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Susceptibility Pattern of Pseudomonas Aeruginosa Against Various Antibiotics Along with Computational Analysis

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Abstract

P. aeruginosa, an increasingly prevalent opportunistic human pathogen, is the most common gram-negative bacterium responsible for the nosocomial and community acquired infections. The excessive use of antibiotics has not only led to treat the infections but also the emergence of antibiotic resistance. Multidrug resistant P. aeruginosa development is currently one of the greatest challenges. Total 100 clinical isolates of P. aeruginosa from inpatient and outpatient were studied. Uropathogenic P. aeruginosa infections were higher in females than males, ratio was found more among young and elderly debilitated patients. 99% of the clinical isolates were resistant to six commonly used antibiotics with the most resistant pattern being Ampicillin (100%), Amoxycillin (99%), Co-trimoxazole (99%), Tetracycline (99%), Cefazoline (99%) and Cefuroxime (100%). The invitro sensitivity pattern of 100 isolates of P. aeruginosa showed Imipenem (97%), Amikacin (79%), Tobramycin (70%), Ceftazidime (62%), Ciprofloxacin (73%), Cefoperazone (60%), Piperacillin (65%), Gentamycin (34%) and Cefotaxime (14%) sensitivity.

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ESBLs producing strains (33%) were also less in number but were much more resistant to β-lactam and other antibiotics. Docking of both effective drugs against bacteria (Amikacin and Imipenem) with least resistance 21% and 3%, respectively, analyzed how these drugs interact with envelope protein to stop its growth.

Keywords: P. Aeruginosa; Antibiotics; Antibiotic Resistance; Nosocomial Infections; Docking

1. Introduction

P. aeruginosa is a versatile bacterium of the class Schizomycetes, order Eubacteriales, family Pseudomonadaceae, and genus Pseudomonas [1]. P. aeruginosa is nearly ubiquitous in nature and is quite innocuous in most environments. It can be frequently isolated from soil, water [2] and occasionally from normal human skin [3]. It can inhabit the nasopharynx and lower digestive tract up to 6%. Normally human faecal carriage of P. aeruginosa is low, around 3% [4]. However, carriage increases with the length of stay in hospital, reaching 30%-50% after 3 weeks and thus can present a distinct risk of endogenous infection [5]. P. aeruginosa, an increasingly prevalent opportunistic human pathogen, is the most common gram-negative bacterium found in nosocomial and community acquired infections. It can infect almost any external site or organ, and therefore, can be isolated from various body fluids such as sputum, urine, wounds, eye or ear swabs and from blood [6].

P. aeruginosa is the only gram-negative bacillus capable of producing two types of water soluble pigments, pyocyanin, a bluish green distinctive non-fluorescent pigment and pyoverdin, a fluorescent greenish-yellow pigment [7]. Pyoverdin is produced abundantly in media of low-iron content, and could function in iron metabolism in the bacterium, whereas, Pyocyanin (from "pyocyaneus") refers to "blue pus" which is a characteristic feature of suppurative infections caused by P. aeruginosa [8]. Aside from pyoverdin and pyocyanin, other pigments may also be produced by some strains of P. aeruginosa, including pyorubrin (red), and pyomelanin (brown) pigment that's why also called as Pseudomonas polycolor [9].

P. aeruginosa infections are multifactorial and complex to initiate [10]. It usually requires a substantial break in first-line defenses. Such a break can result from breach or bypass of normal cutaneous or mucosal barriers (e.g., trauma, surgery, serious burns, or indwelling devices). P. aeruginosa infections begin with bacterial attachment and superficial colonization of cutaneous or mucosal surfaces. Most P. aeruginosa infections are both invasive and toxinogenic. Under favourable nutrient conditions, the mucoid strains of P. aeruginosa produce an extracellular hyperviscous exopolymer glycocalyx (Co-polymer of O-acetylated β-1, 4 linked D-Mannuronic acid and L-Guluronic acid) in copious amounts that effectively protects bacterial cells from opsonization by antibodies, complement deposition, and phagocyte engulfment by providing a barrier against antimicrobial agents and the immune system, thereby increasing its virulence and antibiotic resistance [11, 12]. P. aeruginosa also releases two extracellular proteases; elastase and alkaline protease [13].

P. aeruginosa produces three other soluble proteins: a cytotoxin and two hemolysins. The cytotoxin is a pore-forming protein. It was originally named leukocidin because of its effect on neutrophils, but it appears to be cytotoxic for most eukaryotic cells. Of the two hemolysins, one is a phospholipase and the other is a lecithinase. They appear to act synergistically to break down lipids and lecithin [14].

P. aeruginosa produces two extracellular protein toxins, Exoenzyme S and Exotoxin A. Exoenzyme S is an exotoxin produced by bacteria growing in burned tissue having ADP-ribosylating activity for a variety of eukaryotic proteins and may act to impair the function of phagocytic cells in the bloodstream and internal organs to prepare for invasion by *P. aerugionsa* [15, 16]. Exotoxin A has exactly the same mechanism of action as the diphtheria toxin; it causes the ADP ribosylation of eukaryotic elongation factor 2 leading to inhibition of protein biosynthesis and cell death. Exotoxin A is responsible for both local and systemic diseases [17, 18].

Antibiotics are chemotherapeutic agents that are secondary metabolites of microorganisms. The word antibiotic is derived from Greek word that means "against life". Antibiotics that effectively inhibit bacterial cell wall synthesis include the ß-lactams, penicillins, cephalosporins, monobactams and carbapenems. Many drugs interfere with protein biosynthesis. These include 50S ribosome inhibitors such as clindamycin, chloramphenicol, and the macrolides. The 30S ribosome inhibitors include the tetracyclines and aminoglycosides. Within the last two decades, DNA directed RNA polymerase inhibitors such as rifampin for Mycobacterium tuberculosis and rifabutin for Mycobacterium avium have also become available. There are a large number of DNA gyrase inhibitors such as ciprofloxacin and ofloxacin. The brilliant work of George Hitching and Gertrude Elliot showed that a combination of trimethoprim with a sulfonamide (TMP/SMX) would synergistically inhibit bacterial folic acid metabolism [19].

Unfortunately, as fast as new antibiotics have appeared and they greatly reduced the effects of infectious diseases, microorganisms have very cleverly figured out ways to escape their effects. *P. aeruginosa* may develop several mechanisms of resistance against a variety of antibiotics. The major mechanism of resistance to β-lactam antibiotics is beta-lactamase production. More than 340 β-lactamase enzymes have been detected to date.

These drug inhibitory agents include the emerging class A SHV and TEM-derived extended-spectrum β lactamases (ESBLs), inhibitor resistant enzymes, non-TEM, non-SHV class A ESBLs, and carbenpenemases, class B metallo-β-lactamases and some of their novel inhibitor, plasmid and chromosomally encoded class C enzyme, and finally, the OXA-type oxacillinases ESBLs, and carbapenemases of class D [20] ESBL producing organism pose unique challenges to clinical microbiologists, clinicians, infection control professionals and scientist engaged in finding new antimicrobial agents. ESBLs are enzymes capable of hydrolyzing third and fourth generation cephalosporins such as ceftazidime, ceftoxime and cefepime as well as aztreonam. Currently, carbapenems are regarded as the drug of choice for treatment of infections caused by ESBL-producing organisms [21].

1.1 Study Objectives

To determine magnitude, frequency (prevalence) and current trends of antibiotic resistance development among clinically significant *P. aeruginosa* strains causing various nosocomial and community acquired infections against various commonly used antibiotics.

2. Material and Methods

2.1 Sample Collection and Identification

A total of 100 *P. aeruginosa* isolates were isolated from *Pathology Laboratory, District Headquarters Hospital, Rawalpindi, Pakistan* and identified on the basis of colony morphology according to *Berges Manual of Determinative Bacteriology, 8th edition.*

Samples of pus, urine, blood, different body fluids, and throat, sputum and ear swabs, both from outdoor patients as well as indoor patients from different wards of the hospital were aseptically collected.

2.2 Processing Of Samples

Samples were processed as follow:

2.2.1 Pus Samples

The appearance of Pus samples was noted and were directly inoculated on Blood agar (CM55 and SR50-OXOID) and MacConkey agar (CM7-OXIOD) and incubated for 24-48 hrs at 37^0 C aerobically.

2.2.2 Urine Samples

The appearance (colour, whether clear or cloudy) of freshly collected urine was noted. About 10ml of freshly received well mixed urine samples were centrifuge at 500-1000g for 5 min.By using a sterile calibrated wire loop inoculate a urine on a CLED media (Cystine- Lactose –Electrolyte Deficient (CM 301-OXOID). The plates were incubated for 24 to 48 hours at 37^0 C aerobically. The bacterial growth was Sub-cultured on Blood agar (CM55 and SR50-OXOID) and MacConkey agar (CM7-OXIOD) and incubated for 24-48 hrs at 37^0 C aerobically.

2.2.3 Sputum Sample

Appearance of specimen (purulent, mucopurulent, mucoid, and salivary) was noted. Wash the sputum specimen in about 5ml of sterile physiological saline. Then it was streaked on Blood agar (CM55 and SR50-OXOID) and MacConkey agar (CM7-OXIOD) and incubated for 24-48 hours at 37^0 C aerobically.

2.2.4 Blood Sample

Freshly drawn 3-5mL blood was immediately transferred to 50mL of brain heart infusion (BHI) broth (CM225-OXOID), and incubated at 37^o C for 24 hours. In case of no growth, incubation period was extended for another 24 hours and inspected daily for visible sign of growth. Growth was sub cultured on Blood agar (CM55 and SR50-OXOID) and McConkey's agar (CM7-OXIOD) plates, and incubated at 37^o C for 24 hours. For both with no growth even after 48 hours the plates were further incubated up to 10 days, the samples were considered as negative only if there was no turbidity or growth on tenth day.

2.2.5 Ear swabs

Ear swabs samples were directly swabbed on Blood agar (CM55 and SR50-OXOID) and MacConkey agar (CM7-OXIOD) and incubated for 24-28 hours at 37^0 C aerobically.

2.2.6 Maintenance of Bacterial Isolates

For short term storage, the bacterial isolates were cultured on Tryptic Soy agar slants and petriplates and maintained at 4^0 C and sub cultured monthly for routine use. Whereas, for long term storage mid-exponential phase isolates were taken in tryptic Soy a broth with 20% glycerol in screw capped tubes and kept at -70^0 C.

2.3 Identification and Characterization of Bacterial Strains

2.3.1 Morphological Identification

Pure cultures were obtained and colony size, shape, color, diameter and growth characteristics (abundant, thin, pigment production) were noted. Isolated colonies were initially gram stained by preparing smear from well isolated colonies. Stained smear was examined under microscope to view microscopic characteristics. By using Bergey's Manual of determinative Bacteriology (8^{th} Edition) the isolates were further biochemically characterized and identified.

2.3.2 Biochemical Identification

The primary identification of unknown bacterial isolates were made based on colonial appearance, pigmentation, oxidation-fermentation test, oxidase test, indole test, citrate utilization test, catalase test, hemolysis, ability to grow on cetrimide agar, motility test, ability to grow at 5^0C to 42^0C and other biochemical tests.

2.3.3 Differentiation of mucoid and non-mucoid strains

Using sterile technique, a heavy smear of bacterial isolate on glass slide was prepared , air died , flooded with 1% crystal violet solution , waited for 5 to 7 minutes , the smear was washed with 20% copper sulphate solution , gently blot dried and was examined under microscope to note the presence or absence of capsule(alginate). Precipitation of alginate was used for confirmation of capsule staining. For each isolate, a 48-hour culture was prepared in 100 mL nutrient broth (shaking at 34 – 35°C) and alginate was precipitated by the addition of 50 mL cold ethanol. This mixture was kept at –20°C for 30 min until formation of the alginate precipitate was complete.

2.3.4 Cultured Medias, Antibiotic Discs, Control Strain

Table 1 showed list of chemicals used in this study and their composition. All the antimicrobial Discs used in this study, their codes and strength are given in the Table 2. *Pseudomonas aeruginosa* (ATCC27853) control strain was used during study.

Table 1: Used Culture Medias and Their Composition

Media	Ingredients	Amount(gms/L)
Nutrient Broth	Peptone	10.0
CM-1	Sodium Chloride	5.0
	Meat extract	10.0
Nutrient Agar	Peptone	10.0
CM-3	Sodium Chloride	5.0
	Meat extract	10.0
	Agar	15.0
Muller Hinton Agar	Meat infusion	6.0
CM-337	Casein Hydrolysate	17.5
	Starch	1.5
	Agar	10.0
Blood Agar	Infusion from beef heart	500.0
CM-55	Tryptose	10.0
	Sodium chloride	5.0
	Agar	15.0
	Defibrinated Blood	50.0
Tryptic Soy Agar	Phytone	500.0
CM-131	Tryptose	10.0
	Sodium chloride	5.0
	Agar	15.0

MacConkey Agar	Peptone	20.0
CM-7	Lactose	10.0
	Bile Salt No 3	5.0
	Neutral red	0.075
	Sodium chloride	5.0
	Agar	12.0
Brain Heart Infusion	Infusion from calf brain	200.0
CM-225	Infusion from beaf heart	250.0
	Peptone	10.0
	Dextrose	2.0
	Sodium chloride	5.0
	Disodium Phosphate	2.5
Pseudomonas Cetrimide	Peptone	20.0
Agar	Magnesium Chloride	1.4
CM-559	Potassium Sulphate	10.0
	Cetrimide	0.3
	Agar	13.6

2.3.5 Antimicrobial Susceptibility Testing

The disk diffusion method of Bauer *et al*. (1996) was used for antibiotic susceptibility testing for each bacterial isolate on Muller Hinton agar (CM337-OXOID). Medium was prepared and sterilized by autoclaving at 121◦ for 15 minutes. 25ml of media was poured in 90 mm sterile Petri dishes and incubated at 37ºC overnight to check sterility.

2.3.6 Preparation of 0.5 McFarland Turbidity Standard

McFarland standard was prepared by adding 99.5ml of 1% sulphuric acid and 0.5ml of 1.175% barium chloride.

This McFarland standard provides turbidity comparable to a bacterial suspension containing 1.5 x 10^8 CFU/ml (NCCLS (1993)).

2.3.7 Inoculum Preparation

For inoculum preparation 5ml of Tryptic Soya broth (CM129-OXOID) medium was dispensed in screw-capped test tubes and autoclaved at 121◦ for 15 minutes. The test tubes were cooled and kept in an incubator for 24 hours at 35ºC to check sterility.

Next day one of each identified clinical isolate was inoculated in sterilized test tubes containing media, and place in incubator overnight at 35ºC after 24 hours the turbidity of broth cultures were adjusted according to 0.5

2.3.8 Disc Diffusion (Bauer-Kirby, 1966) Susceptibility test

A sterile cotton swab was saturated by dipping in to standardized bacterial suspension. Inoculum was spread evenly over the entire surface of Mueller –Hinton agar by swabbing back and forth across the agar in three directions to give a uniform inoculum to entire surface. The plates were dry and with in 15 minutes discs of given potencies (table 2) were applied on inoculated plates. Then plates were placed in incubator at 35ºC for 18 hour in an inverted position.

Table 2: Antimicrobial Discs Along With Codes and Potencies Used In Study

Antimicrobial Agent	Antibiotic Group	Code	Disc Potency (µg)
Amoxycillin	Penicillin	AML	25
Cefazoline	Cephalosporin	KZ	30
Co-trimoxazole (Septran)	Sulphonamide	SXT	25
Cefuroxime	Cephalosporin	CXM	30
Ampicillin	Penicillin	AMP	10
Tobramycin	Aminoglycoside	TOB	10
Amikacin	Aminoglycoside	AK	30
Ceftazidime	Cephalosporin	CAZ	30
Cefotaxime	Cephalosporin	CTX	30
Piperacillin	β-lactamase Inhibitor	PRL	100
Gentamycin	Aminoglycoside	CN	10
Tetracycline	Tetracycline	TE	30
Ciprofloxacin	Quinolones	CIP	5
Cefoperazone	Cephalosporin	CFP	75
Imipenem	Carbapenem	IMP	10

2.3.9 Interpretation of Zone Size

After 18 hours of incubation, plates were examined and zones of inhibition were measured. Using the interpretive, interpreted the zone sizes of antimicrobial, and reported the organism sensitive, intermediate, resistant according to National Committee for Control Laboratory standards (NCCLS (1993).

2.4 Computational Analysis

PatchDock server [22, 23] was used for docking analysis. Total 100 runs were carried out to generate best docking complex. The first 10 docked complexes were retrieved and then subjected to FireDock server [24, 25] for further analysis (i.e. refinement and ranking of interactions). Docking interactions were analyzed using LIGPLOT program [26].

2.5 Detection of Extended Spectrum Beta Lactamases (Esbls)

Double disc diffusion method was used to detect the extended spectrum beta lactamases (ESBL). A single, separated colony of the test organism was picked and emulsified in 0.9% normal saline in a test tube, the turbidity of the test organism was matched with 0.5% McFarland's Standard. The suspension of test organism was spread on the Mueller-Hinton agar surface in a petri plate with the help of cotton swab soaked in suspension tube. A disk of co-amoxicilline (20 µg amoxicilline/10µg clavulanic acid) was placed in the center of the agar surface. The discs of cephotaxime ceftriaxone, ceftazidime and aztreonam (30 µg) were arranged in such a way that the distance between the central disc and surrounding discs was approximately 30 mm. The isolates were incubated at 37˚C for 24 hours. After an overnight incubation, the zones around 3rd generation cephalosporins discs and aztreonam were observed. If the inhibition zone around one or more cephalosporins discs was extended on the side nearest to the co-amoxiclave disc, the organism showing this synergism is an ESBL-producer. When there was no extension of zones, the test was repeated by reducing the distance between the cephalosporins and aztreonam, amoxiclave discs to 20 mm or even less. Zones of inhibition were again observed next day .If no extension of 3rd generation of cephalosporins and aztreonam towards co-amoxiclave discs was observed, the organisms were considered as non-producer of ESBLs.

3. Results

To investigate the rate of prevalence of clinically significant *P. aeruginosa* strains and their susceptibilities against various commonly used antibiotics, a total of 200 samples were selected from the specimens received at *Pathology Laboratory. Among total,* 86 were isolated from males, 92 from females and 22 were isolated from environmental sources.

A series of biochemical tests were conducted for identification and Characterization of *P. aeuroginosa* as outlined in the *Bergey's Manual of Determinative Bacteriology (9th Edition).* Culture sensitivity testing of these samples was conducted against 16 most commonly used antibiotics for *Pseudomonal* infections by means of Disc diffusion method [27]. Table 4 indicates the susceptibilities of the 200 isolates. Later on ESBL production and glycocalyx (alignate) formation was also studied in order to understand the virulence of these strains.

Our collected samples comprised of pus, urine, blood, sputum, ear swabs, and also from environmental sources (Table 3).

Table 3: Isolation Sources of *P. Aeruginosa*

Specimen	***P. aeruginosa***
Pus	88
Sputum	10
Urine	64
Blood	18
Ear Swab	8
Environmental	12
TOTAL	**200**

Table 4: Sensitivity Pattern of *P. Aeruginosa*

Antibiotics	**% sensitive**
Ampicillin	0%
Amoxycillin	1%
Septran	1%
Tetracycline	1%
Cefazoline	1%
Cefuroxime	0%
Ceftazidime	62%
Cefotaxime	14%
Tobramycin	70%
Amikacin	79%
Gentamicin	34%
Ciprofloxacin	73%
Piperacillin	65%
Imipenem	97%
Cefoperazone	60%
Ciprofloxacine	39%

P. aeruginosa was found to be the most prevalent organism throughout the sampling period. Although, it is an opportunistic pathogen, responsible for, nosocomial infection throughout the year but the present data showed that most of its occurrence was observed from July-September, covering the months of summer.

In case of gender-wise prevalence *P. aeruginosa* was more prevalent in females (50%) as compared to male (46%) patients (Figure 1). It was observed that indoor patients (84%) were more infected with *P. aeruginosa* infection as compared to outdoor patients (15%).

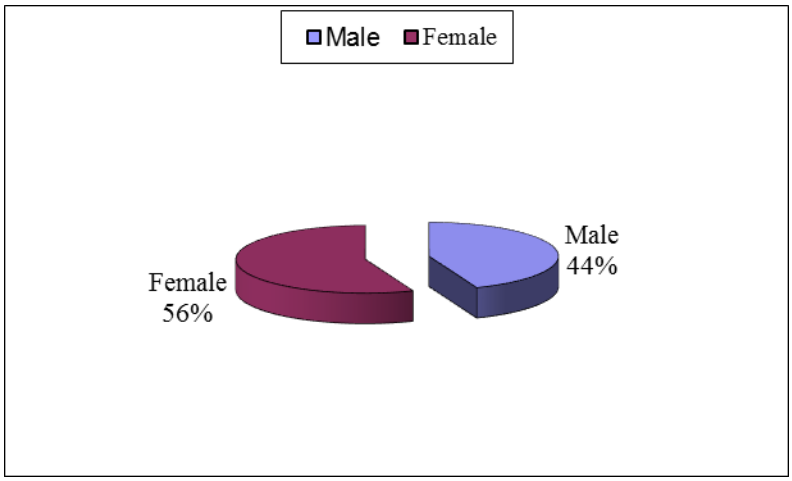

Figure 1: Gender wise Prevalence of ESBL producers among *P. aeruginosa*

Out of collected 100 samples of *P. aerugionsa*, 41 were isolated from pus, 32 from urine, 3 from ear swabs, 4 from blood, 3 from catheter tip, 5 from sputum and 8 from environmental sources (Table 3). Among clinical isolates *P. aeruginosa* was more prevalent in pus (44%) followed by urine (34%), sputum (5%), blood (4%), catheter tips (3%) and ear swabs (3%).

Among 41 isolates of pus, 23 (57%) were from males and 18 (43%) were from females. Among 32 isolates of urine 18 (56%) were from females and 14 (44%) from males. Out of 5 isolates from sputum, 2 (40%) were from males and 3 (40%) were from females.

In case of pus prevalence of *P. aeruginosa* was more in males (57%) as compared to females (43%). In case of urine the prevalence was higher in females (56%) as compare to males (44%). Susceptibility of these clinically significant isolates of *P. aerugionsa* was determined by disc diffusion method against 15 antibiotics. Table. 4 and Figure 2 indicates the susceptibilities of the 100 isolates.

Among Penicillin group (β–lactam antibiotics), for Ampicillin (semisynthetic penicillin, β-lactam) none of isolates were found sensitive and no isolate show intermediate behavior while rest of the isolates showed highly resistant behavior (100%). For Amoxycillin (synthetic penicillin) only 1% isolates showed sensitivity, all of them were resistant. Rate of resistance was 99% in this case.

For Piperacillin (ß-lactamases inhibitor), 65% isolates were sensitive, 3% were intermediate and 32% of isolates showed resistance. Three aminoglycosides, i.e. Tobramycin, Amikacin, and Gentamycin were tested against *P. aeruginosa*. Out of these three aminoglycosides, amikacin showed 79% susceptibility and only 21% resistant behavior was exhibited by the *P. aeruginosa* isolates whereas, Gentamycin showed highest resistance i.e. 66%, no organism exhibited intermediate behavior and only 34% isolates showed sensitivity to this drug.

For Tobramycin, 70 % organisms showed sensitivity and 30% were resistant.

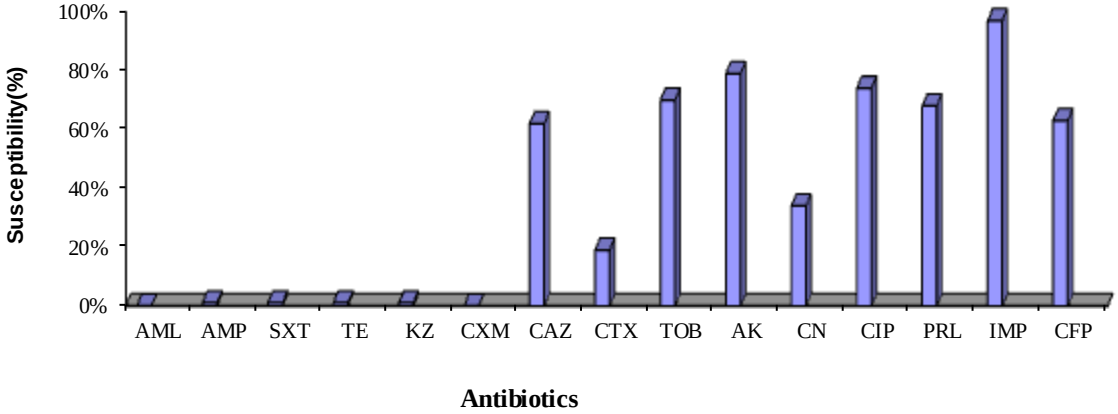

Figure 2: Antimicrobial susceptibility pattern in *P. aeruginosa*

Among the quinolones and floroquinolones, Ciprofloxacin was used. Results showed that 73% organisms were sensitive, 1% were intermediately sensitive and 26% organisms were resistant to it.

Co-trimoxazole (Septran) was also studied to note the susceptibility pattern of *P. aerugionsa* against Sulphonamides. Only 1% of the isolates showed susceptibility to this combination (Septran) and remaining were totally resistant to this antibiotic so resistance was 99%. Among first generation Cephalosporins, Cefazoline showed 99% resistance against all the 100 isolates of *P. aeruginosa.* Cefuroxine, the 2nd generation Cephalosporin also showed 100% resistance for all 100 isolates of *P. aeruginosa.*

Among 3rd generation Cephalosporins, Ceftazidime and Cefotaxime were used to determine the susceptibility pattern. For Ceftazidime, 62% were sensitive and no isolate was intermediate and 38% showed resistance. For Cefotaxime, 14% of the total isolates were sensitive, 5% were intermediate and 81% organisms were found to resistant to this drug. Tetracycline, although it has lost its efficacy against MDR *P. aerugionsa* isolates, but it was also used to determine resistance of *P. aerugionsa* against conventionally used old medicines. For Tetracycline1% of the isolates were sensitive and 99% organisms were found to be resistant to this drug.

Cefoperazone (Extended- spectrum cephalosporin) inhibited 60% of the isolates so 60% isolates were sensitive to it. A total of 3% isolates showed intermediate behavior, while, 37% isolates were resistant to it. Imipenem (Carbapenems) was found to be the most effective antibiotic among all the antibiotics used in this study. The percentage of sensitive organisms was 97%, whereas, only 3% were resistant to Imipenem.

Overall isolates exhibited 100% resistance to Cefuroxime and Ampicillin,, 99% resistance to Tetracycline, Cefazoline, Co-trimoxazole and Amoxycillin 30% resistance to Tobramycin, 21% to Amikacin, 66% to Gentamycin, 38% to Ceftazidime, 81% to Cefotaxime, 32% to Piperacillin, 26% to Ciprofloxacin, 37% to Cefoperazone and 3% to Imipenem (Table 4).

No intermediate resistant strains were found in case of Ceftazidime. No isolate was found sensitive to Cefuroxime. Whereas, Imipenem showed highest number of sensitive organisms (97%) followed by Amikacin (79%) and Ciprofloxacin (73%). Multidrug resistant organisms were found among isolates studied. Few isolates showed complete inhibition to all the antimicrobial agents tested for resistance.

ESBL producing *P. aeruginosa* isolates are a major cause of nosocomial infections. The present study was conducted to determine the prevalence, resistance and phenotypic transfer of ESBLs among *P. aeruginosa* isolates. The detection of ESBL was performed by Double disc diffusion method. Out of 100 isolates, 33 (33%) were found to be ESBL producers (Figure 3).

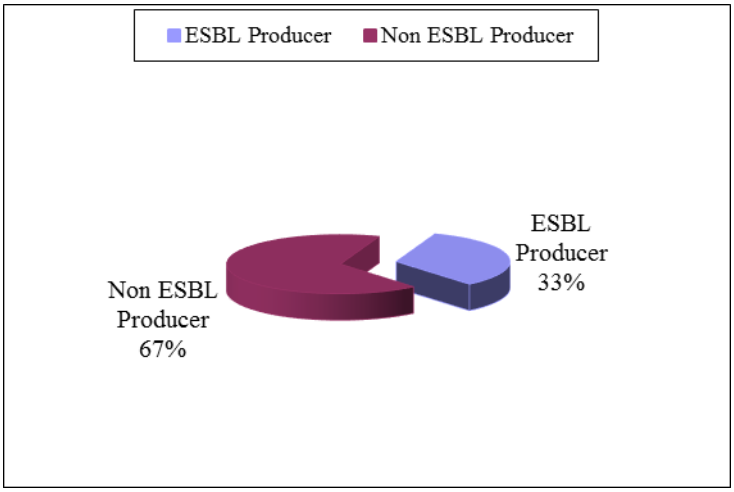

Figure 3: Prevalence of ESBL Producers among *P. aeruginosa*

Out of total 41 pus isolates, 14 (34%) were found to be ESBLs producer, 11 (34%) from urine samples out of 32, 2 (66%) from catheter tip out of 3, 3 (60%) from sputum out of 5, 2 (50%) from blood out of 4 and 4 (50%) from Environmental sources out of 8. No ESBL producing strain was found in ear swab and fluid sample. Now-a-days the newer β-lactams including the third generation cephalosporins in combination with aminoglycosides are frequently used in clinical practice for treatment of nosocomial infections caused by MDR gram negative bacteria (e.g. *P. aerugionsa*). But data on bacterial resistance of clinical isolates from hospital showed that the resistance of *P.* aerugionsa to these newer β- lactams is also increasing.

Protein H1 is envelope protein of Pseudomonas aeruginosa. It protect LPS (Lipopolysaccharide) binding sites from highly cationic antibiotics [28]. Hence docking analysis of this envelope protein (NP_249869.1) was done with drugs Amikacin and Imipenem. These two drugs were selected because both were effective on bacteria with least resistance 21% and 3%, respectively, according to experimental results. These results show that drugs work on this microorganism by affecting this envelope protein. For docking studies receptor H1 envelope protein three dimensional structure was obtained from RCSB-PDB database. Ligands (Amikacin and Imipenem) were obtained from chemspider ligand database. Both ligand molecules have chemspider ID 34635 and 94631, respectively. Docking was performed for both ligands with same receptor protein. Structure of H1 protein receptor has been shown in Figure 4 (a).

Its part b and c shows ligand drugs Amikacin and Imipenem, respectively. Docking complex of receptor with ligands amikacin and imipenem are shown in Figure 4 (part d and e).

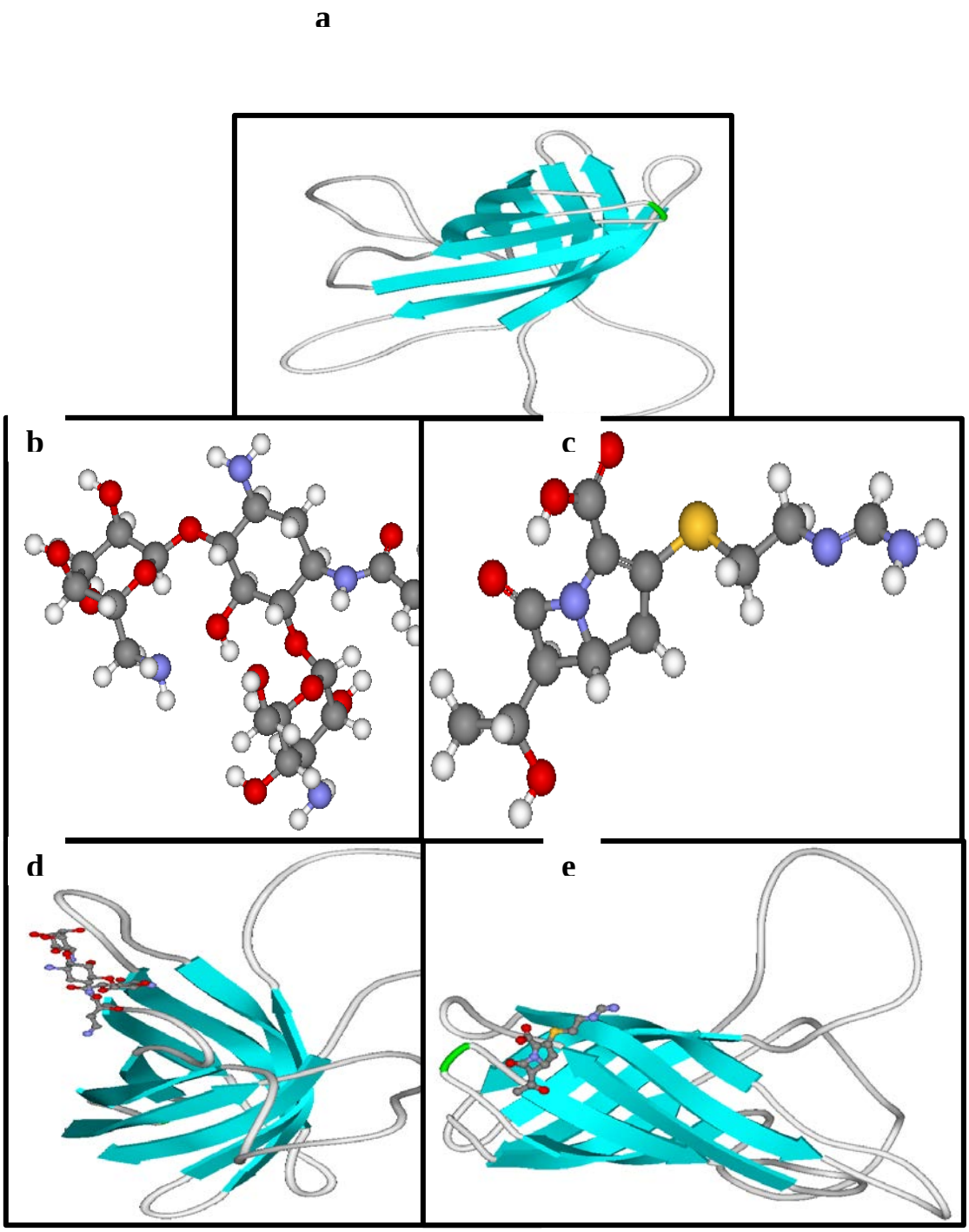

Figure 4: Docking Results: **a)** 3D-Structure of H1 Protein, **b)** Amikacin Ligand, **c)** Imipenem Ligand, **d)** Docking Results for H1 Receptor with Amikacin Ligand. **e)** Docking Results for H1 Receptor with Imipenem Ligand. Viewer: Viewerlite v5.0. H1 Protein color by secondary type and Schematic display style. Ligand: Ball & Stick Display style.

Table 5: Receptor (H1) and Ligand (Amikacin and Imipenem) Residues Involved in Interactions

Receptor-Ligand	Hydrogen Bond Interactions		Hydrophobic Interactions		Figure
	Ligand Atoms	Receptor Residues	Ligand Atoms	Receptor Residues	
H1-Amikacin	O8, O9, O13, N17	Lys70, Leu71, Glu105, Gly108, Lys109, Lys112,	O1, O2, O3, O4, O5, O6, O7, O8, O9, O10, O11, O12, C29, O13, C23, C38, C31, O10, N10, N16, N17, C20, C21, C22, C23, C25, C26, C27, C28, C29, C30, C31, C32, C34, C35, C36, C37, C38	Arg72, Leu104, Gln106, Asp107	
Mutated HR-Imipenem	O4	Gln52	S1, O2, O3, O4, O5, N6, N7, N8, C9, C10, C11, C12, C13, C14, C15, C16, C17, C18, C19, C20	Gln48, Gln49, Phe50, Gly53, Arg54, Tyr55, Tyr80, Asp81, Ala82, Gly95, Gly96, Ala97, Leu123	

Table contains docking results of receptor/ligand residues involved in the interaction for both dockings (HR protein with both ligands). Specific residues or atoms of ligand and receptor are involved in docking interaction. These ligand/receptor residues dock with the help of non-covalent interactions, for example hydrogen bonding and hydrophobic interactions. Residues/atoms of both receptor and ligand involved in hydrogen bonding and hydrophobic interaction are given in Table 5.

Difference in the interaction sites can be estimated through the amino acids and atoms which are involved in the hydrogen bonding and hydrophobic interactions between ligand and binding site of receptor protein. LigPlot results for the docking interactions are shown in Figure 5.

Receptor Residues Involved in Hydrophobic Interactions are shown in black color and represented by brick red spoked arcs ( ). Green dotted lines (.....) show hydrogen Bonding. Receptor residues involved in H-Bonding are shown in Olive Green Color. Ligand Atoms involved in H-Bonding are shown in pink color.

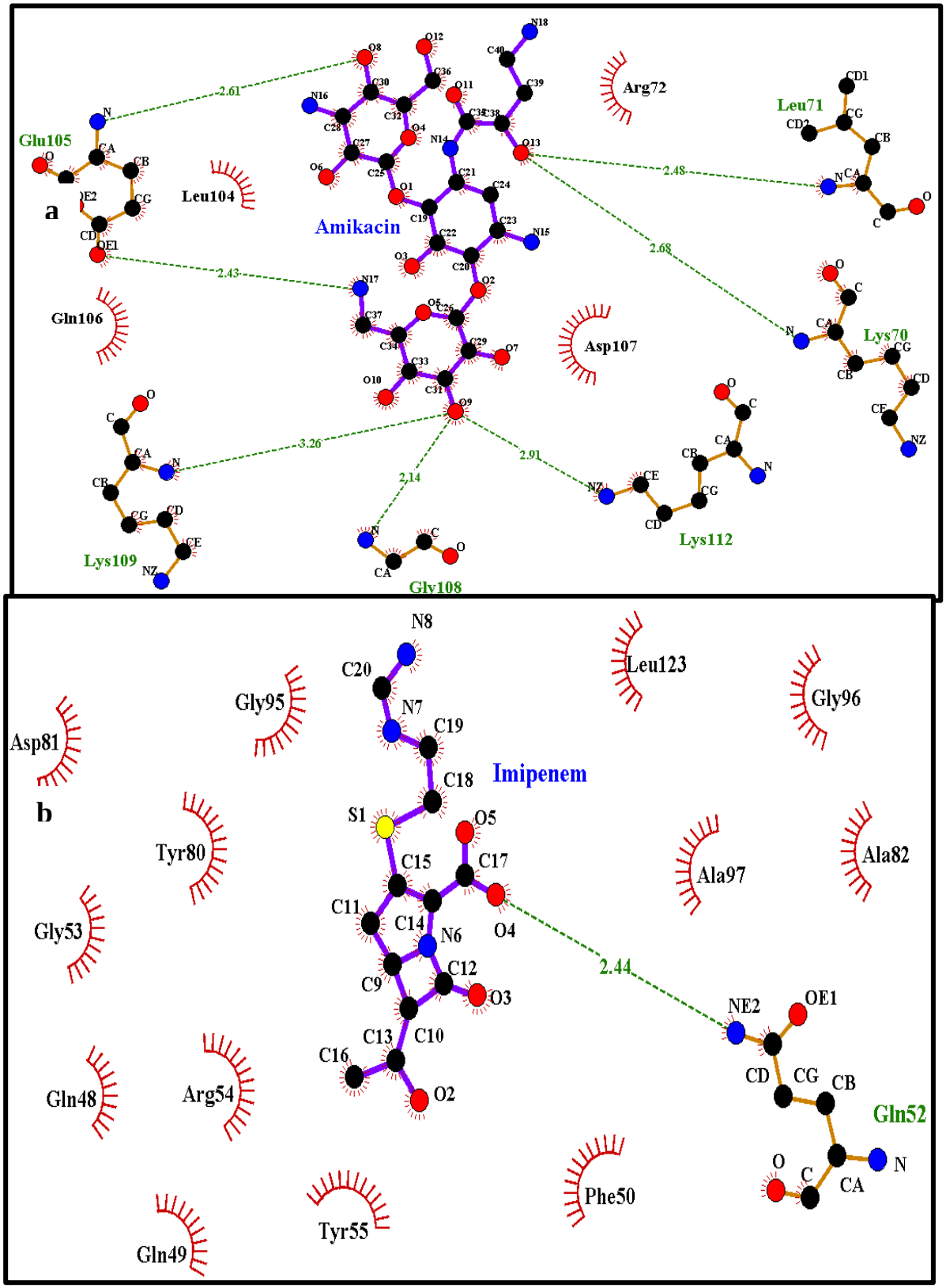

Figure 5: LigPlot Results for Docking Interactions; **a)** H1 with Amikacin, **b)** H1 with Imipenem. Ligand Atoms Involved in Hydrophobic Interactions are shown in blue color and represented by pink spoked arcs ( ).

4. Discussion and Conclusion

Our results showed a significant increase in the number of *P. aeruginosa* strains isolated from pus followed by urine. Similar pattern of isolation of *P. aeruginosa* from pus, urine, ear, nose, wound and other infection sites was reported by [29]. but in a study conducted by [30]. *P. aeruginosa* was mostly isolated from urine samples. This could be attributed due to differences in geographical location and hygienic measures or due to the fact that most patients going for major surgery tend to get catheterised.

In the present study, uropathogenic *P. aeruginosa* was found higher in females than males. Ratio was also found more among young and elderly debilitated patients. The one reason may be that *P. aeruginosa* may be a common inhabitant of lower intestinal tract and in female the distance between anal and vaginal opening is small, thus *P. aeruginosa* through fecal contamination invade and colonized urinary tracts causing infection [31] have suggested that the ascending infection from the lumen of the drainage tube may be the major pathway by which bacteria gain access to the urinary bladder [32] have also documented a higher risk for developing bacteriuria in adult female patients, the elderly and critically ill patients with a urinary catheter. Longer stay in the hospital increases the colonization of skin and environment of the patient and may be responsible for higher incidence of Urinary catheter related infections [33].

In the present study, blood, ear swabs, and sputum samples were so less to comment on, this may be due to the low number of blood, ear swabs and sputum samples sent from wards and OPDs during the study period. However *P. aeruginosa* is said to be responsible for pneumonia and septicaemia with attributable deaths reaching 30% in immunocompromised patients [34, 35, 36].The possibility of aspiration *Pseudomonal* pneumonia cannot be ruled out in post-surgical patients especially if immunocompromised [29].

P. aeruginosa was also isolated from the hands of the nursing staff. This is in agreement with [37] who reported that the hands of nurses working in wards with infected patients often carry *P. aeruginosa*. Similar kind of results was reported by [38].

P. aeruginosa is currently one of the most frequent nosocomial pathogen and the infections due to this organism are often difficult to treat due to antibiotic resistance [39]. The mechanisms of resistance to antibiotics include reduced cell wall permeability, production of chromosomal and plasmid mediated ß-lactamases, [40] aminoglycoside-modifying enzymes [41] and an active multidrug efflux mechanism [42, 43].

In the present study, the susceptibility of 100 clinical isolates of *P. aeruginosa* from pus, blood and miscellaneous samples, towards various antimicrobial agents was determined by disc diffusion method. Highest resistance (100%) was found against cefuroxime and amoxicillin. The next most resistant antibiotics were ampicillin (99%), Septran (99%), Tetracycline (99%) and Cefazoline (99%), Cefotaxime (81%), Gentamycin, (66%) Ceftazidime (38%), Cefoperazone (37%), Piperacillin (32%), Tobramycin (30%), Amikacin (21%) and Imipenem (3%). Among most commonly used cephalosporins, Ceftazidime and Cefoperazone proved to be most effective against *P. aerugionsa*, with resistant rate of (38%) and (37%) respectively. Among first generation cephalosporins, cephradine and cephalexin were tested against *P. aeruginosa* isolates.

Most of the isolates (88%) in this study were cephradine resistant and 80% were resistant to cephalexin. Earlier studies [44, 45] found *P. aerugionsa* to be resistant to these agents.

Among second generation cephalosporins, *P. aerugionsa* showed 100% resistance against cefuroxime [46]. It was previously reported that none of the first or second generation cephalosporins are active against *P. aeruginosa* [47].

Third generation cephalosporin group included commonly used antibiotics cefotaxime, Ceftazidime, Cefoperazone and ceftriaxone, which were tested against *P. aerugionsa* isolates. Among third-generation cephalosporins, ceftriaxone use was found to be less active, as 67% isolates were resistant. Other workers [47, 48] have obtained variable results with this antibiotic.

Among cephalosporins, however, Ceftazidime and Cefoperazone were found to be most effective third-generation cephalosporins as only 38% and 37% isolates were resistant. This finding is consistence with other findings [49, 50].

Among penicillins maximum resistance was noted for ampicillin (99%) and amoxicillin (100%). Similar kind of results had also been reported by [38]. An important striking feature found in this study was increased resistance to gentamycin (66%) whereas the strains were sensitive to amikacin and tobramycin. Various workers have also reported the increased sensitivity of *P. aeruginosa* strains to amikacin and resistance to gentamicin [51, 52].

P. aeruginosa Isolates showed resistance towards various antibiotics such as cephalosporins, tetracycline and gentamicin. Majority of the *P. aeruginosa* strains from the present study was MDR especially the isolates recovered from pus. Such multiple resistance patterns have also been documented earlier [53].

During the study, we observed that the alginate capsules of mucoid strains of *P. aeruginosa* could not act as a barrier against imipenem. This finding is comparable to the results of Slack and Nichol's studies, in which alginate impeded the penetration of all antibiotics except the ß-□□□ lactams [54]However, the alginate glycocalyx provides a barrier against penetration of cefotaxime, and this antibiotic was clearly inferior to imipenem against our *P. aeruginosa* strains. In addition, this reduced susceptibility may be related to the more extensive use of cefotaxime in hospital. On the other hand, additional resistance mechanisms especially production of extended-spectrum β-lactamases (ESBLs) and other enzymes may contribute to ceftazidime resistance [55].

Alginate, an extracellular glycocalyx, probably acts as a barrier against aminoglycosides [56]. The mucoid strains of *P. aeruginosa* were found statistically significantly more resistant to amikacin, gentamicin and tobramycin than the non- mucoid strains by [57]. Similar types of results were found in the present study. Overall, there was moreresistance to gentamycin, followed by tobramycin and amikacin.The results of this study suggest that the capsule may act as a barrier against aminoglycosides [58]. Nevertheless, there is evidence that alginate provides an ionic barrier against penetration of aminoglycoside antibiotics [56]. Reference [54] used antibiotic diffusion through agar as a criterion for direct measurement of the permeability of the alginate layer to antibiotics.

They found that, with the exception of β-lactams, alginate did in fact impede the penetration of antibiotics such as aminoglycosides. However, [59] observed that the alginate-to-anti-biotic ratio could greatly influence the perceived permeability barrier. When this ratio is high, aminoglycosides (but not β-lactams) are retained in the alginate layer. However, low alginate-to-antibiotic ratios quickly result in disruption of the gel structure and faster penetration of amino-glycosides. [57] suggested that high levels of antibiotic saturate the negative charge of alginate and result in a breakdown in the permeability layer.

The development of antimicrobial resistance is a natural process, which cannot be stopped. Resistance means that people cannot be effectively treated, that they are ill for longer period of time and at a greater risk of dying. It also means that epidemics are prolonged and thus that there is a greater risk of infection to others. The development of resistance is accelerated when antimicrobials are misused (http://www.emro.who.net).

Despite the use of potent antibiotics still high mortality exist in case of *P. aeruginosa* infections. Nosocomial multidrug resistant *P. aeruginosa* is an important health care problem worldwide. Antimicrobial resistance prolongs the duration of hospitalization, thereby increasing the cost of patient care. There are multiple factors, which contribute to the global spread of resistance. Decreasing unnecessary antibiotic use, treating with narrow spectrum agents, improving compliance with therapy, decreasing use of antibiotic in animal and agriculture, and improving infection control all have a role in confronting this problem. In addition, immunization may diminish the impact of resistance by preventing infection and also the carriage of transmission.

Amikacin and Imipenem drugs were selected for docking because both were effective on bacteria with least resistance 21% and 3%, respectively, according to experimental results. These results show that drugs work on this microorganism by affecting this envelope protein. Docking results analyzed how these drugs interact with envelope protein to stop its growth.

5. Recommendations

Molecular docking analysis can be performed while working with various microorganisms and some potential drugs can be suggested on basis of resistance. This will lead to more detailed docking analysis and interaction of various drugs.

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