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Detection of Metallo-Beta-Lactamase Genes (Bla_{imp}, Bla_{ndm} and Bla_{vim}) in Imipenem Resistant Bacterial Isolates from Great Kwa River, Nigeria

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ABSTRACT

Bacterial resistance to various antibiotics is mediated by different mechanisms which range from innate factors to genetically acquired traits. Among other factors underlying antibiotics resistance, secretion of Metallo-beta-lactamases (MBL) has a huge impact on effective chemotherapy. *Metallo-beta-lactamase* (MBL) enzyme mediates resistance to carbapenem antibiotics, which are suitable for management of serious infections especially those associated with extended spectrum *beta-lactamases.* The study was aimed at detecting three MBL genes ($bla_{IMP,}$ bla_{VIM} and bla_{NDM}) among imipenem resistant bacterial isolates from Great Kwa River water samples. Ten (10) imipenem resistant bacteria were isolated in this study. The bacteria isolates were subjected to standard antibiotics susceptibility testing procedures and were further screened for MBL genes using polymerase chain reaction and MBL typing. Sequencing of the 16S rRNA genes and construction of a phylogenetic tree for the isolates, the MBL producing bacteria were identified as: *Plesiomonas shigelloides* strain 187, *Enterobacter cloacae* strain S20504, *Photobacterium ganghwense* strain ZR07, *Bacillus licheniformis* strain 60, *Klebsiella pneumoniae* strain DSM 30104, *Plesiomonas sp* strain TIL_TAL_1, *Comamonas testosterone* strain 1, *Enterobacter sacchari* SP1, *Acinetobacter soli* strain MBR7. The bla_{IMP} gene was detected in 7(70%) of the isolates, bla_{NDM} was detected in only one of the isolate while none of the isolates harbored bla_{VIM}. These findings show that genes encoding resistance to imipenem are transferred at an alarming rate among different bacterial species leading to rapid spread of MBL producing bacteria globally.

INTRODUCTION

The *metallo-beta-lactamase* (MBL) confer resistance to all beta-lactam antibiotics except the monobactam and are not deactivated by any known therapeutic agents like clavulanate, sulbactam, and tazobactam (Chika *et al.,* 2017). Several investigations in different countries have shown a high prevalence of MBL producing bacteria isolated from the hospital and environment (Carvalho *et al*., 2005). These MBL producers are associated with the increasing rate of multi-drug resistant infections worldwide and they belong to the 'ESCAPE' group of bacteria and include; *Enterococcus faecium, Staphylococcus aureus, Clostridium difficille, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacteriaceae* species, which show high resistance rate to antibiotics commonly used for treatment (Garbati and Al Godhair, 2013). The discharge of untreated hospital waste into the water bodies and the environment has been linked to observation of antibiotics resistance among bacteria in the environment (Guardabassi *et al.,* 1998).

Due to the widespread occurrence of the MBL producers globally, different methods have been developed for the detection of these bacteria. These detection methods are broadly classified into the phenotypic and genotypic methods (Aghamiri *et al.,* 2014). Phenotypic screening is on the basis of binding of zinc ions (required for MBL activity) by chelating agents (EDTA, thiol groups and dipicolinate) (Souli *et al*., 2008). Though different phenotypic methods for MBL detection exist, no standard phenotypic method has been recommended. Molecular methods, involving use of primers to amplify MBL genes and viewing on agar gel electrophoresis, remain the universal standard.

Meta-beta-lactamases are of different phenotypes and are encoded by several genes .The clinically relevant MBLs include: IMP (Imipenemase), VIM (Verona Integron encoded Metallo-beta-lactamase) and NDM (New Delhi *Metallo-beta-lactamase*) (Meini *et al,* 2014). These enzymes are encoded on extrachromosomal mobile genetic elements and are believed to have evolved from Bc11, a Metallo-beta-lactamase from *Bacillus cereus* and other bacteria harbouring the MBLs on the chromosome (Lim *et al.,* 1988). Ever since the discovery of these enzymes, different variants which require divalent cations for carbapenem hydrolysis have been reported (Lauretti *et al.,* 2009). Considering the broad hydrolytic spectrum of MBLs and the ease of spread and transfer of MBL genes, which are commonly on mobile genetic elements, this investigation was carried out to assess the predominant MBL genes among bacterial isolates from the Great Kwa River.

MATERIALS AND METHODS

Genotypic detection of MBL genes

Based on the method described by Aghamiri *et al* (2014) and Anoar and Omer (2014), Imipenem-resistant isolates were screened for the presence of the following MBL genes: *bla-NDM* *bla-VIM* and *bla-IMP*. The deoxyribonucleic acid (DNA) of the bacterial isolates were extracted by boiling method and assayed for the MBL genes (*bla-NDM* *bla-VIM* and *bla-IMP*) using Polymerase chain reaction (PCR). The isolates were inoculated in Luria Bertani (LB) broth and incubated for 24h. From the LB broth, 5ml was centrifuged at 14000 rpm for 3 min. A suspension of the cells in 500ul of normal saline was boiled at 95^0C for 20 min and subsequently centrifuged at 14000rpm for 3 min after cooling on ice. The clear liquid was decanted to a vial for storage at -20^oC and other analysis. A nanodrop spectrophotometer was used to quantify the DNA in the liquid. For detection of the different genes, each of the *bla-NDM* *bla-VIM* and *bla-IMP* primers (table 1) were added to appropriate quantities of the PCR components to bring it to a final volume of 20μL. The thermocycler was adjusted for initial denaturation to occur for 2 min at 95°C, 30 seconds denaturation at 95°C, annealing for 30 seconds at 48 °C, 30 seconds extension and 2 min final extension at 72 °C. The agarose gel electrophoresis technique was used to detect the MBL genes by comparing the molecular weight of the amplicons with the primers using a 500bp molecular ladder.

Table 1

Primers used in the study

Primer name	Sequence of Primer	Annealing temperature	Target gene	Amplicon in bp
VIM-R	TGGTGTTTGGTCGCAAT	48 °C	*bla VIM*	390bp
VIM-F	CGAATGCGCACCAG			
IMP-R	GGAATAGAGTGGCTTAACTCTC		*bla IMP*	232bp
IMP-F	GTTTAACAAAACAACCACC			
NDM-R	CGGAATGGCTCATCACGATC		*bla NDM*	621 bp
NDM-F	GGTTTGGCGATCTGGTTTTC			

Molecular identification of the Metallo-beta-lactamase (MBL) producing isolatesr RNA Amplification

Amplification of the 16s rRNA region of the rRNA genes was performed in a thermocycler using 27F and 1492R primers with a final volume of *50* µl for 35 cycles. After amplification, resolution of the genes was carried out using gel electrophoresis. The products were viewed

on a UV transilluminator. The genes were sequenced in a BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. Trace edit bioinformatic algorithm was used to edit the gene sequences, the same sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN and were matched using ClustalX. Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987) was adopted for inference of the evolutionary history. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analysed while the evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

RESULTS

Ten bacterial isolates, phenotypically confirmed to be MBL producers were screened for the presence of MBL genes (bla_{IMP}, bla_{VIM} and bla_{NDM}). The purity and quantity of the bacterial genome extracted is shown in Table 1. The purity of the extracted DNA was between the range of 1.9 – 2.4. Specific forward and reverse primers targeting the MBL genes were used in a PCR (Polymerase Chain Reaction) reaction to amplify the MBL gene segments of the bacterial genome of the isolates. The result of the bla_{NDM} and bla_{IMP} gene detection is shown in Plate 1 in an agarose gel electrophoresis photograph. Out of the 10 isolates screened, only one had the bla_{NDM} gene, this is seen in the band showing in lane 8 which corresponds to a molecular weight of 621bp when compared to the 1000bp molecular ladder. For detection of bla_{IMP} gene, the agarose gel electrophoresis in Plate 1 showed that 7 isolates were positive for bla_{IMP} gene. The seven bands seen in the second 1-10 lane correspond to 232bp of bla_{IMP} gene as compared to the 1000bp molecular ladder. From Plate 2, the result of the bla_{VIM} gene detection showed none of the isolates were positive for the bla_{VIM} gene because no bands corresponding to bla_{VIM} gene molecular weight of 390bp were observed.

The 16s rRNA genes of the isolates that harboured the MBL genes were amplified via PCR and subjected to agar agarose gel electrophoresis. The bands as seen on Plate 3 aligns with 1500bp on the molecular ladder and the molecular weight confirms that the bands are 16s rRNA genes. The 16s rRNA genes were sequenced and the obtained 16S rRNA sequences for the isolates matched with the gene sequences on NCBI gene database. The gene sequence of B2 was 99% similar to *Plesiomonas species* and the isolate was closely related to *Plesiomonas shigelloides* strain 187 (KX828296.1) than other *Plesiomonas species* (Figure 1). Isolate B4 was 99% closely related to *Plesiomonas sp strain TIL_TAL_1* (KT99850.1)

than other *Plesiomonas sp*. B1, B9 and B10 had a 100% sequence similarity to *Klebsiella pneumoniae strain DSM 30104* (KX274129.1), *Enterobacter saccharin SP1* (CP007215.3) and *Enterobacter cloacae strain S20504 (*KF956588.1) respectively. Isolates B6 and B7 were 57.6% similar to other species but were closely related to *Photobacterium ganghwense strain ZR07* (KR150790.1). B3, B5 and B8 were 99.8% similar to other species but were very related to *Acinetobacter soli strain MBR7* (JX966425.1), *Comamonas testosterone strain 1* (KX400851.1) and *Bacillus licheniformis strain 60* (KX216385.1). The specific type of MBL genes harboured by the isolates is shown in Table 2, three MBL genes (IMP, VIM and NDM) were screened among the isolates. The bla_{IMP} gene had the highest prevalence, 70% of the gram negative isolates harbored the gene. No bla_{VIM} gene was detected among the isolates and bla_{NDM} gene was detected in only one of the isolates.

TABLE 1

Quantity and purity of bacterial DNA extract from sachet water and Great Kwa River samples

Code No	Quantity of nucleic acid (ng/µl)	Purity (A260/A280)
1	70.50	2.09
2	14.77	1.89
3	52.50	2.01
4	113.86	2.14
5	78.84	2.09
6	20.69	2.02
7	49.62	2.28
8	64.60	1.86
9	116.80	1.98
10	141.16	2.13

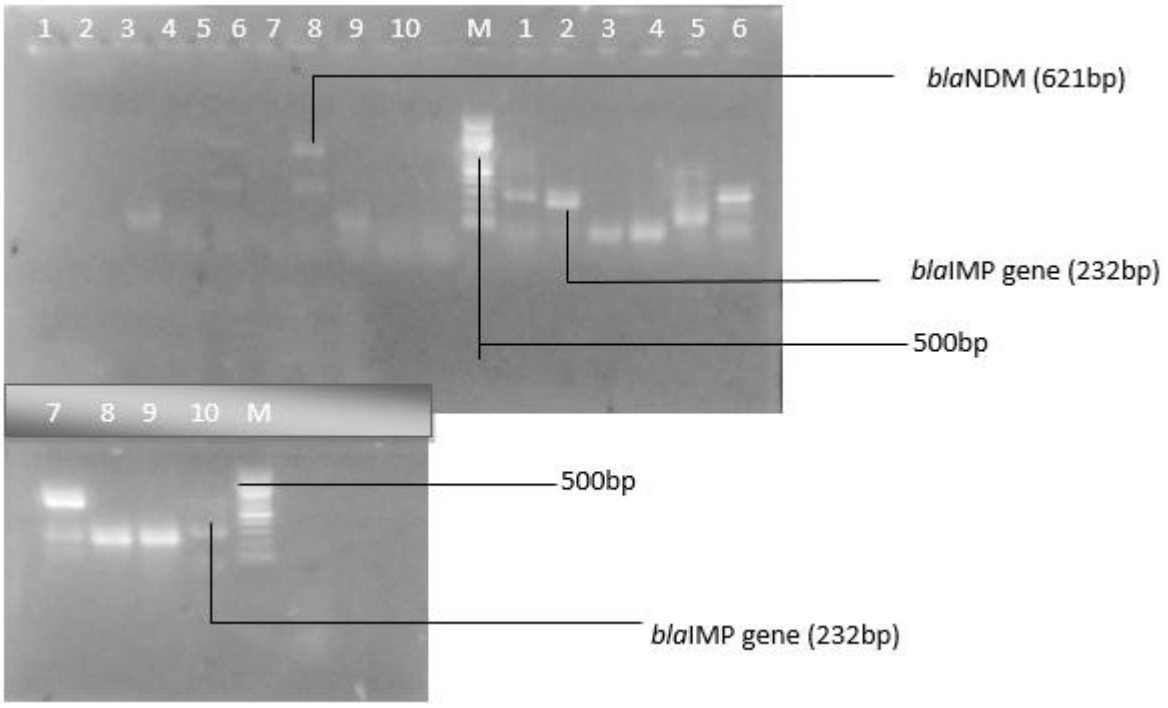

Plate 1: Agarose Gel electrophoresis showing the MBL genes of bacterial isolates from Great Kwa River and sachet water. First Lane 1-10 represents the blaNDM gene detection. Second Lane 1-10 represents blaIMP gene detection while lane M represents the 1000bp molecular ladder.

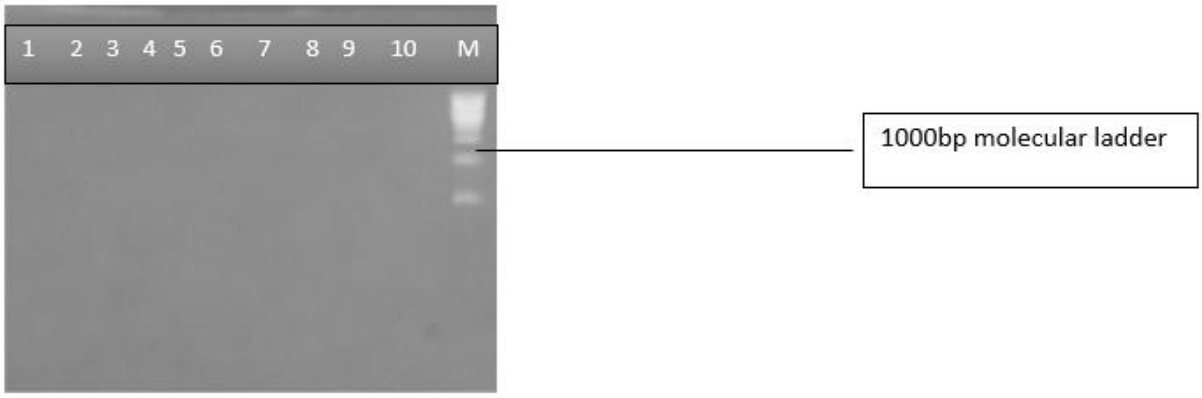

Plate 2: Agarose Gel electrophoresis for detection of *bla*$_{VIM}$ gene among bacterial isolates from Great Kwa River and sachet water. Lane 1-10 represents the samples and lane M represents the 1000bp Quick-Load DNA molecular

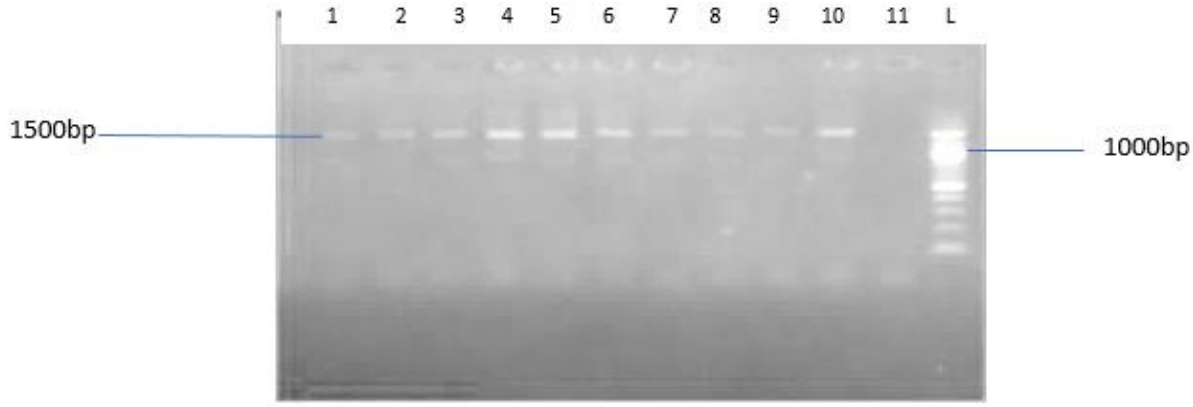

Plate 3 Showing the amplified 16s rRNA gene of the bacterial isolates, Lanes 1-10 represent the 16S rRNA (1500bp), lane 11 represents the negative control while lane L represents the 1500bp molecular ladder.

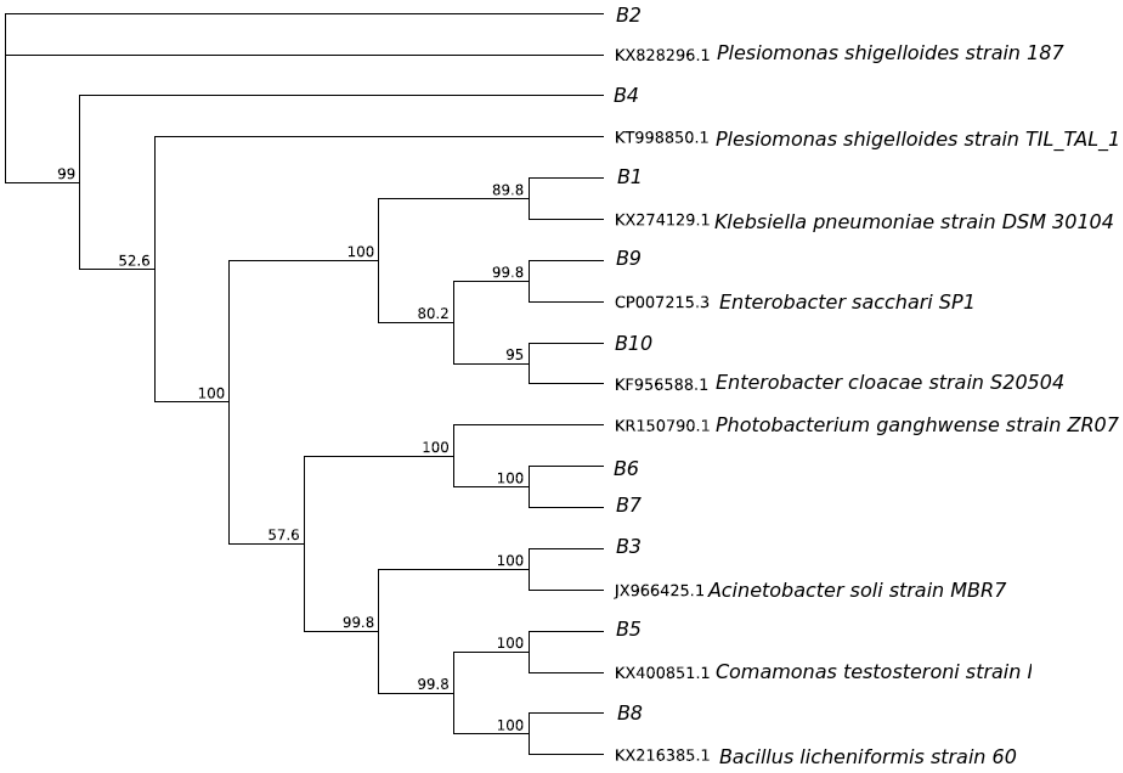

Figure 1: Phylogenetic tree showing the evolutionary distance between the MBL producing bacterial isolates

TABLE 2

Prevalence of *bla_{IMP}*, *bla_{VIM}* and *bla_{NDM}* MBL genes among the isolates

S/N	SAMPLE CODE	PROBABLE ORGANISM	*blaIMP*	*blaVIM*	*blaNDM*
1	B1	*Klebsiella pneumoniae* strain DSM 30104	Positive	Negative	Negative
2	B2	*Plesiomonas shigelloides* strain 187	Positive	Negative	Negative
3	B3	*Acinetobacter soli* strain MBR7	Negative	Negative	Negative
4	B4	*Plesiomonas spp* strain TIL_TAL_1	Negative	Negative	Negative
5	B5	*Comamonas testosterone* strain 1	Negative	Negative	Negative
6	B6	*Photobacterium ganghwense* strain ZR07	Positive	Negative	Negative
--7	B7	*Photobacterium ganghwense* strain ZR07	Positive	Negative	Negative
8	B8	*Bacillus licheniformis* strain 60	Positive	Negative	Positive
9	B9	*Enterobacter sacchari* SP1	Positive	Negative	Negative
10	B10	*Enterobacter cloacae* strain S20504	Positive	Negative	Negative

DISCUSSION

Bacterial resistance to antibiotics can occur through some mechanisms such as: impermeability of the outer membrane, expulsion of the antibiotics by efflux pump, secretion of antibiotics deactivating enzymes and alteration of antibiotics target sites. The detection of the *bla_{NDM}* and *bla_{IMP}* among the isolates showed that the mechanism of resistance to imipenem antibiotics could probably have been by secretion of New Delhi *Metallo-beta-lactamases* (NDM) and *Imipenemases* (IMP). These enzymes are inducible and transcription of the genes (*bla_{NDM}* and *bla_{IMP}*) to Metallo-beta-lactamases occurs only in the presence of the carbapenems. The degradation of the beta-lactam ring of the imipenem by the MBLs result in imipenem resistance. The MBL was discovered in the 1960s and afterwards different types (IMP, VIM, NDM) were subsequently discovered and characterized (Cornaglia *et al.*, 2011). The VIM was first discovered in 1996 from *Pseudomonas aeruginosa* (Lauretti *et al.*, 1999) while NDM was first discovered in a carbapenem-resistant *Klebsiella pneumoniae* strain which haboured a novel gene *bla_{NDM-1}* (Yong *et al*., 2009). The strain was isolated from an ailing Swedish man who was receiving treatment in New Delhi (the capital city of India). Since the discovery of these MBLs, there are reports from different geographical settings of bacterial species harbouring various genes encoding MBLs (Arakawa *et al.*, 2000; Pitout, *et al*., 2005; Ellington *et al.*, 2006). This can be attributed to the rapid rate of gene transfer

among bacterial species and the presence of these genes on the plasmids and other mobile genetic elements (Riccio *et al.*, 2000; Yong *et al.*, 2009).

Different types of MBL genes have been shown to be prevalent in some regions compared to other settings. In our study, the bla_{IMP} and bla_{NDM} genes were more prevalent while the bla_{VIM} was not detected among the isolates. In Iran, Yazdi *et al* (2007) identified bla_{VIM} as the most prevalent MBL gene among 8 bacterial strains from a total of 126 strains while bla_{IMP} was not detected in any of the isolates. Shahcheraghi *et al* (2008) in India also recorded the bla_{VIM} as the most prevalent MBL gene and also none of the 15 *Pseudomonas aeruginosa* strains screened harboured the bla_{IMP} gene. In Iran, Khosravi and Mihani in 2008, detected MBL among 8 *Pseudomonas aeruginosa* strains and found that the most prevalent MBL was the bla_{VIM}. In Brazil, the bla_{IMP} gene was not detected among the isolates (Franco *et al*., 2010). Franklin *et al* (2006) also observed the bla_{IMP} gene as the more prevalent gene among the gram-negative bacterial isolates in their study. Within the African continent, in Ugandan, Okoche *et al* (2015) reported bla_{VIM} as the most predominant MBL gene among Enterobacteriaceae isolates. Perovic *et al* (2016) in South Africa and Abdullahi *et al* (2017) in Nigeria, found bla_{NDM} gene as the more prevalent gene. Findings from this study and observations from other studies show that different bacterial species harbouring the MBL genes are emerging at a rapid rate globally. Hence, treatment of hospital waste before discharge into the environment should be encouraged and monitored in order to curb the widespread of antibiotic-resistant genes in the environment.

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