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Designing of potent inhibitors for metallo-beta-lactamases producing *Escherichia coli* in molecular specification hereto

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Abstract: The rapid spread of Metallo-β-Lactamases producing Gram-negative bacteria in Pakistan is alarming and novel inhibitors with multi inhibition potential are required. In the current study, an effort was made to identify the resistance genes of MBLs producing *E. coli* and single inhibitor was designed having the potential to block all resistant proteins. Results showed that out of 573 clinical isolates, 14.1% MBLs producers have NDM-1 (27.2%) and VIM (13.6%) gene. The isolates were resistant to MEM, AMP, AMC, FEP, CTX, LEV and ATM, while effective antibiotics were TGC, CO, FOS and AK with MICs ranging from 4 to >32µg/ml. RECAP synthesis was used for de-novo discovery of 1000 inhibitors and protein crystal structures were retrieved from PDB. Active sites were identified in each protein and to improve ADMET properties, Lipinski's rules of five was applied. Placement of the ligand was done by London dG algorithm implemented in MOE. For final refinement, GBVI/WSA dG algorithm was used. Based on docking score, visual inspection of ligands interaction with key residues, binding affinity and binding energy of ligands with proteins, 10 compounds were selected for MBLs proteins which presented best ADMET properties, binding energy and affinity than the reported ones.

Keywords: *E. coli*, antibiogram, MICs, MBL genes, drug designing, RECAP synthesis, docking simulation.

INTRODUCTION

Escherichia coli, belonging to Enterobacteriaceae, are Gram-negative rods frequently found in the human gut. Most of the *E. coli* strains, as part of the normal flora, are nonpathogenic but some can cause serious infections in human. The most common infections associated with *E. coli* are; Urinary Tract Infections (UTI), diarrhea, bacteremia, systematic, wound infections and otitis media (Gebre-Sealsssie, 2007).

These infections are usually treated with antibiotics but it's over and misuse has created resistance in *E. coli* which has created serious health issues worldwide (Ventola, 2015). Prevalence and antibiogram profiling of *E. coli* show considerable terrestrial alterations and important variations in different populations of the world (Msolo *et al*., 2016). Antibiogram analysis of clinically isolated *E. coli* revealed high resistance to AMC, AMP, cephalosporins and CIP (Trojan *et al*., 2016; Yengkokpam *et al*., 2007) and in Pakistan, a high rate of antimicrobial resistance has been reported against CIP (49%), cefpodoxime (38%), CTX (36%) and ceftriaxone (34%) (Shah, 2002).

Gram-negative bacteria have developed multidrug resistance, particularly against β-lactam antibiotics due to the production of β-lactamases such as Extended Spectrum-β-lactamases (ESBLs) and MBLs (Sharma *et al*., 2013). Different types of inhibitors are used against ESBLs producing isolates, but carbapenems are still the best option but the appearance of MBLs has limited the use of carbapenems (Rodríguez-Baño *et al*., 2018). The MBLs producing bacteria have the capability to breakdown β-lactam drugs; penicillins, cephalosporins, carbapenems and cephamycins (Denisuik, 2013). In Pakistan, the prevalence of MBLs producing *E. coli* ranges from 34 to 41% among the clinically isolated *E. coli* (Enwuru *et al*., 2011; Nahid *et al*., 2013). A frequency of 18.98% was reported in a study conducted in Nepal while in India, the prevalence of MBLs producing *E. coli* ranges from 8 to 79% (Deshpande *et al*., 2010). These enzymes are carried either on a chromosome or acquired via plasmids. There are four major classes (A, B, C and D) of β-lactamases according to Ambler classification (Bush *et al*., 2010) and most of the MBL enzymes; IMP, VIM, GIM, SIM and NDM-1, belongs to class B (Kazmierczak *et al*., 2016). The carbapenemase, NDM-1 was first reported in 2009 in *K. pneumoniae* and *E. coli* isolates from a Swedish patient who had required medical care in New Delhi, India (Yong *et al.*, 2009) though it was later reported that NDM-1 positive organisms spread in Indian hospitals in 2006 (Castanheira *et al*., 2011). The high production of MBLs among the members of Enterobacteriaceae, mainly *E. coli* and *K. pneumoniae*, which are the most frequent causes of

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infections among humans, is a serious global public concern. Due to limited treatment options, for the infections caused by MBLs producing bacteria, it is very difficult to treat such infections often resulting in treatment failure (Kazmierczak *et al*., 2016). The availability of limited options, colistin and tigecycline are drug of choices against MBL producers (Antachopoulos *et al*., 2017; Holman *et al*., 2017). After completion of human genome project, a variety of new potent drug have been discovered to overcome antibiotic resistance but the quest is still continuous because of the emergence of multidrug resistance. Various techniques like Nuclear Magnetic Resonance (NMR), high throughput protein purification and crystallography are providing structural information of proteins and protein ligand complexes. In the drug discovery process, the combinatorial chemistry is a well-established strategy for generation of new lead molecules. The RECAP analysis and synthesis is a new computational tool used to generate fragments molecules and recombine these molecules in a variety of ways to synthesize reasonable novel chemical structures (Lewell *et al.*, 1998). Molecular Docking (MD) is one of the most common methods for structural based drug designing. It estimates the exact binding modes and bio-affinity of the ligands with target receptor. The basic tools of MD are to search algorithm and scoring functions for producing and analyzing conformations of the ligands (Guedes *et al.*, 2014). It has ability to give compounds with high hit rates during screening libraries of in-silico compounds in contrast to traditional large scale screening (Yi *et al.*, 2018). Thus, with the help of available computational tools the antibiotic resistance can be efficiently handled and will support the clinicians to overcome the resistance mechanisms.

The current study is therefore designed to determine the antimicrobial susceptibility patterns of MBL producing *E. coli*, and search for novel inhibitors, as it is necessary on regular basis for adequate clinical treatment and management.

MATERIALS AND METHODS

Samples collection

A cross sectional study was conducted prospectively between August 2017 and September 2019 in the Pathology Department of Khyber Teaching Hospital (KTH) and Center of Biotechnology and Microbiology, University of Peshawar (COBAM, UOP), Pakistan. All non-duplicate clinical samples including blood, Cerebrospinal Fluid (CSF), urine, pus, sputum and infected wound swabs were aseptically collected from patients of different age groups and transported immediately to the microbiology laboratory for culture and susceptibility testing. Patients undergoing antibiotic therapy and/or suffering from parasitic or fungal infections were excluded from the study. All of the patient's necessary information including history of infection and treatment summary were obtained from hospital and laboratory records. The study was approved by the Institution Research and Ethical Review Board (IREB) of Khyber Medical College, Peshawar (Document No. 122/ADR/KMC).

Bacterial culture and identification

All the collected samples were transported to the microbiology laboratory of KTH and were inoculated on different culture media. Urine samples were inoculated on Cysteine Lactose Electrolyte-Deficient medium (CLED) and MacConkey agar (Oxoid, UK) and incubated over night at 37°C. Specimens like pus, wound swabs and sputum were inoculated on blood agar and MacConkey agar plates and incubated over night at 37°C. Clinical samples like blood (5ml), CSF (5ml) or other body fluids (5ml) were added to each screw capped sterile bottle of automated blood culture system (Versa Trek, USA) containing 45ml medium. The inoculated bottles were subjected for incubation for 5 days at 37^{o}C and observed for the gas production. Pure colonies were analyzed for morphology and Gram staining. Later on, pure cultures of bacteria were further subjected to different biochemical tests using Analytical Profile Index (API) 10S strips (Biomerieux, France) for confirmation (Alnahass *et al.*, 2016).

Phenotypic detection of MBLs

The MBL detection was performed using disc diffusion method. The MBL production was confirmed using double disc potentiation method using inhibitor Ethylene Diamine Tetra Acetic acid (EDTA). The test organism (0.5 McFarland) was inoculated on the Mueller Hinton Agar (MHA) plate and two MEM discs (10µg) were placed while 7µl of EDTA solution (0.5M) was added to one MEM disc. Increase in zone size (>7 mm) in EDTA containing discs represented the MBL production (Asthana *et al.*, 2014).

Antimicrobial susceptibility testing

All isolates were subjected to antimicrobial susceptibility testing against selected antibiotic discs by Kirby Bauer disc diffusion technique as per Clinical and Laboratory Standard Institute 2019 (CLSI-19) guidelines. Briefly, bacterial suspension was adjusted to 0.5McF and spread on MHA. The selective discs were applied on the inoculated culture plates and incubated overnight incubation at 37˚C. The results were interpreted by measuring the inhibition zone diameter according to CLSI-19 guidelines (Cusack *et al.*, 2019). The identified resistant isolates were preserved in 20% glycerol supplemented with Tryptone Soya Broth (TSB) and stored at -80^{o}C (Bashir *et al.*, 2012).

Determination of minimum inhibitory concentrations

The MICs test strips were used for the determination of MICs of selected antibiotics. The test strips containing

exponential gradient of antimicrobial agents were placed on the surface of an inoculated MHA medium. These plates were incubated for 24hrs at 37°C. The MICs were recorded immediately from the scale (µg/mL) at the point where the edge of the inhibition ellipse intersects the MIC test strip (Delma *et al.*, 2020).

Genomic DNA extraction and preservation

The freezed isolates were sub-cultured on MacConkey agar and incubated overnight at 37˚C. Chromosomal DNA was extracted by using GeneJet genomic DNA purification kit (Thermo Scientific, USA). The DNA integrity was determined by gel electrophoresis in 1% agarose (w/v) and purified DNA was stored at -20°C (Bashir *et al.*, 2012; Chen *et al.*, 1998).

Molecular identification of E. coli

The *E. coli* isolates were confirmed by amplifying *β*-D-glucouronidase (*uidA*) gene using specific primers (Bashir *et al.*, 2012). The final Polymerase Chain Reaction (PCR) was done in 27µl final reaction volume containing; 12.5µl Taq master mix (Bioron, Germany), 0.5µl of 50pM forward primer, 0.5µl of 50pM reverse primer, 2µl of DNA template and PCR grade water. Amplification was carried out by using specific primer sequences under the conditions as mentioned in table 1. The gel electrophoresis was done for amplified products and subsequently, gel documentation was done for the visualization of bands.

Molecular characterization of MBLs gene(s)

Molecular identification of MBLs genes were carried out by PCR using the specific primers. All the isolates were screened for MBLs resistant genes; NDM-1 and VIM in pathogenic *E. coli* (Pitout *et al.*, 2005; Poirel *et al.*, 2011; Shanthi *et al.*, 2014). The list of primers and optimized PCR conditions are presented in table 1. The amplified products were electrophoresed at 110volts for 40 to 60 minutes on 1.5% agarose suspended in 1X Tris-acetate-EDTA (TAE) buffer. Gels were stained with Ethidium Bromide solution. Bands were visualized and photographed by gel documentation system (Bio Rad Milan, Italy) using GeneSnap software. The amplicon sizes were determined by comparing them with a 100-bp DNA ladder (Thermo scientific) (Aboderin *et al.*, 2009).

Designing of potent MBLs inhibitors

For drug designing, crystal structures were retrieved from protein data bank (https://www.rcsb.org). NDM-1 PDB-ID 5YPL (Feng *et al.*, 2017) and VIM PDB-ID 4UWR (Leiros *et al.*, 2015) were selected having the refine resolution. Each crystal structure was refined using the MOE2019.0102 auto refinement method to remove the hydrogen bond clashes and gaps in the structure. The side chain refinement was done using Maestro from Schrodinger suite (Schrödinger, 2013).

Data analysis

To visualize the crystal structures, Molecular Operating Environment (MOE2019-0102), PyMOL software, Maestro from Schrodinger suite were used (Bello, 2018; DeLano, 2002).

Electrostatic maps

For the prediction of electrostatically preferred locations of H-bond acceptor, H-bond donor and hydrophobic atoms in the active site, electrostatic Maps were generated for each protein using 4.5 Å receptor grids in MOE2019.0102.

RECAP analysis and synthesis

To generate novel inhibitors database, a total of 21 reported *β*-lactamases inhibitors were recovered from Pubchem server (https://pubchem.ncbi.nlm.nih.gov/) having a unique pubchem CID and were kept in the house database in mdb format. RECAP Analysis and Synthesis implemented in MOE 2019 were applied on the above reported inhibitors as part of a de novo discovery methodology. 1000 novel structure were generated and were saved in mdb format. All the structures in the database were energy minimized and the hydrogens were added to it using the MOE2019.0102.

ADMET properties

To select inhibitors having good ADMET properties, Lipinski's rules of five were applied on output database generated from the RECAP synthesis (Lipinski, 2004).

Docking validation and simulations

Before docking of the novel ligands in the proteins active site, the docking protocol of MOE2019.0102 was validated through redocking of the reported inhibitors in the crystal structures. The method of Rigid body pose generation was used for Protein ligand docking. The validation of docking was reported in RMSD of the redock and reported ligand superimposition. The retrieved output database from RECAP analysis and synthesis was docked in the active sites of each protein after the docking validation. In order to generate tens of thousands of poses of ligand around the restraints to restrict binding site region, the tool of Fast Fourier Transforms (FFT) was selected. The (rigid body) top 10 poses were refined with R-Field electrostatics and the final Refine (top 10 poses) with full GBVI/WSA dG solvation model was generated as output. The docked compounds were saved in mdb format. Using the proxy triangle algorithm, 1 conformation was saved for each ligand using London dG scoring methodology in MOE2019.0102 for refinement (Labute, 2008). Final 10 compounds were selected on the basis of highest docking score, interaction with key residues visual inspection, binding energy and binding affinity, having the potential to inhibit all the MBLs proteins. These 10 final hits have greater binding energy and binding affinity from the crystal structures reported inhibitors.

Calculations of binding energy and binding affinity

The binding affinities were calculated by applying Generalized Born/Volume Integral (GB/VI) algorithm in MOE2019-0102 to determine the most potent ligand for each *β*-lactamases protein complex with ligands. All the non-bonded interaction energies are Generalized Born interaction between the protein residues and the ligand molecules consist of coulomb electrostatic interaction. The binding energy and binding affinity were calculated and described in unit (Kcal/Mol) for each hit after energy minimization (Wadood *et al.*, 2017).

STATISTICAL ANALYSIS

The IBM SPSS Statistics (version 23.0.0) software was used for the calculation of different frequencies and percentages in the current study.

RESULTS

Phenotypic results of MBLs producing E. coli

A total of 81 (14.1%) *E. coli* isolates were positive for MBLs production i.e. resistant to MEM antibiotic disc. Of the isolates, 27 (33.3%) patients were males while 54 (66.7%) were females. The highest rate of MBLs production 26(32.1%) was observed among the age group 41-60 years followed by age-group 21-40 years 21(25.9%), 15(18.5%) in age group 11–20years, 12(14.8%) in age group >60 years and 07(8.6%) in age group 00–10years. It was observed in the current study that majority of the MBLs-Ec isolates were recovered from urine 50 (61.7%) followed by Pus 27 (33.3%) and others 04 (4.9%). The results of the different clinical isolates of MBLs-Ec revealed that 51 (63%) patients had UTIs and 30 (37%) patients had systemic infections (SSIs). The results of the current study revealed that most MBL positive isolates were detected in hospitalized patients 44(54.3%) as compared to OPD patients 37 (45.7%) as shown in table 2.

Antimicrobial resistance in MBLs-Ec isolates

The results of the antibiogram revealed that all isolates were found sensitive (100%) to TGC and CO while resistant (100%) to antibiotics; AMP, FOX, AMC, MEM, IPM, FEP, CTX, ATM and CAZ. The highest resistance was observed in isolates tested against SXT 77(95.1%), CIP 76(93.9%), LVX 75(92.6%), TOB 72(88.9%), CN 69(85.2%), C 61(75.3%), AK 53(65.4%), DO 49(60.5%) and FOS 35(43.2%) as shown in table 3.

Determination of minimum inhibitory concentrations

The MBLs producing isolates were highly resistant to *β*-lactam drugs; CTX 100% (MIC_{50} ≥128; MIC_{90} ≥256 µg/ml), CAZ 100% (MIC_{50} ≥64; MIC_{90} ≥256 µg/ml) and Meropenem 100% (MIC_{50} ≥4; MIC_{90} ≥32 µg/ml). These isolates were also resistant non *β*-lactam drugs; SXT 95% (MIC_{50} ≥32; MIC_{90} ≥32 µg/ml), CIP 92.6% (MIC_{50} ≥32; MIC_{90} ≥256 µg/ml), CN 85.2% (MIC_{50} ≥16; MIC_{90} ≥16 µg/ml) and DO 61.7% (MIC_{50} ≥16; MIC_{90} ≥192 µg/ml) while all the isolates were susceptible (100%) to TGC (MIC_{50} ≥0.5; MIC_{90} ≥1.5 µg/ml) as shown in table 4.

Molecular characterization of MBLs gene (s)

Of the isolates, the targeted genes for MBLs were identified in 27(33.3%) isolates. Genetic analysis detected the MBLs genes; NDM-1 22(27.2%) and VIM 11(13.6%) as shown in table 2 and figure 1. The positive isolates of the current study were confirmed by Sanger sequencing to verify the resistant genes of *E. coli*. The sequencing results of MBLs genes were aligned with reference sequences; Bla-NDM-1 gene with accession number AB604953 and Bla-VIM gene with accession number KR296661.

Designing of potent MBLs inhibitors

Resistance to antibiotics is an issue of concern and has involved interests for the researchers worldwide. In the past, Insilico approaches have been used to predict strategies like targeting the protein crystal structures to design novel inhibitors to overcome the mechanism of resistance (Hassan Baig *et al.*, 2016).

To design multi inhibition small molecules for MBLs proteins, crystal structures of the selected proteins were retrieved from the RCSB PDB server. The Bla-NDM-1 protein PDB ID 5YPL surface representation is reported in figure 2A and Bla-VIM PDB ID 4UWR in figure 2B. The Bla-NDM-1 electrostatic map of the active site is shown in figure 3A. The hydrogen bond acceptor represented in red, hydrogen bond donor atoms in blue and the hydrophobic interaction atoms are represented by green color.

Docking validation

The docking validation was done by superposing co-crystalline and redocked are shown in fig. 4. The NDM-1 redocked and reported superposed ligand has suitable range of RMSD 0.5644 A˚. The crystal structure reported ligand re-docking observed that our docking procedures were satisfactory and can be valid to dock other compounds in the active site of the proteins.

Docking Simulation, prediction of binding energy and binding affinity

The residues interacting with the reported ligand in the crystal structure were selected as active pocket of the proteins. These potent compounds generated from RECAP synthesis were docked with the active pocket residues. The docked compound of 20% based on the lowest docking score was selected for visual inspection, to identify the best interacting compounds. Based on docking score, binding energy and binding affinity, top 10 compounds were selected, which have promising inter-

Table 1: Primers sequence used for confirmation and molecular characterization of MBLs-Ec

Target genes	Gene Primers (5'→3')	Size of product (bp)	Annealing (°C/s)
uidA	F: ATCACCGTGGTGACGCATGTCGC R: CACCACGATGCCATGTTCATCTG	486	54°C/30s
NDM -1	F: GGGCAGTCGCTTCCAACGGT R: GTAGTGCTCAGTGTCGGCAT	475	54°C/30s
VIM	F: GTTTGGTCGCATATCGCAAC R: AATGCGCAGCACCAGGATAG	389	52°C/60s

Table 2: Characteristics of the MBLs producing *E. coli* in different types of clinical isolates

Parameters	MBL positive isolates	Percent (%)
Total Positive Isolates	81	14.1
Gender		
Male	27	33.3
Female	54	66.7
Age Group		
00 – 10	07	8.6
11 – 20	15	18.5
21 – 40	21	25.9
41 – 60	26	32.1
> 60	12	14.8
Specimen Type		
Pus	27	33.3
Urine	50	61.7
Others	4	4.9
Clinical Features		
UTI	51	63.0
SSIs	30	37.0
PIs	0	
Patients Status		
OPD	37	45.7
IPD	44	54.3
MBL Genes		
NDM-1	22	27.2
VIM	11	13.6

interactions with selected proteins. To rank the final compounds, binding energy and binding affinity were calculated for each compound with the selected proteins shown in table 5.

Binding energy and binding affinity for NDM-1 protein

For NDM-1, compound 848 and 870 have binding energy greater than -200 kcal/mol and binding affinity greater than -15 kcal/mol. While compound 783, 155, 7 and 121 have binding energy between -195 to -198 kcal/mol and binding affinity between -13 to -15 kcal/mol. Compounds 77, 176, 149 and 825 have binding energy between -181 and -184 kcal/mol as shown in table 5. The 10 selected compounds interaction with the NDM-1 protein active pocket residues is reported in figure 4A. The interactions bonds of the lead compounds with the key residues of the NDM-1 are shown in table 6. All compounds have also ionic interaction with the ligands which increase the binding energy. The residues interaction population in figure 5A shows that Lys211 have 90% interaction with all the compounds followed by Asn220.

Binding energy and binding affinity for VIM protein

The compound 848 interactions also for VIM have highest binding energy -181.69 kcal/mol and binding affinity -12.13 kcal/mol. The compound 783 have binding energy of -62.74 kcal/mol while compound 870 has -57.51 kcal/mol. The compound 121 and compound 7 have binding energy of -54 kcal/mol with binding affinity of -8 to -7 kcal/mol. The followed six compounds 176, 149, 155, 17 and 825 have binding energy between -46 to -51 kcal/mol having binding affinity of -7 kcal/mol as showed in table 5.

The novel compounds interaction with key residues of the Bla-VIM protein is reported in fig. 7. The Bla-VIM key

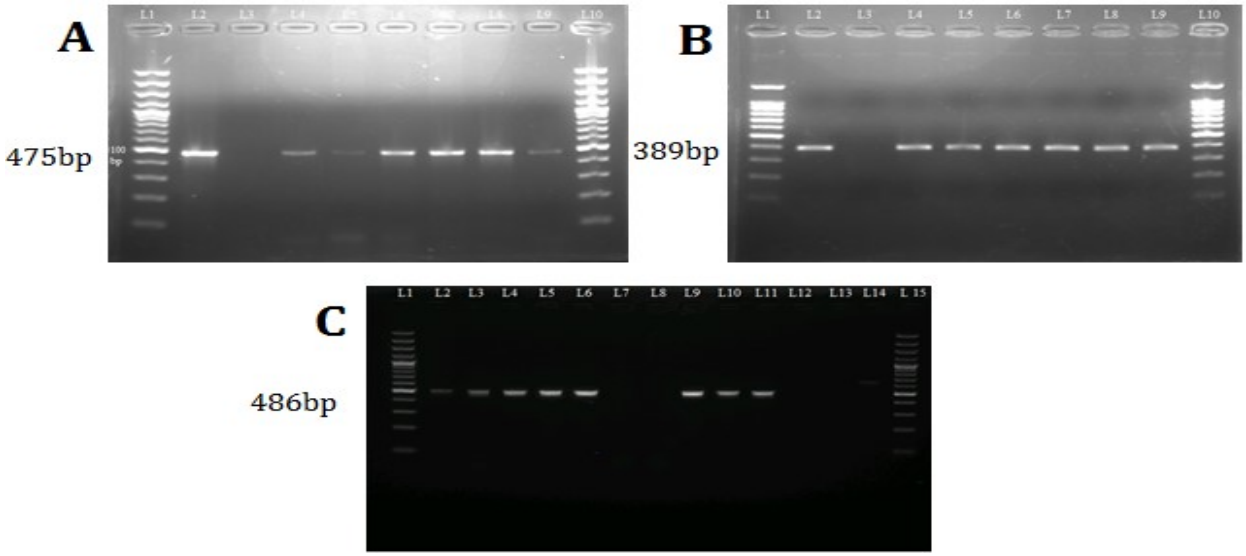

Fig. 1: Gel electrophoresis of PCR products of A) NDM-1 B) VIM C) *uid A* gene

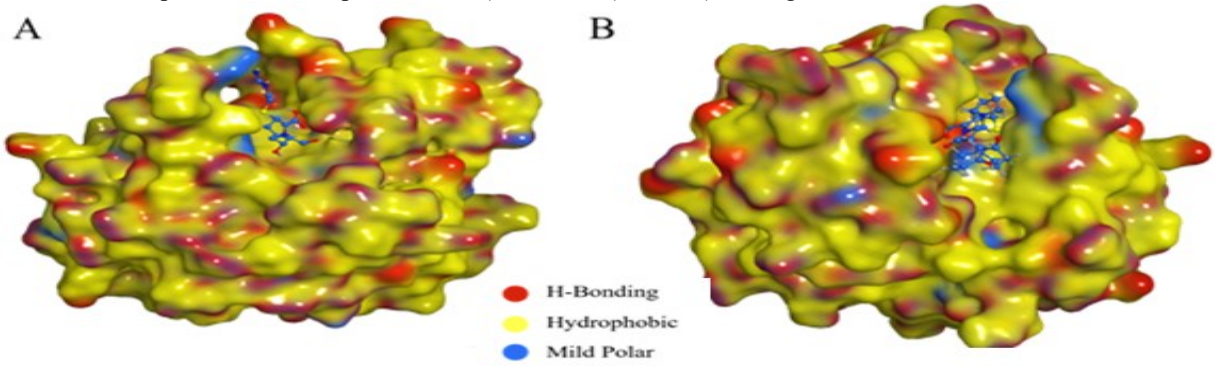

Fig. 2: Surface representation of the selected proteins with crystalized ligands, (A) Bla-NDM-1 protein PDB ID 5YPL and (B) Bla-VIM protein PDB ID 4UWR.

residues interactions bonds are represented in table 7. The residues population in fig. 8 shows that the highest interacting residue His263 have 60 percent interaction with all compounds, followed by other residues of the pocket.

DISCUSSION

Antibiotics resistance in *E. coli* has been observed globally and the increasing resistance among *E. coli* is a major health problem worldwide (El Kholy *et al.*, 2003). Carbapenems are the drugs of choice in case of extended spectrum *β*-lactamase producing *E. coli*. The use of these drugs is also under threat due to the emergence of carbapenemases, mainly the class B metallo *β*-lactamases (Walsh *et al.*, 2005). The current study reported MBLs production in 14.1% of isolates which is lower (34.4%) than reported study from Pakistan (Nahid *et al.*, 2013). The same results (18.98%) were also reported in a study conducted in Nepal (Bora *et al.*, 2014). The high frequency of MBLs-Ec might be due to unhygienic conditions, self-medication, over and misuse of drugs which are becoming ineffective to carbapenems drugs.

The results of the current study showed that female patients dominated (66.7%) as compared to male patients (33.3%). Furthermore, most of the MBLs-Ec isolates were recovered from urine (61.7%) followed by Pus (33.3%) and high MBLs production was detected in hospitalized (54.3%). The study conducted in Nepal also reported the same results; 84% isolates were recovered from urine and 7.9% from pus (Nepal *et al.*, 2017). The same results were documented in another study in which high number of isolates were obtained from urine (64%) and predominantly the isolates were from female patients (63.32%) and majority samples were from hospitalized patients (18%) (Mubarak *et al.*, 2014). The reason might be that a larger number of samples were obtained from hospitalized patients.

The present study revealed that MBLs-Ec isolates were highly resistant to *β*-lactam antibiotics; MEM, IPM,

Table 3: Antibiogram of MBLs-Ec isolates against selected antibiotics (n = 81)

Antibiotics	Generic Names	Sensitivity		Resistance	
		Frequency	Percentage	Frequency	Percentage
AMP	Ampicillin	00	00	81	100
FOX	Cefoxitin	00	00	81	100
AMC	Amoxicillin-Clavulanate	00	00	81	100
SCF	Cefoperazone-Sulbactam	08	9.9	73	90.1
TZP	Piperacillin-Tazobactam	09	11.1	72	88.9
FEP	Cefepime	00	00	81	100
CTX	Cefotaxime	00	00	81	100
CAZ	Ceftazidime	00	00	81	100
ATM	Aztreonem	00	00	81	100
MEM	Meropenem	00	00	81	100
IPM	Imipenem	00	00	81	100
CN	Gentamicin	12	14.8	69	85.2
TOB	Tobramycin	9	11.1	72	88.9
AK	Amikacin	28	34.6	53	65.4
DO	Doxycycline	31	38.3	50	61.7
CIP	Ciprofloxacin	5	6.1	76	93.9
LVX	Levofloxacin	6	7.4	75	92.6
SXT	Trimethoprim-Sulphamethoxazole	4	4.9	77	95.1
C	Chloramphenicol	20	24.7	61	75.3
FOS	Fosfomycin	46	56.8	35	43.2
TGC	Tigecycline	81	100	00	00
CO	Colistin	81	100	00	00

Table 4: Minimum Inhibitory Concentration of selected antibiotics against MBLs-Ec isolates

Antibiotics	Breakpoints			MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	MIC Range (µg/ml)
	S	I	R			
CTX	≤ 1	2	≥ 4	128	256	4-256
CAZ	≤ 4	8	≥ 16	64	256	16-256
MEM	≤ 1	2	≥ 4	4	32	3-256
CN	≤ 4	8	≥ 16	16	16	4-16
AK	≤ 16	#	64	16	256	1-256
DO	≤ 4	8	≥ 16	16	192	1-256
CIP	≤0.25	0.5	≥ 1	32	256	0.094-256

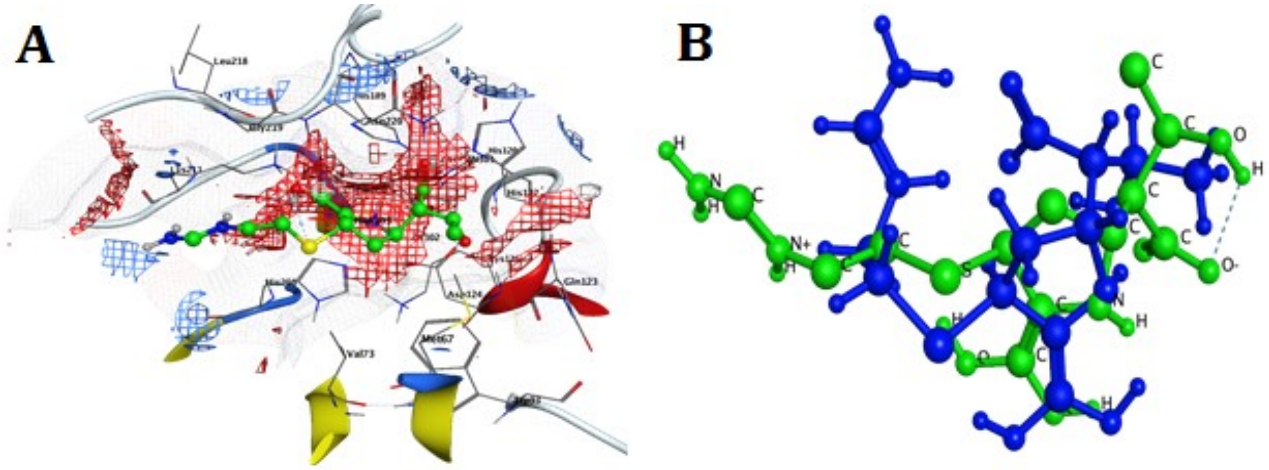

Fig. 3: **A)** Electrostatic maps represent red H-bond acceptor, blue H-bond donor and green hydrophobic atoms of (A) NDM-1) and **B)** Docking validation of (A) Green Bla-NDM-1 protein ligand Hydrolyzed Imipenem superposed with Blue redock.

Table 5: Docking score, binding energy and binding affinity of 10 selected compounds for NDM-1 and VIM proteins

Bla-NDM-1 protein				
S. No	Compound No	Docking Score	Binding Energy (Kcal/mol)	Binding Affinity (Kcal/mol)
1	7	-12.35	-195.53	-15.18
2	17	-11.69	-183.24	-14.56
3	121	-8.61	-195.21	-13.17
4	149	-11.71	-182.90	-14.36
5	155	-12.13	-195.86	-15.64
6	176	-11.93	-183.23	-14.34
7	783	-12.24	-197.95	-15.65
8	825	-11.54	-181.26	-13.89
9	848	-8.91	-205.40	-16.32
10	870	-12.17	-204.70	-15.52
ref			-164.12	-12.76
Bla-VIM protein				
1	7	-5.26	-54.23	-7.89
2	17	-5.69	-48.44	-6.94
3	121	-6.39	-54.49	-8.21
4	149	-5.92	-50.38	-7.52
5	155	-5.30	-48.58	-7.48
6	176	-5.48	-51.87	-7.80
7	783	-7.19	-62.74	-9.05
8	825	-5.27	-46.43	-7.07
9	848	-8.45	-118.69	-12.13
10	870	-5.47	-57.51	-8.66

Table 6: Interacting residues bonds type of Bla-NDM-1 protein active pocket with selected novel 10 compounds.

Comp No	Ala74	His122	Asp124	Lys211	Asn220	His250
7	-	-	-----	AAII	AAaa-	--
17	-	-	--aa-	AAII	--aa-	--
121	-	-	DD---	----	AA---	R-
149	-	R	D----	AAII	AAaa-	--
155	-	-	D-a--	AAII	--aa-	--
176	-	R	----I	AA--	AAa-R	RR
783	-	-	----I	AAII	--aa-	--
825	a	-	----I	AAII	--aa-	--
848	-	-	--aa-	AAI-	A-aa-	--
870	-	-	D-aa-	AAI-	A-aa-	--

* "-" bit not set, "D" sidechain hydrogen bond donor, "A" sidechain hydrogen bond acceptor, "d" backbone hydrogen bond donor, "a" backbone hydrogen bond acceptor, "O" solvent hydrogen bond, "I" ionic attraction, "C" surface contact

Table 7: Interacting residues bonds type of Bla-VIM protein active pocket with selected novel 10 compounds.

Comp No	Ocs221	Glu225	Thr229	Ser230	Ala231	Gly232	Asn233	His263
7	D----	--aa-	--	-	d	-	-----	-----
17	-----	-----	--	-	-	a	-----	AA--R
121	-----	--aa-	--	-	d	-	-----	AA---
149	-----	-----	aa	-	d	-	-----	----R
155	D---I	-----	--	-	-	-	-a---	--II-
176	-----	DD---	--	-	-	a	---RR	-----
783	-----	-----	aa	-	-	-	-----	AA---
825	DDdd-	D---I	a-	d	-	-	-----	A----
848	-----	--aa-	--	-	-	a	-a---	A----
870	-----	D---I	--	-	-	-	Aaa--	A----

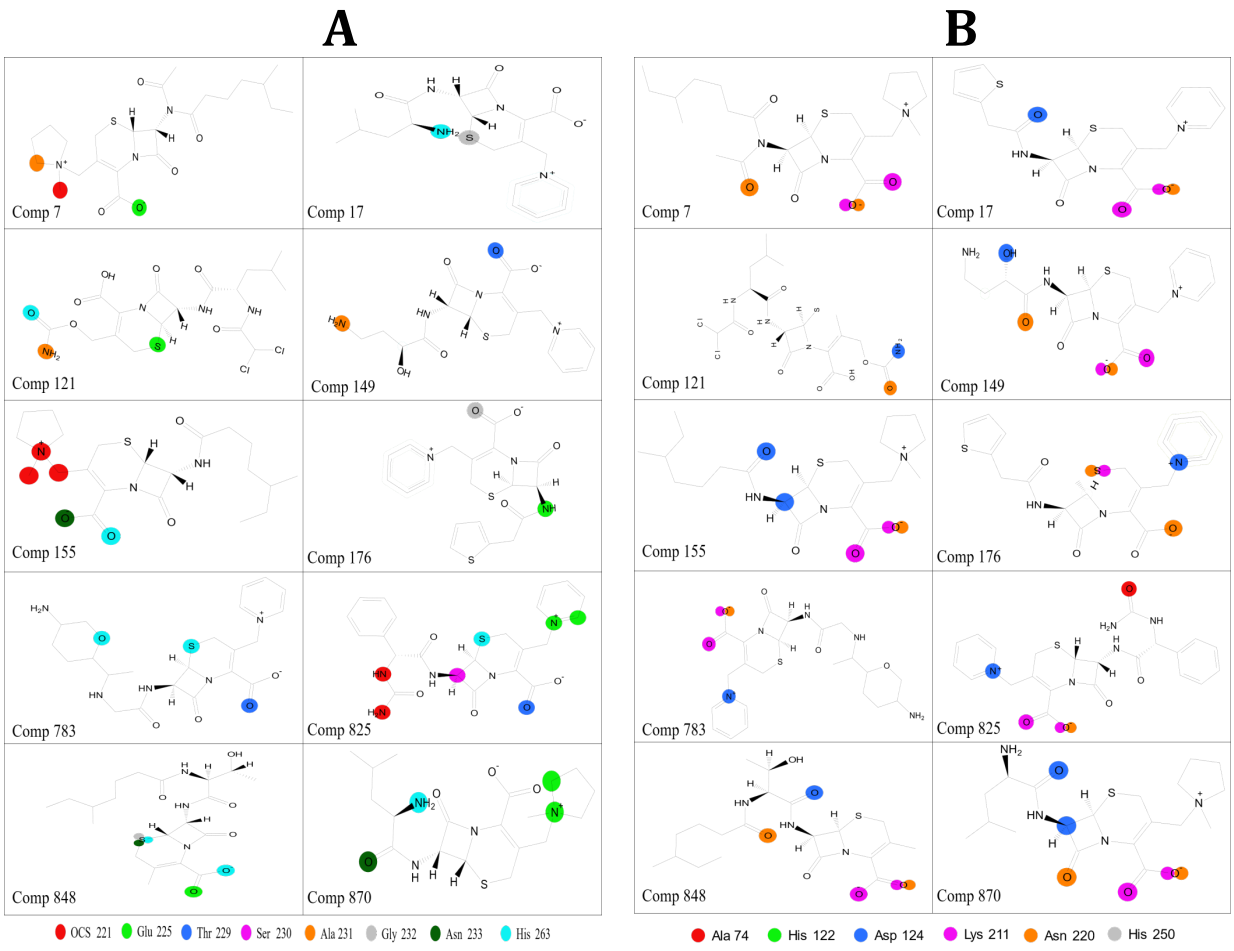

Fig. 4: Predicted top 10 compounds interaction with A) key residues of NDM-1 and B) VIM protein.

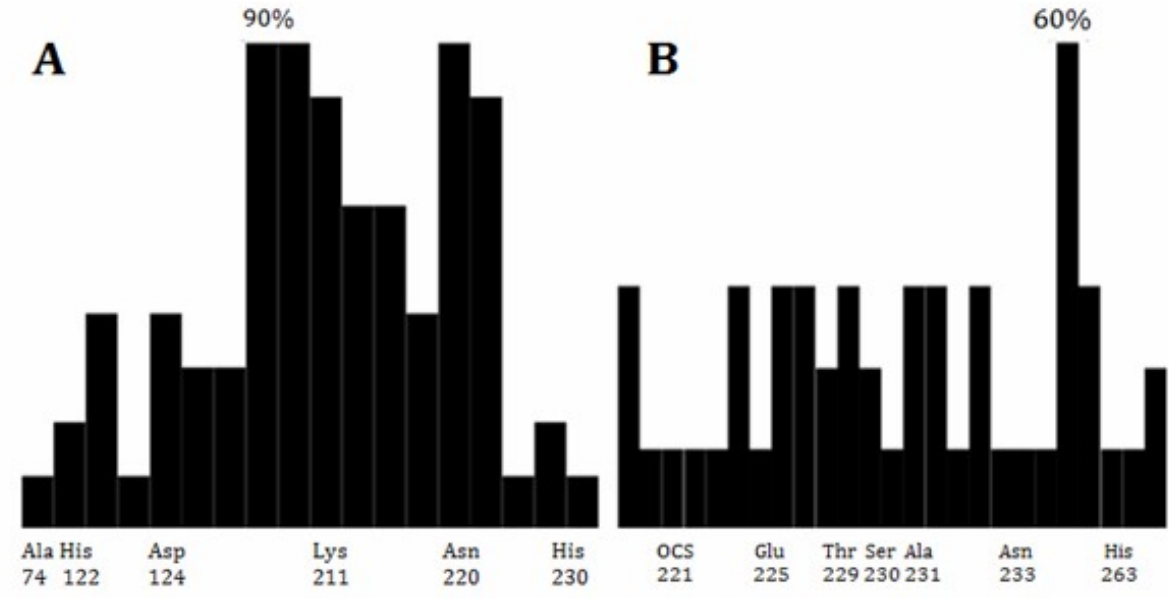

Fig. 5: A) NDM-1 and B) VIM pocket residues interaction population with selected hits compounds.

AMP, AMC, FEP, CTX, ATIM and CAZ as well as to *β*-lactam antibiotics; to SXT, CIP, C, AK and DO. The incidence of resistance to carbapenems drugs were also reported in a study conducted in Pakistan which is accordance to our study (Mubarak *et al.*, 2014), another study confirmed our results that all the MBLs producers were resistant to carbapenems and all *β*-lactam agents (Bora *et al.*, 2013). The same findings were also observed in a study in which all the *E. coli* isolates with MBLs production were resistant to IPM and MEM. These isolates also showed high resistance to the penicillins, cephalosporins, cephamycins, and ATM, as well as to different antibiotic/inhibitor combinations tested in the study (Bora *et al.*, 2014). The MBLs producers were highly resistant to both *β*-lactam and non-*β*-lactam drugs with higher MIC_{50} and MIC_{90}. A similar study was conducted in Turkey in which all the isolates of *Enterobacteriaceae* showed resistance with increased MICs to tested antibiotics which are accordance to our study (Baran *et al.*, 2016). Another study also reported an increase in resistance with higher MICs against carbapenems (64μg/ml) in India which confirmed our results (Khajuria *et al.*, 2014). These variations may be affected by several factors; types of MBLs, reduced permeability, ESBLs and AmpC *β*-lactamases and efflux pumps (Stuart *et al.*, 2010).

This study detected 33.3% of target genes for MBLs; NDM-1gene was the most prevalent (27.2%). The same results (28%) of MBLs-Ec with NDM-1 identified in 50% isolates including VIM (10.6%) were reported in India (Lascols *et al.*, 2011). Another study from Pakistan also reported NDM-1 gene in 23.6% of isolates, 25.1% possessed VIM gene (Shahid *et al.*, 2012). This variation in the incidence rates may be due to the sample size studied.

The current study used in-silico approach for designing new potent inhibitors for the resistant proteins of MBLs-Ec. The reported ligand for NDM-1 protein is hydrolyzed imipenem showed binding energy of (-164.12kcal/mol) and binding affinity (-8.37kcal/mol). The selected 10 compounds for the NDM-1 showed that compound-848 have the highest binding energy (-205.40kcal/mol) and binding affinity (-16.32kcal/mol). The 2nd highest binding energy showed by compound 870 was -204.70kcal/mol and binding affinity -15.52 kcal/mol. In -195 to -200 binding energy range, 4 compounds were reported. All the reported compounds for NDM-1 protein showed best binding energy and binding affinity as compare to the reported ligand hydrolyzed imipenem.

The reported ligand of the VIM protein was not present in the protein crystal structure. The compound 848 interactions also for Bla-VIM have highest binding energy (-181.69 kcal/mol) and binding affinity (-12.13 kcal/mol). The reported 10 compounds for the VIM protein showed best interactions, binding energy and binding affinity to the protein VIM.

CONCLUSION

The spread of MBLs-Ec resistant genes to other members of *Enterobacteriaceae* family increases the frequency of multidrug resistance creating major health problems. Due to the occurrence of resistance mechanism, the treatment options for clinicians are limited. The MBLs-Ec proteins are responsible to cause different infections in both hospitalized and general population. In the current study, the multi resistant proteins producing genes were identified on molecular level and the resistance was verified. To overcome the resistance, novel multi inhibition inhibitors were design to block the resistant MBLs proteins; NDM-1 and VIM. Top 10 compounds based on docking score, binding energy and binding affinity were selected which can inhibit the selected proteins. These hits compounds have unique scaffolds and predicted to be a starting point for the development of novel and potent inhibitors for 2 antibiotic resistant proteins and can be subjected to the experimental procedures.

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