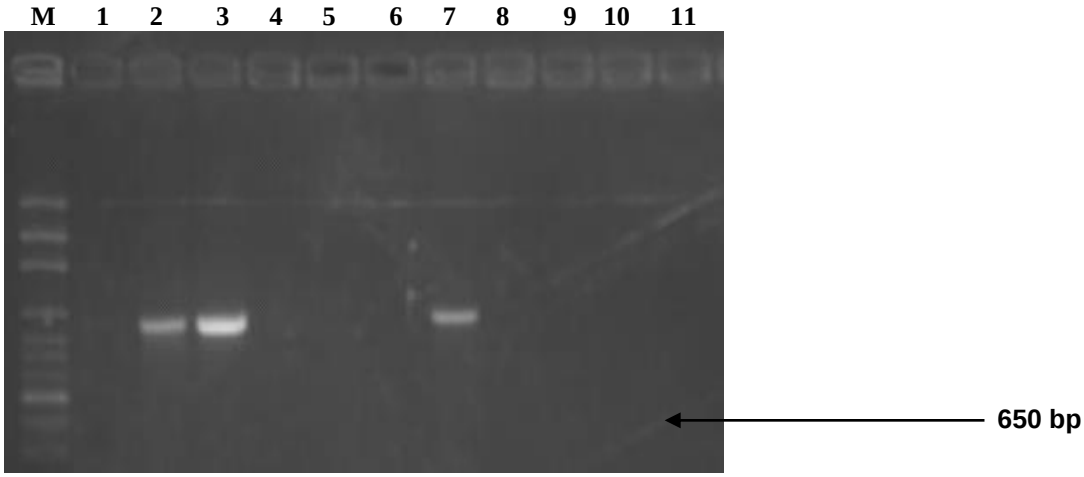

Figure 3: Agarose gel showing bla$_{CTX-M}$ gene amplified from the *B.cepacia* isolates Lane M = 100 bp DNA ladder markers; Lanes 1 – 11 = bacterial isolates

4. DISCUSSION

The isolation and characterization of ESBL-producing *Burkholderia* sp in Abeokuta Ogun State was undertaken to highlight its public health importance in the clinic and environment. Out of one hundred and eighty eight (188) samples, 15(8 %) isolates were identified as *B. cepacia.* This is in agreement with the reports of Mohammed *et al.,* [16] and El Chakhtoura *et al*., [17] but not in alignment with Rastogi *et al.,* [18] who reported that the incidence of nosocomial *B. cepacia* was low in hospital. This was attributed to low socio-economic status, sexual intercourse, poor environmental conditions, where the subjects resides in terms of lack of personal and environmental hygiene among Nigerian men and women in Abeokuta. In these findings, the *B.cepacia* were more abundant in the sputum and urine of diabetic patients who were hospitalized. These results are in agreement with Sarah *et al.,* [19] who reported that *B.cepacia* were also detected in the sputum of diabetic patients Matthaiou *et al.,* [20] reported that patients with *B. cepacia* bacteremia, the majority had serious comorbidities including diabetes mellitus and chronic obstructive pulmonary disease (COPD) but contradicts the reports of Nancy *et al.,* [11], that most of *B. cepacia* isolates were identified from the surgical wards at Burn unit. This study showed that the highest prevalence 4(13.3 %) was recorded among patients of age group 46-55 years while the least prevalence of (0 %)was among patients of age group 6-15 and 86-95 years. Similar result have been documented by Rastogi *et al.,* [18] who reported that the median age of the infected patients was ranged 16–71 years Matthaiou *et al.,* [21] also reported that Chronic *B. cepacia* infection was more common in adults than in children but contradicts Ramsay *et al.,* [22] who reported, that older age group are associated with a higher incidence of infection. In relation to sex, this study shows that more females 9(6.7 %) were infected than males 4(3 %) However, this result is inconsistent with a greater proportion of male patients reported by [Rastogi *et al.,* [18] and Ramsay *et al.,* [22] male sex are associated with a higher incidence of infection Ramsay *et al.,* [22], although the reason for the male predominance is not clear. In this study, all the 15 *B.cepacia* were resistance to Cefuroxime and Augmentin (100 %). Similarly, significantly high rate were recorded against Ceftazidime (93.3 %), Gentamycin (60 %). About 53.3 % were recorded against Ofloxacin, cefixime and ciprofloxacin. Least resistance was recorded against Nitrofurantoin (26.7 %) respectively. Eighty one (67.5 %) of the isolates were multidrug resistance (MDR). Being resistance to 4 classes of Antibiotic. This is similar to other reports from Dervla et al., [23] and Mohammed *et al.,* [16] in which resistance to ciprofloxacin and ceftazidime were variable across the species. This study did not agree with Nancy *et al.,* [11] according to the results reported, isolates of *B cepacia* were most susceptible to meropenem followed by ceftazidime, tobramycin, chloramphenicol, piperacillin/ tazobactam and tetracycline. All strains were resistant to both co-trimoxazole and ciprofloxacin. . They all agreed that this organism was highly resistant to multiple antibiotics. *Gautam et al.,* [24] tested 30 isolated strains of *B cepacia* and they found that the isolates were susceptible to piperacillin– tazobactam, levofloxacin, ceftazidime and tetracycline. Amongst these isolates; maximum resistance was against meropenem and co-trimoxazole.This study has shown that *B. cepacia* isolates were resistant to 4 classes of antibiotcs which implied multidrug resistant. This makes the treatment of *B.cepacia* difficult and it may pose a serious danger across the globe. ESBLs are enzymes that act by inhibiting the action of beta-lactam antibiotics Gautam *et al.,* [25]. The existence of ESBL-producing *B cepacia* is of

serious concern as it stands for a pathogen with the capacity to acquire resistance to a wide range of clinically important antimicrobials and it can lead to numerous infections. This study revealed that 3(20 %) of the *Burkholderia cepacia* isolates habour *blaCTX-M* genes. This is in agreement with the findings of Ferreira *et al.,* [4] who reported that *CTX-M* gene were detected in clinical isolate but contradicts the reports of Nonhlanhla *et al.,* [26] who reported detection of NDM-1 (New Delhi metallo-β- lactamase-1), This may be due to its presence on highly mobile genetic elements that facilitates its spread among bacteria and that it is one of first genes to have developed to mediate resistance to ESBL drugs.

5. CONCLUSION

The results of this study showed that *Burkholderia sp* are present in clinical and environmental sources in Abeokuta Ogun state which could therefore represent an environmental reservoirs and potential sources of *Burkholderia* infection. Isolates obtained were multidrug resistant and harbor bla_{CTXM} gene. This calls for further surveillance *Burkholderia* sp in Abeokuta

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Conflict of Interest

The Authors report no conflict of interest

Contribution of the Authors

ICU carried out the experimental work and wrote the manuscript, BAS, IEW and OEO supervised the research work and proofread the manuscript.

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