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Characterization of *bla*$_{OXA-232}$ carrying carbapenem-resistant *Klebsiella pneumoniae* (CRKP) & their expression profiles under selective carbapenem pressure: An in-depth study from India

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***Background & objectives*: OXA-232 is a five amino acid substitution variant of OXA-48 and is reported in carbapenem-resistant *Klebsiella pneumoniae* (CRKP), which is associated with nosocomial infections among immunocompromised patients in the intensive care unit. This study aimed to characterise *bla*$_{OXA-232}$ in CRKP of clinical origin and investigate its transcriptional response against sub-inhibitory levels of carbapenems.**

***Methods*: CRKP was isolated from blood (pathogens) and stool cultures (colonisers) of neonates and was characterized for *bla*$_{OXA-232}$. Co-existing resistance determinants were investigated in *bla*$_{OXA-232}$ positive isolates, followed by horizontal gene transferability assay and PCR-based replicon typing (PBRT). Cloning of *bla*$_{OXA-232}$ was performed, and expression of *bla*$_{OXA-232}$ in the isolates and their clones under sub-inhibitory concentrations of carbapenems was checked *via* RT-PCR. Mobile genetic elements associated with *bla*$_{OXA-232}$ were investigated, followed by DNA fingerprinting through enterobacterial repetitive intergenic consensus (ERIC) PCR.**

***Results*: *bla*$_{OXA-232}$ with co-carriage of extended-spectrum beta-lactamases (ESBLs), sulphonamides and quinolones were identified in seven CRPK isolates recovered from blood samples of neonates. Transformation and cloning of *bla*$_{OXA-232}$ was successful. The sub-inhibitory concentration of carbapenems induces elevated expression of this resistant determinant. IS*Ecp1* was associated with *bla*$_{OXA-232}$ in the upstream region within two haplotypes of CRKP isolates of clinical origin.**

***Interpretation & conclusions*: Selective carbapenem pressure resulted in higher expression of this gene, which could account for treatment failure. With frequent reports of occurrence among clinical isolates, monitoring and further investigation of this novel variant are necessary to understand its transmission dynamics and to thwart its further dissemination.**

Key words Antimicrobial resistance - CRKP - IS*Ecp1* - neonatal septicaemia - OXA-232

[#]Equal contribution

Over the last two decades, *Klebsiella pneumoniae* (*K. pneumoniae*) has emerged as a clinically significant infectious agent due to its association with nosocomial infections, often causing considerable morbidity and mortality. Several cases of infections have been documented to date, particularly in patients with impaired immune systems in the intensive care unit (ICU), where this pathogen has successfully adapted itself through various mechanisms[1]. Treating such infections is a major challenge to clinicians worldwide because of resistance towards multiple drugs, including carbapenems, the last resort antibiotic in treating multidrug-resistant (MDR) infections[2-4]. Carbapenem-resistant *K. pneumoniae* (CRKP) has been grouped under 'critical priority' pathogens owing to its pathogenicity, multidrug resistance, transmission and dearth of available treatment options by the World Health Organization[5]. Even in the Indian scenario, CRKP has been recognized as a pathogen of great concern[6]. The investigators' lab has characterised CRKP and hypervirulent *K. pneumoniae* (hvKP) belonging to ST5235 from cases of neonatal sepsis with high mortality occurring during multiple outbreaks of *K. pneumoniae* in the neonatal intensive care unit (NICU) of a tertiary referral hospital[7,8].

Since their first report in 2009, the New Delhi metallo-beta-lactamase (NDM) and its variants, became the most widely reported carbapenemase and were predominant in the Indian subcontinent[9]. However, of late, the resurgence of reports of OXA-48-type enzymes worldwide among Carbapenem-Resistant Enterobacterales (CRE), including *K. pneumoniae* has posed new challenges in their management[10]. OXA-232, a five amino acid substitution variant of OXA-48, the class D carbapenemase responsible for carbapenem resistance was first reported within CRKP from France in 2013. It was found localized to a 6.1 kb non-conjugative ColE plasmid[11,12]. Since its first report, this OXA-48 variant has been detected in Singapore, USA, Italy, China and India within CRKP of clinical origin in neonates as well as adult patients suffering from severe infections[10,12-18].

In comparison to OXA-48, the variant shows lower hydrolytic activity against carbapenems. Despite this, several *K. pneumoniae* isolates harbouring the $bla_{OXA-232}$ gene have been reported to be carbapenem-resistant[19]. Hence, it is important to understand the behaviour of these isolates when exposed to sub-inhibitory levels of carbapenems. With recent reports of this emerging variant from India and paucity of information available about the factors triggering its adaptive response, the present study investigates the incidence of $bla_{OXA-232}$ within CRKP of clinical origin and its transcriptional response against concentration-dependent carbapenem stress at sub-inhibitory levels.

Material & Methods

This was a descriptive study characterizing the CRKP isolates collected from outbreaks of neonatal sepsis, with special reference to $bla_{OXA-232}$ gene. This research was carried out in the department of Microbiology, Assam University, Silchar. Thirty five non-duplicate clinical strains of *K. pneumoniae* exhibiting non-susceptibility towards anyone of the carbapenem were received from the Infection Control Section, department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi after obtaining approval from Institutional Ethical Committee.

Study setting and bacterial isolates: The isolates were part of the surveillance and outbreak control practices initiated in the neonatal intensive care unit (NICU) during the period 2017-2022. Briefly, during this period, three outbreaks of *K. pneumoniae* were reported from the NICU. As a part of the hospital infection control protocol, every outbreak was investigated with microbiological environmental surveillance, stool/anal swab culture surveillance and determination of clonality of the isolates either through phenotypic antibiogram typing or genotypically through enterobacterial repetitive intergenic consensus (ERIC) PCR. For this study, only those isolates from the blood or stool of neonates were included, which were non-susceptible to any one carbapenem (imipenem/meropenem/ertapenem). Only one or two isolates (in case of monoclonal outbreak) from each cluster were considered in the study. Inappropriately stored isolates and those without adequate clinical data were excluded. The isolates were phenotypically identified by biochemical methods and were further confirmed *via* VITEK®2 Compact System (Biomerieux, France).

Antibiotic susceptibility testing: Kirby-Bauer disc diffusion method was used to determine the susceptibility of the study isolates against cefepime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), aztreonam (30 µg), imipenem (10 µg), meropenem (10 µg), ertapenem (10 µg), piperacillin/tazobactam (100/10 µg), amikacin (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg) and levofloxacin (5 µg) (HiMedia Laboratories Pvt. Ltd.). The susceptibility patterns were

interpreted according to the Clinical and Laboratory Standard Institute (CLSI) 2022 breakpoint criteria for Enterobacterales. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls.

Broth microdilution method (BMD) was performed as per CLSI 2022 guidelines to establish the Minimum Inhibitory Concentrations (MICs) of imipenem (Merck, France), meropenem (AstraZeneca, UK) and ertapenem (MSD, France) against the study isolates[20].

Screening of carbapenemase production: Carbapenemase production among the study isolates was phenotypically investigated using Rapidec® Carba NP (Biomerieux, France) in accordance with the manufacturer's directives. *E. coli* ATCC 25922 was used as a negative control for the experiment.

Molecular characterization of $bla_{OXA-232}$: DNA templates for the PCR assay were extracted by the boiling centrifugation method[21]. The primer pair OXA-232F (5′-ATTATCGGAATGCCAGCGGT-3′) and OXA-232R (5′-AGGGCGATCAAGCTATTGGG-3′) designed using the NCBI primer blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to amplify 200 bp fragment of $bla_{OXA-232}$ gene. The reaction conditions for the PCR assay were 95˚C for 2 min, 95˚C for 20 sec, 52˚C for 40 sec, 72˚C for 1 min and 72˚C for 5 min. The PCR was carried out for 35 cycles. The volume of a single reaction mixture for the PCR assay was of 25 µl and contained 2X GoTaq® Green Master Mix (12.5 µl, Promega, Madison, USA), primer forward and reverse 1 µl each (10 pmol/µl), DNA template 2 µl (~100 ng/µl) and nuclease-free water.

Detection of other carbapenemases: The isolates harbouring $bla_{OXA-232}$ were also investigated for other carbapenemase-encoding genes belonging to class A (bla_{KPC}, $bla_{IMI/NMC}$ and bla_{SME}), class B (bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{GIM}, bla_{SIM} and bla_{SPM}) and class D (bla_{OXA-23}, $bla_{OXA-24/40}$, bla_{OXA-48}, bla_{OXA-58}, $bla_{OXA-134}$, $bla_{OXA-143}$, $bla_{OXA-198}$, $bla_{OXA-211}$, $bla_{OXA-213}$, $bla_{OXA-214}$, $bla_{OXA-228}$ and $bla_{OXA-211}$) carbapenemases through multiple PCR assays using previously described primer pairs and reaction conditions[22].

Detection of co-existing resistance determinants: The carriage of ESBL genes (bla_{TEM}, bla_{CTX-M}, bla_{SHV}, bla_{OXA-2}, bla_{OXA-10}, bla_{PER}, bla_{GES} and bla_{VEB}), aminoglycoside modifying enzyme (AMEs) genes (*ant(2")-Ia*, *ant(3")-I*, *ant(4')-Ia*, *aac(3)-I*, *aac(3)-IIc*, *aac(6')-Ib*, *aac(6')-II*, *aph(2")-Ib*, *aph(2")-Ic*, *aph(2")-Id*, *aph(3')-I*, *aph(3')-IIb*, *aph(3')-IIIa*, *aph(3')-VI a*, and *aph(4)-Ia*), acquired 16S methyltransferase genes (*rmtA*, *rmtB*, *rmtC*, *rmtD*, *armA* and *npmA*), sulphonamides resistance genes (*sul1*, *sul2* and *sul3*) and quinolones resistance genes (*qnr*A, *qnr*B, *qnr*C, *qnr*S, *qnr*D, *qep* and *aac*(6')-*lb-cr*) were also investigated in the study isolates through multiple PCR assays which were performed using primer pairs and reaction conditions described earlier[23].

Horizontal gene transferability assay: As per manufacturer's instructions, plasmid from $bla_{OXA-232}$ harbouring isolates was extracted using QIAprep Spin Miniprep Kit (Qiagen, Germany). The transformation assay was carried out by the heat-shock method using *E. coli* DH5α as a recipient strain[24]. The $bla_{OXA-232}$ transformants were selected on Luria Bertani agar (HiMedia Laboratories Pvt. Ltd.) containing 0.5 µg/ml of imipenem (Merck, France). Conjugation experiments were performed to assess the self-transferability of $bla_{OXA-232}$. *E. coli* J53 (sodium azide resistant) was used as recipient strain for the experiment. Liquid-mating assay was performed using the donor and recipient cultures and the transconjugants were selected on Luria Bertani agar (HiMedia Laboratories Pvt. Ltd.) medium supplemented with 0.5 µg/ml of imipenem (Merck, France) and 100 µg/ml of sodium azide (HiMedia Laboratories Pvt. Ltd.)[25].

Incompatibility typing of plasmid harbouring $bla_{OXA-232}$: Plasmids from $bla_{OXA-232}$ transformants were extracted and used as template DNA for PCR-Based Replicon Typing (PBRT). Eighteen plasmid incompatibility types (FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA) were targeted through multiple PCR assays using previously described primer pairs and reaction conditions[26].

Cloning of $bla_{OXA-232}$: Cloning was performed with the primer pair OXA-232WF (5′-GCAGTTTGCTAGGGAATGAGAGG-3′) and OXA-232WR (5′-GCATCAGCATTTTGTCCA TATAC-3′) designed using the NCBI primer blast tool (*https://www.ncbi.nlm.nih.gov/tools/primer-blast/*) from the flanking region of the $bla_{OXA-232}$ gene to amplify the whole gene (957 bp) including its native promoter region. For amplification, 50 µl reaction volume was used containing 25 µl of 2X GoTaq® Green Master Mix (Promega, Madison, USA), 1 µl of

each primer (10 pmol/µl), 2 µl of DNA template (~100 ng/µl) and nuclease-free water. The amplification conditions included a 2 minutes initial denaturation at 95˚C followed by 35 cycles of 95˚C for 20 seconds, 52˚C for 40 seconds and 72˚C for 1 minute with a single final extension cycle of 72˚C for 5 minutes.

The amplified PCR products were then purified using the MinElute® PCR Purification Kit (Qiagen, Hilden, Germany) and ligated into pMD20-T vector as per the manufacturer's protocol (Mighty TA-cloning Kit, TaKaRa, Japan). The recombinant plasmid was then transformed into *E. coli* DH5α by the heat-shock method and the clones were selected by blue-white screening on LB agar medium (HiMedia Laboratories Pvt. Ltd.) containing ampicillin (100 µg/mL) and imipenem (0.25 µg/ml) and were further confirmed by colony PCR.

Antibiogram of clones: The susceptibility of the $bla_{OXA-232}$ clones towards ertapenem, imipenem and meropenem were investigated *via* Kirby-Bauer Disc Diffusion method and BMD following the CLSI 2022 guidelines[20].

Transcriptional expressional of $bla_{OXA-232}$ under carbapenem stress: For expression study, $bla_{OXA-232}$ harbouring isolates and their corresponding clones were grown in LB broth (HiMedia Laboratories Pvt. Ltd.) containing 0.5 µg/mL and 1 µg/ml ertapenem, imipenem and meropenem. In accordance with the manufacturer's instructions, mRNA was extracted using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) followed by preparation of cDNA and its quantification *via* QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) and Picodrop (Pico 200, Cambridge, UK), respectively. To analyse the expression level $bla_{OXA-232}$ gene under carbapenem stress, quantitative Real-Time PCR (qRT-PCR) was performed in triplicate form in StepOnePlus™ Real-Time PCR system (Applied Biosystems, USA) using PowerUp™ SYBR® green PCR Master Mix (Applied Biosystems, USA) and the primer pair OXA-232RTF (5′-CCCAATAGCTTGATCGCCCT-3′) and OXA-232RTR (5′-TATCACGCGTCTGTCCATCC-3′) was designed using the NCBI primer blast tool (*https://www.ncbi.nlm.nih.gov/tools/primer-blast/*). The relative fold change in the expression level of the $bla_{OXA-232}$ gene was measured by the ΔΔCT method, and Ct value of each test sample was normalised against the housekeeping gene *pmr*A used as an endogenous control for the reaction. Relative quantification (RQ) values of $bla_{OXA-232}$ gene were compared with the reference sample *E. coli* ATCC 25922 without carbapenem stress and after exposure to varying concentrations of carbapenems.

Association of $bla_{OXA-232}$ with mobile genetic elements: To investigate the linkage of $bla_{OXA-232}$ with the insertion sequences IS*Aba1*, IS*Aba4*, IS*Aba125* and IS*Ecp1*, four sets of PCR assay were performed using the forward primers of IS*Aba1* (5′-CGACGAATACTATGACAC-3′), IS*Aba4* (5′-ACTCTCATATTTTTTCTTGG-3′), IS*Aba125* (5′-GAAACTGTCGCACCTCATGTTTG-3′) and IS*Ecp1* (5′-TTCAAAAAGCATAATCAAAGCC-3′) and the reverse primer of $bla_{OXA-232}$ (5′-AGGGCGATCAAGCTATTGGG-3′)[27,28]. The reaction was performed in T100™ Thermal cycler (Bio-Rad, USA) and a single PCR reaction mixture was of 50 µl volume and contained 2X GoTaq® Green Master Mix (25 µl, Promega, Madison, USA), primer forward and reverse 2 µl each (10 pmol/µl), DNA template 2 µl (~100 ng/µl) and nuclease-free water. The amplification conditions included an initial denaturation of 2 min at 95˚C followed by 34 cycles of 95˚C for 20 sec, 44˚C for 45 sec and 72˚C for 2 min with a single final extension cycle of 72 ˚C for 7 min. The amplified PCR products were then sequenced, and the results were analysed using NCBI BLAST suite programme (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*).

Typing of isolates by ERIC PCR: The clonal relatedness of the $bla_{OXA-232}$ harbouring isolates was determined by ERIC PCR using the universal primers ERIC-F (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC-R (5′-AAGTAAGTGACTGGGGTGAGCG-3′). The PCR assay was carried out using amplification conditions described previously[29]. A dendrogram was created using NTSYS-pc version 2.0 software based on the banding patterns of the amplified products observed in agarose gel through Gel Doc™ EZ Gel Documentation System (Bio-Rad, California, USA).

Results

Carbapenem resistance in the isolates: All 35 *K. pneumoniae* isolates, comprising 14 isolates from blood and 21 isolates from anal swab culture surveillance were phenotypically confirmed for carbapenemase production by Carba NP test. The study isolates exhibited resistance towards all the antibiotics used in antibiotic susceptibility testing (Table I). MICs against carbapenem group of antibiotics were above breakpoint (≥64 µg/ml).

Table I. Resistance profile of *Klebsiella pneumoniae* isolates harbouring $bla_{OXA-232}$

Isolates ID	Antimicrobial resistance profile											
	CPM	CRO	CAZ	ATM	IPM	MEM	ETP	PIT	AMK	GEN	CIP	LVX
BKp1	R	R	R	R	R	R	R	R	R	R	R	R
BKp4	R	R	R	R	R	R	R	R	R	R	R	R
BKp5	R	R	R	R	R	R	R	R	R	R	R	R
BKp8	R	R	R	R	R	R	R	R	R	R	R	R
BKp15	R	R	R	R	R	R	R	R	R	R	R	R
BKp18	R	R	R	R	R	R	R	R	R	R	R	R
BKp22	R	R	R	R	R	R	R	R	R	R	R	R

CPM, cefepime (30 µg); CRO, ceftriaxone (30 µg); CAZ, ceftazidime (30 µg); ATM, aztreonam (30 µg); IPM, imipenem (10 µg); MEM, meropenem (10 µg); ETP, ertapenem (10 µg); PIT, piperacillin/tazobactam (100/10 µg); AMK, amikacin (10 µg); GEN, gentamicin (10 µg); CIP, ciprofloxacin (5 µg); LVX, levofloxacin (5µg); R, resistant

Table II. Resistance profile of carbapenem-resistant *Klebsiella pneumoniae* harbouring $bla_{OXA-232}$

Isolates ID	Carbapenemase	Other co-resistance	ERIC type
BKp1	$bla_{OXA-232}$	$bla_{CTX-M-15}$, *sul2*	I
BKp4	$bla_{OXA-232}$	*aac*(6')*lb-cr*	I
BKp5	$bla_{OXA-232}$	$bla_{CTX-M-15}$, *sul1*, *sul2*	I
BKp8	$bla_{OXA-232}$	bla_{TEM}	I
BKp15	$bla_{OXA-232}$	$bla_{CTX-M-15}$, *qnr*B, *qnr*D	II
BKp18	$bla_{OXA-232}$	$bla_{CTX-M-15}$, *sul2*	I
BKp22	$bla_{OXA-232}$	$bla_{CTX-M-15}$, *sul2*	I

ERIC, enterobacterial repetitive intergenic consensus

Carbapenemase genes in the isolates: PCR assay detected seven *K. pneumoniae* isolates harbouring $bla_{OXA-232}$ gene. In these seven isolates, no other carbapenemase genes were detected. All seven isolates were from blood samples of neonates isolated from different outbreaks of CRKP in the NICU.

Co-carriage of other genes: Additionally, co-carriage of different resistance genes was observed in the $bla_{OXA-232}$ harbouring isolates. In one isolate, a combination of $bla_{CTX-M-15}$, *qnr*B and *qnr*D genes was detected, while in another isolate, co-carriage of $bla_{CTX-M-15}$, *sul1* and *sul2* was observed. In another isolate, $bla_{CTX-M-15,}$ along with, *sul1* was detected, while $bla_{CTX-M-15}$ and *sul2* were identified in two isolates. One isolate each was co-harbouring bla_{TEM} and *aac*(6')*lb-cr* along with $bla_{OXA-232}$. The profile of these isolates are summarised in Table II.

Horizontal gene transfer analysis and cloning of $bla_{OXA-232}$: Transformants harbouring plasmid with $bla_{OXA-232}$ were successfully selected on the medium containing imipenem and were confirmed by PCR assay. However, the gene was located on a non-conjugative plasmid as the conjugation experiment could not produce any transconjugant. Analysis of transformants *via* PBRT for replicon types yielded no results. Cloning of $bla_{OXA-232}$ was successful, which was confirmed by colony PCR. The clones were non-susceptible towards ertapenem, imipenem and meropenem.

Expression studies of $bla_{OXA-232}$ *level*: Analysis of qRT-PCR results revealed that the $bla_{OXA-232}$ level was increased by two-folds and three-folds in the isolates under sub-inhibitory concentrations of imipenem and ertapenem, respectively. While in their clones, a substantial escalation in the transcriptional level of $bla_{OXA-232}$ was observed under sub-inhibitory concentrations of these carbapenems. There was an increase in the levels of $bla_{OXA-232}$ in the isolates with increase in meropenem sub-inhibitory concentrations. However, in the clones, the expression decreased with an increase in meropenem concentration. The average relative quantification values ($2^{-\Delta\Delta CT}$) of the isolates harbouring $bla_{OXA-232}$ and their respective clones against sub-inhibitory concentrations of imipenem, ertapenem,

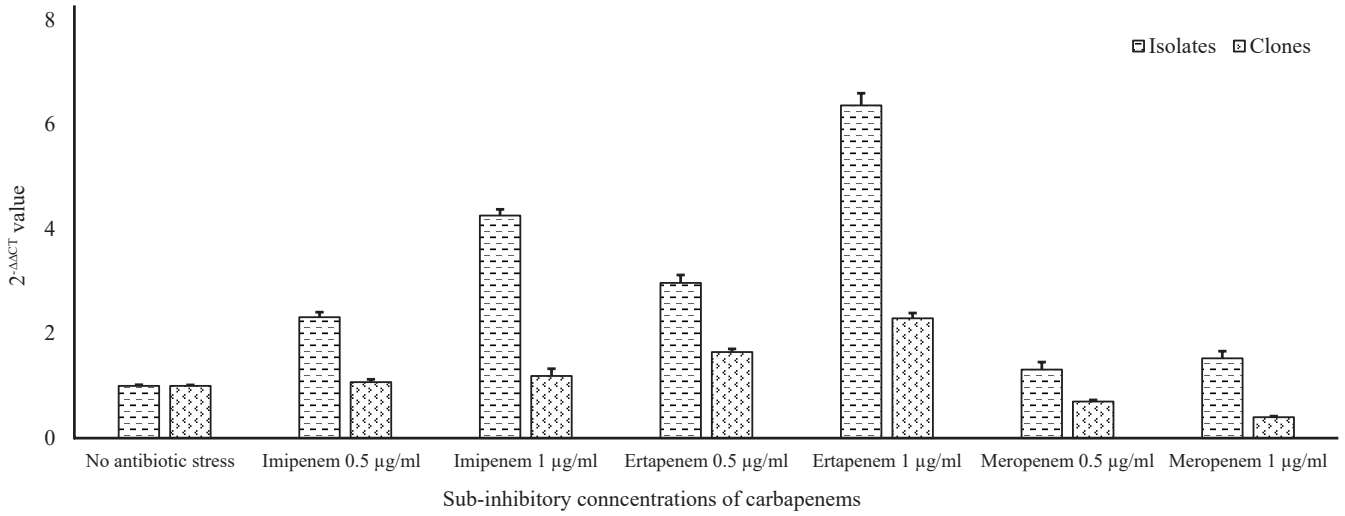

Fig. 1. Transcriptional expression of $bla_{OXA-232}$ in wild types and clones under sub-inhibitory pressure of carbapenems.

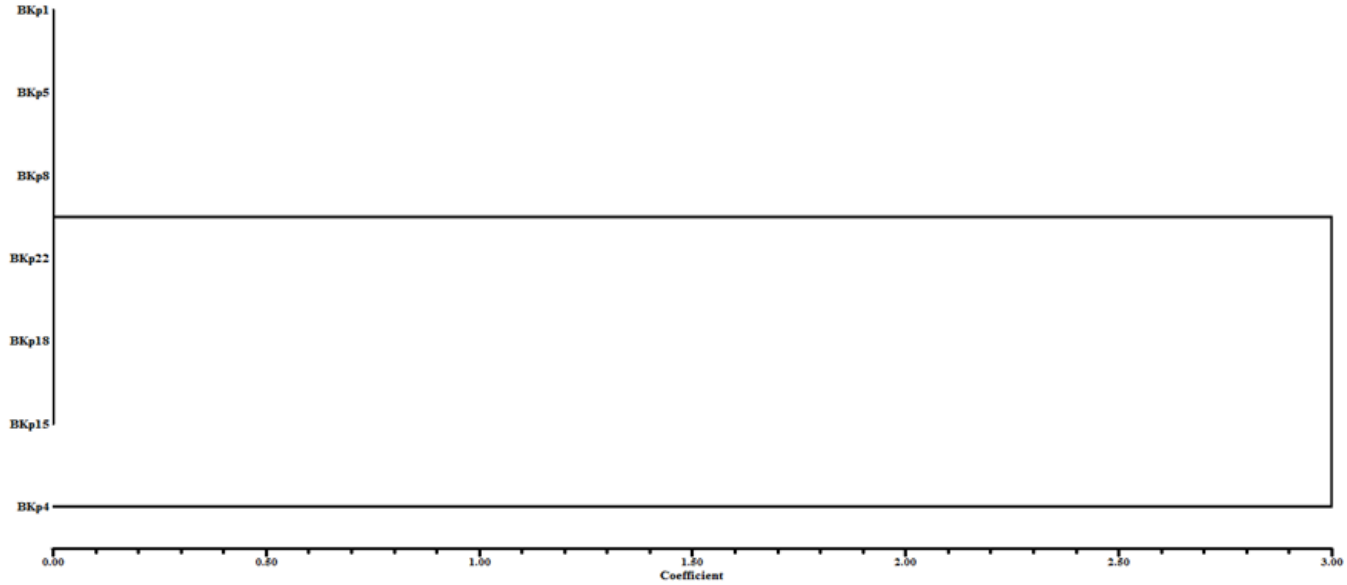

Fig. 2. Clonal relatedness of *Klebsiella pneumoniae* isolates harbouring $bla_{OXA-232}$.

and meropenem have been summarized in Figure 1 and Supplementary Table I.

Insertion sequence and DNA fingerprinting: Genetic analysis of $bla_{OXA-232}$ revealed the association with the insertion sequence IS*Ecp1*. In all seven $bla_{OXA-232}$ harbouring isolates, the gene was found to be associated with IS*Ecp1* in the upstream region. Two different haplotypes of *K. pneumoniae* were identified in DNA fingerprinting by ERIC PCR, which were associated with the carriage and dissemination of the $bla_{OXA-232}$ gene (Figure 2 and Supplementary Table II).

Discussion

This study reported seven CRKP with the carriage of $bla_{OXA-232}$, which is presently the dominant variant of OXA-48 like carbapenemases in Southeast Asian countries. OXA-48-like carbapenemases are considered endemic to countries in the Middle East, Mediterranean regions and the Indian subcontinent[30]. The first report of OXA-232 from India was in 2019. The gene was identified in diverse sequence types of *K. pneumoniae* isolates of clinical origin and was found localized on ColKP3- and ColE-type plasmids[12]. In 2020, a hypervirulent *K. pneumoniae*

belonging to ST23 associated with neonatal sepsis was identified with the carriage of $bla_{OXA-232}$[17]. In line with the previous studies, we also noticed *K. pneumoniae* harbouring $bla_{OXA-232}$ exhibiting a high level of resistance towards carbapenems and other antibiotics. Several instances of *K. pneumoniae* harbouring OXA-232 and co-producing bla_{NDM-1} have been reported previously[10,14,31]. However, in the present investigation, no other carbapenemase genes in $bla_{OXA-232}$ harbouring isolates were found, which were in agreement with studies conducted earlier[12,13,15-18]. The most probable reason for the absence of other carbapenemase genes could be due to the reduction in fitness cost of these isolates as previous studies already found that isolates co-harbouring $bla_{OXA-232}$ with metallo-β-lactamase bla_{NDM} had different plasmids carrying the carbapenemase gene[14]. As these isolates were also co-carrying other multiple resistant genes, it could affect their fitness. Additionally, in these isolates, OXA-232 expression was associated with high-level carbapenem resistance; therefore, added carbapenem mechanisms were not required. Further studies should confirm these speculations, and were not within the scope of the present investigation. Otherwise, there has been a recent shift in the bla_{OXA-48} endemicity from the existing bla_{NDM}, thus accounting for their predominance[12].

K. pneumoniae harbouring $bla_{OXA-232}$ with co-carriage of ESBLs, aminoglycoside-modifying enzymes, sulphonamides and quinolone resistance genes were described in several studies conducted earlier. Mukherjee *et al*[17] in their study reported occurrence of $bla_{OXA-232}$ in hypervirulent *K. pneumoniae* co-producing bla_{TEM}, $bla_{CTX-M-15}$, *rmtF, armA, aph(3")-Ib, sul1*, *sul2* and *aac*(6')-*Ib-cr.* Similarly, Weng and his team 18 in 2020 reported the co-carriage of bla_{TEM}, bla_{CTX-M}, *aph(3)-IIa, aph(3")-Ib, aph(6)-Id* and *rmtD* in $bla_{OXA-232}$ harbouring *K. pneumoniae*[18]. In our study, we also observed the occurrence of $bla_{CTX-M-15}$, bla_{TEM}, $bla_{CTX-M-15}$, *sul1*, *sul2*, *qnr*B, *qnr*D and *aac*(6')-*Ib-cr* among the $bla_{OXA-232}$ positive isolates. The presence of such diverse genes conferring resistance to a wide range of antibiotics warrants a major concern to public health as it significantly limits therapeutic options. In our study, the transformation of plasmid with $bla_{OXA-232}$ was successfully carried out, though the conjugation assay was unsuccessful. This might be due to the carriage of this resistance determinant within the non-conjugative plasmid in the studied isolates. This assumption is supported by various previous findings where the gene was located in a non-conjugative plasmid[11,16-18].

In this study, sub-inhibitory concentrations of carbapenems were used to determine the effects of selective carbapenem pressure on the expression of $bla_{OXA-232}$ in the isolates and their clones. It was revealed that sub-inhibitory concentrations of ertapenem, imipenem and meropenem had significant effects on the expressional level of $bla_{OXA-232}$ when compared with the unexposed isolates as well as their respective clones. This finding implies that selective carbapenem pressure induces an adaptive response and contributes to the elevated expression of this resistance determinant thereby adding to carbapenem resistance. As heavy carbapenem use in hospitals, especially in developing countries, is already an established risk factor for the emergence of carbapenem-resistant organisms, the selective pressure thus caused could result in such adaptations in these isolates[32]. Studies have revealed the association of diverse insertion sequences and transposons with $bla_{OXA-232}$[12]. In our study, the insertion sequence *ISEcp1* was found associated with $bla_{OXA-232,}$ emphasising the role of this mobile genetic element in the maintenance and dissemination of this carbapenem resistance determinant within the two haplotypes of *K. pneumoniae*. Though clonal expansion with the determination of sequence types could not be done in this study, it was revealed that transmission of these isolates could be facilitated by this insertion sequence under antibiotic pressure in the hospital environment.

Overall, the study characterised $bla_{OXA-232-}$ carrying CRKP isolates of clinical origin and clearly demonstrated that higher expression of this gene occurred under sub-inhibitory antibiotic pressure, which could lead to inducible resistance during therapy. With the emergence of further reports on this carbapenemase variant and its adaptations against various factors in the hospital environment, urgent monitoring of this variant should be done to understand its transmission dynamics and to limit further dissemination.

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***Conflicts of Interest*:** None.

***Use of Artificial Intelligence (AI)-Assisted Technology for manuscript preparation*:** The authors confirm that there was no use of Artificial Intelligence (AI)-Assisted Technology for assisting in the writing of the manuscript and no images were manipulated using AI.

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