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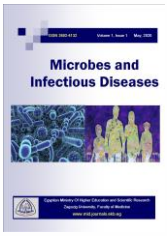

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Original article

Phenotypic and molecular detection of carbapenemase producing *Escherichia coli* and *Klebsiella pneumoniae*

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ABSTRACT

Background: Detection of carbapenemases properly and rapidly is vital in the fight against the emergence and spread of carbapenem resistant bacteria. This study was carried out to phenotypically and molecularly detect carbapenemase producing *Escherichia coli (E. coli)* and *Klebsiella pneumoniae (K. pneumoniae)*. **Methods:** A total of 123 isolates consisting 70 isolates of *E. coli* and 53 isolates of *K. pneumoniae* were screened for carbapenem resistant isolates (CRIs). The CRIs were then screened phenotypically for carbapenemase production using the Modified Hodge Test (MHT), Carba NP test and modified Carbapenem Inactivation Method (mCIM). The CRIs were also screened molecularly for carbapenemase genes by PCR and the carbapenemase genes detected were sequenced. **Results:** Out of the 123 isolates screened for carbapenem resistance, 6 (4.88%) comprising of 2 isolates of *E. coli* (2/70; 2.86%) and 4 isolates of *K. pneumoniae* (4/53; 7.55%) were carbapenem resistant isolates. Phenotypically, all the 6 CRIs (100.00%) were positive for carbapenemase by mCIM. However, 5 (83.33%) were positive by CarbaNP and 4 (66.66%) were positive by MHT. Carbapenemase genes were detected in five out of the six carbapenem resistant isolates screened. The most frequently detected carbapenemase gene was bla_{OXA} gene (57.14%) followed by bla_{NDM} gene (42.86). bla_{KPC} gene was not detected (0.00%). The detection rates of OXA and NDM carbapenemases were found be to 100.0% by Carba NP test and mCIM while the rates of OXA and NDM carbapenemases by MHT were found to be 75.0% and 33.33% respectively. **Conclusion:** Carbapenemase producing *K. pneumoniae* and *E. coli* were detected both phenotypically and molecularly. The carbapenem resistant determinants were bla_{OXA} and bla_{NDM} gene.

Introduction

Carbapenem resistance in *Enterobacteriaceae* has emerged as a threat of public health concern, since carbapenems were regarded as one of the few drugs of last resort for the treatment of infections caused by multidrug resistant pathogens [1].

Carbapenem resistant *Enterobacteriaceae* (CRE) was assigned the highest threat level in 2013 and declared public health threat that requires urgent attention by the Centers for Disease Control and Prevention (CDC) [2]. Infections caused by CRE and other carbapenemase producers are associated with increased cost of treatment, long hospital stay and high mortality [3].

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Detection of carbapenemases usually involves preliminary screening for carbapenem resistance followed by phenotypic detection of carbapenemases and confirmation by PCR detection accompanied by gene sequencing. Several phenotypic tests have been developed and used by various researchers for rapid detection of carbapenemases [4]. The gold standard for carbapenemase detection is genotypic detection of the carbapenemase genes by PCR and sequencing [2].

One of the widely used phenotypic tests for carbapenemases detection in *Enterobacteriaceae* is Modified Hodge test (MHT) [5]. However, this test may give false positive results for isolates that are producers of extended spectrum beta lactamases (ESBL) and β-lactamases other than carbapenemases [6].

Carba NP test is a rapid chromogenic biochemical assay used in the detection of carbapenemases. This test is sensitive, specific and accurate in the detection of carbapenemases in *Enterobacteriaceae* [7].

Modified Carbapenem Inactivation Method (mCIM) is a phenotypic test based on the enzymatic hydrolysis of carbapenems by carbapenemases [8]. It is a rapid and simplified phenotypic test for carbapenemase detection [9]. Rapid and accurate detection of carbapenemases is vital in the choice of appropriate antibiotic therapy and control of the infections caused by carbapenemase producing pathogens.

Materials and Methods

Bacterial isolates

One hundred and twenty three isolates were screened for carbapenem resistance. The isolates consist of 70 *Escherichia coli (E. coli)* and 53 *Klebsiella pneumoniae (K. pneumoniae)* isolated from urine of patients attending selected hospitals in Zaria, Nigeria.

Screening for carbapenem resistant *E. coli* and *K. pneumoniae*

Screening for carbapenem resistant isolates of *K. pneumoniae* and *E. coli* was carried as follows; the isolates were standardized by comparing their turbidity with that of 0.5 McFarland (1.5×10^8 CFU/mL) standard and then subjected to antibiotics susceptibility test on Mueller Hinton agar by modified Kirby-Bauer disc diffusion technique using imipenem (10 µg) and ceftriaxone (30 µg) antibiotic discs.

Using the published Clinical and Laboratory Standards Institute (CLSI) break point [10], the susceptibility or resistance of the isolates to each of the antibiotics tested was determined. Isolates that were non-susceptible to imipenem and ceftriaxone were further screened for carbapenemase production phenotypically and molecularly.

Phenotypic detection of carbapenemase producing isolates

Modified Hodge Test (MHT)

A 0.5 McFarland standard suspension of the indicator organism (*Escherichia coli* ATCC 25922) was prepared in normal saline and then a 1:10 dilution of it in normal saline was inoculated on Mueller Hinton Agar plate as a lawn. The plate was allowed to dry for 10 mins. Meropenem disc was then placed at the middle of the inoculated Mueller Hinton Agar plate. Using a sterilized wire loop 5 colonies of test isolates grown overnight was picked and inoculated in a straight line out from the edge of the disc. Following incubation at 37 ^{o}C for 20 hrs, the MHA plate was examined for enhanced growth of the indicator organism around the test isolates at the intersection of the streak and the zone of inhibition. Enhanced growth of the indicator organism (*Escherichia coli* ATCC 25922) means the test isolate is positive for carbapenemase production while no enhanced growth of the indicator organism means the isolate is negative for carbapenemase production [11].

Carba NP test

The Carba NP test was performed following the protocol described in CLSI [12]. Briefly, bacteria was grown overnight on Mueller-Hinton agar (MHA). The bacterial colony was scraped off with a sterilized wireloop and suspended in a 1.5 mL Eppendorf tube containing 100 µL of 20 mMTris-HCl lysis buffer and mixed using a vortex device for 5 secs. This lysate was mixed with 100 µL of an aqueous indicator solution consisting of 0.05% phenol red with 0.1 mmol/L $ZnSO_4$, previously adjusted to pH 7.8 and 12 mg/mL imipenem-cilastatin injectable form (equivalent to 6 mg/mL of imipenem standard powder) (reaction tube). The control tube was prepared as above but without imipenem. The tubes were then incubated at 35 °C and monitored throughout 2 hrs for color change from red to orange/yellow in the antibiotic-containing tube, which was interpreted as a positive result.

Modified Carbapenem Inactivation Method

The Modified Carbapenem Inactivation Method was carried out as described in the manual of CLSI [12]. For each isolate to be tested, an overnight culture of the isolate on blood agar was emulsified in 2 mL trypticase soy broth (TSB) and then vortexed for 15 secs. Meropenem disc (10µg) was then added to each tube using sterile forcep or a single disc dispenser. The entire disc was immersed in the suspension and then incubated at 35 °C ± 2 °C in ambient air for 4 hrs ± 15 mins. Just before or immediately following completion of the TSB-meropenem disc suspension incubation, a 0.5 McFarland suspension of *E. coli* ATCC 25922 in normal saline was prepared. The standardized inoculum of *E. coli* ATCC 25922 was inoculated on MHA plate as for the routine disc diffusion procedure making sure the inoculum suspension preparation and MHA plate inoculation steps are each completed within 15 minutes. The plates were allowed to dry for 10 mins before adding the meropenem discs. The meropenem disc was removed from each TSB-meropenem disc suspension using a sterilized wire loop by placing the flat side of the loop against the flat edge of the disc and using surface tension, the disc was pulled out of the liquid. The disc was carefully dragged and pressed along the inside edge of the tube to expel excess liquid from the disc. After removing the disc from the tube it was placed on the MHA plate previously inoculated with the meropenem-susceptible *E. coli* ATCC 25922 indicator strain. The MHA plates were inverted and incubated at 35 °C ± 2 °C in ambient air for 18–24 hrs. Following incubation, the zones of inhibition was measured as for the routine disc diffusion method using CLSI, 2019 manual [10].

Isolates with zone diameter of 6-15 mm or presence of pinpoint colonies within a 16-18 mm zone were considered carbapenemase positive isolates. If the test isolate produces a carbapenemase, the meropenem in the disc was hydrolyzed and there was no inhibition or limited growth inhibition of the meropenem-susceptible *E. coli* ATCC 25922.

Isolates with zone diameter of ≥ 19 mm (clear zone) were considered carbapenemase negative isolates. If the test isolate does not produce carbapenemase, the meropenem in the disc was not hydrolyzed and growth of the meropenem-susceptible *E. coli* ATCC 25922 was inhibited.

Isolates with zone diameter of 16–18 mm, zone diameter of ≥ 19 mm and the presence of pinpoint colonies within were considered carbapenemase indeterminate isolates.

Molecular detection of carbapenemase producing isolates

Crude genomic DNA for PCR was extracted from the isolates using the heat lysis method. Briefly, colonies from overnight culture of the isolates were transferred into a test tube containing 1 mL of nuclease-free water and boiled at 100 °C for 10 minutes in a water bath and subsequently frozen at 20 °C for 10 mins. This was followed by centrifugation for 10 minutes at 3000 rpm [13]. Five microliters (5 µL) of the supernatant was used for PCR. All isolates were screened for the carbapenemase resistance genes encoding KPC, NDM and OXA by PCR assay using previously described primers [14-16]. Polymerase chain reaction and sequencing was performed in accordance with Inqaba Biotec's in-house protocol. The amplicons were visualized after running at 100 V for 90 mins on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye.

The carbapenemase gene sequences obtained were compared with those in NCBI database. A minimum sequence percent identity of ≥ 98.00% and 100.00 coverage was used to confirm the genes. Sequences of the carbapenemase genes were edited, aligned with reference sequences from the GenBank using BioEdit version 7.2.5.

Results

Out of the 70 *E. coli* isolates screened for carbapenem resistance, 2 were found to be carbapenem resistant *E. coli* giving an occurrence of 2.86%. So also, 4 isolates of *K. pneumoniae* were found to be carbapenem resistant *K. pneumoniae* out of the 53 *K. pneumoniae* isolates screened giving an occurrence of 7.55%. A total of 6 isolates out of the 123 isolates screened were carbapenem resistant **(Figure 1)**.

All the 6 carbapenem resistant isolates (100.00%) were positive for carbapenemase production by Modified Carbapenem Inactivation Method (mCIM). Four of the six carbapenem resistant isolates (66.67%) were positive for carbapenemase production by Modified Hodge Test (MHT) while five of the carbapenem resistant isolates (83.33%) were positive for carbapenemase production by the Carba NP test **(Table 1)**.

Plate I represents the agarose gel electrophoresis result of PCR amplicons for

carbapenemase genes. *bla*KPC gene was not detected in any of the carbapenem resistant isolates screened. While *bla*OXA gene (amplicon size of 597 bp) and *bla*NDM gene (amplicon size of 550 bp) were detected in 4 and 3 carbapenem resistant isolates respectively.

The most frequently detected carbapenemase gene was bla_{OXA} gene (57.14%) followed by bla_{NDM} gene (42.86). bla_{KPC} gene was not detected (0.0%) in this study **(Figure 2)**.

The detection rates of OXA and NDM carbapenemases were found be to 100.0% by Carba NP test and mCIM while the rates of OXA and NDM carbapenemases by MHT were found to be 75.0% and 33.33% respectively **(Table 2)**.

The occurrence of carbapenemase producing *Escherichia coli* was found to be 1.43%, 2.86%, 2.86% and 2.86% by MHT, mCIM Carba NP and PCR respectively while the occurrence of carbapenemase producing *Klebsiella pneumoniae* was found to be 5.66%, 7.55%, 5.66% and 5.66% by MHT, mCIM Carba NP and PCR respectively. The overall occurrence of carbapenemase producing isolates was 3.25%, 4.88%, 4.07% and 4.07% by MHT, mCIM, Carba NP and PCR respectively **(Table 3)**.

Figure 1. Occurrence of carbapenem resistant **a.** *Escherichia coli* and **b.** *Klebsiella pneumoniae.*

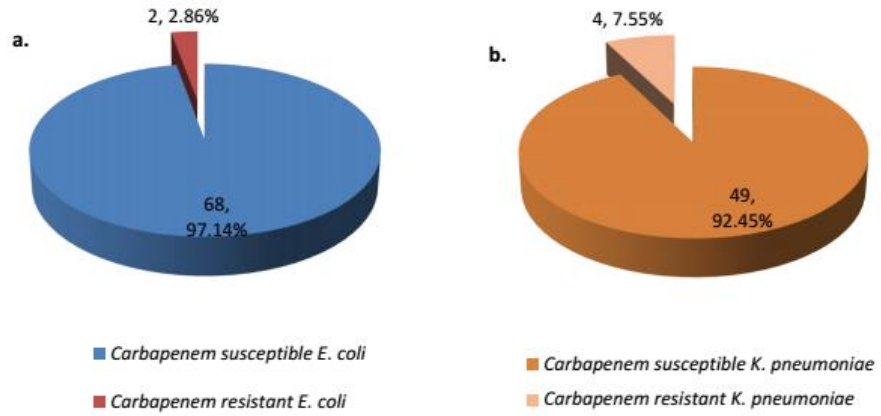

Figure 2. Percentage distribution of carbapenemase genes among carbapenem resistant isolates.

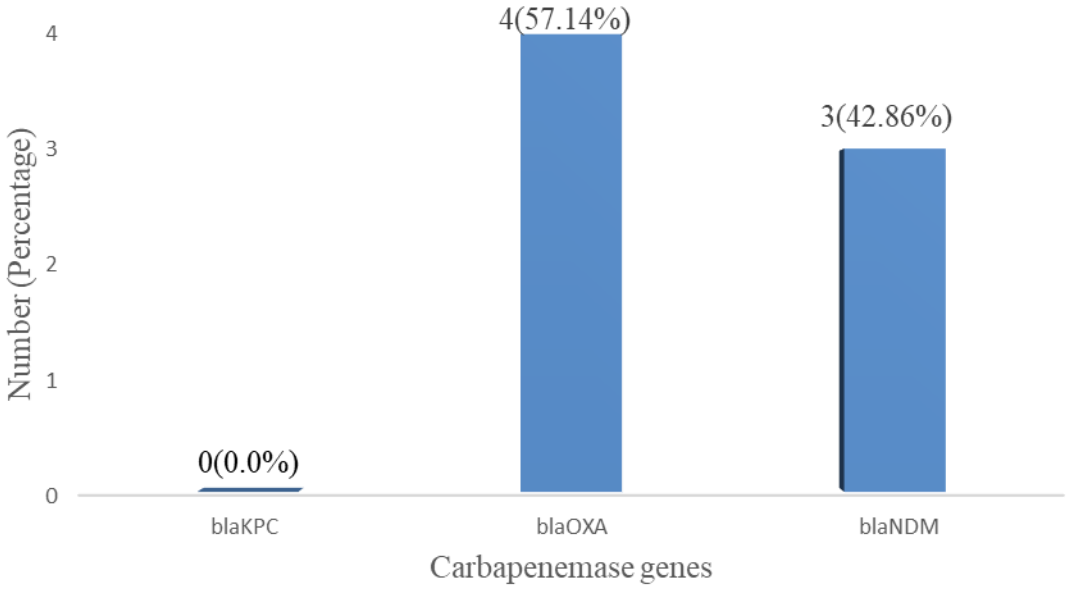

Plate I a. Carba NP Test result

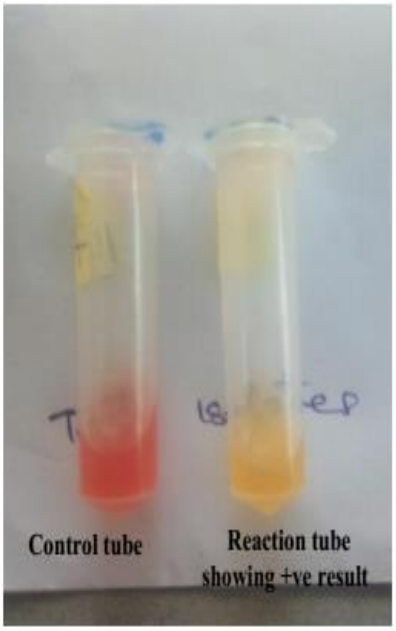

Plate I b. mCIM result

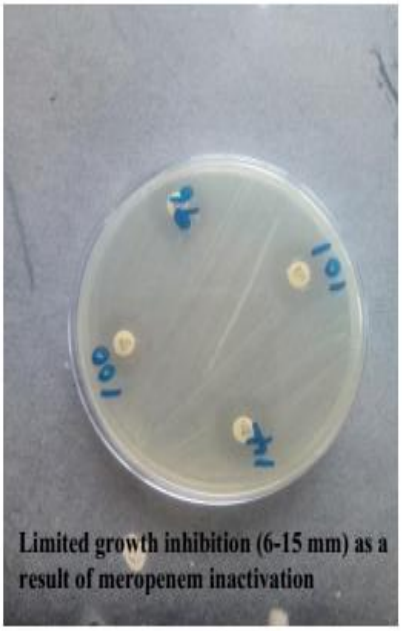

Plate I c. MHT result

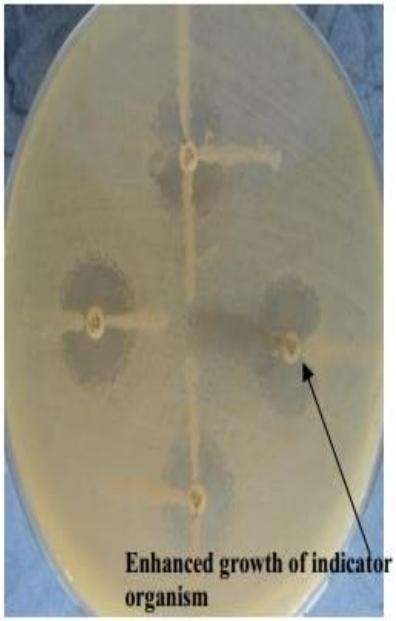

Plate I. Agarose gel eletrophoregram of amplicons of carbapenemase genes. Lane M is 100 bp molecular ladder. Lanes 8, 9, 10 and 11 had bands corresponding to 597 bp (*bla*OXA) while lanes 15, 16 and 17 had bands corresponding to 550 bp (*bla*NDM). Lanes 7, 14 and 22 were negative controls.

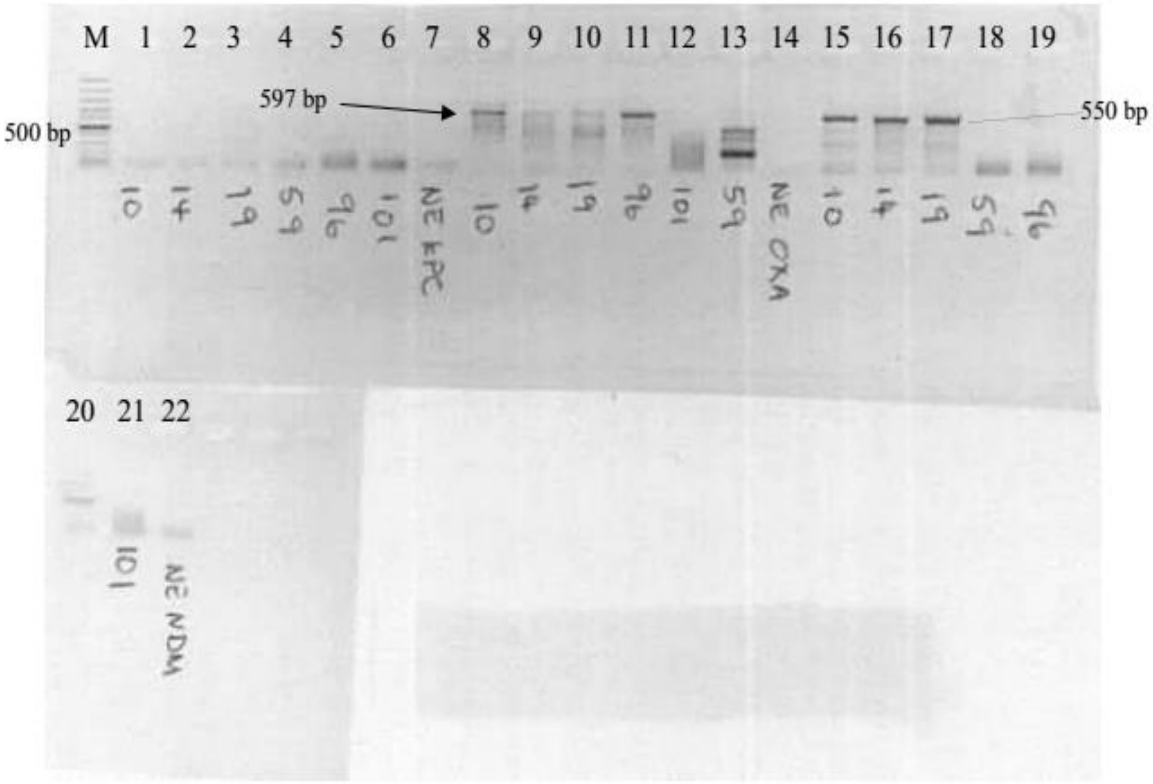

Table 1. Carbapenemase production using different detection techniques.

Isolate code	Isolate identity	Carbapenemase production		
		MHT	mCIM	Carba NP
GUM015	*Klebsiella pneumoniae*	-	+	+
MUF002	*Escherichia coli*	+	+	+
MUF012	*Escherichia coli*	-	+	+
AUM023	*Klebsiella pneumoniae*	+	+	+
GUF078	*Klebsiella pneumoniae*	+	+	-
GUF084	*Klebsiella pneumoniae*	+	+	+
	Percentage positive (%)	66.66 (4/6)	100.00 (6/6)	83.33 (5/6)

Key: MHT = Modified Hodge Test; mCIM = Modified Carbapenem Inactivation Method; Carba NP = Carba NP test; + = carbapenemase producer; - = non carbapenemase producer.

Table 2. Distribution of OXA and NDM among the isolated bacteria using different phenotypic tests.

Carbapenemases	**No. (%) detected**		
	MHT	**mCIM**	**Carba NP**
OXA (4)	3 (75.00)	4 (100.00)	4 (100.00)
NDM (3)	1 (33.33)	3(100.00)	3 (100.00)

Table 3. Occurrence of Carbapenemase Producing *Escherichia coli* and *Klebsiella pneumoniae* based on the different detection methods

Methods	***Escherichia coli* (n = 70)**		***Klebsiella pneumoniae* (n = 53)**		**Overall (n = 123)**	
	No. of CP	**Occurrence (%) of CP**	**No. of CP**	**Occurrence (%) of CP**	**No. of CP**	**Occurrence (%) of CP**
Phenotypic:						
MHT	1	1.43	3	5.66	4	3.25
mCIM	2	2.86	4	7.55	6	4.88
Carba NP	2	2.86	3	5.66	5	4.07
Molecular:						
PCR	2	2.86	3	5.66	5	4.07

Key: CP = Carbapenemase producers; n = number of isolates; MHT = Modified Hodge Test; mCIM = Modified Carbapenem Inactivation Method; Carba NP = Carba NP test; PCR = Polymerase Chain Reaction .

Discussion

Carbapenem resistant *E. coli* and *K. pneumoniae* were detected at the rate of 2.86% and 7.55% respectively with carbapenem resistant *K. pneumoniae* having higher occurrence rate. Higher occurrence of carbapenem resistant *K. pneumoniae* observed in this study might be linked to its ability to acquire and accumulate antibiotic resistance genes as reported by WHO [17]. Other researchers such as **Oduyebo et al.** [18], **Ssekatawa et al.** [19] and **Ne Gelband et al.** [20] have also reported similar phenomenon.

Detection of carbapenem resistant isolates in this study raises concern because carbapenems are usually reserved as last drug of resort for the treatment of infections caused by multidrug resistant GNB and are not commonly prescribed and used in the selected hospitals. This might as a result of international travel of patients into these regions from countries where CRE is endemic [21].

Four (66.67), five (83.33%) and six (100.00%) of the carbapenem resistant isolates (CRIs) were positive for carbapenemase production by MHT, Carba NP test and mCIM respectively. The differences observed in the detection rates might be due to difference in principle, sensitivity, specificity and accuracy of the methods in the detection of carbapenemases.

Occurrence of carbapenemase producing isolates in hospital setting has negative health implications as it can easily spread among patients and health workers. So also the carbapenemase gene can spread to other pathogens because they are located on highly mobile genetic elements [22]. This could also result to prolonged hospital stay and increased cost of treatment due to treatment failure.

The occurrence of carbapenemase producing *E. coli* was 1.43% (as detected by MHT) and 2.86 (as detected by Carba NP test and mCIM). While the occurrence of carbapenemase producing *K. pneumoniae* was 7.55% (as detected by mCIM) and 5.66% (as detected by MHT and Carba NP test). The emergence of carbapenemase producing *E. coli* and *K. pneumoniae* is of great clinical concern because these bacteria are known as the major cause of nosocomial infection [23].

The higher occurrence of carbapenemase producing *K. pneumoniae* compared to carbapenemase producing *E. coli* observed in this study is in line with the report of **Mohammed et al.** [15], **Landman et al.** [24], **Yusuf et al.** [25] and **Yusuf et al.** [26].

Genes that code for carbapenem resistance were detected in five CRIs. The carbapenem resistance determinants were bla_{OXA48} (57.14%) and bla_{NDM} (42.86%). Detection of OXA carbapenemases in *Enterobacteriaceae* is of major public health concern due to their ability to mutate rapidly thereby resulting in expanded spectrum of activity [27, 28].

The detection rate of carbapenemase genes in *E. coli* and *K. pneumoniae* was 2.86% and 5.66% respectively. Higher occurrence of carbapenemase genes in *K. pneumoniae* might be due its permeability to mobile genetic elements hence the high frequency and diversity of resistance genes observed in it. Higher occurrence of carbapenemase genes in *K. pneumoniae* compared to *E. coli* was also reported by **van der Zwaluw et al.** [8] in the Netherlands.

Carba NP test and mCIM had higher detection rates for both OXA and NDM carbapenemases compared to MHT. The low detection rate of NDM by MHT compared to Carba NP test and mCIM observed in this study is similar to the finding of **Zhou et al.** [2] who also reported low detection rate of NDM by MHT.

Ethical Approval: Ethical Approval was not required.

Conflict of Interest: None to declare.

Funding: This research work was self-sponsored

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