Frequency of multi drug resistant *Pseudomonas aeruginosa* in different wound types of hospitalized patients

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Abstract: *Pseudomonas aeruginosa* colonization is one of the major complications of wound infection leading to higher risk of morbidity and mortality. The trend of antibiotic resistant against *Pseudomonas aeruginosa* is increasing day by day due to irregular and extensive use of antibiotics. The main aim of this cross- sectional study is to detect the frequency of MDR *Pseudomonas aeruginosa* among various types of wounds during January to December 2018. In this study total 532 clinical samples were collected from wounded patients and subjected to the isolation and identification of *Pseudomonas aeruginosa* by standard microbiological techniques. Molecular identification of the isolates was done through PCR by using specific primers against Oprl, OprL and PA-SS genes of *Pseudomonas aeruginosa*. Antibiotic susceptibility testing and minimum inhibitory concentration was done by disc diffusion method and broth dilution assay respectively. PCR was performed for the molecular detection of ESBL and MBL genes using specific primers. Out of total 532 clinical samples 203 (38%) samples were identified as *Pseudomonas aeruginosa*. Out of positive samples 119 (58.6%) were confirmed MDR *Pseudomonas aeruginosa.* Out of 119 MDR positive samples, burn wounds showed the highest percentage 43 (36%), while least percentage 4 (3%) of MDR *Pseudomonas aeruginosa* was found in surgical wounds ($P<0.05$). All the selected isolates were resistant to β-lactams drugs and most effective drugs were tigecycline and colistin. Highest prevalence in the infected wound patients is blaNDM 14 (25.9%) producing *P. aeruginosa* and least blaKPC 1 (1.8%) producing *P. aeruginosa*. Results of the study concluded that surgical wounds showed the highest prevalence of MDR *P. aeruginosa*, suitable measures should be adopted to restrain this public health menace.

Keywords: Wounds, *Pseudomonas aeruginosa*, multi drug resistance, antibiotic susceptibility profile.

INTRODUCTION

Wounds are defined as discontinuity of body tissue (Brito *et al.,* 2013) and it found to be common and serious problem around the globe (Guo and DiPietro, 2010) specially when the wounds become infectious. Healing of wounds can be delayed due to presence of bacteria which enhances the inflammatory process (Dhall *et al.,* 2014). The most important bacteria found in the various types of wounds are *P. aeruginosa* and it is an opportunistic pathogen (Kidd *et al.,* 2009). The *P. aeruginosa* is mainly found in the patients of burn, accidental, surgical and pus wounds along-with urinary tract and ear infection patients (Xu *et al.,* 2004; Khalid *et al*., 2017).

Irregular and extensive use of antibiotics results into multiplication of resistant *Pseudomonas* isolates. Resistance of *P. aeruginosa* to commonly used antibiotic is increased day by day and it reached at alarming level (Ahmed, 2016; Naveed *et al*., 2013). *P. aeruginosa* have the capacity to resist against these antibiotics through different processes including efflux pump, antibiotic inactivating enzymes, low permeability of membrane and biofilm formation (Kalantar *et al.,* 2012). Various classes of Extended-spectrum beta-lactamase (ESBL) are produced by different strains of *Pseudomonas aeruginosa* which than exhibit high resistance against variety of antibiotics (Shacheraghi *et al*., 2010; Siddique *et al*., 2018). The extended spectrum β-lactamase (ESBL) and Metallo β-lactamase (MBL) mediated resistance mechanisms are important in emerging resistance mechanisms in *P. aeruginosa* (Strateva & Yordanov, 2009). The ESBLs and MBLs producing *Pseudomonas aeruginosa* are reported all over the world (Aggarwal *et al*., 2008).

In Pakistan, 60% of the population has rural background where unhygienic practices to treat wound infections are a huge risk factor for the colonization of MDR pathogens. To date there is no published data on presence of MDR *pseudomonas aeruginosa* in infected wound patients from Pakistan. Keeping in view this fact and increasing trend of antibiotic resistance, the present study was planned for molecular detection and frequency of MDR *P. aeruginosa* from various types of wounds in hospitalized patients.

MATERIALS AND METHODS

Sample collection

In present cross-sectional study, a total of 532 samples were collected aseptically from the different types of wound including burn wound, surgical wound, accidental wound and pus wound of infected patients from the DHQ and Allied Hospital of District Faisalabad (Scotton *et al.,*

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2014; Rasool *et al*., 2016) by non-probability sampling technique. The samples were collected during January to December 2018 by obtaining a written consent from each patient after describing them the purpose of study.

Ethical approval

This study was approved by Institutional Ethics Review Committee under code GCUF/ERC/173, dated December 21, 2017 and the samples were collected in accordance with international safety rules and ethical standards.

Isolation and Identification of P. aeruginosa

The swab samples were streak on the selective media pseudomonas cetrimide agar (Oxoid Uk) plates and incubated at the 37^{o}C for 24 hours. Identification of the isolates was made based on morphology and biochemical characteristics including catalase, oxidase and citrate (Quinn *et al.,* 2011).

Molecular identification of the isolates

DNA was extracted from all the bacterial isolates by using GeneJET Genomic DNA Purification Kit (Thermo Scientific, UK). The extracted DNA was polymerized by using the specific primers (table 1) against Oprl, OprL and PA-SS genes. The conditions of Polymerase Chain Reaction (PCR) were used include initial denaturation 94°C for 4 minutes, then total 30 cycles with following (denaturation 94°C for 45 seconds, annealing 57°C for 1 minute and polymerization 72°C for 1 minute) and one final extension cycle of 72°C for 5 minutes. PCR amplified product was visualized in ethidium bromide stained 1% agarose gel under the gel doc system (Bio-Rad, UK) (douraghi *et al.*, 2014).

Antimicrobial susceptibility testing

All the clinical isolates were subjected to antibiotic susceptibility testing by using the Kirby-Bauer disc diffusion assay according to CLSI 2017 guidelines. For this assay Mueller Hinton agar (MHA) (Oxoid, UK) was used, different antibiotics were used for the susceptibility testing which includes cefoperazone, cefixime, amikacin, polymyxins-B, cefepime, amoxicillin-clavulanic acid, aztreonam, imipenem, levofloxacin, ciprofloxacin, piperacillin-tazobactam, tigecycline, doxycycline, meropenem and ceftrizone, (Oxoid, UK). For the MIC (µg/mL) different antibiotics were subjected against clinical isolates using broth dilution assay. According to CLSI 2017 guidelines results were interpreted (Patel *et al*., 2016).

Combined Disk Diffusion test (CDDT)

Pseudomonas aeruginosa suspension was swabbed on Mueller Hinton Agar and antibiotic discs of cefotaxime (CTX) 30µg, and cefotaxime-clavulanic acid (30/10µg), ceftazidime (CAZ) 30µg and ceftazidime-clavulanic acid (30/10µg), were placed at distance of 20mm on inoculated Muller Hinton agar. Incubated for 24 hours at 37°C and after 24 hours incubation zone of inhibition was observed.

Genotypic detection of ESBL and MBLs genes

The isolates resistant to cefotaxime (CTX), and ceftazidime (CAZ), were tested for the genotypic detection of ESBL and MBL genes. Phenotypically confirmed ESBL and MBL producing isolates of *Pseudomonas aeruginosa* were tested for the genes encoding blaOXA, blaKPC, blaNDM, blaIMP and blaVIM by PCR with specific primers as shown in (table 1). After DNA extraction by phenol-chloroform method PCR was performed in a thermocycler in specific conditions denaturing temperature 94^{o}C for 4 minutes; 45 seconds at 52^{o}C, 35 cycles of 1 minutes at 94^{o}C and final extension for 4 minutes at 72^{o}C.

RESULTS

Isolation and molecular characterization of Pseudomonas auroginosa

On the basis of phenotypic and molecular characteristics out of 532 specimens 203 (38%) isolates were confirmed *P. aeruginosa*. From 203 positive isolates 119 (58.6%) were recorded MDR as they showed resistance against various antibiotics. Prevalence of MDR *P. aeruginosa* was found significantly higher (36%) in burn wound patients followed by accidental (32%) pus (27%) and surgical wounds (3%) respectively. Detailed percentage prevalence of MDR *P. aeruginosa* is shown in the table 2.

Antibiotic susceptibility testing

In present study multiple antibiotics including Amikacin (AM), Cefixime (CFM), Ceftrizone (CTR), Polymyxins B (PB). Aztreonam (AZT), Cefepime (FEP), Amoxicillin-clavulanate (AMC), Cefoperazone (CFP), Ciprofloxacin (CIP), Gentamicin (CN), Piperacillin-tazobactam (PTZ), Doxycycline (DOX), Imipenem (IPM), Meropenem (MEM), Tigecycline (TGC), and Levofloxacin (LEV) were used against all the isolated *P. aeruginosa.* All the isolates were found resistant to all the cephalosporin antibiotic used in this study, whereas 108 (90%) of isolates were found sensitive to tigecycline and polymyxin B. Detail susceptibility pattern of the isolates against various antibiotics is given in fig. 1.

Phenotypic and Genotypic detection of ESBLs and MBLs in Pseudomonas aeruginosa

Out of 119 the 54 (45.37%) resistance isolates of *Pseudomonas aeruginosa* were positive for ESBL and MBL production checked by combined disc diffusion test. The zone of inhibition was observed ≥5mm increase in the diameter for ceftazidime/clavulanic acid (30/10µg) and cefotaxime/clavulanic acid (30/10µg) and compared with zone of inhibition without clavulanic acid. The prevalence of ESBL and MBL genes were recorded as follow blaOXA 05 (9.5%), blaKPC 1 (1.8%), blaNDM 14 (25.9%) blaVIM 9 (16.5%) and blaIMP 6 (11.11 %) (table 3). The detection of genes was performed by polymerase chain reaction.

Table 1: Sequence of Gene specific primers

Gene Targeted	Forward Primer	Reverse Primer	Unit Length (bp)	Reference
Oprl	5'-ATG AAC AAC GTT CTG AAA TCT GCT-3'	5'-CTT GCG GCT GGC TTT TTC CAG-3'	209 bp	(Douraghi *et al*., 2014)
OprL	5'-ATG GAA ATG CTG AAA TTC GGC-3',	5'-CTT CTT CAG CTC GAC GCG ACG-3'	504 bp	
PA-SS	5'-GGG GGA TCT TCG GAC CTC A -3'	5'TCC TTA GAG TGC CCA CCC G -3'	956 bp	
blaOXA	5'-GCG TGG TTA AGG ATG AAC AC -3'	5'-CAT CAA GTT CAA CCC AAC CG -3'	438	(Poirel *et al*., 2011)
blaKPC	5'-CGT CTA GTT CTG CTG TCT TG-3'	5'-CTT GTC ATC CTT GTT AGG CG-3'	798	
blaNDM	5'-GGT TTG GCG ATC TGG TTT TC-3'	5'-CGG AAT GGC TCA TCA CGA TC-3'	621	
blaIMP	5'-GAA GGA GTT TAT GTT CAT AC-3'	5'-GTA CGT TTC AAG AGT GAT GC-3'	587	(Pitout *et al*., 2005)
blaVIM	5'-GTT TGG TCG CAT ATC GCA AC-3'	5'-AAT GCG CAG CAC CAG GAT AG-3'	382	

Table 2: Percentage prevalence of *Pseudomonas aeruginosa* in various types of wounds

Source of Sample	Total No of Sample	Positive for MDR *P. aeruginosa*	Percentage of Positive samples
Burn Wound	178	43	36%
Accidental Wound	139	39	32%
Pus Wound	142	33	27%
Surgical Wound	73	04	03%
Statistically Significant at (P<0.05)			

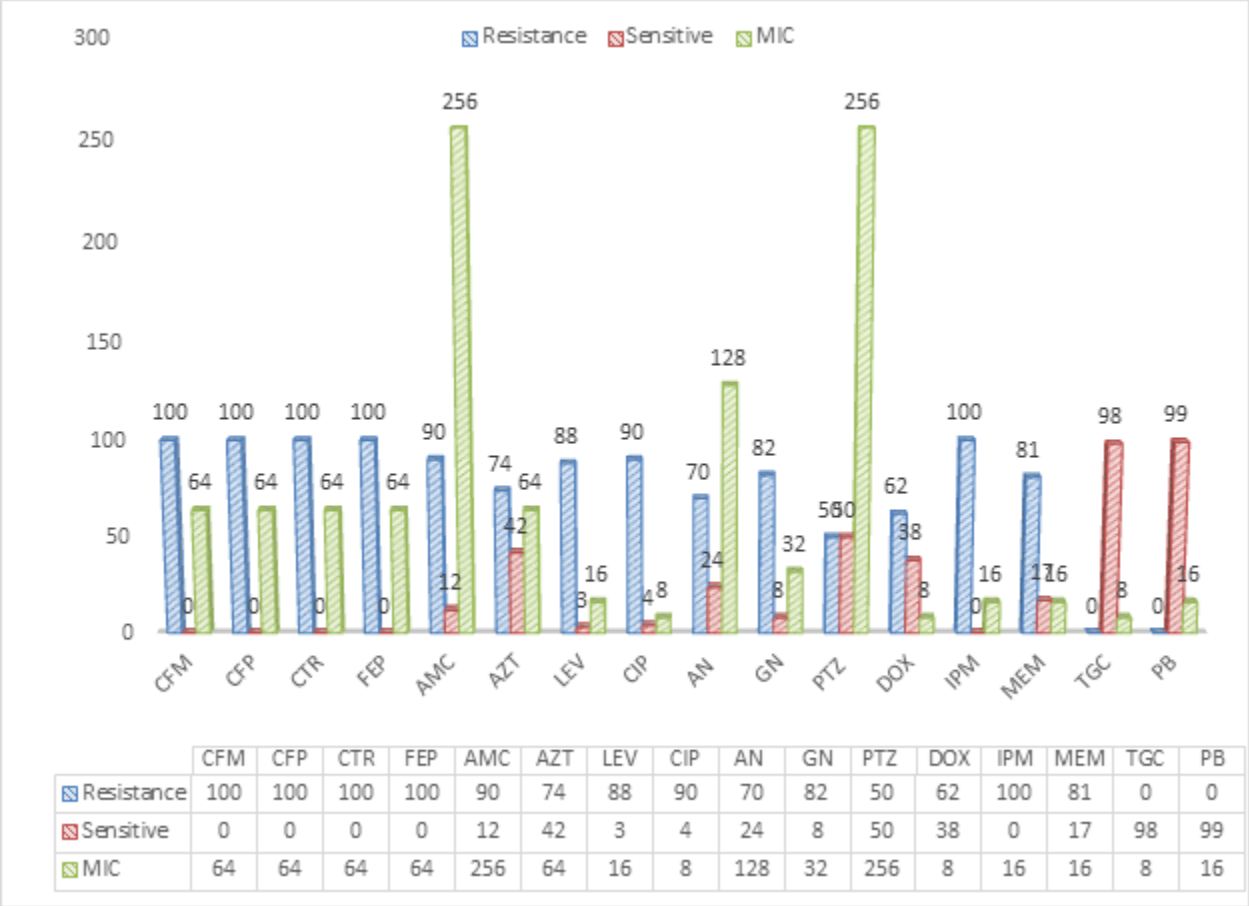
Table 3: percentage prevalence of β-lactams producing *Pseudomonas aeruginosa*

Name of Genes	Positive sample numbers	Percentage prevalence
blaOXA	05	9.5%
blaKPC	01	1.8%
blaNDM	14	25.9%
blaIMP	06	11.11
blaVIM	09	16.5%

DISCUSSION

One of the most important pathogens that require minimal requirement for growth is *Pseudomonas aeruginosa* and it may survive on various types of surfaces. The *P. aeruginosa* are opportunistic pathogens and it has greatest concern for infections in the various types of wounds, immunocompromised patients, and intensive-care units' patients (ICUs) (Bukholm *et al.,* 2002). The main objective in the present study was to estimate the prevalence of MDR *P. aeruginosa* in the different types of wounds along with the identification of the isolates based on species-specific primers through PCR. In the current study we used the species-specific primers including OprL, Oprl and PA-SS for the identification of the *P. aeruginosa* against all the isolates and 203 isolates were confirmed *P. aeruginosa*. In the past a study was conducted in Iran and they also used the OprL and Oprl species specific primers for the identification of the *P. aeruginosa* (douraghi *et al.,* 2014).

In the present study significant prevalence (38%) of *P. aeruginosa* was estimated in the various types of wound. In comparison with the previously conducted study Prevalence of *P. aeruginosa* is slightly high in the present study due to high contamination, insanitary hospital environment and inappropriate management of the wounds. Almost similar results were found in a study conducted in the past which showed the 32% of positive isolates of *P. aeruginosa* (Anupurba *et al.,* 2006). In another study same results were recorded with the 33.3% prevalence of *P. aeruginosa* (Turner *et al.,* 2014) in wound patients, while 27.78% prevalence of *P. aeruginosa* was estimated by Hani and his colleagues from wound patients.



	CFM	CFP	CTR	FEP	AMC	AZT	LEV	CIP	AN	GN	PTZ	DOX	IPM	MEM	TGC	PB
Resistance	100	100	100	100	90	74	88	90	70	82	50	62	100	81	0	0
Sensitive	0	0	0	0	12	42	3	4	24	8	50	38	0	17	98	99
MIC	64	64	64	64	256	64	16	8	128	32	256	8	16	16	8	16

Fig. 1: Antibiotic susceptibility pattern of different antibiotics against *Pseudomonas aerugenosa*

Current findings suggested that Multidrug-resistant (MDR) *P. aeruginosa* are frequently found in the various types of infectious wounds and its highest percentage recorded in the burn wound patients (36%) and least in the surgical wound patients (3%). In the present study percentage of MDR *P. aeruginosa* in burn wound patients is high due to miss management, ability of the *P. aeruginosa* to colonize in tissue, negligence of the patients and unhygienic environment of the hospital. A study was conducted earlier which estimated the prevalence of MDR *P. aeruginosa* in burn wound patients is much greater than other wounds and it dependent on age, sex, and duration of the stay in hospital (Ahmed, 2016; Buhl *et al*., 2015).

As the definition of Multi Drug Resistant (MDR) bacteria are those who show resistance to minimum two specific representative of minimum two classes of antibiotics (Park *et al*., 2011). *P. aeruginosa* was found resistant to various antibiotics which were used in the current study like Cefixime, Cefoperazone, Ceftrizone, Cefepime, Amoxicillin-clavulanate, Aztreonam, Levofloxacin and Ciprofloxacin, while *P. aeruginosa were* found sensitive to other antibiotics including Tigecycline and Polymyxins B. It may be due to the reasons of inappropriate use of antibiotics, mutation in *P. aeruginosa*, genetic background of the organism and environment condition of the specific region. Same studies were conducted all around the globe, in Russia 75% isolates were found resistance to gentamycin (Montero *et al.*, 2009) while a study reported from Bangladesh revealed that 49% of *P. aeruginosa* were sensitive to Tobramycin and 79% to ciprofloxacin (Ansary *et al.*, 1994).

The resistance of ESBL and MBL against *P. aeruginosa* carbapenems have reached frightening level. The resistance in *P. aeruginosa* mainly produced by different mechanisms including enzymes, efflux pumps and porin loss. The results of present study revealed that *P. aeruginosa* were highly resistance against β-lactam drugs, same results were found in study that was conducted in China which demonstrated that *P. aeruginosa* showed 100% resistance to β-lactam drugs (Wang *et al*., 2010). Another study was reported from Pakistan which revealed that clinical isolates of *P. aeruginosa* were found 100% resistant to cephalosporins (Khan *et al*., 2014). Two more studies were conducted in the past which reported the same results, they isolated 100% and 87% resistant *P. aeruginosa* against carbapenems from various clinical samples (Rodriguez-Martinez *et al*., 2009, Hirsch and

Tam, 2010). The reason of high resistance may be illogical, irregular and one-self use of antibiotics in our zone. Various ESBL genes which were observed include blaNDM, blaVIM, blaIMP, blaKPC and blaOXA with following percentage prevalence 25.9%, 16.5%, 11.22%, 1.8% and 9.5% respectively. Similar results were found in a study which was reported from India, they found 18.6% prevalence of bla_{VIM} in *P. aeruginosa*. In Pakistan, data on β-lactam producing *P. aeruginosa* is limited. However, one study from Islamabad recorded 25% prevalence of β-lactam producing *P. aeruginosa.* In clinical settings we observed various malpractices regarding infection management during the study. Key associated risk factors in local health care settings which play a vital role in the transmission of resistant pathogens include inadequate sterilization facilities, negligence of medical personals and poor sanitization.

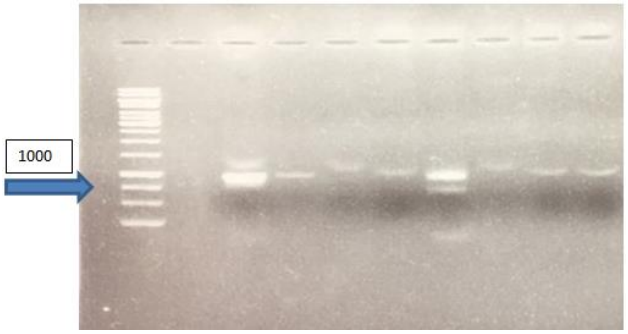


Fig. 2: PCR based detection of PA-SS rDNA genes (956bp). Lane 1: 100bp ladder, Lane 2: Negative Control, Lane 3-10 Positive samples for *P. aeruginosa*

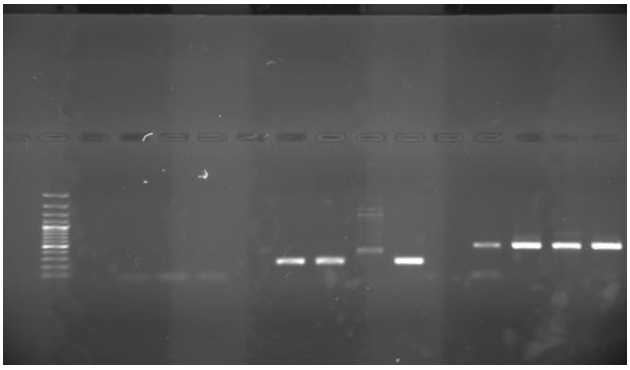


Fig. 3: PCR based detection of *oprL* (504) and *oprl* genes (249bp). Lane 1: 100bp ladder, Lane 2: Negative Control, Lane 3-6 Negative Samples Lane 7-10 Positive samples for *oprl* genes confirming *Pseudomonas,* Lane 11-14 Positive samples for *oprL* confirming *Pseudomonas aeruginosa*

Taking together it is concluded that, in the proposed study significant prevalence of *MDR P. aeruginosa* in hospitalized wound patients was estimated. Irrational use of antibiotics, unhygienic environment and practices from health associated personals were found as the influencing factors which may increase the prevalence of *MDR P. aeruginosa* in hospitalized patients. To restrain the dissemination and distribution of *MDR P. aeruginosa* in hospital settings a comprehensive control strategy should be crafted and implemented.

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