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Research Article

IN-SILICO ANALYSIS AND MOLECULAR DOCKING STUDIES OF SHV GROUP OF EXTENDED SPECTRUM B-LACTAMASES

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ABSTRACT

SHV enzymes are one of the Extended spectrum β- lactamases (ESBL) family which was emerged in *Enterobacteriaceae* causing infections in health care in recent past clinical microbiology scenario. Current study encompasses *In-silico* molecular docking of SHV variants; SHV -5, SHV -12, SHV -28 and SHV-38 with the eight β-lactam drugs and four β-lactam inhibitors. Evolutionary relationship using Multiple Sequence alignment and Phylogenetic tree construction were performed. Swiss model was used for elucidating tertiary models of enzyme sequences and the structures, with confirmed stability using Ramachandran plot. Molecular docking of SHV variants were carried out with drugs and inhibitors to understand the crucial amino acids and significant bonding which play roles in antibiotic responses. In the current study, Sulbactam and Ceftazidime were identified as the drugs having higher negative energy and Fosfomycin found to have least negative energy in the study group. Certain conserved sites of SHV group as well as the mutant site Lys/Glu 235 was found to participate in enzyme-drug/inhibitor binding process. Van der waal's interaction appeared to have significant role in total binding energy, thus making sulbactum to be having highest affinity in docking. The results conclude that the combination of cephalosporin with inhibitors would be more effective for treatment of patients.

KEY WORDS: Molecular docking, SHV variants, β lactamases, Drug, Inhibitor, Antimicrobial resistance.

INTRODUCTION

Extended spectrum β- lactamases (ESBL) production is one of the main mechanisms of resistance to broad spectrum β-lactam antibiotics (Poirel, Decousser and Nordmann, 2003). Among them, SHV enzymes have emerged in Enterobacteriaceae causing infections in health care settings in the last decades of the twentieth century. Most likely having originated from a chromosomal penicillinase of *Klebsiella pneumoniae*, SHV β-lactamases currently encompass a large number of allelic variants including ESBL, non-ESBL and several not classified variants. SHV enzymes have evolved from a narrow- to an extended- spectrum of hydrolyzing activity, including monobactams and carbapenems, as a result of aminoacid changes that altered the configuration around the active site of the β-lactamases(Liakopoulos, Mevius and Ceccarelli, 2016). In this study, molecular docking was done and analysed for SHV group of enzymes which includes SHV -5, SHV -12, SHV -28 and SHV – 38 with the β-lactam drugs and β-lactam inhibitors. Molecular docking is a tool that helps in predicting the orientation of one molecule to a second when they are bound to each other forming a stable complex. Docking finds an important role in national drug designing through the prediction of orientation between drug molecules and their protein targets. (Rasool *et al.*, 2018). SHV-5 and SHV-12 are classified under SHV subgroup 2be, which compromises ESBLs that can also hydrolyze one or more oxyimino β-lactams (cefotaxime, ceftazidime and aztreonam). SHV- 28 & SHV- 38 is classified as new variants whose dissemination was restricted to limited cases. (http://www.lahey.org/studies).

MATERIALS AND METHODS

Retrieval of sequences

Translated nucleotide sequences were retrieved from GENBANK withIDsEU441170(SHV5), EU441172 (SHV28)(Jemima and Verghese, 2009), EU979559 (SHV38) and MG701326 (SHV12).

Multiple Sequence Alignment and Phylogram

Phlylogenetic relationships between four sequences were investigated with ClustalWandwith BLOSUM weight matrix and Phylogram was made with neighbour-joining (NJ) algorithm. Phylogenetic analysis pipeline by GenomeNet (https://www.genome.jp/tools/ete/) (Huerta-Cepas *et al.*, 2016) was used. Alignment and phylogenetic reconstructions were performed using the function "build" of ETE3 v3.0.0b32 (Huerta-Cepas *et al.*, 2016)as implemented on the GenomeNet (https://www.genome.jp/tools/ete/).Alignment was performed with MAFFT v6.861b with the default options (Katoh and Standley, 2013).A distance-based tree was inferred with the BioNJ algorithm (Gascuel, 1997) using PhyML v20160115 (Guindon *et al.*, 2010) ran with model JTT and parameters: -f m --pinv e -o lr --alpha e --nclasses 4 --bootstrap -2.

Retrieval of Drug structures

Protein Data Bank (PDB) structures of drugs and inhibitors such as Cefotaxime [DB00493], Ceftazidime [DB00438], Cefepime [DB01413], Cefixime [DB00671], Meropenem [DB00760], Imipenem [DB01598] , Fosfomycin [DB00828], Aztreonam [DB00355], Clavulanic acid [DB00766], Tazobactam [DB01606], Sulbactam [DB09324], Avibactam [DB09060] were retrieved from DrugBank.(Wishart *et al.*, 2008)

Molecular Modelling and Confirmation of models

SwissModel(Schwede, 2003), was used for generating 3D PDB structures from protein sequences. Swissmodel is the automated comparative protein modelling server to construct homology models from the retrieved sequences. The modelled structures wereconfirmed the stability through online server Probity (http://molprobity.biochem.duke.edu/)(Williams *et al.*, 2018), and the z score was calculated by ProSA (https://www.came.sbg.ac.at/prosa.php).(Wiederstein and Sippl, 2007)

Molecular docking

iGeMdock(Hsu *et al.*, 2011), the graphical docking software is used for docking, the inbuilt tool, Rasmol for viewing and exporting the docked structure. Various output files, of interactions and energies were saved and analysed. The software utilizes empirical scoring functions; it calculates the total binding energy of the protein–ligand interaction by the combination of H-bonding, electrostatic and van der Waals interactions (Fitness = vdW + Hbond + Elec, where fitness is the total energy). iGemDock has following settings:Standard Docking (Population Size: 200, Generation:70 ; Number of populations:2), Stable docking (Population Size: 300, Generation:80 ; Number of populations:10), Accurate Docking (Population Size: 800, Generation:80 ; Number of populations:10), Drug Screening (Population Size: 200, Generation:70 ; Number of populations:3), Quick Docking (Population Size: 150, Generation:70 ; Number of populations:1). Accurate docking (very slow docking) was chosen for the analysis, with population size, 800 is set with 80 generations and 10 solutions. After the completion of the docking, the post docking analysis was performed to find the docking pose, energy values, interactive energies and interacting aminoacids.

RESULTS

Retrieval of sequences

The sequence with Genbank IDs EU441170.1, EU441172, EU979559 and MG701326 representing SHV5, SHV28, SHV38 and SHV12 respectively, were retrieved from *Klebsiella pneumoniae*. All amino acid sequence lengths of retrieved IDs were found to be 286 aa.

Multiple Sequence Alignment and Phylogram

Phylogenetic relationship between SHV5, SHV12, SHV28 and SHV38 were investigated with the help of ClustalW. Aminoacid changes are observed in positions, 3 (F only in SHV28), 31(L in SHV28 and SHV38), 142 (V only in SHV38), 234(S in SHV5 and SHV12) and 235 (E in SHV28 and SHV38) [Fig 1]. Phylogram was made with BioNJalgorithm (Gascuel, 1997) with distance matrix method [Fig 2]. Values obtained are the Chi2-based parametric values return by the approximate likelihood ratio test run by the program.

Fig 1: Multiple sequence alignment of SHV variants. Left side marked with different enzyme variants. The amino acids numberings are according to ABL scheme at right side. Asterisks indicate identical amino acids. "-"indicates the variable region of proteins.

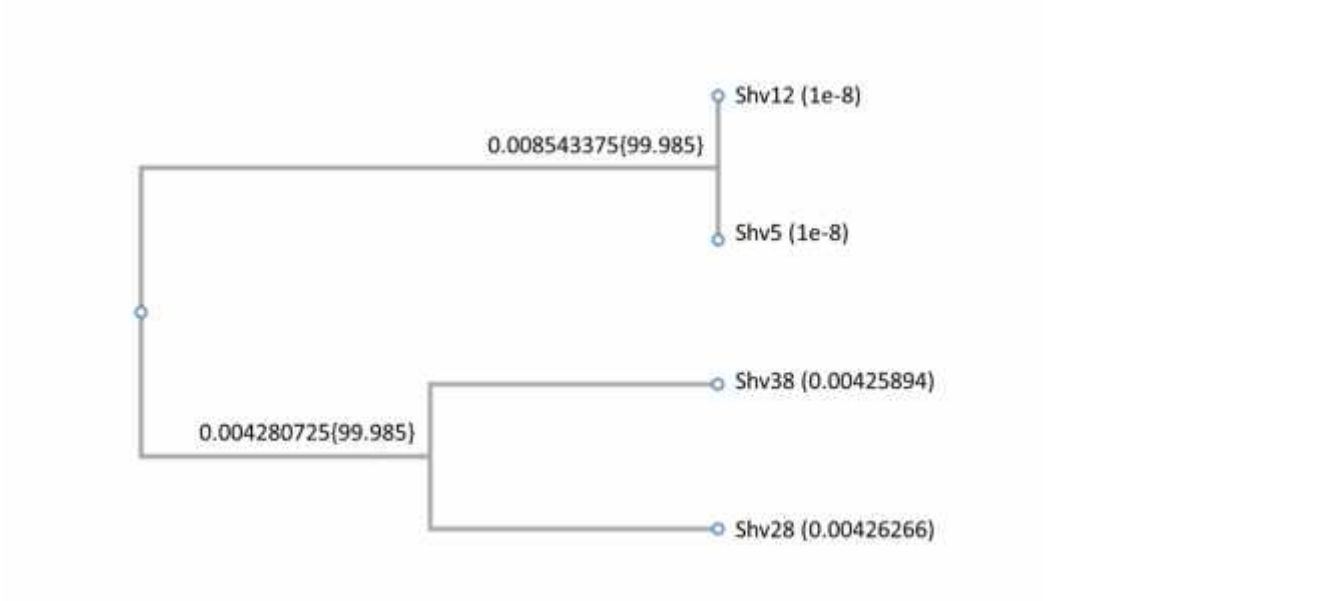

Fig 2: Phylogenetic analyses based on SHV gene sequences obtained from *Klebsiella pneumoniae.* The values represent the distance matrix between enzymes.

Retrieval of Drug structures

PDB structures of third generation Cephalosporin drugs such as Cefotaxime, Cefixime and Ceftazidime and

fourth generation Cephalosporin Cefepime were downloaded. Carbapenems like Imipenem and Meropenem, Inhibitors like Clavulanicacid,Tazobactam, Sulbactam, Avibactam and drugs like Aztreonam and Fosfomycin were considered for docking studies.[Fig 3]

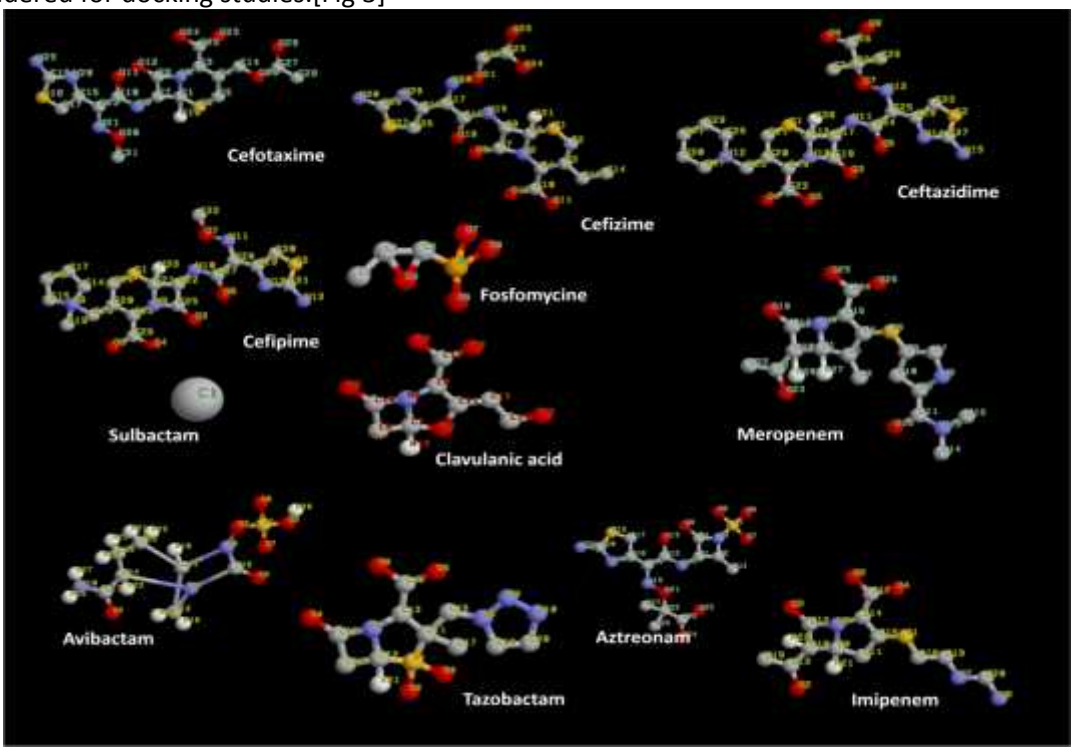

Fig 3: Downloaded PDB structures of eight drugs and four inhibitors. Grey represents carbon (C); Red represents Oxygen(O) ; Purple represents Nitrogen (N); Yellow represents Sulphur (S).

Molecular Modelling and Confirmation of models

Tertiary structures of protein models were developed using Homology model with Swiss model. Structure assessments of the modelled structures were studied for all the enzymes. [Fig 4]PDB structures along with Ramachandran plot analysis of enzymes were done with the online server MolProbity(Williams *et al.*, 2018)(http://molprobity.biochem.duke.edu/). Ramachandran plot confirms that above 90% residues were in favoured and allowed regions, thus confirming the stability of protein structures.Further Z score was calculated by ProSA(Wiederstein and Sippl, 2007)(https://prosa.services.came.sbg.ac.at/).

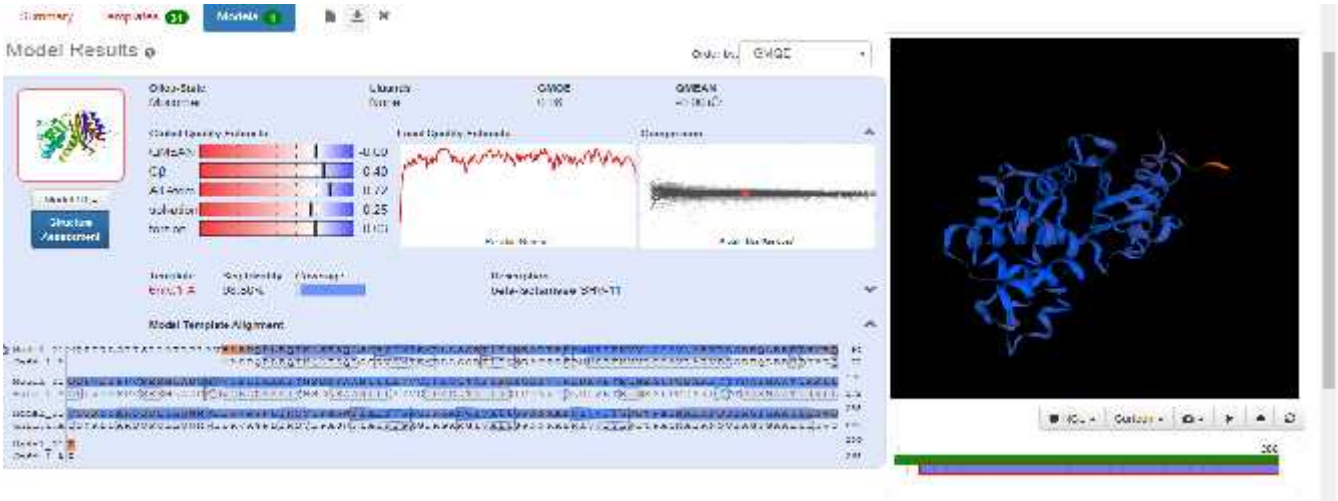

Fig 4: Swiss model result page for SHV variants with protein sequences. The blue cartoon structure depicts the SHV28 enzyme.

Problematic parts of a model are identified by a plot of local quality scores and the same scores are mapped on a display of the 3D structure using colour codes. The z-score graphs for the structures are portrayed in [Fig 5].

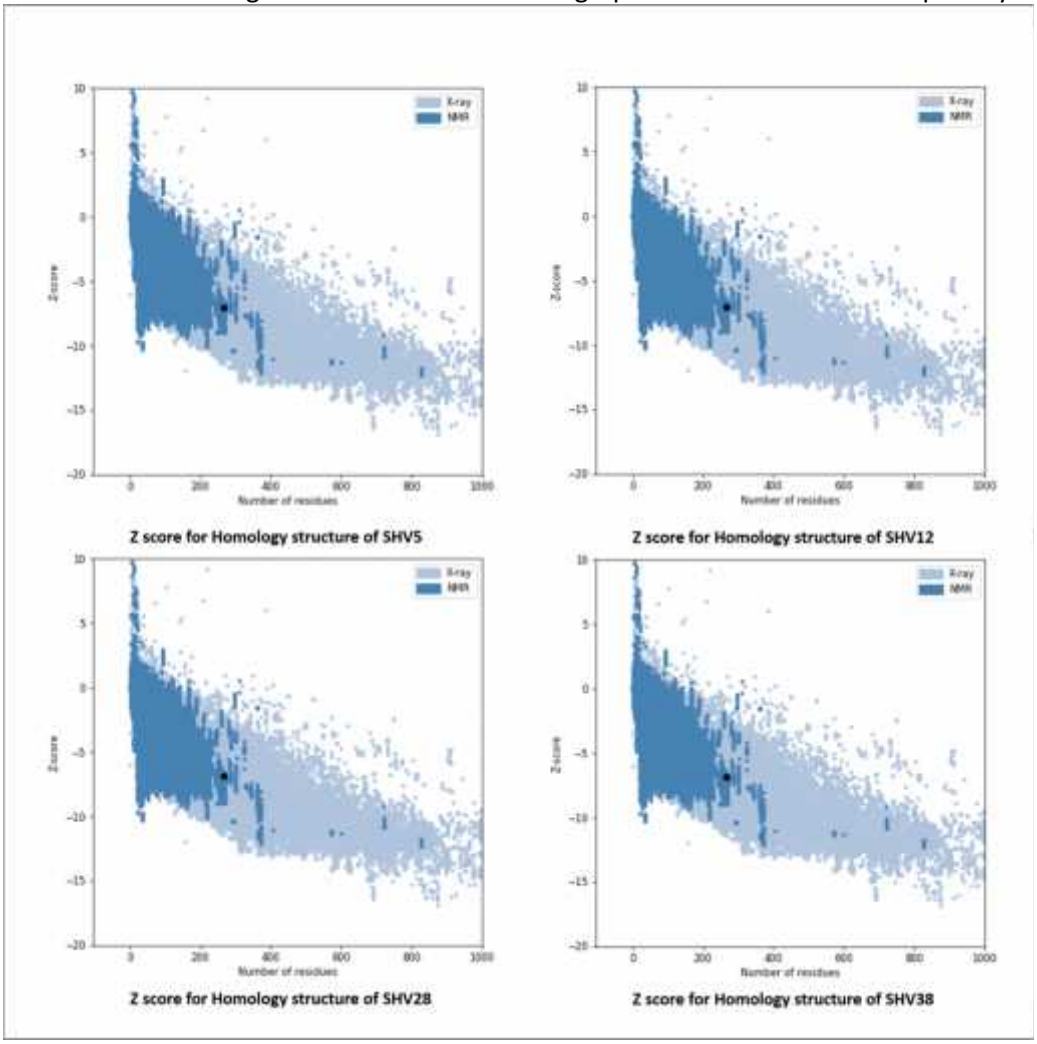

Fig 5: The plot represents groups of structures from different sources (X-ray, NMR) are distinguished by Light blue which indicates X-ray and Dark blue indicates NMR spectroscopy. The black dot represents over-all z-score of the protein structure.

The z-score indicates overall model quality where positive values refer to problematic or erroneous parts of the input structure, while negative values correspond to quality of structure model. The statistics from both analyses correspond that the modelled protein structure is high quality ones, hence eligible for docking studies. Table 1 provides the results from Ramachandran plot and z-score identification.

Table 1: Confirmation of homology modelled PDB Structures with Ramachandran Plot and Z score for structure modality

Protein Structures	Residues in favoured regions	Residues in allowed regions	Z score
SHV5	256/263(97.3%)	263/263 (100%)	-7.05
SHV12	256/263(97.3%)	263/263 (100%)	-7.05
SHV28	260/265 (98.1%)	265/265 (100%)	-6.85
SHV38	260/265 (98.1%)	265/265 (100%)	-6.78

Visualization of PDB structures

All models from SHV variants were visualized in Rasmol 2.7.3, to see the structural configurations. Table 2 describes the structural characteristics of each model.

Table 2: Visualization and characterization of 3D structure of enzymes with Rasmol

	SHV5	SHV12	SHV28	SHV38
Number of Chains	2	2	-	-
Number of Groups	265	265	267	267
Number of Atoms	2027	2027	2039	2041
Number of H Bonds	186	186	179	179
Number of Helices	13	13	13	12
Number of strands	10	10	10	10
Number of Turns	25	25	26	26

Alpha helices are seen in red ribbons, Beta sheets are in cartoons, coloured as green, beta turns are in purple strands for all the PDB structures. [Fig 6]

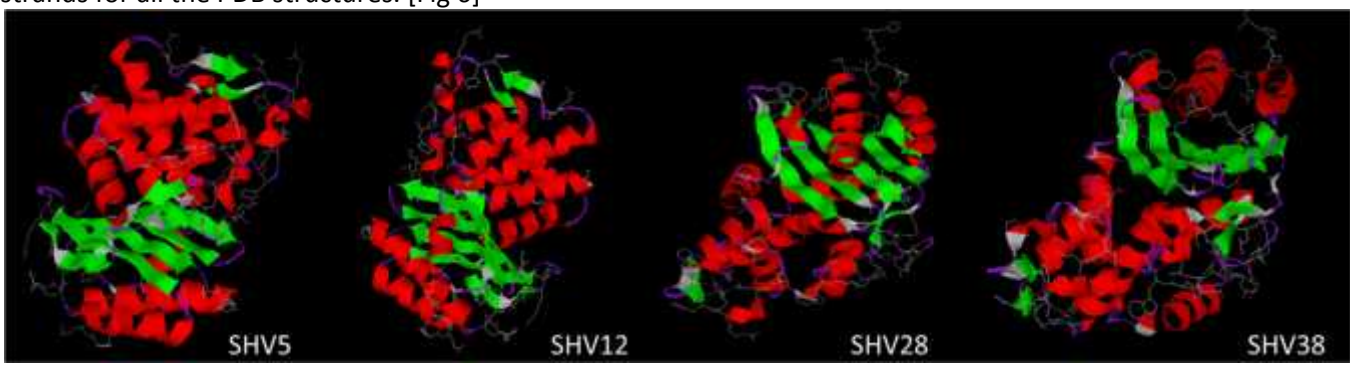

Fig 6: Visualization of modelled structures of SHV5, SHV12, SHV28 and SHV38 using Rasmol software. Alpha helices are seen in red ribbons, Beta sheets are shown in cartoons coloured as green, beta turns are in purple strands for all the PDB structures

Molecular Docking

Molecular docking was done by iGemdock. (Hsu *et al.*, 2011) The outputs described the aminoacid interactions and fitness score depicted as total energy. Unique interactions from all enzyme-Drug/Inhibitor combinations were studied and found that SER-66, ASP-100, TYR-101, SER-126, ASN-128, ARG-160, THR-163,GLU-167, ASN-166, ALA-168, LEU-169, ASP-172, ,ARG-174, THR-231, ALA-233, ARG-239, were present in all four enzyme docking interactions. Interactive energies have been tabulated in Table 3 and the graph is depicted in Fig 7. Sulbactam has maintained constant relatively high binding energy with all the drugs and inhibitors. Sulbactam and Fosfomycin were found at different extremes, in energy graph, yet both shared same binding site with common aminoacids;ARG-133, GLU-140, ALA-141,LEU-142, ASP-145, ARG-147.(Table 4) The other exclusive aminoacids involved in sulbactam were not contributing much in total binding energy. Hence, we have also observed the contribution of various interactions like Hydrogen, Van Der Waals and electrostatic bonding with amino acids and atoms in drug.It can be observed that Van Der Waal's force interactions are prominently leading the binding energy total and thereby increasing the affinity of drugs.

Table 3: Interactive energies of docked enzyme-drug/inhibitor complexes

	SHV5	SHV12	SHV28	SHV38
FOSFOMYCINE	-70.1	-60.8	-68.9	-68.9
CLAUVULANICACID	-73.6	-73.5	-74.4	-74.4
AVIBACTAM	-85.9	-85.9	-87.9	-87.9
IMIPENEM	-91.5	-89.7	-83.7	-83.7
TAZOBACTUM	-92.5	-92.5	-97.8	-97.8

CEFOTAXIME	-98.6	-101	-100.4	-100.5
CEFIXIME	-99	-89.3	-96.5	-96.5
AZTREONAM	-100.2	-100.2	-98.6	-98.6
MEROPENEM	-103.7	-84.4	-90.1	-82.9
CEFIPIME	-108	-81.2	-118.1	-118.1
CEFTAXIDIME	-108.1	-101.8	-108.5	-108.5
SULBACTAM	-162.5	-162.5	-161.7	-161.6

Table 4: Energy comparison with Fosfomycin and Sulbactum

Enzymes	**Drugs**	**VDW**	**Bond**	**Elec**
shv5	Fosfomycin	-39.0404	-31.6751	0.545076
	Sulbactam	-105.942	-57.1282	0.582637
shv12	Fosfomycin	-35.2053	-22.358	-3.24817
	Sulbactam	-105.942	-57.1282	0.582637
shv28	Fosfomycin	-36.7605	-32.2723	0.137445
	Sulbactam	-114.701	-47.4705	0.507243
shv38	Fosfomycin	-36.8772	-32.1888	0.140932
	Sulbactam	-114.72	-47.4201	0.507115

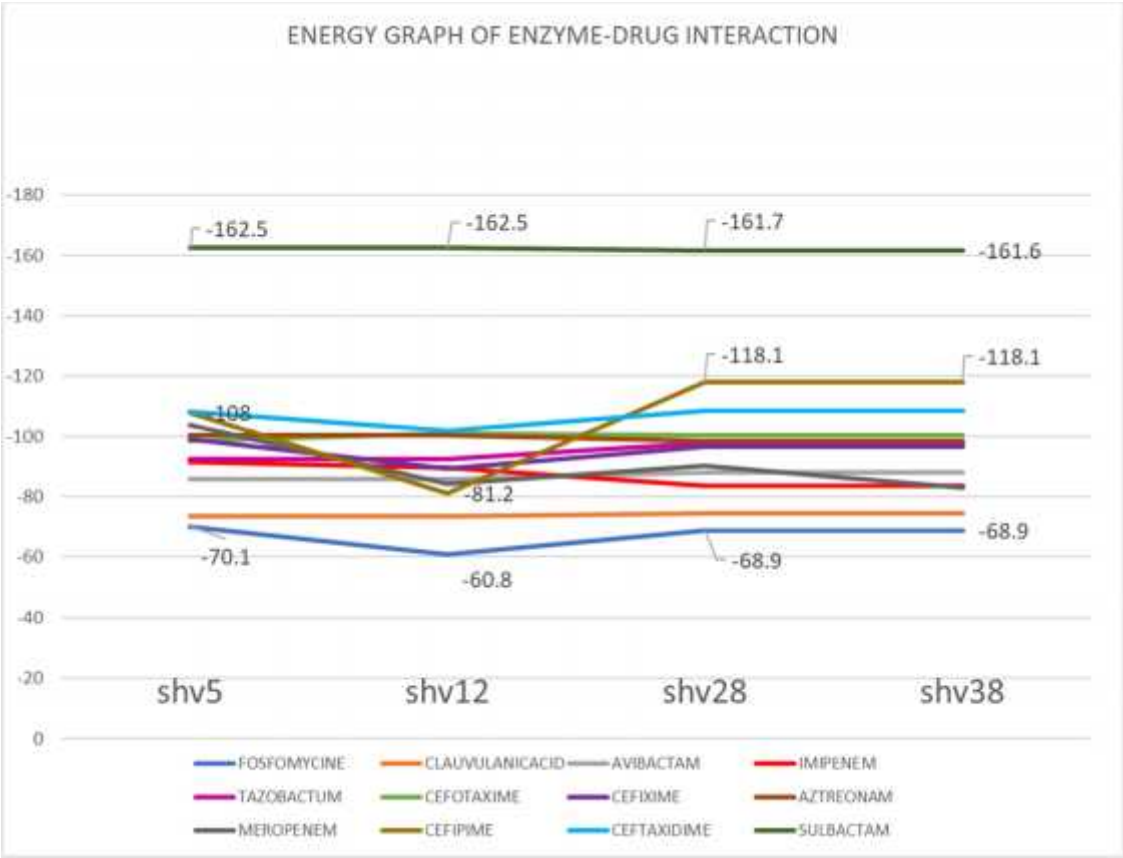

Fig 7: Line-Graphical depiction of docking energy values displayed by enzyme-drug/inhibitor interactions. Enzyme-ligand combination with highest interactive energy were picked up among SHV variants in Fig 8,9 and 10. From the Table 3 and Fig 8 and Fig 10, β lactamase inhibitors had more energies with SHV28 and SHV38, while carbapenems had more affinity with SHV5. Cephalosporins [Fig 9] had mixed response with all considered SHV variants.

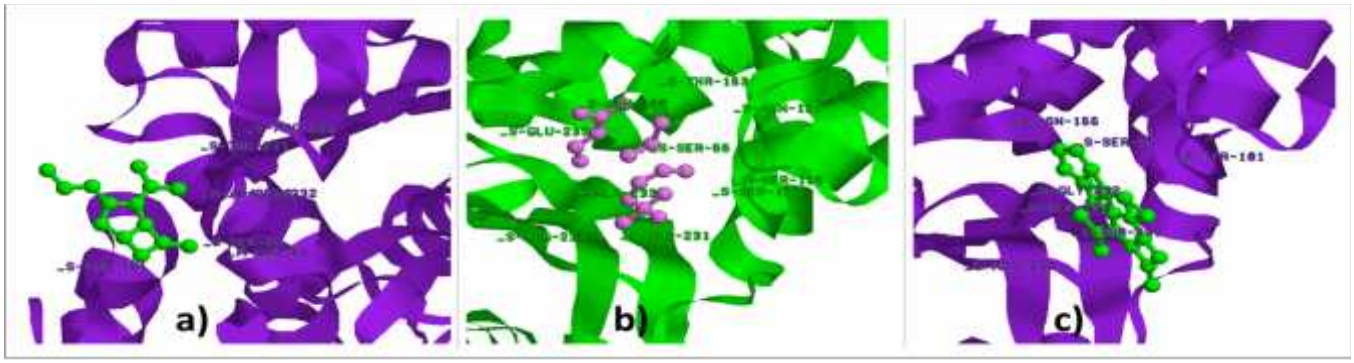

Fig 8: Docking pose of SHV variants with β- lactamase inhibitors showing highest negative energy. a) SHV28 with clavulanic acid b) SHV38 with Avibactam and c) SHV28 with Tazobactam

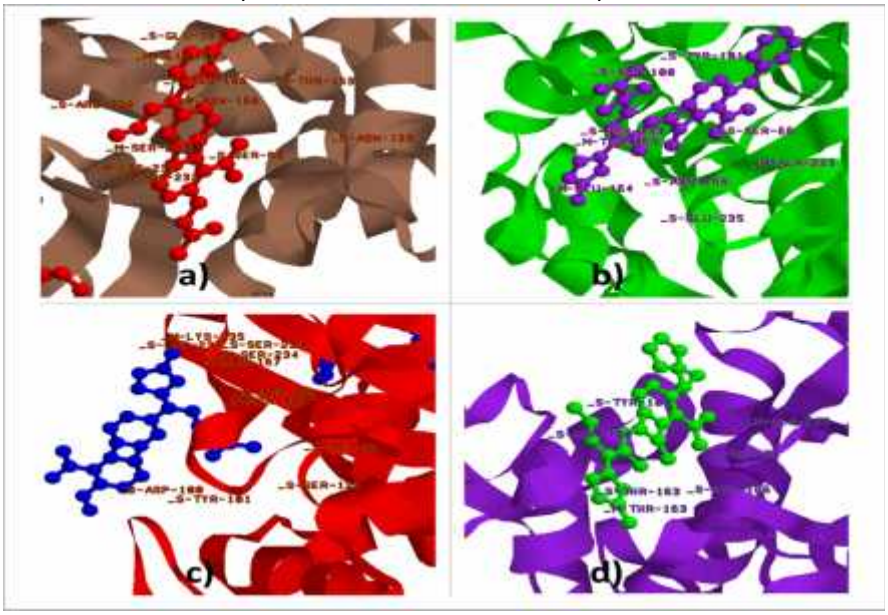

Fig 9: Docking pose of SHV variants with Cephalosporins showing highest negative energy. a) SHV12 with Cefotaxime b) SHV38 with Ceftazidime c) SHV5 with cefixime and d) SHV28 with Cefipime

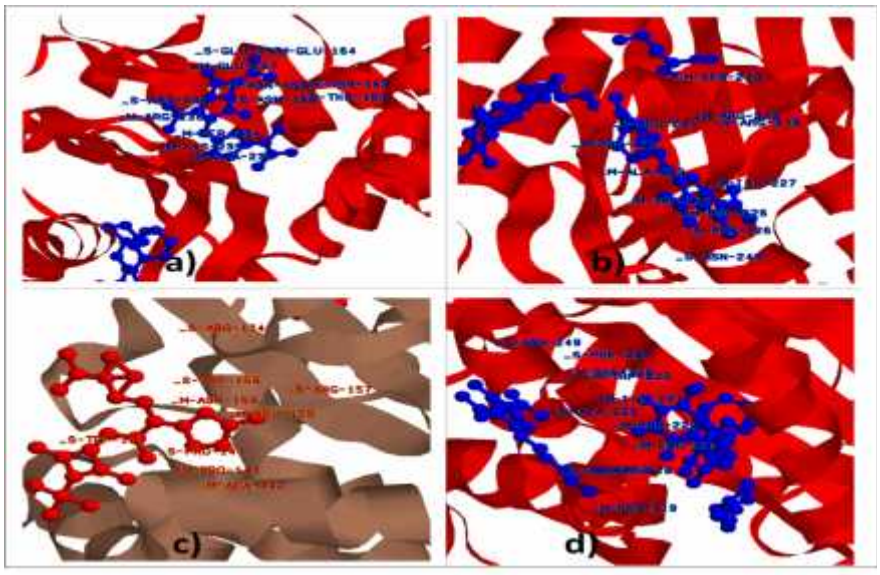

Fig 10: Docking pose of SHV variants with Carbapenems and monobactam showing highest negative energy. a) SHV5 with meropenem b) SHV5 with Imipenem c) SHV12 with Aztreonam d) SHV5 with Aztreonam

Protein-ligand binding site at conserved sites

From the conserved motifs of SHV enzymes, Serine-threonine-phenyalanine-lysine tetrad (S-T-F-K) at positions 66-69 and lysine-threonine-arginine (K-T-G) at positions 230-232.In the present study, the aminoacid positions like Ser66, Thr231 and Gly232 are found involved in atleast one protein-ligand complex of SHV variants. (Jemima and Verghese, 2009)

Most frequently found aminoacid interactions

Most frequent amino acids are found to be ALA-233, ASN-166 and SER-66, occurring seven times in various combination of each enzyme. Second most frequently involved aminoacids, are TYR-101 and GLU-167 occurring six times [Fig 11].

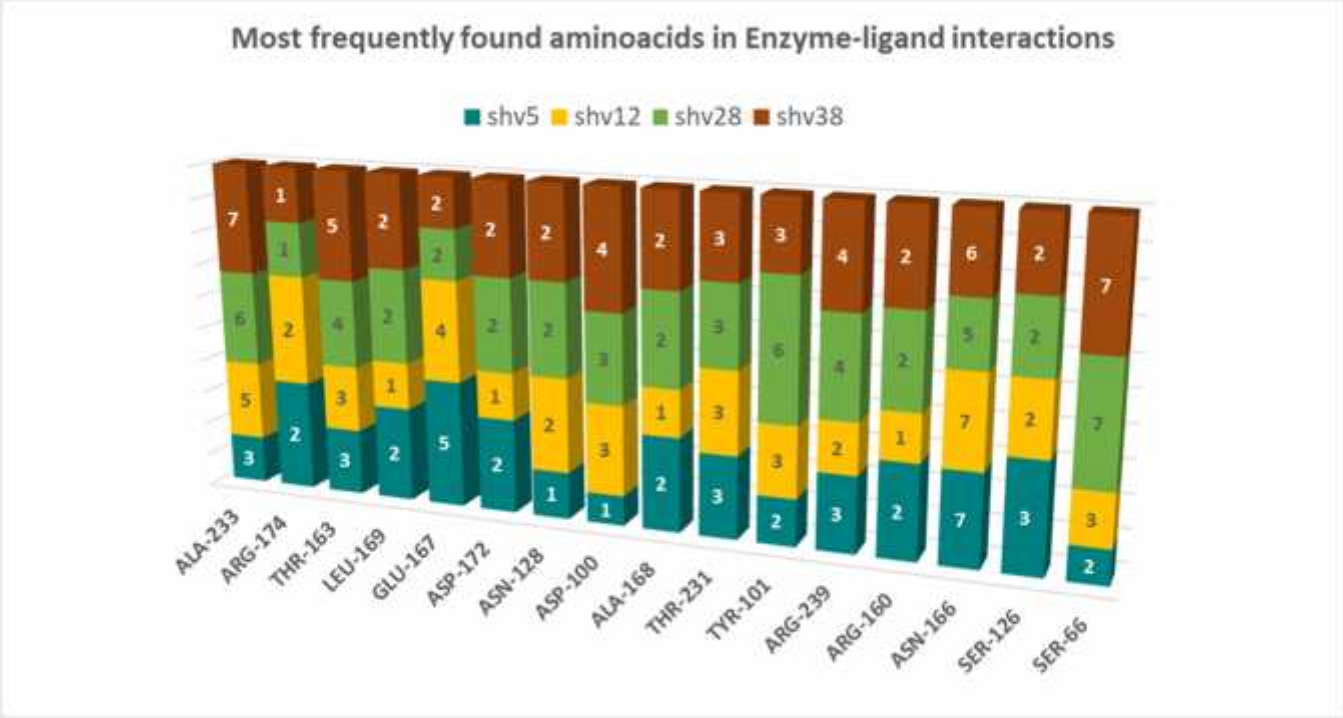

Fig 11: 3-D column bar graph represents repeated frequency of amino acid in enzyme-ligand interactions. Ser-66 in SHV28 and SHV38: Ala-233 in SHV38; ASN-166 in SHV5 and SHV12 showed the maximum frequency of seven times occurrence.

Binding of Ligands on Mutant sites

Each Amino acid interactions were colour coded to see the patterns and common blocks ofaminoacidin various classifications of drugs and inhibitors Fig 12. The mutant site Lys-235, coded as red colour was found mostly in third generation cephalosporins and Avibactam with SHV28 and SHV38.

Figure 12: Colour-coded spreadsheet displays amino acid positions involved enzyme-ligand interactions. Red boxes represent the mutant sites. Other colours used for unique identification.

DISCUSSION

In the present study we tried to analyze the type of resistant gene from the extracted SHV enzymes. Docking analysis was carried out with drugs as well as inhibitors. The docking showed the aminoacid residues crucial to the interaction of each enzyme structure. While docking higher negative energy is considered as an indicator of effective and rigid binding of an enzyme – inhibitor-complex. (Kumar, Sharmila and Murugan, 2014) In this study, it was found that Sulbactam and Ceftazidime had higher negative energy with -162.5, -162.5,-161.7,-161.6 and -108.1, -101.8, -108.5 and -108.5 for SHV5, SHV12, SHV28 and SHV38 respectively. Whereas it was understood that Fosfomycin had lower negative energy with -70.1,-60.8, -68.9 and -68.9 against studied SHV enzymes. From the Multiple sequence alignment and from the phylogenetic tree construction, SHV5 was similar to SHV1 where as SHV28 is similar to SHV38 which was also comparable to the reporting done byHeritage*et al,*Bush K and Jacoby GA, Liakopoulos A*et al*.(Bush and Jacoby, 2010)(Heritage *et al.*, 1999)(Liakopoulos, Mevius and Ceccarelli, 2016) Liakopoulos*et al* have compared most of the SHV variants, which include SHV5, SHV12 and SHV38 under extended β-lactamases (2be)sub group while SHV28 was reported as unclassified allele.(Liakopoulos, Mevius and Ceccarelli, 2016)Gutmann et al in 1989 reported that the SHV-5 was able to hydrolyze broad spectrum cephalosporins and monobactams(Gutmann *et al.*, 1989). From India, we have reported SHV28 as first Indian report from a clinical isolate of *Klebsiella pneumoniae.* The conserved motifs, Serine-threonine-phenyalanine-lysine tetrad (S-T-F-K) at positions 66-69 and lysine-threonine-arginine (K-T-G) at positions 230-232 were mentioned in the same study. (Jemima and Verghese, 2009) Gutmann et al in 1989 reported that the SHV-5 was able to hydrolyze broad spectrum cephalosporins and monobactams. Homology models developed from four SHV variant sequences were confirmed with Ramchandran plot and z-score modality validation. More than 98% of the regions belonged to favoured region as per Ramachandran plot, thus reliable for docking with the drugs and inhibitors. The z-score indications from ProSA program approves the overall model quality. This graph also helped us to check whether the modelled structures are comparable with the similar proteins which are belonging to two experimentally derived sources, i.eXRay and NMR.(Wiederstein and Sippl, 2007)

We have also attempted to study about tertiary protein confirmations like alpha helix, Beta sheets and turns. This structural information about clinically relevant enzymes are a valuable source of information on favourable interactions which would stabilize the ligand's association with the binding pocket of the protein. From the protein visualization analysis, chain, atom groups, Hydrogen bonds, helices, strands, turns and sheets were compared. More or less, all enzymes had similar number of turns (~25), strands (10), helices (~12). Hydrogen bonds were found more in SHV5 and SHV 12, *viz*186 while 179 in SHV28 and SHV38 [Table 2]. Mutant site in SHV28 and SHV38, Glu235 instead of Lys235, interacting with cephalosporins must be impacting the resistance of SHV variants towards the drugs [Fig 12]. Glu235 is also found interacting with Avibactam-SH28/SHV38 in this analysis. The resistance for Cephalosporin/Avibactam is reported in many studies with KPC producing enzymes.(Göttig*et al.*, 2019)(Zhang *et al.*, 2018)To our knowledge it would be the first study to model the rare variant SHV28 and to investigate the enzyme-ligand interactions. Further *in vitro*and *in vivo s*tudies for bactericidal activity of cephalosporins-avibactam against SHV variants would provide more insights regarding the resistance. Our docking studies and analysis have concluded that Sulbactum has been possessing high interactive energies with every SHV variant. As the SHV have evolved from narrow to an extended spectrum of hydrolyzing activity, more described molecular studies and analysis will enhance the importance of newer drug development. From our analyses, SHV variants were interacting with inhibitors with more energies and more affinity towards carbapenems, which are theoretically sensitive in nature. Cephalosporins interacted in a mixed response towards all SHV variants, where the average interactive energy is -100. This result concludes that the combination of cephalosporin and inhibitors would be more effective for patient treatment. Sulbactam is one of four β-lactamase inhibitors in current clinical use to counteract drug resistance caused by degradation of β-lactam antibiotics by these bacterial enzymes. Sulbactam is susceptible to degradation by β-lactamases.*In-silico* docking suggests H-bonding, hydrophobic interaction, charge interaction, aromatic interaction, and Van der waal forces responsible for stabilizing enzyme-inhibitor complex.(Harer and Bhatia, 2014)*In-silico*analysis predicted differential antibiotic pattern among SHV variants. Hence early detection of antibiotic resistance gene variants could guide the choice of optimal antibiotic therapy for successful treatment.

As stated by Tzouvelekis and Bonomo,(Tzouvelekis and Bonomo, 1999) it will not be surprising if SHV enzymes will continue to expand their substrate spectrum as long as the current antibiotics, or novel ones derived from the basic β-lactum structure, are used. SHV enzymes have kept a stable role in antibiotic resistance over the years. Definite identification of these variants is important for surveillance, epidemiological purpose and for newer drug development.

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