Original Research Article

Detection of carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* Associated Urinary Tract Infection

ABSTRACT

Background and Objectives:
Carbapenemase producing bacteria are super bugs that make UTI difficult to treat with drug of last resort such as carbapenem and other antibiotic thus limiting the treatment options. Carbapenemase production is increasing in clinical isolates of *E. coli* and *K. pneumoniae*, their potential to spread widely among patients necessitates molecular detection of **carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* Associated Urinary Tract Infection**

pneumoniae
Methodology
A total of twelve (12) non-repeated clinical isolate of *Escherichia coli* (E1, E2, E3, E4, E5, E6, E7) and *Klebsiella pneumoniae* (K8, K9, K10, K11, K12) selected based on their *in vitro* phenotypic resistant to carbapenem antibiotics from selected patients diagnosed with urinary tract infection at Alex Ekwueme Federal University Hospital Teaching Hospital, Abakaliki (AE-FEUTHA) Ebonyi Sate Nigeria. *Escherichia coli* and *Klebsiella pneumoniae* were further confirmed using standard routine microbiological technique for isolation and identification. *Escherichia coli* and *Klebsiella pneumoniae* strains were further screen for carbapenemase producing gene by PCR specific primer.
Result
PCR analysis with specific primer for carbapenemase gene revealed the presence and predominat of *bla*KPC in *Escherichia coli and Klebsiella pneumoniae* accounted 12(100 %) followed by bla_{NDM} 11(91.7 %), bla_{IMP} 7(58.3 %) and bla_{VIM} 2(16.7) as the least carbapenemase producing gene in *Escherichia coli and Klebsiella pneumoniae*. bla_{KPC} was predominant in *Escherichia coli* 7(58.3 %) followed by bla_{NDM} 6(50.0 %) and bla_{IMP} 5(41.7 %) while both bla_{OXA} and bla_{VIM} (16.7 %) were the least detected carbapenemase gene. *Klebsiella pneumoniae* harbor high proportion of bla_{NDM} and bla_{KPC} both recording 5(41.7 %) followed by bla_{OXA} and bla_{IMP} both recording 2(16.7 %) but bla_{VIM} gene was not documented in all the isolate.
Conclusion
The findings of this study highlight the occurrence of carbapenemase-producing gene in *Escherichia coli* and *Klebsiella pneumoniae* in UTI. Since these genes are carried on the bacteria plasmid there is a tendency of cross-species dissemination over time. The detection of carbapenemase gene call for prompt epidemiological surveillance and preventive strategies to limit the spread of these carbapenemase resistant genetic determinant and the need for antibiotic susceptibility testing of available antibiotic agent.

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Keywords: Urinary Tract Infection, Carbapenemase-producing, *Escherichia coli, Klebsiella pneumoniae*

1. INTRODUCTION

A carbapenemase producing *Escherichia coli* or *Klebsiella pneumoniae* is a bacterium that is resistant to carbapenem antibioitics in susceptibility testing or has a carbapenemase gene [1]. In 2017, the World Health Organization designated carbapenemase-producing *E. coli* and *Klebsiella pneumoniae* as 'critical' and 'high priority pathogens' [2]. Since then, carbapenem resistant *Escherichia coli* and *Klebsiella pneumoniae* is a growing concern worldwide [3, 4] due to rapid evolution and spread of carbapenemase producing genotype e.g., New Delhi metallo-β-lactamase (bla_{NDM}), Imipenemases (bla_{IMP}), Verona integron Metallo-beta-lactamase (bla_{VIM}), Oxacillinase (bla_{OXA}) and *Klebsiella pneumoniae* carbapenemase (bla_{KPC}) which are increasingly being reported among health care associated complicated UTIs owing to their ability to truncates the action of carbapenem and other beta-lactam antibiotics [4, 5, 6]. Urinary Tract Infections (UTIs) are infectious diseases commonly caused by enterobacteriaece such as *Escherichia coli* and *Klebsiella pneumoniae* [4, 7, 8]. These bacteria invade and colonize any part of the urinary tract [4, 9] producing symptoms such as fever, burning sensations while urinating, lower abdominal pain (LAP), itching, blister and ulcer formation in the genital area, genital and suprapubic pain, and pyuria. These are generally determined by the age of the person infected and the location of the infected urinary tract. UTIs are common bacterial infections that affect approximately 150 million people worldwide each year, posing a significant financial burden on the community and health-care system [6]. The prevalence and rapidly evolving carbapenemase gene from *Escherichia coli* and *Klebsiella pneumoniae* make UTI difficult to treat with drug of last resort such as carbapenem thus limiting the treatment options. These resistance genes containing bacteria are super bugs and are termed as carbapenemase producing *Escherichia coli* and *Klebsiella pneumoniae* challenging the empiric treatment of UTI worldwide. Globally, these gene is progressively spreading among clinical isolates of *E. coli and K. pneumoniae* widely amongst patients and has necessitate the need for molecular **investigation of carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* sssociated with Urinary Tract Infection.**

2. MATERIALS AND METHODS

2.1 Characterization of *Escherichia coli* and *Klebsiella pneumoniae*

Non-repeated clinical isolate of *Escherichia coli* (E1, E2, E3, E4, E5, E6, E7) and *Klebsiella pneumoniae* (K8, K9, K10, K11, K12) were collected from selected patient diagnosed with Urinary tract infection at Alex Ekwueme Federal University Teaching Hospital, Abakaliki (AE-FEUTHA) Ebonyi State Nigeria. The clinical isolate were selected based on their *in vitro* phenotypic resistant to carbapenem antibiotics. All the information obtained from the studied subjects was coded to maintain confidentiality and in accordance with the World Medical Association (WMA) declaration of Helsinki on the principles for medical research [10, 11]. The twelve clinical isolate of *Escherichia coli* and *Klebsiella pneumoniae* were further confirmed using standard routine microbiological Technique [12, 13].

2.2 Molecular screening for carbapenemase genes using Polymerase Chain Reaction (PCR)

2.2.1 DNA extraction

Genomic DNA Extraction of *Escherichia coli* and *Klebsiella pneumoniae* was performed using ZR Fungal/Bacterial DNA Miniprep™ (Manufactured by Zymo research, cat number: D6005) kit according to the manufacturer's protocol. Exactly 2 ml of bacterial cells broth was added to a ZR Bashing TM lysis tube and 750 µg lysis solutions was added to it. This was secured in a bead fitted with 2 ml tube holder assembly and processed at a maximum speed for 5 minutes. The ZR bashing bead Tm lysis tube was centrifuged at > 10,000 x g for 1 minute. Up to 400 µg of supernatant was transferred to a Zygomo-Spin TM IV Filter in a collection tube and centrifuged at 7,000 x g for I minute. Exactly 1,200 µg of bacterial DNA binding buffer was added to the filtrate in the collection tube. Exactly 800 ul of the mixture from step 5 was transferred to a Zygomo-spinTM IIC column in a collection tube and centrifuged at 10,000 x g for 1 minute. The flow through from the collection tube was discarded. Then 200 ul DNA Pre-washed buffer was added to the Zymo-Spin TM IIC column in a new tube collection and centrifuged at 10,000 x g for 1 minute. About 200 µg of bacterial DNA Buffer was added to the Zymo-Spin TM IIC column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin TM IIC column was transferred to a clean 1.5 ml micro-centrifuge tube and 100 µl (35 µlminimum) DNA was added directly to the column matrix. Centrifuge was done at 10,000 x g for 30 seconds to elute DNA [11, 14].

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2.2.2 Electrophoresis for DNA and PCR Product

Exactly 1 g of agarose was measured (for DNA) and 2 g of agarose for PCR products. Agarose powder was mixed with 100 ml 1xTAE in a microwave flask. This was microwaved for 1-3 min until agarose completely dissolved. The agarose solution was allowed to cool to about 50 ^{o}C. Then 10 µg EZ vision DNA stain was added. The agarose was poured into a gel tray with the well comb in place. The newly poured gel was placed at 4 ^{o}C for 10-15 mins until it has completely solidified [11, 14].

2.2.3 Loading Samples and Running an Agarose Gel.

A loading buffer was added to each DNA sample of PCR products. Once it got solidified the agarose gel was placed into the gel box (electrophoresis unit). The gel box was filled with 1xTAE buffer until the gel was covered. A molecular weight ladder was carefully loaded into the first lane of gel and the samples were carefully loaded into the additional wells of gel. The gel was run at 80-150 V for about 1-1.5 h. The power was turned off, and the electrodes disconnected from the power source and then the gel was carefully removed from the gel box. The DNA fragments or PCR fragments was visualized under UV trans-illuminator [15, 16].

2.2.4 PCR Mix Components

The PCR mix components was made up of 12.5 µl of Taq 2 x Master Mix from New England Biolabs (M0270); 1 µl each of 10µm forward and reverse primer (Invitrogen, U. S. A™) (Table 1); 2 µl of DNA template and then made up with 8.5 µL Nuclease free water. The PCR reactions was executed as previously described by Edemekong *et al.* [14].

2.2.5 Cycling Conditions

Initial denaturation at 94˚ C for 5 mins, followed by 36 cycles of denaturation at 94˚ C for 30 secs, annealing at 55˚ C for 30 second and elongation at 72 ˚C for 45 seconds. Then a final elongation step at 72˚ C for 7 minutes and hold temperature at 10°C. Initial denaturation at 94˚C for 5mins, followed by 40 cycles of denaturation at 94˚C for 30sec, annealing at 37˚C for 30secs and elongation at 72˚C for 45 sec. Followed by a final elongation step at 72˚C for 7 minutes and hold temperature at 10 ˚C [15, 16, 17].

Table 1: The following Primer Sequences was use for detection of Cabapenemase resistance genes [15, 16].

Primers	Sequence (5'–3'),	Amplicon size (bp)	Annealing Temperature
bla_{KPC}	F: CGTCTAGTTCTGCTGTCTTG R: CTTGTCATCCTTGTTAGGCG	500	**56° C**
bla_{OXA}	F:GCGTGGTTAAGGATGAACAC R: CATCAAGTTCAACCCAACCG	550	**56° C**
bla_{IMP}	F:CTACCGCAGCAGAGTCTTTGC R: ACAACCAGTTTTGCCTTACC	232	**45° C**
bla_{NDM}	F:GCAGCTTGTCGGCCATGCGGGC R: GGTCGCGAAGCTGAGCACCGCAT	800	**52° C**
bla_{VIM}	F:AAAGTTATGCCGCACTCACC R: TGCAACTTCATGTTATGCCG	250	**56° C**

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3. RESULT

Molecular detection of Carbapenemase-producing gene in UTI patients harboring Uropathogenic *Escherichia coli and Klebsiella pneumoniae*

The higher proportion of carbapenemase-producing gene in all the bacterial isolate in this study was bla_{KPC} 12(100 %), followed by bla_{NDM} 11(91.7 %), bla_{IMP} 7(58.3 %) and bla_{VIM} 2(16.7). The bla_{KPC} was predominant in *Escherichia coli* 7(58.3 %) followed by bla_{NDM} 6(50.0 %) and bla_{IMP} 5(41.7 %) while both bla_{OXA} and bla_{VIM} (16.7 %) were the least detected carbapenemase gene. *Klebsiella pneumoniae* harbored higher proportion of bla_{NDM} and bla_{KPC} both recording 5(41.7 %) followed by bla_{OXA} and bla_{IMP} both recording 2(16.7 %) while *bla*VIM gene was not detected as shown in Table 2.

Table 2: Molecular detection of Carbapenemase producing gene in Uropathogenic *Escherichia coli and Klebsiella pneumoniae*

Carbapenemase Class	Genes	Uropathogenic (n=12)		
		E1-7 (%)	**K8-12 (%)**	**Frequency (%)**
A	bla_{KPC}	7(58.3)	5(41.7)	**12(100)**
B	bla_{IMP}	5(41.7)	3(25.0)	**7(66.7)**
B	bla_{VIM}	2(16.7)	0(0.0)	**2(16.7)**
B	bla_{NDM}	6(50.0)	5(41.7)	**11(91.7)**
D	bla_{OXA}	2(16.7)	2(16.7)	**4(33.3)**

Key: **n**-Number of isolate, ***bla*KPC**-*Klebsiella pneumoniae* carbapenemase, ***bla*IMP**-Imipenemases, ***bla*VIM**-Verona integron Metallo-beta-lactamase, ***bla*NDM**-New Delhi Metallo-beta-lactamase, ***bla*OXA**-Oxacillinase

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4. DISCUSSION

The molecular detection of carbapemase-producing genes among the isolates in this study revealed that the *bla*$_{\text{KPC}}$ in *Escherichia coli* and *Klebsiella pneumoniae* accounted for 12(100 %). The plasmid-encoded KPC enzyme was first identified in a *K. pneumoniae* isolate from North Carolina, USA [18]. Since 2001, these isolates have spread across the USA, with reports of KPC-producers across 38 states [19]. Although KPC-producers are now being identified at an alarming rate across Europe, facilitated mostly by clonal dissemination, to the best of our knowledge there is no published report of bla$_{\text{KPC}}$-positive isolates from this area of study (Abakaliki) while elsewhere of Nigeria such as Maiduguri, northeast Nigeria and in two Chinese studies, bla$_{\text{KPC}}$ has been reported as the predominant carbapenemase gene [16, 20, 21].

The *bla*$_{\text{NDM}}$ 11(91.7 %) was the second most predominant carbapenem gene, despite the fact that NDM-1 bearing gene is increasingly reported worldwide, NDM-1 has been most commonly identified in *E. coli* and *K. pneumoniae* [6, 21, 22, 23, 24, 25] as evidence in this study. This study inferred that the frequent switch of predominant carbapenemase genotype might result from introduction of those strains from different sources (like some regions, animals or food) with different popular carbapenemase genes, or transformation of some mobile elements that carried carbapenemase genes between species [26, 27, 28]. Although the driving mechanism for this situation remains unclear, it further highlights the importance of long-term active resistance surveillance of CR-isolate in community and hospital setting. Although existing literature highlight India and Pakistan being considered to be the main reservoir for NDM-producing isolates which were detected in this study, it has been suggested that the Middle East region might be a secondary reservoir for the spread of bla$_{\text{NDM}}$-1 isolates as there is a high frequency of population movement between Saudi Arabia and the Indian subcontinent [29, 30, 31, 32]. This study speculate that population movement where *bla*$_{\text{KPC}}$ and *bla*$_{\text{NDM}}$ is endemic and was first identified, might explain the high occurrence of isolates with *bla*$_{\text{KPC}}$ resistance gene in this setting, although the absence of data on patient travel history to those endemic area precludes any firm conclusions being drawn.

bla$_{\text{OXA}}$ recorded 4(33.3 %) but not were predominantly detected as commonly speculated as being the most frequently detected gene in *K. pneumoniae* and *E. coli* and other Enterobacterales. The production of OXA is one of the main mechanisms of carbapenem resistant in most bacteria. This finding correspond with Alizadeh *et al*. [33] who reported percentage of carbapenemase genes in *K. pneumoniae*; blaOXA-like (78%) while different *E. coli* clones have successfully emerged in the world; examples are *E. coli* O15:K52:H1-D of sequence type 393 (ST393), *E. coli* ST131, and *E. coli* ST38, which is characterized by the production of the OXA β-lactamase and is related to strains of the Mediterranean basin countries [34, 35]. The role of bla$_{\text{OXA}}$ strain in UTI in this study can't be underestimated but virulence has been linked to the presence of *bla*$_{\text{OXA–48}}$ in clinical *E. coli* and *K. pneumoniae* isolates. Several studies reported on clinical isolates with an unusual high lethality in murine infection models as well as the presence of genes associated with virulence or host colonization, but the specific role of OXA-48 had not been addressed [36, 37, 38].

To the best of our knowledge this study tends to be the first to report the present of *bla*$_{\text{VIM}}$ 2(16.7) producing *E. coli* in Abakaliki while isolate positive for the bla$_{\text{VIM}}$ gene has been found by other researchers [20, 23, 39, 40, 41] and in one study it was located on an *IncI1* plasmid of a novel sequence type (ST 297) [42]. Since this gene are carried on plasmid there is a tendency of cross-species dissemination over time.

Although bla$_{\text{IPM}}$ 66.7 % was reported in this finding, notably this genes is not commonly reported among *E. coli* and *K. pneumoniae* but the co-existence of Amber class A such as bla$_{\text{IPM}}$ with Amber class B and D are known to confer resistance to oxyiminocephalosporins (ceftriaxone and ceftazidime) and cephamycins (cefoxitin), and transfer of the resistance has been confirmed by transconjugation [43, 44, 45]. Therefore, it should be noted that carbapenemase reservoirs in healthcare workers, patients, or the hospital environment may be a principal mode of spread in nosocomial outbreaks.

5. CONCLUSION

This finding echoes a successfully emergence of carbapenemase producing genes in *E. coli* and *K. pneumoniae* associated UTI. Although, *bla*VIM gene was absent in *K. pneumoniae*, the virulence role of carbapenemase producing genes in UTI in this study can't be underestimated as they can lead to persistence of UTI among patients due to treatment failure. The detection of carbapenemase gene call for prompt epidemiological surveillance and preventive strategies to limit the spread of these carbapenemase resistant genetic determinant and the need for antibiotic susceptibility testing of available antibiotic agent .

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CONSENT
As per international standard or University standard, patients' written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL
In compliance with international standard or University standard written ethical approval with number SMOH/ERC/042/21 has been collected and preserved by the author (s).

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