ISOLATION, IDENTIFICATION AND ANTIMICROBIAL ANALYSIS OF PSEUDOMONAS AERUGINOSA FROM DIFFERENT CLINICAL SAMPLES AND TO STUDY THE INTERACTIONS OF TARGET AND RESISTANT PROTEINS WITH CEFEPIME AND IMIPENEM THROUGH DOCKING

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ABSTRACT

Objective: The present study aims towards gender wise distribution of P. aeruginosa and detection of antimicrobial analysis against various isolates from different clinical samples and to better understand the interaction of antibiotics with the resistant proteins and sensitive proteins of this pathogen using Docking.

Study Design: Analytical/Experimental study.

Place and Duration of Study: The research study was carried out at Microbiology laboratory of Shaheed Benazir Bhutto Women University, Peshawar from July to October 2014.

Methodology: A total of 79 different clinical samples comprising of fourty-five early morning mid stream urine samples, twenty-eight pus samples, five blood and one stool specimens were processed. MacConkey agar and CLED (Cysteine Lactose Electrolyte Deficient) agar were used as growth media for the culturing of microorganisms. Microorganisms were then identified through Gram staining followed by microscopy. Biochemical tests such as Urease, TSI (triple sugar iron), simmon citrate and Oxidase were carried out. Antibiogram analysis was accomplished using Kirby- Bauer antibiotic disk diffusion method.

Results: The prevalence rate of P. aeruginosa in the present study was found to be 5.06% where female patients constituted a larger group with 75% as compared to male (25%). Antibiotic susceptibility test results showed that all of the P. aeruginosa isolates were fully resistant (100%) to augmentin, imipenem, and erythromycin. They exhibited moderate resistant to cefotaxime and were fully sensitive (100%) to piperacillin-tazobactam, cefepime, cefobid, amikacin, gentamycin, and norfloxacin, while 75% sensitivity was shown to ciprofloxacin and 50% sensitivity was shown against ceftriaxone, aztreonam and moxifloxacin. GLN547, GLU568, GLU550 and THR565 of penicillin binding protein of P. aeruginosa are involved in providing sensitivity to cefepime. Whereas TRP87, ASP119, GLU225 and ASN233 of Metallo β-Lactamase of P. aeruginosa are involved in developing resistance to imipenem.

Conclusion: It was concluded that P. aeruginosa was present in high percentage in female ant it showed resistance towards many first generation drugs. Developing resistance towards third generation drugs was also noticed. Docking showed strong interactions among selected drugs and respective proteins.

Keywords: Antibiotic susceptibility, Pseudomonas aeruginosa, Penicillin Binding Protein, Metallo β-Lactamase.

INTRODUCTION

Pseudomonas aeruginosa commonly abbreviated as P. aeruginosa is a Gram negative, non spore forming, straight or slightly curved rod-shaped bacterium that occurs as an isolated bacterium or in pairs and incidentally in short chains.[1] The asporogenous, motile, non-fermenting, obligate aerobic, saprophytic, and oxidase positive bacilli is a free living bacterium and an opportunistic pathogen that is widely distributed in nature, predominantly in humid places and inhabit environments including plants, soil, water, sewage and intestinal tract.[2-3] By nature, this organism is provided with weak pathogenic capacity. However, its capability to endure on inert materials, minimum nutritional demand, tolerance to a wide range of physical conditions and resistance to various antimicrobial agents and antiseptics, contributes a lot to its ecological achievement and its role as an effective and an efficient opportunistic pathogen.[4] P. aeruginosa and P. pseudomallei are very critical clinical strains, while Pseudomonas maltophillia is occasionally medicinal and clinically important strain

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and other Pseudomonas species may rarely cause disease.[5]

P. aeruginosa is a common nosocomial pathogen[6] that mostly targets debilitated or immunosuppressed hosts admitted in intensive care units, HIV-infected patients, and causes infections with a high mortality rate[7] in hospitalized patient specifically in respiratory diseases, burn patients, orthopedic related infections, and catheterized patients.[8] Hence, pseudomonal infections can develop in skin, subcutaneous tissue, eyes, ears, bones, and urinary tract. The infection site may varies with the route of entry and the host's susceptibility.[9] In addition, it is one of the noteworthy opportunistic bacteria that is isolated from wounds.[10]

According to the National Nosocomial Infections Surveillance System, P. aeruginosa is ranked as the third most common pathogen that accounts for 10.1% of all nosocomial infections.[11] It has been recognized as the second most common cause of ventilator associated pneumonia, the fourth most frequent organism causing urinary tract infections that are catheter-associated, the fifth known cause of surgical infections and the seventh notable cause of bloodstream infections.[12] The most common tools used for detection of P. aeruginosa infections are standard microbiological techniques that comprises of phenotypic and biochemical profiles. Although such commercial tests tend to prove unsatisfactory, unreliable and lengthy.[13]

The most frequent pathogens found in the pus samples were Pseudomonas and E. coli spp.[14] Basu et al.[15] also reported Pseudomonas and E. coli spp. to be the most commonly occurring Gram Negative Rods (GNR) in wound infection. In addition, P. aeruginosa along with Escherichia coli, Proteus mirabilis, Klebseilla pneumoniae and Streptococcus faecalis is the most common organism responsible for catheter-associated UTIs.[16]

Antibiotic resistance is one of the common problems, affecting human well being. Common factors that accounts for resistance are overuse and misuse of antibiotics, patient associated factors, improper prescriptions by the physicians, common use of broad spectrum antibiotics and self medications.[17] P. aeruginosa are most commonly responsible for life-threatening infections that are difficult to cure due to high resistance to many antimicrobial agents.[18] Basic mechanisms that accounts for resistance in P. aeruginosa relies on i.e. cell wall's low permeability, chromosomal changes, transmission of resistance genes via plasmids, transposones, and bacteriophages and large size of genomic material with an ability to express a wide-ranging resistance mechanisms.[19]

Mostly P. aeruginosa are found resistant to penicillins, ampicillin, amoxicillin-clavulanate, narrow and extended spectrum cephalosporins, tetracyclines, rifampin, macrolides and chloramphenicol.[20] The most frequently encountered clinical resistance was through β-lactamase enzymes. Some researchers dectected metallo β-lactamase production rate from 5% to 62% in P. aeruginosa strains.[21] Resistance to imipenem is generally associated with changes in the structure or loss of OprD outer membrane protein and rarely due to production of metallo-beta-lactamases. AmpC enzymes of P. aeruginosa may also play role in providing decreased susceptibility to imipenem. Gutie´rrez et al, [22] reported an association between carbapenem resistance and AmpC overproduction in P. aeruginosa.

Cefepime is most frequently reserved for the treatment of severe nosocomial pneumonia and infections caused by P. aeruginosa. Cefepime is demonstrated as the most active third generation cephalosporin against P. aeruginosa that is consistent with reports from several groups cited by Gad.[23]

Molecular docking is a basic tool in structural molecular biology and computer-assisted drug design. The goal of ligand-protein docking is to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure.[24]

The present study aimed towards the isolation of P. aeruginosa from various clinical samples and to study the antimicrobial activities of different antibiotics against these isolates. The interaction of antibiotics with the resistant and sensitive proteins of this pathogen were checked through bioinformatics tools such as Docking that helped a lot in understanding the binding sites of these drugs with the proteins of the pathogen.

MATERIALS AND METHODS

The research study was carried out at Microbiology laboratory of Shaheed Benazir Bhutto Women University, Peshawar (SBBWU) from July to October 2014. Samples were collected randomly from patients visited routinely different Government hospitals including Khyber Teaching Hospital (KTH), Hayatabad Medical Complex (HMC) and Lady Reading Hospital (LRH) of Peshawar.

Fourty-five early morning mid stream urine samples, twenty-eight pus samples, five blood and one stool specimens were collected. Relevant information including informed consent, name, age, gender, address, antibiotic usage and other relevant laboratory findings were jot down on the request forms and were taken into consideration.

Samples of urine, blood, pus and sputum were collected. Before collection of urine sample, individuals were instructed on how to collect the samples observing all aseptic conditions such as washing the penis and distal urethra with sterile water or with mild alcohol and avoiding the penis and distal urethra from making contact with the container. Urine samples were collected aseptically in sterile plastic bags with screw caps sterile. For collection of sputum specimen, the

patients were provided with a clean, dry, wide necked, leak proof container. Patient coughed deeply to produce sputum. Samples of blood were collected aseptically, transferred to sterile glass bottles and were immediately transported to the laboratory. Pus samples were collected aseptically with sterile cotton swabs. Urine, pus, and sputum were processed directly after transportation while blood sample were initially incubated at 37◦C for 10 days. The samples were brought to the Microbiology laboratory of SBBWU. The samples of clinical isolates were processed for morphological, colonial, biochemical identification. Antimicrobial susceptibility test was also done for identification.

Routine culture media such as MacConkey agar[25] and CLED agar were used as growth media for the culturing of microorganisms that were prepared on the bases of manufacturer's instructions and sterilized by autoclaving for 15 minutes at 121 ^{0}C. Media's were allowed to cool down to 45 ^{0}C, then poured in the Petri plates. MacConkey agar is used as a selective media which promote the growth of gram negative bacteria while inhibit the growth of gram positive bacteria. MacConkey agar favor the growth of both lactose and non-lactose fermenting bacteria. CLED agar is used as a differential media used for the isolation, differentiation and identification of urinary tract organisms. Each sample was then inoculated on two plates containing CLED and MacConkey agar. After streaking, all the plates were incubated at 37 °C for 24 hours. For further identification of microorganism, a battery of tests were performed that included gram's staining (microscopic analysis), colony morphology (macroscopic analysis), and biochemical tests such as oxidase[26], TSI agar [27], Simmon citrate and Urease tests for the confirmation of the isolates as P. aeruginosa.

The susceptibility tests of clinical isolates of P. aeruginosa were determined by Kirby-Bauer antibiotic disk diffusion method by using Muller Hinton Agar. Criteria developed by National Committee for Clinical Laboratory Standards (NCCLS) were used for interpretation of the susceptibility results of bacteria to antimicrobial agents, whether being resistant or sensitive. The commonly used standard commercial disks with their concentrations were as follows: Augmentine (30μgm), Piperacillin-tazobactam (110μg), Cefotaxime (30μg), Ceftriaxone (30μg), Cefobid (75μg), Cefepime (30μg), Aztreonam (30μg), Imipenem (10μg), Amikacin (30μg), Gentamycin (10μg), Erythromycin (15µg), Moxifloxacin (5μg), Norfloxacin (10μg) and Ciprofloxacin (5μg).

BIOINFORMATICS TOOLS

Retrieval of Target Protein and Drug Structure from PDB and Drug bank Criteria used for selection of drug for molecular docking is based on completely resistance towards antibiotic shown by pathogen and the antibiotic towards which the pathogens showed complete sensitivity. Accession number of penicillin binding protein and metallo β-lactamase was searched in UniProt KB (www.uniprot.org) that was entered in RCSB (www.rcsb.org) searching bar. Structure of such sensitive and target proteins were retrieved from RCSB. Structure of the cefepime and imipenem was downloaded from drug bank database (www.drugbank.ca). HexServer software was used for docking. The docking result was interpreted using Discovery studio viewer (DS viewer) software.

RESULTS

Out of the 79 samples processed during study period, only 4(5.06%) isolates were of P. aeruginosa, while other 75(94.94%) isolates represented other bacterial genera. Most of the isolates were obtained from pus samples (10.71%) and urine (2.22%). Results are shown in (Table I).

Table I: Percentage Distribution of Pseudomonas aeruginosa among Clinical isolates

Samples	No. of samples	Species identified	% identification
Urine	45	1	2.22 %
Pus	28	3	10.71%
Blood	5	0	0%
Sputum	1	0	0%

Table II: Gender wise Distribution of Clinical Isolates of Pseudomonas aeruginosa

Sample	No of samples	Female	Male	Female %	Male %
Pus	28	2	1	7.14%	3.6%
Urine	45	1	0	2.22%	0.0%

Table III: Tabular Result of Cultural Characteristics

Species Name	Differential colonial Characteristics on	
	MacConkey agar	EMB Agar
Pseudomonas aeruginosa	Colourless Colonies	Colourless Colonies

Table IV: Result of Biochemical Test

Specie Name		Pseudomonas aureginosa	Interpretation
Chemical Nature		Non-Lactose Fermenter	Do not ferment the lactose.
Oxidase Test		Positive	Purple colour appears indicating oxidation of phenylenediamine present in the reagent.
Simmon Citrate Agar		Positive	Appearance of blue colour indicates consumption of citrate by bacteria as a source of carbon due to presence of citrate.
Urease Agar Slant		Negative	No appearance of pink colour indicates that the bacteria the organism lack urease enzyme that split urea in the presence of water to release ammonia and carbon dioxide.
TSI Agar	Slope	Alkaline	No appearance of yellow colour on slope indicating that no sugar was consumed by bacteria due to which no formation of acid occurred and medium remains alkaline.
	Butt	Alkaline	No appearance of yellow colour on slope indicating that no sugar was consumed by bacteria, so, no acid formation.
	H2S	No H2S Production	No appearance of black colour indicating no production of H2S.
	Gas	No gas Production	No gas production because no appearance of CO2 & O2 bubbles.

Table V: Antibiotic Susceptibility of Clinical P. aeruginosa Isolates

Clinical Specimen					Pus and Urine Samples											
Patient ID Number					Sample1(S1),S2,S3,S4											
					Susceptibility Measurement											
					Resistant				Intermediate				Sensitive			
S.No	Antibiotic Agent	Code No.	Disc Potency (µg /disc)	Standard Sensitivity	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4
1.	Moxifloxacin	MXF	(5µg)	17									24	20	20	20
2.	Amikacin	AK	(30µg)	17									30	31	34	28
3.	Augmentin	AMC	(30µg)	18	10		12	10		16						
4.	Aztreonam	ATM	(30µg)	22	14							20		28	25	
5.	Cefoperazone	CFP	(75µg)	21									18	25	27	22
6.	Cefotaxime	CTX	(30µg)	23					20	22		16			25	
7.	Ciprofloxacin	CIP	(5µg)	21						20			30		32	28
8.	Erythromycin	E	(15µg)		10	18	13	10								
9.	Gentamycin	CN	(10µg)	15									24	22	25	21
10.	Cefepime	FEP	(30µg)	18									25	30	34	32
11.	Norfloxacin	NOR	(10µg)	17									30	34	28	30
12.	Ceftriaxone	CRO30	(30µg)	21					17		18			22		25
13.	Tazocin	TZP110	(110µg)	21									25	30	31	27
14.	Imipenem	IPM10	(10µg)	16	12	10	13	10								

In this study, gender wise distribution of P. aeruginosa included 3(75%) samples from females while 1(25%) was from male. On the whole, female population was found more infected than males (Table II and Figure I).

When grown on EMB Agar, P. aureginosa produced colourless colonies with no lactose fermenta-

Table VI: Interaction of Imipenem with Metallo β-Lactamase

Pathogen	Drug	Protein	Protein Residues	Residue Atom	Drug Atom	Hetatoms	Distance in °A
Pseudomonas aeruginosa	imipenem		TRP87	NE1	O2		2.540
			TRP87	HE1	O2		2.077
			ASP119	H	H31		1.449
			ASP119	H	C16		2.273
			GLU225	N	H25		5.073
		Metallo β-Lactamase	GLU225	OE2	H26		3.453
			ASN233	HD21	O4		1.456

Table VI: Interaction of Cefepime with Penicillin Binding Protein (PBP)

Pathogen	Drug	Protein	Protein Residues	Residue Atom	Drug Atom	Hetatoms	Distance in °A
Pseudomonas aeruginosa	Cefepime	PBP	GLN547	HE22	S		2.416
			GLU568	CG	H		2.091
			GLU568	OE2	H		0.817
			GLU568	OE2	C		1.481
			GLU550	OE2	H		0.841
			GLU550	OE2	N		1.336
			THR565	OG1	O		2.730
			THR565	HG1	O		2.441
			THR565	CB	H		5.097
			THR565	CB	C		6.062
			GLU568	OE1	C		1.194
			GLU550	OE2	C		0.976
			GLU550	OE2	C		1.714
			GLU550	CD	N		1.533
			GLU550	OE1	C		1.648
			GLU550	OE1	N		1.618

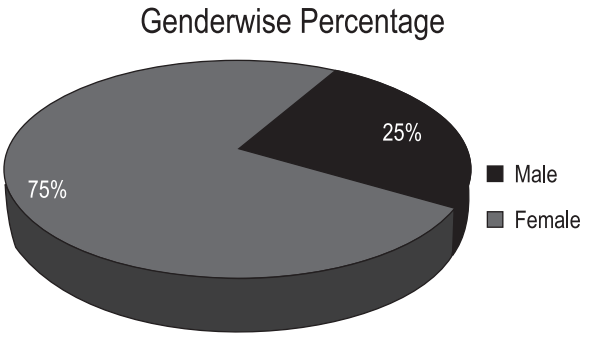

Figure I: The sex wise distribution of the isolated P. aeruginosa specimens among the study population(n=4).

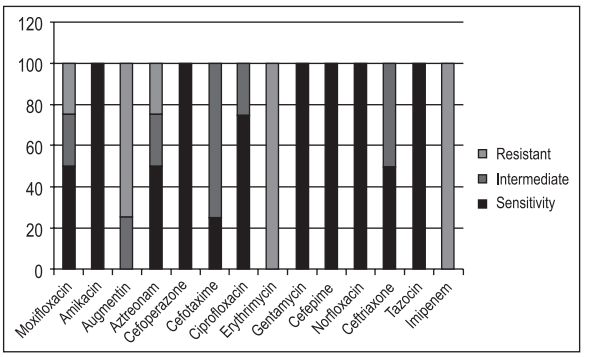

Figure II: Graphical Presentation of Antibiotic Susceptibility of Clinical P. aeruginosa Isolates

tion. When grown on Mac Conkey agar P. aureginosa produced small, round, flat, irregular slightly raised colonies with no lactose fermentation (Table III). When P. aureginosa was subjected to Gram reaction, under microscope, it showed rod shape, gram negative bacilli. The selected isolates were further characterized by different biochemical tests. The results of the different biochemical tests performed for the four isolates are given in Table IV.

After characterization the samples for P. aeruginosa were subjected to antibiotic susceptibility test for antibiogram analysis. Table V and Figure II shows the susceptibility measurement while the graph shows percentage sensitivity and resistivity.

Docking was carried out to determine and understand the interaction between selected drugs and proteins.

Table VI and VII given below show interaction of imipenem with Metallo β-Lactamase (Resistance Providing Protein) and cefepime with penicillin binding protein (PBP) (Target Protein for the drug).

DISCUSSION

The present research work was conducted to isolate and identify P. aeruginosa from various clinical samples such as urine, pus, blood and stool. In addition, the prevalence of P. aeruginosa isolates in clinical specimens was also examined over the study period. The prevalence rate of P. aeruginosa in the present study was found to be 5.06%, this level is relatively high when compared with Nkang [28] studies that have prevalence level of 2.1%.

In present study, the sex-wise distribution rate of P. aeruginosa was compared with other studies. In present study, female patients constituted a larger group with 75% as compared to male (25%). Chander[29] also found high frequency of P. aeruginosa in females (55.17%) than males (44.83%). This result support existing study with slight difference.

Susceptibility measurement measures the capability of different antibiotics to retard or inhibit microorganism growth. Increasing resistance to various anti-pseudomonal drugs particularly among clinical strains has been reported worldwide.[29] The antimicrobial susceptibility profiles of P. aeruginosa to fourteen antimicrobial agents were tested. In this study, all the P. aeruginosa isolates were found to be 100% sensitive to piperacillin-tazobactam, cefoperazone and cefepime. Results in Gad's paper demonstrated cefepime as the most active cephalosporin against P. aeruginosa that is consistent with reports from several groups cited by Gad.[23] 50% sensitivity with 25% resistance was observed against aztreonam and 50% strains of P. aeruginosa were found sensitive with 50% intermediate sensitivity to Ceftriaxone. Toroglu [21] reported that third generation Ceftriaxone showed 66% susceptibility to clinical isolates. Variation in the resistance may be caused by factors such as exposure to antibiotics, population under observation, and form of clinical sample examined. 75% intermediate susceptibility was observed against cefotaxime that showed similarity with Haleem[30] work.

100% resistance was analyzed in hospital strains of P. aeruginosa against three most commonly prescribed antibiotics i.e. augmentin, imipenem and erythrocin. Bacterial resistance to beta-lactam antibiotics is mainly due to the production of beta-lactamases. Resistivity pattern showed 100% resistance of amoxicillin/clavulanate in the study conducted by Amadi [31]

The resistance pattern of P. aeruginosa to erythromycin was in consistent with report of Javiya [32] that showed 98.1% and 100% resistance to erythromycin. In the study conducted by Rodriguez-Martinez [33] 87.5% of the selected isolates were resistant to imipenem that agreed with our result with slight difference. Resistance to imipenem is generally associated with changes in the structure or loss of OprD outer membrane protein and rarely due to production of metallo-beta-lactamases. AmpC enzymes of P. aeruginosa may also play role in providing decreased susceptibility to imipenem. Gutie´rrez [22] reported an association between carbapenem resistance and AmpC overproduction in P. aeruginosa.

Aminoglycosides especially gentamicin are well-known frontline antibiotics in the treatment of infections caused by gram negative bacterias. Antimicrobial susceptibility of P. aeruginosa to gentamicin reported in this study was 100% that is comparable to what was reported by Nkang[28] who reported the similar sensitivities of gram negative isolates to gentamicin. In the present study, 100% susceptibility was observed toward amikacin that was almost similar with values reported in other studies, with sensitivity of 81.4% in Chander[29] report.

The fluoroquinolones are a family of broad spectrum antibacterial agents that are very effective against gram negative organisms, including P. aeruginosa. In recent study, 75% of isolates were found sensitive to ciprofloxacin that agreed the result of conducted Chander[29] 73.3 %. P. aeruginosa strains in this study exhibited a high rate of sensitivity to the norfloxacin (100%), moxifloxacin 50% and slightly lesser rate of sensitivity to norfloxacin than the existing strain reported by Gad[23] 62% and 62.5% in study reported by Haleem.[30]

Through docking, it was concluded GLN547, GLU568, GLU550 and THR565 amino acids of penicillin binding protein of P. aeruginosa were involved in providing sensitivity to cefepime. Whereas, TRP87, ASP119, GLU225 and ASN233 of Metallo β-Lactamase of P. aeruginosa were involved in developing resistance to imipenem as these are responsible in developing interactions with this drug, thus depriving it to bind with the target protein. Mostly strong interactions were observed that lies within range of 4°A.

CONCLUSION

It was concluded that P. aeruginosa was present in high percentage in female as compared to males. In addition, P. aeruginosa showed resistance towards many first generation drugs such as augmentin, erythromycin. Developing resistance towards third generation drugs such as imipenem was also noticed. Docking showed strong interactions among selected drugs (cefepime, imipenem) and respective proteins (penicillin binding protein, metallo β-lactamase) and if resistance develops against the cefepime, it may depict that the targeted amino acids have undergone evolution.

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