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PHENOTYPIC CHARACTERIZATION OF METALLO-B-LACTAMASE AMONG CARBAPENEM-RESISTANT *ENTEROBACTERIACEAE* IN SOUTHWESTERN NIGERIA AND ACCURACY IN MEANS OF DIAGNOSIS

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Abstract

This study investigated the occurrence of Metallo- β -lactamase in Carbapenem-resistant Enterobacteriaceae (CRE) and the accuracy of some CRE diagnostic methods. CRE were isolated from clinical samples using Blood Agar and MacConkey Agar. Phenotypic screening for carbapenemase production performed using Carbapenem Brilliance Agar (CBA), Modified Hodge Test (MHT) and Carbapenem Inactivation Method (CIM). Accuracy of tests was determined by comparing with MHT as the gold standard. CRE were screened for Metallo- β -lactamase production using ethylenediaminetetraacetic acid (EDTA) inhibition test. Statistical analysis was done using Statistical Product and Service Solutions (SPSS) version 20. Sixty-six of the 110 isolates obtained were Enterobacteriaceae. The overall prevalence of CRE was 22.7%. The prevalence of Metallo- β -lactamases among CRE and Enterobacteriaceae was 56.0% and 21.2% respectively. CBA, MHT, and CIM tests detected 40.9%, 37.9%, and 28.6% positive cases respectively. The sensitivity of CBA was higher (>90%) than CIM (<70%) while the specificity of both CBA and CIM was above 90%. High prevalence of CRE was dominated by Metallo- β -lactamases and MHT, CBA and CIM are accurate tests for CRE diagnosis.

Keywords: Carbapenem, Diagnosis, Enterobacteriaceae, Prevalence, Metallo- β -lactamase

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Abbreviations:

CRE - Carbapenem-resistant Enterobacteriaceae

CBA - Carbapenem Brilliance Agar

MHT - Modified Hodge Test

CIM - Carbapenem Inactivation Method-CIM

TP = True positive, FN = False negative, FP = False Positive, TN = True Negative

Introduction

Carbapenems are the effective first line of antibiotics that react against causative agents of a wide range of diseases. (Hu *et al.*, 2012). Carbapenem antibiotics are some of the most efficient antibacterial agents used in the treatment of multidrug-resistant bacterial infections (Cui *et al.*, 2019). Carbapenem-Resistant Enterobacteriaceae (CRE) are mainly members of the gram-negative class of microorganisms that are not susceptible to carbapenem antibiotics (Niu and Chen, 2018). They include Enterobacteriaceae that have been noted to defend themselves by the production of carbapenemase through a phenotypic or molecular assay, regardless of in-vitro carbapenem vulnerability (CDC, 2015). The major mechanisms for carbapenem resistance are the production of carbapenemase enzyme and horizontal transfer of carbapenem-resistant genes. Also, alterations in the permeability of the outer membranes caused by loss of porins and/or upregulation of the efflux system are also part of the mechanism for carbapenem resistance in the gram-negative group of organisms (Bialvaei *et al.*, 2016).

Carbapenemase production in an organism can be observed either by phenotypic and/or genotypic means in medical laboratories. Based on the classification of Ambler, carbapenemases can be grouped into classes A, B, and D. Class A and Class D carbapenemases are serine β -lactamases which possess serine at their active site, while Class B carbapenemases are Metallo- β -lactamases (MBLs) which have zinc at their active site (Ambler, 1980). Metallo- β -lactamases have a wide substrate spectrum that can catalyze the hydrolysis of virtually all β -lactam antibiotics; excluding monobactams (Drawz and Boromo, 2010).

CRE have emerged as a major class of bacterial pathogens, which harbor a significant amount of threat to global public health. CRE are usually resistant to all beta-lactam antibiotics and sometimes, possess a higher degree of resistance against other antimicrobial agents, hence, reducing the option of available treatments (Chen, 2014). The timely and effective detection of CRE, particularly, carbapenemase-producing Enterobacteriaceae, becomes an urgent necessity

to avert clinical manifestation and to aid accurate therapy. Hence, this study determined the occurrence of Metallo- β -lactamase in Carbapenem-resistant Enterobacteriaceae and the accuracy of some CRE diagnostic methods.

Materials and Methods

Sample collection

Samples of cerebrospinal fluid, stool, and urine were obtained from 110 hospitalized patients in Sacred Heart Hospital Abeokuta, Federal Medical Centre Abeokuta, and isolates were obtained in a government Hospital in Oyo state Nigeria. The samples collected were transported to the laboratory for analysis. Ethical approvals were obtained from the institutional ethical review boards of the hospitals. The study was conducted between February and December 2019.

Isolation and characterization of Carbapenem-Resistant Enterobacteriaceae

Samples were inoculated on sterile CRE Brilliance Agar (Oxoid, UK), Blood Agar (Oxoid, UK), and MacConkey Agar (Oxoid, UK) plates and incubated aerobically at 37°C for 24 hours. On CRE Brilliance Agar (CBA), the typical colour appearance was used in bacterial identification while colonial morphology and attributes on other agars were noted and distinct pure isolates were Gram-stained (Winn *et al.*, 2006). Gram-negative rods were subjected to the motility test, the Indole test, and Citrate test. All Gram-negative rods were further confirmed by the Microbact Gram-negative identification system™ 24E kit (Oxoid, UK).

Phenotypic Screening for Carbapenemase Production

Modified Hodges Test (MHT)

CRE isolates obtained from CBA were screened by Modified Hodge Test (MHT) for confirmation of carbapenemase production. The tenth dilutions of 0.5 McFarland suspension of *E. coli* ATCC 25922 were cultured onto Mueller-Hinton Agar (Oxoid, UK) plates, and a 10 μ mipenem disk (Oxoid, UK) placed at the center of the plate. The suspected CRE isolates

were later streaked from the edge/end of the disk to the edge of the plate forming a straight line of thick inoculum and were incubated for 24 hours. The growth of susceptible *E. coli* in the background towards the disk, creating a cloverleaf-like appearance indicated the production of carbapenemase by the test isolate (CLSI, 2017).

Carbapenem Inactivation Method (CIM)

CRE isolates obtained from CBA were used for the CIM test. A 10 µg meropenem disk was incubated in a suspension of the test strain (*E. coli* ATCC 25922) at 37°C for 120 minutes. After this incubation, the meropenem disk was removed from the solution and placed on a Mueller Hinton Agar plate already seeded with susceptible *E. coli* ATCC 25922 as with the MHT. Provided carbapenemase is produced, the meropenem in the disk would have been inactivated during the initial incubation, allowing for uninhibited visible growth of the susceptible test strain (*E. coli*). Thus, giving the impression that the *E. coli* is resistant. However, if a clear inhibition zone is formed, it implies that there is no carbapenemase production (Van der Zwaluwet *al.*, 2015).

Screening for Metallo-β-lactamase Production

The CRE isolates that were positive for MHT were screened for Metallo-β-lactamase production using ethylenediaminetetraacetic acid (EDTA) inhibition test. An EDTA solution was added to a carbapenem disk and then placed on a

sterile Mueller Hinton agar plate that has been seeded with the test strain of interest. The presence of MBL showed a larger zone of inhibition around the EDTA-carbapenem disk compared with plates without EDTA solution that served as the control for the test (Iovleva, 2017).

Data Analysis

Data analysis was done using Statistical Package for Social Sciences now known as Statistical Product and Service Solutions (SPSS) version 20. Wilcoxon rank-sum test was used to compare the diagnostic methods. The sensitivity, specificity, and predictive values of the diagnostic techniques were calculated at a 95% confidence interval.

RESULTS AND DISCUSSION

The percentage occurrence of all bacterial isolates with respect to sex is presented in Figure 1. It was revealed that the most prevalent bacterium in this study was *Escherichia coli* and its prevalence was slightly higher among the females compared to males ($p > 0.05$). Following *E. coli* was *Klebsiella pneumoniae* with a significantly higher prevalence ($p < 0.05$) among the males compared to the females. *Shigella* was the least encountered bacterium in this study and it was only found in the male subjects used in the study.

Figure 1. Percentage occurrence of bacterial isolates in relation to sex

Note: Values with asterisk (*) connotes significant difference ($p < 0.05$)

Out of the 66 Enterobacteriaceae isolates, *Escherichia coli* was the most prevalent organism, followed by *Klebsiella pneumoniae*. This finding is similar to other studies from Nigeria including Hamza and Abdulhadi, (2016) and Oluwafemi *et al.*, (2018). This outcome could be attributed to the fact that *Escherichia coli* exists as normal flora in the human intestine. The fact that both *Klebsiella pneumoniae* and *Shigella sp.* were significantly higher in males compared to females is in line with the findings of Xia *et al.*, (2011) and Infar *et al.*, (2017). This male preponderance may be attributed to the working population proportion between the genders. Moreover, males do more

out-door jobs. Hence, there is a greater chance of exposure to infections.

The prevalence of carbapenem-resistant Enterobacteriaceae and Metallo- β -lactamase in the study populations are presented in Table 1. The overall prevalence of 24.5%, 22.7%, and 17.3% of CRE were recorded using CBA, MHT, and CIM respectively. In addition, the CRE prevalence of 40.9%, 37.9%, and 28.8% were recorded among the Enterobacteriaceae isolates using CBA, MHT, and CIM respectively. The prevalence of Metallo- β -lactamase production among the CRE was 56.0% using MHT as the gold standard with a prevalence of 21.2% among the Enterobacteriaceae.

Table 1: Prevalence of CRE and Metallo- β -lactamases in CRE

Prevalence Level	CBA (%)	MHT (%)	CIM (%)	EDTA (MBL %*)
Overall	27(24.5)	25(22.7)	19(17.3)	14(12.7)
Out of 66	27(40.9)	25(37.9)	19(28.8)	14(21.2)
Out of CRE	-	-	-	14(56.0)*

In this study, the prevalence of MBLs was 21.2% in patients that were clinically diagnosed for CRE. Studies from other parts of Nigeria reported prevalence rates ranging from 4.1% to 21.2%. For instance, in Kaduna/Kano, Lagos, Calabar, and Enugu using the ethylenediaminetetraacetic acid (EDTA) disk synergy method gave a prevalence of 21.2% (Yusuf *et al.*, 2012), 8.8% (Eyo *et al.*, 2013), 4.1% (Aibinu *et al.*, 2007) and 10% (Chika *et al.*, 2014) respectively. Diagnostic methodology differences, geographical locations, and different sampled populations may partly be considered as a factor for variances in prevalence results. Although, Zubair and Iregbu (2018) reported that the EDTA disk synergy method is of lower specificity than the disk potentiating.

The occurrence of different carbapenemase-producing *Enterobacteriaceae* in stool and urine samples of the patients was presented in Table 2. *Klebsiella pneumoniae* was the most occurring *Enterobacteriaceae*, followed by *E. coli* and *Pseudomonas aeruginosa*, while *Proteus mirabilis* was the least occurring. The results showed that all the *Enterobacteriaceae* occurred more in the urine samples compared with stool samples. The percentage of occurrence in the urine samples ranged from 66.7% for *E. coli* to 100% in *Proteus mirabilis*. On the other hand, the percentage occurrence of *Enterobacteriaceae* in the stool samples ranged from 16.7% for *Pseudomonas aeruginosa* to 33.3% for *E. coli*, while *Proteus mirabilis* was not found in any of the stool samples.

Table 2: Occurrence of different Carbapenemase-producing *Enterobacteriaceae* in Stool and Urine samples of the infected participants

Type	No. Detected	Stool	Urine
<i>Escherichia coli</i>	6	2(33.3)	4(66.7)
<i>Klebsiella pneumoniae</i>	10	2(20.0)	8(80.0)
<i>Pseudomonas aeruginosa</i>	6	1(16.7)	5(83.3)
<i>Proteus mirabilis</i>	3	0(0.0)	3(100.0)
Total	25	5(18.5)	20(81.5)

Among 66 *Enterobacteriaceae* isolates, 25(37.9%) were detected to be CRE. Similar results have been documented in studies from the USA (21%) (Lascos *et al.*, 2013); Nigeria (33.5%) (Yusuf *et al.*, 2012), and Iran (14.5%) (Haji *et al.*, 2012). However, this was higher than the reported prevalence from Morocco (2.8%) (El Wartiti *et al.*, 2012), Bangladesh (4.8%) (Hayder *et al.*, 2012), and Taiwan (2.5%) (Lai *et al.*, 2013). Variation in the prevailing rate of Carbapenemase-producing *Enterobacteriaceae* in different studies may be

due to the abuse in the utilization of carbapenems, cultural values/beliefs, and vertical transfer of infections.

The prevalence of CRE with respect to Clinical Diagnosis was shown in Table 3. The majority of the patients who were positive of CRE were clinically diagnosed for UTIs. Out of the total CRE detected by CBA, MHT, and CIM; 62.5%, 60%, and 68.8% were respectively diagnosed for UTI. In the same vein, 57.1% of the patients who tested positive to Metallo- β -lactamase were clinically diagnosed for UTI.

Table 3: Prevalence of CRE detection in relation to Clinical Diagnosis

Clinical Diagnosis	CBA	MHT	CIM	EDTA
Acute diarrhea	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Acute pyelonephritis	4(16.7)	3(15.0)	2(12.5)	2(14.29)
Diarrhea	3(12.5)	3(15.0)	1(6.25)	3(21.43)
Dysentery	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Urosepsis	2(8.3)	2(10.0)	2(12.5)	1(7.14)
UTI	15(62.5)	12(60.0)	11(68.8)	8(57.1)
Total	24(100.0)	20(100.0)	16(100.0)	14(100.0)
	p<0.05	p<0.05	p<0.05	p<0.05

Carbapenemase-producing Enterobacteriaceae were found more in urine having *Klebsiella pneumoniae* and *E. coli* being the most prevalent. This finding is similar to the result of Eshetie *et al.*, (2015), which documented that many of the carbapenemase producers were *K. pneumoniae* followed by *E. coli* and *Enterobacter* spp. The same situations were also noticed in the findings from Turkey and Morocco which indicated that *K. pneumoniae* were the principal isolate followed by *E. coli* and *K. oxytoca* (Lascols *et al.*, 2013; Nazik *et al.*, 2012). Documented reports indicated that *Escherichia coli* and

Klebsiella pneumoniae are most implicated among patients with UTI (Melaku *et al.*, 2012; Sharma and Paul, 2012).

Table 4 revealed a CRE prevalence of 40.9%, 37.9%, and 28.8% using CBA, MHT, and CIM respectively. All the isolation methods detected a higher number of *K. pneumoniae* while CIM seemed not to be very sensitive in detecting *E. coli* as only four isolates of *E. coli* were detected by CIM compared with CBA and MHT, which detected 10 and 9 isolates of *E. coli* respectively.

Table 4: Comparison of detection using different methods of isolation

Isolates	Methods of Isolation			
	CBA N	MHT N	CIM N	EDTA N
<i>Escherichia coli</i>	7	7	4	4
<i>Klebsiellapneumoniae</i>	10	9	7	6
<i>Pseudomonas aeruginosa</i>	7	6	4	3
<i>Proteus mirabilis</i>	3	3	4	1
TOTAL (out of 66 clinically diagnosed patients)	27 (40.9)	25(37.9)	19(28.8)	14(21.2)

All the three methods were able to simultaneously detect 16 (55.2%) samples out of 29 distinctly positive cases. While CBA was able to detect 23 positive cases simultaneously with the gold standard, CIM detected 17 cases.

CBA detected two positive cases alone, while MHT detected one positive case alone. In addition, CBA and CIM detected two positive cases together (Figure 2).

Figure 2: Relationship between the three diagnostic tests using positive cases

Note: Addition of the two arrows gives the true positive cases for CRE using MHT as gold standard. Adding remaining values in the circle for CRE gives the false positive value.

All the three methods were able to simultaneously detect 81 (85.3%) samples out of the universal set of 95 distinctly negative cases. While CBA was able to detect 82 negative cases simultaneously with the gold standard, CIM

detected 83 cases. CBA detected one negative case alone, while MHT detected two positive cases alone. Furthermore, CIM detected eight positive cases alone (Figure 3).

Figure 3: Relationship between the three diagnostic tests using negative cases

The two by two contingency table showed that CIM recorded higher false-negative results (8 of 91) compared with CBA (2 of 83). Consequently, CBA recorded higher false-positive results (4 of 27) compared with that of CIM (2 of 19) (Table 5).

Table 6 shows the Sensitivity, Specificity, and Accuracy of CBA when compared with MHT as the gold standard. The result showed that both the sensitivity and specificity of CRE were greater than 90% with a test accuracy of 94.69% (95% CI = 88.80% - 98.03%).

Table 6: Sensitivity, Specificity and Accuracy of CRE when compared with MHT as the Gold Standard

Statistic	Value	95% CI
Sensitivity	92.00%	73.97 - 99.02
Specificity	95.45%	88.77 - 98.75
Positive Likelihood Ratio	20.24	7.72 - 53.09
Negative Likelihood Ratio	0.08	0.02 - 0.32
Disease prevalence (*)	22.12%	14.86 - 30.90
Positive Predictive Value (PPV)	85.19%	68.67 - 93.78
Negative Predictive Value (NPV)	97.67%	91.74 - 99.37
Accuracy (*)	94.69%	88.80 - 98.03

Contrarily, a sensitivity and specificity of 68% and 97.65% was recorded for CIM when compared with MHT as the gold standard. The result further showed that the accuracy of CIM in the diagnosis of carbapenemase-producing

Enterobacteriaceae is 90.91% (Table 7). Positive predictive values were higher than 80%, while negative predictive values were greater than 90% in both methods.

Table 7: Sensitivity, Specificity and Accuracy of CIM when compared with MHT as the Gold Standard

Statistic	Value	95% CI
Sensitivity	68.00%	46.50 - 85.05
Specificity	97.65%	91.76 - 99.71
Positive Likelihood Ratio	28.9	7.16 - 116.69
Negative Likelihood Ratio	0.33	0.18 - 0.58
Disease prevalence (*)	22.73%	15.28 - 31.70
Positive Predictive Value (PPV)	89.47%	67.80 - 97.17
Negative Predictive Value (NPV)	91.21%	85.41 - 94.84
Accuracy (*)	90.91%	83.92 - 95.55

In this study, 27 (40.9%), 25 (37.9%), and 19 (28.6%) positive cases were detected by CBA, MHT, and CIM respectively. The probability of both CBA and CIM methods to correctly classify a patient (given by a test of accuracy) is greater than 90%. This high sensitivity and specificity of CBA are evident in previous studies (Bracco *et al.*, 2013; Stuart *et al.*, 2013). In the same vein, the low sensitivity and high specificity demonstrated by CIM is also reported in past related studies. Elifet *et al.*, (2016) documented the sensitivity and specificity values of 78% and 100% for CIM.

Conclusion

This study focused on the phenotypic characterization of Metallo- β -lactamase in Carbapenem-Resistant *Enterobacteriaceae* (CRE) in Southwestern Nigeria. The prevalence of Metallo- β -lactamase production among the CRE was 56.0% using MHT as the gold standard with a prevalence of 21.2% among the *Enterobacteriaceae*. This study also evaluated the accuracy of three methods of detection of CRE; being CBA, MHT, and CIM. All three methods evaluated simultaneously detected CRE in the samples, however, both CBA and CIM

detected CRE with high (90%) accuracy. This study recommends CBA and CIM as simple and cost-effective methods for the diagnosis of CRE in clinical laboratories.

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Conflicts of Interest

This research study declares no conflicts of interest.

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