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## **Phenotypic and Genotypic Characterisation of Carbapenem-Resistant Gram-Negative Bacilli Pathogens from Hospitals in Ghana**

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## **ABSTRACT**

In Ghana, surveillance efforts on antibiotic resistance so far have not covered carbapenem resistance. In this study, our aim was to apply phenotypic and genotypic methods to identify and characterise carbapenem-resistant (CR) Gram-negative bacteria from the hospital environment in Ghana. A collection of 3840 isolates of Gram-negative bacilli infections from various clinical specimens was screened for carbapenem resistance by disc diffusion for imipenem, meropenem and doripenem. Minimum Inhibitory Concentration (MIC) of the CR isolates was determined by E-test for the three carbapenems. All the CR isolates were further screened for carbapenemase activity by modified Hodge and Boronic-acid disc synergy tests. The CR isolates were investigated for the presence of carbapenemase and extended spectrum beta-lactamase genes by PCR and confirmed by sequencing. The overall prevalence of carbapenem-resistant isolates was 2.9% (111/ 3840). Based on the disc diffusion test, prevalence of resistance to carbapenems were doripenem (75%), imipenem (66.7%) and meropenem (58%). The highest measurable MIC levels ( $\geq 32$   $\mu\text{g/ml}$ ) were observed in 56.8% of CR isolates with the non-fermenters *Pseudomonas aeruginosa* (24.3%) and *Acinetobacter* species (18.9%) disproportionately represented. Phenotypic identification of carbapenemase activity occurred in 18.9% of the CR isolates by the modified Hodge test and 2.7% by Boronic acid-disc synergy test; 21.6% exhibited carbapenemase production by both methods. All the CR isolates carried ESBL genes (*bla*TEM and *bla*SHV) while 23.4% were carriers of carbapenemase genes of which 14.4% were *bla*NDM-1, 7.2% *bla*VIM-1 and 1.8% *bla*OXA-48. Phylogenetically, the CR isolates were diverse and showed limited relatedness to isolates from other geographical regions.

## **KEY WORDS**

Carbapenem, Ghana, Gram-negative bacilli, *Pseudomonas aeruginosa*, *Acinetobacter* species

## 1.0 BACKGROUND

Gram-negative bacilli are common colonizers of the human intestinal tract and are implicated in a wide range of diseases such as pneumonia, meningitis, septicaemia and urinary tract infections.<sup>1-5</sup> They include enterobacteriaceae as well as non-fermenters such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The latter tend to be more associated with opportunistic infections in seriously ill, hospitalized and immunocompromised patients. Carbapenems are considered a last-resort treatment of Gram-negative infections, and emergence and spread of carbapenem resistance constitute a very important public health problem.<sup>1</sup> The mechanisms of carbapenem resistance in Gram-negative bacilli involves (i) hyper production of ESBL combined with reduced outer membrane permeability and/or (ii) carbapenemase production.<sup>1,6</sup> Following the discovery of NmcA in *Enterobacter cloacae* as the first carbapenemase in 1993,<sup>7</sup> carbapenemases have gained worldwide recognition due to their association with outbreaks and the ease with which they can spread.<sup>1,8</sup> The enzymes have been characterised as various classes of carbapenemases including the most common types: the New Delhi metallo- $\beta$ -lactamases (NDM), Verona integron-encoded metallo- $\beta$ -lactamases (VIM), oxacillinase-48 (OXA-48), imipenem-resistant *Pseudomonas*-1 (IMP-1)  $\beta$ -lactamase and *Klebsiella pneumoniae* carbapenemases (KPCs). The genes encoding these enzymes are *bla*NDM, *bla*VIM, *bla*OXA-48, *bla*IMP, and *bla*KPC, respectively.<sup>1,5,8</sup>

Although carbapenemase-producing organisms are a global problem, the majority of the epidemiological data on the subject relate to the developed world. Recently, a few African countries have extensively investigated carbapenemase producers among Gram-negative bacilli to find out the prevailing types and distribution in their locations such as

in South Africa,<sup>2</sup> Kenya,<sup>3</sup> Morocco,<sup>4</sup> Tanzania,<sup>5</sup> and Nigeria.<sup>6</sup> More importantly, in terms of resistance to carbapenems, in sub-Saharan Africa there are limited data available to ascertain the extent of the spread of carbapenemase gene carriers. Current studies show there is a lack of interest, poor implementation of surveillance studies and an unsystematic approach to carrying out research studies on carbapenemase-producing bacteria in this part of the sub-region.<sup>8</sup> Recently, a nationwide laboratory based antimicrobial resistance study carried out in Ghanaian health care facilities by Opintan *et al.*<sup>9</sup> reported no data on the prevalence and distribution of CRE, despite the fact that there are increased numbers of multidrug-resistant organisms in the country. To fill this research gap, we applied various techniques to identify and characterise carbapenem-resistant (CR) Gram-negative organisms and their resistance genes from the hospital environment in Ghana.

## **2.0 METHODS**

### **2.1 Study Sites and Bacteria Isolates**

A prospective study was carried out in four selected hospital laboratories in Ghana from September 2012 to September 2014. The study hospitals included Korle-Bu Teaching Hospital in the Greater Accra Region (KBTH), Effia-Nkwanta Hospital (ENH) in the Western Region, AngloGold Mines Hospital (AMH) in the Ashanti Region and Ho Regional Hospital (HRH) in the Volta Region. These hospitals were selected to represent the different types of hospitals in Ghana including tertiary hospitals (KBTH), regional hospitals (HRH and ENH) and district hospitals (AMH).

A total of 3840 Gram-negative bacilli isolates from various clinical specimens in the study hospitals were collected, and Figure 1 provides a schematic representation of the investigations carried out on the isolates. Multidrug resistant CR isolates were identified

using the disc diffusion technique of the British Society for Antimicrobial Chemotherapy (BSAC) method. Multidrug resistance (MDR) was defined as resistance to three or more classes of antimicrobial agents.<sup>10,11</sup> The CR study isolates were identified to the species levels by the use of Vitek 2 automated Compact system (BioMérieux, France). Quality control strains including *Escherichia coli* ATCC 25922, a known susceptible strain to carbapenems, and *Klebsiella pneumoniae* carbapenemase positive NCTC 13438 were included as controls in the identification process.

## **2.2 Antimicrobial Susceptibility Testing**

All the MDR isolates were re-tested using three carbapenem discs (Oxoid Ltd, Basingstoke, UK); imipenem, meropenem and doripenem according to the BSAC disc diffusion method<sup>10</sup> on Mueller-Hinton agar (MHA), (Biotec Ltd, UK). A reference strain of *Escherichia coli* ATCC 25922 was included as a negative control organism. Zone sizes were interpreted based on the BSAC breakpoints.<sup>11</sup> For isolates with diameters  $\leq 15$  mm, minimum inhibitory concentrations (MICs) were determined using the E-test method<sup>11</sup>, and resistance was defined as isolates with MIC against the same carbapenems of  $\geq 4$   $\mu\text{g/ml}$  according to BSAC breakpoints. Additionally, E-test strips (BioMérieux, France) for imipenem, meropenem and ertapenem showing MIC values greater or within 2 to 4, 2 to 4, and 1  $\mu\text{g/ml}$  levels, respectively, were considered carbapenemase producers.<sup>12</sup> The non-fermenting bacilli were excluded for ertapenem disc and E-test strip determination due to lack of activity against these organisms.

## **2.3 Phenotypic based methods for detection of carbapenem-resistant organisms**

All the CR isolates were screened for carbapenemase activity by modified Hodge and Boronic-acid disc synergy tests<sup>13,14</sup> and beta-lactamase activity by nitrocefin assay.<sup>15</sup> The modified Hodge test was performed according to the method described by

Anderson *et al.*<sup>16</sup> Briefly, the indicator organism *Escherichia coli* ATCC 25922 was prepared by obtaining an overnight broth culture which was adjusted to 0.5 McFarland turbidity standard followed by a 10-fold dilution in saline. This broth was then used to inoculate the surface of plates of Mueller-Hinton agar (Biotec Ltd, UK) by swabbing and meropenem (10 $\mu$ g) was placed at the centre. Three to five colonies of the test organisms were used to inoculate the plate in a line straight from the edge of disc to the end of the plate and incubated overnight at 35-37°C for 16-24 hours. The plate was read and interpreted as positive test when a clover-leaf like indentation of the *Escherichia coli* ATCC 25922 grew along the test organism growth streak within the disc diffusion zone.

The Boronic-acid disc synergy test was performed as described by Doi *et al.*<sup>17</sup> Carbapenem discs containing 10 $\mu$ g each of imipenem, meropenem and ertapenem were supplemented with 20  $\mu$ l of 20 mg/ml 3-aminophenyl-boronic acid (APB) and dissolved in dimethyl sulphoxide (DMSO; Sigma-Aldrich Company Ltd Dorset, UK) to yield a 400  $\mu$ g/ml final concentration. A suspension of the test organism was prepared according the same protocol described above and then used to swab the surface of a MHA plate. The supplemented discs of imipenem, meropenem and ertapenem containing 400  $\mu$ g/ml each and un-supplemented discs of each antimicrobial respectively were placed far apart on the plate and incubated at 35-37°C in air for 16-18 hours. A positive test for KPC enzyme production was recorded when the measured diameter of the growth-inhibitory zone around each carbapenem disc with boronic acid was  $\geq 5$  mm more than that around the disc containing the carbapenem alone.

In the evaluation of beta-lactamase activity of the CR isolates by nitrocefin assay, preparation of  $\beta$ -lactamase extracts from the isolates was first done using the technique described by Dai *et al.*<sup>15</sup> Subsequently, the  $\beta$ -lactamase concentration of the extracts

was determined by a microplate assay as follows. In each well, 10  $\mu$ l of collected extract was added to a 96-well plate. Total volume was adjusted to 50  $\mu$ l per well with  $\beta$ L Assay Buffer (100 mM Sodium phosphate, pH 7.0). Reaction mixture was prepared according to the manufacturer's instructions. In each well, a total reaction mix of 50  $\mu$ l was prepared comprising  $\beta$ L Assay Buffer of 48 $\mu$ l and 2 $\mu$ l of nitrocefin solution. Reaction mix was added to the extract in each well, and was then thoroughly mixed, and the absorbance of the solution was measured at 390 and 486 nm every minute for a 15-minute period using a plate reader (Tecan Infinite<sup>®</sup> 200 PRO Männedorf, Switzerland). Positive controls were prepared in  $\beta$ L Assay Buffer and 10  $\mu$ l of the diluted positive control pipetted into the desired well. Double distilled water was included as a negative control. The volume was adjusted to 50  $\mu$ l with  $\beta$ L Assay Buffer. For the blank, 10  $\mu$ L sodium phosphate buffer was used and adjusted in the same way as the positive control. The mixture was then incubated at 30°C for 1 hour.

#### **2.4 Analysis of Carbapenemase and Extended Spectrum $\beta$ -lactamase Genes**

DNA extraction of the CR isolates was done using the QiaAmp mini Kit (Qiagen, Hilden, Germany). All the 111 CR isolates were investigated for the presence of carbapenemase genes by PCR using primers from the five most common genes: New Delhi metallo-beta-lactamase-1 (NDM-1), *Klebsiella pneumoniae* carbapenemase-1 (KPC-1), Verona integron-encoded metallo- $\beta$ -lactamase-1 (VIM-1), Oxacillinase-48 (OXA-48) and Imipenem-resistant *Pseudomonas*-1 (IMP-1). The primers were identified from Poirel *et al.*<sup>18</sup> (Supplementary Table 1). Primers identified by Schlesinger *et al.*<sup>19</sup> were also used to amplify regions of the extended spectrum  $\beta$ -lactamase genes (*bla*TEM and *bla*SHV) in the CR study isolates (Supplementary Table 1). Different PCR cycling conditions were employed for carbapenemase and extended spectrum  $\beta$ -lactamase genes (Supplementary Table 1). Two  $\mu$ l of all the PCR reaction mixtures were separated by 2.0% agarose gel electrophoresis, visualized with ethidium



bromide staining and UV illumination, and then photographed with a gel documentation system (Gel Doc 2000; Bio-Rad, UK). Purified 26 positive carbapenemase products including positive control strains were prepared and sent to Eurofins Genomics, Ebersberg, Germany for sequencing.

## **2.5 Data Analysis**

Data from the study were entered into Microsoft Excel and analyzed in Stata (v 7.0; Stata Corp, College Station, TX). Descriptive statistics were carried out on the data and the results summarized in tables and graphs. The descriptive statistics included frequencies and prevalence of various Gram-negative bacteria, their antibiograms, and distribution of carbapenemase resistance genes. The sensitivity and specificity of Modified Hodge and Boronic Acid-Disc Synergy tests in the detection of carbapenemase activity compared with PCR-based detection of carbapenemase genes, were evaluated based on the formula described by Pasteran *et al.*<sup>20</sup>

## **2.6 Ethical Considerations**

The study was approved by the Ethical Committee of the School of Biomedical and Allied Health Sciences, University of Ghana (Manuscript Ethics Identification Number: SAHS-ET/SAHS/PSM/ML/05/AA/26A/2012-2013). As the samples used in the study were archived isolates, we could not obtain patients' consent for use of their clinical data. However, all patients' data and isolates were de-identified to ensure anonymity.

## **3.0 RESULTS**

### **3.1 Carbapenem Resistant Isolates Demographics and Characteristics**

A total of 111 CR isolates were identified from screening 3840 Gram-negative bacilli isolates. The CR isolates were from 55 male and 56 female patients with ages between 3 days and 91 years. The 111 CR isolates comprised 51 *Pseudomonas aeruginosa*, 31

*Acinetobacter* species, 12 *Escherichia coli*, 7 *Pseudomonas putida*, 3 each of *Klebsiella pneumoniae* and *Enterobacter cloacae*, and 1 each of *Cronobacter sakazakii*, *Providencia stuartii*, *Shigella sonnei* and *Sphingomonas paucimobilis*. The organisms were isolated from 10 specimen types but most of the isolates were from wound (47) and urine (31).

### **3.2 Antibiogram of Carbapenem Resistant Isolates**

Based on the disc diffusion test, prevalence of resistance to carbapenems were doripenem (75%, n=83), imipenem (66.7%, n=74) and meropenem (58%, n=64). The highest measurable MIC levels were observed in 56.8% (63) of CR isolates at  $\geq 32$   $\mu\text{g/ml}$ , of which relatively high prevalence of 24.3% (12) and 18.9% (6) were detected in *Pseudomonas aeruginosa* and *Acinetobacter* isolates, respectively (Figure 2).

### **3.3 Carbapenemase activity based on phenotypic tests**

Details of the carbapenemase activity among the CR isolates are shown in Table 2. Phenotypic identification of carbapenamase activity was revealed in 21/111 (18.9%) of the CR isolates by the modified Hodge test (MHT) and 3/111 (2.7%) by Boronic acid-disc synergy test (BADST). None of the CR isolates were positive for both MHT and BADST phenotypic test. The three *Enterobacter cloacae* isolates included in the study were the only isolates, which showed positivity from the BADST screen.

### **3.4 Phenotypic and genotypic correlation of carbapenem-resistant isolates**

The phenotypic and genotypic correlation of CR isolates is shown in Table 1. The 21 MHT positive isolates included 13 *Acinetobacter* spp., 6 *Pseudomonas* spp. and 2 *Klebsiella pneumoniae* isolates, none of which were detected by BADST screen as positive. The two *Klebsiella pneumoniae* MHT positive isolates were detected by PCR as OXA-48 producers, while one of the MHT positive *Acinetobacter* spp. was a NDM-1 producer. The only remaining CR *Klebsiella pneumoniae* isolate included in the study,

showed a negative result for MHT, BADST as well as KPC resistance gene. Carbapenemase resistance genes, VIM-1, KPC-1 and IMP-1 were undetected by MHT as carbapenemase-producing isolates. A correlation was observed between MHT positivity and PCR assay for OXA-48 gene positivity. There was no correlation between BADST positivity and PCR-based results. Clearly, phenotype-based tests were less efficient in detecting carbapenemase activity considering the percentage sensitivity and specificity values when compared with the PCR-based assay results obtained in this study (Table 2).

### **3.5 Distribution of Carbapenemase-producing Genes and Phylogenetic Analysis**

Based on the PCR assay, 26/111 (23.4%) of the bacterial genomic DNA samples were PCR-positive for carbapenemase genes, of which 14.4% (16) were *bla*NDM-1, 7.2% (8) *bla*VIM-1 and 1.8% (2) for *bla*OXA-48 genes. As shown in Figure 3, the highest carbapenemase gene prevalence was from *Acinetobacter* species (9 NDM-1 positives) and *Pseudomonas aeruginosa* (2 NDM-1 and 7 VIM-1), followed by *Escherichia coli* (3 NDM-1), *Klebsiella pneumoniae* (2 OXA-48), one VIM-1 for *Pseudomonas putida* isolate, and one each NDM-1 for *Providencia stuartii* and *Shigella sonnei*, respectively. No carbapenemase encoding genes were found in the isolates identified as *Enterobacter cloacae*, *Cronobacter sakazakii* and *Sphingomonas paucimobilis*.

Of the 26 carbapenemase producers, 23 (88.5%) were isolated in KBTH and these included producers of NDM-1 and VIM-1 by several Gram-negative organisms (*Acinetobacter spp.*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Pseudomonas putida*, *Providencia stuartii*) but not OXA-48 (Table 3). The other three hospitals each had one carbapenemase producer which included one *Klebsiella pneumoniae* OXA-48 producer each at ENRH and AGAMH, and one NDM-1 *Shigella sonnei* producer at HRH (Table 3).

Phylogenetic analyses were carried out for OXA-48, VIM-1 and NDM-1 of the CR isolates in comparison with GenBank established strains using control strains. Unfortunately, two of the PCR-positive carbapenemase genes, VIM-1 positive *Pseudomonas putida* and NDM-1 positive *Acinetobacter* species, including the positive NDM-1 control strains failed to yield substantial sequenced data to be added for their genetic relationships. The phylogenetic analyses showed an unevenly high level of heterogeneity among the CR isolates harbouring carbapenemase encoding genes; cluster of the Ghanaian isolates were distinctive from the controls used and those in the GenBank.

#### **4.0 Discussion**

This study had an overall CR prevalence of 2.9% from total Gram-negative bacterial pathogens while 23.4% of the CR isolates were PCR positive for known carbapenemase genes. It is worth noting that carbapenem antimicrobials are currently excluded from the Ghana Ministry of Health essential drug list following strict adherence to antimicrobial usage policy,<sup>21</sup> and so carbapenem use in Ghana is very low. However, the effectiveness of meropenem and its extended use for children was later recommended for patients in hospitals with serious infections around 2011. Presumably, the low-level usage of carbapenems may have contributed to this remarkably low prevalence rate of resistance in the country's hospitals. The present overall prevalence of carbapenem resistance was relatively low when compared with a similar study in tertiary hospitals in Nigeria where, 5.5% (10/182) Gram-negative isolates were carrying recognised carbapenemase genes, mainly, metallo- $\beta$ -lactamase carbapenemases.<sup>6</sup> While the relative prevalence of carbapenem resistance in Ghana is low, it is troubling that there is a

measurable carriage rate in a country with exceptionally low levels of carbapenem usage.

We observed high levels of resistance among some CR isolates in this study, which could have serious public health implications, as carbapenems are considered as a last-resort for treatment for bacterial infections. For example, in the disc diffusion test, resistance to meropenem, imipenem and doripenem was observed in all the CR isolates of *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Shigella sonnei*. Additionally, in the MIC E-test assays, 56.8% (63) of CR isolates showed complete resistance to imipenem, meropenem and ertapenem at  $\geq 32$   $\mu\text{g/ml}$ . The prevalence of ertapenem resistance among the carbapenemase producers was 2.7% and all of the ertapenem-resistant isolates were susceptible to meropenem in the E-test. This concurs with the observation of Anderson *et al.*<sup>16</sup> that in vitro ertapenem resistant isolates may remain susceptible to other carbapenems.

Sensitivity and specificity of phenotype-based tests (MHT and BADST) in this study showed no corresponding results with the molecular assay (PCR-based) employed. This concurs with studies by Nordmann *et al.*<sup>1</sup> and Doyle *et al.*<sup>22</sup> who confirmed low sensitivity of <12% for MBL detection by the phenotype-based tests, partly due to false-positivity resulting from ESBLs and AmpC co-existing with porin which mimic carbapenemase activity. Previous studies by Hara *et al.*<sup>12</sup> and Galani *et al.*<sup>23</sup> also confirmed MHT was unable to detect weak VIM and NDM carbapenemase genes, though the majority of VIM-1 and NDM-1-producing genes were detectable in *Pseudomonas aeruginosa* and *Acinetobacter* CR isolates.<sup>12,13,23</sup> Pasteran *et al.*<sup>24</sup> described the boronic acid test as the most specific and sensitive (with meropenem and imipenem discs) for detection of KPC in *Klebsiella pneumoniae* isolates, only when the

corresponding organisms existed without the presence of AmpC  $\beta$ -lactamase enzyme.<sup>13,24</sup> The inconsistency could be due to the presence of metallo- $\beta$ -lactamases in strains harbouring *AmpC* genes (ACC, CIT, DHA, EBC, FOX and MOX), which according to Hara *et al.*<sup>12</sup> are largely undetectable by MHT and BADST techniques.

In this study, 21.6% of the CR isolates harboured Class B carbapenemases (NDM-1 and VIM-1 genes), particularly *Pseudomonas aeruginosa* and *Acinetobacter* species. By comparison, a study in Iran showed that 33% of CR *Pseudomonas aeruginosa* harboured a *blaVIM* gene<sup>25</sup> while another study in India showed that 36% of CR *Acinetobacter* species carried a *blaIMP-1* gene.<sup>26</sup> No IMP-1 and KPC-1 genes were detected from Ghanaian CR strains. Detection of IMP-1 genes in Africa have been described as rare since their presence have been predominantly observed in China, Japan, and Australia.<sup>27</sup> A study by Lee *et al.*<sup>28</sup> suggested that success of *blaKPC* spread depends on certain clones of plasmids that are particularly suited to *Klebsiella pneumoniae* isolates compared with other Gram-negative bacterial pathogens. In the current study, there were only three *K. pneumoniae* isolates, which could have reduced the chance of detecting the KPC-1 gene. Interestingly, two of the *K. pneumoniae* isolates harboured the OXA-48 gene. This is the first time this important carbapenemase gene has been observed in Ghana. OXA-48 gene was first detected in Turkey in 2004 and had since spread to Middle East and beyond,<sup>29</sup> including known European countries such as Belgium,<sup>30</sup> France,<sup>31</sup> the Netherlands,<sup>32</sup> and recently in Denmark, Hungary, Romania and Spain.<sup>33</sup> A study carried out in Senegal identified the emerging danger of OXA-48 genes in the country's hospitals and community which was attributed to movement of the same major OXA-48 encoding plasmid spreading across the borders of Africa and the Middle East.<sup>34</sup> In the current study, the two OXA-48 producing *Klebsiella pneumoniae* strains were isolated from Effia-Nkwanta Regional

Hospital in the Western region of Ghana and AngloGold Ashanti Mines hospital in the Ashanti region. The two hospitals are about 200 kilometres apart, suggesting that OXA-48 producing strains may be widespread in Ghana. As shown by the phylogenetic analysis, carbapenemase genes harboured by the CR isolates appear to be highly diverse, signalling that further studies on carbapenemase genes in Ghana could reveal novel variants from the genes detected in this study. Diversity of carbapenemase genes observed in this study probably indicates lack of clonal expansion of carbapenem resistance in Ghana, which is a very positive thing for the Ghanaian public health system. There is however the need for surveillance of carbapenem resistance in Ghana using both phenotypic and genotypic methods.

There are a few limitations of the study. There were relatively small numbers of isolates included for certain Gram-negative bacteria such as *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Shigella sonnei*, which was unexpected. Furthermore, we did not screen for all known carbapenemase genes which could explain why for some isolates, carbapenemase genes were not observed even though the isolates concerned were carbapenem-resistant. For example, one of the three *K. pneumoniae* did not contain any carbapenemase genes screened, though it did carry ESBLs. It should also be noted that although ESBLs do not readily deactivate carbapenems, they could confer carbapenem resistance in combination with chromosomal porin mutations that prevent increase of  $\beta$ -lactam agents in the bacteria [35].

We conclude that, in Ghana, the occurrence of carbapenem resistance is relatively low with high numbers of ESBL carriage, coupled with a small number of PCR-positive carbapenemase gene detections. The CR isolates are diverse and show limited relatedness to isolates from other geographical regions. This is the first report of the

occurrence of three significant carbapenemase genes; NDM-1, VIM-1 and OXA-48 in Ghanaian hospitals. This study thus provides an insight of the emerging resistance genes belonging to the Ambler Class B, two MBL-types; NDM-1 and VIM-1 and one non-MBL-type, OXA-48 in the Class D group.

### **Competing interests**

The authors declare no competing interest.

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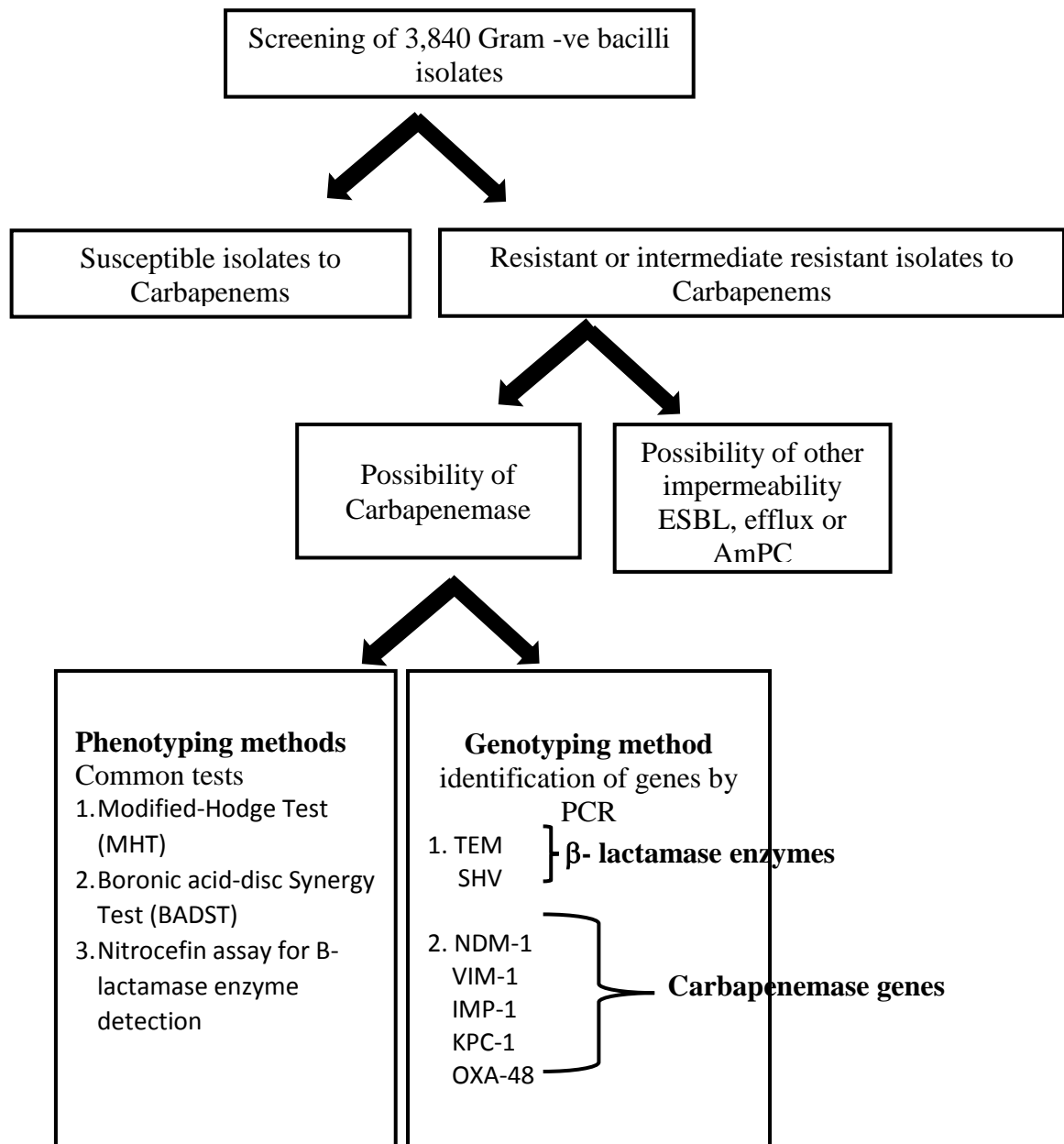
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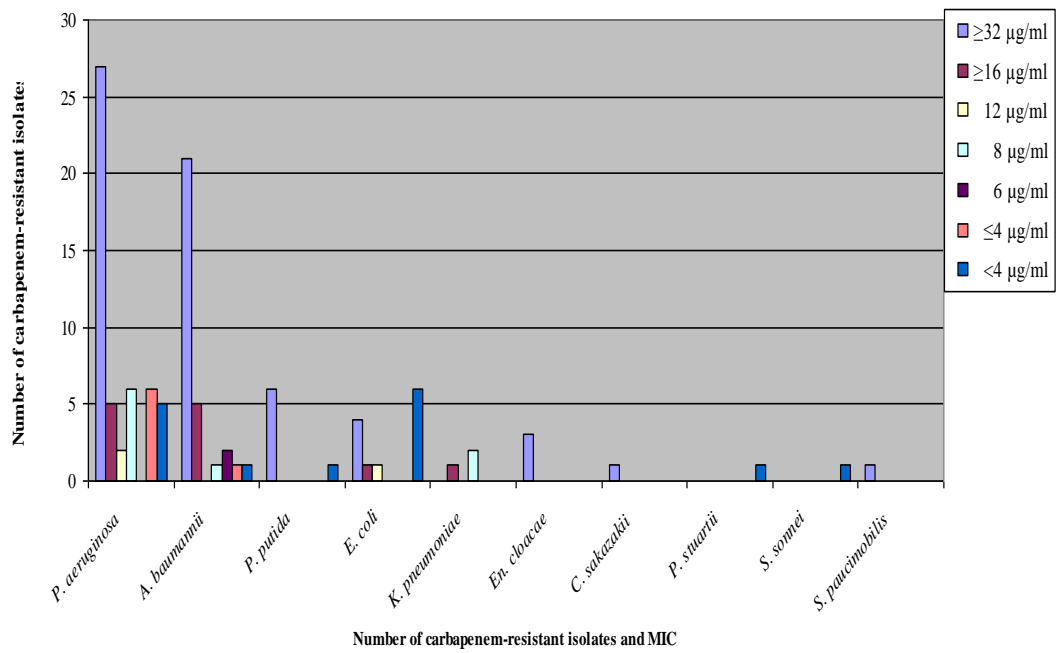
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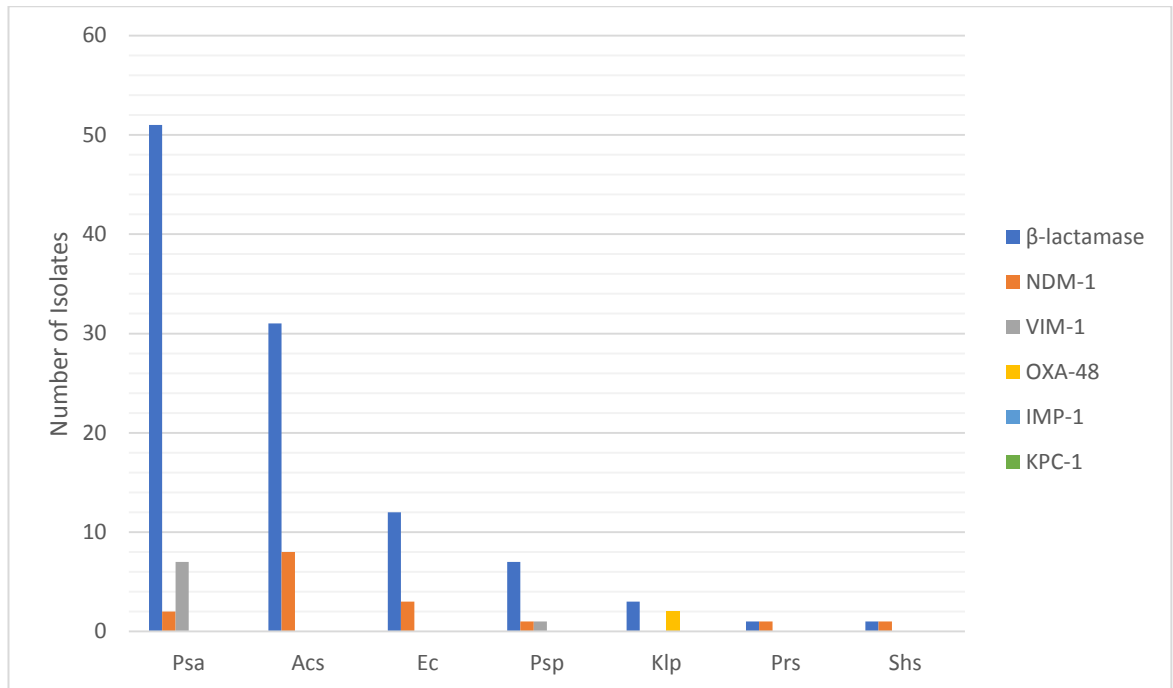
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**Figure 1: Schematic representation of the investigations carried out on the study isolates**



**Figure 2: Minimum inhibitory concentration (MIC) of carbapenem-resistant isolates**



**Figure 3. Distribution of carbapenemases and beta-lactamase in the study isolates**

Note: NDM-1= New Delhi metallo- $\beta$ -lactamase-1, VIM-1= Verona integron-encoded metallo- $\beta$ -lactamase-1, OXA-48= oxacillinase-48, IMP-1= imipenem-resistant *Pseudomonas*-1, KPC-1= *Klebsiella pneumoniae* carbapenemase-1, Psa= *Pseudomonas aeruginosa*, Acs= *Acinetobacter* species, Ec= *Escherichia coli*, Psp= *Pseudomonas putida*, Klp= *Klebsiella pneumoniae*, Prs= *Providencia stuartii*, Shs= *Shigella sonnei*.

**Table 1: Distribution of presumptive carbapenemase producers in phenotypic and genotypic assays among carbapenem-resistant isolates**

Bacterial isolate		Phenotypic test		Type of gene				
Organism	CR total	MHT	BADST	KPC-1	NDM-1	VIM-1	OXA-48	IMP-1
<i>P. aeruginosa</i>	51	5	-	-	2	7	-	-
<i>Acinetobacter spp</i>	31	13	-	-	9	-	-	-
<i>E. coli</i>	12	-	-	-	3	-	-	-
<i>P. putida</i>	7	-	-	-	-	1	-	-
<i>K. pneumoniae</i>	3	3	-	-	-	-	2	-
<i>En. cloacae</i>	3	-	3	-	-	-	-	-
<i>C. sakazakii</i>	1	-	-	-	-	-	-	-
<i>P. stuartii</i>	1	-	-	-	1	-	-	-
<i>S. sonnei</i>	1	-	-	-	1	-	-	-
<i>S. paucimobilis</i>	1	-	-	-	-	-	-	-
<b>Total</b>	111	21	3	-	16	8	2	-

Note: - = not found

**Table 2: Prevalence of carbapenemase resistance genes and the tested assays**

Carbapenem-resistant isolate (n) <sup>a</sup>	Frequency (%) by:				
	MHT (n=21)		<sup>b</sup> BADST (n=3)		PCR amplification assay
Carbapenemase genes (26)	SE	SP	SE	SP	
NDM-1 (16)	6.3 <sup>c</sup>	78.9	0	0	16 (100)
VIM-1 (8)	0	79.6	0	0	8 (100)
OXA-48 (2)	100 <sup>d</sup>	82.6	0	0	2 (100)
IMP-1 (0)	0	0	0	0	0 (0)
KPC-1 (0)	0	0	0	0	0 (0)

<sup>a</sup> Total number of CR isolates (111)

<sup>b</sup> None was detected by PCR assay

<sup>c</sup> Only one MHT positive (n=1) was detected by PCR assay

<sup>d</sup> All MHT positives (n=2) were detected by PCR assay

Note: MHT= modified Hodge test, BADST= boronic acid-disc synergy test, n= number, SE= sensitivity, SP= specificity



**Table 3: Distribution of carbapenemase-positive genes in the study hospitals**

Hospital	Type of carbapenemase-positive genes									
	<i>P. aeruginosa</i>	<i>Acinetobacter Spp</i>	<i>E. coli</i>	<i>P. putida</i>	<i>En. Cloacae</i>	<i>K. pneumoniae</i>	<i>C. Sakazakii</i>	<i>P. Stuartii</i>	<i>S. sonnei</i>	<i>S. paucimobilis</i>
<i>KBTH, GA/R</i> (23) <sup>a</sup>	2 NDM-1 7 VIM-1	9 NDM-1	3 NDM-1	1 VIM-1	-	-	-	1 NDM-1	-	-
<i>ENRH, W/R</i> (1)	-	-	-	-	-	1 OXA-48	-	-	-	-
<i>AGAMH, A/R</i> (1)	-	-	-	-	-	1 OXA-48	-	-	-	-
<i>HRH, V/R</i> (1)	-	-	-	-	-	-	-	-	1NDM-1	-

(<sup>a</sup>) Total number of carbapenemase-positive gene(s) obtained from each hospital

-= no carbapenemase-positive gene found; KBTH, GA/R= Korle Bu Teaching Hospital, Greater Accra Region; ENRH, W/R= Effia Nkwanta Regional Hospital, Western Region; AGAMH, A/R= AngloGold Ashanti Mines Hospital, Ashanti Region; HRH, V/R= Ho Regional Hospital, Volta Region.

**Supplementary Table 1: Primer sets for amplification of carbapenemase and extended spectrum  $\beta$ -lactamase genes**

Gene	Primer sequence (5'→3')	Amplicon size (bp)	PCR cycling conditions	Reference
<i>blaIMP</i>	Forward - GGAATAGAGTGGCTTAAYTCTC Reverse -  GGTTTAAAYAAAACAACCACC	232	Initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 30 seconds, and elongation at 72°C for 1 minute 30 seconds, followed by a final elongation step at 72°C for 10 minutes	Poirel <i>et al.</i> <sup>18</sup>
<i>blaVIM</i>	Forward - GATGGTGGTTTGGTCGCATA Reverse - CGAATGCGCAGCACCAG	390		Poirel <i>et al.</i> <sup>18</sup>
<i>blaOXA-48</i>	Forward - GCGTGGTTAAGGATGAACAC Reverse -  CATCAAGTTCAACCCAACCG	438		Poirel <i>et al.</i> <sup>18</sup>
<i>blaNDM</i>	Forward - GGTTTGGCGATCTGGTTTTTC Reverse -  CGGAATGGCTCATCACGATC	621		Poirel <i>et al.</i> <sup>18</sup>
<i>blaKPC</i>	Forward - CGTCTAGTTCTGCTGTCTTG Reverse -  CTTGTCATCCTTGTTAGGCG	798		Poirel <i>et al.</i> <sup>18</sup>
<i>blaTEM</i>	Forward - TCAACATTTTGTCTCGTCG Reverse - CTGACAGTTACCAATGCTTA	860	Initial denaturation 15 minutes at 95°C and 35 cycles of 1 minute at 94°C, 1 minute at an annealing temperature of 47°C and 50°C designed for each primer set for TEM and SHV respectively, and 1 min at 55°C, followed by 10 minutes at 72°C for the final extension.	Schlesinger <i>et al.</i> <sup>19</sup>
<i>blaSHV</i>	Forward - TTTATCGGCCYTCCTCAAGG Reverse - GCTGCGGGCCGGATAACG	930		Schlesinger <i>et al.</i> <sup>19</sup>

Note: IMP, imipenem-resistant *Pseudomonas*; VIM, Verona integron-encoded metallo- $\beta$ -lactamase; OXA-48, oxacillinase-48; NDM, New Delhi metallo- $\beta$ -lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; TEM-1, Temoniera-1; SHV-1, sulphhydryl variable-1