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Resistance profile of genetically distinct clinical *Pseudomonas aeruginosa* isolates from public hospitals in central Pakistan

Sidra Saleem, Habib Bokhari *

Department of Biosciences, COMSATS University, Islamabad, Pakistan

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ABSTRACT

Introduction: Pseudomonas aeruginosa (member of ESKAPE group) is predominantly responsible for emerging nosocomial infections and poses serious health concern due to ever-increasing drug resistance trends. The current study investigates the prevalence of such highly resistant *P. aeruginosa* in major hospital settings and further characterizes and compares them for genetic heterogeneity.
Materials and methods: Samples of patients (n = 108) with wound infections, bacteremia and burn injuries from major hospitals of Rawalpindi and Islamabad during 2017 to 2018 were collected for the present study. The samples were processed in the COMSATS Microbiology and Public Health lab and screened for the *P. aeruginosa* by routinely used biochemical tests, drug susceptibility tests and rapid molecular approaches.
Results: The results suggested that most of the isolates (88/108) are indeed *P. aeruginosa* (81.4%) underpinning the need of its active surveillance in hospital settings. Further analysis suggested that 32 of these 88 microbes are multi-drug resistance (36.3%), 16 (18.1%) are extensively drug resistance and 4 (4.5%) are pan-drug resistance. Moreover, double disc synergistic test suggested that 16 (18.1%) are positive for metallo-β-lactamase production. Molecular screening confirmed that 2 (12.5%) and 3 (18.75%) of these 16 isolates are positive for VIM and NDM gene respectively while all the studied isolates were positive for AmpC β-lactamase. PAP17 isolate harbors both VIM and NDM genes. ERIC PCR profiling showed that majority of MDR bacteria fall in cluster II and III similarly XDR bacteria also fall in cluster II and III while PDR bacteria fall in cluster IV.
Conclusion: This study revealed that majority of the isolates are multi drug resistant MDR and extensively drug resistant (XDR). However, the presence of some pan drug resistant (PDR) isolates among such small sample size screened is of utmost concern. Molecular typing of extremely resistant *P. aeruginosa* revealed high genetic diversity. Therefore, we suggest that regular monitoring and surveillance of such highly resistant *P. aeruginosa* in hospital settings will help to control their transmission and hence reduce the disease burden.

Introduction

Multi-drug resistant (MDR) bacterial infections among hospitalized patients are currently one of the most serious global concerns and results in high mortality rate and rising hospital costs. According to World health organization and US centers for disease control and prevention (CDC), ever rising antimicrobial resistance is an imminent threat to human health taking us back to pre-antibiotic era [1,2]. There are number of bacterial pathogens which have been labelled as extremely drug resistant pathogens and among these the members of ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*) are of significant importance in connection with the emerging nosocomial infections. *P. aeruginosa* is one of the most important members of ESKAPE group and it frequently acquires resistance traits as well as transfer it due to horizontal gene transfer (HGT) and hence such population of *P. aeruginosa* successfully 'escape' antibiotics treatment [3,4]. Since last decade carbapenems (imipenem and meropenem) have been used as an effective treatment for *P. aeruginosa* infections, however, during the last few years bacteria acquired resistance to these

Abbreviations: PBS, phosphate buffer saline; EDTA, ethylene diamine tetra acetate; MBL, metallo beta lactamase; MDR, multi drug resistance; GIM, German imipenemase; IMP, imipenem; NDM, New-Delhi metallo-β-lactamase; SIM, Seoul imipenemase; SPM, Sao Paulo metallo-β-lactamase; VIM, Verona integron-encoded metallo-β-lactamase.

* Corresponding author.
E-mail addresses: Sidrasaleem65@yahoo.com (S. Saleem), habib@comsats.edu.pk (H. Bokhari).

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antibiotics [5] and more recently, colistin or polymyxin B are considered as a last resort for the treatment of XDR *P. aeruginosa* [6], however, resistance to colistin has also been reported recently [7,8].

β-lactams based antibiotics, being broad host spectrum and less toxic, penetrate the gram-negative bacteria through outer membrane channel proteins called porins are considered as a relatively safe option for treatment [9,10]. *P. aeruginosa* exhibits antibiotic resistance by three different mechanisms i.e. Intrinsic resistance (presence of AmpC β-lactamase, MexAB-OprM & MexXY-OprM efflux pump), acquired resistance (overexpression of AmpC β-lactamase and Mex efflux pump system due to certain mutations in regulatory factors and plasmid mediated resistance), and adaptive resistance (unstable form of resistance due to sustained antibiotic pressure) [11–13]. *P. aeruginosa* acquires resistance to a wide range of β-lactams by producing β-lactamases enzymes and more specifically due to the production of metallo-β-lactamases (MBLs): IMP (Imipenem), VIM (Verona integron-encoded imipenemase), SPM (Sao Paulo imipenemase) and GIM (German imipenemase) [14]. These MBLs hydrolyze antipseudomonal cephalosporins and carbapenems effectively and their activity is not suppressed by the most commercially available existing β-lactamase inhibitors [15]. The prevalence of IMP, VIM and NDM-1 has increased significantly throughout the world, specifically in Southeast Asia including Pakistan [16]. These genes mostly spread by mobile elements such as integrons that reside on the plasmid or on genomic DNA of bacteria [17].

In current study, we screened 108 samples (for the presence of *P. aeruginosa*) gathered from hospitalized patients and patients (with pus wound, bacteremia, and ear infection) visiting the associated diagnostic laboratories during the period of one year i.e. 2017–2018. The informed consent was obtained as per standard procedure from the patients. *P. aeruginosa* isolates this obtained were further subjected to detailed characterization for their resistance properties and gene conferring resistance and for their possible epidemiological association or link using simple and cost-effective ERIC-PCR approach.

Materials and methods

Isolation and characterization of P. Aeruginosa

The samples for the current study were collected after necessary approval from ethical committee of Department of Biosciences, COMSATS University during the period of 2017–2018 from the major hospital settings and associated laboratories (Rawalpindi & Islamabad). Out of 88 samples 66 (61.1%) samples were cultured from pus and wounds, 13 (12.03%) from blood, 1 (0.92%) from urine and 8 (7.4%) from catheter tips (Supplementary table). To trace the possible transmission source 20 samples from Operation Theatres, wards and water were also collected from respective hospital settings and processed as per standard microbiological procedures. Colony morphology and fluorescence production was determined on Pseudomonas Cetrimide agar (Oxoid, Basingstoke, UK), structural identification (shape and cell wall) was determined by gram staining and recommended biochemical tests i.e. Oxidase test, Catalase test, Indole test, Simmon's citrate test and gelatin liquification [18].

DNA extraction

For molecular identification DNA was extracted with the help of Wiz bio gDNA tissue/ bacteria/ blood isolation kit (W17000-000, Wizbiosolutons Inc, Korea). Nano drop (Berthold Detection System GmbH, Germany) was used to quantify the concentration of DNA and it was ran on 0.8% agarose gel to further check its integrity.

Molecular identification

Molecular identification of *P. aeruginosa* on genus and species level was performed using PCR by employing specific primer sets i.e. *OprI* (lipoprotein I) and *OprL* (lipoprotein L)(Table 1) as reported previously [18]. *P. aeruginosa* ATCC 27853 was used as a reference strain. The PCR products were visualized using UV transilluminator (Bio-Rad Laboratories, Inc. USA) after running them on 2% agarose gel.

Screening of drug resistant P. Aeruginosa

Bacterial isolate that acquired resistance to at least one agent in two or three categories of antimicrobial agents are considered as multi drug resistance bacteria (MDR). Similarly, XDR was defined as bacterial isolates that remain susceptible to at least one or two antimicrobial categories whereas PDR was defined as non-susceptible to all the agents in all antimicrobial categories [19]. Kirby Bauer disc diffusion method was used for the susceptibility of antibiotics against *P. aeruginosa* isolates [20,21]. *P. aeruginosa* ATCC 27853 was used as a reference strain. CLSI and EUCAST 2017 recommended drugs were used for screening all *P. aeruginosa* and following antibiotic impregnated discs were used: Ciprofloxacin (CIP: 5 μg), Ceftazidime (CAZ: 30 μg), Aztreonam (ATM: 30 μg), Imipenem (IMP: 10 μg), Tigecycline (TGC: 15 μg), Amikacin (AK: 30 μg), Colistin (CT: 10 μg).

IMP-EDTA (Ethylenediaminetetraacetic) disc diffusion method

Metallo- β-lactamase production was confirmed by IMP-EDTA double disc synergistic test. Pure isolated CFU of bacteria was suspended in PBS (Phosphate buffer saline) and turbidity value set to 0.5 McFarland's standard. Inoculum prepared in PBS was plated on freshly prepared Muller-Hinton agar (Oxoid, Basingstoke, UK) with the help of sterile cotton swab to make a lawn plate [21]. Two imipenem (IMP: 10ug) discs were placed at the distance of 20 cm on bacterial lawn and 0.5 μl (0.5 M) EDTA was added to one of the two imipenem disc and incubated for 18 h at 37 °C. Ethylenediaminetetraacetic EDTA serves as chelating agent by removing zinc ions from active site of metallo-β-lactamase. Ethylenediaminetetraacetic treatment conferred sensitivity to *P. aeruginosa* against carbapenems. After 18 h of incubation at 37°C zone diameter was measured for both imipenem alone and imipenem-EDTA, (in comparison to reference strain ATCC 27853), difference in zone diameter ≥7 mm was considered as MBL positive isolates [22].

Multiplex PCR for class B metallo-β-lactamases

For class B metallo-β-lactamases (MBL) detection, 16 shortlisted carbapenem resistance and Imipenem-EDTA positive isolates were used. Multiplex PCR for detection of class B metallo-β-lactamases (MBL) (IMP, VIM, SPM, SIM and GIM) was employed as described previously [23,24]. In house available well characterized *K. pneumoniae* BK1 was used as positive control [25]. Similarly, PCR for a new subgroup of MBL gene New Dehli metallo-β-lactamase was performed for screening all phenotypically resistant *P. aeruginosa* isolates [26]. In house available *K. pneumoniae* NK2 is used as positive control [25]. The PCR products were separated using 2% agarose gel and primers (for each family) used during current study are shown in Table 1.

PCR for blaampC *and* oprD *genes*

Gene specific PCR for *blaampC* (AmpC β-lactamase), and *oprD* (gene that encode outer membrane channel proteins) that facilitate the entry of antibiotics inside bacterial cell), was performed for

Table 1
List of primers used during current study.

Sr. no	Gene		Primer	Product size	Reference
1.	OprI	F R	5′-ATGAACAACGTTCTGAAATTCTCT -3′ 5′- CTTGCGGCTGGCTTTTTCCAG -3′	249 bps	Douraghi et al. [19]
2.	OprL	F R	5′- ATGGAAATGCTGAAATTCGGC -3′ 5′- CTTCTTCAGCTCGACGCGACG -3′	504 bps	Douraghi et al. [19]
3.	IMP	F R	5′- GGAATAGAGTGGCTTAAYTCTC-3′ 5′- CCAAACYACTASGTTATCT-3′	188 bps	Ellington et al. [23]
4.	VIM	F R	5′- GATGGTGTTTGGTCGCATA-3′ 5′- CGAATGCGCAGCACCAG-3′	390 bps	Ellington et al. [23]
5.	GIM	F R	5′- TCGACACACCTTGGTCTGAA-3′ 5′- AACTTCCAACTTTGCCATGC-3′	477 bps	Ellington et al. [23]
6.	SPM	F R	5′- AAAATCTGGGTACGCAAACG -3′ 5′- ACATTATCCGCTGGAACAGG-3′	271 bps	Ellington et al. [23]
7.	SIM	F R	5′- TACAAGGGATTCGGCATCG-3′ 5′- TAATGGCCTGTTCCCATGTG -3′	570 bps	Ellington et al. [23]
8.	NDM-1	F R	5′- GGGCAGTCGCTTACGGT-3′ 5′- GTAGTCCTCAGTGTCGGCAT-3′	477 bps	Shenoy et al. [26]
9.	AmpC	F R	5′-CTTCCACACTGCTGTTCGCC -3′ 5′-TTGGCCAGGATCACCAGTCC-3′	1063 bps	Rodríguez-Martínez et al. [24]
10.	OprD	F R	5′-GCTCGACCTCGAGGCAGGCCA -3′ 5′-CCAGCGATTGGTCGGATGCCA-3′	242 bps	Rodríguez-Martínez et al. [24]

screening all phenotypically resistant *P. aeruginosa* isolates [24,27]. *P. aeruginosa* PAO1 was used as a positive control for *oprD* gene. The PCR products were visualized under the gel doc system after running on 2% agarose gel. Primers used in current study are listed in Table 1.

ERIC PCR for species diversity

For studying species diversity among *P. aeruginosa* isolates, ERIC PCR was performed for 23 MDR, XDR and PDR isolates by using following set of primers: ERIC PCR, (ERIC1: 5′-ATGTAAGCTCCTGGGGATTCAC-3′ & ERIC2: 5′-AAGTAAGTGACTGGGGTGAGCG-3′) [28]. Each PCR reaction mixture contains DNA (6 μl), primer each (2 μl), wiz bio 2X master mix (12 μl), and PCR water (5 μl). The PCR products were separated on 2% agarose gel and visualizes as above. The clustering was done by using software DendroUPGMA (genomes.urs.cat/UPGMA) [29,30].

Results

Isolation and characterization of P. Aeruginosa

Out of 108 samples all 88 clinical samples were positive for *P. aeruginosa*, while none of environment sample (collected from operation theatre, ventilator, water samples, ward samples) were positive on selective media i.e. Pseudomonas Cetrimide agar. All 88 samples were cetrimide, oxidase, catalase, and simmon's citrate positive. All samples liquefied gelatin and have shown motility in Sugar, Indole, and Motility (SIM) medium while shown negative results for indole and hydrogen sulfide production.

Molecular identification

PCR results confirmed *OprI* and *OprL* amplicon of predicted size in all *P. aeruginosa* isolates (Fig. 1).

Screening of drug resistant P. Aeruginosa

Antibiotic susceptibility testing of all (88) isolates of *P. aeruginosa* suggested that majority of isolates (73%) were sensitive to ciprofloxacin, followed by amikacin (57%), imipenem (57%), and colistin (56%) respectively. However, noticeable resistance was observed against most commonly used drugs like ceftazidime (98%) and in particular to tigecycline (94%) (Fig. 2).

IMP-EDTA (Ethylenediaminetetraacetic acid) disc diffusion method

The results suggested that 16 out of 26 isolates have shown ≥ 15 mm zone of inhibition with EDTA containing imipenem discs, while plain imipenem disc showed no zone of inhibition and were positive for MBL production. 10 isolates have shown ≤7 mm zone difference with EDTA-IMP discs as compared to imipenem alone. These 10 isolates were considered as MBL negative isolates.

Multiplex PCR for class B metallo-β-lactamases

Multiplex PCR results revealed that only 2 isolates were positive for VIM gene whereas none of isolate was positive for IMP (188 bps), GIM (477 bps), SPM (271 bps) and SIM (570 bps) (Fig. 3). PCR for NDM gene revealed that only 3 isolates were positive for NDM-1 gene (477 bps). The results are shown in (Fig. 3).

PCR for blaampC *and* oprD *genes*

The presence of *blaampC* gene and *oprD* genes in all studied isolates were confirmed by PCR i.e. amplification of 1068 bps for Amp-C lactamase gene and 249 bps for OprD gene respectively (Fig. 4).

ERIC-PCR

ERIC-PCR results have shown the distinct patterns of DNA reflecting species diversity of *P. aeruginosa* among pus wound infections, burn and bacteremia cases. Phylogenetic clustering suggested the presence of four distinct clusters from I-IV. While majority of isolates are present in cluster III with total 11 isolates and 6 are present in cluster II, 5 isolates share cluster IV and 1 isolate is present in cluster I (Fig. 5). Cluster II are majorly occupied by MDR isolates. XDR bacteria fall in cluster II and III and PDR bacteria fall in Cluster IV.

Discussion

The gram–negative bacterium *P. aeruginosa* is an opportunistic human pathogen responsible for many types of infectious diseases, particularly affecting the immuno-compromised individuals and more specifically found in association with the patients with pulmonary tract, urinary tract, burns, wounds, and blood infections

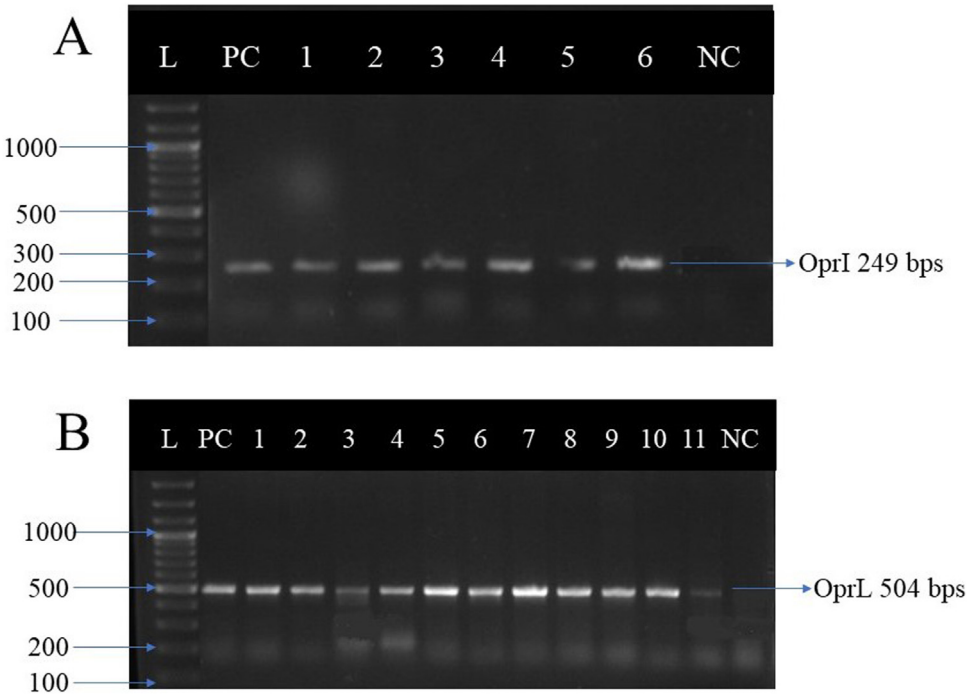
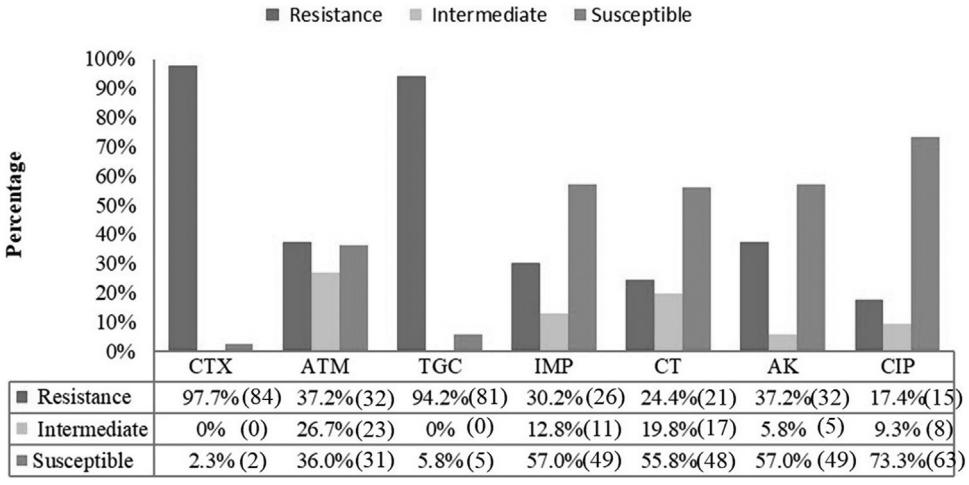


Fig. 1. PCR based identification of *Pseudomonas aeruginosa* at genus and species level. (A) OprI 249 bps amplification for genus identification, (B) OprL 502 bps fragment amplification shown species identification. L represent 100 bps ladder, PC (positive control *P. aeruginosa* ATCC 27853), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 are different isolates of *P. aeruginosa,* NC is negative control.



	CTX	ATM	TGC	IMP	CT	AK	CIP
Resistance	97.7%(84)	37.2%(32)	94.2%(81)	30.2%(26)	24.4%(21)	37.2%(32)	17.4%(15)
Intermediate	0% (0)	26.7%(23)	0% (0)	12.8%(11)	19.8%(17)	5.8% (5)	9.3% (8)
Susceptible	2.3% (2)	36.0%(31)	5.8%(5)	57.0%(49)	55.8%(48)	57.0% (49)	73.3%(63)

Fig. 2. Antibiogram showing the percentage activity of different antibiotics against *P. aeruginosa* isolates.

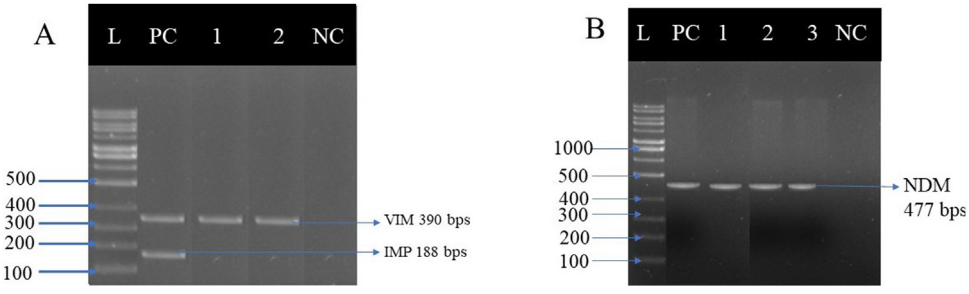


Fig. 3. (A) Metallo-beta-lactamase (*blaIMP, blaSPM, blaGIM, blaVIM* & *blaSIM*) screening through multiplex PCR revealed the presence of 390 bps *blaVIM* gene in 2 isolates, 1 = PAP17 & 2 = PAP21. (B) PCR amplification of 477 bps fragment indicates the presence of bla^{NDM} gene 3 isolates, 1 = PAH4, 2 = PAT1 and 3 = PAP17, isolates PAP17 shows co-existences of VIM and NDM.

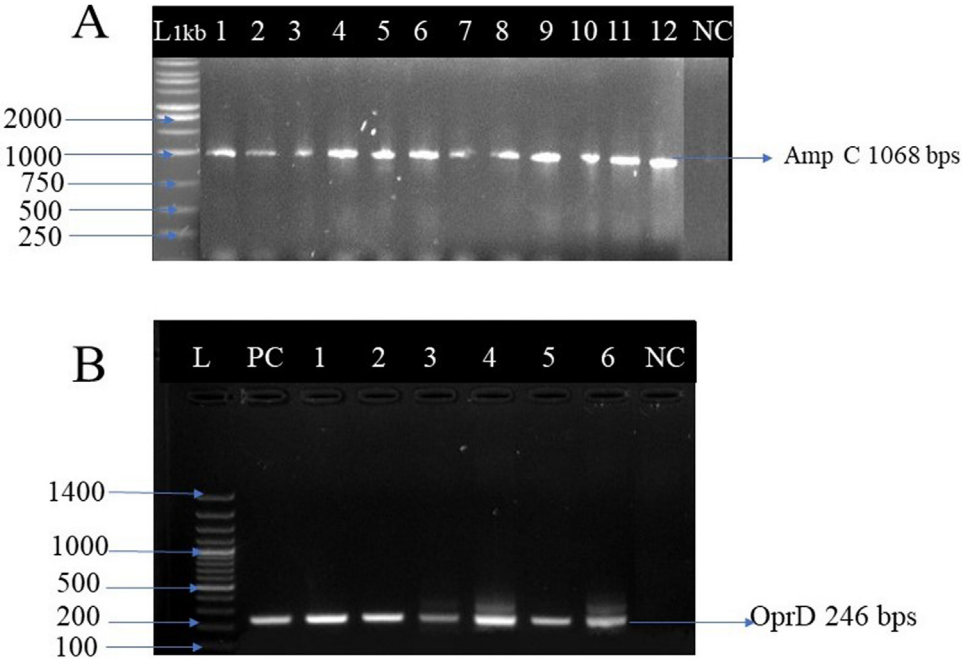


Fig. 4. (A) Amp-C lactamase gene (1068 bps) amplified from various *P. aeruginosa* isolates, NC (negative control). (B) PCR for OprD (outer membrane protein) has shown the presence of 242bps amplified product. PC (positive control *P. aeruginosa* PAO1), 1, 2, 3, 4, 5, 6, 7 are different isolates of *P. aeruginosa*, NC is negative control.

[31]. The major public health concern is to find suitable treatment for *P. aeruginosa* infections keeping in view the alarming trends of developing drug resistance against multiple antibiotics, leading to higher prevalence of MDR, XDR and PDR isolates. To treat MDR and XDR isolates carbapenems (imipenem, meropenem) are most effective drugs but unfortunately bacteria developed resistance against these drugs mainly due to their inappropriately excessive use [5,12]. This is evident by the fact that current study has shown that 4 out of 88 clinical isolates were PDR, 16 isolates were XDR and 32 isolates were MDR. Moreover, all isolates are 100% resistant to macrolides class of antibiotics while 90% isolates are resistant to cephalosporins and tetracycline antibiotics. Alarmingly, 30% and 17% isolates have shown resistance to carbapenem and fluoroquinolones, respectively. Keeping in view the previously reported trends from the region [32–35], the current study has shown increasingly high frequency of prevailing MDR isolates.

Moreover, there was not a single study from Pakistan where the susceptibility data was correlated with the molecular data and with the subsequent genetic diversity of circulating *P. aeruginosa* and hence to elucidate any epidemiological links (transmission patterns). We employed simple molecular typing method ERIC-PCR [28] to find out the genetic relatedness among extremely resistant pathogens and to study how they compare with each other. ERIC-PCR, a PCR based technique was previously used for studying *P. aeruginosa* endemicity and being rapid and cost-effective approach, it can be used in resource deficient facilities and can also give some clues for the possible transmission patterns of drug resistant pathogens [29,30].

There is an increased risk that patients with PDR, XDR or MDR *P. aeruginosa* infections get inappropriate antibiotic treatment, and delay in well targeted treatment may also lead to harmful effect on patient recovery. The pattern of resistance varies from place to place and region to region, so it is recommended that antibiotics used for the treatment of infection are selected based on local susceptibility data or more specifically based on susceptibility data of patient's specific culture [36]. Hence, in some circumstances antibiogram can also be used to compare the relatedness among isolates [37].

Epidemiological typing for diversity analysis by ERIC-PCR revealed the occurrence of four distinct clusters among *P. aeruginosa* isolates with majority of isolates belonging to cluster II, III and IV. Epidemiological typing profiles during current study have indicated that these isolates did not share much genetic relatedness and suggested independent origins of dissemination like previous reports [29,30]. Nineteen isolates from pus wound infection are scattered among all four clusters. Out of these nineteen isolates majority of isolates resides in cluster II and III. While from three blood samples, one sample show genetic similarity with pus wound isolates and share a same branch on the sub-cluster of cluster III and remaining two belongs to different branches of same cluster. Cluster IV is occupied by three PDR isolates while one PDR isolate reside on cluster III. One isolate in cluster I and two isolates in cluster IV are XDR and three Isolates from cluster II and seven isolates from cluster III belonged to XDR group while rest of the isolates present at cluster II and III are MDR pathogens. Surprisingly, according to National AMR Action Plan for Pakistan 2017–2018, it is stated that *P. aeruginosa* have shown less resistance to carbapenems (6.5%) as compared to *K. pneumoniae* (30%) whereas current study based on small dataset suggests that resistance trends for *P. aeruginosa* are alarmingly than expected (https://www.nih.org.pk/wp-content/uploads/2018/08/AMR-National-Action-Plan-Pakistan.pdf).

Increased resistance to β-lactam antibiotics in case of nosocomial infections is a serious health crisis and different mechanisms are involved in resistance development. This includes acquisition of *bla* genes in the genome of bacteria through horizontal gene transfer resulting in production of enzymes that degrade the structure of β-lactam antibiotics [38]. Class B metallo-β-lactamases are most important class of β-lactamases and in this class bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SPM} and bla_{SIM} genes are included [23]. The current investigation revealed significant number of isolates positive for MBL production i.e. 16 isolates were found positive for MBL production out of 26 carbapenem resistant isolates by EDTA double disc synergistic test.

Genotypic detection of carbapenem positive isolates for MBL genes revealed that 2 out of 16 isolates were positive for VIM, however 14 isolates were negative for VIM and other metallo-β-

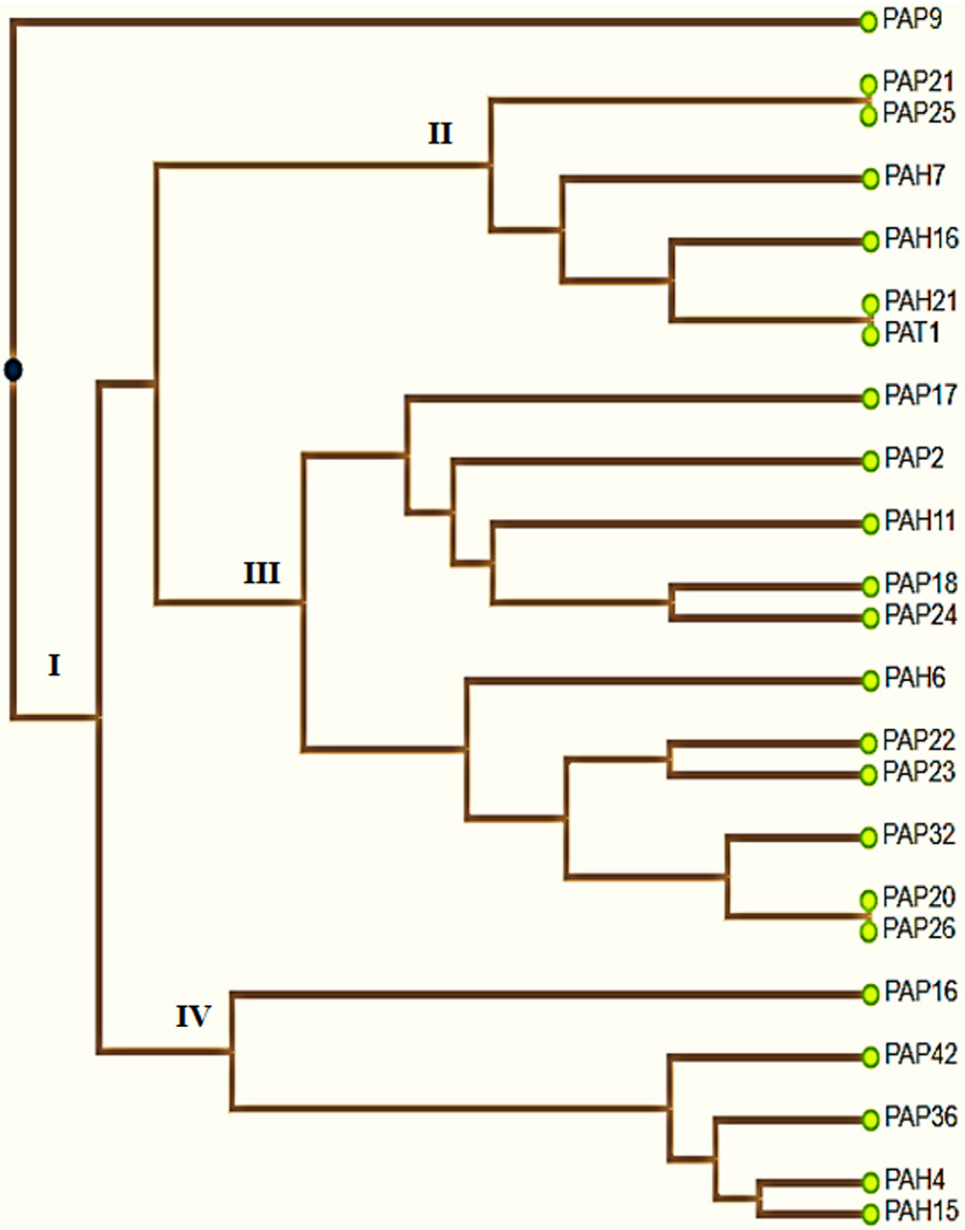


Fig. 5. Dendrogram based on UPGMA cluster analysis of ERIC-PCR. The names of the analyzed strains are mentioned with their phylogenetic groups mentioned as (I, II, III, and IV).

lactamases i.e. IMP, GIM, SPM, and SIM. Similarly, only 3 isolates were positive for NDM gene among 16 isolates screened. The current study has shown less prevalence of MBL genes among *P. aeruginosa* isolates when compared with some of other recent studies from India (12.5% and 10.3%) and China (35.1%) [38–40]. In the absence of high prevalence of metallo-β-lactamases it is stated that AmpC β-lactamase is the possible source of resistance to beta-lactam antibiotics [13]. Since AmpC β-lactamase acts as antagonistic to *oprD* gene (encode outer membrane protein) as increased production of AmpC β-lactamase negatively influence the outer membrane channels in the outer membrane of bacteria. These are the channels in the membrane of bacteria that facilitate the entry of carbapenem antibiotics [11]. It is known that when production of AmpC β-lactamase production increases under antibiotics stress it reduces the number of (*oprD* gene) channels in the outer membrane, as a result bacterium show resistance to β-lactam antibiotics [27].

The recently rapidly rising trends in resistance against wide variety of antibiotics seen among variety of *P. aeruginosa* isolates are mainly due to indiscriminate use of antibiotics to treat different kinds of infections ranging from bacteremia, ear infections, burn patients, trauma and surgical patients [41,42]. Moreover, the transmission of such highly resistant pathogens within the hospital facility and its different wards can further complicate the control of infection. Hence, rapid and cost-effective epidemiological investigations in resource deficient facilities may help in tracking highly resistant *P. aeruginosa* outbreaks and developing effective surveillance and control strategies.

In this study, we used ERIC-PCR to find the genetic diversity of such *P. aeruginosa* isolates and the results of our findings suggest that the genetically diverse isolates are associated with different sources or isolation sites. We also found pan-drug resistance (PDR) and extensively drug resistance (XDR) isolates of *P. aeruginosa* along with a high percentage of MDR that is an alarming situation

and demands a public health emergency to deal with the emergence of such superbugs. Moreover, we identified that 2 (2.22%) *P. aeruginosa* isolates were positive for metallo-β-lactamase while 3 (3.48%) were positive for New Delhi metallo-β-lactamase. Fortunately, bla_{NDM}, bla_{VIM}, bla_{IMP} gene was not detected in all isolates. Our study is one of the few reports regarding prevalence of *P. aeruginosa* from Pakistan and first report where the distribution of the resistance pattern along with the genetic diversity was evaluated among patients from tertiary care hospital settings. The study suggested that majority of extremely drug resistance isolates are circulating among pus wound infections while some of them belong to bacteremia and ear infections. Moreover, the genetic elements such as AmpC β- lactamase, and MBL were screened and were mostly seen in association with *P. aeruginosa* members present in cluster III with 54.54 and 4.54 percentage of occurrence respectively. Furthermore, the genetic diversity study in general reflected that these MDR isolates were highly prevalent among *P. aeruginosa* isolates seen in cluster III underpinning the need for further investigation of their pathogenic potential. To best of our knowledge this is the first report from Pakistan where the phenotypic drug resistance traits as well as genetic traits such as AmpC β-lactamase were investigated and correlated with the prevalent types of *P. aeruginosa.* However, status of other element such as Class A -β-lactamases and Class D -β-lactamases like TEM, SHV, CTX, and OXA etc. will further help in understanding the resistance spectrum in association with clinically circulating *P. aeruginosa*.

Conclusion

The ever-increasing antibiotic resistance among *P. aeruginosa* ranging from different sources has been demonstrated in the current study and incidences of patients infected with XDR and PDR isolates are also reported. However intrinsic resistance is more prevalent among *P. aeruginosa* as compared to acquired resistance as we found all isolates carried AmpC-lactamase as compared to metallo-β-lactamases (VIM, GIM, IMP, SPM, SIM and NDM). This is the first study from this region that not only have shown the presence of metallo- β-lactamases among *P. aeruginosa* which predominantly are not only extremely drug resistant due to metallo-β-lactamase producing *bla* MBL genes but also are genetically diverse. This is certainly a growing public health concern and poses a serious threat for widespread transmission of antibiotic resistance encoding genes to other less resistant or susceptible community of *Pseudomonas aeruginosa* or other bacterial pathogens. It is envisioned that in future epidemiological studies, genotype data along with the spectrum of drug resistance will be more tightly linked to understand the dissemination patters of such highly drug resistant pathogens. However, expanding this to other hospital settings will surely help in clearly understanding the disease burden due to extremely resistant *P. aeruginosa* in Pakistan.

Consent for publication

Patient's consent was taken for publication of information about them.

Availability of data and materials

All data and materials of this research are available.

Authors' contributions

HB conceived and designed the experiments. SS performed the experiments and wrote the manuscript. SS and HB analysed the data.

Authors information

SS is MS research student at Microbiology Lab; and HB is Professor of Microbiology & Immunology at Department of Biosciences at COMSATS University.

Funding

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Competing interests

None declared.

Ethical approval

Clinical samples from neonates, geriatrics and burn patients were collected after informed and written consent from patients. The project was subjected to evaluation and subsequently approved by the Ethical Review Committee of the Department of Biosciences, COMSATS University during the period September 2017 – February 2018 under ethical grant number 2017159.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jiph.2019.08.019.

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