ANALYSIS OF CLINICAL SAMPLES FOR ESCHERICHIA COLI ALONG WITH MOLECULAR DOCKING OF PBP1B WITH AMIKACIN AND CTX-M 14 WITH CEFTAZIDIME

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ABSTRACT

The present study was undertaken to determine the prevalence of E. coli and its antibiogram analysis using disc diffusion method and to study the interactions between target and resistant proteins with drugs through docking. A total of 80 samples were obtained from different Hospitals of Peshawar and were processed in the Laboratory of Microbiology Department, Shaheed Benazir Bhutto Women university Peshawar, from July to September, 2014 for E. coli identification. All samples were streaked on MacConkey and Blood agar while urine was streaked on CLED agar. E. coli identification was based on cultural characteristics, gram staining and biochemical tests. Analysis of clinical samples revealed no growth on Sputum and blood sample while puss had different bacterial growth in which account of E. coli was much lower i.e. 12% while higher percentage of this isolate was found in urine 21.4%. E. coli occur more frequently with enhanced percentage in females than males. Antibiotic sensitivity was performed using 14 antibiotics by disc diffusion method in which E. coli show maximum susceptibility towards imipenem (91.6%), Amikacin (83.3%) and meropenem (83.3%). While maximum resistance was seen towards erythromycin (100%), Ceftazidime (83.3%) and cefotaxime (75%). To measure the distances, docking of Amikacin and Ceftazidime with PBP 1b and CTX-M 14 enzyme were performed. Minimum distance of docked complex of Amikacin-PBP1b showed active action of drug that target the major component of cell wall synthesis while smallest distance between Ceftazidime and CTX-M 14 showed the attack of this enzyme to drug for the purpose of inactivation. This finding is useful for the selection of effective drug for empirical treatment and for prevention and control of the infection.

Keyword: Urinalysis, UTI, Uropathogenic Escherichia coli, Antibiogram analysis, PBP1b, CTX-M 14, Molecular docking parameters, Amikacin, Ceftazidime

INTRODUCTION

Escherichia coli is a common inhabitant of the human, animal gut and in the lower intestine of warm-blooded organisms but can also be found in water, soil and vegetation. When strains of E. coli are outside their normal habitat of the gut, they can cause serious infections. E. coli exist as normal flora and provide the pool for instigation of UTI and when it get enter into the bladder, it starts multiplying in urine causes UTI and probably cause 75 - 90% of uncomplicated UTIs[1] with serious health problem affecting millions of people each year both in the society and hospital settings and reported in all age groups in both male and females. Pathogenic E. coli is associated with intestinal and extraintestinal infections. UPEC strains are responsible for UTI having virulence factors that adhere to uroepithelial cells and ability to resist phagocytosis and bactericidal action[2,3,4]. UTI is a general term referring to the infection anywhere in the urinary tract and is a commonly caused by bacterium E. coli [5].

Several aspects on male UTI are unclear or poorly described and estimated that one-third of all 80-year-old men will have had an incident of bacturia[6]. The higher prevalence in females as compared with males is attributable to the conciseness of the female urethra and is more liable to infectivity during sexual activity[7,8].

The ESBL producing E. coli are increasingly causing urinary tract infections and making therapy of UTI difficult and promoting greater use of expensive broad spectrum antibiotics, such as carbapenems[9]. These enzymes are small proteins that are produced by bacteria and make them resistant to common antibiotics that are used in hospitals. The ESBL producing E. coli is well-known to protect against effect of penicillins, cephalosporins and monobactams on their cell wall synthesis. They have the ability to hydrolyze oxyimino-cephalosporins (for example, cefotaxime, Ceftazidime and ceftriaxone) and monobactams (for example, aztreonam), but not cephamycins or carbapenems[10,11]. Among ESBL, CTX-M beta lactamases are rapidly growing type and are mostly found in E. coli and poses a serious threat towards cephalosporin and various infections. The primary factor responsible for the development and spread of bacterial resistance is the injudicious use of antimicrobial agents[12].

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Molecular docking is three-dimensional construction of a bimolecular complex and hence plays an important role in the rational design of drugs[13]. During the docking process, the ligand-protein molecules adjust their conformation to achieve an overall "best-fit" and this kind of conformational adjustments resulting in the overall binding is referred to as "induced-fit"[14].

This study is conducted for the identification of E. coli from different clinical sample and to know the most common and widely occurrence of the disease associated with E. coli that is UTI. The main objective is to study the effects of various antibiotics on E. coli and to determine which antibiotic is most effective for inhibiting its growth. To study the interacting points, docking is carry out which would reveal the best interacting drug and allow us to characterize the behavior of small molecules (drug) in the binding site of target proteins. This will help to understand the structure-activity relationship of target proteins and resistant proteins against antibiotics at molecular level.

MATERIAL AND METHODS

This study was conducted in the Laboratory of Microbiology Department, Shaheed Benazir Bhutto Women university Peshawar from July to September 2014 based on isolation of E. coli from different clinical samples including urine, puss, blood and sputum and discus the most common infection caused by it i.e. Urinary Tract Infection, as E. coli is the major causative agent of UTI. The patients were under the treatment in a ward of Medical C ward, Children B, ward, PRA, Gynae OPD, OPD, Medical E Ward, SBW, Medical D Ward, Medical A Ward, Children A Ward.

A total of 80 cases were studied, in which samples were collected from children young and old age patients who stayed for the clinical treatment in a ward of the hospital between the ages of 5 to above 30 years, patients who have not attained the age of 5 years were excluded. The details of each patient were recorded including name, age, gender and ward. Fresh mid-stream urine (from both asymptomatic and symptomatic patients being tested for UTIs), puss, blood, and sputum specimens were collected in sterile disposable containers which were labeled properly with specific codes and were processed immediately within one hour of collection at room temperature for microbiological analysis. They were then examined for the presence of E. coli.

The media for identification were, Nutrient Broth (NB), Cysteine Lactose Electrolyte Deficient (CLED) agar, MacConkey agar and Blood agar.

CLED is the differential media used for the differentiation of urinary organisms. Only urine samples were cultured on medium CLED agar. MaCconkey agar is selective medium used for growth of both lactose fermenter and non fermenter gram negative microorganisms. Blood agar is used to check production of hemolysin which breaks down red blood cells. Each sample was then cultured on three plates containing CLED, MacConkey and Blood agar. After streaking, the plates were incubated at 37 °C for 24 hours.

Bacterial detection was based on standard culture and biochemical characteristics of isolates. E. coli were identified through Gram staining which is used to characterize bacteria as gram positive or gram negative. For confirmation, relevant Biochemical tests including Citrate, Triple Sugar Iron (TSI) and Urease tests were performed. TSI agar slant is used to determine the ability of an organism to attack a specific carbohydrate incorporated into a basal growth medium, production of gas and hydrogen sulphide. Urease test is used to determine the ability of organisms to degrade urea by means of the enzyme urease. Simmons Citrate Agar is used for the differentiation of microorganisms on the basis of citrate utilization. Culture from the media plates was taken by means of standard loop and inoculated by stabbing the butt and streaking the slant. Slants were then incubated for 24 hours at 37◦C.

Antibiotic susceptibility of Escherichia coli was performed on Mueller-Hinton agar using 14 different antibiotics that were placed at distance of regular intervals. Bacterial suspension of Escherichia coli was made in normal saline/nutrient broth and then spread on Mueller-Hinton agar. After overnight incubation at 37oC, the diameter of every zone of inhibition was measured in millimeters. The test was interpreted according to the standards established by Clinical and Laboratory Standards Institute (CLSI).

Bioinformatics Tool

Selection of Target and Resistance Protein

The PDB ID for crystal structure of penicillin binding protein 1 b with entry code P02919 and beta lactamase with entry code Q9L5C7 was taken from Uniprot. The structures were then retrieved from protein data bank. The protein molecules were visualized by using software Accelrys Discovery Studio.

Retrieval of Drug

For the selection of ligand, amikin and fortam are selected to interact with PBP1b and beta lactamases CTX-M 14 respectively. Structures were retrieved from the Drug Bank..

Molecular Docking

Protein docking was performed using the Hex server which is the first Fourier transform (FFT) based protein docking server to be powered by graphics processors where docking is done by the server itself. In the first step the protein and ligand structure in PDB format were uploaded and submitted to the server.

After submission server produces a ranked list of 1000 docking predictions for subsequent refinement. The docking result was downloaded and visualize through Discovery Studio Viewer where the distance of interactions between protein and drug were measured.

RESULTS

A total of 80 clinical specimens were investigated for presence of E. coli. The higher percentage was obtained from urine samples followed by puss samples and no growth was observed in blood and sputum samples. In this study only positive specimen for E. coli was included. Recorded data revealed that Females are more susceptible to this organism than males. The Higher percentages of E. coli in urine were isolated from the patients in the age group of above 30. In puss samples, E. coli is less isolated bacterium and is commonly found in patients with age group 16-30. Table 1 summarizes percent identification according to age and gender.

During Microbiological analysis growth of E. coli was confirmed by large, elevated and yellow colonies on CLED agar and pink smooth colonies were observed on MacConkey medium. Gray colonies with smooth margins were seen on blood agar. When gram staining was performed, the gram negative rods were appeared under microscope that had taken stained pink which indicates the confirmation of E. coli.

E. coli was confirmed through biochemical identification revealed as it had given positive test for lactose fermenting and Triple Sugar Iron agar test, as TSI agar slant changes from red to yellow color, yellow butt and slant that indicate the fermentation of sugars and production of acid. The negative biochemical tests are citrate and Urease, results shown in Table 2.

Antibiotic sensitivity tests were performed using different antibiotics. After overnight incubation, zone of inhibition was measured according to CLSI (Clinical and Laboratory Standards Institute) criteria. From the results it was observed that E. coli showed high resistance when they were tested against erythromycin (100%), Ceftazidime (83.3%) and ceftriaxone (75%). In comparison, low resistance rates were found against moxifloxacin (50%), ciprofloxacin (41.6%). High sensitivity was observed for Amikacin (83.3%), imipenem (91.6%), meropenem (83.3%), augmentine (75%), cefoperazone (66.6%) and norfloxacine (58.3%), while low sensitivity was observed against tazocine (50%), aztreonem (41.6%). High moderate sensitivity rates were observed for cefepime (75%). Table 3 and Figure 4 summarizes all antibiotics with their generic and brand name.

Interaction distance of amino acid with Amikacin was measured in angstrom (Å). Amino acid (PRO158, LYS437, and PHE215) at position O, HZ3, and H interact with drug atom H46, O11, and N18 and makes a distance of 2.321 Å, 2.325 Å, and 1.860 Å respectively (see figure 1). Table 4 summarizes all interactions with their specific positions.

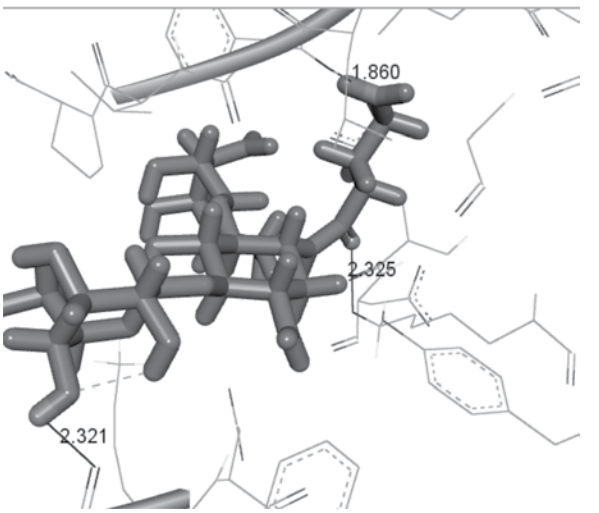

Figure 1: Interaction of Amikacin and Penicillin Binding Protein 1B.

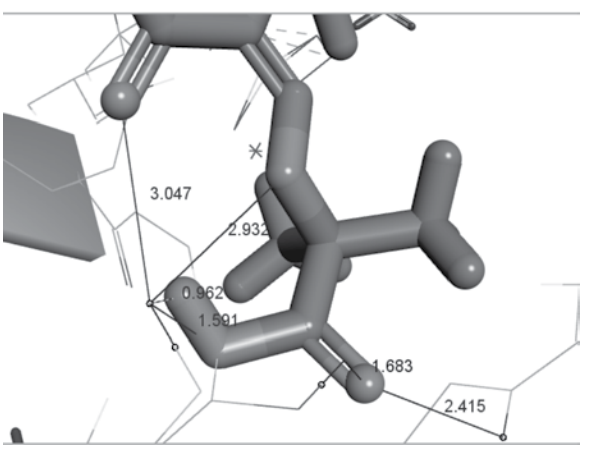

Figure 2: Direct Interaction of CTX-M 14 with Ceftazidime

Strong connections were seen between the Ceftazidime and resistant protein CTX-M-14 (see figure 2) in which Protein residue SER274 interact with drug atom O6, O7, O8, O9 and H39 at position OG and HG by making distance 3.047, 2.932, 1.591, 1.683 and 0.962 respectively while ARG276 interact with drug atom O9 at position HE and makes a distance of 2.415.

CTX-M 14 also indirectly interacts with Ceftazidime through water atoms. The residues that indirectly interact with drug atom through water molecules are SER237, SER274, ASP240, and THR244 (See Figure 3). All direct and indirect interactions are summarized in table 5.

(A) Protein Residue SER237 connected to drug atom O5 through Hetatom HOH2118.

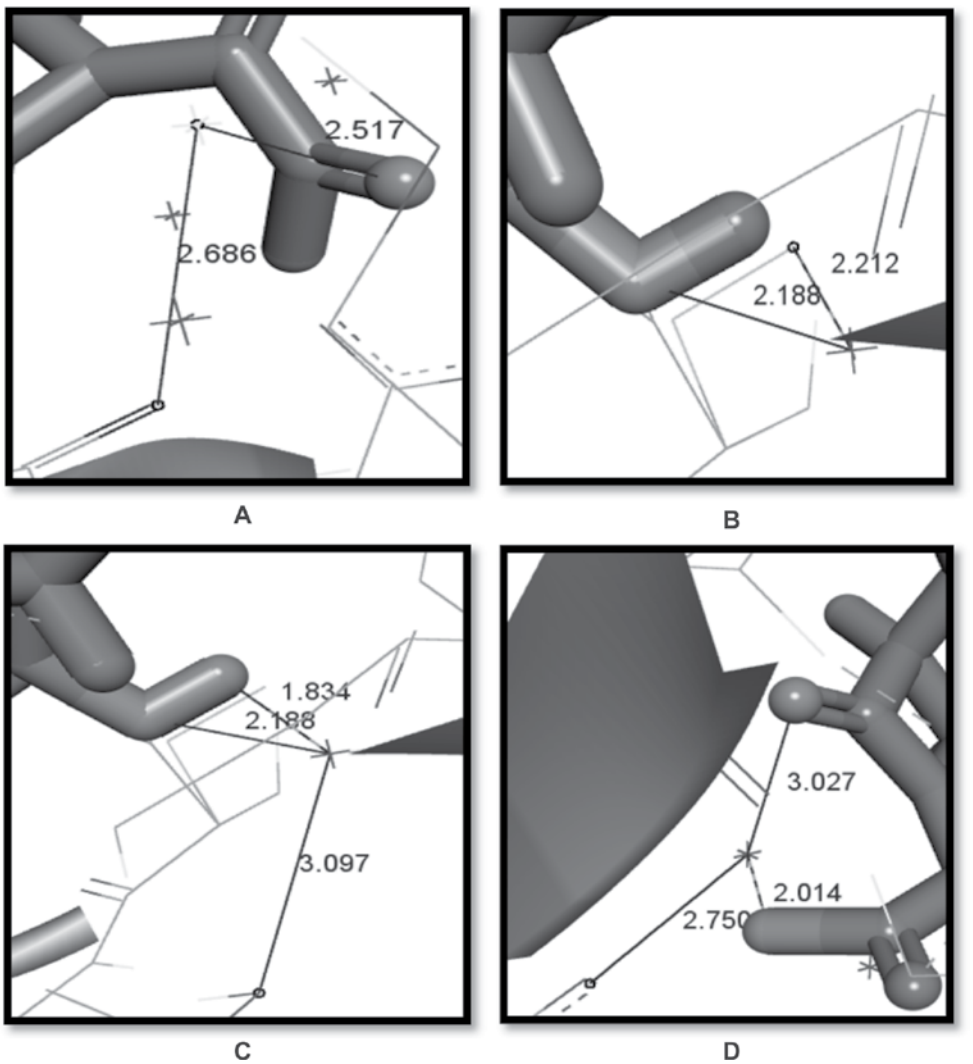

Figure 3: Indirect Interactions of CTX-M 14 through Hetatoms with Ceftazidime

(B) Protein Residue SER274 connected to drug atom O8 through hetatom HOH2314.

(C) Protein residue THR244 at position OG1 connected to two drug atoms (H39, O8) through Hetatom HOH2314. (D) Amino acid ASP240 at position OD1 connected to two drug atoms (O3, O4) through Hetatom HOH2353. Note: hetatoms are colored red.

DISCUSSION

Results from data demonstrated that higher percentage 21.4% (9) was found in urine specimen so they had proven urinary tract infection while least 12% (3) found in puss sample. This is conformity with the study of Mohanalakshmi et al., 2014 who identified more E. coli isolates in urine than puss. This indicates that Uropathogenic E. coli (UPEC) possess various virulence factors which enable the bacteria to survive and multiply in urine. The main reason behind lower percentage of E. coli in pus sample is because Gram positive bacteria present in greater number in puss than Gram negative bacteria and among Enterobacteracae family the percent of E. coli is much lower because E. coli merely causes skin or soft tissue infection and it is a less common route of entry for this isolate. The percent similarity of this isolate in puss was seen in study of Poonam verma, 2012 who isolate 16% E. coli isolates and the differences between this percent rate is due to change in number of samples.

The prevalence of UTI in relation to gender showed that 14.3% women population suffered from this infection than males (7.1%), as urine infections are rare in men under age group of 50 but are common in old age around 60s, this observation was also found in the study of Mohammed Akram et al., 2007 and Ziad Daoud et al., 2011.

UTI in relation to age showed that prevalence was higher in individuals who are in age group of above 30 (9.5%) including both male and female while high inci-

Table 1: Isolation of E. coli in Relation to Age and Gender

NO. OF E. COLI IN URINE SAMPLES				
Age	**Males (n=19)**	**Females (n=23)**	**Total (n=42)**	**Percentage**
5-15	1	1	2	4.7
16-30	-	3	3	7.1
>30	2	2	4	9.5
Total Percent	3 7.1%	6 14.3%	9	21.4%
PUSS				
Age	**Males (n=12)**	**Females (n=13)**	**Total (n=25)**	**Percentage**
5-15	**-**	-	**-**	**0**
16-30	1	2	3	12%
>30	-	-	-	0
Total Percent	1 4%	2 8%	3	12%

Table 2: Biochemical Identification for E. coli

Bio-chemical test	**Reaction on slants**				**Interpretations**
Triple Sugar Iron	**Slope**	**Butt**	**H2S**	**Gas**	Appearance of yellow color indicates the consumption of three sugars and production of acid at slop and butt. No appearance of CO2 and O2 bubbles and black color at the butt hence no production of Gas and H2S.
	Yellow	Yellow	Negative	Negative	
Simon citrate test	No reaction				No appearance of blue color indicated that a bacterium doesn't consume citrate as source of carbon and the test is negative
Urease test	No reaction				No, appearance of pink color indicated that bacteria doesn't produce urease enzyme and the test is negative.

dence in female were found in age group of 16-30 with percent of 7.1%. The female cases examined by age demonstrate that majority of positive cases fall under middle-age. This is the agreement with the report of Ojo et al., Farhat Ullah et al., and Irum Shaifali et al. This is because women in middle-age group are grown-up and sexually active.

E. coli isolated either from urinary infection or from all body parts infections always show a similar pattern of susceptibility but the percentage is more enhanced with urinary isolates[19]. Susceptibility of E. coli found remarkable result with aminoglycosides, carbapenem and four generation cephalosporin (cefepime). Figure 4 show that out of the 12 isolates it is most susceptible towards Amikacin (83.3), meropenem (83.3) and imipenem (91.6) while high moderate sensitivity were obtained with cefepime that is 75 % Similar results found in study of Zahrani and Akhtar, 2005 and Farhat ullah et al. The commonly administered drug in Khyber Teaching Hospital is Amikacin and results of Ioana mos et al., 2010 are closely similar with respect to susceptibility rates of Amikacin against E. coli which were seen as 75-78.1%, and was concluded that aminoglycosides, carbapenem and four generation cephalosporin are highly sensitive to this isolate. Mohammad Younas et al., 2009 also suggested that Amikacin is the best empirical antibiotic for all age groups.

Aminoglycosides are available in lesser price and also have long record of safety and effectiveness. The Docked complex of Amikacin and PBP1b found interacting points of H46, O11 and N18 atoms of drug closely interact to the amino acid residue PHE215, LYS437 and

Table 3: Antibiotic Susceptibility Results of E. coli

ANTIBIOTICS	Brand name	Disk Conc.	Zone Diameter interpretive criteria R I S			Sensitive%	Moderate sensitive%	Resistant%
Aminoglycosides								
Amikacin	Amikin	30	≤14	15-16	≥17	10(83.3)	2(16.6)	0
Carbapenem								
Imipenem	Tienem	10	≤19	20-22	≥23	11(91.6)	1(8.3)	0
Meropenem	Meronem		≤19	20-22	≥23	10(83.3)	1(8.3)	1(8.3)
Monobactam								
Aztreonem	Azactam	30	≤17	18-20	≥21	5(41.6)	4(33.3)	3(25)
Cephalosporin								
Cefepime	Maxipime	30	≤18	19-24	≥25	2(16.6)	9(75)	1(8.3)
Ceftazidime	Fortam	30	≤17	18-20	≥21	2(16.6)	0	10(83.3)
Ceftriaxone	Rocephin	30	≤19	20-22	≥23	1(8.3)	2(16.6)	9(75)
Cefoperazone	Cefobid	75	≤15	16-20	≥21	8(66.6)	2(16.6)	2(16.6)
Fluoroquinolone								
Moxifloxacin	Avelox	5	≤14	15-17	≥18	2(16.6)	4(33.3)	6(50)
Ciprofloxacin	Ciprocin	5	≤20	21-30	≥31	3(25)	4(33.3)	5(41.6)
Norfloxacin	Norfloxacin		≤12	13-16	≥17	7(58.3)	3 (25)	2 (16.6)
Macrolide								
Erythromycin	Erythromycin	15	≤13	14-22	≥23	0	0	12(100)
B-lactamase inhibitors combination								
Tazobactam/ piperacillin	Tazocin	110	≤17	18-20	≥21	6(50)	4(33.3)	2(16.6)
Amoxicillin/ clavulanic acid	Augmentin	30	≤13	14-17	≥18	9(75)	0	3(25)

R = Resistance, I = Intermediate, S = Sensitive

PRO158 of target protein with making a distance of 2.321, 2.325, and 1.860 respectively and displayed the highest interaction and was found to be most efficient inhibitor. It was noted that these three amino acids are more sensitive to attach the drug where the PRO 158 bind very closely to active drug atom N18.

E. coli resistance towards Cephalosporin, Monobactam, Fluoroquinolones and Macrolid showed 100% resistance to Erythromycin (macrolid). Resistant cephalosporin includes Ceftriaxon 75% and Ceftazidime 83.3%. These resistances occur mainly due to the beta lactamase producing E. coli strains. Fluoroquinolones are about 50% resistant to this isolate except norfloxacin which is 58% susceptible. This is because quinolones are extensively used antibiotics therefore these have been associated with raising level of resistance. Farhat ullah et al., recorded resistance of about 62% to ceftriaxone and 65% resistance to Ceftazidime and fluoroquinolones were recorded about 61% resistant in his study. Iraj pakzad et al., 2011 recorded high antibiotic resistance to Ceftazidime and lower resistance to aztreonem towards E. coli. Antibiotic resistance is due to beta lactamases enzymes and has ability to hydrolyze extended spectrum cephalosporin including ceftriaxone and Ceftazidime and monobactam. This hydrolysis is due to the attack of serine, which is present at the active site of enzyme, to the amide bond of antibiotic ring that cause hydrolysis. The most common type of Extended Spectrum Beta Lactamase is CTX-M. E. coli strains that produce this enzyme will probably a major cause of UTI.

Different studies have been shown much resistance to Ceftazidime. Docked complex of Ceftazidime with CTX-M 14 showed interacting amino acid SER274, ASP240, SER237, THR244, ARG276 which are crucial to

Table 4: Docking Results of CTX-M 14 with CEFTAZIDIME and Penicillin Binding Protein1b with AMIKACIN

Drug	Protein	Protein residue	Residue atom	Hetatoms	Drug atom	Distance in °A
Amikacin	PBP1b	PRO158	O		H46	2.321
		LYS437	HZ3		O11	2.325
		PHE215	H		N18	1.860
Ceftazidime		SER274	OG		O6	3.047
		SER274	OG		O7	2.932
		SER274	OG		O8	1.591
		SER274	HG		O9	1.683
		SER274	OG		H39	0.962
		ARG276	HE		O9	2.415
		SER237	O	HOH2118		2.686
					O5	2.517
		SER274	OG	HOH2314		2.212
					O8	2.188
		THR244	OG1	HOH2314		3.097
					H39	1.834
					O8	2.188
		ASP240	ODI	HOH2353		2.750
					O3	3.027
					O4	2.014

Drug-Enzyme interaction by making minimum distances and causes its hydrolysis as serine attack the amide bond of drug. This showed that ligand bind efficiently to the protein.

CONCLUSION

From the results it can be concluded that E. coli concentration was higher in urinary infection. It is still a most common uropathogen causing UTIs both in male and female of all age groups. Docking results concluded that magnifying view at molecular level shows all interactions below 3Å that indicates strong interaction as Amikacin bind effectively with one of the class of Penicillin Binding Proteins i.e. PBP 1b and attacks amino acids of PRO158, LYS 437, PHE 215 the, thus E. coli is highly sensitive to aminoglycosides. Docked complex of Ceftazidime and CTX-M 14 make smallest distances i.e. below 4Å, represent the inactivation of this drug by enzyme due to the attack of serine amino acid (SER274) residue to the drug atom.

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