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ORIGINAL ARTICLE

# Molecular Characterization and Computational Modelling of New Delhi Metallo-β-Lactamase-5 from an *Escherichia coli* Isolate (KOEC3) of Bovine Origin

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**Abstract** Emergence of antimicrobial resistance mediated through New Delhi metallo-β-lactamases (NDMs) is a serious therapeutic challenge. Till date, 16 different NDMs have been described. In this study, we report the molecular and structural characteristics of NDM-5 isolated from an *Escherichia coli* isolate (KOEC3) of bovine origin. Using PCR amplification, cloning and sequencing of full *bla*NDM gene, we identified the NDM type as NDM-5. Cloning of full gene in *E. coli* DH5α and subsequent assessment of antibiotic susceptibility of the transformed cells indicated possible role of native promoter in expression *bla*NDM-5. Translated amino acid sequence had two substitutions (Val88Leu and Met154Leu) compared to NDM-1. Theoretically deduced isoelectric pH of NDM-5 was 5.88 and instability index was 36.99, indicating a stable protein. From the amino acids sequence, a 3D model of the protein was computed. Analysis of the protein structure elucidated zinc coordination and also revealed a large binding cleft and flexible nature of the protein, which might be the reason for broad substrate range. Docking experiments revealed plausible binding poses for five carbapenem drugs in the vicinity of metal ions. In conclusion, results provided possible explanation for wide range of antibiotics catalyzed by NDM-5 and likely interaction modes with five carbapenem drugs.

**Keywords** Metallo-β-lactamase · *bla*NDM-5 · Structure · Carbapenem · Cleft · Docking

D. Purkait, A. Ahuja and U. Bhattacharjee have contributed equally for this study.

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## Introduction

Emergence of antimicrobial resistance is an evolving public health crisis [1]. Among many available antimicrobials, β-lactam antibiotics had been the preferred choice for clinical application due to their efficacy, low cost and minimal toxicity [2]. β-lactamases constitute most important resistance mechanisms for inactivating the β-lactam antibiotics by hydrolysis of the β-lactam ring [3]. Of the two classes of β-lactamases, serine-β-lactamases and metallo-β-lactamases, the latter poses the stiffest clinical challenge by inactivating almost all β-lactams, except for monobactams [3, 4]. New Delhi metallo-β-lactamase (NDM) is the most recent addition in the list of these difficult enzymes and is associated with extensive resistance in Gram negative bacteria [5, 6]. Since first report of NDM in 2009, 16 variants of NDM enzymes (NDM-1 to -16) have been discovered and assigned [7].

Occurrences of NDM have been reported from many countries including Asian countries such as China, Bangladesh, India, Vietnam [3, 8–16]. Among many variants, NDM-5 has been reported to possess increased resistance to carbapenems and broad-spectrum cephalosporins and was first reported in 2011 in an *Escherichia coli* isolate from United Kingdom [10]. Since then, an increasing number of human infections with organisms harbouring NDM-5 have been reported from many parts of the world [3] including Spain [17], China [9], Algeria [18], Japan [19], South Korea [20] and India [11]. Evidences of animal infections with NDM-5 are rare, though infections with organisms carrying NDM-1 have been reported in companion animals [21].

Structural characterization (such as modelling, docking) of protein molecules, provides insight into molecular functions and biological interactions, thus providing a platform for identification of plausible antibacterial agent [22]. However, unlike NDM-1, till date, there have been no reports on the structural characteristics of the NDM-5. In a previous report, we reported the presence of *bla*NDM, from an *E. coli* isolate (KOEC3) of bovine origin [23]. Since NDM-5 is known to possess more resistance to carbapenems, we intended to investigate the molecular and structural basis of carbapenem inactivation by NDM-5 through a combined wet lab and in silico approach.

## Materials and Methods

### Full Gene Amplification, Cloning and Characterization of Transformed Cells

Full gene of *bla*NDM from KOEC3 isolate was amplified by PCR using the reported primers [10] at 58 °C for annealing and 72 °C for 45 s for extension. Amplification was checked by agarose gel (1.5 %) electrophoresis. The PCR product was purified by QiaQuick PCR purification kit (Qiagen, USA) as per manufacturer's instruction. The purified PCR product was then ligated into a pTZ57R/T vector using InsTAclone PCR cloning kit (Thermo Scientific, Waltham, USA) as per manufacturer's instructions with negative control of only vector (without insert) and positive control of 'control fragment' supplied with the kit. Following ligation, *E. coli* DH5α cells were transformed using the ligated vector (InsTAclone kit, Thermo Scientific). Transformed cells, with recombinant plasmid were selected through Blue-White colony screening on X-Gal (20 µg/ml)—IPTG (24 µg/ml)—ampicillin (50 µg/ml) containing agar plates. For transformation control, plasmids (with and without insert) provided in the kit were used as positive and negative control, respectively. Presence of *bla*NDM gene in cloned sample was confirmed by colony PCR as described above. Orientation of the insert was checked by colony PCR employing forward primer of NDM and T7 promoter universal primer. In order to eliminate the background resistance effect of the vector pTZ57R/T on the final result, *E. coli* DH5α cells were transformed only with the vector without any insert.

Transformed *E. coli* cells and the KOEC3 isolate were subjected to antibiotic susceptibility test by Pheonix$^{TM}$ 100 (Becton–Dickinson, Singapore) or by broth dilution method as per EUCAST guideline [24]. Results were interpreted according to manufacturer's instructions/EUCAST recommendation.

### Sequencing

Purified recombinant plasmids from transformed *E. coli* cells were subjected to bi-directional sequencing by using the BigDye Terminator cycle sequencing kit (Applied Biosystems, USA) in ABI 3500xL Genetic analyzer automated sequencer (Applied Biosystems, USA) as per the manufacturer's instructions.

### Sequence Analysis

The sequences obtained through bidirectional sequencing were then assembled and homology was searched against the *bla*NDM sequences available at Lahey Clinic database [7] using the NCBI blastn tool (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Nucleotide sequence was further translated into corresponding amino acid sequence using the EMBOSS Transeq tool and was analysed for primary information using the ExPASyProtParam tool.

## 3D Model Building

3D model of the enzyme was built through homology modelling using Swiss-Model Workspace available at http://swissmodel.expasy.org/. Due to unavailability of crystal structure of NDM-5 in the protein data bank an initial template search was made and three structures of NDM-1 (PDB IDs 4eyb.1.A, 4h0d.1.A, 3q6x.1.A) were chosen. NDM-5 models were built using these templates and the final model was selected based on their QMEAN score.

Quality of the computed model was analysed at QMEAN server available at http://swissmodel.expasy.org/qmean/cgi/index.cgi. Ramachandran plot analysis, all atom clash score, and protein geometry of the structure were estimated using the MolProbity tool available at http://molprobity.biochem.duke.edu/.

Computed model was also put to molecular dynamic simulation for 2.5 ns using NAMD software [25]. For simulation, the model was minimized, solvated, added with sodium and chloride ions at 0.15 mol/l concentration.

### Structure Analysis

Model structure was analysed at the PDBsum server available at http://www.ebi.ac.uk/pdbsum/. Secondary structure was analysed using ProMotif, and cleft analysis was conducted using SurfNet1.4 and metal binding was investigated using the Ligplot available at the same server. During analysis computed model, two other previously reported structures (PDB ID: 4EXY and 4EYL) were also included. Since our computed model was a monomer, only monomeric structures were chosen up to a resolution of 2 Å. In case(s) where ligand was already complexed, the same was removed and further processed.

### Binding Study

Binding of five carbapenem antibiotics (doripenem, ertapenem, faropenem, imipenem, meropenem) were studied using the SwissDock server [26] under accurate mode. Ligand molecules (PubChem IDs: doripenem-73303; ertapenem-150610; faropenem-65894; imipenem-104838; meropenem-441130) were prepared using UCSF Chimera tool 1.10 [27]. Since energy minimization was known to optimize molecules towards stable state, the receptor molecule (NDM-5) was prepared by addition of H-ions and minimization of energy by AMBER force field 99SB using Chimera 1.10. Resulting molecule was submitted to SwissDock server for blind docking and results were visualized with Chimera 1.10. Similarly two other structures (PDB ID: 4EXY, 4EYL) of NDM-1 was also included in the analysis for further comparison.

## Results and Discussion

### Molecular Characterization

Molecular characterization of the *E. coli* isolate (KOEC3) targeting *bla*NDM gene resulted in amplification of an expected product with a molecular weight of 813 bp. Colony PCR of transformed cell resulted in amplification of an approximately 850 bp indicating correct orientation (Supplementary Figure 1). Alignment of obtained sequence with *bla*NDM sequences listed at Lahey database and NCBI nucleotide database [7] revealed 100 % sequence alignment with *bla*NDM-5 (JN104597, KF408074, KF408073, KF408072, KM598665). Therefore, the *bla*NDM gene of KOEC3 isolate was inferred as *bla*NDM-5. The sequence was deposited to NCBI (KC769583.2).

The isolate (KOEC3) was resistant to almost all antibiotics (19/22) tested (Table 1). The isolate was also resistant to cephalosporins (cefazolin, cefuroxime, ceftazidime, cefepime->16 μg/ml; ceftriaxone->32 μg/ml), carbapenems (imipenem, meropenem, doripenem, ertapenem->8 μg/ml; faropenem->16 μg/ml), other β-lactams (ampicillin, amoxicillin-clavulanate, aztreonam) but was susceptible to tetracycline, colistin and chloramphenicol. In contrast, transformed *E. coli* DH5α cells were resistant only to cefazolin (>16 μg/ml), ampicillin (>16 μg/ml) and amoxicillin-clavulanate (>16/8 μg/ml; Table 1). Similar results were also obtained for transformed *E. coli* DH5α cells without the insert. The loss of resistance to a majority of β-lactams in the transformants was also

**Table 1** Minimum inhibitory concentrations (MIC) of *E. coli* isolate KOEC3 and transformants for various antimicrobials (μg/ml)

| Antimicrobials | KOEC3 | Transformed *E. coli* DH5α (with *bla*NDM-5 insert) |
|---|---|---|
| Amikacin | >32 | ≤8 |
| Gentamicin | >8 | ≤1 |
| Tobramycin | >8 | ≤1 |
| Imipenem | >8 | ≤1 |
| Meropenem | >8 | ≤1 |
| Doripenem | >8 | ≤1 |
| Faropenem | >16 | ≤1 |
| Ertapenem | >8 | ≤0.5 |
| Cefazolin | >16 | >16 |
| Cefuroxime | >16 | 8 |
| Ceftazidime | >16 | ≤0.5 |
| Ceftriaxone | >32 | ≤2 |
| Cefepime | >16 | ≤1 |
| Aztreonam | >16 | ≤2 |
| Ampicillin | >16 | >16 |
| Amoxicillin-Clavulanate[a] | >16/8 | >16/8 |
| Piperacillin-Tazobactam[b] | >64/4 | >64/4 |
| Trimethoprim-sulfamethoxazole | >2/38[c] | ≤0.5/9.5[d] |
| Nitrofurantoin | 32 | ≤16 |
| Ciprofloxacin | >2 | ≤0.5 |
| Levofloxacin | >4 | ≤1 |
| Moxifloxacin | >4 | ≤1 |
| Tetracycline | ≤2 | ≤2 |

[a] Amoxicillin (16 μg/ml) and clavulanate (8 μg/ml) in combination
[b] Piperacillin (64 μg/ml) and tazobactam (4 μg/ml) in combination
[c] Trimethoprim (2 μg/ml) and sulfamethoxazole (38 μg/ml) in combination
[d] Trimethoprim (0.5 μg/ml) and sulfamethoxazole (9.5 μg/ml) in combination

reported previously [10] suggesting the role of native promoter in expression of NDM-5. In the present study, the absence of native promoter in the transformed DH5α cells might have resulted in susceptibility to several antibiotics. Moreover, common cloning vectors are known to contain resistance marker genes under separate promoters, but to our knowledge none is known to contain carbapenem resistance as 'resistance marker' for selection of transformed cells. Therefore, the loss of resistance to carbapenems drugs was most likely due to absence of native promoter.

## Amino Acid Sequence

Primary sequence of NDM-5 gene consisted of 270 amino acids, with molecular weight of 28495.4 and theoretically determined isoelectric point of 5.88 which was similar to NDM-4 [28]. Calculated instability index (36.99) indicated the protein to be stable as proteins with an instability index below 40 were considered as stable [29]. Comparison of amino acid sequence of NDM-5 (KOEC3) with other NDM sequences listed at Lahey database revealed varying degree of amino acid substitutions ranging from 1 to 7 (Supplementary Table 1). Two substitutions (Val88Leu, Met154Leu) observed in NDM-5 (KOEC3) was also reported previously [10]. Interestingly, the presence of leucine at position 88 was unique to NDM-5 and might serve as a signature for NDM-5. Though this substitution is believed to confer increased resistance to carbapenems [10], in our subsequent docking studies we did not observe any direct interaction between the drug molecule and the leucine residue at position 88.

## Modelling of NDM-5

3D modelling of NDM-5 yielded three models and the final model (Fig. 1a, b) was chosen based on the highest QMEAN4 score of 0.79. Model quality checking (Supplementary Table 2) revealed that all atom clash score was 3.38 (97th percentile; 100th-best; 0th-worst) and overall MolProbity score was 1.18 (99th percentile) indicating reliable model quality.

Further quality check against non-redundant set of protein data bank structures at QMEAN server, indicated acceptable model quality (Supplementary Figure 2a, 2b, 2c) with QMEAN score of 0.796 and overall Z-score of 0.29. Per residue error plot of the model was visualized on a colour scale of blue (errors <1 Å) to red (errors above 3.5 Å). While our computed model was quite reliable, two error prone regions were also discovered.

Ramachandran plot analysis of NDM-5 structure revealed that 97.9 % of all residues were in favoured regions and 99.6 % of residues were in Ramachandran allowed regions with one outlier (phi, psi) at 90 Asp (Supplementary Figure 3).

Molecular dynamic simulation of computed NDM-5 model revealed consistent RMSD values (Supplementary Figure 4) which indicated reliable structure.

The NDM-5 chain was a monomer with two zinc atoms attached (Fig. 1a). Surface topology of the NDM-5 chain was computed using Chimera 1.10. The total solvent accessible surface area (SASA) was 10105 $Å^2$ and solvent excluded surface area was 9057.37 $Å^2$. SASA was also calculated from the molecular weight of the protein by the equation:

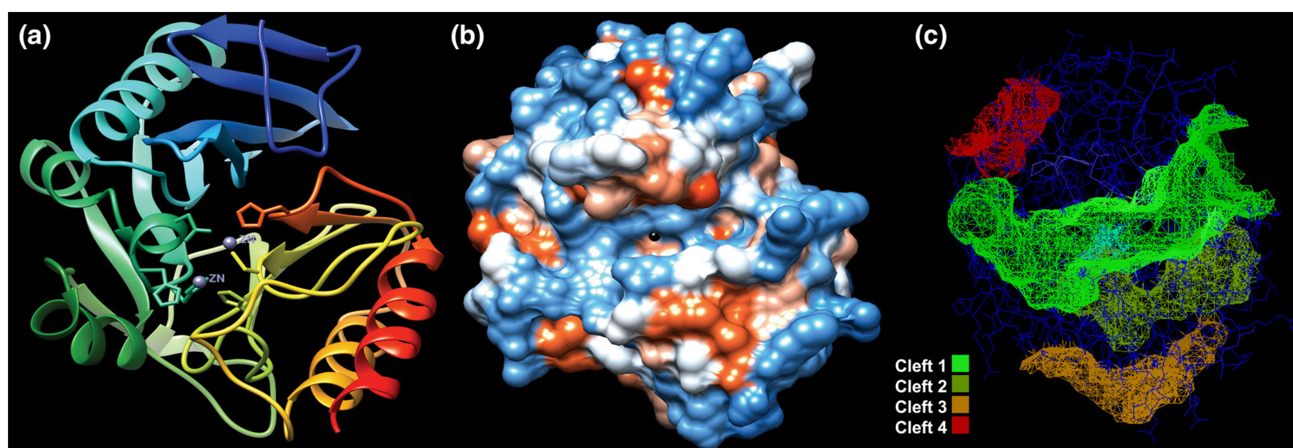


**Fig. 1** Computed three dimensional structure of NDM-5 (created with UCSF Chimera 1.10). **a** Ribbon structure of NDM-5 coloured in rainbow style. Metal ions coloured *grey*. **b** Surface topology of NDM-5 coloured by Coulombic surface colouring. Surface calculated using AMBER ff 14SB charge model on three colour scale of *red*, −10 kcal/(mol *e*), *white*, 0 kcal/(mol *e*), and *blue*, 10 kcal/(mol *e*), where *e* is unit electron charge. **c** Predicted binding clefts of NDM-5

$$SASA = 4.84M^{0.760}$$

where, M is the molecular weight of protein [30]. Calculated SASA was 11761.8. From these two estimates we calculated relative SASA value for NDM-5 which was 0.86. Since relative SASA was known to be a dependable predictor of flexibility of monomeric proteins, our results indicated that our computed model of NDM-5 was reasonably flexible allowing conformational changes during catalytic activity of the enzyme [30]. Conformational changes in metallo-β-lactamases leads to a closure of loop 3 that aids in substrate interaction and can increase the substrate range [3].

Secondary structure analysis revealed that the NDM-5 chain (residue 30–270) was composed of 2 sheets (A and B), 4 beta–alpha–beta motifs, 12 strands, 8 helices. There were 24 instances of β-turns, 6 β-hairpins, 4 β-bulges, 1 γ-turn and 5 helix–helix interactions (Supplementary Figure 5).

Metal ions (zinc) are important for the activity of metallo-β-lactamases [3]. Results (Supplementary Figure 6) of metal binding analysis revealed that both the zinc atoms were coordinated in trigonal bipyramid geometry at His and Cys site. While the zinc atom at Cys site was held by aspartic acid (124), cystine (208) and histidine (250, also the closest; 2.03 Å), the other zinc atom at His site was coordinated by three histidine residues located at 120, 122 (closest at 1.96 Å), 189 position In our model for NDM-5, the distance between two zinc atoms was 4.54 Å which was less than a previous report on NDM-1 [31].

To identify, the most probable binding sites, we undertook cleft analysis of our computer generated model. Our analysis showed existence of four major clefts (Fig. 1c, Supplementary Table 3). The volume of the largest cleft was 2958.19 $Å^3$ (bright green) followed by the second largest cleft with a volume of 934.45 $Å^3$ (dull green). In case of single chain proteins, possession of large cleft and the ratio (ideally >2.0) of two largest clefts (R1) are important functional requirements as ligands tend to preferentially bind to the largest cleft [32]. Therefore, the largest cleft (cleft 1) is presumably the most probable binding site for our computed model of NDM-5. In case of our model of NDM-5, the ratio was 3.17, indicating that cleft 1 was the most probable and also the preferential binding site for ligands. Cleft analysis was also performed for two previously published structures of NDM-1 (PDB ID: 4EXY, 4EYL) employing same web server (PDBsum). Results revealed that compared to NDM-5 (KOEC3), the largest clefts of 4EXY and 4EYL were smaller by 4.35 and 13 %, respectively. Moreover, largest clefts of 4EXY and 4EYL were also less deep. Therefore, the results of our study indicating existence of a comparatively larger and deeper cleft in NDM-5 (cleft 1), along with higher R1 ratio, flexibility and stability of the molecule (see previous sections), possibly explained the greater catalytic activity by NDM-5 against wide range of antibiotics. Inclusion of bulky side chain to antibiotic molecule is a common strategy for circumventing antibiotic hydrolysis by bacterial enzymes. To investigate the possibility of such approach, we compared the molecular sizes of the carbapenem molecules (Doripenem −341.0 $Å^3$, Ertapenem −390.2 $Å^3$, Faropenem −223.6 $Å^3$, Imipenem −261.2 $Å^3$, Meropenem −326.5 $Å^3$) with the predicted largest binding cleft (cleft 1) volume. Our analysis revealed very low drug volume-to-cleft volume ratio ranging from 0.076 for faropenem to 0.132 for ertapenem. These results indicated that due to existence of a quite large binding cleft the tactic to increase the bulk of the drug molecule may not be suitable for development of novel antibiotics against NDM-5 producing bacteria and other alternate strategies need to be explored.

## Ligand Binding

Prediction of ligand binding (docking) by in silico molecular docking is a useful approach to study molecular interactions [22]. This has been successfully applied for identification of novel antimicrobial targets [33], putative antifungal analogues [34]. However, limitations of computational docking should not be ignored while interpreting results [35].In the present study we employed computational docking for analysis of interactions between NDM-5 and five carbapenems antibiotics.

Docking studies were performed for five carbapenem antibiotics at the SwissDock server using CHARMM force field parameters [26]. We assumed that the most likely ligand BMs should be the ones that bind within the largest cleft on the protein surface with best available full fitness value returned by the docking experiments. Based on this we attempted to identify most probable BMs for the antibiotics (Fig. 2a–e). Overall, our results corroborated with previous results on NDM-1 [36]. Free energy values (kcal/mol) and full fitness scores of ligands (Supplementary Table 4) revealed lowest ΔG for meropenem (−8.65) followed by imipenem (−8.24) and doripenem (−8.18). But highest full fitness score was obtained for doripenem (−1059.9) followed by imipenem (−1052.4) and meropenem (−917.5). The results indicated that among all BMs, BM for imipenem was most consistent.

Further inspection of the BMs, revealed that the rim of the binding pocket was formed by Ile35, Gly36, Gln37, Lys214, Ala215 and Lys216. Residues at the rim of the binding pocket are known to influence the binding between the enzyme and drug molecules by van der Waals interactions and hydrogen bonding thus securing the drug molecules in binding cleft [31]. Due to

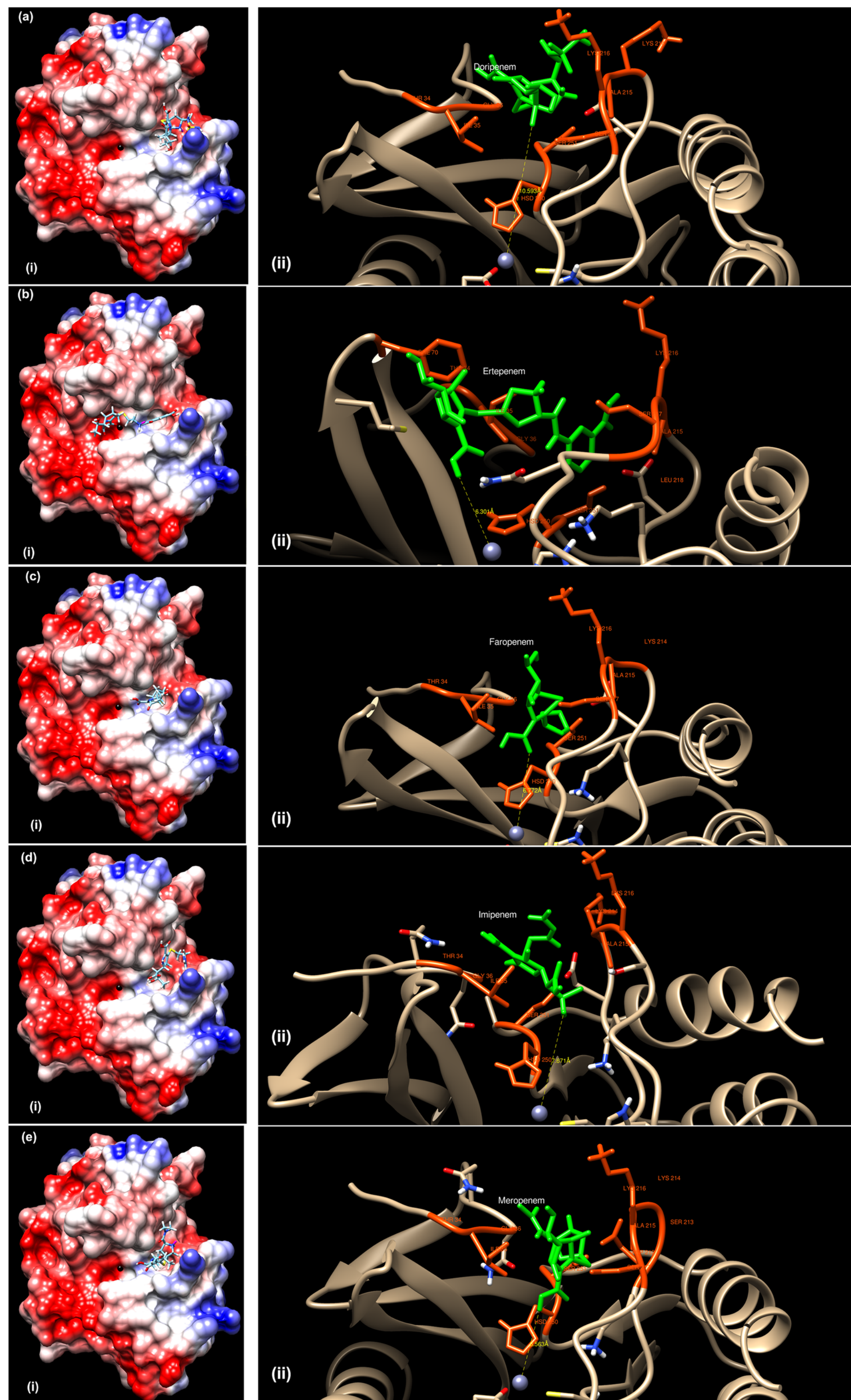


**Fig. 2** Binding of various antibiotic molecules with NDM-5. **a** Doripenem, **b** Ertapenem, **c** Faropenem, **d** Imipenem, and **e** Meropenem. (*i*) and (*ii*) in each panel indicate overview and involvements of residues (*orange*) and metal ions (*grey*), respectively

unavailability of similar studies with NDM-5, we compared our results with previous report available for crystal structure of NDM-1 complexed with hydrolyzed meropenem [37]. In both cases, bindings were mediated by Ala215, Gly219, His250, and Lys216. However differences were observed from a previous study on NDM-1 [31], where binding of imipenem and meropenem were predicted closer (within 5 Å) to the His site of zinc

coordination. However, comparison of docking results of NDM-5 (KOEC-3) with the docking results of two other previously reported structures (4EXY, 4EYL) revealed that the minimum distances over which the ligand binding took place were variable (Supplementary Table 5). While doripenem interacted with NDM-5 at farthest (10.593 Å), faropenem and imipenem interacted closer to NDM-5 compared to NDM-1 molecules (4EXY, 4EYL). Distances for ertapenem and meropenem were in between respective values for 4EXY and 4EYL.

Binding of multiple diverse ligands at a particular protein site is an emerging conceptual framework in protein science [38]. Proteins, if allowed to interact with an ensemble of ligands, might bind to multiple ligands with altered shapes and sizes [38]. In the present study, we observed a large preferentially binding cleft (cleft 1) on the NDM-5 molecule and the cleft volume was much greater than the molecular volumes of the ligands (carbapenem drugs) studied. Therefore, it is tempting to explore the possibility of binding of multiple ligands. However, it should be kept in mind that ligand binding in metallo-β-lactamases are mediated through zinc atoms which play critical role in initiation of catalysis and stabilization of hydroxide ion for nucleophilic attack on the carbonyl group of the drug molecule [3]. Unless, the zinc atoms are freed, binding of multiple ligands might not have biological implication.

## Conclusions

In conclusion, our study reported molecular and structural characterization of the *bla*NDM-5 previously isolated from an *E. coli* isolate (KOEC3) of bovine origin. While molecular characterization indicated the likely importance of native promoter for expression of NDM-5, computer aided structural analysis generated a stable three dimensional structure of the protein with four major predicted binding sites, coordination of the metal atoms (zinc) at His and Cys sites. Existence of large binding cleft on the molecule posed hindrance for development of novel antimicrobial by incorporating bulky side chain into the existing carbapenems drugs. Computer aided ligand binding studies, on the other hand, identified possible binding poses and interactions of five carbapenem antibiotic molecules (ligand) with the receptor (NDM-5).

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