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Genotypic, phenotypic, and *in silico* analysis of carbapenem-resistant *Klebsiella pneumoniae*

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Due to an increase in serious infections and a lack of efficient therapies, *Klebsiella pneumoniae* has recently gained more recognition. The production of carbapenemases is one of the most common strategies by which *K. pneumoniae* acquire resistance to carbapenems which is considered the last resort of antibiotics. Previously collected isolates from different clinical settings and on the basis of their genetic profile, mainly the absence and presence of single or dual carbapenemases (OXA-181, OXA-232, NDM-1, NDM-5, NDM-5+OXA-181, and NDM-1+OXA-232), mutations in porins, and efflux pumps, seven isolates (M40, M52, M39, J20, M53, M49, and M17B) were selected. Its phenotypic resistance against two carbapenem drugs (ertapenem and meropenem) was checked and we found NDM-5 followed by OXA-181 and NDM-5+OXA-181 carrying isolates showed high MIC values. Further, no significant differences were observed either in the presence of efflux pumps or mutations in porins among isolates. By molecular docking, among single amino acid differences between OXA-181 and OXA-232 and with two amino acids differences between NDM-1 and NDM-5, OXA-232 and NDM-5 showed a higher binding affinity than OXA-181 and NDM-1 with both antibiotics. It is concluded that the presence of specific carbapenemases or combinations of the same can drastically increase MIC values. The presence of NDM-5, and OXA-181, or their combinations is more fatal than NDM-1+OXA-232.

Keywords: Carbapenemases, *Klebsiella pneumoniae*, Minimum inhibitory concentration (MIC), Molecular docking, Whole genome sequencing

Resistance to carbapenem is one of the growing concerns in healthcare-associated infections caused by *Klebsiella pneumoniae* (*Kp*). The World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) identified carbapenem-resistant *Enterobacteriaceae* (CRE) as one of the most significant dangers[1]. Extended-spectrum-lactamase-producing (ESBLs) *Enterobacteriaceae*, particularly *K. pneumoniae*, are the most common cause of healthcare-associated infections, and carbapenem therapy is their primary treatment[2]. Due to a limited number of drugs available for the carbapenem-resistant strains, carbapenem-resistant *Kp* and other CRE are grouped under the critical priority pathogens.

In particular, some distinct molecular mechanisms that may cause carbapenem resistance in *K. pneumoniae* could arise due to carbapenemase production, efflux pumps, and porin mutations[3]. The carbapenemases are divided into Ambler classes A (serine penicillinases), B (metallo-β-lactamases), and D (oxacillinases), which are the primary cause of carbapenem resistance in *Enterobacteriaceae*[4]. The majority of carbapenem resistance is linked to the presence of carbapenemases, the most prevalent of which are found in *Enterobacteriaceae* as Class A (*bla*KPC), Class B (*bla*IMP, *bla*NDM, *bla*VIM), and Class D (*bla*OXA-48-like) types[5]. A recent study from India reported the dominance of OXA-48-like (*bla*OXA-232 & *bla*OXA-181) and NDM type (*bla*NDM-5 & *bla*NDM-1), while other carbapenemases such as *bla*KPC, *bla*VIM, and *bla*IMP were rarely detected[6].

With the exception of monobactams, New Delhi metallo-lactamase (NDM), a carbapenemase, that has the ability to gravely endanger global health, was first discovered in a Swedish patient who had previously been hospitalised in India[7]. NDM-5, which was first discovered in *E. coli*, had Valine at position 88 and Methionine at position 154 replaced by Leucine[8]. Compared to NDM-1, NDM-5 exhibits higher

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hydrolytic activity toward carbapenems, cefotaxime, cephalothin, and ceftazidime[8].

In 2001, a patient in Istanbul, Turkey, had a carbapenem-resistant *K. pneumoniae* isolate that led to the discovery of OXA-48[9]. According to the literature, the "OXA-48-like" subfamily was formed after a number of variations in the oxacillinase-type enzyme OXA-48 were discovered since its initial description. Variants differ from OXA-48 by one to five deletions or substitutions of amino acids. OXA-181 (four changes at Thr104Ala, Asn110Asp, Glu168Gln, and Ser171Ala), and OXA-232 (one substitution at Arg214Ser) are two of the more prominent members[10].

Recently, few studies have shown the presence of dual carbapenemase producers (NDM+OXA)[11-14]. However, detailed studies on the correlations between the genotypic presence of single carbapenemase producers and dual carbapenemase producers to the phenotypic MIC values are lacking. In this research, we looked at the presence of carbapenemases (single/dual) and their effect on the MIC values in *Klebsiella pneumoniae*. Further, we performed the molecular docking of NDM and OXA to group I and group II carbapenems in order to model the interaction and correlate the obtained MIC values. Here, it was discovered that a high MIC value is directly correlated with carbapenemase genes, specifically OXA-181 and NDM-5, regardless of efflux pumps and mutations in porins.

Materials and Methods

Isolate selection and Bioinformatics analysis

Previously reported Six whole genome sequenced *Klebsiella pneumoniae* isolates from our lab were selected for this study[6]. Isolates M40 (strain: DGL2), M52 (strain: DGL10), M39 (strain: SBS11), M53 (strain: DGL11), M49 (strain: DGL7) & M17B (strain: SBS5) were selected to represent; no NDM or OXA-48-like gene/s (M40), OXA-232 harbouring (M52), NDM-1 harbouring (M39), NDM-5 producing (M53), OXA-181 & NDM-5 harbouring (M49), and OXA-232 & NDM-1 harbouring (M17B). Additionally, a new isolate (J20) was included to represent the OXA-181 producers, and its genome sequence was also submitted to NCBI (Biosampleno: - SAMN35994701).

Carbapenemase genes (*bla*NDM and *bla*OXA-48-like), loss or mutations of porins, and efflux pumps related to carbapenem resistance were detected using the Comprehensive Antibiotic Resistance Database (CARD)[15] and ResFinder[16] from Center for Genomic Epidemiology (CGE) tool box (*https://www.genomicepidemiology.org/*). Briefly, Resistance Gene Identifier of the CARD was used to upload the genome assembly, and the results were downloaded. Further the presence of single/dual carbapenemase genes and efflux-related genes were shortlisted. Genome assembly was uploaded to ResFinder tool by selecting the chromosomal point mutations option in order to find mutations in OmpKs. Further, mutations in OmpK35, OmpK36, and OmpK37 relevant to carbapenem were shortlisted.

Minimum inhibitory concentration (MIC) determination using broth microdilution method

Minimum inhibitory concentrations (MIC) of all seven isolates were determined using CLSI's guidelines[17]. Briefly, all isolates were grown overnight in Mueller Hinton Broth (MHB-Himedia) at 37°C. A Multiskan GO microplate spectrophotometer (Thermo Scientific, USA) was used to create bacterial inoculums, which were then adjusted to 0.5 McFarland standard (0.08-0.13 OD at 600 nm). The culture was diluted by 1:100 in Mueller Hinton broth (MHB). Bacteria diluted in MHB (100 µL) was poured in all 96 wells. The ertapenem and meropenem concentration range of 512 µg/mL to 1 µg/mL was used from stock solution, individually. Only MHB and *K. pneumoniae* with MHB were taken as negative and positive control, respectively. These bacteria with either ertapenem or meropenem were incubated at 37°C temperature for 18-24 h. All experiments were performed in triplicates. According to CLSI guidelines (2021)[17], breakpoints for the determination of minimum inhibitory concentration (MIC) were: Ertapenem susceptible <= 0.5 µg/mL, resistant >=2 µg/mL while meropenem susceptible <=1 µg/mL, resistant >= 4 µg/mL.

Molecular Docking

The 3D structure of target proteins OXA-181 (PDBID: 5OEO), OXA-232 (PDBID:5HFO), NDM-1 (PDBID:6TTC), and NDM-5 (PDBID:6MGY) were obtained from the RCSB Protein Data Bank. (*https://www.rcsb.org/*). Energy minimization of3D structure of the protein was performed in Swiss PDB viewer (*http://www.expasy.org/spdbv/*).

Carbapenem drugs (meropenem and ertapenem) were used for this docking study. The coordinates of meropenem (PubChem CID441130) and ertapenem (PubChem CID150610) were obtained using PubChem

compound database (*https://pubchem.ncbi.nlm.nih.gov/*)[18]. Protein preparation was done using AutoDock tools (version, 1.5.7)[19], and AutoDock vina (The Scripps Research Institute)[20] was used to generate protein-ligand interactions. The docking results were analysed by AutoDock Tools and Discovery studio visualizer[21].

Results

Genotypic analysis of Carbapenem resistance

Carbapenemase genes

There was no carbapenemase gene detected in isolate M40, single carbapenemases; *bla*OXA-232, *bla*NDM-1, *bla*OXA-181, and *bla*NDM-5 were detected in M52, M39, J20, and M53, respectively. Presence of dual carbapenemase was detected in M49 and M17B harbouring *bla*NDM-5+*bla*OXA-181 and *bla*NDM-1+*bla*OXA-232, respectively (Table 1).

Loss or Mutations of Porins

In this study, three porins, mainly ompK35, ompK36 and ompK37 were studied for the detection of loss of porins or mutations in porins. The well reported mutation for carbapenem resistance, ompK35 was not detected in any of the seven isolates. A single mutation in amino acid, p.A217S was detected in all isolates for ompK36 except M52, in which loss of this porin was detected. An additional ompK mutation p.N218H was detected only in M17B. Major mutations were detected in ompK37 that reflect resistance toward carbapenem antibiotics. Dual mutation p.I70M and p.I128M was detected in all isolates, except M39. An additional mutation m233_None234insQ was detected in both M40 and M52, while p.N230G was only detected in M52 (Table 1).

Efflux pumps

Here, four efflux pumps related to carbapenem resistance were found to be distributed among isolates, of which LptD, KpnG, KpnH, and marA all four were detected in M40, M52, and M49. Isolate M39 and M17B both had KpnG & marA, while additionally KpnH was also detected in J20, M53, and M17B. KpnG and marA were found to be most frequent and detected in all isolates (Table 1).

Except, p.N230G (found in M52), all other porin mutations were found to be common between the susceptible isolate (M40) and the carbapenem resistant isolates (M39, J20, M53, M49, and M17B). No difference in the occurrence of efflux pumps was found across all seven isolates.

Table 1 — List of known carbapenemase genes, mutations or loss of porins, and efflux pumps related to carbapenem resistance.

Isolates	Carbapenemase genes	Loss or mutations of Porins			Efflux pumps
		ompK35	ompK36	ompK37	
M40	None	None	p.A217S	p.I70M, p.I128M, m233_None234insQ	LptD, KpnG, KpnH, marA
M52	*bla*OXA-232	None	Gene not found (Loss of Porin)	p.I70M, p.I128M, p.N230G, m233_None234insQ	LptD, KpnG, KpnH, marA
M39	*bla*NDM-1	None	p.A217S	None	KpnG, marA
J20	*bla*OXA-181	None	p.A217S	p.I70M, p.I128M	KpnG, KpnH, marA
M53	*bla*NDM-5	None	p.A217S	p.I70M, p.I128M	KpnG, KpnH, marA
M49	*bla*NDM-5, *bla*OXA-181	None	p.A217S	p.I70M, p.I128M	LptD, KpnG, KpnH, marA
M17B	*bla*NDM-1, *bla*OXA-232	None	p.A217S, p.N218H	p.I70M, p.I128M	KpnG, KpnH, marA

Phenotypic analysis of Carbapenem resistance

Ertapenem and meropenem belonging to group 1 and group 2 carbapenem drugs, respectively, were used for the MIC determination. Isolate M40 was chosen as control isolate as its MIC values with ertapenem and meropenem were 8 µg/mL and 1 µg/mL. Though the rest of the isolates (M52, M39, J20, M53, M49, and M17B) were resistant to both ertapenem and meropenem, the MIC values were tremendously high for ertapenem compared to meropenem (Fig. 1). The MIC values of single carbapenemase producers, M52 (*bla*OXA-232), M39 (*bla*NDM-1), J20 (*bla*OXA-181), and M53 (*bla*NDM-5) were 128 µg/mL, 16 µg/mL, >512 µg/mL, and >512 µg/mL, respectively, for ertapenem, and 64 µg/mL, 4 µg/mL, 128 µg/mL, and 512 µg/mL for meropenem, respectively. Lastly, the MIC values of dual carbapenemase producers, M17B (*bla*OXA-232+*bla*NDM-1) and M49 (*bla*OXA-181+*bla*NDM-5) were 128 µg/mL, >512 µg/mL for ertapenem and 8 µg/mL, 128 µg/mL for meropenem (Fig. 1). Isolates containing either single carbapenemase (*bla*OXA-181 and *bla*NDM-5) or dual carbapenemase (*bla*OXA-181+*bla*NDM-5) exhibited higher MIC values for both antibiotics compared to single carbapenemase (*bla*OXA-232 and *bla*NDM-1) or dual carbapenemase (*bla*OXA-232+ *bla*NDM-1), which was an interesting finding.

Molecular Docking

The 3D structure of proteins (NDM-1, NDM-5, OXA-181, and OXA-232) was retrieved and docking was done with carbapenem drugs (meropenem and ertapenem). On the basis of binding energy, hydrogen bonding, and RMSD value, docking analysis was obtained as shown in (Table 2).

Interaction of Meropenem with NDM-1, NDM-5, OXA-181, and OXA-232

The docking score for meropenem with NDM-1, NDM-5, OXA-181, and OXA-232 were -6.5, -6.2, -

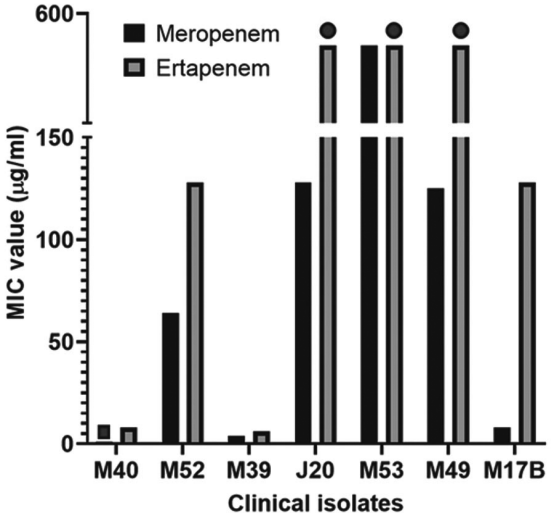

Fig. 1 — Minimum inhibitory Concentration of *K. pneumoniae* isolates; M40 (no carbapenemase gene), M52 (*bla*OXA-232), M39 (*bla*NDM-1), J20 (*bla*OXA-181), M53 (*bla*NDM-5), M49 (*bla*NDM-5+*bla*OXA-181), and M17B (*blaNDM-1*+*bla*OXA-232). Square filled with blue colour indicates MIC value <1 µg/mL, Circle filled with blue indicates MIC value >512 µg/mL

Table 2 — Docking results of meropenem and ertapenem with NDM-1, NDM-5, OXA-181, and OXA-232

No.	Compound	Protein	RMSD	Binding energy (kcal/mol)	No. of H bonds (Drug-Enzyme)	Amino acid involved in interaction
1.	Meropenem	NDM-1	0.000	-6.5	4	Phe177, Gly178, Leu180, Lys181, Ala174, Pro175, Asn176, Pro171, Val169
		NDM-5	0.000	-6.2	4	Ser217, Lys216, Asp212, Ala215, Lys214, Leu218, Gly219, Lys211, Ser251, His250, His189, Cys208, Trp93
		OXA-181	0.000	-6.9	3	Tyr117, Ile102, Trp105, Ser118, Thr213, Arg214, Leu158, Ser70, Val120, Tyr211, Ala69, Lys116, Ala104
		OXA-232	0.000	-7.5	5	Tyr211, Ala69, Ser70, Gly210, Arg250, Ser118, Thr209, Leu247, Lys208, Lys116, Tyr117, Leu158, Val120
2.	Ertapenem	NDM-1	0.000	-7.5	4	Val182, Val169, Leu180, Lys181, Pro171, Ala174, Phe177, Gly178, Phe240, Asp199, Pro175, Asn176, Trp168
		NDM-5	0.000	-8.0	4	Ala239, Pro241, Ile203, Gly197, Ala204, Phe240, Ile198, Ala243, Lys242, Asp199, Lys181, Thr201, Asp202, Val182, val169, Pro171, Trp168
		OXA-181	0.000	-7.6	3	Ser70, Ser118, Arg250, Tyr211, Gly210, Trp105, Ile102, Tyr117, Gln251, Thr209, Lys208, Trp222, Met115, Ala207, Thr197, Lys116, Ala104
		OXA-232	0.000	-9.1	8	Lys116, Tyr117, Leu247, Arg250, Lys208, Gly210, Ser118, Ser70, Ala69, Tyr211, Val120, Leu158, Trp105, Ala207, Thr209, Thr197, Trp222

6.9, and -7.5kcal/mol, respectively. As elicited in (Fig. 2A), Hydrogen Bonding, van der Waals interaction and other interactions (Unfavorable Acceptor-Acceptor and Pi-Sigma) were involved which is responsible for the binding. The lower binding energy (-7.5) of OXA-232 indicates greater binding affinity as compared to the more binding energy (-6.5) of NDM-1, which indicates lower binding affinity towards meropenem drug.

Interaction of Ertapenem with NDM-1, NDM-5, OXA-181 and OXA-232

The docking score (expressed as binding affinity) for ertapenem with NDM-1, NDM-5, OXA-181, and OXA-232 was -7.5, -8.0, -7.6 and, -9.1 kcal/mol, respectively. Ertapenem had lower binding energy than meropenem towards all four proteins. Results also indicate, a greater number of amino acids was also involved in interaction shown in (Fig. 2B).

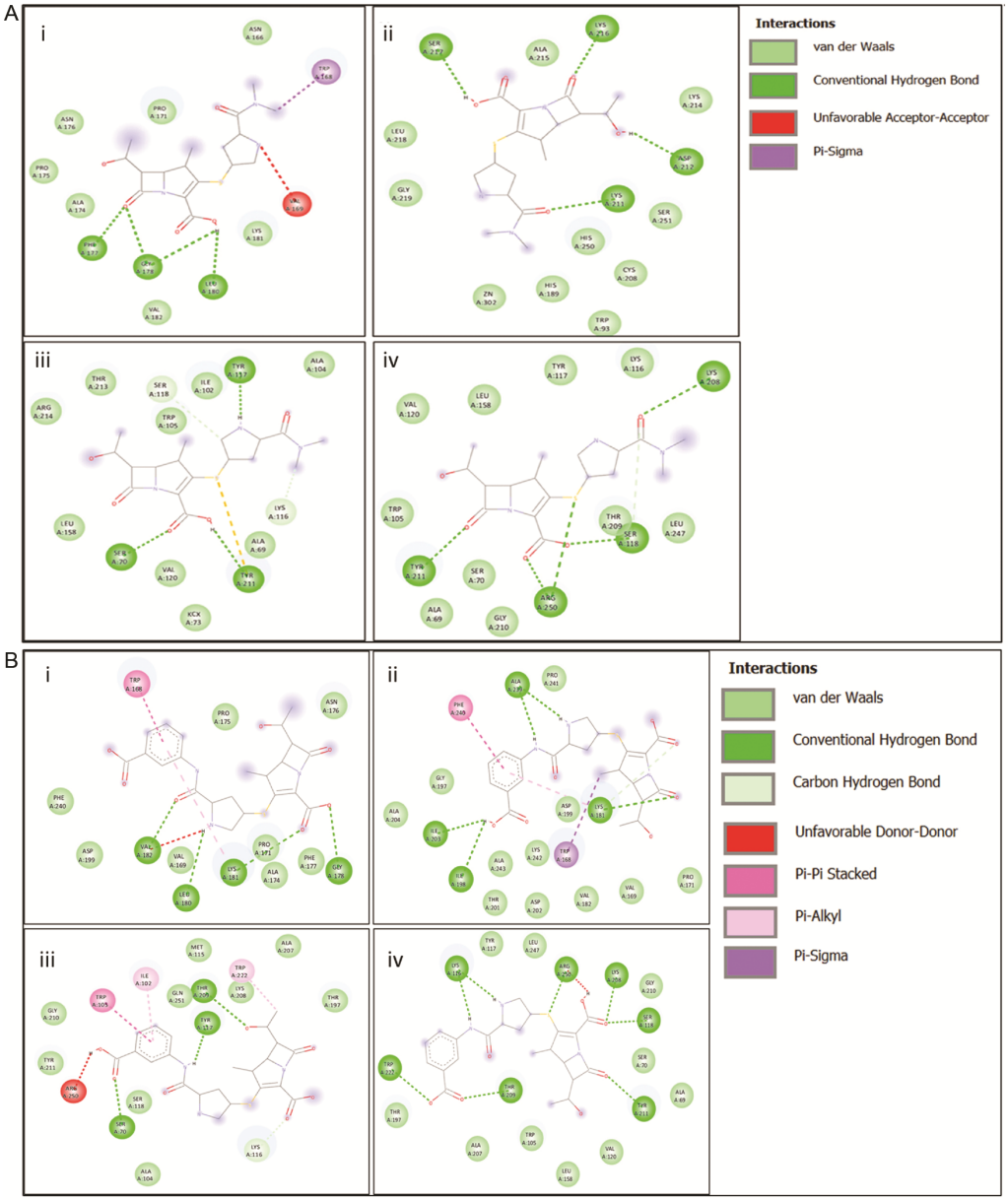

Fig. 2 — (A) Two-dimensional interaction diagram of meropenem with carbapenem-resistant proteins: low energy docking conformations of meropenem docked with (i) NDM-1 (ii) NDM-5, (iii) OXA-181, and (iv) OXA-232. Amino acid residues involved in hydrogen bonding (green) and van der Waals interaction (light green) are shown. Green dashed line representing hydrogen bonding; and (B) Two-dimensional interaction diagram of ertapenem with carbapenem-resistant proteins**:** low energy docking conformations of ertapenem docked with (i) NDM-1 (ii) NDM-5, (iii) OXA-181, and (iv) OXA-232.The amino acid residues involved in van der Waals interaction (light green) and hydrogen bonding (green) are illustrated. Green dashed line representing hydrogen bonding

Discussion

The present study addresses the important question of which carbapenemase (*bla*OXA or *bla*NDM) leads to high MIC values and whether a particular combination of dual carbapenemases leads to extremely high MIC values. We choose to study seven isolates of *Klebsiella pneumoniae* isolates harbouring no, single, or dual carbapenemase genes. The genome sequence of all isolates was compared and no differences were found in the mutations in porins. And also, there was no significant difference in the occurrence of efflux pumps between the susceptible and resistant isolates. From this analysis, we concluded that the carbapenemases (*bla*NDM-1, *bla*NDM-5, *bla*OXA-181, and *bla*OXA-232) contributing to the carbapenem resistance in the studied *K. pneumoniae* isolates. Since the focus of the study was to correlate the presence of single and dual carbapenemase producers with the MIC values, we discuss the same accordingly below.

K. pneumoniae isolates with *bla*OXA and *bla*NDM are very frequently found and many studies have determined the MIC[11,22-25]. However, since the breakpoint for resistant isolate is >2 µg/mL for ertapenem, and >4 µg/mL for meropenem, most reports show resistance and values beyond the break point are not determined. In the present study we conducted the MIC upto512 µg/mL to find the differences within the carbapenemases.

The carbapenemases, OXA-181 and OXA-232 only differed by a single amino acid, arginine at position 225 to serine, despite OXA-181carrying isolates having high MIC values and similarly, two amino acid difference between NDM-1 and NDM-5 valine at 89th position to leucine and methionine at 156th position to leucine was detected, although NDM-5 harbouring isolates were showing high MIC value. This single or double amino acid difference may contribute to enhancing the binding affinity of carbapenemases with both the antibiotics which could be the major possible reason for showing high MIC values. In addition to that, isolate harbouring OXA-181 (J20) demonstrated a higher MIC in this investigation, supporting a better hydrolytic activity towards carbapenems. In recent studies, comparison to OXA-48 and OXA-181, OXA-232 has been shown to have weaker hydrolytic activity towards carbapenems but a stronger ability to hydrolyzepenicillins[24-25]. Also, isolate with NDM-5 (M53) was showing greater MIC, similarly a study in which they reported, variants of NDM such as NDM-4, NDM-5, and NDM-7 had enhanced action towards carbapenems in comparison to NDM-1[26]. Overall, among all four individual carbapenemase producers, NDM-5 was showing Greater MIC (≥512 µg/mL and 512 µg/mL for ertapenem and meropenem, respectively), for both the carbapenem drug.

Next, we compared the dual carbapenemase producers, and we found that the combination of*bla*NDM-5+*bla*OXA-181 producer (M49) showed extremely high MIC values than the combination of *bla*NDM-1+*bla*OXA-232 (M17B). In a study from Norway, Oman, and Singapore, it was discovered that a dual carbapenemase-producing *K. pneumoniae* with a unique combination of (*bla*OXA-181+*bla*NDM-1) had >32 µg/mL MIC values for both meropenem and ertapenem[27-29]. A different report from Nepal revealed that the coproducer of *bla*NDM-1+*bla*OXA-232/181 had a MIC range of 128-512 µg/mL in *K. pneumoniae* isolates[23].

The present study's drawback is that NDM-5+OXA-232 and NDM-1+OXA-181 dual carbapenemase producers were not available. Hence, studies are warranted to understand the detailed analysis of these combinations.

Lastly, we did the docking studies to validate our genotypic and phenotypic analysis. It allows to analyse the conformation and orientation (referred as “pose”) of carbapenem drugs into the binding site of a macromolecular target[30]. Low energy docking conformations of drugs with carbapenem-resistant proteins showing high binding affinity.OXA-232 demonstrated the lowest binding energies to meropenem and ertapenem compared to OXA-181, NDM-5, and NDM-1, indicating a high binding affinity, while NDM-1 had a lowest binding affinity than the OXA-232 and NDM-5. Overall, with the exception of OXA-232, no other carbapenems were found to have any discernible differences in binding energy. Due to the exploitation of antibiotics, there is a need of an alternative treatment option such as; quorum sensing inhibitors[31], synthesis of nanoparticles (gold/silver etc.)[32], and screening of novel targets[33].

Conclusion

From this study, it is concluded that isolates with a single carbapenemase, *bla*NDM-5 (M53) followed by *bla*OXA-181 (J20) and a dual carbapenemase *bla*NDM-5+*bla*OXA-181 (M49) is a more deadly carbapenemase determinant than a single carbapenemase producer *bla*NDM-1 (M39) and *bla*OXA-232 (M52) or a dual carbapenemase *bla*NDM-1+*bla*OXA-232 (M17B) against both the antibiotics, regardless of the

presence or absence of efflux pumps and porin mutations. Further, the study should be confirmed with a greater number of isolates to confirm our findings.

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Conflict of interest

All authors declare no conflicts of interest.

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