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An Analysis of High Frequency and Resistance Pattern in *Pseudomonas Aeruginosa* Isolated from Clinical Specimens Obtained from Tertiary Care Hospital

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Abstract: Resistance in pathogenic bacteria against antibiotics has remained a challenge to our clinicians in managing various infections and *Pseudomonas aeruginosa* among it. This clinical study aims to determine the antimicrobial resistance against *Pseudomonas aeruginosa* isolated from the clinical specimens to fill the scientific gap present in our area. The pathogen has been cultured from isolates collected from clinical specimens, patients admitted to Jinnah hospital Lahore, province of Punjab, and the susceptibility to various antimicrobial drugs studied. Overall, 1159 samples of urine, wound swabs, sputum, blood, tissue, and pus were collected from infected patients of age groups ranging from 20-70 years and included both female and male patients. *Pseudomonas aeruginosa* was identified using biochemical tests and staining procedures, as confirmed by API20NE. Susceptibility to different antimicrobial agents was then performed using the Kirby-Bauer method. Almost 22.0% of the clinical specimen came out to be positive for *Pseudomonas aeruginosa*, with a slightly higher percentage in female patients than males. Department-wise isolation of *Pseudomonas aeruginosa* was surgery n = 94 (36.8%), medicine n = 66 (25.9%), orthopedics n = 34 (13.3%), ICU n = 29 (11.4%), ENT n = 14 (5.5%) and Gynaecology n = 18 (7.0%) ($p \leq 0.001$). Sample-wise isolation of *Pseudomonas aeruginosa* was wound swabs n = 89 (34.9%), urine n = 71 (27.8%) and sputum n = 35 (13.7%), blood n = 30 (11.7%), pus n = 18 (7.05%) and tissue n = 12 (4.7%). The age group of 40-49 showed the highest frequency of *Pseudomonas aeruginosa*. These clinical isolates were then tested against different antibiotic drugs, amongst which the highest resistance was found against ceftazidime. This study showed a high prevalence of infection caused by *Pseudomonas aeruginosa* in hospitalized patients admitted to a tertiary care hospital, whereby this microbe exhibited multidrug resistance against various antibiotics. The emergence of antimicrobial-resistant strains of *Pseudomonas aeruginosa* is largely attributed to excessive usage of antibiotic drugs. The highest resistance was exhibited against ceftazidime.

Keywords: *Pseudomonas aeruginosa*, nosocomial infection, antimicrobial susceptibility, pathogen, Kirby-Bauer method.

三級醫院臨床標本分離的銅綠假單胞菌高頻及耐藥模式分析

摘要：病原菌對抗生素的耐藥性一直是我們臨床醫生管理各種感染和其中的銅綠假單胞菌的挑戰。本臨床研究旨在確定對從臨床標本中分離的銅綠假單胞菌的抗菌素耐藥性，以填補我們地區存在的科學空白。病原體是從臨床標本中收集的分離株中培養的，旁遮普省拉合爾真納醫院收治的患者，以及研究的各種抗菌藥物的敏感性。總體而言，從20-70歲年齡組的感染患者收集了1159份尿液、傷口拭子、痰液、血液、組織和膿液樣本，包括

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女性和男性患者。革蘭氏陰性非腸桿菌科的24至48小時鑑定證實，銅綠假單胞菌使用生化測試和染色程序進行鑑定。然後使用柯比鮑爾方法測定對不同抗菌劑的敏感性。幾乎22.0%的臨床標本顯示銅綠假單胞菌呈陽性，女性患者的比例略高於男性。銅綠假單胞菌的科室隔離是手術n等於94(36.8%)，內科n等於66(25.9%)，骨科n等於34(13.3%)，重症監護室n於29(11.4%)，耳鼻喉科n等於14(5.5%)和婦科n等於18(7.0%)(p≤.001)。銅綠假單胞菌的樣本分離是傷口拭子n等於89(34.9%)、尿液n等於71(27.8%)和痰n等於35(13.7%)、血液n等於30(11.7%)、膿n等於18(7.05)和組織n等於12(4.7%)。4049歲年齡組表現出銅綠假單胞菌的最高頻率。然後針對不同的抗生素藥物對這些臨床分離株進行了測試，其中發現對頭孢他啶的耐藥性最高。這項研究表明，在三級醫院住院患者中，銅綠假單胞菌引起的感染率很高，因此這種微生物對各種抗生素表現出多藥耐藥性。銅綠假單胞菌耐藥菌株的出現很大程度上歸因於抗生素藥物的過度使用。對頭孢他啶表現出最高的耐藥性。

关键词：銅綠假單胞菌，醫院感染，抗菌藥物敏感性，病原體，柯比-鮑爾法。

1. Introduction

Hospital-acquired infections are more commonly labeled as nosocomial infections. These infections may be systemic or localized4 and usually develop within 48-72 hours after hospital admission [26]. Nosocomial infections are a major cause of morbidity and mortality in hospitalized patients and increasing complications in inpatient treatments, thus leading to a prolonged hospital stay [28]. Hospital-acquired infections most commonly occur in the bloodstream, surgical site wounds, respiratory tract, and urinary tract [12]. These infections initiate the pathogenic invasion into body tissues, thereby causing damage. They disrupt the immune pathways leading to the production of pus, thus creating hindrance in the wound healing process [15]. The Developing hospital-acquired infection among immune-deficient patients was 2.34 times higher [1]. The incidence of such infections is greater in old age, malnourished individuals, and smokers [19]. *Pseudomonas aeruginosa* is one of the most commonly isolated pathogens in nosocomial infections [3]. A surgical wound infection occurs following a surgical procedure resulting in various complications [20]. The tissue damage that occurs during the invasion by a microbe occurs because of the superantigens and toxins by bacteria, along with an increased number of T cells [9]. *Staphylococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa* mark the common causative agents for infections at surgical wounds [15].

Pseudomonas aeruginosa is a gram-negative rod. It is a ubiquitous microbe occurring commonly in the environment. The different culture media employed to identify *Pseudomonas aeruginosa* are MacConkey agar, Cetrimide Agar, and blood agar [6]. The biochemical tests used in identifying *Pseudomonas aeruginosa* are the oxidase test, catalase test, citrate utilization test, and gelatin liquefaction test. It has been reported that *Pseudomonas aeruginosa* is resistant to many antimicrobial drugs like chloramphenicol, tetracycline, and quinolones [14]. However, the pattern of multidrug resistance differs worldwide. This research study has been conducted to isolate, identify and create an antibiogram of *Pseudomonas aeruginosa* isolated from clinical samples.

2. Materials and Methods

2.1. Materials

MacConkey culture media (Oxoid, United kingdom), cetrimide agar (Oxoid, United kingdom), Blood culture (Oxoid, United Kingdom), and Mueller-Hinton agar (Oxoid, United Kingdom) was followed by identification through API20NE. Antibiotic discs used were amikacin (30µg), piperacillin/tazobactam (100µg), gentamicin (10µg), cefoperazone/sulbactam (75-10µg), imipenem (10µg), aztreonam (10µg), ciprofloxacin (5µg), meropenem (10µg), cefoxitin (30 µg) and ceftazidime (30µg) [16] Antibiotic susceptibility testing was done on Mueller-Hinton agar using the disc diffusion method by measuring the inhibition zones.

2.2. Methods

2.2.1. Study Design

In total, clinical samples from 1159 patients in the age range of 20-70 years admitted to a tertiary care hospital in Lahore were studied, as shown in Table 1.

Table 1 Number of patients testing positive in both genders (male, female) (n = 1159)

Female	Male	+ve Female	+ve Male	Total +ve (%)	Total -ve (%)
523	636	145	110	255 (22%)	904 (77.9%)

2.2.2. Isolation and Biochemical Characterization of Bacteria

Samples were collected after proper consent [8]. These samples were inoculated on the Nutrient agar for activation. Following incubation for 24 hours at 37°C, gram staining of selected colonies was performed. The colonies were then streaked on cetrimide agar for selectively growing *Pseudomonas aeruginosa* [11] proceeded with inoculation on MacConkey agar for lactose fermentation plus blood agar for evaluating hemolysis. All these media were incubated at 37°C for 24 hours after streaking [13]. These isolated colonies were then confirmed using biochemical tests that included the oxidase test, catalase test, indole test, methyl red-Voges Proskauer test, and motility test [22].

2.2.3. Antibiotic Susceptibility Test

Kirby-Bauer method was performed for antibiotic susceptibility against amikacin (30 µg), piperacillin/tazobactam (100 µg), gentamicin (10 µg), cefoperazone/sulbactam (75-10 µg), imipenem (10 µg), aztreonam (10 µg), ciprofloxacin (5 µg), meropenem (10 µg), cefoxitin (30 µg) and ceftazidime (30 µg) [16].

2.2.4. Statistical Analysis

The frequency and percentage of resistance were evaluated according to the ward and type of sample. A Chi-square test was used for determining significance. A p-value ≤ of 0.05 was considered to be statistically significant.

3. Results

3.1. Isolation and Identification of *Pseudomonas Aeruginosa* from Clinical Samples

Out of a total of 1159 collected samples of urine, wound swabs, blood, tissue, pus, and sputum, the isolation rate of *Pseudomonas aeruginosa* was 22.0% (255/1159). Out of 255 isolates, 145 isolates were from females, and 110 were obtained from males. *Pseudomonas aeruginosa* were gram-negative rods as seen under oil immersion (100X) lens after gram staining. Colonies exhibiting β-hemolysis on blood agar reveal non-lactose fermenting pale clear colonies on MacConkey agar. Growth on Cetrimide agar media revealed yellow-green pigments. Pale clear colonies grew on MacConkey agar after incubation for 24 hours at 37°C. A hazy appearance was observed in the test tube inoculated with *Pseudomonas aeruginosa*, indicating that it is a motile microbe. The appearance of the green color showed that the citrate utilization test was positive. Negative results were noted for the triple sugar iron test.

3.2. Antibiotic Susceptibility Test

Pseudomonas aeruginosa showed 45% resistance against amikacin, 60% - against ceftazidime, 55% - against ciprofloxacin, 58% - against cefoperazone/sulbactam, 55% - against gentamicin, 51% - against meropenem, 53% - against imipenem, 40% - against piperacillin/tazobactam, 45% - against cefoxitin and 50% - against aztreonam as shown in Table 6 and Fig. 1.

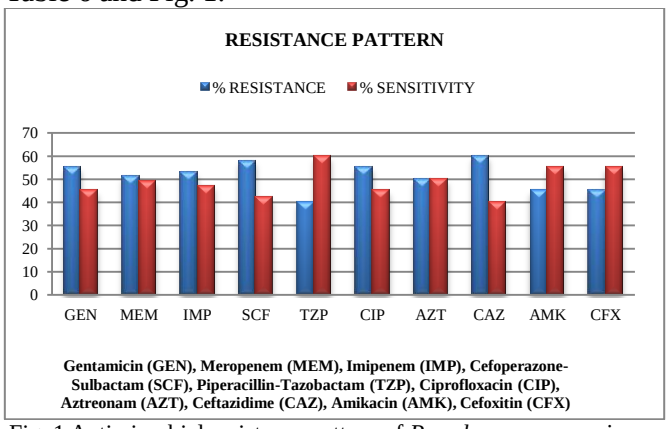

Fig. 1 Antimicrobial resistance pattern of *Pseudomonas aeruginosa*

Sample wise isolation was wound swabs n = 89 (34.9%), urine n = 71 (27.8%) and sputum n = 35 (13.7%), blood n = 30 (11.7%), pus n = 18 (7.05), and tissue n = 12 (4.7%) as shown in Table 3.

Of the specimens from different wards, 36.8% were from surgery (surg), 25.9% from medicine (med), 13.3% from orthopedics (ortho), 11.4% from intensive care unit (ICU), 5.5% from otolaryngology (ENT), and 7.0% from gynecology department came out to be positive for *Pseudomonas aeruginosa* as shown in Table 5.

Table 2 Number of isolates with relation to age groups

Age groups (years)	Total isolates	Female	Male
20-29	28	5	23
30-39	57	36	21
40-49	65	55	10
50-59	63	39	24
60-69	42	10	32

Table 3 Distribution of specimen with positive *Pseudomonas aeruginosa* isolates

Site/Source	Number of isolates	% of isolates
Wound	89	34.9
Urine	71	27.8
Sputum	35	13.7
Blood	30	11.7
Pus	18	7.05
Tissue	12	4.7
Total	255	100 resistance rate

Table 4 Results of different biochemical tests performed to confirm *Pseudomonas aeruginosa*

Biochemical tests for *Pseudomonas aeruginosa*	**Results**
Catalase	Positive
Oxidase	Positive
Simmon's citrate	Positive
Urease	Negative
Indole	Negative
Motility	Positive
Methyl red	Negative
Voges-Proskauer	Negative
Gel liquefaction	Positive
Glucose	Negative
Lactose	Negative
Sucrose	Negative
Acid	Negative
H_2S gas	Negative

Table 5 Isolates of *Pseudomonas aeruginosa* with relation to different wards

Ward	**Specimen type**						**Total**
	Urine	**Wound**	**Sputum**	**Blood**	**Pus**	**Tissue**	
Surgery	30	35	10	9	6	4	94(36.8%)
Medicine	26	18	8	7	4	3	66(25.9%)
Orthopedic	4	15	7	5	1	2	34(13.3%)
ICU	4	15	5	3	1	1	29(11.4%)
ENT	3	4	1	2	3	1	14(5.5%)
Gynecology	4	2	4	4	3	1	18(7.0%)
Total	71	89	35	30	18	12	255(100%)

Table 6 Antimicrobial resistance pattern observed for *Pseudomonas aeruginosa*

Antibiotic	**Wards**						**Total**	**P-value**
	Surg	**Med**	**Ortho**	**ICU**	**ENT**	**Gynae**		
Gentamicin	51	44	25	10	4	6	140(55%)	0.001
Meropenem	56	42	4	14	8	6	130(51%)	0.000
Imipenem	62	27	21	9	7	9	135(53%)	0.005
Cefoperazone-Sulbactam	76	39	11	10	5	7	148(58%)	0.000
Pip-Tazo	35	25	19	16	3	4	102(40%)	0.048
Ciprofloxacin	51	51	22	8	4	4	140(55%)	0.000
Aztreonam	39	32	28	18	5	6	128(50%)	0.001
Ceftazidime	65	35	16	14	10	13	153(60%)	0.062
Amikacin	51	35	5	9	5	10	115(45%)	0.001
Cefoxitin	43	30	10	24	2	5	114(45%)	0.000

4. Discussion

Nosocomial infections are causative for high mortality and morbidity in hospitalized patients. Many of these infections are caused by bacteria, but viral, fungal, and protozoal infections are also common. *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Enterococci* are some bacteria that cause a huge number of infections [17]. *Pseudomonas aeruginosa* is a major cause of hospital-acquired infections. Major pathogenic factors include exotoxin A, lipopolysaccharides, proteases, and leukocidin [5]. The rapid emergence of multidrug-resistant strains of *Pseudomonas aeruginosa* is doubtlessly globally alarming while increasing morbidity and mortality rates [7]. The strong intrinsic resistance mechanisms that are possessed by *Pseudomonas aeruginosa* such as β-lactamase enzyme production, major efflux pumps, having enzymes that modify aminoglycosides, poor membrane antibiotic permeability, plus topoisomerase II and IV alteration that makes *Pseudomonas aeruginosa* quinolone resistant. Unfortunately, all these mechanisms exist simultaneously, giving rise to MDR strains of *Pseudomonas aeruginosa*. All these mechanisms are attributed to multidrug resistance in *Pseudomonas aeruginosa* [25].

Antibiotic susceptibility testing is important in deciding the most suitable antibiotic that should be given for nosocomial infections caused by *Pseudomonas aeruginosa*. The present study has been carried out to isolate and identify *Pseudomonas aeruginosa* from clinical samples obtained from 1159 patients admitted to a tertiary care hospital in Lahore and study the antibiogram of *Pseudomonas aeruginosa* against commonly used antibiotic drugs. Both genders were included in this study ranging in age from 20-70 years. Approximately 22.0% of patients were positive for *Pseudomonas aeruginosa* (Table 1). The high frequency of *Pseudomonas aeruginosa* was isolated from patients in the age group of 40-49 years, with females in total having a higher number of infections with *Pseudomonas aeruginosa* ($p \leq 0.001$) (Table 2).

Pseudomonas aeruginosa showed 45% resistance against amikacin, 60% - against ceftazidime, 55% - against ciprofloxacin, 58% - against cefoperazone/sulbactam, 55% - against gentamicin, 51% - against meropenem, 53% - against imipenem, 40% - against piperacillin/tazobactam, 45% - against cefoxitin, and 50% - against aztreonam. Of all the specimens, 34.9% isolates of *Pseudomonas aeruginosa* were from wound specimens, 27.8% - from urine specimens, 13.7% - from sputum samples, 11.7% - from blood samples, 7.05% - from pus samples, and 4.7% - from tissue specimens. Of the specimens from different wards, 36.8% were from surgery, 25.9% - from medicine, 13.3% - from orthopedics, 11.4% - from ICU, 5.5% - from ENT, and 7% - from the gynecology department came out to be positive for *Pseudomonas aeruginosa* as shown in Table 5. These findings are very similar to the results of a study carried out by Rajat and colleagues in India who isolated *Pseudomonas aeruginosa* as the major infection causing microbe, and the high frequency of

these infections was in the age group 20-41 years [21].

A similarly higher prevalence of *Pseudomonas aeruginosa* (23.33%) has also been reported in yet another research [16]. The current study reports a 22% rate of culture positivity for *Pseudomonas aeruginosa* isolates compared to 9-32%, as reported in previous studies [21, 24]. This variation in prevalence might be due to the studied population, geographical location, type of hospital, and the variation of received clinical samples. These results are somehow per the previous studies where urine and pus and urine samples were common sources [27].

Certain drugs among cephalosporins are especially acknowledged for their strong anti-pseudomonal activity, cefoperazone, and ceftazidime. *Pseudomonas aeruginosa* isolates have shown high resistance against ceftazidime in this study of 60%. These findings are in concordance with previous studies, which have reported similar findings [2, 29]. Nonetheless, exceptionally high resistance rates equalling 56-97% have also been reported [23]. Among carbapenems, imipenem marks as a potent inhibitor of cell wall synthesis. Similar to β-lactam antibiotics, imipenem also produces therapeutic effects by crossing the cell wall by porins and ultimately binding to penicillin-binding proteins (PBP) present in the cell membrane. Porin OprD mutation in *Pseudomonas aeruginosa* in combination with production of AmpC plus acquisition of MBL genes by the microbe becomes the cause of resistance against imipenem [30]. These differences in the resistance rates are most possibly in association with the differences in antibiotic usage in different settings plus the selective pressure. Amongst carbapenems, the current study has shown high resistance to imipenem at 53% and meropenem at 51%. Quite similar findings of higher resistance rates have been reported as 43% by Ullah et al. [27], as 49% by Ameen et al. [30], as 59% by Qadeer et al. [31], and as 60% by Khan et al. [10].

A significant aspect of the results obtained in this study is the resistance against fluoroquinolone. Second-generation fluoroquinolones showed 55% resistance against isolates of *Pseudomonas aeruginosa* that is quite comparable to the study conducted by Shah *et al*. and Ali *et al*. [2, 23]. They reported a 50% and 60% resistance rate, respectively. Similarly, a much-augmented rate was reported in previous studies of 66% and 75% [17, 18].

Many studies have contrasting results where these antimicrobial drugs were more effective against *Pseudomonas aeruginosa* isolates [2]. Variation in resistance rate is credited to the differences in sample size, type, duration, and study settings. By the National AMR Action Plan for Pakistan 2017–2018, it has been stated that *Pseudomonas aeruginosa* has shown lesser resistance against carbapenems (6.5%) in comparison to *Klebsiella pneumonia* (30%). In contrast, the current study suggests that resistance trends for *Pseudomonas aeruginosa* are quite alarming than was previously expected [32].

5. Conclusion

Our study found that almost all of the commonly prescribed antibiotics had more than a 40% resistance rate against *Pseudomonas aeruginosa*. In comparison, ceftazidime had the highest among all (60%), and surprisingly these antibiotics are commonly prescribed in surgical wards (36.8%) followed by medical wards (25.9%). Awareness should be provided to the clinicians of concerned departments where these antibiotics are frequently being used so that the rate of drug resistance can be reduced and alternate medicines should be administered. Also, it is better to perform an antimicrobial susceptibility test as this will reduce treatment costs and help overcome the high load of treatment failures.

6. Limitations and Further Study

The limitation of this study is the small number of datasets. However, this can be solved by using the cross-validation method. Another weakness is the collection of samples from a single center. A multicenter study should be conducted to validate the findings of our study.

Abbreviations

Gentamicin (GEN), Meropenem (MEM), Imipenem (IMP), Cefoperazone-Sulbactam (SCF), Piperacillin-Tazobactam (TZP), Ciprofloxacin (CIP), Aztreonam (AZT), Ceftazidime (CAZ), Amikacin (AMK), Cefoxitin (CFX).

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Declaration

This study is part of the Ph.D. thesis of Maria Muddassir.

Ethics Approval

This study was approved by the Ethical Committee of the University of Lahore (Ref # IMBB/UOL/20/138).

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