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Conjugative plasmid transfer among CTX-M-15 *Klebsiella pneumoniae* clinical isolates

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ABSTRACT: Conjugational transmission of antibiotic resistance genes across bacterial species and genera has aggravated the problem of *Klebsiella pneumoniae* antibiotic resistance. This study aimed to determine the conjugative plasmid transfer among CTX-M-15 *Klebsiella pneumoniae* isolates. A total of 121 *K. pneumoniae* isolates were identified using conventional bacteriological techniques and production of Extended Spectrum Beta Lactamase was confirmed using double disk synergy test. Molecular detection of antibiotic resistance genes and plasmid replicons were done by PCR and plasmid based replicon typing methods respectively. Plasmid transfer capacity of the isolates harboring the CTX-M-15 genes was assessed using broth mating method. Out of the 121 isolates screened for ESBL production, only 48 (39.7%) of the *K. pneumoniae* isolates were ESBL producers. CTX-M-15 (n = 38, 86.4%) was the most frequently occurred ESBL gene detected. FIIK was the most prevalent replicon identified in 14(36.8%) isolates. Conjugation experiment was successful for 19(62.5%) out of 32 CTX-M-15 *K. pneumoniae* isolates selected as donors for conjugation based on their plasmid replicons. The replicons detected in the transconjugants included FIIK (n=10; 34.4%), FIB (n=5; 15.6%) and then FIA and R (n=4; 12.5%). A statistically significant association (P = 0.03) between the presence of CTX-M-15 and plasmid replicons was observed in this study. The spread of $bla_{CTX-M-15}$ gene is driven mainly by the horizontal transfer of IncF group of plasmids which is inferred from the predominance of these plasmids among the isolates.

Keywords: Antibiotic resistance, CTX-M-15, Extended Spectrum Beta Lactamase, *Klebsiella pneumoniae*, plasmids replicons.

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen related to both community- acquired and healthcare associated infections, such as pneumonia, pyogenic liver abscess, urinary tract infections, sepsis, burns and wound infections worldwide (Odewale *et al*., 2023). The increasing prevalence of multidrug-resistant *K. pneumoniae* has led to it being classified as a major public health concern (Caneiras *et al*., 2019; Sa'id *et al*., 2020). However, *K. pneumoniae* has become a clinically important microorganism, particularly in last two decades due to its tendency to develop antibiotic resistance and cause lethal outcomes (Cao *et al*., 2014; WHO, 2017). High frequency of infections caused by *K. pneumoniae* in the clinical setting in Nigeria has affected many departments particularly intensive care units, post-operative and pediatrics (Giwa *et al.,* 2018). *K. pneumoniae* isolates are frequently resistant to broad-spectrum beta lactam antibiotics, due to the production of Extended-Spectrum β-Lactamases (ESBLs), thus, an evidence of the role of *K. pneumoniae* as a reservoir for plasmids carrying antibiotic resistances genes (Hennequinn *et al.,* 2012; Ferreira *et al*., 2019). The production of extended-spectrum lactamases (ESBL) in this organism contributes to the emergence and dissemination of multidrug resistant *K. pneumoniae* strains (Caneiras *et al*., 2019).

The most common ESBL genes isolated from clinical specimens are Cefotaximase-Munich (CTX-M), Sulfhydryl Variant (SHV) and Temonera (TEM) (Paterson and Bonomo, 2005; Xu *et al*., 2019). During the past decade, CTX-M-type has represented the most rapidly growing group of ESBLs; specifically, CTX-M-15 has recently emerged as the dominant type of cefotaximase in Enterobacteriaceae responsible for imparting drug resistance resulting to outbreaks in hospital settings worldwide (Iroha *et al*., 2011; Xu *et al*., 2019). Multidrug resistance *K. pneumoniae* is well known as a reservoir of these resistance genotypes encoded on plasmids which are responsible for the transfer of antimicrobial resistance across same species and other different species (Navon-Venezia *et al*., 2017). The IncF group of plasmids are typical plasmid types that greatly contributes to the spread of antibiotic resistance genes in Enterobacteriaceae with CTX-M-15-positive IncFIIK plasmids oftenly characterized in *K. pneumoniae* (Dolejska *et al*., 2012; Johnson and Nolan, 2009; Wyres *et al*., 2019). These plasmids are responsible for the intracellular accumulation and intercellular transfer of antibiotic resistant genes through the mechanism of conjugation (Jordt *et al*., 2020). In such cases, even in the absence of selection pressure, high rates of conjugation ensure steady and long-term persistence of plasmids and associated resistant genes in minimal fitness costs (Hawkey *et al*., 2022). Consequently, the dissemination of these resistant strains has ruined the use of β-lactams which are considered the most available, safest and currently used antibiotics for treatment of bacterial infections worldwide (Agyepong *et al*., 2019). Data on such circumstances are limited in the North eastern region of Nigeria. However, this study determined the conjugative plasmid transfer among CTX-M-15 *Klebsiella pneumoniae* isolates. The research was carried out with great hope that it could improve our understanding on the dissemination of IncF plasmid among ESBL *K. pneumoniae* strains and also contributes to the establishment of a modified-therapeutic-antimicrobial strategy to hinder or even eliminate the transmission and spread of such pathogenic strains in the hospital settings.

MATERIALS AND METHODS

Study sites and sources of clinical isolates

This study was carried out at the Modibbo Adama University Teaching Hospital, Yola (former Federal Medical Centre, Yola) and Specialist Hospital, Jimeta. The two hospitals are both referral and tertiary health care facilities located in Yola, capital city and administrative center of Adamawa State in the North-East region of Nigeria..

The sample was collected and processed from February, 2021 to July, 2022. A total of 121 clinical *Klebsiella pneumoniae* were isolated and identified by conventional bacteriological tests (Podschun and Ullaman, 1998). Fifty eight of the *K. pneumoniae* isolates were from urine samples, 44 from sputum samples and 19 were from wound samples. They were further confirmed by microscopy and biochemical tests using API 20 E kit (BioMerieux, France) for identification of *K. pneumoniae.*

Ethical considerations

Ethical clearance were received from the Research Ethical Committee of Modibbo Adama University Teaching Hospital, Yola, Yola (Ref. No.: FMCY/SUB/96N/T/36) and Specialist Hospital, Jimeta (Ref. No.: ADS/SHY/SUB/301/VOL.). Samples from the participants were only included in the study after the consent was taken from each participant.

Phenotypic detection of ESBL production

Phenotypic detection of ESBL was carried out by first screening the isolates using standard disc diffusion method following the guidelines of Clinical Laboratory Standard Institute (CLSI, 2021). The beta lactam antibiotic discs used for the screening included cefotaxime, ceftazidime, cefpodoxime and aztreonam. Isolates with the following zone diameters to these antibiotics; ceftazidime zone ≤ 22 mm, cefpodoxime zone ≤ 17 mm, aztreonam zone ≤ 27 mm and cefotaxime zone ≤ 27 mm shows comparatively high level of resistance and are suspicious of ESBL production.

The Isolates were then subjected to the double disc synergy test (CLSI, 2021) to confirm for the presence of ESBL producing enzymes. Briefly, isolates suspicious of ESBL production were cultured overnight on nutrient agar. A suspension prepared to match a 0.5 McFarland turbidity standard was inoculated using a sterile cotton swab onto the entire surface of each of the Mueller Hinton agar plates. Amoxicillin (20 µg)/clavulanic acid (10 µg) combination disc was placed at the center of each inoculated Mueller Hinton agar plate. Cefotaxime (30 µg) and ceftazidime (30 µg) single discs were then placed 20 mm (center to center) from the amoxycillin/clavulanic acid disc and incubated at 37ºC for 18 hours. Enhancement of the zones of inhibition of either cefotaxime or ceftazidime disc towards the amoxycillin/clavulanic acid disc was taken as evidence of ESBL production.

Extraction of the plasmid deoxyribonucleic acid (DNA)

To extract plasmid DNA, bacterial cultures were processed using the Qiagen kit according to the manufacturer instructions (Qiagen Biotech. South Africa). The bacterial plasmids were run on 1% agarose gel and the results were

Table 1. Nucleotide sequences of the primers used for the detection antibiotic resistance genes.

Primer name	5` - 3` Primer sequence	Annealing temp. (0C)	Product size (bp)	References
CTX-M-1 F	CGATGTGCAGTACCAGTAA	57	585	Soge *et al*., 2006
CTX-M-1 R	TTAGTGACCAGAACAGCGG			
CTX-M-3 F	GTGACAAAGAGAGTGCAACG	60	1200	Soge *et al*., 2006
CTX-M-3 R	ATGATTCTCGCCGCTGAAGCC			
CTX-M-9 F	CGATGTGCAGTACCAGTAA	60	857	Gharrah *et al*., 2017
CTX-M-9 R	TTAGTGACCAGAACAGCGG			
CTX-M-15 F	GTGATACCACTTCACCTC	60	800	Gharrah *et al*., 2017
CTX-M-15 R	AGTAAGTGACCAGAATCAG			

read using ultra violet light in gel documentation system for detection of their pattern. The DNA was stored at -20°C until further use.

Molecular detection of ESBL genes by polymerase chain reaction (PCR)

All ESBL producing isolates were screened for the presence of antibiotic resistance genes encoding CTX-M-1, CTX-M-3, CTX-M-9 and CTX-M-15. The plasmid DNA extracted was used as a template for the molecular detection of these target genes using conventional PCR technique. The primer sequences, annealing temperature and the product size used for the amplification were listed in Table 1. Gel electrophoresis was run to visualize the PCR products and the sizes of the PCR products were estimated by comparison with the mobility of a molecular weight ladder that ran alongside experimental samples in the gel.

Plasmid Identification by PCR-based replicon typing

The CTX-M-15 isolates were assessed by PCR-based replicon typing (PBRT) to detect the replicon types of CTX-M-15 encoding plasmids (Carattoli *et al.*, 2005). A commercially available PBRT kit was used (Diatheva, Fano P, UItaly). The kit is based on eight multiplex PCR reactions. Gel electrophoresis was run to visualize the amplicons by loading dye (5.0 µl) and 2.5% agarose gel directly to the PCR product. The gel was run on 120 volt for 70 minutes. Visible bands were compared with the positive control and identified.

Generation of ESBL-producing transconjugants

Conjugation was carried out by broth mating method to test the transmissibility of ESBL plasmid from *K. pneumoniae* isolates as donors to a recipient *E. coli* J53 AzR strain (plasmid free and azide resistant). The donors and recipients-plasmid free, azide resistant strains were incubated both on broth culture (100 µL of the donor's isolate cultures was added to 1 ml of the recipient isolate i.e. mixed in the ratio of 1:10 donors : recipient strains) at 37°C for 18 hours. The transconjugants were selected on nutrient agar medium supplemented with cefotaxime 2 µg/ml and azide 100 µg/ml to inhibit the growth of the donor and recipient respectively. Controls were prepared for donor and recipient isolates by inoculating both of them onto nutrient agar with the same antibiotics.

Data analysis

Data from the work were entered in the worksheet of SPSS version 20.0. Frequency and percentages were analyzed as descriptive findings. Two way ANOVA was used to compare the mean differences in ESBL production and conjugative transfer capacity of the CTX-M-15 encoding plasmid replicons. The significant difference was evaluated at $p \leq 0.05$.

RESULTS

Out of the 121 *K. pneumoniae* isolates screened for ESBL production, 48 (39.7%) were found to be ESBL producers (Table 2). The results indicated that the isolates were able to enhance the inhibition zones of cefotaxime and ceftazidime on the site facing the amoxicillin-clavulanate disk. This enhancement towards the amoxicillin-clavulanate disk was interpreted as a positive test.

Characterization of CTX-M ESBL genes

The frequency of occurrence of the CTX-M ESBL genes detected among the ESBL *K. pneumoniae* isolates showed that only 44 (91.7%) out of the 48 ESBL producers were found to possess CTX-M genes (Table 3). The CTX-M genes found were as follows: CTX-M-15 (n = 38, 86.4%) was the most prevalent ESBL gene detected, followed by CTX-M-3 (n = 3, 6.8%). CTX-M-9 (n=2, 4.5%) and CTX-M-1 (n=1, 2.3%) were detected at low rate (Table 3).

Table 2. ESBL production ability of the isolates.

β-lactam antibiotics	SDDT		DDST	
	Resistance n (%)	Susceptible n (%)	+ve n (%)	-ve n (%)
Cefotaxime	98 (81.0)	23 (19.0)	48 (39.7)	50
Ceftazidime	98 (81.0)	23 (19.0)	48 (39.7)	50
Cefpodoxime	94 (77.7)	27 (19.8)	NT	NT
Aztreonam	78 (64.5)	43 (35.5)	NT	NT

Keys: SDDT: Standard disk diffusion test; DDST: Double disk synergy test; +ve: positive; -venegative; NT: not tested; n: no of isolates; %: percentage of occurrence.

Table 3. Occurrence of ESBL-encoding genes in *K. pneumoniae* Isolates by PCR.

ESBL- encoding Genes	n (%)
CTX-M-15	38 (86.4)
CTX-M-3	3 (6.8)
CTX-M-9	2 (4.5)
CTX-M-1	1 (2.3)
Not Detected	4 (9.1)
Total	48(100)

Keys: n- Number of isolates; %- Percentage occurrence.

Table 4. Results of PCR-based replicon typing (PBRT) of the plasmids carried by CTX-M -15 encoding clinical *K. pneumoniae* isolates.

Replicon types	Replicon number	n (%)
FIIK	1	14(36.8)
FIA		2(5.3)
FIB		3(7.9)
R		5(13.2)
FIIK + R	2	2(5.3)
FIIK + FIA		1(2.6)
FIIK + FIB s		1(2.6)
FIA + FIB		2(5.3)
FIB + R		1(2.6)
R+I1		1(2.6)
Non typeable		6(15.8)
Total		38

Key: n=Number of Isolates; %= Percentage of occurrence.

Plasmid replicon typing in CTX-M- 15 *K. pneumoniae* isolates

Out of the 38 CTX-M-15 *K. pneumoniae* identified, plasmid replicon types (FIIK, FIA, FIB, R and I1) occurring either as single or accompanied by a different replicon were detected in 32 isolates and 6 were found to be non typeable. The number and type of plasmid replicons detected in each isolate were shown in Table 4. FIIK was the most prevalent replicon identified and was found alone in 14(36.8%) isolates. Other plasmid replicons detected were R in 5(13.2%), FIB in 2(7.9%) and FIA in 1(5.3%) isolates.

Transmissibility of antibiotic resistance plasmids

Despite repeated attempts, conjugation experiment was successful for only 19 (59.4%) out of 32 CTX-M-15 *K. pneumoniae* isolates which were selected as donors for conjugation based on their possession of plasmid replicons (either single or double). The transfer of replicons failed to occur in the remaining 13 (40.6%) isolates.

Conjugation experiments were not successful for all donors carrying the FIIK alleles. The replicons detected in the transconjugants included FIIK (n=10; 34.4%), FIB (n=5; 15.6%) and then FIA and R (n=4; 12.5%) (Table 5). A statistically significant association ($p = 0.03$) between the presence of CTX-M-15 and FIIK was observed in this study as FIIK replicon was the dominant replicon detected in 10 out of 19 CTX-M-15 harboring transconjugants.

DISCUSSION

The increased prevalence of ESBL *K. pneumoniae* in different regions of the world especially the developing countries is disturbing. This problem may be as a result of multiple facts such as inappropriate use of antibiotics for febrile infections as well as limited infection control measures to reduce the spread of multidrug resistant strains. In this study, the reason for the occurrence of ESBL (39.7%) in *Klebsiella pneumoniae* is due to greater chance of acquiring multidrug resistance determinants, thereby, the tendency to cause healthcare associated infections (Mohammed *et al*., 2016; Hymavathi *et al*., 2018; Sa'id *et al*., 2020).

Although the dominant variants of CTX-Ms are geogra-

Table 5. Characteristics of the donor *K. pneumoniae* isolates selected for conjugation experiments.

Replicon types	Replicon number	n (%)	Transfer ability
FIIK	1	14(36.8)	9(Y) 5(N)
FIA		2(5.3)	1(Y) 1(N)
FIB		3(7.9)	2(Y) 1(N)
R		5(13.2)	3(Y) 2(N)
FIIK + R	2	2(5.3)	0(Y) 2(N)
FIIK + FIA		1(2.6)	1(Y) 0(N)
FIIK + FIB		1(2.6)	0(Y) 1(N)
FIA + FIB		2(5.3)	2(Y) 0(N)
FIB + R		1(2.6)	1(Y) 0(N)
R+I1		1(2.6)	0(Y) 1(N)
Total		32	19(Y) 13(N)

Key: n=Number of Isolates; %= Percentage of occurrence; Y: successful conjugation; N: failed conjugation.

phically different, CTX-M-15 is the most common variants detected worldwide in clinically important pathogens. The rate of detection of CTX-M-15 among the 48 ESBL positive *K. pneumoniae* isolates was 44 (86.4%) in the present study. This high frequency of CTX-M-15 was in line with the results of several studies worldwide (Soge *et al*., 2006; Gharrah *et al*., 2017; Caneiras *et al*., 2019; Minja *et al*., 2021; Odewale *et al*., 2023).

The replicons detected in our plasmids indicates the complexity of multidrug resistant *K. pneumoniae* isolates harboring CTX-M-15 ESBL gene. The existence of more than one replicon within the same plasmid may enhance the possibility of genetic reassortment and recombination events and also contribute to the plasmid diversity by taking on new resistance genes into the plasmid framework (Coelho *et al*., 2010). In this study, all the isolates were found to carry either 1or 2 replicon on their plasmid and this finding implies the existence of highly diverse plasmids carried by the CTX-M-15 *K. pneumoniae* isolates. The FIIK replicon was the most prevalent replicon type detected as a single replicon in 14(36.8%) isolates or accompanied by another replicon in 3(10.5%) of the 32 isolates investigated in this study. *K. pneumoniae* isolates carrying FIIK replicon has been documented to have great capability to diffuse and persist in time (Coelho *et al*., 2010), mainly because the bacteria are provided with both virulence and antibiotic resistance determinants on the FIIK plasmids (Villa *et al*., 2010; Marisa *et al*., 2015). R replicon was the second most common replicon found in this study detected in 5 (13.2%) of the isolates occurring alone or 3 (10.5%) harboring other replicons. Previous studies have described both FIIK and R replicons identified on a resistance plasmid to have an association with CTX-M-15 in Spanish and Malaysian *K. pneumoniae* isolates (Coelho *et al*., 2010; Almarzooq *et al*., 2015).

Conjugation is the most common mechanism of horizontal dissemination of resistance plasmids. In this study, ability to transfer ESBL plasmids to *E. coli* strain J53 AzR recipient strain was only successful in 19 (62.8%) of 32 *K. pneumoniae* donor isolates. Approximately similar rate (61%) of conjugation has been documented for multidrug resistant *K. pneumoniae* isolates investigated in a Czech study (Dolejska *et al*., 2012). The replicative and transferability properties of plasmids are linked to their incompatibility groups and there is also the existence of relationship between FIIK allele and conjugative efficiency of the plasmids (Canton *et al*., 2007). IncF plasmids are characterized by extensive mutations, insertions, deletions and recombination events which may affect the tra genes encoding transferase proteins for mating aggregation and DNA movement into the recipient cell and, consequently, plasmids conjugative efficiency (Harajly *et al*., 2010; Hawkey *et al*., 2022). All plasmids that were not successfully transferred to the *E. coli* recipient strain may occur due to loss or mutations in tra genes present in the plasmids carrying such alleles (Partridge *et al*., 2011). The association between the presence of CTX-M-15 and FIIK plasmid replicon on transconjugant plasmids was noted in this study, in concordant with other previous reports (Coelho *et al*., 2010; Villa *et al*., 2010; Dolejska *et al*., 2012; Minja *et al*., 2021).

Plasmids are of particular concern in the public health sector, as they often carry multiple different antimicrobial resistance genes, and so can facilitate rapid and mass introduction of a broad range of antimicrobial resistance phenotypes via cellular uptake of only a single plasmid (Thomas and Nielsen, 2005; Barlow, 2009). Individual *K. pneumoniae* strains can carry multiple large plasmids of different incompatibility types, with clinical isolates often harboring two or more plasmids (Mathers *et al*., 2015; Hawkey *et al*., 2022). Hence the possibility of strains arising with multiple antimicrobial resistance plasmids is of

concern, due to the accumulation of large numbers of antimicrobial genes in individual plasmids (Conlan *et al*., 2016; Ramirez, 2014). Although the possibility of isolates carrying antibiotic resistant encoding plasmids is a serious concern, the occurrence is now very common.

Conclusion

Antibiotic resistance has been observed to be transferred by conjugation thus limiting the treatment options of infectious diseases caused by ESBL producing *K. pneumoniae* isolates. The spread of $bla_{\text{CTX-M-15}}$ is driven mainly by the horizontal transfer of the IncF group of plasmids which is inferred from the predominance of these plasmids and the conjugative nature of these plasmids among the isolates. FIIK was the dominant replicon type plasmids among the isolates derived from the study and was evidenced to be associated with $bla_{\text{CTX-M-15}}$. This study highlighted the importance on the need to select modalities to overcome the challenge in the dissemination of multidrug resistance associated with infections caused by CTX-M-15 *K. pneumoniae* and improve infection therapy.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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