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Research Article

DOCKING OF CTX-M-9 GROUP OF ENZYMES WITH DRUGS AND INHIBITORS AND THEIR EVOLUTIONARY RELATIONSHIP

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ABSTRACT

Background: Among the type of ESBLs, CTX-M-type ESBLs represent a new and rapidly growing family of molecular class-A ESBLs. The prevalence of CTX-M-type ESBLs poses a serious threat to the clinical use of third generation cephalosporins for the treatment of severe infections.
Objective: The objectives of the present study are computational study of CTX-M-9, 14 and 27 of blaCTX-M sequences. Based on resistance to organisms, docking of drugs (cefotaxime, cefixime and cefepime) as well as inhibitors (clavulanate, sulbactam and tazobactam) with CTX-M-9, 14 and 27 for the identification of amino acid residues crucial to the enzyme-drug and enzyme-inhibitor interaction.
Method: Bioactivity analysis was done using Pubchem, and docking of drugs and inhibitors with CTX-M-9 group of enzymes were done using Schrodinger to identify the amino acid residues that are crucial for interaction. Phylogenetic analysis was constructed by Neighbor-Joining method for calculating minimum distance and bootstrapping values was done for 1000 replicates to obtain >70% recombination.
Results: Schrodinger analysis revealed the amino acid residues that interacted with CTX-M-9, 14 and 27 and they were found to be ASP 101, ASN 136, LYS 137, ASP 101, GLU 166, SER 130, ASN 132, THR 235, SER 237, and ASP 240 respectively. Among the drugs, cefotaxime, cefixime and among inhibitors clavulanic acid and tazobactam were found to interact with CTX-M-9, 14 and 27. Minimum distance obtained from Neighbor-Joining analysis was 0.02 evolutionary rate of divergence, and only two nodes connecting (CTX-M-3, 55 and CTX-M-9, 16, 51) of bootstrapping values revealed 100 and 88% respectively, whereas other CTX-M-9 group of enzymes showed less than 70% recombination.
Conclusion: The current study revealed the amino acid residues crucial to 'CTX-M-drug' and 'CTX-M-inhibitor' interactions among CTX-M-9 group of enzymes using different bioinformatics tools which would be useful for the development of a versatile CTX-M-inhibitor.

Keywords: ESBL, blaCTX-M, Docking, Neighbor-Joining

INTRODUCTION

Antimicrobial resistant organisms have emerged to cause global health problems that will likely to form into one of the most significant challenges facing medical practice in today's world [1]. The first report on ESBLs capable of hydrolyzing the extended spectrum cephalosporins based on genetic and functional characteristics was reported in *Klebsiella pneumoniae* from Germany in 1983 [2]. ESBLs are plasmid-associated enzymes that hydrolyze oxy-imino cephalosporins and monobactams [3]. CTX-Ms have evolved from chromosomal genes of *Kluyvera sp* and the first isolate was reported in *E. coli* from Munich, Germany in 1989. Especially these enzymes hydrolyzes cefotaxime than ceftazidime, hence the name (Cefotaximase, Munich). The CTX-M group consists of different members CTX-M-1 (22 members), CTX-M-2 (10 members), CTX-M-8 (3 members), CTX-M-9 (18, 19, 38, 45, 14, 46, 48, 50, 47, 49, 17, 24, 65, 27, 16, 9, 51, 21, 3, and 55), and CTX-M-25 (4 members) where the spread of these groups are based on the geographical localization [4]. The different methods employed for phylogenetic reconstruction for analysis of sequence datasets are: parsimony methods [5] distance based methods [6], and maximum likelihood methods [7], [8]. Previous research reports have revealed the three dimensional structures of CTX-M-15, SME-1 and IMI-1 with tazobactam viewing the interaction of amino acid residues Ser70, Ser130, Lys235, Thr236 and Gly237 using GOLD [9], while the present study depicts the docking of CTX-M-9 which interacted efficiently with tazobactam using Schrodinger. However, the docking study of CTX-M-15 with clindamycin and piperacillin-tazobactam revealed the amino acid residues interacting in the prominent sites Gly26, Ala28, Ala47, Met48, Thr51, Val54, Pro63, Met166, Ala167, Leu191 and Ala26, Val27, Thr51, Val54, Met55, Leu173, Met166, Pro163 and Leu191 using Discover studio 3.1 respectively [10]. On the other hand, the modelled structure of CTX-M-15 was docked with cefotaxime using Hex 5.1 that revealed the amino acid residues Asn132, Glu166, Pro167, Val172, Lys234 and Thr235 in the docked complex [11], whereas, in this study CTX-M-14 interacted well with cefotaxime at active sites using Schrodinger.

To the best of our knowledge, there are no studies reported on CTX-M-9 group (CTX-M-9, 14, and 27) of enzymes and their docking with drugs (cefotaxime, cefepime, and cefixime) and inhibitors (clavulanate, sulbactam, and tazobactam). In view of the current background, the mode of interaction of CTX-M-9 group with drugs and inhibitors are studied with the following objectives: The computational study of CTX-M-9, 14 and 27 of *bla*CTX-M sequences and structures from *Enterobacteriaceae* members, docking of drugs (cefotaxime, cefixime, and cefepime) as well as Inhibitors (clavulanate, sulbactam, and tazobactam) with CTX-M- 9, 14 and 27, identification of amino acid residues of CTX-M- 9, 14, 27 crucial to the enzyme-drug and enzyme-inhibitor interaction, comparative effect of β-lactamase inhibitors against CTX-M-9, 14, 27 on the basis of interaction energies by *insilico* approach and phylogenetic reconstruction of CTX-M-9 group of enzymes using MEGA 5.1 [12].

Materials and Methods

The database maintained exclusively for β-lactamase enzymes (www.lahey.org/studies) was searched for CTX-M enzyme sequences submitted across the globe. The ESBL- types in this study include CTX-M-9 group (mostly CTX-M-9, CTX-M-14, and CTX-M-27) of enzymes. The sequences used in the present study appear in GenBank as [GenBank: AF174129], [GenBank: AF252622], and [GenBank: AY156923]. Protein Data Bank (PDB) IDs of the deposited structures are shown in Table 1. PDB structures of drugs (cefotaxime, cefepime, cefixime) as well as inhibitors (clavulanic acid and tazobactam) were retrieved from DrugBank (www.drugbank.ca/search/chemquery). In case any of the structure (e.g. sulbactam) was not available in pdb format with DrugBank, it's 2 - D structure was retrieved from Pubchem (www.pubchem.ncbi.nlm.nih.gov/search). The structural analysis

was done on ceftazidime like boronic acid bound with CTX-M-9 and 14 enzymes, for the identification of putative targets using PubChem, whereas for CTX-M-27 is not reported. Bioactivity analysis for the ligand CB4-pinacol**[[2-Amino-Alpha-(1-Carboxy-1-Methylethoxyimino)-4- Thiazoleacetyl] Amino] Methaneboronate]**, for the identification of targets based on the bioassay. It also gives the structure activity relationship of the ligand CB4- pinacol **[[2-Amino-Alpha-(1-Carboxy-1-Methylethoxyimino)-4-Thiazoleacetyl] Amino] Methaneboronate]** with the targets.

Molecular docking: The ligand (either inhibitor or drug) was docked into each of the enzyme – structures using Schrodinger software (Schrodinger Software Solutions, USA). In the current work, Schrodinger software was used to dock the ligand with the target. The ligand was (drug and inhibitor) docked against target protein (CTX-M-9, 14 and 27). The steps involved for docking using Schrodinger, i) Ligprep, ii) Protein preparation wizard, iii) Glide grid generation, iv) Docking.

Ligprep: Ligprep is a method designed to prepare high quality, all-atom 3D structures for large numbers of drug-like molecules, the resulting 2D or 3D structures can be saved in SD or Maestro format.

Protein preparation wizard: The typical structure file from PDB is not suitable for immediate use in molecular modeling calculations. A typical PDB structure file consists heavy atoms, metal ions, co crystallized ligand, water molecules, and cofactors. Schrodinger has therefore assembled a set of tools to prepare proteins in a form that is suitable for modelling calculations.

Glide grid generation: Glide looks for a favourable interaction between one or more ligand molecules and a receptor molecule (protein). The shape and properties of the receptor are represented on a grid by several different sets of fields that provide more accurate scoring of the ligand poses.

Docking: Protein-Ligand docking is a molecular modelling technique used to predict the orientation and confirmation of binding of ligands with proteins. The increase in negative value of glide score more is the interaction between the ligand and target protein. This shows that the ligands (drug or inhibitor) can bind effectively to the protein. The docking parameters used are:

(a) Docking Using SP (Standard Precision)

The workflow of Standard Precision (SP) is:

Import structure ⟶ impref_protein_out.mae

Applications ⟶ Glide ⟶ Ligand docking

Settings ⟶ Import receptor grid files ⟶ SP ⟶ Dock flexibly

Ligands ⟶ Selected entries ⟶ Start

Output file ⟶ glide-dock_SP_protein_pv.maegz

(b) Docking Using XP (Extra Precision)

The workflow of Extra Precision (XP) is:

Import structure ⟶ impref_protein_out.mae

Applications ⟶ Glide ⟶ Ligand docking

Settings ⟶ Import receptor grid files ⟶ XP Dock flexibly

Ligands ⟶ elected entries ⟶ Start

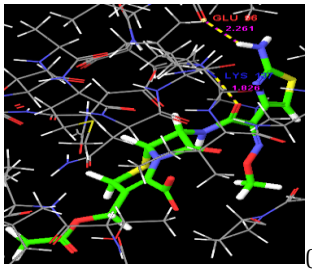
Output file ⟶ glide-dock_XP_protein_pv.maegz

The CTX-M-9 group of sequences were retrieved from GenBank using their ID's contained in www.lahey.org/studies. Multiple sequence alignments were performed for the CTX-M-9 group of sequences using ClustalW program in MEGA and the output was saved in .mas format. The Neighbour – joining tree was constructed using construct/Test Neighbor-Joining Tree under phylogeny option in MEGA 5.1 and tested with bootstrapping values upto 1000 replicates along with complete deletion of gaps in the analysis preferences window, minimum evolution (ME) criterion is computed.

Results and Discussion

Docking results

The drugs (cefotaxime, cefepime and cefixime) as well as inhibitors (clavulanic acid, tazobactam and sulbactam) were docked into each of the enzyme – structures. Figures (Figure 1 Figure 2 Figure 3) shows the amino acid residues crucial to the interaction of each of the enzyme structures completed with cefotaxime, cefepime, cefixime, clavulanic acid, tazobactam and sulbactam, separately. Schrodinger analysis of the docked structures revealed that with respect to the CTX-M- 9 and 14, cefotaxime interacted with the residues GLU 96, LYS 137, GLN 141. Similarly, cefixime interacted with ASP 240, ASN 132, SER 130, and ALA 272. PDBsum revealed the binding pockets for the proteins (1YLJ, 1YLZ, and 1YLP) to be docked with ligands using ligplot. These interactions might help the researchers for the future development of a versatile CTX-M-resistant antibiotic.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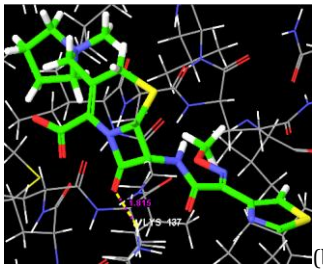
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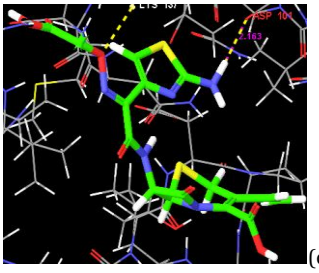
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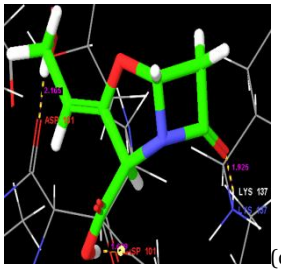
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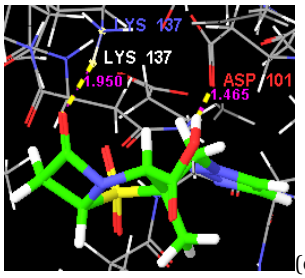

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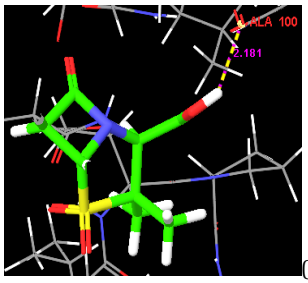


(f)

Figure 1: Interaction of CTX-M-9 with (a) cefotaxime (b) cefepime (c) cefixime (d) clavulanic acid (e) tazobactam (f) sulbactam

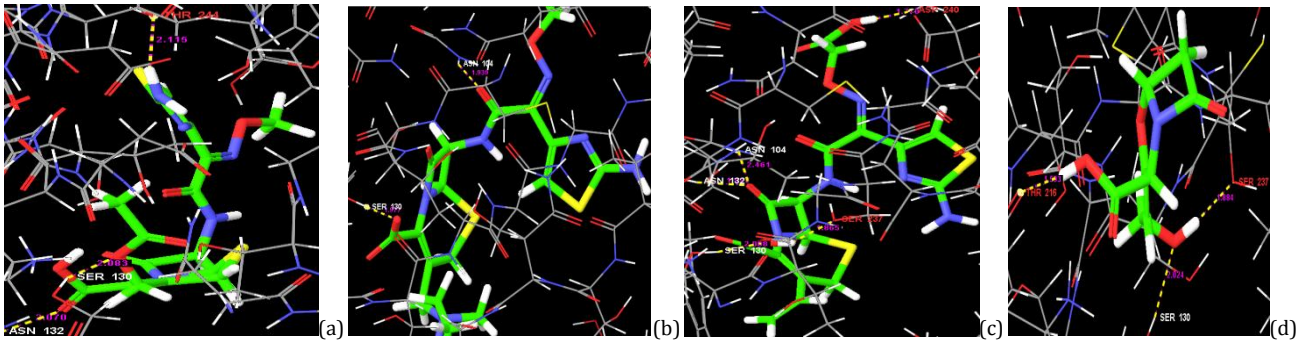


Figure 2: Interaction of CTX-M-14 with (a) cefotaxime (b) cefepime (c) cefixime (d) clavulanic acid (e) tazobactam (f) sulbactam

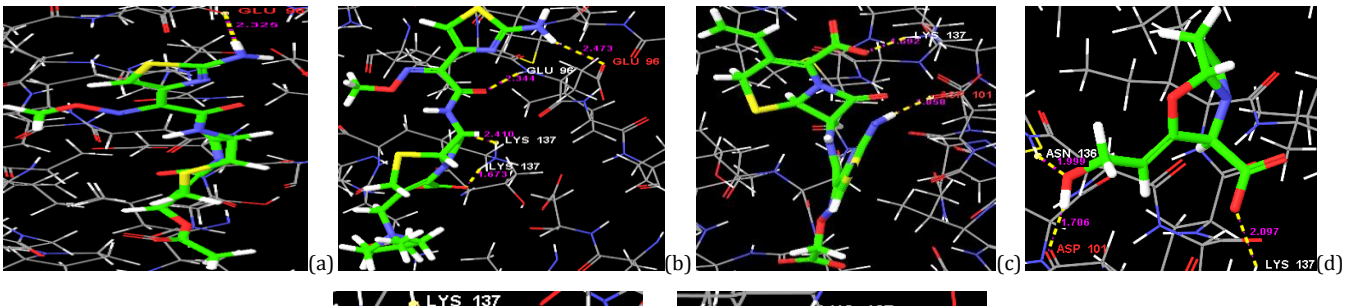


Figure 3: Interaction of CTX-M-27 with (a) cefotaxime (b) cefepime (c) cefixime (d) clavulanic acid (e) tazobactam (f) sulbactam

***In silico* comparison of efficacies of traditional β-lactamase inhibitors against CTX-M-9 group of enzymes**

A β-lactam antibiotic when given in combination with β-lactamase-inhibitor against antibiotic resistant bacteria it becomes susceptible to this combination, since the active site which is occupied by the inhibitor makes the enzyme ineffective. Higher (negative) energy is considered as an indicator of effective and rigid binding of an enzyme – inhibitor – complex. Among the studied enzyme inhibitor – complexes, the complexes involving clavulanic acid and tazobactam displayed high interaction energies (Table 1). Schrodinger analysis revealed the binding of CTX-M-9, CTX-M-14 and 27 with ligand where amino acid residues ASP 101, ASN 136, LYS 137, ASP 101, GLU 166, SER 130, ASN 132, THR 235, SER 237, and ASP 240. Hence tazobactam and clavulanic acid were found to be the most efficient inhibitors for CTX-M-9 and CTX-M-14 respectively.

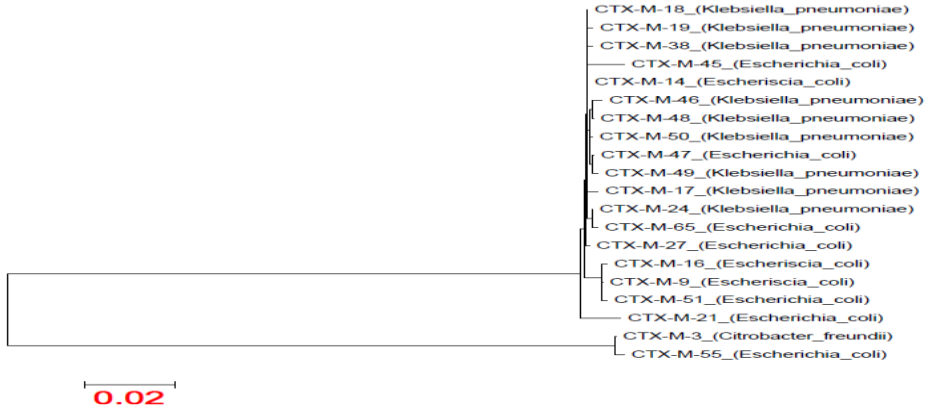
Table 1: Bioinformative details of the CTX-M structures and interaction energies of the docked enzyme-inhibitor-complexes

Name of CTX-M/ GenBank Accession Number	CTX-M-9/ AF174129	CTX-M-14/ AF252622	CTX-M-27/ AY156923
PDB ID of the CTX-M	1YLJ	1YLZ	1YLP
Docking with Clavulanic acid (All energies are in Kj/mol)	Glide energy= -22.430 Glide gScore= -4.683	Glide energy= -26.921 Glide gScore= -5.785	Glide energy= -21.621 Glide gScore= -5.534
Docking with Sulbactam (All energies are in	Glide energy= -23.953 Glide gScore= -3.978	Glide energy= -23.390 Glide gScore= -4.233	Glide energy= -19.118 Glide gScore= -4.184

Kj/mol)			
Docking with Tazobactam (All energies are in Kj/mol)	Glide energy= -31.228 Glide gScore= -4.749	Glide energy= -25.320 Glide gScore= -3.600	Glide energy= -23.763 Glide gScore= -3.860
Docking with Cefotaxime (All energies are in Kj/mol)	Glide energy= -29.787 Glide gScore= -3.502	Glide energy= -38.870 Glide gScore= -4.922	Glide energy= -31.618 Glide gScore= -3.241
Docking with Cefepime (All energies are in Kj/mol)	Glide energy= -24.935 Glide gScore= -2.675	Glide energy= -44.303 Glide gScore= -4.690	Glide energy= -28.077 Glide gScore= -2.509
Docking with Cefixime (All energies are in Kj/mol)	Glide energy= -26.3125 Glide gScore= -2.762	Glide energy= -49.818 Glide gScore= -5.357	Glide energy= -30.737 Glide gScore= -3.438

Phylogenetic Analysis

Phylogenetic analysis was constructed for the CTX-M-9 group of enzymes using neighbor-joining. The evolutionary divergence for CTX-M-9 group of enzymes was calculated. The tree revealed that the average rate of divergence as 0.02 (Figure 4).



Bootstrapping was performed for 1000 replicates among CTX-M-9 group to obtain more than 70% recombination. The nodes connecting CTX-M-3, CTX-M-55 and CTX-M-16, CTX-M-9, CTX-M-51 showed 100% and 80% recombination, whereas CTX-M-14 and CTX-M-27 showed 8% and 23% recombination (Figure 5).

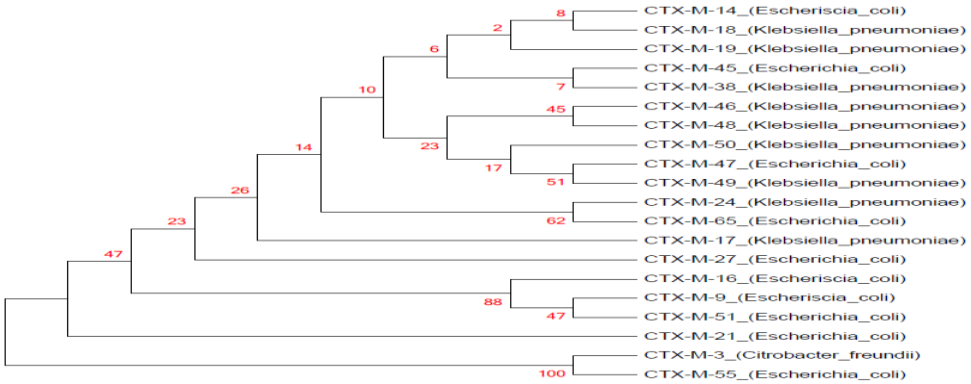


Figure 5: Bootstrapping Tree was tested and the numbers at the major branch points refer to the number of times that a particular node was found in 1,000 bootstrap replications

CTX-Ms have become dominant ESBLs in hospitals and in the community. The bioactivity analysis of the compound (CB4) pinacol was done to identify the targets using PubChem analysis. Han et al. (2009) reported that each compound was analysed by counting their targets with biological activity [13]. The Bioactivity summary reported by Wang et al includes assay targets such as West Nile virus NS2bNS3 proteinase, Factor XIa, kallikrein-related peptidase 5, Cathepsin G and Factor XIIa [14]. The targets identified using Bioassay summary in this study are SHV-5 and SHV-1. For docking of CTX-M-9 group enzymes (9, 14, and 27), the structures were retrieved from www.lahey.org/studies using their GenBank ID's (AF174129, AF252622, and AY156923). Among the drugs (cefotaxime, cefepime, and cefixime) and inhibitors (clavulanic acid, tazobactam, and sulbactam), cefotaxime, tazobactam and cefixime, clavulanic acid interacted with CTX-M-9, CTX-M-14 and 27 with amino acid residues ASP 101, ASN 136, LYS 137, ASP 101, GLU 166, SER 130, ASN 132, THR 235, SER 237, and ASP 240 using Schrodinger. Clavulanic acid is a suicide inactivator, which forms an irreversible complex with β-lactamase enzymes [15]. Among the studied inhibitors clavulanic acid and tazobactam displayed higher energy. In a similar study, Shakil and Khan (2009) using Discovery Studio, resulted that ceftazidime interacted with A226, G227, L228, P229, A/T230, S231, W232, R285, T288, D289, G290 and L/Y291 of CTX-M-type 2009 variants [16]. In this study, sulbactam displayed the highest (negative) interaction energies and was found to be the most efficient inhibitor [17]. The CTX-M-9 group showed clavulanic acid and tazobactam as better inhibitors. Similarly, Asad in 2010 reported the amino acid residues interacting with cefotaxime and CTX-M-15 enzyme are ASN 104, ASN 132, GLY 227, THR 235, GLY 236, and SER 237 using AutoDock [18]. Ramathilagam et al in 2013 reported ligand-receptor interactions of

indole derivative (code: CR1) against target enzyme human renin complexed with inhibitor (PDB ID: 2IKO) using Molegro virtual Docker that interacted with residues THR 80, SER 225 and SER 79 [19]. Chen et al reported that in CTX-M, Ser 237 interacted with oxyiminoside chains of third-generation cephalosporins and is partly responsible for the ESBL activity of these enzymes [20]. The role of amide linkage recognized by the canonical site in CTX-M that typically interacts with the conserved R1 amide side chain of β-lactam drugs ASN 132 and ASN 104 [21]. Bethel et al reported the aminoacid residue ARG 276 in CTX-M-9 plays a crucial role in the recognition of C_3 carboxylate of inhibitors and the location of positive charge to a region of the active site rather than a specific residue represented as a evolutionary strategy used by β-lactamases [22]. Kumaran et al reported docking of inhibitor (1,2-benzenedicarboxylic acid) against active site of the MEGA protein for the inhibition of lung cancer [23]. Ma et al. (1998) reported that the unique feature of CTX-M enzymes is that they are better inhibited by tazobactam than by sulbactam or clavulanic acid [24]. Thus, the findings of the present study are concomitant with the previous findings. It can be explicated by the information that higher negative interaction energy for enzyme-antibiotic complex is an indicator of more stable and effective between the two during binding. Phylogenetic analysis deals with the evolutionary histories of living organisms, and represents the evolutionary divergence. Phylogenetic tree was constructed using Neighbor- Joining tree [25] as a distance tree method using MEGA. In this study, we found 0.02 divergence among CTX-M-9 group and bootstrapping value was observed to be 100% identity. Both Valverde et al (2009) and Vervoort et al (2012) reported 0.1 divergence tested with 1000 replicates using neighbour-joining for CTX-M-25 group and relaxase proteins of several plasmids [26]. In another study Gaze et al reported neighbor-joining tree for chromosomal genes KLUC-1, KLUA-1, and KLUG-1 that are progenitors of CTX-M-1, CTX-M-2, and CTX-M-8 groups [27].

CONCLUSION

This is the first study to report among CTX-M-9 group of enzymes and their docking with drugs and inhibitors to the best of our knowledge. Docking of drugs (cefotaxime, cefixime and cefepime) as well as inhibitors (clavulanate, sulbactam and tazobactam) with CTX-M- 9 group (9, 14 and 27) for the identification of amino acid residues crucial to the enzyme-drug and enzyme-inhibitor interaction. Among the drugs, cefotaxime and cefixime and among inhibitors clauvlanic acid and tazobactam were found to interact with CTX-M-9, 14 and 27. Phylogenetic analysis was constructed by Neighbor-Joining method for calculating minimum distance and bootstrapping values was done for 1000 replicates to obtain >70% recombination. Minimum distance obtained from Neighbor-Joining analysis was 0.02 evolutionary divergence, and only two nodes connecting (CTX-M-3, 55 and CTX-M-9, 16, 51) the bootstrapping values revealed 100 and 88% respectively, whereas other CTX-M-9 group of enzymes (CTX-M-14 and 27) showed less than 70% recombination. The current study revealed the amino acid residues crucial to 'CTX-M-drug' and 'CTX-M-inhibitor' interactions among CTX-M-9 group of enzymes using different bioinformatics tools which would be useful for the development of a versatile CTX-M-inhibitor.

ABBREVIATION

CTX-M	cefotaxime
ESBLs	Extended Spectrum of Beta Lactamases
MEGA	Molecular Evolutionary for Genetic Analysis
PDB	Protein Data Bank
KLUC	*Kluyvera cryocrescens*
KLUA	*Kluyvera ascorbata*
KLUG	*Kluyvera georgiana*
ME	Minimum Evolution

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