



Occurrence of Carbapenem Resistant *Klebsiella pneumoniae* in Clinical Samples from Some Selected Hospitals in Zaria, Kaduna State

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Authors' contributions

This work was carried out in collaboration between all authors. All authors designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript, managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

Aims: The aim of the study was to determine the occurrence of carbapenem resistant *Klebsiella pneumoniae* in clinical samples from some selected hospitals in Zaria, Kaduna state.

Study Design: Hospital based cross sectional study. The study was carried out over a period of 6 months from June to November 2015.

Methodology: A total of 150 clinical samples were collected from which 19 *Klebsiella pneumoniae* were isolated. Antibiotic susceptibility testing was carried out for all the isolates. The isolate that was resistant to Imipenem was screened for *K. pneumoniae* carbapenase production using the Modified Hodge Test.

Results: Out of the 19 isolates screened, only one was intermediately resistant to Imipenem. This isolate was screened for *Klebsiella pneumoniae* carbapenemase (KPC) production using the Modified Hodge Test (Cloverleaf test). The isolate was a non KPC producer, suggesting the

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resistance to Imipenem is likely due to other mechanism such as decreased outer membrane permeability, over expression of β -lactamases, production of cephalosporinase and porin loss but not due to carbapenemase production. Carbapenem resistant *Klebsiella pneumoniae* in Zaria as seen in this study occurs at the rate of 0% and that of KPC producing *Klebsiella pneumoniae* occurs also at 0%.

Conclusion: Even though the level of carbapenem resistance was low and none of the isolates was a KPC producer, most of the isolates were multidrug resistant isolates and this is alarming.

Keywords: Carbapenems; *Klebsiella pneumoniae*; carbapenemase; Modified Hodge Test; imipenem; resistance.

1. INTRODUCTION

Klebsiella pneumoniae is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium found in the normal flora of the mouth, skin and intestines. In the recent years, *Klebsiella pneumoniae* has become important pathogen in nosocomial infections. *Klebsiella pneumoniae* is most frequently recovered from clinical specimens and can cause a classic form of primary pneumonia.

Carbapenems are a class of β -lactam, broad spectrum antibiotic which act by inhibiting the cell wall synthesis and are known to be most effective against Gram negative infections. Carbapenem in combination with other agents, remain a mainstay of therapy in patients with serious hospital acquired infections. The introduction of carbapenem into clinical practice represents a great advancement for the treatment of β -lactam resistant bacteria. Due to their broad spectrum of activity and stability to hydrolysis by most beta lactamases, the carbapenem have been the drug of choice for treatment of infections caused by penicillin or cephalosporin resistant Gram negative bacilli [1].

Carbapenems exhibit bacteriocidal activity by binding to the penicillin binding proteins (PBP), thus preventing the linking of peptidoglycan strands and further synthesis of the bacterial cell wall [2]. Resistance to carbapenem is produced through 3 mechanisms: reduced permeability, efflux and synthesis of carbapenem β -lactamases [3].

Until recently, carbapenems were the choice for the therapeutic management of multidrug-resistant Gram-negative bacterial infections. Currently, the spread of carbapenem-resistant bacteria has caused grave concern due to the limited choice in antibiotics for treating infections caused by Gram negative bacilli [4]. Resistance

in bacteria to carbapenems mainly is due to the production of carbapenem hydrolyzing enzymes called carbapenemases. These bacteria have the potential to spread rapidly within the hospital environment and also across continents [5].

In 2001, the first KPC-producing *K. pneumoniae* isolate was reported in North Carolina, USA [6]. The enzyme (KPC-1), an Ambler class A beta-lactamase, was not the first carbapenemase to be detected in *K. pneumoniae*, as isolates harboring Ambler class B metallo-beta-lactamases capable of hydrolyzing carbapenems had previously been reported in Japan as early as 1994 [7].

Klebsiella pneumoniae carbapenemase production is an important mechanism of resistance for an increasingly wide range of Gram-negative bacteria and is no longer limited to *K. pneumoniae*. KPC-producing bacteria are often misidentified by routine microbiological susceptibility testing and incorrectly reported as sensitive to carbapenems; however, resistance to the carbapenem antibiotic ertapenem is common and a better indicator of the presence of KPCs. Carbapenem antibiotics are generally not effective against KPC-producing organisms. The common drugs of choice based on *in vitro* susceptibility testing are the polymyxins, tigecycline, and less frequently the aminoglycosides [8].

In 2012, [9] conducted a study on carbapenem resistant *Klebsiella pneumoniae* isolated from in-patients at the Lagos University Teaching Hospital over a period of 6 months. Out of the 153 *Klebsiella pneumoniae* isolates from in-patients, 8 were resistant to carbapenem while only 4 of the 8 CRKP were recognized by Modified Hodge Test as carbapenemase producers.

Resistance mechanisms have been found for every class of antibiotics [10]. The metallo

β -lactamase in Gram negative bacilli is becoming a therapeutic challenge, as this enzyme usually possesses a broad hydrolysis profile that includes the carbapenems and other β -lactam antibiotics [11]. In Nigeria there have been reports of carbapenemase producing clinical isolates of enteric bacteria particularly among *E. coli* and *Klebsiella* spp [12].

Infections caused by KPC-producing *K. pneumoniae* have been associated with increased cost and length of stay as well as frequent treatment failures and death [13]. Risk factors for infection include advanced age [14], being severely ill [15], previous treatment with antibiotics [16], organ or stem-cell transplantation, mechanical ventilation, and long hospital stays [17].

KPC-producing bacteria present a significant problem in clinical situations where administration of effective empiric antibiotics is essential to preventing mortality. This applies to serious infections such as bacteremia, but also extends to other infections in patients undergoing organ transplants and cancer treatment, where the immunocompromised status of patients requires effective empiric antibiotics [8]. Mathers et al. [18] reported two cases of orthotopic liver transplant recipients that died as a result of infections caused by KPC-producing *K. pneumoniae*. Both patients were initially treated with meropenem based on the results of routine susceptibility testing.

Accurate and timely detection of these resistance mechanisms (i.e. carbapenemase production) is very important in deciding the appropriate treatment schedule. Detection of the resistance mechanisms is always a serious challenge to the clinical laboratories [19].

Enterobacteriaceae are among the leading causes of nosocomial infections [20]. Early identification of KPC-producing bacteria with *in vitro* testing is of paramount importance to the success of infection control efforts [13]. In the appropriate setting, active surveillance can improve infection control by detecting colonization and preventing horizontal spread [21].

CRE are known to harbor additional drug-resistance genes to other antimicrobial drug classes, which may also be carried on mobile genetic elements. *K. pneumoniae* sequence type 258 strains are KPC-producing clones that

harbor Tn4401-bearing plasmids. These clones are highly effective in plasmid transfer across bacteria and are known to carry other plasmid-based antimicrobial drug resistance genes such as those that encode resistance to trimethoprim/sulfamethoxazole, aminoglycosides, and fluoroquinolones [22].

On 23rd August 2011, the Disease Daily reported an outbreak of an infection caused by a *Klebsiella pneumoniae* carbapenemase producing *Klebsiella pneumoniae* among patients at Panama's Social Security Hospital. The death toll rose to 50 with 71 patients infected within a month [23]. In the same year, U.S National Institutes of Health Clinical Center experienced an outbreak of Carbapenem Resistant *Klebsiella pneumoniae* that affected 18 patients, 11 of whom died. Integrated genomic and epidemiological analysis traced the outbreak back to three independent transmissions from a single patient who was discharged 3 weeks before the next case became clinically apparent [24].

The term "carbapenem" is defined as the 4:5 fused lactam ring of penicillins with a double bond between C-2 and C-3 and substitution of carbon for sulfur at C-1. The stereochemistry of the hydroxyethyl side chain of carbapenem is a key contributor of carbapenems and is important for activity [25]. Carbapenems like other members of β -lactams, are not easily diffusible through the bacterial cell wall [26].

The carbon atom at C-1 position plays a major role in the potency, spectrum of carbapenems and in their stability against β -lactamases. Also the strategically positioned hydroxyethyl side chain aids in resistance to hydrolysis by β -lactamases. The *trans* configuration the β -lactam ring at C-5 and C-6 results in the stability against β -lactamases [25].

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is resistant to almost all antimicrobial agents, is associated with substantial morbidity and mortality, and poses a serious threat to public health. The ongoing worldwide spread of this pathogen emphasizes the need for immediate intervention [27].

The objective of the research was to screen *Klebsiella pneumoniae* isolated from clinical samples for carbapenem resistance and further check for KPC production by the carbapenem resistant isolates.

2. MATERIALS AND METHODS

2.1 Study Area/Geographical Site

The study was conducted in Zaria. It is a major city in Kaduna State in northern Nigeria, as well as a Local Government Area. It was a formerly known as Zazzau and also one of the original seven Hausa city-states. Zaria lies within the coordinates 11°04'N 7°42'E / 11.067°N 7.700°E (<https://en.m.wikipedia.org/wiki/zaria>).

2.2 Inclusion Criteria

Patients sent to the Microbiology laboratory for suspected cases of Urinary Tract Infection, Respiratory Tract Infection or wound infection and who consented.

2.3 Sample Collection and Inoculation

A total of 150 samples of sputum, urine and wound swab were collected from patients sent to Microbiology laboratory of the selected hospitals in Zaria using convenience sampling technique. All clinical samples were collected and processed according to standard operating procedure. The samples were then inoculated on MacConkey agar and incubated overnight at 37°C [28].

2.4 Cultural Identification

The isolates were identified by their morphological characteristics on MacConkey agar. Isolates that appear as pink mucoid colonies on MacConkey after incubation at 37°C for 18–24 hours were processed for Gram staining [28].

2.5 Biochemical Characterization

The following biochemical tests were carried out to characterize the isolates: Indole production, Methyl Red - Voges-Proskauer test, Citrate utilisation test, Urease test, Glucose, Lactose, Sucrose fermentation and Hydrogen sulphide production using TSI Agar, Motility and Aesculin hydrolysis test [28].

The biochemical characteristics of *Klebsiella pneumoniae* are: indole production (-); Methyl Red (-) Voges-Proskauer test (+), Citrate utilisation test (+), Urease test (+), Glucose (+), Lactose (+), Sucrose (+) fermentation and Hydrogen sulphide production (-) using TSI Agar, Motility (-) and Aesculin hydrolysis test (+).

2.6 Antibiotic Susceptibility Test

Biochemically identified isolates of *Klebsiella pneumoniae* were standardized and subjected to antibiotics susceptibility test on Mueller Hinton agar by modified Kirby-Bauer disc diffusion technique using the following antibiotic discs (oxid): Tetracycline (30 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Cefotaxime (30 µg), Ampicillin (30 µg), Cotrimoxazole [Trimethoprim-Sulfamethoxazole] (1.25/23.75 µg), Gentamicin (10 µg) and Imipenem (10 µg).

Briefly, the inocula were standardized by using a sterilized wire to pick four or five isolated colonies of the test organism and then suspending the organism in 2 ml sterile normal saline. The suspension was then mixed properly and the turbidity was adjusted to a 0.5 McFarland standard. A sterile swab was dipped into the inoculum tubes and then excess fluid was removed by rotating the swab against the side of the tube. The Mueller Hinton agar was then inoculated by streaking the swab stick three times over the surface of the agar, rotating the plate approximately 60° each time to ensure even distribution of the inocula. The plates were allowed to set at room temperature for 3-5 minutes for the surface of the agar to dry.

Using a sterile forcep the discs were placed one at a time on the plates and pressed gently to ensure complete contact with the agar surface. The plates were then allowed to set at room temperature for 5 minutes before incubation at 37°C for 24 hours. The sizes of the zone of inhibition were measured with the aid of a ruler to the nearest millimetre. Using the published Clinical Laboratory Standards Institute [CLSI] guidelines, the susceptibility or resistance of the organism to each of the drug tested was determined.

Isolates resistant to Imipenem was screened for KPC production by the Cloverleaf test/Modified Hodge Test as recommended by the Clinical Laboratory Standards Institute [29].

2.7 Detection of *Klebsiella pneumoniae* Carbapenemase (KPC) Production Using Cloverleaf Test or Modified Hodge Test (MHT)

This test is based on the inactivation of a carbapenem by either whole cells or cell extracts of the test organisms, which enables a

carbapenem susceptible indicator strain (*Escherichia coli* ATCC 25922) to extend growth towards a carbapenem disk, along the streak of inoculum of the test strain.

A 0.5 McFarland standard suspension of the indicator organism (*Escherichia coli* ATCC 25922) was Prepare 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 3 to 10 minutes. Imipenem disk was then placed at the middle of the inoculated Mueller Hinton Agar plate. Using a sterile loop 3 to 5 colonies of MHT positive quality control strain of *Klebsiella pneumoniae* (ATCC BAA-1705) and test isolates grown overnight were picked and inoculated in a straight line out from the edge of the disk. Following incubation at 37°C for 16 to 20 hours, 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 KPC production while no enhanced growth of the indicator organism means the isolate is negative for KPC production [29]. The ATCC strains were provided by Dr. Yahaya Mohammed, Department of Medical Microbiology, Faculty of Basic Medical Sciences, College of Health Sciences, Usmanu Danfodiyo University, Sokoto, Sokoto state, Nigeria.

3. RESULTS AND DISCUSSION

Out of the 150 clinical samples collected from selected hospitals in Zaria comprising of 68 urine samples, 44 wound swabs and 38 sputum

samples, 19 *Klebsiella pneumoniae* were isolated giving a prevalence of 12.67%.

Result of the antibiotic susceptibility pattern of the isolates is presented in Fig. 2. All the isolates screened were resistant (100%) to Ampicillin and Cefotaxime. The isolates screened showed 5.3%, 21.1%, 36.8% and 42.1% resistance to Imipenem, Gentamicin, Ciprofloxacin and Chloramphenicol respectively. A moderately high and high resistance to Tetracycline (57.9%) and Cotrimoxazole (78.9%) respectively were recorded in this study.

Emergence and spread of carbapenemases account for the resistance of bacteria to carbapenems which were the “drug of last resort” in the treatment of infection caused by multidrug resistant Gram negative bacteria. The only treatment option that remains potentially toxic to carbapenems resistant bacteria is polymyxin B and colistin [30] and Tigecycline [31]. Due to the increase and spread of carbapenems resistant bacteria, in a 2013 Threat Report on Antimicrobial Resistance, the CDC prioritized CRE as an urgent threat (the highest level), requiring concerted commitment and action, and noted that ≈50% of hospitalized patients with bloodstream infection caused by CRE die from the infection [32,33]. As such it is necessary to know the prevalence of carbapenems resistance in the clinical isolates. Failure to identify them may lead to inappropriate therapy, treatment failure, spread of KPC producing organisms among patients and may result in increased mortality.

High level of susceptibility was demonstrated to Imipenem (94.7%). The high level of susceptibility demonstrated to Imipenem,

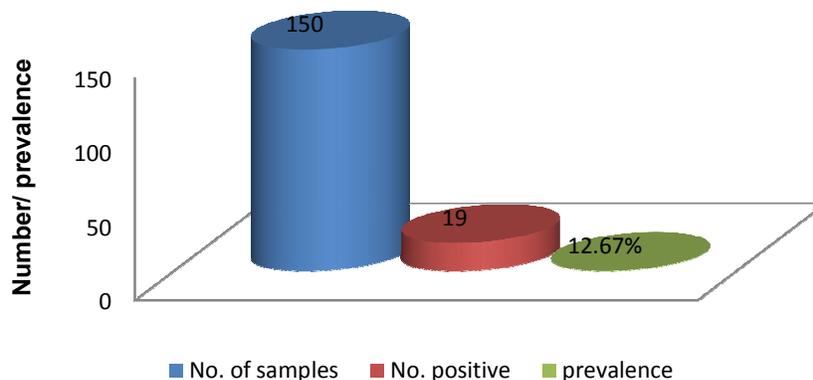


Fig. 1. Prevalence of *Klebsiella pneumoniae* isolated from clinical samples in Zaria

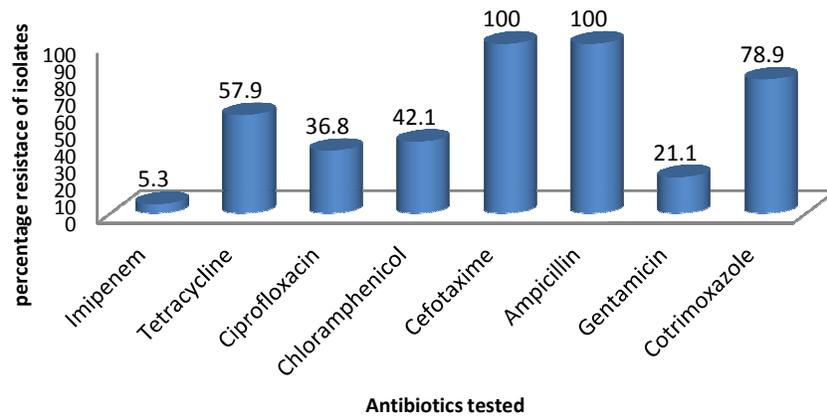


Fig. 2. Percentage resistance of *Klebsiella pneumoniae* to the antibiotics tested as determined by modified Kirby-Bauer disc diffusion technique according to CLSI cut points

Gentamicin and Ciprofloxacin in this study agrees with the study of Osundiya et al. [34], Chikwendu et al. [35] and Prado et al. [36]. The high susceptibility level (94.7%) to Imipenem recorded in this study is lower than that recorded Enwuru et al. [37] where they reported a 100% susceptibility of *Klebsiella pneumoniae* to Imipenem in Lagos. Yusuf et al. [38] reported 6-9% resistance of *Klebsiella pneumoniae* to Imipenem in Kano.

Antibiotic resistance has no border to cross especially in setting where proper infection control is not in practice. It doesn't discriminate between the specimen from which the organism was isolated or even the gender and age of the patient from which it was isolated as demonstrated by the isolates screened in this study. Table 1 shows the antibiotic susceptibility pattern of *Klebsiella pneumoniae* isolated from clinical samples in Zaria. All the isolates screened were resistant (100%) to Ampicillin. As for Cefotaxime, 94.7% resistance was recorded.

The isolates screened showed 94.7%, 78.9%, 63.2% and 57.9% susceptibility to Imipenem, Gentamicin, Ciprofloxacin and Chloramphenicol respectively. A moderately high and high resistance to Tetracycline (57.9%) and Cotrimoxazole (78.9%) respectively were recorded in this study.

The occurrence of carbapenem resistant *Klebsiella pneumoniae* in Zaria is shown in Table 2. Out of 19 *Klebsiella pneumoniae* isolates from 150 clinical samples, 1 was intermediately resistant (non-susceptible) to imipenem hence the occurrence of carbapenem resistance was 0.0%.

The occurrence of carbapenem resistant *Klebsiella pneumoniae* in this study is 0%. The low occurrence rate of carbapenem resistant *Klebsiella pneumoniae* and low level of resistance demonstrated by the isolates to Imipenem in this study could be due to the fact that (i) Imipenem (and other carbapenems)

Table 1. Antibiotic susceptibility pattern of *Klebsiella pneumoniae* isolated from clinical samples in Zaria

Antibiotic (disk content)	*n = 19		
	Susceptible	Intermediate	Resistant
Imipenem (10 µg)	18(94.7)	1(5.3)	0(0.0)
Tetracycline (30 µg)	8(42.1)	0(0.0)	11(57.9)
Ciprofloxacin (5 µg)	12(63.2)	2(10.5)	5(26.3)
Chloramphenicol (30 µg)	11(57.9)	1(5.3)	7(36.8)
Cefotaxime (30 µg)	0(0.0)	1(5.3)	18(94.7)
Ampicillin (10 µg)	0(0.0)	0(0.0)	19(100.0)
Gentamicin (10 µg)	15(78.9)	0(0.0)	4(21.1)
Cotrimoxazole (1.25/23.75 µg)	4(21.1)	0(0.0)	15(78.9)

*Total number of isolates screened (n = 19)

Table 2. Occurrence of carbapenem resistant *Klebsiella pneumoniae* isolated from clinical samples in Zaria

Number of samples screened	Number of isolates	Number of carbapenem resistant isolates	Percentage of carbapenem resistant isolates
150	19	0	0%

usage is still low in Nigeria (ii) they are expensive as such they are not subjected to abuse. (iii) They are not readily available and are reserved for life threatening Gram negative infections (iv) they are administered intravenously (v) they are not frequently prescribed.

According to CLSI [29], Carbapenemase-producing isolates usually test intermediate or resistant to one or more carbapenems and usually test resistant to one or more agents in cephalosporin subclass III (e.g. Cefotaxime). The isolate with the above definition was then screened for KPC production using the Modified Hodge test (Clover leaf test). Since the isolate shows no enhanced growth of the indicator strain (*Escherichia coli* ATCC 25922) it is negative for KPC production. Pictures 1 and 2 show the results of the screening test for KPC production using MHT.

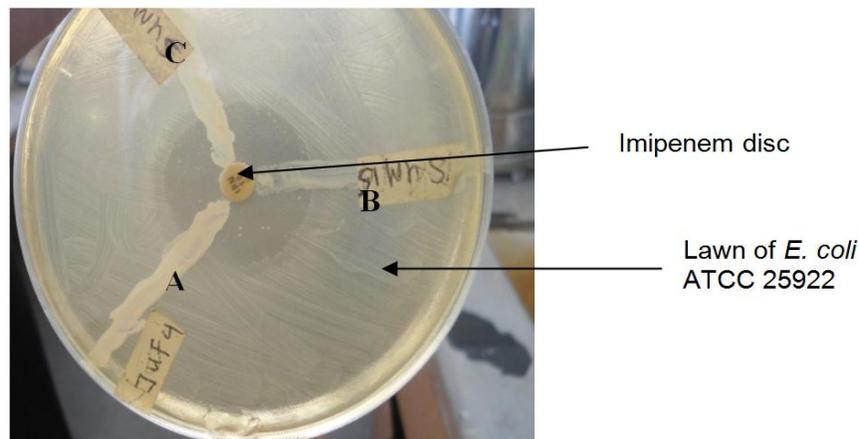
The occurrence of *Klebsiella pneumoniae* carbapenemase (KPC) producing *Klebsiella*

pneumoniae in Zaria is presented in Table 3. Only one out of the 19 isolates screened was intermediately resistant to Imipenem. This isolate gave a negative Modified Hodge Test hence the occurrence of KPC producing *Klebsiella pneumoniae* in this study is 0%.

Klebsiella pneumoniae carbapenemase (KPC) production is one of the mechanisms of resistance used by *Klebsiella pneumoniae* to resist Imipenem. In this study the isolate that was resistant to Imipenem and Cefotaxime (a third generation Cephalosporin) was screened for KPC production using the Modified Hodge Test as recommended by [19]. This isolate was found to be a non *Klebsiella pneumoniae* carbapenemase (KPC) producer, giving an occurrence rate of 0.0% for KPC producing *Klebsiella pneumoniae* in this study. This suggests that the resistance is either due to porin loss which limits the entry of the carbapenems into the cell thereby resulting in decreased outer membrane permeability or over expression of

Table 3. Occurrence of *Klebsiella pneumoniae* carbapenemase (KPC) producing *Klebsiella pneumoniae* in Zaria

Number of samples screened	Number of isolates	Number of carbapenem intermediately resistant isolate(s)	Number of KPC producers (%)
150	19	1	0(0%)



Picture. 1. Result of test for the detection of *Klebsiella pneumoniae* carbapenemase (KPC) production using Modified Hodge Test (Cloverleaf test). A, B and C are the test isolates



Picture. 2. Result of the Modified Hodge Test using the positive control strain and 9 of the isolates

β -lactamases (such as AmpC β -lactamases and ESBLs) possessing low-level carbapenemase activity [19].

The occurrence rate of KPC producing *Klebsiella pneumoniae* observed in this study (0.0%) is lower than a prevalence of (29.9%) reported by [31] among patients in intensive care unit and surgical wards of tertiary health care centers in Kano. The reason for the higher prevalence in their study is because they were dealing with hospitalized patients who form one of the populations at risk of harbouring KPC organisms. [39] reported that 10.2% of the 28 carbapenem resistant isolates screened for carbapenemase production by MHT were MHT positive (*Klebsiella pneumoniae* carbapenemase (KPC) producer) in Maiduguri.

Carbapenems resistance traits such as decreased outer membrane permeability, over expression of β -lactamases, production of cephalosporinase and porin loss are not transferable, unlike most of the carbapenemase genes. This explains why carbapenem-resistant isolates that do not produce carbapenemases are considered to be much less important from a public health perspective than carbapenemase producers. The spread of carbapenemase producers is by far the most important current clinical issue in antibiotic resistance in Gram-negatives, and must be strictly controlled [40].

4. CONCLUSION

The discovery of carbapenems was a major breakthrough in infectious disease therapeutics because of their ability to inhibit Penicillin Binding Protein (PBP) and β -lactamase. The carbapenems are regarded as the agents of “last resort” for many complicated bacterial infections. As Multidrug resistant pathogens continue to emerge, there is need to screen isolates for resistance to carbapenem and confirm them by Modified Hodge Test.

Antibiotic resistance is on the increase in Nigeria and worldwide. The occurrence of carbapenem resistant *Klebsiella pneumoniae* in Zaria according to this study is 0.0% while that *Klebsiella pneumoniae* carbapenemase producer was 0.0%. The low level of resistance recorded for Imipenem is an indication that it still remains one of the antibiotics of “last resort” for infections caused by multidrug resistant pathogens. The occurrence of carbapenem resistant organism is on the increase even though it remains low and is less common when compared to the occurrence of extended-spectrum β -lactamase (ESBL) and AmpC producing organisms.

The Modified Hodge Test is a readily available confirmatory test for KPC production. It can be performed in many clinical microbiology laboratories. Since it is less expensive and requires less expertise. Studies have shown that

MHT is very sensitive and reliable for the detection of carbapenemases.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

Ethical approval was gotten from the ethical committee of Kaduna state Ministry of Health. All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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