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DETECTION OF METALLO-BETA-LACTAMASE AMONG IMIPENEM RESISTANT BACTERIAL ISOLATES FROM GREAT KWA RIVER, NIGERIA

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Abstract: **The carbapenems are used as drugs of last resort for treatment of severe infections associated with the Extended spectrum beta lactamases (ESBL). The emergence of Metallo-beta lactamase (MBL) producing bacteria is a threat to the efficacy of these drugs due to the broad spectrum of activity of the MBLs on carbapenems and other beta lactams. This study was undertaken to detect MBL among imipenem resistant isolates from the Great Kwa River and the antibiogram. A total of 30 surface water samples were randomly and aseptically collected from three sites of Great Kwa River and transported to the laboratory within 18h for analysis. The surface plating technique was used to isolate bacteria from these samples after a 5-step ten fold serial dilution. Following standard guidelines for antibiotics susceptibility testing, the imipenem resistant isolates were subjected to double disk synergy test (DDST) and combined disk test (CDT) for detection of MBL. From a total of 42 imipenem resistant bacterial isolates, 18(42%) showed MBL production using the DDST and CDT phenotypic methods. With the DDST, 51% of the isolates were positive for MBL production while 71% showed MBL production with the CDT. There was no significant difference between these phenotypic tests (p>0.05). The species frequency of MBL production among the isolates were *E.coli* (5%), *Citrobacter sp* (5%), *Aeromonas sp* (11%), *Salmonella sp* (5%), *Plesiomonas sp* (16%) and *Proteus sp* (16%). All the MBL producing isolates were resistant to at least three antibiotics; the number of antibiotics all the isolates showed resistance include: *Salmonella sp* (7), *Aeromonas sp* (0), *E.coli* (8), *Serratia sp* (0), *Plesiomonas sp* (2), *Proteus sp* (0). The widespread emergence and spread of MBL producing bacteria is a threat to human health because of the multiple antibiotics resistance nature. Early detection of these isolates and regulation of carbapenem usage should be implemented.**

Keywords: **imipenem resistant isolates, Extended spectrum beta lactamases (ESBL), drugs, treatment.**

1. INTRODUCTION

The Great Kwa River is a reservoir to antibiotics resistant bacteria and genes coding for resistance to several antibiotics (Abu and Egenonu, 2008 Edet *et al*., 2017). This can be attributed to the anthropogenic activities and discharge of untreated waste and antibiotics from the hospital settings and other industries into the water bodies (Deng *et al*., 2016). The presence of different classes of antibiotics in the aquatic ecosystem has resulted in the emergence of bacteria which show resistance to various classes of antibiotics through natural selection process (Xi *et al*.,2009; Bergeron *et al*., 2015 Khan *et al*., 2016). This natural water body is a source of drinking water for many who cannot afford a potable or treated water source and also serves as a raw water source for various water treatment facilities (Eni *et al*., 2014). This can contribute to the transmission of water borne diseases and pathogenic bacteria which can be resistant to different classes of antibiotics via mechanisms which include alteration of antibiotics target sites, efflux pump mechanism, membrane impermeability and production of antibiotics deactivating enymes such as aminoglycosidases and beta lactamases (Wilke et al., 2005; Lin *et al*., 2015).

The metallo-beta-lactamases belong to the group B class of beta lactamases based on Ambler beta lactamase classification scheme (Ambler *et al*., 1991) and degrades the beta lactam antibiotics by destroying the beta lactam ring structure (Walsh *et al*., 2005). Unlike other beta lactamases, the metallo-beta-lactamases degrade carbapenems (which are used as antibiotics of last resort for treatment of severe bacterial infections) and all classes of beta lactams except the monobactams (Zhanel, *et al.*, 2007; Papp-Wallace *et al*., 2011). Due to the broad hydrolytic spectrum of this enzyme, limited antibiotics (toxic polymyxins B and colistin) are available for treatment and management of infections caused by the MBL producing bacteria (Mwinga *et al*., 2018). The widespread prevalence of the MBL producing bacteria is alarming and constitute a huge threat to public health and therapy.

Early detection of the enzyme is vital for effective treatment of infections associated with MBL producing bacteria. Phenotypic methods for detection of metallo-beta-lactamases include double disk synergy test (DDST) using imipenem (IPM) and EDTA discs or 2-mercaptopropionic acid (2-MPA), double disc synergy test (DDST) using ceftazidime (CAZ) and EDTA discs or 2-mercaptopropionic acid (2-MPA) (Lee *et al*., 2003), the combined IPM-EDTA disk test (CDT) (Yong *et al*., 2002), the MIC (Minimum Inhibitory Concentration) reduction of four-fold with imipenem EDTA combination (Migliavacca *et al*., 2002) and the E-test. These methods are all based on the use of chelating agents which deactivate the enzyme by binding to divalent cations. This study was carried out to assess the antibiotics resistance profile and detection of MBL producing bacteria from Great Kwa River.

2. MATERIALS AND METHODS

Study area and Sample collection:

The samples were collected from three different sites (Esuk Atu, Anantigha beach and Esuk Nsidung) of the Great Kwa River which drains the east side of the city of Calabar. (Figure 1). Fifteen (15) surface water samples were aseptically and randomly collected in duplicate from three sites of Great Kwa River (Obufa Esuk Atu beach, Nsidun beach and Anantigha beach) into sterile bottles and transported to laboratory within 18 h for analysis. From each of the location, 5 surface water samples were randomly collected from different points (approximately 50m apart) and analysed within 18 h of collection.

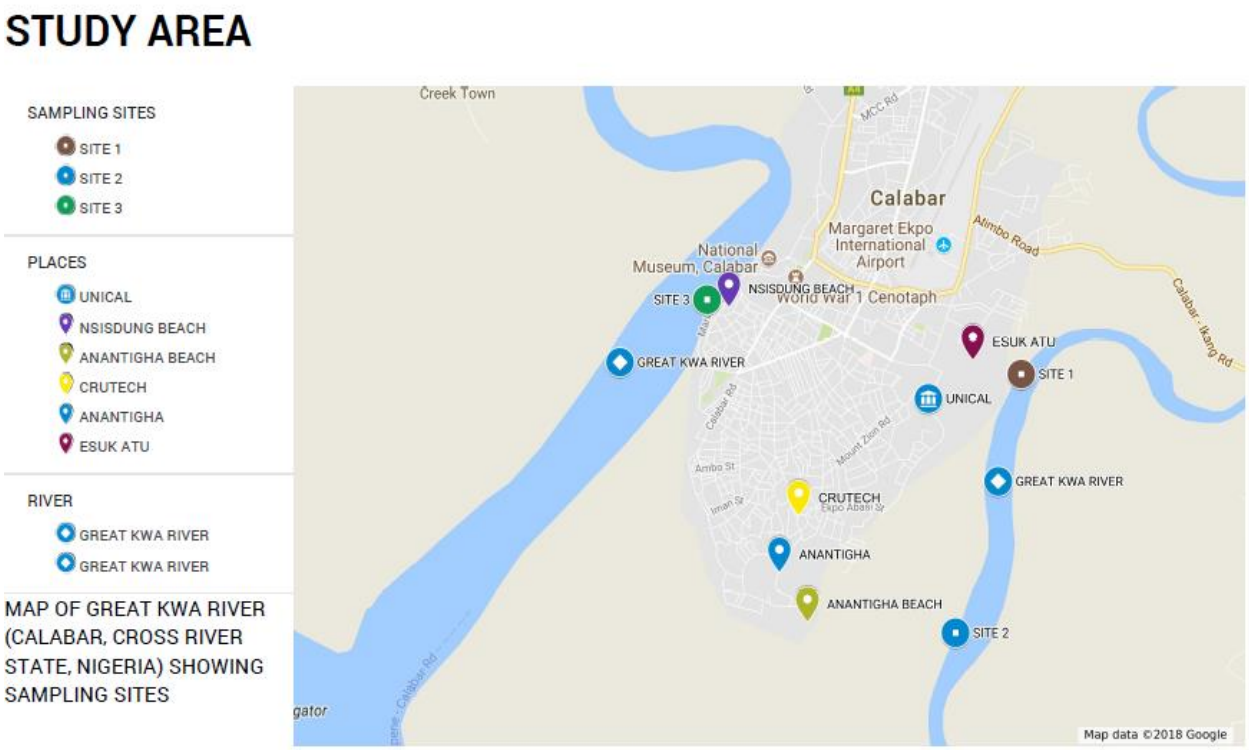

Figure 1: Map of Great Kwa River (Calabar, Cross-River State) showing sampling sites

Isolation and identification of isolates:

As described by Oladipo *et al.* (2009), a 5-step ten fold serial dilution was performed on the Great Kwa River samples and 1ml of the last two dilutions were plated using surface plating technique on the following media: sterile MacConkey and Nutrient agar. Following the procedures for bacterial identification in Bergey's manual of determinative bacteriology, the following biochemical tests were performed to identify the isolates: Grams staining, oxidase, sugar fermentation, citrate, urease, catalase, Motility Indole Ornithine (MIO), and Methyl Red (MR) tests. The macroscopic, cultural, physiological and biochemical characteristics results were compared with the Bergey's manual in order to identify the isolates.

Screening for imipenem resistant isolates:

According to Clinical laboratory standard guidelines (CLSI) guidelines for antibiotics susceptibility testing, a freshly prepared pure broth culture of the isolates which corresponds with 0.5 Macfarland was swapped on a Mueller Hinton agar plate. A 10 µg imipenem disk was placed on the agar surface and incubated for 24h at 37°C. Imipenem resistance was determined by measuring the diameter of the zones of inhibition and comparing with the standard ranges in CLSI manual (2006).

Phenotypic detection of Metallo-beta-lactamase (MBL) producing bacteria:

Two phenotypic methods (imipenem double disk synergy test (DDST) and combined disk tests CDT) were used to detect metallo-beta-lactamase (MBL). The DDST result was interpreted based on a synergy between the blank disk (incorporated with EDTA) and the imipenem disk which were placed 20mm apart. A synergy between the two disks showed MBL production and vice versa. For the CDT, imipenem disk, incorporated with 10µL of EDTA solution and imipenem disk without EDTA were used; a 7mm difference in the two zones of inhibition was interpreted as MBL positive result.

3. RESULTS

A total of 42 imipenem resistant bacterial isolates were screened for metallo-beta-lactamase production using two phenotypic tests (Table 1). With the Imipenem double disk synergy test (DDST) and combined disk test (CDT), 57% and 71% of the isolates showed MBL production respectively while 42% were MBL positive using both phenotypic methods. Among these MBL producing bacteria, the *Serratia sp* had the highest frequency of MBL production of 42% followed by *Plesiomonas sp* (16%) and *Proteus spp* (16%); for other isolates, the percentages of MBL production were: *E.coli* (5%), *Citrobacter sp* (5%), *Aeromonas sp* (11%), *Salmonella sp* (5%) (Figure 2). These isolates showed resistance to at least two antibiotics (Table 3); the highest resistance was observed among the *E. coli* isolates followed by the *Salmonella sp* which showed resistance to at least 8 out of 13 antibiotics used in the study.

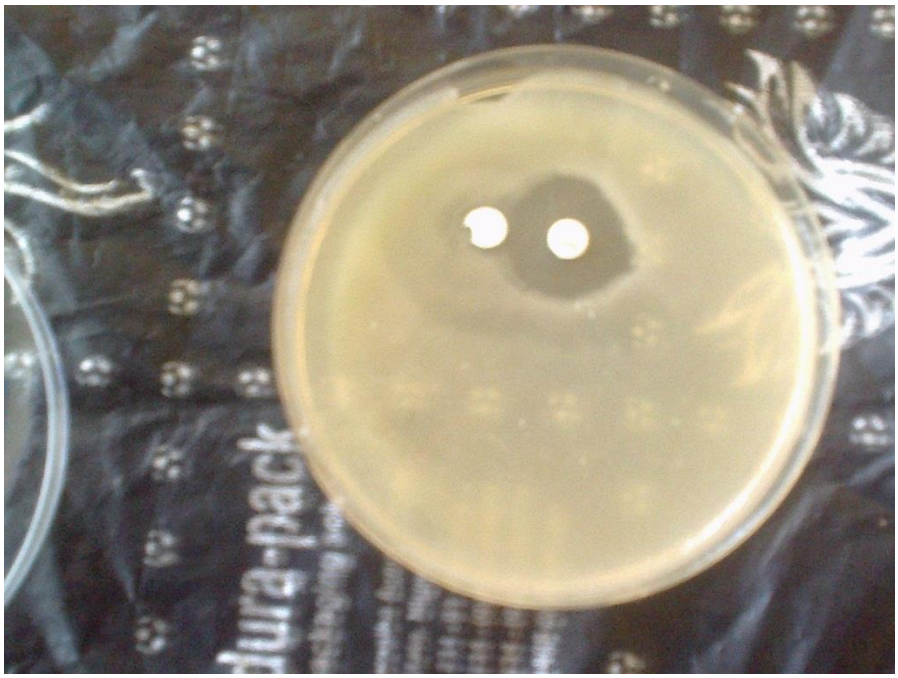

Plate 1: Metallo-beta lactamase Detection using Imipenem double disk synergy test

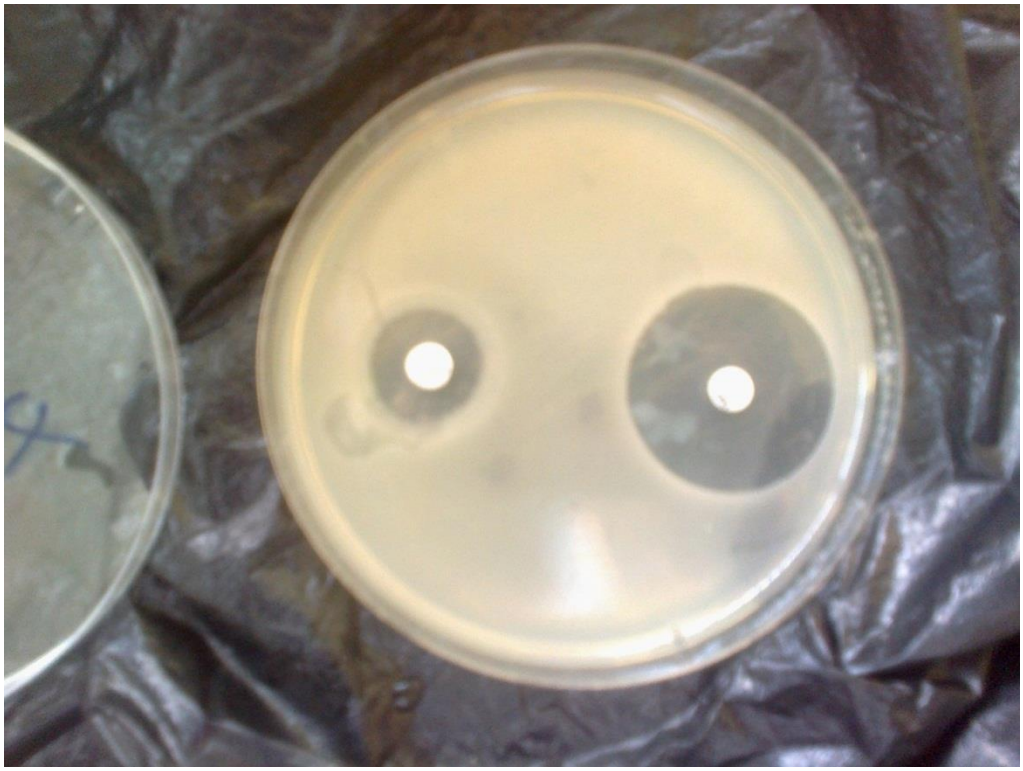

Plate 2: Metallo-beta lactamase Detection using imipenem combined disk test (CDT)

Table 1: Metallo-beta-lactamase (MBL) phenotypic screening

Samples	Total number of imipenem resistant isolates	DDST POSITIVE n(%)	CDT POSITIVE n(%)	DDST and CDT Positive isolates(%)
Great kwa River	42	24(57)	30(71)	18(42)

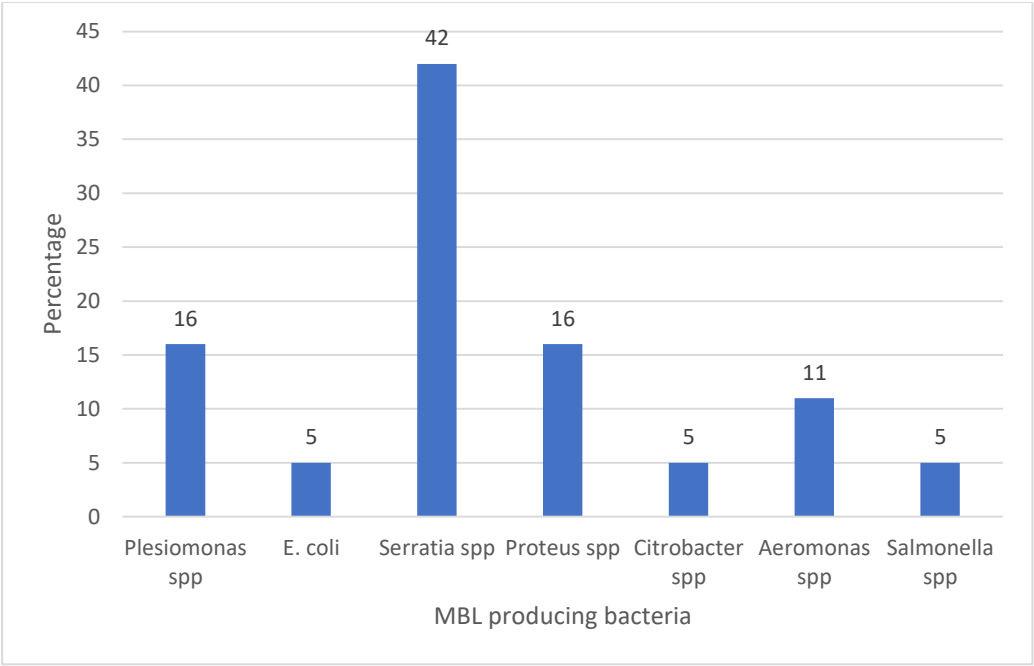

Figure 2: Frequency of MBL production among the different species of bacteria

Table 2: Antibiotics resistance profile of Metallo-beta-lactamase (MBL) producing bacteria from Great Kwa River

Antibiotics	*Salmonella spp* (n=1)	*Aeromonas spp* (n=2)	*E.coli* (n=1)	*Serratia spp* (n=8)	*Plesiomonas spp* (n=3)	*Proteus spp* (n=3)	*Citrobacter spp* (n=1)
OFX	1(100)	0(0)	1(100)	4(50)	3(100)	2(67)	0(0)
PEF	0(0)	0(0)	1(100)	3(38)	0(0)	0(0)	0(0)
CN	1(100)	0(0)	1(100)	0(0)	1(33)	2(67)	0(0)
AU	1(100)	0(0)	1(100)	4(50)	2(67)	2(67)	0(0)
AM	0(0)	0(0)	0(0)	0(0)	1(33)	0(0)	0(0)
CPX	0(0)	0(0)	0(0)	1(13)	0(0)	0(0)	0(0)
SP	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
CH	1(100)	0(0)	0(0)	1(13)	2(67)	2(67)	0(0)
SXT	0(0)	0(0)	1(100)	1(13)	3(100)	1(33)	0(0)
S	0(0)	0(0)	1(100)	0(0)	2(67)	2(67)	0(0)
CXM	1(100)	0(0)	0(0)	3(38)	1(33)	2(67)	0(0)
AZT	1(100)	1(50)	1(100)	2(25)	2(67)	1(33)	1(100)
CAZ	1(100)	1(50)	1(100)	6(75)	2(67)	2(67)	1(100)

4. DISCUSSION

Metallo-beta-lactamase (MBL) producing bacteria are a major threat to an effective antibacterial chemotherapy. Unlike other types of beta lactamases which have known inhibitors, the MBLs have no clinical inhibitors and they hydrolyse many beta lactam antibiotics. MBLs are known to mediate resistance to carbapenem antibiotics such as imipenem, meropenem ertapenem etc. MBL production among the gram-negative bacteria have been observed worldwide; the rapid genetic transfer and the poor therapeutic outcomes associated with infections caused by MBL producing pathogens are major concerns (Walsh et al., 2005). MBLs have been detected in different bacterial species, especially among *Enterobacteriaceae* which are responsible for various infections in man and animals. Due to the broad spectrum of MBLs, the MBL producing bacteria are often resistant to several antibiotics.

In this study, *Serratia sp* had the highest MBL production rate compared to other isolates. Isolates such as *Plesiomonas spp, Escherichia coli, Serratia sp, Proteus sp, Citrobacter sp* and *Aeromonas sp* are all opportunistic pathogens except for *Salmonella sp* which are potential pathogens. Production of MBL among these isolates is a clear indication of the spread and uptake of MBL genes among diverse bacterial species. These isolates have been linked to some nosocomial infections within the hospital settings (Jarvis and Martone, 1992). Some of these isolates have been noted for their multiple antibiotics resistance nature and this can be linked to production of MBL which degrades several beta lactam antibiotics. In other studies, the percentages of *Enterobacteriaceae* isolates producing MBL were 8.5%, 5.2%, 85.5% and 100% respectively as observed by Yusf *et al* (2014), Oduyebo *et al* (2015) and Chika *et al* (2016). In other countries like Morocco, 2.8% (EL Wartiti *et al.*, 2012), 35.24% in Tanzania (Mushi *et al.*, 2014) and 8.6% in Taiwan (Lai *et al.*, 2013) were recorded.

Results from phenotypic detection of MBL showed that with the Imipenem combined disk test, a higher number of MBL producing bacteria was detected compared to the imipenem double disk synergy test. The difference in the results from both tests, though not statistically significant ($p>0.05$), shows that the Imipenem CDT is more sensitive for detection of MBL (Yan *et al*., 2004). These phenotypic tests are based on inactivation of metallo-beta-lactamases in the presence of chelating agents such as Ethylene Diamine Tetra acetic acid (EDTA) or other chelators which can bind zinc, the cation that enhances activity of MBLs (Walsh *et al*., 2005). These phenotypic tests have been used globally to effectively detect MBLs and the results confirmed with molecular methods aimed at detecting the presence of genes encoding MBL production (Franklin *et al.*, 2006; Owlia *et al.*, 2008; Behera *et al.*, 2008; Picao *et al*., 2008 Qu *et al*., 2009). Consequently, these phenotypic tests can be adopted in Clinical laboratories for screening of MBL producing bacteria as opposed to molecular methods which is expensive. Early detection of MBLs phenotypically can help curb the spread of MBL producing bacteria as well as improve outcome of antibiotics chemotherapy (Franklin *et al*., 2006).

MBL producing isolates in this study all showed resistance to at least three antibiotics of different classes. Similar findings have been reported in other studies among different bacterial species showing varying resistance to more than one class of antibiotics such as gentamycin, ciprofloxacin, nalidixic acid, ofloxacin, ceftazidime and cefoxitin (Nishio *et*

al., 2004 ; Mishra *et al.*, 2012; Miradar and Roopa, 2015; Chika *et al.*, 2016; Shirani and Roshandel, 2016; Chika *et al.*, 2017). The production of MBLs can contribute to multiple antibiotics resistance which can undermine the efficacy of antibiotics. As a result, limited options (such as polmyxin B and colistin) are available for treatment of infections associated with MBL producing pathogens (Behera *et al*., 2008). This is due to the broad hydrolytic spectrum of MBLs, which degrade various beta lactams. Apart from intrinsic mechanisms such as up regulation of efflux pumps and outer membrane impermeability, the presence of other genes which encode resistance to other classes of antibiotics can also contribute to the multiple antibiotics resistance nature of these isolates.

5. CONCLUSION

The isolates in this study have been shown to be MBL producing bacteria using phenotypic detection methods. Antibiogram of these isolates showed resistance to different antibiotics of various classes. This shows that MBL producing bacteria are a major threat to health and chemotherapy and as such, adoption of cheap detection methods for detection of MBLs in clinical laboratories is vital for effective treatment of infections associated with MBL producing pathogens.

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